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Co-transcriptional Assembly of Chemically Modified RNA Nanoparticles Functionalized with siRNAs

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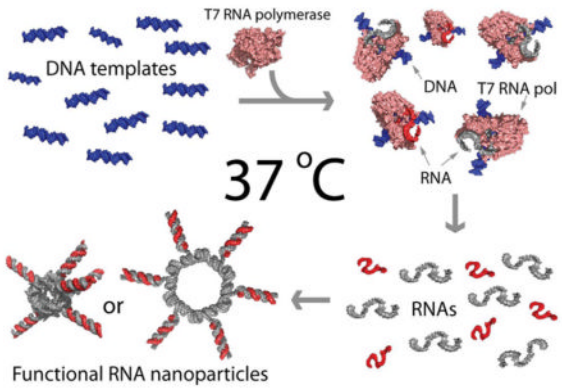
Abstract



We report a generalized methodology for the one-pot production of chemically modified functional RNA nanoparticles during in vitro transcription with T7 RNA polymerase. The efficiency of incorporation of 2′-fluoro-dNTP in the transcripts by the wild type T7 RNA polymerase dramatically increases in the presence of manganese ions, resulting in a high-yield production of chemically modified RNA nanoparticles functionalized with siRNAs that are resistant to nucleases from human blood serum. Moreover, the unpurified transcription mixture can be used for functional ex vivo pilot experiments.

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

Extended Materials and Methods section, RNA sequences used in this project, and additional Figures S1–S7. This material is available free of charge via the Internet at http://pubs.acs.org.

Supporting Information

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Keywords

RNA nanotechnology; self-assembly; siRNA; chemical modifications; transcription; T7 RNA polymerase

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RNA molecules can be designed to form a wide variety of compact and thermodynamically stable three-dimensional structures (nanoparticles or NP) suitable for a broad range of nanotechnological and biomedical applications.[1–3] Currently, two main strategies are used to design various RNA NP.[4] In one strategy, based on the principles of RNA architectonics,[5–9] the single-stranded RNAs (ssRNAs) form monomers with prefolded structural motifs followed by bottom-up assemblies via intermolecular tertiary hydrogen bond formations (e.g., hexameric nanorings[10,11]). In another strategy,[12] ssRNAs are designed to avoid stable internal secondary structures, and their assemblies are built solely from intermolecular interactions independent of any tertiary bindings (e.g., nanocubes[4,13]). Fusion of the individual RNAs participating in NP formation with functional/therapeutic RNAs (ribozymes, small interfering RNA (siRNAs), aptamers, and so forth) allows precise control over the stoichiometric organization and simultaneous delivery of different RNA functionalities into cells.[1,3] However, biomedical integration of such functional RNA NP is somewhat limited by at least three obstacles:

i. *The cost and size limitations associated with chemical synthesis of RNA*. The addition of functional groups to RNA scaffolds typically involves increasing the length of the individual RNA strands entering into the composition of the RNA nanoparticles. This strategy often requires the synthesis of RNAs in lengths that exceed what is currently available by commercial synthesis (i.e., RNAs that are greater than 60-nt). Furthermore, because chemical synthesis of RNA is relatively expensive compared to DNA, long RNAs are usually prepared by in vitro transcription with bacteriophage T7 RNA polymerase.

ii. *The complexity of RNA NP production*. Given that the synthesis of DNA oligos are relatively inexpensive, RNA synthesis usually relies on the use of DNA templates coding individual strands of RNA NP which are transcribed in vitro. Resulting RNAs are gel purified, recovered from purification, and combined at equimolar quantities. Thermal denaturation and specific refolding protocols are used to ensure the desired NP formation. Denaturation and refolding conditions strongly depend on NP design strategies as well as the sequences of individual RNAs. Therefore, each NP requires an optimization of the assembly protocol.[4]

iii. *Low retention time of RNA NP in the patient bloodstream due to their susceptibility to nuclease degradation*. Inclusion of dNMPs chemically modified at the 2′-position of the ribose sugar into the RNA strands of RNA NPs offers a promising way to increase the retention time of functional RNA NPs in the bloodstream.[1] Production of chemically modified RNA NPs has been previously achieved through transcription of individual RNA strands by mutant bacteriophage T7 RNA polymerase in the presence of 2′-fluorinated dUTP, and unmodified ATP, GTP, and CTP. This Y639F mutant enzyme (defective in discrimination between rNTP and dNTP substrates[14]) is commercially available and relatively expensive. The purified RNA strands were used for NP assemblies[15] by thermal denaturation and refolding. Notably, the overall yields of fluorinated RNAs, produced by the mutant polymerase according to the manufacturer's protocol, are significantly lower than the amount of unmodified transcripts (data not shown), precluding the large-scale production of chemically modified NPs. Apparently, the enzyme mutation does not completely alleviate the inefficient incorporation of several

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modified residues in a row, which sometimes is essential for formation of the full-length transcript.

