

Fabrication of Stable and RNase-Resistant RNA Nanoparticles Active in Gearing the Nanomotors for Viral DNA Packaging

Jing Liu,^{†,§,||} Songchuan Guo,^{‡,||} Mathieu Cinier,^{†,||} Luda S. Shlyakhtenko,[¶] Yi Shu,[†] Chaoping Chen,[⊥] Guanxin Shen,[§] and Peixuan Guo^{*,†}

[†]Nanobiomedical Center, SEEBME, College of Engineering & College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267, United States, [‡]Kylin Therapeutics, Inc., West Lafayette, Indiana 47906, United States, [§]Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, People's Republic of China, [⊥]Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523, United States, and [¶]Department of Pharmaceutical Sciences College of Pharmacy, University of Nebraska Medical Center, Nebraska Medical Center, Omaha, Nebraska 68198, United States. ^{||}These authors contributed equally.

Living organisms produce a wide variety of highly ordered or patterned structures such as smart nanomachines and elegant arrays that are made up of macromolecules to perform diverse biological functions. RNA and DNA share certain common features via their unique properties of strand complementarities and self-assembly, which can serve as powerful building blocks for bottom-up fabrication of nanostructures and nanodevices. A pioneering concept introduced by Ned Seeman 30 years ago has led to an explosion of knowledge in DNA nanotechnology.^{1–3} RNA can be manipulated with simplicity characteristic of DNA, while possessing non-canonical base-pairing, versatile function, and catalytic activity similar to proteins. Typically, RNA molecules contain a large variety of single-stranded stem-loops for inter- and/or intramolecular interactions. These loops can serve as mounting dovetails, and thus, external linking dowels might not be needed in fabrication and assembly.^{4–8}

Although the concept of RNA nanotechnology has been developed for more than 10 years^{6,9–13} (for a review, see ref 14), standing in awe of RNA to RNase degradation has made many scientists hesitant to apply RNA nanotechnology. The popularity in the study of RNA nanostructure has emerged only recently, as reflected in the fact that 90% of publications in RNA nanostructure were published on or after 2006. However, RNA instability and degradation

ABSTRACT Both DNA and RNA can serve as powerful building blocks for bottom-up fabrication of nanostructures. A pioneering concept proposed by Ned Seeman 30 years ago has led to an explosion of knowledge in DNA nanotechnology. RNA can be manipulated with simplicity characteristic of DNA, while possessing noncanonical base-pairing, versatile function, and catalytic activity similar to proteins. However, standing in awe of the sensitivity of RNA to RNase degradation has made many scientists flinch away from RNA nanotechnology. Here we report the construction of stable RNA nanoparticles resistant to RNase digestion. The 2'-F (2'-fluoro) RNA retained its property for correct folding in dimer formation, appropriate structure in procapsid binding, and biological activity in gearing the phi29 nanomotor to package viral DNA and producing infectious viral particles. Our results demonstrate that it is practical to produce RNase-resistant, biologically active, and stable RNA for application in nanotechnology.

KEYWORDS: 2'-F modification · pRNA · RNase resistant · dimer formation · phi29 DNA-packaging nanomotor · viral assembly · viral DNA packaging · nanobiotechnology · bionanotechnology

has continued to significantly hinder the advancement of this field.

Discovery of siRNA has provided another venue for RNA-mediated therapeutics.¹⁵ Several RNA-based therapeutic approaches using small interfering RNA (siRNA),^{16–20} ribozymes,^{21–24} and antisense RNA^{25,26} have been shown to down-regulate specific gene expression in cancerous or viral-infected cells. Recent discoveries of novel functionality of small regulators or noncoding RNA such as ribozyme, tRNA, mRNA, rRNA, snoRNAs, microRNAs, siRNAs, piRNAs, snRNA, SmY RNA, scaRNA, gRNA, Y RNA, telomerase RNA, and riboswitch also appear as a result of the expanding interest in the RNA field.^{27–30} Although gene silencing with high efficacy and specificity

*Address correspondence to guopn@ucmail.uc.edu. Phone: (513) 558-0041. Fax: (513) 558-6079.

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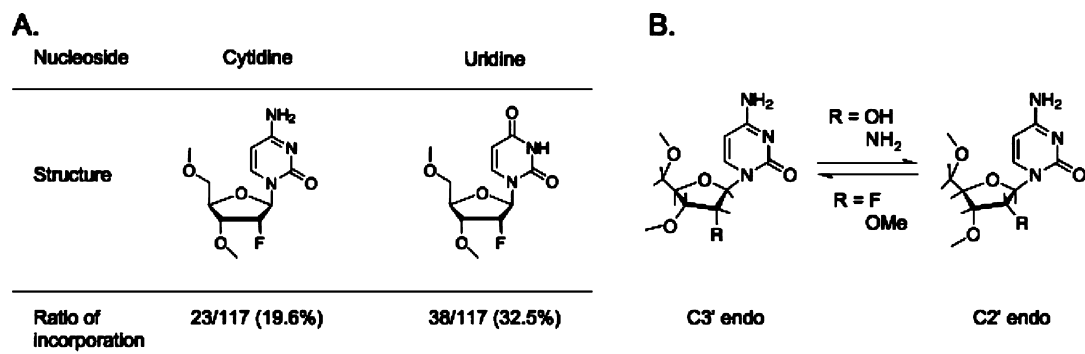


Figure 1. Structure of the 2'-modified ribonucleosides. (A) Location of modification and ratio of incorporation in the pRNA Aa' sequence. (B) Illustration of the equilibrium between the C2' endo and C3' endo conformation of the sugar ring following the 2' substituent.

has been achieved *in vitro*, the effective use of RNA as a therapeutic in clinical application remains challenging due to degradation of RNA in serum after entering the body.

