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Controllable Self-Assembly of Nanoparticles for Specific Delivery of Multiple Therapeutic Molecules to Cancer Cells Using RNA Nanotechnology

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Abstract

By utilizing RNA nanotechnology, we engineered both therapeutic siRNA and a receptor-binding RNA aptamer into individual pRNAs of phi29's motor. The RNA building block harboring siRNA or other therapeutic molecules was fabricated subsequently into a trimer through the interaction of engineered right and left interlocking RNA loops. The incubation of the protein-free nanoscale particles containing the receptor-binding aptamer or other ligands resulted in the binding and co-entry of the trivalent therapeutic particles into cells, subsequently modulating the apoptosis of cancer cells and leukemia model lymphocytes in cell culture and animal trials. The use of such antigenicity-free 20–40 nm particles holds promise for the repeated long-term treatment of chronic diseases.

Introduction

Nanotechnology has brought about an unprecedented variety of revolutionary approaches for the detection and therapy of diseases. Because of their small size, nanoparticles can interact readily with biomolecules either on the surface of or within cells. To take advantage of this, it is desirable for one to develop multifunctional engineered, targeted complexes capable of bypassing biological barriers to deliver multiple therapeutic agents directly to specific cells or tissues. Because of their easy access to many areas of the body, multivalent nanoparticles offer the possibility of a wealth of innovative tools with the potential to combine detection and therapy in ways previously unimaginable.

At different stages of cancer development, the abnormal and malfunctioning cells express a variety of cellular factors, signaling molecules, markers, receptors, and other specific antigens. Earlier detection and treatment of cancer with unique signatures by multivalent therapeutic agents or detection sensors could benefit patients greatly and save lives.¹ However, developing the ability to detect stage-specific characteristics of cancer cells and thereby produce targeted imaging and multiple-drug delivery systems remains a challenge. It is difficult for particles

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larger than 100 nm to enter cells. Molecules smaller than 20 nm could move out of blood vessels or kidney during circulation and have a shorter retention time in the body.

Small interfering siRNA,^{2–6} ribozymes,⁷ and anti-sense RNA^{8,9} all show significant potential in new molecular approaches to down-regulate specific gene expression in cancerous or viral-infected cells.¹⁰ The successful application of siRNAs and ribozymes for the treatment of cancer and infectious diseases requires overcoming the following obstacles: (1) difficulty entering the cell because of the size limit for membrane penetration; (2) degradation by exonucleases within the cell; (3) trafficking into the appropriate cell compartment; and (4) correct folding of the ribozymes or siRNA in the cell if fused to a carrier. At this time, the development of a safe, efficient, specific, and nonpathogenic nanoparticle for the delivery of multiple therapeutic RNAs is highly desirable.

A bacteriophage phi29-encoded small RNA has been shown to play a novel and essential role in packaging DNA into procapsids;¹¹ this RNA is termed packaging RNA or “pRNA”. pRNA forms dimers, trimers, and hexamers with sizes of 10–30 nm (Figure 1) via hand-in-hand interaction through the base-pairing of two interlocking left- and right-hand loops.^{12–15}

A computer model of the three-dimensional structure of pRNA building blocks has been constructed¹⁶ on the basis of experimental data derived from photoaffinity cross-linking,¹⁷ chemical modification interference,^{18,19} complementary modification,^{20,21} nuclease probing,²² oligo targeting,²³ competition assays,^{24,25} and cryo-atomic force microscopy (AFM).^{18,19,26} pRNA hexamer docking with the connector crystal structure reveals an impressive match with available biochemical, genetic, and physical data concerning the 3D structure of pRNA.¹⁶

Recently, we used the bottom-up assembly characteristics of the building blocks of pRNA to produce a variety of structures and shapes including rods, triangles, twins, tetramers, and 3D arrays up to several micrometers in size.^{13,27} Such a process occurs as a result of the interaction of programmed helical regions and loops. Arrays produced by this method have been shown to be resistant to a variety of environmental stresses including a wide range of salt concentrations, temperatures, and pH levels.^{13,27}

The 117-nucleotide pRNA monomer contains two functional domains: the intermolecular-interacting domain and the double-stranded helical DNA-packaging domain (Figure 1). The intermolecular-interacting domain is located in the central section of the pRNA molecule and contains two interlocking left and right loops that can be engineered for bottom-up assembly, whereas the double-stranded helical DNA-packaging domain is located at the 5'/3' paired ends. Available data suggests that these two domains fold independently of one another. Structural studies have confirmed that the 5' and 3' ends of pRNA are proximate and pair to form a double-stranded helix.^{16,27} This double-stranded region (with more than 30 nucleotides) is an independent domain, and the addition or deletion of nucleotides at the 5' end preceding nucleotide #23 and at the 3' end following nucleotide #97 does not affect the correct folding of the intermolecular interaction domain. Complementary modification studies have revealed that altering the primary sequence of any nucleotide of the helix does not impact pRNA structure and folding if the two strands are paired.

