Published in final edited form as:

Nat Protoc. ; 6(12): 2022–2034. doi:10.1038/nprot.2011.418.

Design and self-assembly of siRNA-functionalized RNA nanoparticles for use in automated nanomedicine

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Abstract

Individual genes can be targeted with siRNAs. The use of nucleic acid nanoparticles (NPs) is a convenient method for delivering combinations of specific siRNAs in an organized and programmable manner. We present three assembly protocols to produce two different types of RNA self-assembling functional NPs using processes that are fully automatable. These NPs are engineered based on two complementary nanoscaffold designs (nanoring and nanocube), which serve as carriers of multiple siRNAs. The NPs are functionalized by the extension of up to six scaffold strands with siRNA duplexes. The assembly protocols yield functionalized RNA NPs, and we show that they interact *in vitro* with human recombinant Dicer to produce siRNAs. Our design strategies allow for fast, economical and easily controlled production of endotoxin-free therapeutic RNA NPs that are suitable for preclinical development.

INTRODUCTION

One of the goals of bio-nanotechnology is the rational development and synthesis of nanoscaffolds that allow precise positioning of different therapeutic agents or biosensors in three-dimensional (3D) space. The myriad biomedical applications of such nanoscaffolds derive from their capacity to selectively deliver specific therapeutics to targeted human tissues with controlled stoichiometry and presentation of the delivered drugs^{1,2}. Recently, siRNAs have shown considerable therapeutic potential to downregulate specific gene expression in cancerous or virus-infected cells, and RNA aptamers have shown promising

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Note: Supplementary information is available via the HTML version of this article.

AUTHOR CONTRIBUTIONS K.A.A., W.W.G., B.A.S. and L.J. conceived and designed the experiments. K.A.A., W.W.G., E.B., B.A.S. and L.J. contributed to the sequence and 3D model design. K.A.A., W.W.G. and F.M.W. performed self-assembly PAGE and dicing experiments. K.A.A. and F.M.W. performed DLS experiments. K.A.A., W.W.G., B.A.S. and L.J. analyzed the data. M.A.D. and K.A.A. performed the LAL assay. K.A.A., W.W.G., B.A.S. and L.J. co-wrote the paper.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

targeting, therapeutic and diagnostic properties^{1,3–5}. Furthermore, current research suggests that pooling siRNAs together has the advantage of mitigating off-target effects associated with individual siRNAs and increasing therapeutic potency^{6,7}. Therefore, RNA-based scaffolds, capable of packaging multiple siRNAs together in one NP, offer an attractive and valuable building material for engineering functional therapeutics.

Nucleic acid NPs are a convenient method for packaging specific siRNAs in an organized and programmable manner^{3,4,8,9}. Our recent achievements in RNA nanotechnology^{8,10} introduced two orthogonal strategies for the production of 3D RNA scaffolds (nanocubes and nanorings) with the potential for broad use in nanotechnological and biomedical applications. These two nanodesign strategies use alternative assembly principles, which can be interchanged depending on the particular task or nanotechnological application. Previously, the nanoring and the nanocube, which are described herein, were functionalized with siRNAs for RNA interference (RNAi) therapeutics; the nanocube was also functionalized with malachite green aptamers, which, once triggered by assembly, can be used for fluorescent visualization^{8,10}. In contrast to other systems that rely on peptide conjugation, liposomal encapsulation or the use of synthetic nanomaterials as scaffolds to coat or embed siRNAs^{11–14}, our system applies the simplicity and biocompatibility unique to an all-RNA NP to package multiple siRNAs, thus eliminating the need to use sophisticated chemistry or materials, which can cause concerns with respect to toxicity^{15–17}. In many ways, our scaffolds are similar to the packaging RNA assemblies that were derived from the DNA-packaging motor of bacteriophage phi29, which has been used previously as a scaffold for both siRNAs and aptamers^{4,18}. However, because our strategy uses oligonucleotides that fall within the scope of commercial RNA synthesis (~80 nucleotides), it offers a greater degree of flexibility with respect to the ability to make chemical modifications and the potential for scaling up and automation while avoiding the need to construct bipartite modules¹⁸.

In this manner, the following protocol offers a low-cost and simple method for synthesizing and assembling RNA NPs with the ability to package multiple siRNAs. Depending on the protocol, the total assembly process requires no more than 25 min, resulting in high-yield NP production. To the best of our knowledge, this is the fastest and simplest technique for nucleic acid-based functional NP assembly. In addition, quality control experiments (native PAGE and/or dynamic light scattering) do not require highly sophisticated instrumentation or expertise to conduct.

The two types of NP scaffolds described offer complementary strategies for siRNA functionalization. Although the nanocube offers the advantage in terms of control of chemical and thermodynamic stabilities through the addition of DNA hybrid strands¹⁰, the overall sequence constraints associated with the scaffold were optimized independently of any functionalized siRNA sequences. As a consequence, the RNA sequences that make up the core of the nanocube may not be optimal for accommodating every potential siRNA. Additional parameters, such as adding heating and cooling steps, raising the temperature at which the cube is assembled or optimizing the sequence of the scaffold together with the potential siRNA strands, may be necessary to produce functionalized NPs depending on the desired siRNA sequence modifications. However, the nanocube, owing to the fact that it avoids the use of tertiary contacts, may not always require the use of divalent metal ions for assembly and can withstand future treatments of elevated temperatures, salt gradients and organic solvent extractions to a greater degree than the nanoring. In addition, the nanocube design is able to accommodate DNA hybrid strands, improving the chemical stability of the NP. Despite these advantages, owing to the simplicity of the design, the nanoring as a scaffold has fewer sequence constraints, giving it the potential to be optimized more easily

with respect to any particular siRNA sequences and thus making it preferable for general use.

Experimental design

By extending the 3' ends of each of the scaffold strands with a corresponding siRNA antisense (or sense) sequence, both the nanoring and nanocube scaffolds can be modified to package up to six siRNAs. The six siRNAs may or may not be of the same siRNA sequence, and they may vary depending on the experimenter's desired purpose. Hybridization of the corresponding complementary siRNA strand to the scaffold extension produces the desired siRNA duplexes. In most cases, functionalizing the RNA NPs requires minimal design steps (described later in RNA scaffold and siRNA sequence design), and following the preformed siRNA duplex protocol (described later in Cassette-based assembly protocols for functionalized NPs) helps to prevent misfolding of the scaffold strands. According to the NP design, the corresponding DNA templates and primers are purchased to produce the appropriate DNA oligonucleotides from which the corresponding RNA can be transcribed.

