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## Preface

RNA nanotechnology is a young field with many potential applications. The goal is to utilize designed RNA strands, such that the obtained constructs have specific properties in terms of shape and functionality. RNA has potential functionalities that are comparable to that of proteins, but possesses (compared to proteins) simpler design principles akin to DNA. The promise is that designed RNA complexes may make possible novel types of molecular assemblies with applications in medicine (as therapeutics or diagnostics), material science, imaging, structural biology, and basic research.

Indeed, using this approach, scientists have shown that they can design RNAs that self-assemble into predefined shapes (such as rings, cubes, tetrahedrons, or lattices). Furthermore, designed RNAs can be programmed to impart different functionalities such as gene knockdown via RNA interference, temperature-specific behavior or RNA-based logic or multi-functional assemblies.

These successes, however, are typically only possible due to the use of specialized computational and experimental approaches. Repeating achievements based on regular research papers is frequently challenging if the methods are described only briefly. It is therefore particularly useful that detailed protocols provided by leading experts in the field are compiled as a unit, thus making the current state of the art accessible to scientists entering the field. Here we present in 23 chapters a spectrum of computational and experimental protocols pertaining to the creation, characterization, and utilization of RNA nanostructures.

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## Content

Computational approaches are an integral part of RNA design. Sun and Chen describe the use of a coarse-grained computational model in order to predict interactions between metal ions and the three-dimensional structure of RNAs (Chapter 1). Next, the use of RNA motifs for the generation of RNA ring structures is presented by Parlea et al. (Chapter 2). This is accomplished by an enumeration of motifs and linker helix lengths. It is shown how to process the obtained ring structures using a web interface. Coarse-grained models can be refined utilizing all-atom simulations in general and molecular dynamics simulation in particular. Kim et al. present a protocol for the simulation of RNAs and RNA lipid interactions using molecular dynamics and the Amber software package (Chapter 3). Jang and Ahn describe the use of circular DNA templates for the self-assembly of multimeric RNAi structures via rolling circle transcription (Chapter 4). The resulting RNAi transcripts can be hybridized with DNA strands that are conjugated with folate, leading to nucleic acid nanoparticles that can target folate-expressing cancer cells. Raghava describes the use of a computational approach for the prediction of the immunogenicity of RNA structures with respect to toll-like receptors TLR7 and TLR8 (Chapter 5). This is particularly interesting for predicting the immunogenicity of RNA nanostructures because an immune response is, depending on the application, to be avoided or desired.

Designed RNAs are frequently generated from DNA using RNA polymerase. Kireeva et al. demonstrate how the conditions for utilizing T7 RNA polymerase can be optimized for the efficient production of RNA nanostructures (Chapter 6). This allows for the incorporation of chemically modified nucleotides, resulting in RNA nanostructures that are resistant to ribonuclease degradation.

An important aspect of RNA nanotechnology is the ability to characterize the generated particles. An important quantity for characterizing the stability of RNA nanostructures is the melting temperature. Benkato et al. describe the use of thermal gradient gel electrophoresis for the determination of the melting temperatures of RNA complexes (Chapter 8). Boerneke and Herrmann demonstrate that X-ray crystallography can be used to determine the structure of RNA complexes at atomic resolution (Chapter 9). Their protocol includes detailed steps for the design of the RNA structures (an RNA square and two types of RNA triangles), experimental preparation of the RNA as well as analysis of X-ray crystallography results. Dabkowska et al. describe the self-assembly of RNA scaffolds on the surface of lipid bilayers (Chapter 7). The authors utilize a variety of technologies (such as atomic force microscopy, ellipsometry, and confocal fluorescence microscopy) in order to characterize the RNA nanostructure in the context of an interacting lipid bilayer. Interestingly, the cationic lipid promotes the adsorption and assembly of the polyanionic RNA.

One potential obstacle with respect to biomedical applications is the efficient delivery of nucleic acid material into cells. A variety of contributions pertain to the delivery of nucleic acid nanostructures into cells. While delivery agents differ widely, they have in common, shielding of the electrophilic nature and the negative charge of RNA during cell entry, and protection from environmental factors such as nucleases.

Lokesh et al. provide a protocol for the generation and validation of X-aptamers. X-aptamers can be viewed as oligonucleotide-inspired biopolymers that offer, compared to RNA and DNA, an extended sequence alphabet. X-aptamers have applications in targeting proteins and in cellular delivery of small molecules (Chapter 10).

Kruspe and Giangrade present aptamer-siRNA chimeras (AsiCs) (Chapter 11). Such aptamer-siRNA chimeras have potential applications against cancer if the aptamer part is chosen to bind to cell surface receptors and to facilitate uptake by tumor cells, and the conjugated siRNA is chosen to downregulate an oncogene.