Recently, it was reported that 2'-modifications can enhance the serum stability of siRNA^{31,32} without compromising double-helix formation and the effect of

siRNA in gene silencing.^{33–39} Earlier studies showed that, while the A-form helix is very important, the 2'-OH is not required for siRNA activity.⁴⁰ Most studies on 2'-hydroxyl group modification^{38,39,41–43} (Figure 1) focused on double-stranded siRNA.^{33–36,44} Even though the use of 2'-modifications in siRNA gene silencing studies has been popular and 2'-deoxy-2'-fluoro modification (2'-F)

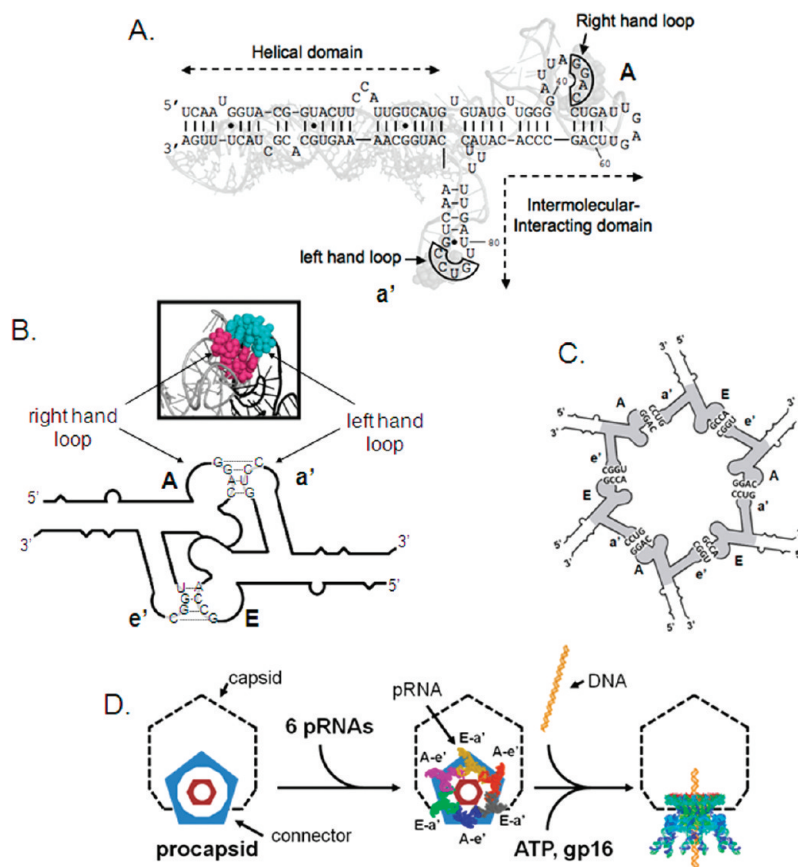


Figure 2. Sequence and secondary structure of phi29 DNA-packaging RNA (pRNA). Upper-case letters represent the upper loop and lower-case letters the lower loop of pRNA. A pair of upper and lower case for the same letters indicates a pair of complementary loops, whereas a pair of upper and lower case from different letters indicates noncomplementary loops. For example, pRNA Aa' refers to a pRNA with complementary right loop A (^{5'}G₄₅G₄₆A₄₇C₄₈) and left loop a' (^{3'}C₈₅C₈₄U₈₃G₈₂), which can form a homodimer by itself. pRNA Ae' containing noncomplementary upper loop A (^{5'}G₄₅G₄₆A₄₇C₄₈) and lower loop e' (^{3'}C₈₅C₈₄U₈₃U₈₂) can form a heterodimer with pRNA Ea' containing left-hand loop a' (^{3'}C₈₅C₈₄U₈₃G₈₂) and right-hand loop E (^{5'}G₄₅C₄₆C₄₇A₄₈). (A) Superposition of the 2D and 3D structure of the pRNA Aa'. The four bases in the right and left loops, which are responsible for inter-RNA interactions, are boxed. (B) Diagram depicting pRNA dimer formation and 3D model of the hand-in-hand interaction. (C) pRNA hexamer. (D) Packaging of phi29 DNA through the motor geared by six pRNA.

has also been applied to SELEX,^{45,46} their effects on the folding and biological function of modified RNA related to the unique properties of RNA, which involve noncanonical base-pairing, have not been demonstrated.

2'-F modification has been demonstrated to support the C3' endo conformation of the sugar ring that plays a role in the formation of the A-form configuration in RNA (Figure 2B).⁴⁷ This explains why 2'-F modification enhances the stability of RNA/RNA duplex as long as the annealing temperature (T_m) is monitored. Besides the alternation of ribose structure, which is involved in ribonuclease action, the indirect resistance against nuclease or base degradation of the phosphodiester bond is also correlated with its potential to increase the duplex stability.⁴⁸

Although the improved nuclease resistance provided by 2'-modification is now well established, there is still a discrepancy about the effect of such modifications on the activity of chemically modified siRNA. Some reports demonstrate that 2'-modification of the sense strand generates a stable complex that retains gene silencing function.^{33–37} Other reports show that a large number of 2'-modifications (in either strand) decreases siRNA activity.³⁹ The mechanism of siRNA action involves the unwinding of the siRNA in the RISC complex and the binding of the antisense strand to the mRNA.⁴⁹ It is expected that due to the requirement for siRNA double-strand formation, 2'-modifications on the sense strand and not the antisense strand did not affect helix formation and protects the siRNA from degradation *in vivo* during the delivery process, thus enhancing the siRNA function. However, the escort of the siRNA from degradation during *in vivo* transportation would not justify a conclusion that 2'-modification does not affect the protein/RNA interaction in the RISC complex, since the sense strand is not present in the protein/RNA complex. Others have shown that 2'-modification of the antisense strand also resulted in a gene silencing effect. However, all of these studies used only 50% (U and C, not G and A) of the 2'-modified nucleotide; the discrepancy might be due to the case-by-case effect, since some locations of modification might affect the protein/RNA interaction, while other locations might not.

