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Editorial

RNA Nanotechnology: Methods for synthesis, conjugation, assembly and application of RNA nanoparticles

Nanotechnology addresses the creation and application of materials at the nanometer scale using either top-down approaches or bottom-up assembly. In particular, the notion of using DNA as a nanomaterial has led to several publications of exciting research. As an alternative to DNA, RNA has recently catapulted into place as a nanotechnology platform due to its diversity in structure and function. RNA can acquire a wide variety of secondary structures and complementary property which rival and surpass that of DNA. RNA nanoparticles can be fabricated with a level of simplicity characteristic of DNA, yet they possess versatile tertiary structure and catalytic functions that can mimic some forms of proteins. RNA is unique in comparison to DNA by virtue of several properties: higher thermodynamic stability; both canonical and noncanonical base pairing ability as well as a variety of single stranded loops suitable for inter- and intra-molecular interactions; base stacking; and distinct *in vivo* attributes. Subsequent to the pioneering demonstration that RNA dimer, trimer and hexamer can be fabricated by re-engineering of RNA molecules [1], novel properties of RNA nanoparticles have been explored for treatment and detection of diseases and various other applications. Over the last 5 years, there has been an explosion of publications on RNA nanostructures relating to the rapid emergence of RNA nanotechnology [2].

The construction of RNA nanoparticles (Fig. 1) involves a multistep process [2] beginning with the conception step, whereby the desired properties of the nanoparticles are defined and the global structure of the particle and application are considered. A computational approach is then applied to predict the folding of the building blocks and the consequences of inter-RNA interactions in the assembly of the final RNA quaternary structure. After the monomeric building blocks are generated either by *in vitro* transcription using RNA polymerase or chemical synthesis, the individual subunits are mixed in stoichiometric ratio to assemble into quaternary architectures by either templated or non-templated methods. The resulting RNA nanoparticles are characterized by atomic force microscope (AFM), electron microscope (EM), gel electrophoresis or chromatography to ensure proper folding consistent with the desired structural and functional capabilities. After thorough assessment, RNA nanoparticles are used for a variety of applications including the treatment and diagnosis of diseases and the regulation of cellular functions. In this special issue of Methods, leading experts were invited to address these six steps in RNA nanotechnology.

1. Conception of final structure/function of RNA nanoparticles

RNA nanoparticle construction requires the design of building blocks to self-assemble in a predefined manner to form larger quaternary structures harboring chemical moieties, therapeutics modules and/or other functionalities [2]. Thus the first step involves formulating a design strategy with regards to the final structure and function of the nanoparticle. The reviews by Shu et al. [3], Dua et al. [4], Zhou et al. [5], and Ishikawa et al. [6] and coworkers describe the strategies in the design of building blocks, incorporation of functional entities, and assembly of nanoparticles for the delivery of therapeutics and detection markers for the treatment and/or diagnosis of cancer and viral infections. Dua and coworkers [4] review the SELEX process for identification of aptamers targeted at cell-surface disease-associated membrane proteins and how to incorporate them into RNA nanoparticles. Ishikawa and coworkers [6] review natural and artificially generated GNRA/receptor interacting modules which are extensively involved in long-range tertiary interactions and play a critical role in catalytic RNAs and riboswitches. Shu and co-workers [3] describe the application of the pRNA of bacteriophage phi29 DNA packaging motor for constructing multivalent chimeric pRNA for targeted delivery of siRNA, ribozyme, and other drugs. Zhou and coworkers [5] describe the use of phi29 pRNA for the delivery of dual inhibitory anti-gp120 aptamer–siRNA chimera in which both aptamer and siRNA components have potent anti-HIV activities.