Numerous studies have indicated that siRNA is a double-stranded (ds) RNA helix.^{2–5,28} To test whether it is possible to replace the pRNA helical region with double-stranded siRNA, we engineered a variety of chimeric pRNAs.^{28b} For this report, we found that replacement of pRNA's 3'/5' helical region with siRNA or connection of the CD4-binding RNA aptamer, folate, or other chemical components in this region did not interfere with the folding of the pRNA and siRNA or the function of the inserted moiety, nor did it impact the formation of trimers. It was found that three reagents engineered into the trimer were co-delivered

simultaneously to specific cancer cells, guided by one of the chimeric pRNA building blocks that harbored the CD4-binding RNA aptamer or folate that bound the cell surface receptor.

Being able to use nanoparticles with sizes of about 20 nm avoids the problem of the short half-life of small molecules in vivo because of short retention time. In addition, the problem of having molecules larger than 100 nm that are poorly delivered to cells is solved. It is well-accepted in the scientific community that RNA has a very low or undetectable level of immunogenicity except when complexed with protein.^{29,30} Our system does not contain protein or peptides, and thus the use of such protein-free nanoparticles to avoid immune response would allow for long-term administration in the treatment of chronic diseases.

Results

Nomenclature of RNA building blocks: To simplify the description of bottom-up assembly using engineered RNA building blocks, we will use uppercase letters to represent the right-hand loop of pRNA and lowercase letters to represent the left-hand loop (Figure 1). The same letters in upper and lower cases indicate complementary sequences for loop/loop interaction, whereas different letters indicate noncomplementary loops. For example, pRNA A-b' represents pRNA where right loop A (5'G₄₅G₄₆A₄₇C₄₈) is complementary to left loop a' (3'C₈₅C₈₄U₈₃G₈₂) of pRNA E-a'.¹² "pRNA/aptamer(CD4)" denotes a pRNA chimera that harbors an aptamer that binds CD4, whereas pRNA/siRNA-(survivin) represents a pRNA chimera that harbors an siRNA targeting the anti-apoptosis factor survivin.

1. Engineering of Chimeric RNA as Building Blocks for the Fabrication of Trimers as Vehicles for the Delivery of Therapeutic Molecules

First, the possibility of engineering chimeric pRNA building blocks harboring receptor-binding aptamer was tested. For specific delivery of therapeutic vehicles to cells, it is necessary to incorporate a chemical moiety that can recognize cell surface markers. In comparison to antibodies and phage-displaying peptides, RNA aptamer is an attractive alternative because it offers the advantage of avoiding the induction of immune responses.^{29,30} A powerful technique to obtain RNA aptamers that selectively bind to specific receptors with high affinity is based on in vitro screening of RNA molecules from a library that contains random RNA sequences.^{31,32} A number of aptamers have been obtained that specifically recognize a particular cell surface receptor such as CD4 by using this SELEX approach.³³ One CD4-binding RNA aptamer was incorporated into the pRNA via connection to its original 5'/3' end (Figure 1). The pRNA vector was engineered and reorganized into a circularly permuted form, with the nascent 5' and 3' end relocated to residues 71 and 75, respectively, of the original pRNA sequence. The 71/75 end has been shown to be located in a tightly folded area,¹⁶ which buries and protects the ends from exonuclease degradation.⁷ The chimeric pRNA building block with this aptamer also contained the appropriate right and left loops required for the engineering and fabrication of the trimeric complex.

The pRNA/aptamer(CD4) was labeled with FITC and assayed for its ability to bind the CD4 receptor by fluorescent microscopy to test whether the pRNA/aptamer chimera was able to bind to specific cells. The binding assay using a CD4-overexpressing engineered thymic T cell line, D1 (CD4^{hi}) (see below), and the CD4 negative parental line, D1 (CD4^{neg}), from which it was derived³⁴ revealed that the chimeric pRNA-FITC/aptamer (CD4) was able to bind CD4^{hi} T cells efficiently. Binding and internalization was observed by confocal microscopy using the "section" technique. A layer of T cells in the confocal microscope image displayed as a green circle, confirming binding of the FITC-label chimeric pRNA/aptamer (CD4) to the spherical T cells (Figure 2-II). The binding specificity of the aptamers was investigated using a variety of controls, including CD4 receptor-negative D1 cells (Figure 2E–H and P), fluorescent pRNA trimer without CD4 aptamer (not shown), and fluorescent dye alone without

RNA (not shown), all of which resulted in minimal fluorescence detection. Both CD4^{hi} and CD4^{neg} internalized the transferrin-Texas Red positive control, suggesting that the cells were competent for the endocytosis of membrane-bound molecules (Figure 2N).