Nanodesign strategy 1 (nanoring scaffold)—The first strategy is based on the principles of RNA architectonics¹⁹. It achieves a remarkable degree of structural control by using prefolded RNA structural motifs as building blocks for bottom-up assemblies. Strategy 1 uses the rational design of artificial 3D RNA architectures formed by predetermined folding processes^{19,20}. In this approach, structural RNA tertiary fragments (motifs) are extracted from known X-ray and NMR structures of natural RNA molecules, and then recombined into novel RNA architectonic building blocks by computer 3D modeling^{21–28}. This strategy has been successfully used to design numerous platforms, including modular RNA units forming dimers^{20,29,30}, functionalized closed trimers⁹, self-assembling squares^{31,32}, 1D³³ and 2D^{32,34} arrays, and 3D objects³⁵. Most recently, this strategy was used to design and create thermostable hexameric rings^{8,27,28}, which are used in this work as one example of a functionalized nanoscaffold.

Strategy 1 is used when there are a certain number of fixed constraints on the structure imposed by a given sequence so that the 3D tertiary conformation of the desired motif remains uninterrupted. In terms of the assembly protocols, the use of natural RNA tertiary fragments in strategy 1 typically requires prefolding of all individual monomer units before their assembly. In the case of the nanoring, heating the mixture of strands at 95 °C for 2–3 min followed by immediately placing the samples on ice before the addition of the assembly buffer assures the formation of the thermodynamically favored helices within the nanoring, which occur much more quickly than the formation of intermolecular hydrogen bonding between complementary regions on different strands of RNA. This first step in self-assembly is essential for facilitating the correct folding and formation of the desired secondary structure of RNA so that the tertiary interacting and structural motifs in each monomer unit can also occur.

Although they have advantages in terms of structural control and they have reasonably high thermal and chemical stabilities, building blocks of RNA architectonics generally consist of relatively long oligonucleotides (~100 nucleotides), which may not always be suitable for some nanotechnology applications. This is why it is desirable to have alternative RNA nanoscaffolds made of shorter sequences, which are amenable to chemical synthesis and customized chemical modifications. However, because the nanoring is made from two exceptionally simple motifs, namely a helix capped by two terminal loops, this limitation does not apply.

Nanodesign strategy 2 (nanocube scaffold)—The second strategy is based only on canonical Watson–Crick interactions and uses relatively short (26–52 nt) single-stranded RNAs¹⁰. This approach uses a computer-aided technique to engineer different RNA cubes as a second type of functionalized nanoscaffold^{10,23}. The computational method is described in the supplementary material of the original publication¹⁰, and therefore will be only briefly summarized here. The computational approach is an *in silico* optimization of RNA sequences that proceeds by minimizing an objective function. The objective function consists of numerical terms that describe the similarity between predicted and desired RNA secondary structures, as well as terms containing sequence design rules. Alternative methods for computational RNA sequence design are INFO-RNA³⁶, NUPACK³⁷ and RNAinverse³⁸.

In contrast to strategy 1, monomers in strategy 2 are designed to avoid stable internal secondary structure, and their self-assembly does not rely on any tertiary interactions. In this manner, the sequence constraints associated with the NP design do not fall on one particular sequence or region of a sequence, but are spread over the entire system. As a result, the assembly protocol for strategy 2 constructs does not require monomer prefolding steps and uses only one incubation step at the proper assembly temperature.

To ascertain the potential compatibility of each design strategy with automation, we developed and optimized three assembly protocols for each type of nanodesign strategy (Fig. 1a,b), which we refer to as ‘one-pot’, ‘stepwise’ and ‘preformed siRNA duplex’ assemblies. For each protocol, we introduce the idea of using premade cassettes. The cassettes referred to herein indicate test tubes with equimolar stocks of individual RNA NP components (single-stranded RNAs or RNA duplexes) in 1× assembly buffer (Fig. 1c). Given the current advancements in micropumps and microfluidic devices, these cassette-type assemblies—relying on the equimolar addition of stock components—greatly facilitate automated engineering of designer-functionalized RNA NPs. Moreover, the cassette ‘preformed siRNA duplex’ assembly method (Fig. 1a,b) is identical for both strategy 1 and strategy 2 construct formation, simplifying the use for potential robotic systems.

RNA scaffold and siRNA sequence design

As the RNAi pathway in mammals is induced by siRNAs of 21–22 nt in length³⁹, chemically synthesized analogs of these duplexes for the diagnosis, prevention and treatment of human disease have an equivalent size of 21–25 nt with 2-nt 3′-end overhangs⁴⁰. These molecules serve to induce the post-transcriptional gene-silencing pathway of RNAs; however, longer siRNAs (up to 30-nt long) are thought to induce post-transcriptional gene silencing with increased potency because of Dicer’s cleaving processivity and the subsequent involvement of the RNA-induced silencing complex^{41,42}. As a demonstration of this concept, we functionalized cubes and rings with elongated 25-bp siRNA duplexes engineered to silence the expression of EGFP⁴³. All the sequences we used are presented in Supplementary Data 1. Sequence design can be performed via the RNA secondary structure prediction and sequence design web server at <http://matchfold.abcc.ncifcrf.gov/> (ref. 44). The starting sequences and desired secondary structures should be provided in a text format, as explained on the web site. Nucleotides that are represented as lowercase characters are not modified during the sequence optimization process. After submitting a sequence optimization compute job, the job ID returned by the web server should be saved or bookmarked because (depending on the size and number of nucleotide sequences) it can take several hours or more than a day for the stochastic sequence optimization algorithm to terminate. Once the sequence optimization is completed, one should compare the predicted secondary structures of the optimized sequences with the desired target structure of the RNA complex.

By using a linker of either two As or two Us, either sense or antisense sequences are programmed into the cube or ring nanoscaffolds as an extension of either their 3' or 5' ends (Fig. 2a,b and Supplementary Fig. 1). The scaffold cube and ring strands used in this project are listed in Supplementary Data 1 and named A–F. Three-dimensional models of the functionalized scaffold units (sA–sF) are shown in gray in Figure 2. The corresponding antisense units (An) are shown in red. By incorporating multiple RNAi effectors into a single carrier, the six-stranded cube or hexagonal ring scaffolds offer a novel delivery approach for combinatorial RNAi in which multiple genes expressions may be shut down at one time.