All previous studies on 2'-modification of siRNA proved only that 2'-modifications increase the thermal stability of the helical structures of the double-stranded RNA. The questions of whether the 2'-modified RNA folds correctly as wild-type RNA, which is unique in noncanonical nucleotide interactions, and if the 2'-modified RNA retained biological function have not been reported. To address these questions, we synthesized 2'-F-modified RNA to study the effect of such modifications on the RNA structure, folding, and biological function, taking advantage of our highly sensitive *in vitro* bacteriophage phi29 virion assembly assay.

The bacteriophage phi29 DNA-packaging nanomotor⁵⁰ is comprised of (1) a central protein core,^{51,52} (2) six copies of packaging RNA (pRNA),^{9,10,53,54} and (3) an

energy conversion enzyme gp16 (Figure 2). The central core of the motor is called the connector, containing a dodecamer channel that acts as a path for the translocation of dsDNA (Figure 2D). The pRNA forms dimers via the inter-RNA interaction with one pair of the right- and left-hand interlocking loops (Figure 2A, B). The dimer configuration is the building block in hexamer formation^{9,10,55–57} (Figure 2C). The unique features of the pRNA^{9,53} of the bacteriophage phi29 DNA-packaging motor to form dimers, trimers, hexamers, and patterned superstructures via loop–loop interactions^{9,10,55–57} make it a promising vehicle for escorting therapeutics such as siRNA for targeting to specific cancer or viral-infected cells.^{7,8,14,58–60}

Here we report the production of highly stable pRNA molecules, even in the presence of fetal bovin serum (FBS), through the 2'-modification. The 2'-modified pRNA was synthesized by *in vitro* transcription with Y639F mutant T7 RNA polymerase^{61–63} in the presence of 2'-modified pyrimidine. Our data illustrated that incorporation of 2'-F-modified pyrimidine in the pRNA sequence did not affect its folding for dimer formation and motor binding. The resulting modified pRNAs retained biological function close to its nonmodified counterpart in gearing the phi29 DNA-packaging nanomotor and generated the infectious virion even in the presence of high concentrations of RNase.

RESULTS AND DISCUSSION

The effects of pyrimidine modifications with 2'-F derivatives were evaluated using a highly sensitive phi29 DNA-packaging and virion assembly system. The phi29 packaging RNA (pRNA) forms a hexameric ring through inter-RNA interactions to gear the bacterial virus phi29 DNA-packaging motor via defined steps of conformational changes (Figure 2). The six pRNAs form a hexagon via intermolecular base-pairing between the right loop (bases 42–45) and the left loop (bases 82–85) (Figure 2B).^{9,10,57,64–66} pRNA dimers are the building blocks of hexamers^{10,67} (Figure 2C), with the pathway for building a hexamer being dimer → tetramer → hexamer.^{67,68} Phylogenetic analyses of pRNAs⁶⁹ from several phages show few conserved bases but similar secondary structures,⁵⁵ and single base mutation in the helical domain renders the pRNA inactive,⁷⁰ suggesting the high specificity of pRNA structure. The indicators for assessing RNA folding, structure, and function are dimer formation, procapsid binding, and pRNA biological activity in DNA packaging and phi29 virion assembly.

pRNA Dimer Formation Is Not Affected by 2'-F Modification.

Since dimer formation relies on correct folding of individual RNA subunits and the chemical structure of these two loops for interaction, the efficiency in dimer formation could serve as a parameter to evaluate the effect of 2'-modification in RNA folding, structure, and inter-

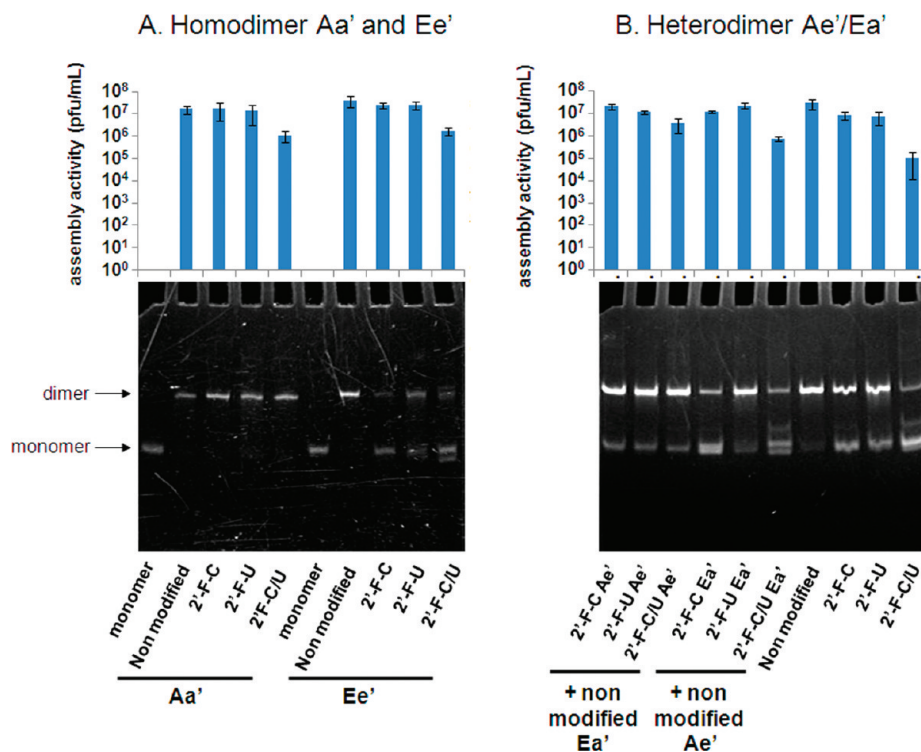


Figure 3. Native PAGE gel showing the formation of dimer nanoparticles of pRNA and a histogram of the virion assembly activities associated with the nanoparticle (A) homodimers Aa' and Ee', or (B) heterodimer Ae'/Ea'.