Then, the engineering of chimeric pRNA building blocks harboring receptor-binding folate was also tested. Folate receptors are overexpressed in various types of tumors and have been used to deliver therapeutic reagents specifically into cancer cells.^{35,36} Therefore, selective targeting of folate-conjugated pRNA nanoparticles to the folate receptor is a useful method for targeting cancer cells. A chimeric RNA harboring pRNA and folate (folic-pRNA) was engineered by covalently linking a folate to the 5'-end of the pRNA. Specific cell binding of folic-pRNA was demonstrated by flow cytometry using a folic-pRNA labeled with FITC.^{28b} When folate receptor-positive human nasopharyngeal epidermal carcinoma cells were incubated with this dually labeled pRNA, nearly all of the cells were FITC-positive. Adding free folate to the incubation buffer decreased the FITC positive cells to less than 1%, suggesting that the binding of pRNA to cells is specific and folate-dependent. As a control, FITC-labeled pRNA without folate conjugation did not exhibit binding.

2. Determination of the Sequence Requirement of the Hand-In-Hand Loops in Chimeric Trimer Formation

Bases 45–48 and 82–85 of wild-type pRNA in the left- and right-hand loops, respectively, were found to engage in pRNA/pRNA interactions.^{12,14,18} When all four nucleotides were paired, at least one G/C pair was required. The maximum number of base pairings between the two loops to allow optimal multimer formation was five. The minimum number of nucleotides needed for pRNA/pRNA interaction in the right and left loop was five and three, respectively. Our results suggest that a 75-nucleotide RNA segment, nucleotides 23–97, is a self-folded independent domain involved in RNA/RNA interaction in pRNA trimer formation, whereas nucleotides 1–22 and 98–120 were dispensable for trimer formation.

The mechanism of pRNA trimer formation by interlocking loop/loop interaction was utilized for the fabrication of the trimer of chimeric pRNA harboring receptor-binding RNA aptamer and/or therapeutic siRNA (Figure 1). An individual chimeric pRNA building block was engineered to carry one daughter RNA molecule such as siRNA or receptor-binding aptamer. Each building block was designed intentionally to have specific right or left loops, such as A-b'(right-left), to interact with other building blocks. The possibility of appropriate folding of pRNA and their competency in forming trimers were tested. Mixing of individual chimeric pRNAs with counterpart partners with appropriate interlocking loops resulted in the efficient formation of the desired trimer, as documented by gel electrophoresis, AFM imaging, and sucrose gradient sedimentation (Figure 3). The monomer building block migrated more rapidly in native gels, whereas the trimer complex composed of three chimeric pRNA (A-b'), (B-e'), and (E-a') (Figure 1-III) migrated more slowly in native gels (Figure 3A). This suggests that RNA trimers were generated from the monomeric building block despite the replacement of the 5'/3' helix with ds-siRNA or the connection of the 5'/3' end to a CD4-binding aptamer. The correct folding of pRNA chimera was also confirmed by phi29 in vitro assembly inhibition assay,^{11,14} based on the finding that correct folding of pRNA chimeras will ensure competitive binding to phi29 procapsid for the pRNA binding site and will block phi29 DNA packaging during phi29 replication.

3. Determination of the Length Requirement of the pRNA Vector for the Construction of pRNA Chimera Harboring siRNA

As noted earlier, the intermolecular interacting domain and the double-stranded helical domain of pRNA fold independently (Figure 1). Altering the primary sequence of any nucleotide of the helical domain does not impact pRNA structure and folding as long as the complementarity

is preserved. Additional functional RNA, such as a hammerhead ribozyme, have been conjugated to the double-stranded domain, resulting in enhanced cleavage efficiency of ribozyme.⁷

Extensive studies reveal that siRNA is a double-stranded RNA helix.^{2–5,28} To test whether it is possible to replace the pRNA helical region with double-stranded siRNA and to determine which construct has the optimal function in gene silencing and trimer formation, we constructed several chimeric pRNA/siRNA and their ability in gene silencing was tested. A 29-bp siRNA was connected to nucleotides 29/91 or 18/99 of pRNA, resulting in pRNA/siRNA(GFP)-29/91 and pRNA/siRNA(GFP)21/99, respectively. Two additional uridines were inserted into the three-way junction to increase the flexibility at this region for RNase processing. As revealed by fluorescent microscopy, both chimeric siRNAs showed significant inhibition against GFP expression after introduction into cells by transient transfection (Figure 4). The inhibition was highly specific because a mutant chimeric pRNA/siRNA with mutations at the siRNA sequence did not exhibit any inhibitory effects.

Circularly permuted pRNA chimeras were also constructed to carry siRNA. For these constructs, their 5'/3' ends were relocated within a tightly folded region and therefore not accessed easily by exonuclease, thus increasing the stability of the entire RNA.³⁷ Two circularly permuted pRNA/siRNA chimera, with new 5'/3' termini located at 71/75 or 29/30 positions of original pRNA, were constructed and referred to as pRNA/siRNA(GFP)71/75 and pRNA/siRNA(GFP)29/30, respectively. pRNA/siRNA(GFP)29/30 silenced GFP expression with lower efficiency than pRNA/siRNA(GFP)-21/99, whereas pRNA/siRNA(GFP)71/75 had virtually no effect on silencing GFP expression (Figure 4).