To demonstrate the generality of the developed assembly protocols, we also tested RNA NPs functionalized with six siRNAs having different sequences, aiming to silence six different parts of an mRNA. For this, six RNA sequences of cube and ring nanoscaffolds were concatenated with six antisense siRNA strands targeting different regions of an HIV-1 genome^{45,46}. A combinatorial search was performed, testing all possible ways to concatenate the six scaffold sequences with the six siRNA sequences. Each combination was scored on the basis of an RNA secondary structure prediction, such that the optimal choice (highest score) corresponded to the combination with the fewest predicted base pairings between the scaffold and siRNA regions.

RNA preparation

RNA molecules can be purchased or can be prepared by transcription of PCR-amplified DNA templates, as described in Steps 1–6; briefly, synthetic DNA molecules coding for the sequence of the designed RNA are purchased already amplified by PCR using primers containing the T7 RNA polymerase promoter. PCR products are purified using the QIAquick PCR purification kit, and then RNA molecules are prepared enzymatically by *in vitro* transcription using T7 RNA polymerase⁴⁷. To visualize assembled RNA NP quality control experiments, [³²P]Cp-labeled RNA molecules are used (T4 RNA ligase is used to label the 3' ends of RNA molecules by attaching [³²P]Cp (ref. 29)). In the case of the initial radiolabeled native PAGE assays, we recommend using at least two different radiolabeled RNA scaffolds or concatenated strands individually mixed with other nonlabeled RNAs, followed by the assembly protocol. For dicing functional control experiments, RNA molecules are co-transcriptionally α [³²P]-ATP body labeled^{8,10}.

Essential formulation quality control before safety evaluation for biomedical applications

The use of engineered nanomaterials for biomedical applications has been challenged by concerns over nanomaterial toxicity⁴⁸. It has been shown that chemical impurities (e.g., metal catalysts used in synthesis) and biological contaminants (e.g., bacterial endotoxins) are often responsible for toxicities observed with engineered nanomaterials^{49,50}. All materials intended for medical use in humans must be evaluated for safety before use, including small molecules, biotechnology-derived pharmaceuticals, medical devices^{51,52} and nanomaterials. Recent data from safety evaluations of engineered nanomaterials suggest that endotoxin contamination is a common problem^{53–55}. Endotoxin is a component of the cell walls of Gram-negative bacteria. It is present in tap water, in air, on many surfaces and in many common laboratory reagents and supplies. Endotoxin is a very potent immunostimulant and may confound the results of both toxicity studies and efficacy trials. Nanomaterials have large surface area-to-volume ratios and may have high reactivity, making them prone to interaction and contamination with bacterial endotoxins. In addition, NPs are often synthesized using equipment that is not routinely used for medical purposes (e.g., in materials science labs), resulting in endotoxin contamination being common among many nanoformulations entering preclinical studies. Certain previous clinical uses of siRNA have been halted because of concerns over toxicity, including fever and hypersensitivity

reactions^{56,57}. However, both fever and hypersensitivity reactions can also be caused by endotoxin. It is therefore essential to determine the amount of endotoxin in an siRNA delivery system early in preclinical development and to correctly distinguish observed responses to the siRNA NP from those to endotoxin.

Owing to the recombinant nature of the enzymes used for the preparation of RNA NPs, it is expected that a certain amount of endotoxin may be introduced into the NP formulation during synthesis. As endotoxin may be introduced during any step of the preparation procedure, it is important to take precautions to minimize the amount of this common contaminant at each step. All materials intended for injection into human patients or into animals (during preclinical studies) must comply with the FDA's limit for the allowable amount of endotoxin in drug formulations. The allowable limit is 5 EU kg⁻¹ h⁻¹ (refs. 58,59). Therefore, the lower the level of endotoxin in a given formulation, the higher the dose that can be allowably administered. Preparing endotoxin-free formulations is essential for supporting dose-escalation toxicity studies. As the purpose of this study is to develop protocols for engineering RNA nanotechnology-based siRNA delivery systems that can be translated into biomedical applications, several essential steps for preparation of endotoxin-free RNA NPs have been developed and are listed in Box 1. We also experimentally screened siRNA-containing NPs for the presence of endotoxin and optimized the assembly protocols to minimize endotoxin levels.

Cassette-based assembly protocols for functionalized NPs

Preparation of samples follows one of three cassette-based assembly protocols amenable to complete automation, referred to as 'one-pot assembly', 'stepwise assembly' and 'premade siRNA duplex assembly', as described below (Fig. 1). Please note that all of these protocols lead to the production of high-yield functionalized RNA NP assemblies consisting of 6–12 strands. However, NPs made of a larger number of strands can also be assembled by following these protocols (data not shown). Formation of such constructs is accomplished by using the appropriate combination of cassettes by simple manual mixing of equal aliquots and further incubation (drawing from either the 18 cassettes shown in Figure 1c (top and middle) or the 12 cassettes shown in Figure 1c (bottom)). For example, to produce NPs not containing any concatenated strands, one should use the cassettes from one of the top rows in the images in Figure 1c.

One-pot assembly—A total of 18 cassettes are prepared with all RNA strands (six with scaffold strands, six with siRNA sense-concatenated scaffold strands and six with different concentration equivalents of antisense siRNA strands) at equal concentrations in 1× RNA NP assembly buffer (final concentrations: 89 mM Trisborate buffer (pH 8.3), 2 mM Mg(OAc)₂). The RNA concentration in each cassette should be six times higher than the desirable final concentration of the functional RNA NP. Depending on the desired functional NP composition, corresponding strands are combined at equal volumes (1 μM final); antisense strands are present in amounts equal to that of the sense-concatenated scaffold strands (1–6 μM final). For example, in order to generate NPs containing six siRNAs (identical or different), equal volumes of strands from the sA–sF cassettes should be combined with 6:An in the case of 'one-pot' and 'stepwise' assemblies; in the case of 'duplex' assembly, there should be strands from the cassettes sA:An–sF:An (Fig. 1c). In the case of cubic NPs, samples are heated at 95 °C for 2 min, and then immediately snap-cooled to 45 °C. In the case of ring NPs, samples are heated at 95 °C for 2 min, then immediately snap-cooled to 0 °C and assembled at 30 °C for 20 min.