RNA interactions. In this study, both homodimers and heterodimers were evaluated. Homodimers are composed of two identical pRNA subunits such as pRNA Aa' or Ee'. The heterodimer is composed of two different interlocking pRNA such as pRNA Ae' and Ea' (see Figure 2). Our results indicate that pRNAs with 2'-F pyrimidine modification were competent in pRNA dimer formation, with only C or U modified or both C and U modified (Figure 3). In addition, their migration profile in native PAGE gel was found to be very similar to their unmodified counterpart, which also indicates proper folding (Figure 3).

2'-F Modification of pRNA Has Minimal Impact on phi29 Virion Assembly. *Viral Assembly of 2'-F pRNA Homodimer.* Aa' and Ee' pRNA homodimers that are either 2'-F-C or 2'-F-U modified exhibited 10⁷ pfu/mL activity, which was comparable with nonmodified Aa' and Ee' homodimers. Also, 2'-F-C/U-modified homodimers displayed an assembly activity around 10⁶ pfu/mL (Figure 3A).

Viral Assembly of 2'-F pRNA Heterodimer. To further verify the modification's impact on pRNA function, various combinations of Ae' and Ea' monomers with and without modification were analyzed by the *in vitro* assembly system. Heterodimers formed by a nonmodified monomer and monomer with 2'-F-C or 2'-F-U pyrimidine modified Ae' or Ea' had comparable activity to nonmodified heterodimer at 10⁷ pfu/mL. The same observation has been made when both monomers were 2'-F-C or 2'-F-U modified. Heterodimers with one 2'-F-C/U-modified monomer and one nonmodified monomer also had an activity of 10⁶–10⁷ pfu/mL. A slight de-

crease in virion assembly activity (10⁵ pfu/mL) was observed in the case of heterodimer formed with two 2'-F-C/U monomer Ae' and Ea'.

The number of plaque-forming units per milliliter (pfu/mL) produced in the viral assembly assay^{70–72} was used as a measure of the biological activity of the modified pRNA, which is in direct correlation with the retention of their correct folding. Furthermore, homodimers and heterodimers formed by two 2'-F-C/U-modified monomers displayed only a slight decrease in virion assembly activity despite their high incorporation of modified ribonucleotides. In our model, such virion assembly activities are indicative of a fully functional and correctly folded pRNA molecule.

2'-F-Modified pRNA Aa' Remains Stable in the Presence of RNase A or Fetal Bovin Serum. Phi29 pRNA made of natural nucleotides is sensitive to degradation *in vivo* and *in vitro* by RNases or serum, which contains RNA degradation enzymes. Such instability limits its potential to be used as a building block in bottom-up therapeutic nanoparticle assembly.^{6,56}

We examined the stability of different 2'-F-modified pRNAs Aa' (2'-F-C, 2'-F-U, and 2'-F-C/U) in the presence of RNase A or in the presence of fetal bovine serum, which mimics *in vivo* conditions (Figure 4). Although 2'-F-C and 2'-F-U did not show a significant increase in stability in the presence of either RNase A or FBS (Supporting Information, Figure S1), 2'-F-C/U-modified pRNAs demonstrated resistance to both RNase A and FBS digestion for up to 16 h of incubation (Figure 4).

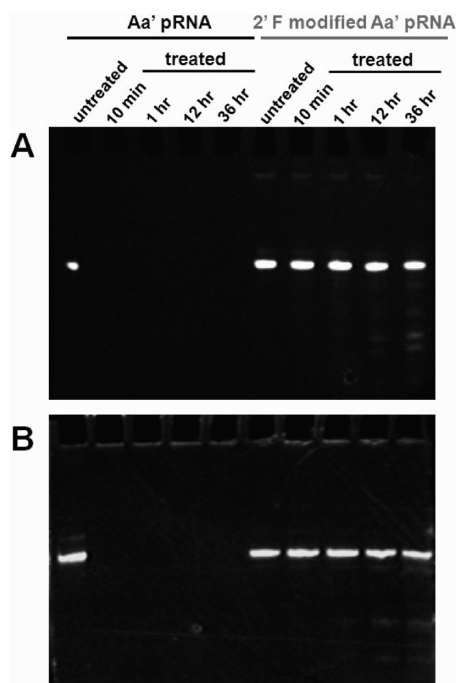


Figure 4. Urea-PAGE denatured gel showing the stability between nonmodified pRNA Aa' and 2'-F-C/U pRNA Aa' after incubation at different time points in the presence of (A) RNase A (1 mg/mL) and (B) fetal bovine serum (10%).

2'-F modifications have been demonstrated to support the C3' endo conformation of the sugar ring, which plays a role in the formation of the A-form configuration in RNA. This explains why 2'-F modification did not interfere or even enhance the stability of the RNA/RNA duplex as far as the T_m is concerned. Besides the alteration of ribose structure, which is involved in ribonuclease action, the indirect resistance against nuclease of the phosphodiester bond is often correlated with the potential of the 2'-modified nucleotide to increase the duplex stability.⁴⁸ Our data suggests that both 2'-F-U and 2'-F-C are required to achieve enhanced improvement in RNase resistance. 2'-F nucleosides were able to be incorporated at high ratios (2'-F-C/U) in the pRNA sequence by the mutant Y639F of the T7 RNA polymerase,^{61–63} leading to highly resistant pRNA even in the presence of FBS. Furthermore, regarding the slight decrease in virion assembly activity discussed above, such a high ratio of 2'-F nucleotide incorporation appears not to be detrimental for the pRNA function or folding. For these reasons, 2'-F-C/U-pRNA was identified as our best option to provide RNase-resistant pRNA that can be used to gear the phi29 DNA-packaging nanomotor or as a building block for nanoparticle assembly. Thus, our study proceeded using only 2'-F-C/U-modified pRNA.