In addition, chimeric pRNA harboring siRNA for luciferase were also constructed (Figure 1-III). Dual reporter assay demonstrated that pRNA/siRNA(Firefly) strongly and specifically inhibited the expression of firefly luciferase proteins without affecting renilla luciferase expression. Specific knockdown was also observed for pRNA/siRNA-(Renilla) in other trials.

4. Demonstrating the Co-Delivery of Three pRNA Chimeric Building Blocks into One Cell

a. Demonstrating Co-Delivery by Confocal Microscopy and Flow Cytometry—

CD4 is a receptor displayed on the surface of certain subsets of T lymphocytes. In T helper cells, CD4 is normally not involved in endocytosis except when overexpressed.³⁸ A murine thymic T lymphocyte cell line D1,³⁴ which depends on IL-7 for growth, was used as a model system for testing the effects of specific gene delivery via CD4. Because D1 cells are immature thymic cells that minimally express CD4, we overexpressed murine CD4 in D1 cells by electroporating them in hypotonic buffer in the presence of a mammalian expression vector containing L3T4 (mouse CD4). Antibiotic selection was used to screen out integrations, and the cells that expressed high levels of CD4 (CD4^{hi}) were further selected by fluorescence-activated cell sorting (FACS). As a result, more than 99% of the CD4^{hi} cells expressed the CD4 receptor.

A trimeric complex composed of pRNA(A-b')/aptamer-(CD4), pRNA(B-e')/FITC, and pRNA(E-a')/Rhodamine (Figure 1-III-C) was assembled and assayed by the section technique using confocal microscopy (Figure 2) to test whether this RNA nanoparticle could serve as a vehicle to concurrently and specifically deliver multiple therapeutic molecules. Binding of the trimer and the co-entry of three chimeric building blocks into the cell via CD4 binding was demonstrated by detection of fluorescence within CD4^{hi} cells (Figure 2A–D and I–L)). Such binding and entry was specific because no fluorescence was observed on CD4^{neg} cells (Figure 2E–H). When an FITC filter was used, the green fluorescent label was visible in the CD4^{hi} T lymphocytes (Figure 2A and I). When a rhodamine filter was used, the red label was visible on the image that overlaps with the FITC label in the CD4^{hi} T lymphocytes (Figure 2B and J).

The similarity and overlap of the FITC image with the rhodamine image, (Figure 2A,B and I,J) and the coappearance of both green and red, representing FITC and rhodamine, respectively, on the same location of the cell confirmed the co-delivery of three chimeric pRNA building blocks into specific cells with the aid of CD4-binding RNA aptamer. These results indicate that all of the pRNA building blocks were co-delivered as a trimer, and that the phi29 pRNA trimer can serve as an effective vehicle for the delivery of multiple therapeutic components.

The co-delivery of three components in the fabricated trimer was further confirmed by analyzing a large population of cells (30 000 cells) using flow cytometry (Figure 5-I). Dual incorporation of both FITC and Rhodamine in 90–95% of the CD4^{hi} T cells indicates successful uptake of the pRNA trimer by almost all of the CD4-expressing cells in the population.

b. Functional Assay for the Co-Delivered Trimer Harboring pRNA/siRNA(CD4), pRNA/aptamer(CD4) and pRNA/FITC—Having demonstrated co-delivery of three components of the pRNA trimer (Figure 2), it is necessary to test if uptake of the pRNA trimer resulted in a biological effect mediated by the delivered components.

CD4^{hi} and CD4^{neg} D1 T cells were incubated, but not transfected, with pRNA trimer containing the building blocks of pRNA/siRNA(CD4), pRNA/aptamer(CD4), and pRNA/FITC (Figure 1-III). Inhibition of CD4 expression by siRNA-(CD4) incorporated in the trimer was demonstrated by measuring surface expression of CD4 with a PE-labeled CD4 antibody. CD4^{neg} D1 cells did not take up detectable amounts of the pRNA trimer when assessed for FITC uptake. This was confirmed by flow cytometry (data not shown). In contrast, 85% of CD4^{hi} D1 cells treated with the pRNA trimer were FITC-positive (Figure 5-II), demonstrating that the CD4 aptamer was selectively targeting CD4-overexpressing cells and delivering the pRNA trimer complex.

Next, to determine whether the siRNA(CD4) delivered by the pRNA trimer complex resulted in the down-regulation of CD4 surface expression, CD4^{hi} D1 cells were incubated with the trimer for 24 h, and then stained with anti-CD4 antibody conjugated with PE and subjected to flow cytometry analysis. FITC-positive and FITC-negative cells were gated from the total cell population (Figure 5-II). The CD4 level in FITC-negative cells was determined to be 42.56%, but the level was reduced to 17.8% in FITC-positive CD4^{hi} D1 cells. Therefore, only in FITC-positive cells (85% of the CD4^{hi} D1 cells) but not FITC-negative cells (15% of the CD4^{hi} D1 cells) was the level of CD4 expression decreased to such an extent. These findings suggest that in CD4-expressing cells the pRNA trimer is internalized, as indicated by the uptake of pRNA/FITC, and is functional in silencing the CD4 gene, as indicated by the co-delivery of pRNA/siRNA(CD4) complex resulting in decreased expression of the CD4 protein in FITC-positive cells.