Stepwise assembly—The same cassettes are used in the stepwise assembly as in the one-pot assembly. Equal volumes of cube/ring scaffold strand cassettes (A–F) and/or sense-

concatenated scaffold strand cassettes (sA–sF) are combined. In the case of cube NPs, samples are heated at 95 °C for 2 min, and then immediately snap-cooled to 45 °C with further incubation for 10 min. In the case of the ring NPs, samples are heated at 95 °C for 2 min, then immediately snap-cooled to 0 °C and assembled at 30 °C for 10 min. Antisense strands from corresponding An cassettes are added and the completed solutions additionally incubated at 45 °C (for cube NPs) or at 30 °C (for ring NPs) for 10 min.

Premade siRNA duplex assembly—Please note that this protocol is identical for both strategy 1– and strategy 2–engineered constructs; for both ring and cube scaffold NPs, cassettes with RNA duplex solutions are mixed and incubated at 45 °C for 20 min. Twelve equimolar cassette solutions are first prepared for six of the scaffold strands as monomers, and six sense-concatenated scaffold strands and antisense-assembled duplexes. Monomer cassettes are prepared on ice by combining double-deionized water (ddiH₂O), 5× RNA NP assembly buffer and RNA (in the order given). Duplex cassettes are prepared by combining ddiH₂O and assembly buffer with equimolar concentrations of sense-concatenated scaffold strands and antisense strands on ice.

Seven different protocols (listed below) of duplex formations were tested (Supplementary Fig. 2). For all preparations, equimolar quantities of sense-concatenated cube strands and complementary antisense RNA strands were first combined in water on ice (4 °C), and the solution was gently mixed.

- *45 °C incubation.* The antisense:sense-concatenated scaffold strand solutions were heated for 2 min at 95 °C, and then snap-cooled to 45 °C. Samples were incubated at 45 °C for 30 min to allow for duplex formation, and then immediately cooled to 0 °C on ice¹⁰. Hybridization buffer (89 mM Tris-borate buffer (pH 8.3), 2 mM Mg(OAc)₂ and 50 mM KCl) was added in one of two ways: either before the initial heating step at 95 °C or 1 min after the transfer from 95 °C to 45 °C.
- *Snap cool.* RNA solutions were combined with hybridization buffer on ice, incubated at 95 °C for 2 min, immediately cooled to room temperature (RT, 21 °C) and kept at RT for 30 min to form duplexes. Mixtures were then cooled to 0 °C on ice.
- *Slow cool.* To allow duplex formation, hybridization buffer was added at 0 °C to the oligonucleotides, and the mixture was incubated in a heat block set to 95 °C for 1 min. This was followed by a gradual temperature decrease to RT over a 30-min period by removing the heat block, still containing the samples, from power. Samples were then cooled to 4 °C on ice.
- *Fast cool.* The oligonucleotide mixtures were incubated in a heat block at 95 °C for 2 min. The block containing the samples was removed from heat, placed on ice and left to cool to 0 °C on ice over a period of 30 min. Hybridization buffer was added to the RNA solutions either before heating or after the step at 95 °C.
- *37 °C incubation.* The RNA solution was combined with hybridization buffer at 0 °C, heated for 5 min at 95 °C and incubated for a 30-min period at 37 °C. Samples were further cooled to 4 °C on ice⁶⁰.
- *68 °C incubation.* Hybridization buffer was added to the RNA samples on ice. Solutions were heated to 68 °C for 10 min, and then slowly cooled to RT to anneal the duplexes. Next, the samples were cooled to 4 °C on ice⁶¹.
- *30 °C incubation.* To allow duplex formation, hybridization buffer was added at 0 °C to the oligonucleotides, and the mixture was incubated at 100 °C for 1 min. This

was followed by a gradual temperature decrease to 30 °C over a 30-min period. Samples were then cooled to 4 °C on ice⁶².

Using a variety of literature sources, the seven approaches listed above for siRNA duplex formation between sense-concatenated scaffold strands and antisense strands were attempted and analyzed in terms of product yield (Supplementary Fig. 2). The fastest protocol (see *Fast cool*) with relatively high yield (~95%) is used in this protocol.

MATERIALS

REAGENTS

▲ **CRITICAL** All experiments should be performed in ddiH₂O or ACC Pyroclear-certified limulus amoebocyte lysate (LAL) reagent water (Associates of Cape Cod, cat. no. WP100C) or Ultra Pure water (Quality Biological, cat. no. 351-029-721).

- Spermidine (Sigma, cat. no. S0266) **! CAUTION** It can cause irritation to the eyes, respiratory system and skin.
- DTT (0.1 M; Invitrogen, cat. no. 18080-093) **! CAUTION** It can cause irritation to the eyes.
- ATP, GTP, CTP, UTP (Sigma, cat. nos. A7699, G8877, C1506, 94370)
- Pyrophosphatase inorganic lyophilized powder (IPP; Sigma, cat. no. I1643)
- T7 RNA polymerase (Promega, cat. no. N2611)
- RNasin (Promega, cat. no. M6101)
- DNase (Promega, cat. no. P2075)
- TE (Tris-EDTA) buffer, pH 8.0 (Quality Biological, cat. no. 351-011-131)
- Boric acid (Fisher, cat. no. A74-3) **! CAUTION** It may affect fertility or cause damage to the unborn child.
- Tris (Sigma, cat. no. T6066) **! CAUTION** It can cause irritation to the eyes.
- Acrylamide-bisacrylamide (37.5:1, wt/wt), 30% (Fisher, cat. no. BP1408-1) **! CAUTION** It is toxic if ingested or absorbed through the skin.
- Urea (Sigma, cat. no. U6504)
- T4 RNA ligase (New England Biolabs, cat. no. M0204S)
- [³²P]Cp (250 μCi mol⁻¹, PerkinElmer, cat. no. BLU019A250UC) **! CAUTION** This is radioactive material.
- α[³²P]-ATP (250 μCi mol⁻¹, PerkinElmer, cat. no. BLU002A250UC) **! CAUTION** This is radioactive material.
- Glycerol (Life Technologies, cat. no. 5514UA)
- Xylene cyanol (Sigma, cat. no. X-4126) **! CAUTION** Avoid contact and inhalation.
- Bromophenol blue (Sigma, cat. no. B-8026) **! CAUTION** Avoid contact and inhalation.
- Ammonium persulfate (Sigma, cat. no. A3678) **! CAUTION** It can cause irritation to the eyes.
- Tetramethylethylenediamine (TEMED, Sigma, cat. no. T7024) **! CAUTION** It may cause respiratory tract irritation if inhaled. It may also cause skin or eye irritation if

absorbed through skin or if it comes in contact with eyes, and is harmful if swallowed.

