HHS Public Access

Author manuscript

ACS Comb Sci. Author manuscript; available in PMC 2019 January 24.

Published in final edited form as:

ACS Comb Sci. 2016 September 12; 18(9): 527–547. doi:10.1021/acscombsci.6b00073.

Cellular Delivery of RNA Nanoparticles

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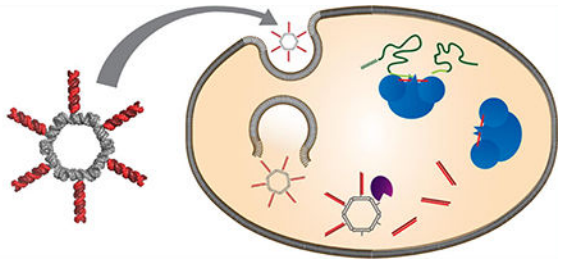
Abstract

RNA nanostructures can be programmed to exhibit defined sizes, shapes and stoichiometries from naturally occurring or de novo designed RNA motifs. These constructs can be used as scaffolds to attach functional moieties, such as ligand binding motifs or gene expression regulators, for nanobiology applications. This review is focused on four areas of importance to RNA nanotechnology: the types of RNAs of particular interest for nanobiology, the assembly of RNA nanoconstructs, the challenges of cellular delivery of RNAs in vivo, and the delivery carriers that aid in the matter. The available strategies for the design of nucleic acid nanostructures, as well as for formulation of their carriers, make RNA nanotechnology an important tool in both basic research and applied biomedical science.

Graphical Abstract



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The authors declare no competing financial interest.

Keywords

RNA; *self-assembly*; *nanoconstruct*; *RNA nanoparticle*; *therapeutics*; *delivery*

■ INTRODUCTION

While protein-based nanotechnology is a well-explored subject by now,[1,2] nucleic acid nanotechnology is a field still in its infancy. Both deoxyribo- and ribonucleic acids (DNA and RNA, respectively) have been used extensively to build various nanostructures,[3−11] some of which were functionalized toward therapeutic or diagnostic applications.[10,12] The suitability of RNAs as a material for use in nanotechnology and nanomedicine is due to their chemical, structural and functional properties. Chemically, not only can RNA store genetic information,[13] but it can also be easily modified.[14] Structurally, RNA can be modulated to form nanostructures of various shapes.[15] RNA is capable of self-assembling under physiological conditions[12,16] through both intramolecular recognition and long-range intermolecular interactions.[17] Functionally, RNA can perform catalytic, enzyme-like activities.[18−21] Furthermore, as a biological molecule, RNA is biocompatible and biodegradable, and thus an excellent contender as a material for therapeutics and medicine.

■ FUNCTIONAL RNAS

RNAs are key molecules in a plethora of cellular processes, ranging from gene regulation to protein synthesis. The central dogma of molecular biology states that DNA encodes for RNA and RNA codes (in the form of mRNA) for proteins, but this is not the entire picture. Cellular noncoding RNAs are involved in various other molecular processes such as splicing, intracellular transport and post-transcriptional regulation of gene expression. Several types of functional RNA or RNA elements have particular interests for the RNA nanotechnology field, as seen below.

Interfering RNAs.

RNA interference (RNAi) is an RNA-mediated, post-transcriptional gene regulation process through which the synthesis of targeted proteins is downregulated by interfering in the expression of the genetically encoded message.[22] The interference is performed by short noncoding RNAs that hybridize with complementary mRNA sequences, resulting in translation inhibition or message degradation. The principal RNAs involved in the RNAi mechanism are endogenous interfering micro RNAs (miRNAs), piwi RNAs (piRNA), and exogenous small interfering RNAs (siRNAs).