2. Computation and prediction of structure and folding

RNA can naturally fold into complex architectures via self assembly mediated by canonical and non-canonical base pairing and more importantly by higher order tertiary interactions, pseudoknots, kissing-loops, stem stacking, etc. Due to the unusual folding properties, the rules that elucidate RNA folding are complex. Currently, using an RNA 2D prediction program by Zuker, experimental data indicates that typically only 70% of the 2D folding prediction is accurate and even less accurate for 3D and 4D structures [2]. Programs for the computation of inter-molecular interactions of RNA subunits for quaternary nanostructure formation are in imperative demand. Kasprzak and coworkers [7] discuss computational approaches for the design of RNA constructs, using tectosquare as an illustration of an experimentally verified nanostructure. RNA junctions with idealized helices (internal and multibranch loops, as well as kissing-loops) can be extracted from the

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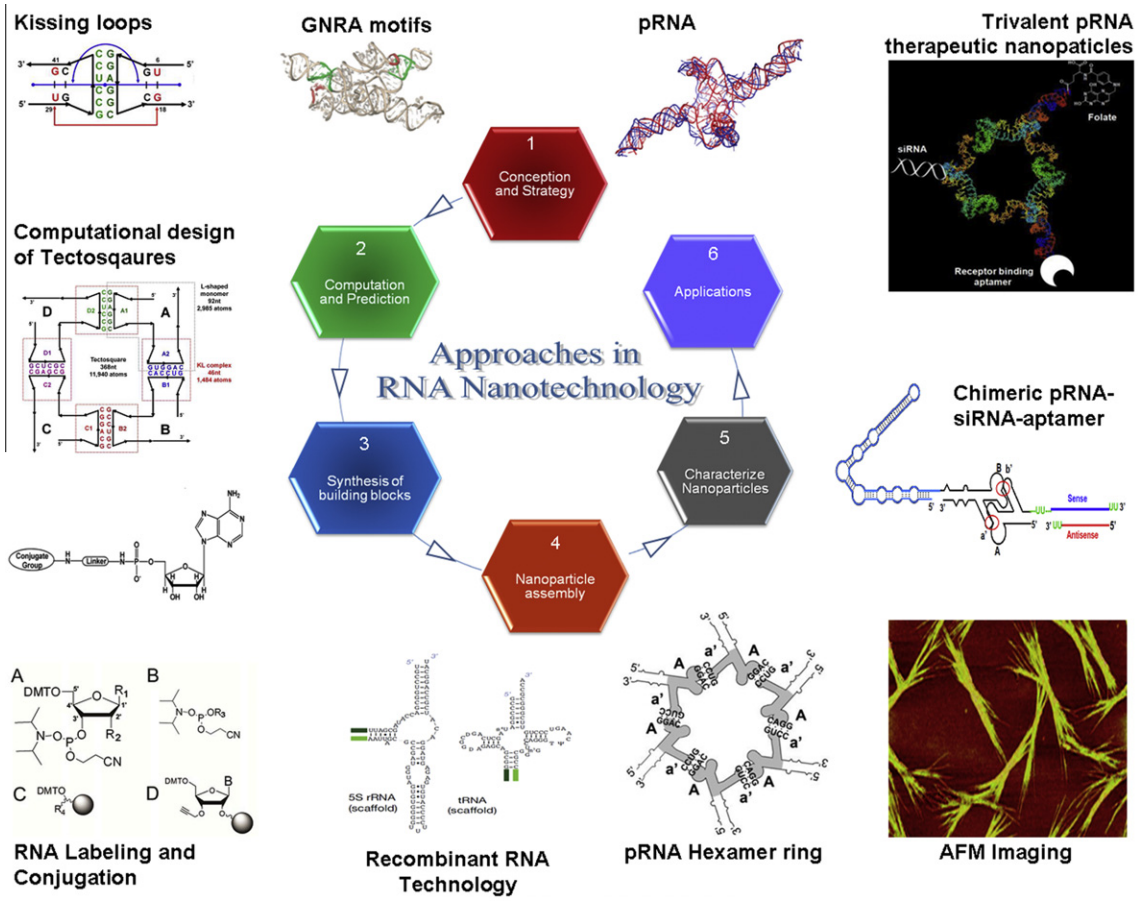

Fig. 1. Approaches in RNA Nanotechnology. All figures were adapted from the nine articles [3–10,12] in this issue of Methods.

RNA Junction database to serve as linkers in building larger nanoconstructs with desired geometry. RNA modeling softwares (such as, RNA2D3D and Nanotiler) and Molecular Dynamics simulations can then be applied to generate the desired 3D nanostructures [7]. Similar methodologies can be applied to design and evaluate modular nanostructures containing various naturally occurring RNA motifs. Other examples include varieties of loop/loop interactions, tertiary architecture contacts, and special motifs (such as 3-way junction, 4-way junction). A rich resource of well-developed databases can be utilized to extract known RNA structural units for construction of novel RNA nanoparticles with desired properties.