Activity of the 2'-F-C/U-pRNA in the RNase Environment. The stability of 2'-F-modified pRNA was further tested by an *in vitro* viral assembly. In the presence of RNase A, the activity of nonmodified pRNA was completely abolished. However, the 2'-F-C/U-modified pRNA retained

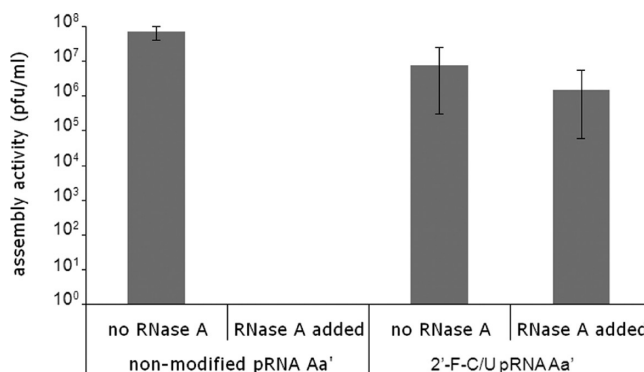


Figure 5. Assembly activities of the 2'-F-C/U pRNA Aa' and its non-modified counterparts in the presence or absence of RNase A.

most of the assembly activity (10⁶ pfu/mL in the presence of RNase A, as compared to 10⁷ pfu/mL in the absence of RNase A) (Figure 5). This result supports the conclusion that 2'-F-C/U modification renders pRNA with RNase A resistance while not interfering with the appropriate folding and biological function of pRNA.

Effect of pRNA 2'-F Modification in DNA Packaging. We also compared DNA-packaging efficiencies between 2'-F-C/U pRNA and nonmodified pRNA with a phi29 DNA-packaging assay. The 1% agarose gel electrophoresis showed that DNA-packaging activity mediated by 2'-F-C/U pRNA was dose-dependent, and the packaging efficiency was similar to or slightly lower than that of nonmodified pRNA (Figure 6). This indicated that 2'-F-C/U pRNA retained correct folding and was competent in DNA packaging.

Characterization of 2'-F-C/U pRNA: pH Stability, Sucrose Gradient Sedimentation, Titration of the Dimer Formation, and Atomic Force Microscopy (AFM). The effect of divalent metal ions and pH of the solution on the dimer formation of 2'-F-C/U pRNA molecules was investigated. Sucrose

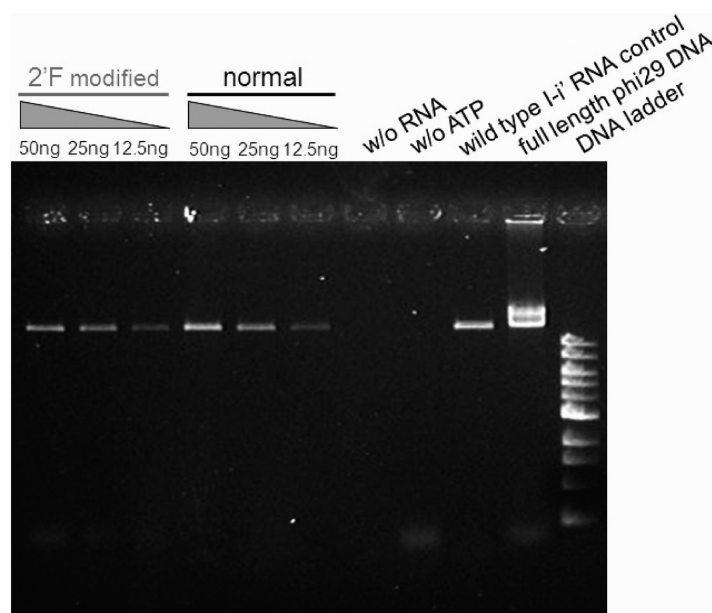


Figure 6. Agarose gel showing the efficiency of the 2'-F-C/U pRNA Aa' in DNA packaging compared to its nonmodified counterparts.

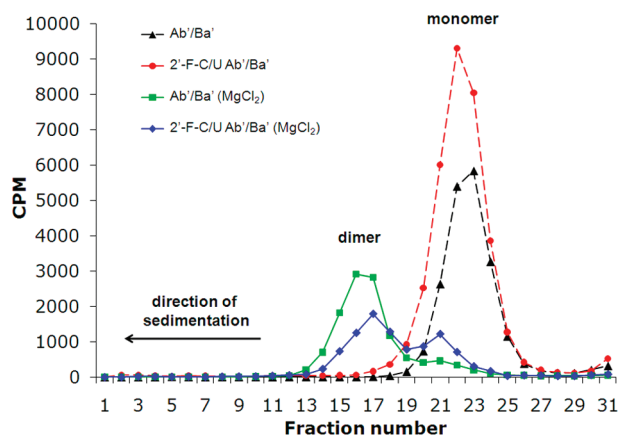


Figure 7. Sucrose gradient sedimentation analysis of the formation of $[^{32}\text{P}]$ pRNA dimer in the presence or absence of MgCl_2 .

gradient sedimentation of nonmodified pRNA revealed a peak at fraction number 22, representing the monomer⁶⁷ in the absence of divalent metal ions (Figure 7). In the presence of MgCl_2 , the peak was shifted to the fraction number 16, corresponding to the dimer⁶⁷ with a higher molecular weight (pRNA dimer, Figure 7). The same profile was obtained with 2'-F-C/U pRNA, except that the dimer formation efficiency was reduced slightly (Figure 7). These data suggest that the incorporation of 2'-F modification slightly decreased the stability of the resulting pRNA dimer complex. Previously, it was demonstrated that pRNA dimer formation was not exclusive to the presence of Mg^{2+} but could also occur in the presence of other divalent metals such as Sr^{2+} and Mn^{2+} .⁶⁷ Interestingly, we also found that Mn^{2+} and Sr^{2+} led to the formation of the dimer of 2'-F-C/U pRNA (Supporting Information, Figure S2), but this was not further investigated.