More than 60% of human genes are miRNA-regulated posttranscriptionally,[23] and dysregulation of miRNAs levels have been associated with cancer.[24] The same miRNA can regulate various genes, and a gene can be regulated by several miRNAs.[25] Their biogenesis begins with their own transcription, or in gene introns. Primary miRNA encoding transcripts (pri-miRNAs) are processed in the nucleus into a single stranded, 60−70 nts long stem loop precursor miRNAs (pre-miRNAs) by Drosha, an RNase III nuclease.[26] miRNAs spliced from introns (mirtrons) bypass the Drosha and Microprocessor complex (associated from

Drosha and DGCR8, the nuclear protein that recognizes double stranded RNA), and a lariate-debranching enzyme aids in generation of a pre-miRNA stem loop.[27] These short hairpin RNAs (shRNAs) are transported by Exportin-5 from nucleus to cytoplasm, where Dicer, an RNase III type enzyme, cleaves the hairpin loop, leaving 22 nucleotides (nts) long mature miRNAs ready to enter the RNA interference pathway.[28] miRNA is unwound by any of the Argonaut proteins *Ago*1−4, yet only *Ago*2 has high dicing activity. The Argonaut protein *Ago*2, a component protein of the RNA-induced Silencing Complex (RISC), is the protein which recognizes the short double stranded RNA, uses one strand to guide the RISC complex to the mRNA, and usually degrades the other strand.[29] The incorporated RNA strand helps guide RISC to the complementary sequence, generally on a 3′UTR region of the mRNA. miRNA typically pairs imperfectly to the mRNA, with only a seed region consisting of nucleotide positions 2−8 required to be complementary base paired to the mRNA. miRNA-RISC complexes tend to stall or repress message translation, by cleaving the polyA tail, destabilizing the mRNA, or sometimes degrading the mRNA message.[30]

piRNAs are another type of endogenously generated interfering RNA, and are longer than mature miRNAs, ranging from 26 to 31 nts. piRNAs complex with piwi Argonaute proteins to silence the expression of certain genetic elements and retrotransposons, and destabilize heterochromatin formation in germline cells.[31]

siRNAs are processed from cytoplasmic double stranded RNAs, regardless of their exogenous or endogenous origin.[32] Cytoplasmic double-stranded RNAs are cleaved by Dicer, which cuts double-stranded RNA into 21−23 nucleotides (nts) long siRNAs. These resulting duplexes are phosphorylated at their 5′ end and hydroxylated at their 3′ end, with two nt 3′ overhangs. The thermodynamically favored end is unwound by endonucleases, and the “guide” strand (or antisense strand) is loaded into RISC and transported to the complementary region of an mRNA, while the passenger strand is degraded.[33] The RISC complex cleaves the mRNA in the middle of the guide siRNA recognition site. While only the seed region of the miRNA is required to be complementary to its mRNA target, the guide strand of an siRNA pairs perfectly to the mRNA. Whereas miRNA-RISC generally reduces the efficiency of message translation, guide siRNA-RISC complexes cleave mRNA site specifically, resulting in a repression of translation.[34]

Because RNA interference is triggered by the presence of short double-stranded RNA, not only do interfering RNAs function as a regulators of endogenous protein expression, but the RISC mechanism also acts as an innate cellular defense mechanism against pathogenic double-stranded RNAs of viral or transposon origin. Due to the ability of RNA interference to temporarily downregulate virtually any target gene, rationally designed siRNAs make RNAi a highly attractive tool for both basic research as well as clinical applications. Indeed, more than 20 clinical trials utilizing RNAi-based drugs have been undertaken or are currently ongoing.[35]

Aptamers.

Aptamers are relatively short oligonucleotides that bind selectively and robustly to their target. These short nucleic acid segments can fold into particular secondary and tertiary structures to bind to specific ligands, small molecules, or proteins. Naturally, they occur in