3. Synthesis of RNA building block or subunits of the nanoparticles

RNA nanoparticles containing different functionalities require conjugation and/or crosslinking of modules, labeling of each subunits, and chemical modification of nucleotides. RNA modifications to incorporate site-specific labels or reactive groups (that can be used for subsequent labeling or conjugation to other biomolecules) can be achieved via synthetic or enzymatic means. Paredes and coworkers [8] provide a detailed review on RNA labeling, conjugation, and ligation techniques that will allow the visualization, structural elucidation, localization, and biodistribution of modified RNA constructs. Enzymatic methods (such as T7 RNA Polymerase) are especially useful for the labeling of *in vitro* or *in vivo* RNA at the 5'- or 3'-end with modified nucleotides. An optimized scalable strategy to conjugate ligands such as folate to the 5'-end of AMP is detailed by Laing and coworkers [9]. The modified AMP can be purified with high yield using boronate affinity gel chromatography and incorporated at the 5'-end during *in vitro* transcription by T7 RNA polymerase. The folate-AMP conjugate provides a means for delivering RNA via endocytosis. This approach can be readily applied to conjugate various amine linkers and functional moieties to AMP for therapeutic/detection purposes. Ponchon and Dardel [10] detail recent *in vivo* RNA expression strategies for the production of recombinant stable RNA.

4. The assembly of RNA nanoparticles

Construction of nanoparticles via self-assembly (templated or non-templated) requires the use of addressable and predictable building blocks [2]. Templated assembly involves the interaction of RNAs with one another under the influence of a specific external force, structure, or spatial constraint. Non-templated assembly involves the formation of a larger structure by individual components without external influence. In this issue, Shu et al. [3], Dua et al. [4], Zhou et al. [5], Ponchon and Dardel [10] and coworkers review the non-templated self-assembly approach. Both papers by Shu et al. [3] and Zhou et al. [5] utilize the structural features of the pRNA of the bacteriophage phi29 DNA packaging motor which uses a hexameric RNA ring to gear the machine. The pRNA has been reengineered to form dimers, trimers, tetramers, hexamers and arrays via hand-in-hand or foot-to-foot interactions between two interlocking loops [11]. These nanoparticles have been used successfully as polyvalent vehicles to deliver a variety of therapeutic molecules as well as for the construction of RNA arrays. Ponchon and Dardel [10] detail recent *in vivo* RNA expression of recombinant stable RNA by camouflaging the RNA of interest within the well conserved scaffold of tRNA and 5S rRNA.

5. Characterization of the resulting RNA nanoparticles

AFM is the method of choice for analyzing the structure and dynamics of nano-assemblies and arrays of DNA/RNA. Lyubchenko et al. [12] provided a review on the various aspects of Atomic-force microscopy (AFM) for reliable imaging of various structures and topologies of DNA and RNA nanostructures. A key step involves the immobilization of the sample for imaging with AFM. A number of surfaces have been investigated for deposition of nucleic acids, and these substrates require extensive surface modifications prior to deposition of the sample.

6. The application of RNA nanoparticles

The versatility of RNA structure, the low free-energy in RNA annealing, the amenability in sequence, the choice in structural control, and the property of self-assembly make RNA an ideal material in nanotechnology applications as demonstrated by Zhou et al. [5], Shu et al. [3], Dua et al. [4] and coworkers in the development of potential therapeutic RNA nanoparticles for the diagnosis and treatment of prostate cancer, lung cancer, ovarian cancer, brain cancer, breast cancer, pancreatic cancer, hepatitis viral infection, and HIV infection. RNA's novel roles in nanomedicine include cell recognition and binding for diagnosis targeted delivery via receptor mediated endocytosis; intracellular control and computation via gene silencing and regulation; nuclear membrane penetration; and brain-blood barrier passing.

7. Conclusions

Natural or synthetic RNA molecules can fold into pre-defined structures that can spontaneously assemble into nanoparticles with multiple functionalities. The field of RNA nanotechnology is emerging but will play an increasingly important role in medicine, biotechnology, synthetic biology and nanotechnology. The research in RNA nanotechnology requires the development of feasible methods, and this special Methods issue serves as the first comprehensive collection of applications in the emerging field of RNA nanotechnology.

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