Extensive study (Figure 5-III) with controls indicated that the reduction of CD4 level in PE staining seen in Figure 5-II is specifically due to gene silencing by pRNA/siRNA-(CD4) in the pRNA trimer and is not caused by CD4-binding-triggered internalization because the CD4 aptamer containing pRNA complexes did not cause CD4 reduction unless the CD4 siRNA was incorporated. The sequence-dependent toxicity or some of the other adverse effects of pRNA chimeras were dramatically mitigated by the conjugation of a small DNA oligo into the complex (manuscript in preparation).

c. Functional Assay for Co-Delivery of siRNAs Targeting Anti- or Proapoptosis Factors Guided by CD4-Binding RNA Aptamer—To evaluate the effectiveness of the preferential delivery system, we engineered RNA nanoparticles harboring siRNA targeting pro- or antiapoptotic factors. Survivin is an antiapoptotic factor that is not expressed in most

normal adult human tissues but is expressed in most human cancers.^{39–41} pRNA/siRNA (survivin) was tested to evaluate the usefulness of inducing apoptosis as a therapeutic means for killing cancer cells. For comparison, chimeric pRNA/siRNAs targeting proapoptosis factors, with BIM as examples, were designed and tested. By preventing apoptosis instead of inducing it, the proapoptosis assay could more clearly determine if the delivery of the chimeric pRNA complex produced a nonspecific toxic response. To this end, two cytokine-dependent cell lines were employed: an IL-3 dependent pro-B cell line (FL5.12A) and the CD4^{hi} or CD4^{neg} IL-7-dependent D1 cell line. Withdrawal of IL-3 or IL-7 could promote the apoptosis of D1 or the FL5.12A cell line, respectively, and induce the expression of BIM in FL5.12A cells.^{42–49}

Extensive testing of the RNA nanoparticles, including monomer (by transfection), dimer (by transfection or incubation), and trimer (by incubation), confirmed the specific binding and entry of pRNA complexes guided by the CD4-binding RNA aptamer. An annexin V-propidium iodide (PI) double-staining method followed by flow cytometry was carried out 12, 24, 48, and 120 h after transfection confirmed the induction of apoptosis by pRNA/siRNA(survivin) in MCF-7 cells (data not shown). It was found that pRNA/siRNA(survivin) and pRNA/siRNA (BIM) silenced their target genes specifically and efficiently. In addition, the introduction of pRNA/siRNA(survivin) caused cancer cells and cytokine-dependent cells to die, whereas the introduction of pRNA/siRNA(BIM) protected IL-3-dependent cells from death in the absence of IL-3 (Figure 6).

d. Co-Delivery of pRNA/siRNA(Renilla) or pRNA/siRNA-(Firefly) in Trimers

Guided by Folate-pRNA—The co-delivery of three components to specific cells conveyed by the RNA nanoparticle was further demonstrated using trimers harboring pRNA/siRNA (Firefly or Renilla). One of the luciferases, for example, renilla luciferase, will actively serve as an internal control for the other luciferase, for example, firefly luciferase, using a dual report assay. The mutual internal control could prove the specific activity of siRNA by eliminating the possible nonspecific effect. Reduction of the specific luciferase activity was demonstrated by the dual assay system by incubation of the human nasopharyngeal carcinoma cells expressing folate receptors on their surface with trimer, confirming the specific delivery of the trimer into the cells guided by folate-pRNA.^{28b}

5. Mechanism of Action, Processing of Chimeric pRNA/siRNA Complex into Individual Double-Stranded siRNA by Cellular Components or Dicer

The chimeric pRNA/siRNA complex functions within the cell in a rolesimilar to specific siRNA in gene silencing. This raises an important question: are the chimeric complexes processed into individual siRNA? To address this question, we incubated the chimeric pRNA/siRNA monomer or the trimeric chimera with cell lysates (Figure 6A–D) and analyzed by denatured gel. The monomer in this study harbored a 29-nucleotide double-stranded siRNA connected to the three-way junction from nucleotides 29–91 (Figure 6A). Two additional uridines were added to the UUU bifurcation bulge to help enhance processing efficiency by increasing the ΔG for the folding of the loop. Incubation with cell lysates resulted in a band with a size equal to 29-nucleotide double-stranded RNA (Figure 6A and C lane c–e), suggesting that the siRNA was released after cleavage in the cell lysates by RNase as expected because the single-stranded bifurcation bulge is much more susceptible than the double-stranded RNA to RNase digestion.

Incubation of the purified trimeric chimera with purified Dicer, a cell component responsible for the processing of siRNA, resulted in a band with a size equal to 21-nucleotide double-stranded siRNA, suggesting that the gene silencing effect induced by the trimer is indeed caused by siRNAs after the processing of the trimer by the Dicer in the cell.