riboswitches,[36] and their structure and conformation change upon binding to a target ligand.[37–39] Aptamers can also be selected in vitro to bind small molecules and peptides with high affinity and specificity.[40–42] In addition to recognition, aptamers can be used in drug therapies themselves.[43,44] Their high selectivity and their potential to be used as effectors make them an invaluable therapeutic tool. Aptamers that affect the corresponding protein's function have been employed as selective response modulators. Activators, or agonistic aptamers, have been selected for insulin,[45] for G-protein coupled receptors' agonistic autoantibody in cardiomyopathy,[46] for neurodegenerative disease's TrkB neurotrophin receptor,[47] or for CD40, CD28, CD8+ presenting immuno-cells.[48] Antagonistic aptamers that bind and block their proteins or receptors can be used for both diagnostic and therapeutic purposes, with several aptamers in various stages of clinical trials, including phase III.[49,50] For example, aptamers binding and inhibiting the human prothrombin protein have been selected. The inhibition of this blood-clotting factor prevents the formation of thrombin, thus implementing an RNA-based anticoagulant.[51] Other antagonist aptamers have been proven effective against arthritis' Interleukin-6 receptor,[52] E-selectin expressing vascular cells,[53] metastatic gastric cancer related receptor periostin,[54] and immune check-points proteins on T cell receptors TIM3.[55] Furthermore, a particular advantage of nucleic acid based aptamer therapies can be exploited by using a nucleic acid strand antisense to the designed functional aptamer that can bind and deactivate the compound. In this way, it is straightforward to design an antidote for the initial aptamer/drug. The existence of an antidote makes the use of higher dose therapies feasible, since the aptamer/drug can be deactivated as soon as needed.[51]

Splicing Modulators.

Aberrant RNA splicing is associated with cancer as well as a variety of congenital conditions.[56,57] RNA splicing can be targeted using a variety of different approaches such as small molecules, antisense oligonucleotides, or modified small nuclear RNAs (snRNAs).[57] Using synthetic antisense oligonucleotides, it has been demonstrated that hearing can be restored in a mouse model of human hereditary deafness.[58]

RNA Switches.

Some RNAs change their conformation upon binding to other molecules or upon changes in temperature. This conformational switch can then be a signal for a subsequent molecular event. Naturally occurring RNA switches, called riboswitches, are segments of mRNA capable of binding a small molecule or ligand and subsequently altering the mRNA's expression pattern.[59]

This switching capability can be combined with RNA functionalities. For example, the specific binding of a ligand to an RNA region upstream of a gene (akin to specific aptamer-ligand binding) can, in some cases, modulate transcription or translation. RNA interference can also be modulated with a switching capability: designed shRNA-based complexes that change their conformation upon binding to a biomarker mRNA were reported recently. This capability can be utilized to convey therapeutic functionality preferentially in diseased cells.[60]

Ribozymes.

A particular class of RNAs capable of catalyzing biological reactions in an enzyme-like manner was discovered more than 30 years ago,[19,20] yet their biological functions are still being uncovered. These RNA enzymes (ribozymes[20]) of unknown evolutionary origin are found in all phyla and are essential to life,[61] supporting the "RNA world" hypothesis.[62,63] The chemical reactions they catalyze include self-cleavage, nucleic acid ligation (both DNA and RNA)[64] and peptide bond formation.[65] Each ribozyme family has its own structure, organized by junctions or pseudoknots, and its own distinct geometry of the active site. The most common reaction they catalyze is self-scission through the acid−base mechanism,[66] promoted by increased pH and mediated generally by divalent cation presence (Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Pb^{2+}) or high concentrations of monovalent cations (Na^{+}). Some ribozymes bind metabolites or other organic molecules, yet the 2′-OH nucleophilic attack on the 3′-phosphate consistently yields two RNAs, a 5′-RNA with a 2′,3′-cyclic phosphate and a 3′-RNA with a 5′-OH.[67] Ribozymes can cis-cleave themselves from transcripts, as in the case of self-splicing introns, or trans-cleave cellular RNAs, such as tRNA and other ncRNAs, as in the case of RNase P-assisted RNA.[68] Recent studies uncovered nine naturally occurring self-cleaving motifs, and six distinct classes of self-scissing ribozymes, catalyzing the transesterification reactions. The involvement of ribozymes goes beyond self-cleaving, and bioinformatics and experimental studies suggest ribozymes catalyze RNA splicing, transfer RNA biosynthesis, viral replication, as well as other molecular processes.[67] In vitro selection allows for retention of synthetic ribozymes with particular catalytic properties, such as self-cleaving,[69] selfreplicating,[70] and self-cleaving riboswitches (aptozymes).[71] Development of sequence-specific self-scissing ribozyme, biosensing, and therapeutic ribozymes is an area of ongoing research.72−75

Exogenous mRNAs.