- Magnesium acetate ($\text{Mg}(\text{OAc})_2$, VWR, cat. no. BOH4170)
- BLOCK-iT Dicer RNAi Kit (Invitrogen, cat. no. K3650)
- Whatman chromatography paper (Fisher, cat. no. 05-716-6A)

EQUIPMENT

- Microcentrifuge for 0.5 ml tubes
- Vortex mixer
- Programmable incubator and/or heating blocks
- Sequencing apparatus (vertical electrophoresis) for high-resolution PAGE, that is, 10% (37.5:1) acrylamide, 1× Tris-borate buffer (pH 8.3), 2 mM $\text{Mg}(\text{OAc})_2$
- Gel dryer
- Phosphoimaging instrument and screen
- Freezer (− 20 °C) and 4 °C refrigerator
- Access to UV spectrophotometer
- Access to dynamic light-scattering (DLS) apparatus such as DynaPro NanoStar (Wyatt Technology)
- Phosphorimaging instrument (e.g., Storm or Typhoon, GE Healthcare)
- ImageQuant (GE Healthcare)

REAGENT SETUP

Transcription buffer (1× final concentration)—Mix 15 mM MgCl_2 , 2 mM spermidine, 50 mM Tris buffer (pH 7.5), 2.5 mM NTPs, 10 mM DTT, 0.1 $\mu\text{g } \mu\text{l}^{-1}$ IPP and 0.8 U μl^{-1} RNasin. This buffer can be stored at − 20 °C for 1 year.

Tris-borate buffer (10× final concentration)—Mix 0.89 M Tris and 0.8 M boric acid (pH 8.3). This buffer can be stored at RT for 1 year.

RNA NP assembly buffer (5× final concentration)—Mix 5× Tris-borate buffer (pH 8.3) and 10 mM $\text{Mg}(\text{OAc})_2$. This buffer can be stored at RT for 2–3 months.

RNA eluting buffer (final concentration)—Mix 300 mM NaCl, 10 mM Tris (pH 7.5) and 0.5 mM EDTA. This buffer can be stored at RT for 1 year.

Native PAGE loading buffer (1× final concentrations)—Mix 50% (vol/vol) glycerol, 1× Tris-borate buffer (pH 8.3), 0.01% (wt/vol) bromophenol blue and 0.01% (wt/vol) xylene cyanol tracking dyes. This buffer can be stored at RT for 1 year.

Native PAGE running buffer (1× final concentrations)—Mix 1× Tris-borate buffer (pH 8.3) and 2 mM $\text{Mg}(\text{OAc})_2$. This buffer can be stored at 4 °C for 1 year.

PROCEDURE

RNA synthesis ● TIMING 16 h

- 1| For 200 μ l of transcription mixture, add 50 μ l of PCR-amplified DNA template (encoding RNA scaffold strand or RNA sense-concatenated scaffold strand or short RNA antisense strand; see INTRODUCTION for further information on RNA preparation) to 150 μ l of 1 $\times$ transcription buffer.
- 2| Incubate for 4 h at 37 °C.
- 3| Stop the transcription by adding 5 μ l of RQ1 RNase-free DNase (10 U μ g⁻¹).
- 4| Incubate for 30 min at 37 °C.
- 5| Purify the transcription mixture on a denaturing urea gel (8% (wt/vol) acrylamide, 8 M urea)^{10,29,30,47}; elute band overnight (~12 h) in RNA elution buffer.

▲ **CRITICAL STEP** Freshly prepared or purchased RNA molecules should be resuspended in a TE or ddiH₂O buffer and kept on ice for several hours or frozen. Please note that if they are frozen, RNA samples must be vortex-mixed for 10 s before use.

- 6| Measure concentrations of all resuspended synthesized or purchased RNAs (scaffold strands, sense-concatenated scaffold strands, and short antisense strands) using a UV spectrophotometer. Extinction coefficients for all strands can be calculated using the nearest neighbor method (http://www.ambion.com/techlib/misc/oligo_calculator.html).

? TROUBLESHOOTING

RNA cassettes preparation ● TIMING ~30 min

- 7| Prepare a 100- μ l volume of RNA cassettes using option A for single-stranded RNAs and option B for duplex RNAs:

(A) Preparation of stock solution cassettes containing single-stranded RNAs

- i. Prepare an 80- μ l solution with all RNA strands (from Step 6) at equal concentrations in ddiH₂O in Eppendorf microcentrifuge tubes.

▲ **CRITICAL STEP** The final RNA concentrations in the cassettes should be six times higher than the desirable concentration of functionalized RNA NPs. For example, if the desirable RNA NP concentration is 1 μ M, the concentrations of RNAs in the cassettes should be 6 μ M.

- ii. Add 20 μ l of 5 $\times$ RNA NP assembly buffer to make a 1 $\times$ final concentration of buffer in a 100- μ l total volume of RNA cassettes.

▲ **CRITICAL STEP** Once they have been prepared, RNA cassettes should not be frozen and can be stored at 4 °C or on ice for several weeks.

(B) Preparation of stock solution cassettes containing duplex RNAs

- i. Mix sense-concatenated strands and corresponding short antisense strands in Eppendorf microcentrifuge tubes at equal

concentrations six times higher than the desirable concentration of functionalized RNA NPs, to a total volume of 80 μ l of solution.