RNA molecules generally hydrolyze in basic environments due to the 2'-OH, which acts as a nucleophilic moiety to attack the phosphodiester backbone. The alkaline hydrolysis of RNA requires a linear geometry between the 2'-OH and the phosphate group, which is prevented by the 3'-endo sugar pucker (Figure 1B) of the helical region.⁴⁸ It has been previously reported that pRNA molecules present an unusual stability in a broad range of pH from 4 to 10 due to its tight and stable

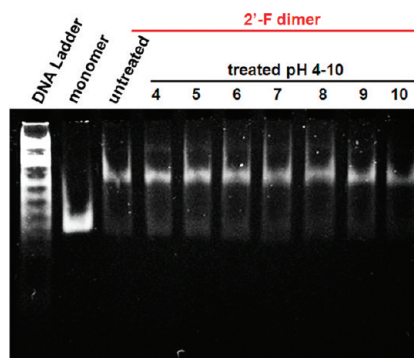


Figure 8. Native PAGE gel showing the stability of the 2'-F-C/U pRNA Aa' homodimer after incubation at different pH ranging from 4 to 10.

folding.^{6,56} The stability of 2'-F-C/U pRNA dimer particles was also explored in a similar range of pH from 4 to 10. In native PAGE, no lower bands, relating to partial hydrolysis of the modified pRNA, were found, indicating that the 2'-F-C/U-pRNA is highly stable, even in a basic environment, up to pH 10 (Figure 8). Moreover, the dimer particle did not dissociate in these conditions, indicating that the 2'-F-C/U pRNA retained its self-assembling property in this broad range of pH (Figure 8).

To continue the characterization of 2'-F-C/U pRNA properties, association constants relative to the dimer formation of $[^{32}\text{P}]$ pRNA Ab' monomer and cold 2'-F-C/U or nonmodified pRNA Ba' were compared (Figure 9). A plot of the dimer formation with the radiolabeled nonmodified pRNA Ab' function of the concentration of 2'-F-modified and nonmodified pRNA Ba' showed exactly the same profile and revealed an association constant between 24 and 8 nM for both. This suggests that 2'-F-C/U pRNA preserved the dimer formation property of pRNA. Consequently, the decrease in stability observed during sucrose gradient sedimentation should not be attributed to a decrease in affinity for hand-in-hand recognition and dimer formation, but rather to a slight increase of the dissociation constant.

Finally, the shape of dimer nanoparticles made of 2'-F-C/U pRNA have been visualized by atomic force microscopy (AFM) (Figure 10). 2'-F-C/U hand-in-hand or 2'-F-C/U foot-to-foot dimer nanoparticles imaged by AFM showed a similar shape compared to the respective nanoparticles prepared with nonmodified pRNA. This showed that 2'-F-C/U pRNA can be used as an efficient building block for nanoparticle assembly, while preserving the original shape of the nonmodified pRNA.

Since the ribose 2'-OH group frequently plays critical roles (hydrogen-bonding and metal ion coordination) in 3D structure formation of functional RNAs, it is surprising that pRNA retains biological activity even after substitution of its C/U with their 2'-F derivatives. Although, there is still a discrepancy considering fluorine can establish hydrogen bonding, it is generally accepted that fluorine has a lower capacity to form a hydrogen bond than oxygen.⁷³ It would be interesting to know the effect of 2'-F substitutions on the 3D structure of the pRNA monomer (Figure 2A), dimer, and hexamer (Figure 2B,C). However, without studies by X-ray diffraction or NMR, the 3D structure of the modified pRNA remains elusive. In this study, we used biological activity assays of the pRNA (dimer formation, virion assembly, and DNA packaging) as an indicator of the effect of 2'-F substitution on the global structure of pRNA. Moreover, 2'-F-C/U-modified pRNA retains its ability to form a dimer in the presence of Mg^{2+} , which demonstrates that 2'-F-modified pRNA retains the capacity of metal ion coordination to a certain extent. Besides the considerations in terms of hydrogen bonding and metal ion coordination, the 2'-OH plays an important role by inducing steric hindrance that gives RNA its specificity

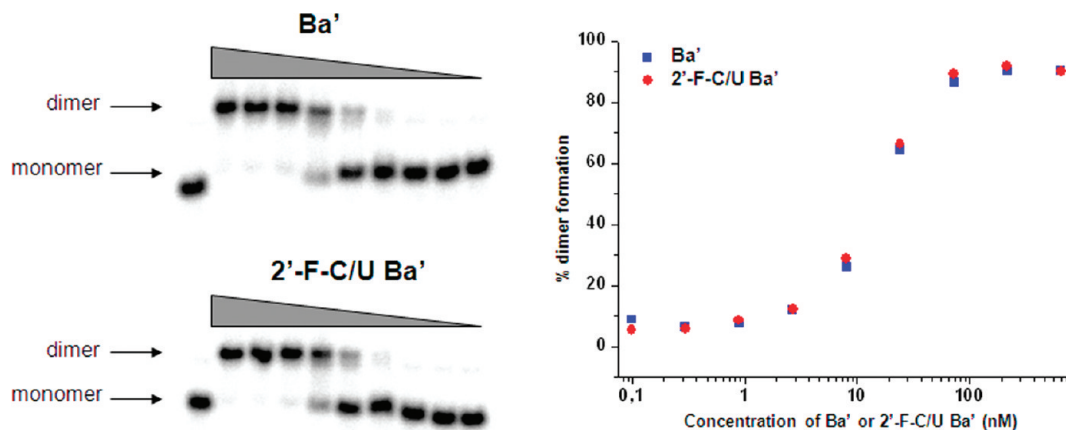


Figure 9. Titration of dimer formation between [^{32}P] pRNA Aa' and cold $2'$ -F-C/U and nonmodified pRNA Ba' (0.1, 0.3, 0.9, 2.6, 8, 24, 72, 216, and 650 nM). (A) Native PAGE gel. (B) Plot showing the percentage of dimer formation function of the concentration of cold $2'$ -F-C/U and nonmodified pRNA Ba' .

in terms of helical conformation (A-form), and the $2'$ -F substitution has already been reported to favor the A-form conformation.^{73,74} Our results suggest that maintaining the A-form helical conformation is more important for pRNA activity than the chemical properties of the $2'$ -group.