6. Animal Trials to Demonstrate Specific Delivery of the Therapeutic pRNA/siRNA Complex to Cancer Cells Conveyed by the RNA Nanoparticle

The potential of this RNA complex to suppress tumor formation was tested in athymic nude mice. The mice receiving only cancer cells developed tumors, whereas the group of mice that received cancer cells pretreated with the pRNA complex containing both receptor-binding ligand and pRNA/siRNA(survivin) did not develop tumors (Figure 7).

Discussion

The potential utilization of self-assembling bionanomaterials is being investigated by an increasing number of researchers.^{50–53} We have reported that phi29 pRNA, via the interaction of programmed helical regions and loops, can be engineered and fabricated at will to form a variety of structures and shapes including twins, tetramers, rods, triangles, and arrays with sizes ranging from nanometers to micrometers.^{13,27} Such fabricated RNA nanoparticles could hold diverse RNA building blocks with controllable stoichiometries ranging from one, two, three, or six copies up to thousands of copies. The beauty of this system is that the size, shape, and stoichiometry of the building blocks and the final product are capable of being manipulated and controlled. The features of multiplicity and assortment make such RNA nanoparticles capable of carrying polyvalent therapeutic molecules to enhance therapeutic efficacy. Using one complex to carry out the actions of several molecules will help one solve the problem of developing multiple factors for a specific therapeutic strategy. We have reported here the co-delivery of three components using the mechanism of pRNA trimer formation. Co-delivery of six components in a hexameric complex is also possible because pRNA forms hexamers as well.^{14–16} One building block of the deliverable RNA complex can be modified to carry an RNA aptamer that binds a specific cell-surface receptor, thereby inducing receptor-mediated endocytosis. The second building block of the hexamer will carry heavy metal, quantum dots, fluorescent beads, or radioisotopes for cancer detection. The third building block of the hexamer will be altered to carry components that will be used to enhance endosome disruption so that the therapeutic molecules are released. The fourth and fifth building blocks of the RNA complex will carry therapeutic siRNA, ribozyme RNA, antisense RNA, or other drugs to be delivered. A sixth building block of the hexamer will be designed to allow for the detection of apoptosis.

Methods

Engineering of RNA Building Blocks and Bottom-Up Assembly of pRNA Trimer

RNAs were prepared as described previously.¹³ DNA oligos were synthesized with the desired sequences and used to produce double-stranded DNA by PCR. The DNA products containing the T7 promoter were cloned into plasmids or used as a substrate for direct in vitro transcription. All pRNA chimera produced by T7 RNA polymerase were treated by calf intestinal alkaline phosphatase to remove the 5'-phosphate.⁵⁴

To engineer pRNA/siRNA(GFP), pRNA/siRNA(luciferase), and pRNA/siRNA(survivin), we replaced the helical region at the 5'/3' paired ends of pRNA with double-stranded siRNA that connects to nucleotides #29 and #91.

To engineer chimeric pRNA harboring CD4-binding aptamer, we connected the sequence of the RNA aptamer for CD4 binding³³ to the 5' and 3' ends of pRNA. The pRNA was reorganized into a circularly permuted form, with the nascent 5'/3'-end relocated at pRNA nucleotides 71/75 located in a tightly folded area.¹⁶ The chimeric pRNA with this CD4-binding aptamer retained the appropriate right and left loops, for example, loop A and b' for pRNA A-b', for the fabrication of trimer.

Physical Characterization of the Fabricated RNA Nanoparticles

The structure and purity of the fabricated RNA nanoparticles were verified by (1) 8% native polyacrylamide gel with 10 mM magnesium but without urea; (2) sedimentation by 5–20% sucrose gradient with 10 mM magnesium; and (3) AFM imaging.^{18,19,26}

Functional Assay for Engineered Chimeric pRNA Building Blocks to Cells

For proB FL5.12A cells, 10^7 cells were resuspended in 500 μ L RPMI with 10% FBS. For D1 cells, the cells were resuspended in the hypo-osmolar buffer. Electroporation was performed with 4-mm cuvettes using an Electro Square Porator ECM 830. FL5.12A cells were electroporated at 200 V, 3 pulses, and D1 cells at 180 V, 1 pulse. After a short incubation on ice, cells were resuspended in 10 mL complete media with cytokine and incubated for 2 h at 37 °C and 5% CO₂. Cell viability was measured microscopically by trypan blue exclusion assay before transfection with Mirus reagent. Cells (10^6) were incubated with the RNA constructs (100 nM) overnight, washed twice with Hanks' balanced salt solution, resuspended in the media with or without cytokine, and incubated at 37 °C + 5% CO₂ for an additional 24–48 h.

For drosophila S2 cells, GFP-expressing plasmid pMT-GFP and various siRNAs were cotransfected into cells in a 24-well plate using Cellfectin (Invitrogen) 24 h after seeding. The expression of GFP was induced by overnight incubation with CuSO₄ at 0.5 mM 24 h after transfection.² Inhibition of GFP expression was observed by fluorescence microscopy.

