

COMMENTARY

Rolling Circle Transcription of Tandem siRNA to Generate Spherulitic RNA Nanoparticles for Cell Entry

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Molecular Therapy–Nucleic Acids (2012) 1, e36; doi:10.1038/mtna.2012.31; published online 7 August 2012

Small interfering RNA (siRNA) holds great promise as a simple, yet powerful therapeutic strategy to knockdown the function of a particular gene. However, the major obstacle to the use of siRNA in clinical applications is the lack of an effective delivery system. Much effort has concentrated on developing methods to package or encapsulate siRNA with polymers or lipids. In a recent issue of *Nature Materials*, Paula Hammond and coworkers present an innovative approach to siRNA delivery based on the production of dense RNA “microsponges” that contain more than a half million copies of an siRNA precursor.¹ The morphology of these microsponges resembles that of spherulites: assemblies of linear polymers in which highly ordered pleated lamellar sheets, interspersed with amorphous regions, radiate outward from a nucleating core to form a roughly spherical aggregate. The innovative strategy demonstrates improvements in RNA production, payload encapsulation, and transport and cell entry.

Many approaches have been employed to tackle the siRNA delivery issue, but challenges remain. These include the issues of chemical and thermodynamic instability, short *in vivo* half-life, unfavorable biodistribution, low yield, high production cost, *in vivo* toxicity and side effects, a lack of specific targeting, and inefficient endosomal escape. The recent application of RNA nanotechnology² has shown promise for enhanced RNA stability,^{3,4} extended half life,⁵ and reduced toxicity.⁵ RNA/DNA nanotechnology has also enabled the 3D arrangement of multiple folate ligands to enhance folate receptor-binding so as to increase delivery of siRNA to cancerous cells.⁶ Nevertheless, vector production, cell entry, and endosomal escape remain challenging.

RNA is a “polynucleic acid”, but its polymeric nature in material and technology applications is often overlooked due to the misconception that it is very unstable. The linear and multimeric properties of RNA are very similar to those of conventional polymers, and RNA strands can be assembled in a controlled and predictive fashion to generate nanoparticles with defined structure and stoichiometry. To date, these properties have been exploited to design and manufacture a variety of polyvalent nanoparticles having distinct structures.² Hammond and colleagues have departed from this approach to nanoparticle design and production with an innovative strategy that generates monodisperse spherulitic

RNA nanoparticles from extremely high molecular weight RNA strands.

The authors produced the RNA interference (RNAi) spherulites using a rolling circle approach to transcribe the RNA. Rolling circle replication is utilized by certain viruses and cells to amplify genetic materials or generate replication intermediates. Recently, it has been exploited for use in genomic DNA amplification using the powerful phi29 DNA polymerase, which displays high processivity and high proof-reading activity.⁷ This efficient method of viral RNA synthesis has also been applied to the production of siRNA and small hairpin RNA.⁸ Hammond and coworkers constructed a circular DNA construct harboring an siRNA gene preceded by a T7 promoter and lacking terminators. As a result, T7 RNA polymerase can act progressively and continuously around the circular DNA hundreds or thousands of times generating multiple copies of tandem RNA units (Figure 1). As the RNA strand is transcribed continuously, it grows in length to become fiber-like, then sheet-like, and finally condenses into dense spherulitic RNA nanoparticles (Figure 2). Based on the microscopic texture of these microspheres, the authors refer to these structures as RNAi microsponges. Lamellae, typically having widths of ~10 nm, pack densely to form spherulites with diameters varying from micrometers to hundreds of micrometers. The resultant semicrystalline structures exhibit characteristic birefringence patterns arising from the regular pattern of lamellae, a property also observed here as the hairpin moieties of RNA polymers organize and pack into microspheres with an average size of ~2 μm. Interestingly, this unique structure appears to protect RNA from degradation in the serum.

In the report, the RNAi-spherulites were shown to produce ~21 nt small RNA fragments after incubation with Dicer. However, pure RNA is negatively charged and direct cellular uptake remains insignificant due to electrostatic repulsion from the negatively charged cell membrane. In order to achieve efficient cellular uptake and subsequent processing by the RNAi machinery the group introduced synthetic polycations, such as polyethylenimine (PEI), into the RNAi-spherulites to serve two purposes: (i) condense the RNAi-spherulite to a nanoscale particle, from 2 μm to 200 nm; and (ii) alter the net charge of the particle from negative to positive.