The upregulation of a gene or the introduction of a novel gene can be accomplished by the delivery of synthetic mRNA. To increase the stability and efficacy of the delivered mRNA, a variety of molecular properties can be tuned, such as the use of modified nucleotides (to increase resistance against nuclease degradation), 5′-cap modifications (to increase eIF4E binding or increase resistance against decapping), optimization of codon usage (to increase translation processivity) or poly-A tail length modifications (to modulate the stability of the mRNA).[76]

CRISPR RNAs.

Another approach that incorporates functional RNAs into a nanoconstruct utilizes CRISPR (Clustered regularly interspaced short palindromic repeats), a system of acquired immunity against phages found in bacteria and archaebacteria.[77] Repurposing this system for biomedical applications is based on the capability of an exogenous Cas9 protein complexed with an RNA guide strand to bind a targeted DNA region and induce DNA single-strand breaks or double strand breaks, as well as DNA methylation. The capability of inducing programmable genomic single-strand or double-strand breaks can be utilized to excise or replace genomic regions. The functionality of the Cas protein to cause targeted DNA methylation can be used for programmed downregulation of gene expression. Furthermore,

it has been shown that Cas proteins with deactivated nuclease activity retain their capability to target a programmed DNA region. Such “dead” Cas proteins (dCas or dCas9) have been used to transport molecular cargo to desired DNA regions. For example, the dCas9-mediated transport of transcription factors can be utilized for programmed gene activation.[78,79] Designed CRISPR systems utilizing RNA nanostructures, such as RNA scaffolds, have been reported.[80]

RNA/DNA Hybrids.

Hybrid structures consisting of duplexed RNA and DNA strands have interesting properties. Because most types of nucleases do not degrade RNA/DNA hybrid duplexes, such structures can have a higher in vivo stability compared to RNA-only equivalents. Pairs of cognate RNA/DNA hybrid structures with toeholds can be delivered separately leading to a reassociation resulting, for example, in RNAi activation in cells.[16,81,82]

■ RATIONAL RNA NANOSTRUCTURE DESIGN

RNA structures can be thought of as being composed of structural modules, or “RNA motifs”. Different motifs can have different functions: some have architectural roles (helices, branched junctions, kink-turns, etc.), and can be interchanged between different molecular contexts, being used as structural units to engineer novel constructs with new properties.[8] Some RNA motifs can interact with other RNA motifs, giving RNA self-assembling properties, or with other molecules, giving RNA molecular recognition functions. RNA motifs have been compiled and cataloged in various databases. Some databases are motif-specific: RNAJunction is a compilation of kissing loops, internal loops, bulges and multiway junctions[83] while the k-turn database provides a detailed collection and analysis of kink-turns.[84] Other RNA tertiary motif databases include SCOR,[85] FRABASE[86] and Motif Atlas.[87] RNA STRAND[88] and RNA CoSSMos[89] are databases geared toward secondary structure analysis and secondary structure searches. They can be used as tools to identify motifs with specific geometries and functions, and potential candidates for interchanging structural modules in a given structure.[9] All these databases can be used as motif pools from which to assemble RNA nanoconstructs, and thus they can serve as a starting point for the RNA nanostructure designer. A unique database of in-silico assembled RNA nanostructures, the Ring Catalog, compiles ring-like RNA nanostructures that are computationally predicted by combinatorial assembly of helices, multiway junctions, single nucleotide internal loops (bulges), general internal loops, and kissing loops found in the RNAJunction database.[90]

RNA nanoconstructs can be used as scaffolds for further functionalization, and the functionalized constructs are generally called RNA Nanoparticles (NPs).[91] There are several advantages to using RNA nanoconstructs as scaffolds for nanoparticles. Naturally, macromolecular complexes such as ribosomes, ribozymes, RNase P, spliceosomes, etc., are RNA-based machines. Hence, RNA rather than DNA is a component of choice for all these “natural nanoparticles”.[92] Artificially, RNA NPs can be engineered to present precise sizes, shapes and stoichiometries. Their synthesis is simple and straightforward, both chemically and enzymatically. RNA scaffolds can be functionalized with a variety of functional moieties, depending on the desired applications. A polyvalent RNA NP can incorporate not

only multiple siRNAs for increased potency, but several functional moieties, such as distinct siRNAs for a synergistic effect, aptamers, fluorescent tags, peptides, etc., for multipurpose effects, such as direct targeting, diagnostics and therapeutics. Thus, due to their chemical, structural and enzymatic properties, RNA is a biologically active material that can be utilized in nanotechnology and biomedical applications.