We have achieved high yields of RNA NP by eliminating the purification step of individual RNA strands and post-transcriptional assembly of RNA NPs. Our methodology is based on in vitro transcription by wild type (wt) T7 RNA polymerase of a mixture of DNA templates encoding RNA strands that go into the composition of the given RNA NP (Figure 1 and Supporting Information Figure S1). As a proof of principle, we demonstrate cotranscriptional assembly of previously characterized RNA NPs, rings and cubes.[4,10,13] These NPs were functionalized at either their 5′- or 3′-ends with siRNAs (0–6 siRNAs per RNA NP) targeted against green fluorescent protein (GFP). When all 6 siRNAs were present, the NPs were composed of 12 RNA strands each (Figure 2a and Supporting Information Figure S2).[4] However, larger complexes are shown to be cot ranscriptionally produced as well (e.g., RNA NP composed of 22 RNA strands shown in Supporting Information Figure S3).

Formation of RNA NP takes place directly in the transcription reaction when equimolar amounts of DNA templates encoding specifically designed RNAs (Supporting Information Figure S1) are introduced. The resulting functional RNA NP, characterized by nondenaturing polyacrylamide gel electrophoresis (native PAGE) and dynamic light scattering (DLS) experiments (Figure 2a and Supporting Information Figure S2a), can be easily separated from other components of the transcription reaction by native PAGE or by membrane filtration (Figure 2b,c Supporting Information Figure S2b). Furthermore, we established experimental conditions when chemically modified RNA NP are obtained with yields comparable to the nonmodified RNAs (Figure 2d,e and Supporting Information Figure S4) even when wt T7 RNA polymerase is used for transcription.

Substitution of UTPs with 2′-F-dUTPs in a conventional transcription buffer dramatically lowers the yields (more than 10-fold) of the transcribed full-length RNAs (Figure 2d and Supporting Information Figure S4b at 0 mM $MnCl_2$). However, in the presence of Mn^{2+} the production of chemically modified RNA molecules as well as co-transcriptionally assembled chemically modified RNA NP becomes possible in high yields with the wt T7 RNA polymerase. In the series of experiments addressing Mn^{2+} effect on individual RNA strand synthesis (Figure 2d, blue curve), we established that the addition of 0.25–0.75 mM Mn^{2+} to the transcription buffer containing 5 mM $MgCl_2$ and unmodified NTPs doubles the yield of RNA chains. Interestingly, the presence of Mn^{2+} at 1 mM or higher concentrations had an inhibitory effect on transcription. When UTP was substituted with 2′-F-dUTP, Mn^{2+} addition resulted in a >10-fold increase of the full-size chemically modified RNA yield (Figure 2d, red curve). The yields of the chemically modified RNAs in the presence of 5 mM Mg^{2+} and 0.5 mM Mn^{2+} were comparable to the unmodified RNAs transcribed in the presence of 5 mM $MgCl_2$. This result agrees with the previously reported effect of Mn^{2+} on incorporation of 8-N_3AMP.[16] It appears that Mn^{2+} relaxes the substrate specificity of T7 RNA polymerase in a manner similar to the Y639F mutation. However, in contrast to the Y639F mutation, Mn^{2+} at 0.25–0.75 mM range promotes, rather than inhibits, the transcription with regular NTP substrates. Notably, Mn^{2+} does not interfere with the cotranscriptional assembly of RNA NP. We have been consistently obtaining similar amounts of regular and chemically modified RNA NP in transcription reactions containing DNA templates encoding all chains required for the NP formation (Figure 2e). In some experiments, the yields of the chemically modified functionalized NPs appeared even slightly higher than the yields of the unmodified NPs (data not shown).

As expected, replacement of UMPs with 2′-F-dUMPs in the RNA strands forming the nanocubes and nanorings results in increased resistance of the RNA NP to ribonucleases from human blood serum (Supporting Information Figure S4c,d). In addition to being

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significantly more resistant to blood serum ribonucleases, fluorinated RNA NP functionalized with siRNAs can still be processed by the human recombinant Dicer (Supporting Information Figure S5).