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PEI is a polymeric transfection agent. It is possible that PEI condenses RNAi-spherulites into nanoparticles with a positively charged outer layer, which can then bind to the anionic cell surface and subsequently be internalized into the cell. Due to the lower pH environment within the endosome, protonation of amine residues of PEI can lower the osmotic potential and cause osmotic swelling, which can result in bursting of the endosome and release of the payload to ultimately generate functional siRNAs derived from the RNAi-spherulites. Upon release into the cytoplasm, the RNA-induced silencing complex can then process the siRNA, which can successfully knockdown expression of target genes, as shown in previous *in vitro* and *in vivo* studies.

Hammond and co-authors demonstrate that fluorescent PEI-condensed RNAi-spherulites can transfect a cancer cell line and silence firefly luciferase expression.

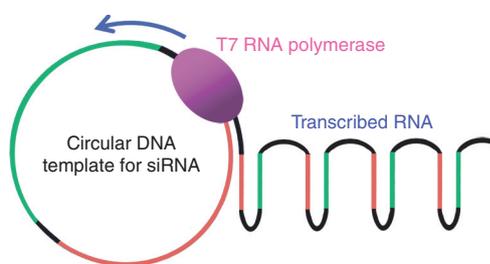


Figure 1 Schematic showing continuous synthesis of RNA by T7 RNA polymerase using rolling circle transcription.

In addition, they evaluated gene expression knockdown *in vivo* with intratumoral injection of the same PEI-condensed RNAi-spherulites designed to produce siRNA for the silencing of firefly luciferase expressed by the cancer cells. After 4 days, firefly luciferase expression was significantly reduced in induced tumors in mice by the RNAi-spherulites.

The feasibility of *in vivo* delivery of PEI-condensed RNAi-spherulites for therapy, however, remains uncertain. Variable mechanisms and routes can lead to nanoparticle uptake by cells. Phagocytosis, macropinocytosis, and clathrin- or caveolae-mediated endocytosis all can lead to the internalization of nanoparticles. Extensive studies in xenograft models have revealed that delivery of polymer complexes or nanomaterials to the tumor require a delicate balance between minimizing particle clearance mediated by the monocyte phagocytic system, which includes lung, spleen, and liver macrophages (Kupffer cells), and extravasation from the porous tumor vasculature. Whether the PEI/RNAi-spherulite can be formulated in a way to evade the monocyte phagocytic system and accumulate in target organs remains to be evaluated.

The targeted delivery into specific cells is the major point of interest that underlies the effective therapeutic use of any RNA nanoparticle. The internalization of RNAi-spherulites in this report does not involve specific targeting. Internalization of particles smaller than 200 nm in diameter involves clathrin-coated pits whereas entry of particles larger than 200 nm is mediated by caveolae. The 200 nm size reported here is large enough that nonspecific cell entry may still be an issue. It would be very interesting to elucidate which route