Gupta et al. demonstrate the use of bolaamphiphiles for cellular delivery of RNA. Bolaamphiphiles are linear molecules that consist of a hydrophobic aliphatic chain and polar hydrophilic head groups on either end (Chapter 12). The cellular delivery of mRNA is particularly interesting and challenging (due to their, compared to small RNAs, dramatically larger size). Li and Dong describe the use of a class of lipid-like (also called lipidoid) nanoparticles that they recently developed for the cellular delivery of mRNA (Chapter 13). They describe the preparation of mRNA-loaded nanoparticles, characterization of their structure (via cryo-TEM), cytotoxicity, and transfection properties. Another moiety for cellular delivery of RNA is based on chitosan. Chitosan is a natural polysaccharide that can form RNA-containing nanoparticles, as described in the protocol contributed by Denizli et al. (Chapter 14). Wang et al. describe the synthesis of poly(lactide-co-glycolide) (PLGA) based polymers, the synthesis of cationic lipid G0-C14 based on 0th generation poly(amidoamine) (PAMAM) dendrimer, and the synthesis of RNA-containing PLGA-PEG/lipid hybrid nanoparticles (Chapter 15). The use and characterization of siRNA-containing oxime ether lipid nanoparticles is described by Puri et al. (Chapter 16). This includes the characterization of the transfection efficiency, stability, and assembly properties of the nanoparticles.

These protocols pertaining to RNA delivery show that there are a variety of options available for accomplishing this goal. Taken together, one can conclude that cellular delivery of RNA and DNA payloads is, while challenging, not a prohibitive obstacle for the development of RNA therapeutics.

A nanopore device can be utilized for the sequence-specific detection of RNA and DNA strands. Tian et al. describe the use of a nanopore in order to selectively identify miRNAs while providing an approach for avoiding “contamination” of the nanopore signal due to the presence of different additional RNAs (Chapter 17). Martins et al. provide a protocol for utilizing RNA/DNA hybrids that can re-associate in target cells into active siRNA and lead to knockdown of target genes. This technology is utilized as potential anti-HIV-1 therapy. They provide detailed protocol steps for the design, purification, and assembly of RNA/DNA hybrids as well as for the inhibition of HIV-1 genes (Chapter 18).

An important extension of the concept of static RNA scaffolds is to utilize dynamic RNA complexes in general and RNA switches in particular. Green describes the design and experimental verification of a prokaryotic RNA switch that operates on the level of translation (Chapter 19). A reporter mRNA is designed such that the translation start codon is hidden from the translation initiation complex via a stable hairpin structure. This hairpin is designed to undergo a structural change upon binding of a biomarker RNA. This then exposes the start codon, facilitating translation of the reporter RNA such as a green fluorescent protein.

Zakrevsky et al. provide the protocol for a two-stranded RNA switch that corresponds to the conditional activation of RNA silencing (Chapter 20). Such a switch is particularly challenging because it involves designing an RNA nanostructure where the rather stable Dicer-substrate helix with more than 21 base pairs is energetically outcompeted via several shorter helices. This was made possible via a novel computational approach that allows the estimation of the free energy of folding of multistranded pseudoknotted RNA complexes.

Tanaka et al. demonstrated that the utilization of motifs and domains in designed RNAs and RNA complexes can be rather large entities: they modified a group I ribozyme so that it undergoes self-dimerization (Chapter 21). They present protocol steps for the rational design and experimental assembly of the RNA complex. They also provide several ribozyme activity assays in order to verify the enzymatic activity of the ribozyme. An exciting possibility is the use of CRISPR: Zalatan presents a protocol that demonstrates the use of CRISPR guide RNA in order to recruit desired proteins and thereby control transcription (Chapter 22). The designed RNA acts in this fashion as a “conductor” for recruiting desired protein factors.

Utilizing RNAs for therapeutic purposes entails challenges not only with respect to cellular delivery but also with respect to biodistribution. Haque et al. demonstrate the different biodistribution properties of a variety of different RNA nanoparticles that are based on the very stable phi29 three-way junction motif (Chapter 23). They not only create a spectrum of different RNA nanostructures but also extensively characterize mouse *in vivo* properties such as biodistribution. This large number of techniques for the production and characterization of a family of different nanostructures represents the current state of the art of RNA nanotechnology.

In summary, we are excited to present this compilation of protocols contributed by renowned experts in the field of RNA nanotechnology. Sharing details not only of the results but also of how to achieve and reproduce those results is important for sustained progress in

this research domain. These are exciting times for the creative application of RNA design because the ground work has been laid and important functionalities are readily available in order to be incorporated into designs. We are confident that this volume and its wide spectrum of contributions will be a valuable aid for the design and production of RNA nanostructures.

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RNA Nanostructures

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