- ii. Add 20 μ l of 5 $\times$ RNA NP assembly buffer to prepare a 1 $\times$ final concentration of buffer in a 100- μ l total volume of RNA cassettes.
- iii. Incubate the mixture in a heat block at 95 $^{\circ}$ C for 2 min.
 ▲ **CRITICAL STEP** Do not incubate the mixture longer as it can promote the degradation of RNAs.
- iv. Remove the block containing the samples and place it on ice.
- v. Let it cool to 0 $^{\circ}$ C on ice (~5 min).
- vi. To check the successful duplex RNA formation, the samples can be subjected to the quality control experiments described in Step 9.

▲ **CRITICAL STEP** Once they have been prepared, RNA cassettes should not be frozen and can be stored at 4 $^{\circ}$ C or on ice for several weeks.

? TROUBLESHOOTING

Functionalized RNA NP assembly from cassettes

- 8| Assemble the functionalized RNA NPs using the preformed cassettes from Step 7 by using option A for the one-pot assembly of cubes, option B for the one-pot assembly of rings, option C for the stepwise assembly of cubes, option D for the stepwise assembly of rings or option E for the duplex assembly of cubes and rings.

(A) One-pot assembly of cubes ● TIMING 22 min

- i. Depending on the final number of siRNAs entering the composition of the desired NP, manually mix equal volumes of corresponding siRNA sense-concatenated strands, antisense strands and appropriate nonconcatenated scaffold strands.
- ii. Incubate the mixture in a heat block at 95 $^{\circ}$ C for 2 min.
 ▲ **CRITICAL STEP** Do not incubate the mixture longer, as it can promote the degradation of RNAs.
- iii. Snap-cool the mixture by rapidly transferring the sample to the heating block set at 45 $^{\circ}$ C; incubate for 20 min.

(B) One-pot assembly of rings ● TIMING 24 min

- i. Depending on the final number of siRNAs entering the composition of desired NP, manually mix equal volumes of corresponding siRNA sense-concatenated strands, antisense strands and appropriate non-concatenated scaffold strands.
- ii. Incubate the mixture in a heat block at 95 $^{\circ}$ C for 2 min.
 ▲ **CRITICAL STEP** Do not incubate the mixture longer, as it can promote the degradation of RNAs.

- iii. Snap-cool the mixture by placing it on ice; incubate for 2 min.
- iv. Bring the temperature to 30 °C in a heating block and incubate for 20 min.

(C) Stepwise assembly of cubes ● TIMING 22 min

- i. Depending on the final number of siRNAs entering the composition of desired NP, manually mix equal volumes of corresponding siRNA sense-concatenated strands, antisense strands and appropriate non-concatenated scaffold strands.
- ii. Incubate the mixture in a heat block at 95 °C for 2 min.
▲ **CRITICAL STEP** Do not incubate the mixture longer, as it can promote the degradation of RNAs.
- iii. Snap-cool the sample by rapidly transferring it to the heating block set at 45 °C; incubate for 10 min.
- iv. Add antisense strands in amounts equal to the molarities of corresponding sense-concatenated scaffold strands.
- v. Incubate the mixture for an additional 10 min at 45 °C.

(D) Stepwise assembly of rings ● TIMING 24 min

- i. Depending on the final number of siRNAs entering the composition of desired NP, manually mix equal volumes of corresponding siRNA sense-concatenated strands, antisense strands and appropriate non-concatenated scaffold strands.
- ii. Incubate the mixture in a heat block at 95 °C for 2 min.
▲ **CRITICAL STEP** Do not incubate the mixture longer, as it can promote the degradation of RNAs.
- iii. Snap-cool the sample by placing it on ice; incubate for 2 min.
- iv. Bring the temperature to 30 °C in a heat block and incubate for 10 min.
- v. Add antisense strands in the amounts equal to the molarities of corresponding sense-concatenated scaffold strands.
- vi. Incubate the mixture for an additional 10 min at 30 °C.

(E) Duplex assembly for cubes and rings ● TIMING 20 min

- i. Depending on the final number of siRNAs entering the composition of desired NP, manually mix equal volumes of corresponding duplexes and appropriate non-concatenated scaffold strands.
- ii. Incubate the mixture in a heat block at 45 °C for 20 min.

Assembled RNA NP quality control experiments

- 9| Perform quality control experiments—use option A for native PAGE or option B for DLS experiments.

(A) Native PAGE ● TIMING 4 h plus time necessary to expose the screen (at least 4 h)

- i. Prepare a sequencing gel for vertical electrophoresis for high-resolution PAGE; i.e., 10% (37.5:1) acrylamide, 1× Tris-borate buffer (pH 8.3) and 2 mM Mg(OAc)₂. To induce gel polymerization, add 1 ml of 10% (wt/vol) APS and 50 µl of TEMED for every 100 ml of sequencing gel solution. We recommend using a vertical gel of dimensions 31 cm × 38.5 cm with a spacer thickness of 0.75 mm.
- ii. Prerun this gel in a cold room or refrigerator (4 °C) in 1× native PAGE running buffer for 30 min at 5 W.
- iii. While prerunning the gel, mix equal volumes of preassembled NPs (final concentration of at least 0.5 µM) and corresponding controls with equal volumes of 1× native PAGE loading buffer, to a final volume of 10 µl.
- iv. Load samples in the individual lanes of a gel (5 µl per lane). For the best separation results, perform electrophoresis for 3 h at 20 W at 4 °C.

? TROUBLESHOOTING

- v. A control sample of a previously characterized NP (such as a nonfunctionalized cube or ring NP) should be loaded for comparison with newly assembled RNA NPs. A ring or cube devoid of any siRNA functionality should migrate further than those with one siRNA functionality; those with one siRNA should migrate further than those with two, and so on.

▲ **CRITICAL STEP** Use a syringe to wash the loading wells several times with running buffer before loading.

- vi. Transfer the gel to the Whatman chromatography paper and dry the gel on a gel dryer.
- vii. Expose the dried gel overnight to a phosphorimaging screen, then scan it using a phosphorimaging instrument (Storm, Typhoon or similar) and quantify bands using two-dimensional densitometry software (ImageQuant or similar).

▲ **CRITICAL STEP** Assembled NPs are expected to migrate as a single band on nondenaturing gels. The presence of any other bands should, in total, comprise no more than 25% of the entire band percentage as determined by following the product instructions for the ImageQuant TL 1D gel analysis software.

▲ **CRITICAL STEP** The gel should be dried completely to prevent band diffusion and phosphorimaging screen damage.