Manual Design of RNA Nanostructures Based on Naturally Occurring RNA 3D Motifs.

Naturally occurring RNAs and RNA motifs can be employed toward generating RNA NPs, as in the case of pRNA. Symmetric, multimeric structures are frequently associated with proteins and rarely encountered in RNA molecules. Yet, the bacteriophage phi29 has a symmetrical structure assembled by long-range tertiary Watson−Crick base pairing between repeated molecular units. This packaging RNA (pRNA) assembles through loop−loop interaction in a hexameric ring and translocates the DNA in the bacteriophage phi29 (Figure 1a.i).[93,94] By manipulating the sequence at the interface between the repeating monomers, various nanoconstructs assembling via “hand-in-hand”, “foot-to-foot”, and “arm-on-arm” orientations were produced.[95] Addition of four nucleotides in the interacting hand loops ensured specific complementarity and allowed for nine predicted interfaces. Seven of the designed loops mediated dimerization in vitro and were used to design a pRNA dimer, trimer, tetramer, pentamer, hexamer, and heptamer (Figure 1a.ii). Addition of short, palindromic sequences to the end of the pRNA sequence allows for “foot-to-foot” dimerization, and combined design permits assembling polymers up to 14 units in length (Figure 1a.iii).[96] The core obtained after removal of the hairpin loops (pRNA-3WJ),[97] and the core engineered by “ligating” two 3WJ in an X-shaped core, were utilized to create branched hexameric constructs (Figure 1a.iv).[98] These scaffolds have been used for functional attachments. pRNAs have been successfully incorporated in RNA NPs and used to deliver therapeutic and diagnostic molecules to diseased cells like cancer and viral-infected cells, see Functionalization of RNA Nanostructures section.[96,97,99]

Computational Design of RNA Nanostructures Using Naturally Occurring RNA 3D Motifs.

3-D Motif-Based Nanostructures.—Another approach to intermolecular assembly of RNA nanostructures involves the use and development of computational tools for exploring and modeling feasible 3D conformations, and for designing strand sequences capable of self-assembling into these designed structures.[100,101] Such computational methods speed up the entire design of the RNA-based nanoparticles and go hand in hand with experimental procedures. Because of the plethora of solved RNA tertiary structures available, detailed bioinformatics analysis has resulted in the identification of recurrent RNA structural motifs. With knowledge of RNA structure and folding characteristics, these structural RNA motifs can be implemented as modular building blocks for designing RNA based nanoconstructs.

The process can start with a collection of RNA motifs, such as n-way junctions and kissing-loops compiled in the RNAJunction database.[83] Modeling programs, such as Nano-Tiler and RNA2D3D[101] can use such building blocks as “corner stones” in the 3D models. Initially the selected building blocks are handled as rigid objects and are roughly fit together. However, as is the case in natural RNAs where larger structural contexts may exert local distortions, deformations can be applied to the elements of the models in order to achieve

full structure backbone connectivity. NanoTiler applies them to idealized helices connecting the junctions, and gives the user a measure of the deformation as feedback. A collection of nanoring-like structures can be found in the Ring Catalog.[82] RNA2D3D facilitates interactive distortions and assists in the relaxation process of the selected fragments via short energy minimizations and/or MD runs, leaving the expert decision-making to the user. Alternative conformations of comprising motifs based on experimental data (X-ray crystallography, NMR, cryo-EM), available in RNAJunction or in any other source for the same basic motif type, can also be used to explore the extent of structure variability and potentially be used “as is” in the modeling process.