CONCLUSION

The presence of $2'$ -F modification did not affect the folding and self-assembling property of pRNA in the

presence of divalent ions. The modified pRNA was stable between pH 4 and 10 and resistant to degradation by RNase A or other degradation enzymes in FBS. The $2'$ -F-C/U-modified pRNA molecule was also demonstrated to efficiently gear the phi29 DNA-packaging nanomotor for DNA packaging and virion assembly, even in the presence of high concentrations of RNase. In conclusion, RNase-resistant and biologically active stable RNA can be constructed by chemical modification for application in nanotechnology.

EXPERIMENTAL PROCEDURE

In Vitro Synthesis of Chemically Modified pRNAs. Chemically modified pRNAs were synthesized following our previously described method for production of the pRNA mutant⁷⁵ with some modifications. Briefly, linear plasmid DNA was used as a template to generate PCR DNA fragments with the appropriate primer pair (TAATACGACTCACTATTAGAATGGTACGGTACTTCC and AGAAAGTAGCGTGTC). The purified PCR DNA fragments were used as templates to synthesize the pRNA Aa' by *in vitro* tran-

scription using Y639F mutant T7 RNA polymerase in a final volume of 20 μ L containing 40 mM Tris-acetate pH 8.0, 5 mM DTT, 1 mM EDTA, 10 mM Mg-acetate, 0.5 mM $MnCl_2$, 8 mM spermidine, 2 μ g of DNA template, $2'$ -F-modified dCTP and/or dUTP (each at 5 mM final, Trilink), ATP, and GTP (each at 5 mM final). After overnight incubation at 37 $^{\circ}C$, the reaction was terminated with the addition of 1 μ L of RNase-free DNase I (1 mg/mL, Sigma). Chemically modified pRNAs were then resolved in 8% polyacrylamide gel with 8 M urea. The corresponding bands

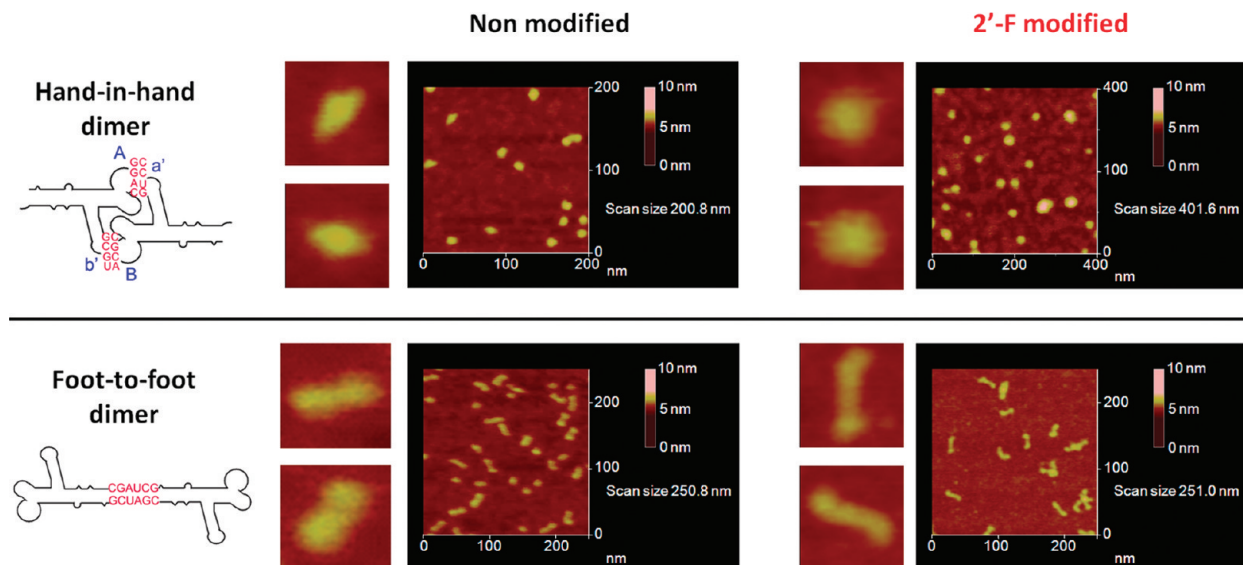


Figure 10. Atomic force microscopy images showing hand-in-hand and foot-to-foot dimer nanoparticles of nonmodified pRNA and $2'$ -F-C/U pRNA, respectively.

were excised and eluted from the gel over 1.5 h at 37 °C in the elution buffer (0.5 M NH₄OAc, 0.1 mM EDTA, 0.1% SDS, and 0.5 mM MgCl₂). pRNAs were then precipitated overnight at -20 °C after addition of a 2.5× volume of 100% ethanol and 1/10 volume of 3 M NaOAc (pH 5.2). The precipitate was pelleted by centrifugation (16500g, 10 min), washed with 70% ethanol, and dried by speed vacuum. Finally, dried RNA was resolved in 0.05% DEPC aqueous solution to constitute our stock solution of pRNA.

Native TBM PAGE for Dimer Detection. Stock solutions of pRNA were diluted in TBM buffer (89 mM Tris, 200 mM boric acid, 5 mM MgCl₂, pH 7.6) to achieve a final concentration of 30 μg/mL. An equal molar ratio of each pRNA was applied to study the formation of dimers, while keeping the total amount of pRNA constant in each lane. Native polyacrylamide gels (12%) were prepared in TBM buffer. Mg²⁺ (5 mM) was included in all buffers to maintain the folding of pRNA and the formation of dimers.^{67,76} After running at 4 °C for 3 h, gels were stained with ethidium bromide and images were captured with an Eagle Eye II system (Stratagene).