? TROUBLESHOOTING

(B) DLS ● TIMING 3–4 h

- i. Centrifuge NPs (a final concentration of at least 0.5 µM and a final volume of at least 55 µl of preassembled RNA NPs) at 15,000g at 4 °C for 3 h.
- ii. Collect the top 50 µl of supernatant for analysis by DLS.

- iii. Place the sample in the DynaPro NanoStar with the temperature set at 25 °C.

▲ **CRITICAL STEP** Samples should be kept on ice until the time of measurement.

- iv. Perform the measurements at a wavelength of 662 nm and under a constant flow of nitrogen gas. The following parameters should be used: correlation filter between 1.50 and 60,000 μ s; peak radius between 0.7 and 1,000 nm; sample type: globular proteins; R_g model: sphere; acquisition time: 5 s; number of acquisitions: 10. The intensity of light scattered by the particles in the sample will be converted to hydrodynamic radius (R_h) measurements by the DynaPro Dynamics software and displayed as a histogram. Experimentally, R_h should appear in highest concentration at the hypothetical R_h values, indicating that the sample consists largely of the desired NP.

? TROUBLESHOOTING

Assembled RNA NP functional control experiments ● **TIMING** 24 h plus time to expose the screen

- 10| To perform functional control experiments, incubate functionalized NPs (final concentration of 3 μ M) for 20 h at 37 °C in the presence of BLOCK-iT Dicer enzyme.
- 11| Quench dicing reaction by adding one-tenth of the volume of Dicer stop solution (provided by the manufacturers).
- 12| Analyze on a 2 mM Mg(OAc)₂ native 7% (wt/vol) PAGE and an 8 M urea/10% (wt/vol) PAGE gel as described in Step 9A(i–iv). The Dicer should cleave 22-nt siRNA sequences from the functionalized cube or ring. On a nondenaturing gel, this will result in a duplex and cube scaffold (to be certain of the results, a nonmodified cube or ring and a preformed duplex should be used as controls, which will migrate to the same location as the digested functionalized NPs). On a denaturing gel, the result will be monomer strands from the NP and 22-nt strands from the sense:antisense control duplex (a nonmodified cube or ring monomer and an antisense monomer should be used as controls, which will migrate to the same location as the digested and denatured strands).

? TROUBLESHOOTING

? TROUBLESHOOTING

Please refer to Table 1 for troubleshooting advice.

● **TIMING**

Steps 1–6, RNA synthesis: ~16 h

Step 7, RNA cassettes preparation: ~30 min

Step 8, Functional RNA NP assemblies: ~25 min

Step 9A, Assembled RNA NP quality control experiments: ~8 h

Step 9B, Assembled RNA NP quality control experiments: 3–4 h

Steps 10–12, Assembled RNA NPs functional control experiments: ~24 h (plus time to expose the screen)

ANTICIPATED RESULTS

Anticipated assembly results—To check the quality of the assemblies, we recommend carrying out native PAGE together with DLS experiments (Fig. 2). These experiments are low in cost and easy to perform. The results presented in Figure 2 and supplementary Figures 3 and 4 show the outputs for three different self-assembly protocols for cube and ring NPs functionalized with up to six siRNAs. Major dark bands correspond to the products of the assemblies. Please note that depending on the number and orientation of siRNA-concatenated scaffold strands, the shapes of NPs and their relative gel shifts may be slightly different (supplementary Fig. 3, cubes functionalized with four siRNA duplexes).

As controls, previously characterized nonconcatenated cubes and rings are used. All bands are quantified using commercially available ImageQuant software; as an alternative, public domain ImageJ software can be used. Equally sized boxes should be drawn around the bands corresponding to the NPs. The yield of a NP is calculated by dividing its corresponding quantified value by the total sum of the values for all other complexes present in the corresponding lane¹⁰.

In this protocol, as an example, quantification of the bands obtained from at least four independent assemblies for each type of NP reveals that, depending on the protocol and the composition of RNA NPs, the average yields for all three protocols are very comparable and range between 75 and 90% (Fig. 2a–c and supplementary Figs. 3 and 4).

By using DLS, the hydrodynamic radii (R_h) for the assembled RNA NPs can be determined (Fig. 2d). The values obtained using the assembly protocols are in good agreement with the predicted radii of circumscribed spheres around CPK models of RNA NPs (3' and 5' concatenated cubes with six siRNAs are 11 and 11.5 nm, respectively; 3' and 5' concatenated rings with six siRNAs are 15 nm each). Overall, PAGE results and DLS data can strongly confirm the successful formation of functionalized NPs.

Anticipated functional control results—To test *in vitro* efficacy of siRNA-concatenated NPs as stimulators of RNAi, the digestion experiments with recombinant human enzyme Dicer (r-Dicer)⁶³ can be used⁸. The digestion of RNA duplexes by r-Dicer is known to result in 21–23-nt Dicer-generated siRNAs capable of specific gene silencing in mammalian cells⁶⁴. Thus, combining r-Dicer with cube and ring NPs containing six siRNAs (Fig. 3) allows for an easy estimation *in vitro* of the suitability of assembled functionalized RNA NPs for potential use *in vivo*. In addition, this provides evidence of all six possible siRNA addendums undergoing dicing, and thus illustrates the possibility of targeting six different gene sequences through co-RNAi. For dicing experiments, we recommend using any two different body-labeled RNA-siRNA concatenated strands (strand E* or F* in Fig. 3) individually assembled with other nonlabeled RNAs (A, B, C, D, F, Ant for E* and A, B, C, D, E, Ant for F*), followed by the assembly protocol. As size-marker controls, radiolabeled RNAs of different lengths (19–24 nucleotides) and RNA duplexes comparable in size to Dicer-generated siRNAs (19–21 bps) can be used.