For example, RNA nanorings were designed to assemble as hexagon-shaped supramolecular structures through the formation of kissing complexes between six “dumbbell” shaped monomer units, that is, helices capped by kissing loops at each end (Figure 1b.i).[11] In this particular design, the kissing loops are sequence variants of the ColE1 kissing complex, a unique assembly motif that exhibits a 120-degree angle between adjacent helices.[102] The use of six distinct kissing complexes to assemble the nanoring scaffold provides the means to have complete control over the functional composition of each heterogeneous monomer unit.[91] The 5′ and 3′ ends of each monomer strand are located at the midpoint of the helix. While other supramolecular RNA architectures have been generated that make use of kissing loops for assembly,[93,103–105] the nanoring is distinct in that the particle’s geometry is defined by the intermolecular assembly interaction, rather than by intricate structural motifs embedded within the core of each monomer. A consequence of this monomer design is robust intramolecular folding and intermolecular assembly. Fully assembled nanorings can be generated from isothermal, single-pot cotranscription reactions with multiple DNA templates that are required to encode the RNA strands for nanoring formation.[12] Cotranscriptional assembly was proven for the nanocube constructs as well, see De Novo Design of RNA Scaffolds section.[10] The capability to cotranscriptionally generate functional nanoring and nanocube assemblies eliminates many of the typical steps associated with RNA nanoparticle synthesis, purification and assembly, greatly reducing the time required to prepare particles for delivery. This cotranscriptional assembly can also be made compatible with use of 2′-modified NTPs. Incorporation of 2′-F-dUTP during cotranscriptional nanoring (or nanocube) assembly greatly increases its resistance to nuclease degradation in blood serum, a desirable trait for applications associated with in vivo delivery.[12]

De Novo Design of RNA Scaffolds.—For some RNA scaffolds of particular shapes and properties, no naturally occurring motifs may be able to be identified. As in the case of the in silico designed RNA cube, the RNAjunction database does not contain any 3-way junction that corresponds approximately to a cube corner.[10,83] In this case, several 3D design strategies present themselves: one approach is to design a 3D structure with the proper 3D motifs (in this case a 3-way junction with 90° interhelix angles) de novo (Figure 1c.i). Alternatively, instead of focusing on the 3D motifs, one may optimize the position of the involved helices and then place bridging RNA strands de novo. Using the latter method, an RNA nanocube was designed in this manner and assembled experimentally.[106]

Atomic-level molecular dynamics simulations may aid in improving the 3D structural model of the nucleic acid nanostructure and may aid in predicting some of its properties.

Alternative conformations of the desired building blocks can also be obtained by subjecting only the building blocks to molecular dynamics simulations.[107] This approach can be used when, because of its size, detailed simulations are not feasible for an entire nanostructure model.[108] Computationally less costly coarse-grained methods, such as those based on the elastic network models,[109−112] can also provide valuable information about the most biologically relevant low frequency motions (normal modes) of the building blocks, as has been shown for nucleic acids and nucleic acids/protein complexes.[11,113–117] Through these methods it is feasible to evaluate the entire nanostructure that atomic resolution methods may still be unable to handle.

A coarse-grained anisotropic network model (ANM) was applied to the characterization of the RNA cubic nanostructures.[106]The cubes have helical edges and single-stranded corner linkers, and the models were created for several cube variants, differing from each other by the length of the single-stranded bridges between the helical edges (0−3 nts). The resulting models predicted different efficiencies of assembly for the considered variants due to steric constraints in the cube corners. These predictions were confirmed experimentally in vitro. However, the sizes of the nanocubes measured in solvent appeared to be larger than the initial models would suggest. ANM simulations were used to demonstrate that the dynamics or distortions of the cubes increase their radii of gyration. This was needed to be considered to reconcile the initial differences between the modeling results and the experimental data. In addition, the ANM simulation results deepened our understanding of the reasons for the differences in the efficiency of assembly and in the melting temperatures of the cube variants. Different sequence designs were tested as well: nanostructures assembling from 6 and 10 RNA molecules were tested.[106,118]

Functionalization of RNA Nanostructures.