Functional Assays for Delivery of the Fabricated RNA Nanoparticle to Cells

For the IL-7-dependent D1 cell line, which was established from CD4-CD8- mouse thymocytes isolated from a p53^{-/-} mouse,³⁴ the cells were grown in complete medium containing RPMI1640 with 10% FBS (fetal bovine serum) and with penicillin and streptomycin at 50 U.I per ml, 0.1% beta-mercaptoethanol, and 50 ng/mL IL-7. FL5.12A cells were grown in complete medium supplemented with 2 ng/mL IL-3. For overexpression of CD4 in the D1 cells, the L3T4 (mouse CD4) insert was subcloned into pcDNA 6/V5-HisB (Invitrogen). D1 cells were transfected with DNA by electroporation. Stable cell lines were selected by antibiotic resistance. D1-CD4^{hi} cells, expressing high levels of CD4, were further isolated by fluorescence-activated cell sorting. The D1-CD4^{hi} cell line was maintained in complete medium supplemented with 50 ng/mL IL-7 and Blasticidin HCl (2.5 mg/mL).

For assaying the delivery of folate-containing trimers, a human nasopharyngeal epidermal carcinoma KB cell line was maintained in folate-free RPMI1640 medium (Gibco BRL) supplemented with 10% FBS and penicillin and streptomycin in a 5% CO₂ incubator. The cells were grown into monolayers, and the serum provided the normal complement of endogenous folate for cell growth. Cell titer was determined by a hemocytometer after trypan blue straining.

The trimeric RNA complex was prepared by mixing pRNA(A-b')/folate, pRNA(B-e')/siRNA (GFP), and pRNA-(E-a')/siRNA(Firefly) in the same molar concentration. The trimer complex was then purified from gel and added into KB cells to allow the binding and entry of RNA. After washing with RPMI medium, the cells were collected and subjected to dual reporter assay (Promega).

To assay for the delivery of chimeric pRNA complex to D1 cells, we seeded CD4^{hi}, CD4^{lo}, and CD4^{neg} cells in a 96-well flat-bottom plate. Then, we washed 5×10^4 cells per sample once in PBS with 10 mM Mg²⁺. Cells were incubated for 30 min at 37 °C in 20 μ L of PBS with 10 mM Mg²⁺ and 400 ng (100 nM) of RNA complex. After incubation, complete medium, with or without cytokines, was added to a final volume of 100 μ L and cells were incubated at

37 °C + 5% CO₂ for 24 or 48 h. Cell viability was measured microscopically by trypan blue exclusion assay.

Chimeric pRNA/siRNA Processing

The processing of RNA by cell lysate was carried out following the procedure used by Bernstein et al.⁵⁵ For the processing of RNA by Dicer, the purified recombinant enzyme was purchased from Gene Therapy Systems.

Confocal Microscopy

Coverslips coated with poly-L-lysine (200 µg/mL) were incubated overnight with cells grown in complete media supplemented with cytokines. CD4-negative pro-B cell line F15.12A (CD4^{neg}), pro-T cell line D1 (CD4^{neg}), and a D1-CD4 overexpressing cell line (CD4^{hi}) were incubated with the chimeric pRNA trimer. The trimer complex was purified from gel using the mixture containing the same molar RNA of pRNA(A-b')/aptamer-(CD4), pRNA(B-e')/FITC, and pRNA(E-a')/rhodamine in the presence of 10 mM Mg²⁺. Cover slips with cells were fixed with 4% paraformaldehyde, washed in PBS with 10 mM Mg²⁺, and mounted in Gel/MountT (Biomed, CA). The images were captured by Zeiss confocal microscope LSM 510 NLO.

In Vivo Animal Trials

Five-week-old male athymic nude mice (Harlan Sprague Dawley) were housed in a pathogen-free environment. Animals were randomly assigned to experimental groups ($n = 8$). Cells were maintained in antibiotic-free medium for one week before injection. On the day of injection, cells were rinsed with PBS twice and incubated with the dimeric pRNA complex at 37 °C for 3 h before being collected by trypsin digestion. For the injection of each mouse, we used 2.5×10^5 cells in 0.1 mL of media. Cells were inoculated subcutaneously at the right axilla of the forelimb. Once xenografts were visible, their size was determined two times per week by externally measuring tumors in two dimensions. Volume was calculated by the following equation: $V = (LW^2) \times 0.5$, where L is the length and W is the width of the xenograft.

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I. 3-D structure of Phi29 pRNA monomer II. pRNA trimer

III. Trimers

Figure 1.

Schematic diagram of the engineering and fabrication of RNA nanoparticles. (I) Size and 3D computer model¹⁶ of phi29 pRNA vector. (II) The fabricated trimer. (III) Six different configurations of trimer designs containing aptamer, siRNA, ligand, and fluorescent labels.

I. Assay for co-entry of 3 RNAs in trimer

II. Control assay to differentiate entry from binding

Figure 2.