Anticipated endotoxin screening results—The results presented in Table 2 show that when the precautions described in Box 1 (to prevent endotoxin contamination) are not followed (assembly of NPs was performed using Picotap high-purity laboratory water), the levels of contaminating endotoxin in the RNA NPs is ~0.6 EU ml⁻¹ per 200 nM for the majority of the formulations, and therefore the maximum allowable dose of these formulations is 1.7 µM kg⁻¹ h⁻¹. However, when the precautions are followed, endotoxin is

not detectable (the measured levels are below the assay lower limit of quantification of 0.01 EU ml⁻¹ per 200 nM). Thus, for these endotoxin-free formulations, the allowable dose can exceed 100 µM kg⁻¹ h⁻¹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported (in part) by the Intramural Research Program of the US National Institutes of Health (NIH), National Cancer Institute, Center for Cancer Research. This work has been funded in whole or in part with federal funds from the National Cancer Institute, NIH, under contract no. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US government. This research was also supported by NIH grant no. R01GM-079604 (to L.J.). We are grateful to B. Neun for technical assistance with the LAL assay and to J. Hall for help with manuscript preparation.

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Box 1 | Steps for preventing endotoxin contamination

1. Wear gloves, spray gloves with 70% (vol/vol) alcohol before touching tubes/reagents etc., and change gloves often during the process.
2. Use pyrogen-free reagents and water (ddH₂O, Lonza LAL grade water or ACC Pyroclear-certified LAL reagent water).
3. Use sterile glassware and pipettes; to depyrogenize glassware, bake it at 250 °C for 2 h or at 200 °C overnight.
4. Perform the assembly in a laminar flow hood.
5. Avoid using cellulose-based filters, as these may be a source of β-glucan, which interferes with the LAL assay.
6. Avoid breathing, coughing or sneezing into the tubes/reaction beakers.
7. Avoid touching your face; wash hands and change gloves immediately if you touch something.

Figure 1.

Schematic representation of three different cassette-based assembly protocols for siRNA-functionalized NPs. (**a–c**) Two nanodesign strategies (nanorings and nanocubes) are represented in **a** and **b**, respectively, and the corresponding cassette compositions for each type of assembly protocol are shown in **c**. Please note that the cassette compositions are the same for one-pot and stepwise assemblies (I and II), and that the assembly protocol for preformed siRNA duplex assembly (III) is identical for both design strategies.

Figure 2.

Quality control experiments for assembled RNA NPs. **(a,b)** Three-dimensional models and assembly quality control experiments of 3' **(a)** and 5' siRNA-concatenated **(b)** RNA NPs with tables showing the yields of assembly for corresponding NPs (nanocubes on the left hand side, nanorings on the right-hand side) concatenated with one to six siRNAs. **(c,d)** Each number represents an average value calculated from at least three independent experiments. Native PAGE **(c)** and DLS results **(d)** for the NP assembly experiments. Please note that each gel represents the results of 21 independent assemblies.

Figure 3.

Functional control experiments for assembled RNA NPs. **(a,b)** Dicing experiment results for cube **(a)** and ring **(b)** RNA NPs concatenated with six siRNAs at the 5' side. Bodies labeled with ^{32}P siRNA sense-concatenated scaffold strands E and F and antisense (Ant) are indicated with asterisks. Native and denaturing PAGE gels were used to identify the dicing products corresponding to the processed cubes and rings (with remaining 5' overhang tails of 8 nt) and siRNA products formed with 21-nt complementary strands using appropriate molecular RNA markers of known lengths. C, control (assembled NPs without any treatment); (+), NPs incubated with Dicer for 20 h at 37 °C; (–), NPs incubated at 37 °C for 20 h in the dicing buffer without the Dicer enzyme.

Table 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
6	Transcription yields are low	Yields of the PCR products are low	Check PCR product by agarose gel electrophoresis before transcription, proceed only after visualization
		Transcription reagents are old	Use freshly prepared reagents such as DTT, which degrades after multiple freeze-thaw cycles
7B(vi), 9A(vii)	Multiple banding on gels because of RNA contamination or degradation	RNase contamination	Check monomers on denaturing gel, resynthesize RNA if necessary
		RNA degradation because of the presence of divalent ions (Mg^{2+})	Keep assemblies on ice to avoid degradation via nonspecific cleavage in the presence of metal ions
9A(iv)	RNA does not migrate or is stuck in the wells	Wells were not washed well enough	Wells have particulate in them: filter the reagents for the gel, clean the comb well with water and ethanol and let it air dry
		Prefolding of the strategy 1 monomers was not achieved	For ring assemblies, heat to 95 °C for 2–3 min and snap cool on ice for 3 min
9A(vii)	No RNA assembly products on PAGE gel	Absence of divalent ions (Mg^{2+})	Verify whether magnesium salt was added to assembly buffer, gel and gel buffer
	Multiple assembly products on native PAGE gel	Monomer single-stranded RNAs tend to stick to the tube walls	Vortex individual monomers well before assembly
		Initial concentrations were not measured correctly	Remeasure concentrations of monomers before assembly
		Alternative folding may prevent the accessibility of kissing loops	Check sequences for complementary kissing loops and/or secondary structure
9B(iv)	Broad DLS spectra or spectra with multiple peaks	Sample is polydispersed because of contamination or poor assembly	Remeasure concentrations of monomers before assembly; centrifuge your sample
12	Assemblies are not processed by Dicer	Sequence design is incorrect	Verify the RNA design so that annealed strands result in 2-nt 3' overhangs
		Assembly problems	Anneal a slight excess (~ 5%) of duplex strands to the NPs
		Inactivation of the enzyme	Check the freshness or activity of Dicer by dicing with short 27/25-nt duplexes ⁴³

Table 2

Endotoxin levels in nanoparticle formulations before and after optimization of synthesis protocol.

Sample description	Endotoxin, EU ml ⁻¹	Endotoxin EU ml ⁻¹
	Before optimization	After optimization
Cube duplex monomer	0.050	BLOQ
Cube	0.572	BLOQ
Cube-6siRNA	0.010	BLOQ
Nanoring (NR)	0.682	BLOQ
NR-6siRNA	0.653	BLOQ
NR monomer	0.597	BLOQ
Buffer	0.714	BLOQ

Nanoparticles were prepared as described in the PROCEDURE and tested for the presence of bacterial endotoxins by kinetic turbidity limulus amoebocyte lysate (LAL). The detailed protocol for LAL is described elsewhere^{58,59,65}. The USP standard BET85 was followed. Each sample was tested at concentration 100-fold above that used in efficacy experiments *in vitro*. Shown is mean result ($n = 3$). BLOQ, below lower limit of quantification. Assay's lower limit of quantification is 0.01 EU ml⁻¹. One milliliter of nanoformulation corresponds to 200 nM RNA.