It is important to have “fast-screening” experiments to quickly assess the functionality of produced RNA NP. Therefore, we addressed if the transcription reaction mixtures containing nonpurified RNA NP are suitable for functional cell culture assays (Figure 3 and Supporting Information Figure S6) using human breast cancer cells stably expressing eGFP (MDA-MB-231/eGFP). In these experiments, cells were cotransfected with different amounts of purified and nonpurified transcription mixtures and three days after, the level of eGFP expression was analyzed with fluorescence microscopy and flow cytometry. All experiments were repeated at least three times. The results demonstrated no eGFP silencing by the transcription mixture containing nonfunctionalized RNA NP. However, when the cells were transfected with the purified and nonpurified cotranscriptionally assembled functionalized RNA NP, a significant level of silencing was detected even at the 100-fold dilution of initial transcription mixtures (Figure 3 and Supporting Information Figure S6). Moreover, chemically modified (with 2′-F-dUMP) functionalized RNA NP caused significant silencing comparable to the nonmodified RNA NP (Supporting Information Figure S7). All these results are in a good agreement with silencing caused by in vitro assembled RNA NP and siRNA duplexes (data not shown).

We demonstrate that two types of RNA NP, nanorings and nanocubes, designed based on intramolecular and intermolecular hydrogen bond formation, respectively, can be produced by cotranscriptional assembly. The RNA NP functionalized at either the 5′- or 3′-side with siRNA duplexes targeting enhanced green fluorescence protein (eGFP)[4,17] efficiently silence its expression when transfected into the cultured cells. Formation of RNA NP carrying functional siRNAs in physiological conditions suggests the possibility of their expression in vivo combining gene therapy and RNA nanotechnology approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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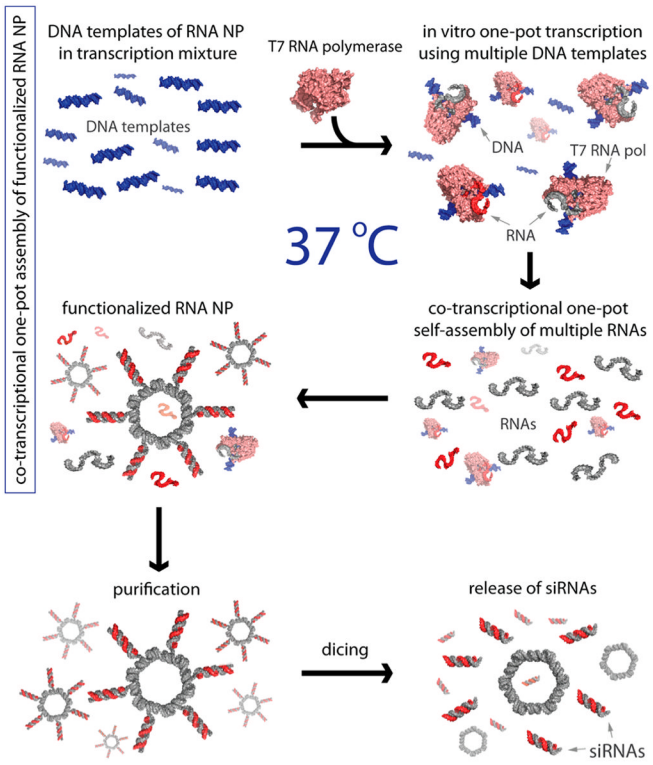

Figure 1.
Cotranscriptional assembly of RNA NPs. Schematic representation of cotranscriptional assembly leading to the formation of RNA NP (nanoring) functionalized with six siRNAs and their further purification. Functional siRNAs can be released by Dicer nuclease.