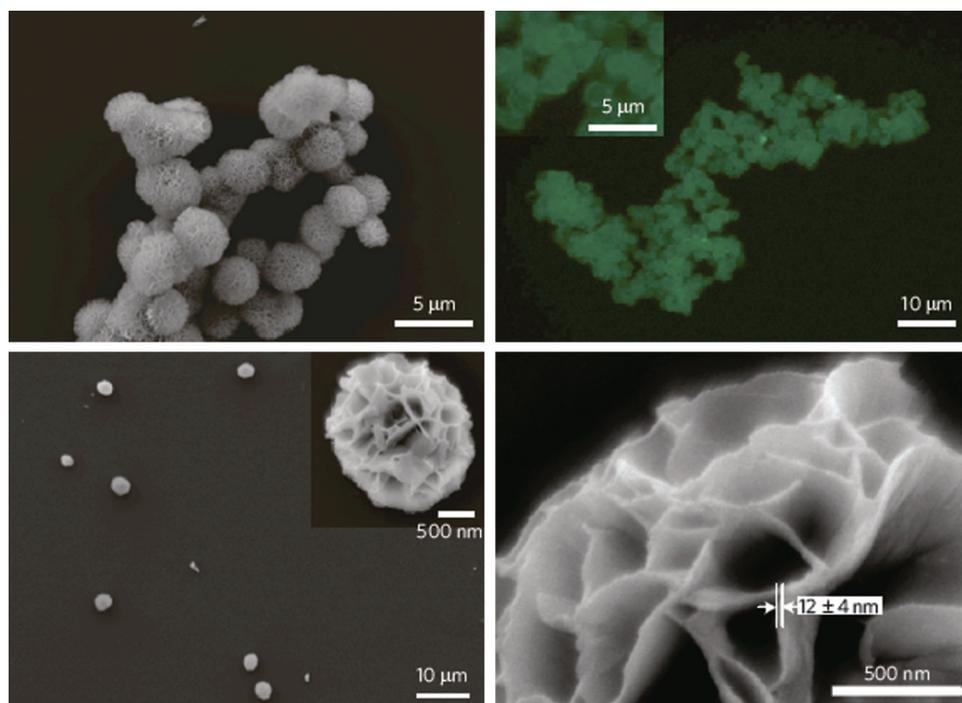


Figure 2 Micrographs of the spherulitic RNA nanoparticles. (Upper left) Scanning electron microscopy (SEM) image of several spherulitic RNA nanoparticles clustered together, bar = 5 µm. (Upper right) Fluorescence microscopy image of spherulitic RNA nanoparticles stained with SYBR II dye, bar = 10 µm, and a higher magnification image in the inset, bar = 5 µm. (Lower left) Low magnification SEM image of spherulitic RNA nanoparticles, bar = 10 µm, and a higher magnification SEM image in the inset, bar = 500 nm. (Lower right) High magnification SEM image showing the sheet-like substructure of the spherulitic RNA nanoparticles, bar = 500 nm. Figures reproduced with permission from ref. 1, © 2012 NPG.

is involved in the uptake of these large and unique nanoparticles. Moreover, the demonstration that intratumoral injection of RNAi-spherulites inhibits the expression of a target gene within tumors raises the prospect for the multifaceted utility of these particles if they could be modified to achieve specific targeting *in vivo*. However, for specific receptor-mediated endocytosis, it is generally believed that the optimal size for therapeutic nanoparticles is in the tens of nanometers; if this innovative design could be reproduced on a smaller scale of particle size, it has the potential to serve as the basis of a highly specific and efficacious cancer therapeutic.

The spherulite self-assembly process is well characterized in synthetic polymers, such as polyethylene, upon slow cooling from the molten state.⁹ Nevertheless, this phenomenon is not restricted to chemical polymers but is ubiquitous in nature, having been observed in crystallization processes involving matter as diverse as metals, mineral aggregates, liquid crystals, and biopolymers such as carbohydrates or proteins, and even DNA.¹⁰ However, Hammond and coworkers are the first to report such behavior in RNA polymers. The elucidation of these polymeric properties of RNA provides new insight for future studies of RNA structures and should inspire others to consider these properties in the context of the design, assembly, function, chemical modification, drug conjugation, and therapeutic delivery of RNA structures.

The emerging field of RNA nanotechnology² has brought about many innovations on the scientific front and holds strong promise for targeted therapies with significantly reduced side effects. RNA has proven versatile and multifaceted; it displays many unexpected functions and features,^{11–15} and it is anticipated that fervent research efforts will continue toward application development of this promising technology. The report by Hammond and coworkers reveals additional features in RNA nanoparticle assembly, provides new insight into siRNA delivery, and opens new directions of research in RNA nanotechnology, while its therapeutic potential requires further evaluation.

Acknowledgments. We thank Markos Leggas, Bruce Shapiro, John Rossi, Mark Behlke, Brent Hallahan, Jayden Smith,

Yi Shu, Hui Li, Farzin Haque, Huaming Fang, and Zhengyi Zhao for insightful comments. The author's research on RNA nanotechnology was supported by National Institutes of Health grant R01 EB003730 and National Cancer Institute Cancer Nanotechnology Platform Partnership Program on RNA Nanotechnology for Cancer Therapy (U01 CA151648). The author is a co-founder of Kylin Therapeutics, Inc., and Biomotor and Nucleic Acids Nanotech Development, Ltd. The author declared no conflict of interest.

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