RNA Activity Assay with the Highly Sensitive phi29 Assembly System. The activity of pRNAs was assayed with the highly sensitive phi29 *in vitro* assembly system.^{71,72,77} The purification of procapsids,^{78,79} gp16,⁸⁰ and DNA-gp3 and the preparation of neck and tail protein extracts have been described previously.^{71,72,77} *In vitro* phi29 assembly was performed as described previously.⁷¹ Briefly, 10 μL of purified procapsid (0.013 μM) was dialyzed on a 0.025 μm VS filter membrane (Millipore Corp.) against TBE buffer (89 mM Tris, 200 mM boric acid, 2 mM EDTA, pH 7.6) for 15 min at RT. Then 1.5 μL of TMS buffer (50 mM Tris, 100 mM NaCl, 10 mM MgCl₂, pH 7.8) was added to a stock solution of pRNAs (50 ng/μL) to provide Mg²⁺ necessary for the dimer formation. Procapsid solution was added to each pRNA solution, and the mixtures were further dialyzed against TMS for 30 min. The pRNA-enriched procapsid solution was then mixed with gp16^{80,81} and DNA-gp3⁷¹ in the reaction buffer (10 mM ATP, 6 mM 2-mercaptoethanol, 3 mM spermidine in TMS) to initiate DNA packaging. After 30 min of incubation, neck, tail, and morphogenic proteins^{72,77} were added to the DNA-packaging reactions to complete assembly of infectious virions. The activity of each pRNA was measured on the sole basis of the number of plaque formations per unit (pfu/mL) produced in the viral assembly assay.^{70–72}

Stability Assay in the Presence of RNase. In a final volume of 20 μL, 2 μg of nonmodified and 2'-F-C/U pRNA Aa' were incubated at 37 °C in RMP1-1640 medium (Gibco) containing either fetal bovine serum (Gibco) or RNase A (RPA grade, Ambion) at a final concentration of 10% and 1 mg/mL, respectively. Aliquots (4 μL) were taken at multiple time points (0, 10 min, 1 h, 12 h, and 36 h) and ran for 2 h at RT in 8% urea PAGE in TBE buffer. After running, gels were stained by ethidium bromide and images were captured with an Eagle Eye II system (Stratagene).

***In Vitro* phi29 DNA Packaging.** The *in vitro* DNA-packaging efficiency of phi29 virion in the presence of pRNA, ATPase gp16, phi29 DNA-gp3, and procapsid, and with a supply of final 1 mM ATP in TMS buffer was assayed as previously described.^{71,72} Briefly, the 10 μL of purified procapsid (0.13 μM) was mixed with pRNA and dialyzed on a 0.025 μm VS filter membrane (Millipore Corp.) against TBE buffer for 15 min at RT and then against TMS for another 30 min. The pRNA-enriched procapsid solution was then mixed with gp16,^{80,81} DNA-gp3,⁷¹ and ATP-containing reaction buffer to initiate the DNA-packaging step. After 1 h, the packaging mixture was treated by 1 μL of 1 mg/mL DNase (Worthington Biochemical Co.) at RT for 20 min, followed by the treatment of 1 μL of 0.5 M EDTA at 75 °C for 15–20 min, 0.5 μL of 0.1 mg/mL RNase A (RPA grade, Ambion) at RT for 10–20 min, and 1 μL of 20 mg/mL Proteinase K at 55 °C for 1 h. The final reaction mixture was assayed by 0.8% agarose electrophoresis at 100 V for 1 h. After running, gels were stained by ethidium bromide and images were captured with an Eagle Eye II system (Stratagene).

Effect of pH on 2'-F-C/U pRNA Dimer Formation. About 250 ng of 2'-F-C/U pRNA Aa' was incubated in phosphate-citrate buffer at different pH values (4, 5, 6, 7, 8, 9, and 10) for 5 min at room temperature and then ran in a native PAGE in TBM buffer. After

running, the gel was then stained by ethidium bromide and imaged with an Eagle Eye II system (Stratagene).

Dimer Formation of 2'-F RNA in Various Conditions Detected by Sucrose Gradient. Linear 5–20% sucrose gradients were prepared in TB buffer (89 mM Tris, 200 mM boric acid, pH 7.6), with or without 10 mM MgCl₂ (Fischer). The pRNA mixtures were then loaded onto the top of the gradient. Samples were spun at 45 000 rpm for 14.5 h at 4 °C in a SW55 rotor. After sedimentation, fractions were collected and subjected to scintillation counting (Packard 1900TR liquid scintillation analyzer).

pRNA Association. [³²P]pRNA Ab' at a final concentration of 16.25 nM was incubated with increasing concentrations of 2'-F-C/U-modified and nonmodified pRNA Ba' (0.1, 0.3, 0.9, 2.6, 8, 24, 72, 216, and 650 nM) for 30 min and then separated by a TBM native gel PAGE. After running, gels were imaged with a Cyclone Storage Phosphor Screen system (Packard) and analyzed with OptiQuant software.

AFM Images of Hand-in-Hand and Foot-to-Foot pRNA Dimer. For all samples, the specially modified mica surface (APS mica) was used. The APS mica was obtained by incubation of freshly cleaved mica in 167 nM 1-(3-aminopropyl)silatrane. The details of APS mica surface modification are described in the literature.^{82,83} Samples were diluted with TMS buffer up to 3–5 nM final concentration. Then the droplets of samples (5–10 μL) were immediately deposited on APS mica. After 2 min incubation on the surface the excess of the sample was washed with DEPC-treated water and dried with the flow of Ar gas. AFM images in air were acquired using a MultiMode AFM NanoScope IV system (Veeco/Digital Instruments, Santa Barbara, CA) operating in tapping mode. Two type of AFM probes were used for tapping mode imaging in air: (1) regular tapping mode silicon probes (Olympus from Asylum Research, Santa Barbara, CA) with a spring constant of about 42 N/m and a resonant frequency of 300–320 kHz; (2) noncontact NSG01_DLC probes (K-Tek Nanotechnology, Wilsonville, OR) with a spring constant of about 5.5 N/m and a resonance frequency of 120–150 kHz.

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Supporting Information Available: RNase stability data for 2'-F-C and 2'-F-U pRNA Aa'; sucrose sedimentation profiles of pRNA in the presence of Mg²⁺, Mn²⁺, and Sr²⁺. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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