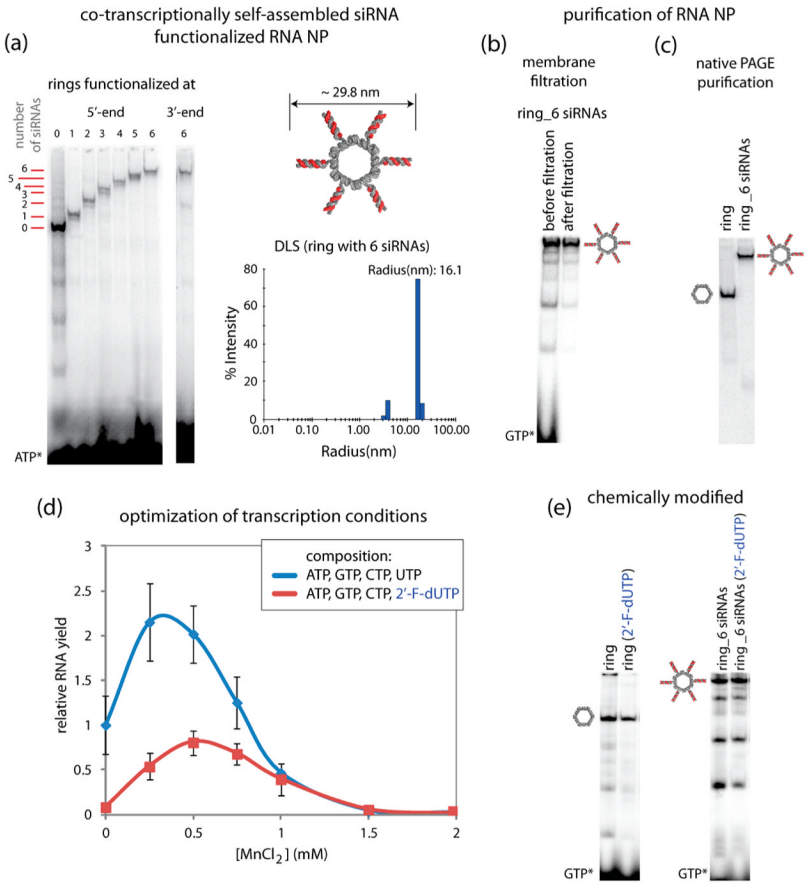

Figure 2.

Co-transcriptional assemblies of RNA nanoparticles (NP) with and without chemical modifications (2′-F-dUTP) and their further purifications. (a) Native-PAGE results representing cotranscriptional body-labeled assemblies leading to the formations of ring RNA NP 5′- or 3′-end functionalized with different numbers of siRNAs (0–6) and dynamic light scattering (DLS) result for assembly. (b) Membrane filtration and (c) native-PAGE purification of cotranscriptionally assembled functional RNA NP. (d) Presence of Mn^{2+} stimulates transcription with regular and chemically modified NTP substrates. The relative amounts of single-stranded RNAs produced in the presence of regular NTPs (blue trace) and ATP, CTP, GTP, and 2′-F-dUTP (red trace) are normalized to the standard yields of the nonmodified full-length transcript produced in the absence of $MnCl_2$ (conventional transcription buffer). The error bars show standard error from nine independent experiments. (e) Native-PAGE results illustrating the formation of cotranscriptionally assembled chemically modified (2′-F-dUTPs) functionalized ring RNA NP. Please note that conventional and 2′-Fl-dUMP-containing nanorings are produced with similar yields.

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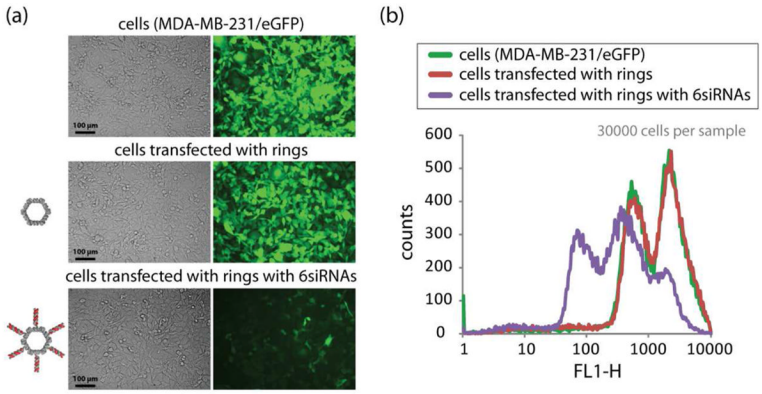

Figure 3.

GFP knockdown assays for human breast cancer cells (MDA-MB-231/GFP) that stably express enhanced GFP (eGFP). Three days after the transfection of cells with cotranscriptionally assembled RNA NP functionalized with siRNA against eGFP, eGFP expression was observed by (a) fluorescence microscopy and (b) statistically (30 000 cells per sample) analyzed with flow cytometry experiments. Please note that the individual nonfunctionalized cotranscriptionally assembled RNA NP cause no decrease in eGFP production.

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