Confocal microscopy showing the specific and simultaneous delivery of three components to CD4-overexpressing cells. (I) Assay for the binding of pRNA trimer containing pRNA(A-b')/aptamer(CD4), pRNA(B-e')-FITC, and pRNA(E-a')-Rhodamine to CD4^{hi} T cells (A–D of left column, and I–L of right column) and CD4^{neg} T cells (E–H of middle column). A, E, and I were imaged with an FITC filter; whereas B, F, and J were viewed with a Rhodamine filter; C, G, and K are overlays; and D, H, and L are DIC images. The right column represents a close-up view of CD4^{hi} cells. Arrows point to the complexes that had entered the cell. (II) Section of confocal microscopy images to differentiate between binding (M) and cell entry (arrows in N and O) as well as negative control (P). Binding of FITC-labeled pRNA trimer containing CD4-binding aptamer to lymphocytes was shown as a circle, and entry was shown as a green spot inside cell (arrow in O). The red color in N is a positive entry control of transferrin labeled with Texas red.

Figure 3.

Native polyacrylamide gel (A), AFM imaging (B), and sucrose gradient sedimentation (C) to detect the formation of pRNA trimers. (A) Native PAGE gel showing pRNA monomer and trimer of pRNA chimeras exhibiting different migration rates. (B) AFM images of pRNA monomer and trimer with low and high magnification. The pRNA monomers folded into a checkmark shape, and the trimer exhibited a triangular shape. The color within each image reflects the thickness and height of the molecule. Brighter (or whiter) color indicates a thicker or taller molecule; darker color indicates a thinner molecule. (C) Separation of pRNA monomers and trimers by 5–20% sucrose gradient sedimentation. All particles were loaded onto the top of the gradient and separated by ultracentrifugation. Sedimentation is from right to left.

Figure 4. GFP knockdown assay of different chimeric siRNA constructs. Two days after cells being transfected with various chimeric pRNA/siRNA constructs, GFP expression was observed by fluorescence microscope.

I. Co-delivery of trimer via CD4 binding aptamer

II. Functional assay of siRNA(CD4) in trimer after delivery

III. Assay for negative controls

Figure 5.

Functional assay of siRNA(CD4) co-delivered via the fabricated pRNA trimer harboring aptamer(CD4), siRNA(CD4), and pRNA/FITC. (I) Positive control showing the co-delivery of pRNA(A-b')/aptamer(CD4), pRNA(B-e')-FITC, and pRNA(E-a')-Rhodamine in trimers (Figure 1-III-A). Incubation, not transfection, of CD4^{hi} T cells with the trimer resulted in 95% of the cells taking up both FITC and Rhodamine fluorescent RNAs mediated by aptamer (CD4), as assessed by flow cytometry. (II) Incubation, not transfection, of CD4^{hi} T cells with pRNA trimer containing pRNA(A-b')/aptamer(CD4), pRNA(B-e')-FITC, and pRNA(E-a')-siRNA(CD4) (Figure 1-III-C) resulted in 85% of the cells taking up FITC. The CD4^{hi} T cells taking up the pRNA trimer were divided into FITC-positive and FITC-negative cells, and CD4 levels were determined by surface staining with a PE-labeled antibody. In the FITC-positive cells, the CD4 level was reduced to 17.8%, in comparison with 42.56% for the FITC-negative cells, demonstrating the delivery and function of the siRNA(CD4) in the trimers. (III) Negative controls showing that incubation of CD4^{hi} T cells with pRNA dimers or trimers harboring aptamer(CD4) but not siRNA-(CD4) did not make difference in CD4 level (all around 90%) and cell viability (all around 70%). The control dimers or trimers contained pRNA/siRNA (BIM) instead of pRNA/siRNA(CD4).

Figure 6.

Processing of chimeric pRNA/siRNA complex into siRNA by cell lysates (C) or purified Dicer (D). The pRNA/siRNA monomer (lane b–e), phi29 pRNA vector (lane f–I, and m–o), or the trimeric chimera (j–l) were labeled with ^{32}P at the 5' end and incubated with cell lysates (C) or Dicer (D) and analyzed by denaturing gel. (E) and (F) show the inhibition of proapoptosis factor BIM with specific pRNA/siRNAs protected lymphocytes from cytokine withdrawal and induced apoptosis. FL5.12A cells are cytokine-dependent lymphocyte cell lines that express BIM, and exhibit apoptosis in the absence of cytokines. Introduction of pRNA/siRNA(BIM) into FL5.12A cells (E) resulted in protection from IL-3 withdrawal-induced cell death. Protein levels of BIM (F) were assayed by Western blot.

Figure 7.

Animal trials for cancer therapy using the fabricated RNA nanoparticles. (A) Injection without the pRNA/siRNA chimera (No RNA); (B) Treatment with RNA chimera containing folate-pRNA and siRNA(survivin); (C) Treatment with RNA chimera containing folate-pRNA and siRNA(survivin) with mutations in the siRNA sequence; (D) Treatment with pRNA-siRNA chimera that does not contain a folate at its 5' end.