Regions of complementary base pairing in RNA-based nanostructures can be used to easily append functional RNA motifs to the scaffold core, without the need for separate covalent linkages and complex chemistries.[91] RNA nanostructures are versatile scaffolds that can be repurposed to harbor various functional RNA elements. Individual monomer molecules of nanoconstructs can be functionalized with additional RNA moieties by extension of either the 5′ or 3′ ends to encode functional elements for specific applications.[12,91,119,120] In fact, design strategies can be devised so the functional elements, such as siRNA, are encoded within the sequence of a scaffolding unit, if so desired.

RNA nanorings present a robust and versatile method for the delivery of siRNA or other functional RNA entities to cells. The RNA nanoring was originally envisioned as a delivery vehicle for Dicer substrate RNA duplexes (DS-RNAs),[121] and indeed DS-RNA functionalized nanorings are processed by Dicer to liberate siRNA duplexes from the ring scaffold (Figure 1b.ii).[91] More recent work has shown the nanoring can be effectively delivered to cells in both tissue culture and xenograft tumor models.[14]

Nanorings functionalized with 6 different Dicer-substrate RNAs (that are processed by Dicer to siRNA) against 6 different targets of HIV-1 showed significant reduction in viral protein expression in transfected cells.[14] Incorporation of single-stranded toeholds extending from each monomer unit further increases the ease with which functional entities can be attached

for given applications by simply incubating the scaffold with new functional elements that harbor complementary toeholds to specific monomers of the ring.

Building on the ease with which siRNA could be conjugated to an RNA scaffold, additional functional RNA moieties including miRNA, aptamers, ribozymes, fluorescent tags, proteins, and other elements can be added to increase the functional diversity of the resulting RNA nanoparticle. Nanorings functionalized with the J18 aptamer against human epidermal growth factor receptor (EGFR) bind to cells which express high levels of EGFR on their surface, while dye labeled nanorings can be used to locate the scaffolds associated with the cells (Figure 1b.iii).[14]

The same functionalization strategies outlined for the nanoring scaffold can be applied to the nanocube scaffold, as well as other related RNA nanoconstructs that have been extensively tested and characterized (Figure 1c.ii).[10,106] The key difference is the assembly process relying on interstrand interactions of multiple RNA chains.

Hybrid Technology in RNA NPs.

In a DS-RNA duplex, one RNA strand can be substituted by a corresponding complementary DNA strand, to confer nuclease resistance to the construct, resulting in an RNA/DNA hybrid. Another complementary construct, a DNA/RNA hybrid, is required to assemble with the first, in order to confer the functionality to the recombined construct (Figure 2a.i). The extension of the hybrid strands, either RNA or DNA, with single stranded n-nucleotide toeholds, offers anchorage for initiation of hybrid strand exchange (Figure 2a.ii). The reassociation resulting in the original DS-RNA duplex, with a double stranded DNA side product (Figure 2a.iii), can be initiated through recognition of complementary toeholds, composed of either RNA or DNA, because of thermodynamic differences between the RNA and the DNA duplexes. Such constructs have the unique property of being conditionally activated only when both complementary hybrids are present. Complementary hybrid constructs can be designed for DS RNAs, and as well as other constructs incorporating multiple functionalities.[81]

Extending the hybrid concept to RNA NPs, the functionalized arms of the nanoparticles can be split into hybrids with either DNA[14] or RNA toeholds.[81] Recent advances show that the functional elements associated with the ring scaffold can be designed for conditional activation in response to a supplemental hybrid construct. For instance, nanorings have been functionalized with RNA/DNA hybrid arms which are able to produce Dicer-substrate RNA duplexes upon interaction with a separate hybrid entity (Figure 2b.i).[14,122] This conditional activation can be extended to other functional entities such as FRET pairs and aptamers,[16] producing even greater control over the degree of functionalization of the constructs.[123] Cubes with hybrid RNA/DNA arms reassociated with their cognate DNA/RNA hybrids to produce conditionally activated functional nanoparticles with DS-RNAs, capable of silencing GFP (Figure 2b.ii).[118]

One advantage of this novel technique is that theoretically many functional RNAs (silencing, activators, aptamers, etc.) can be split with the use of complementary DNAs into the nonfunctional RNA/DNA hybrids, and the initial RNA function can be restored only through

reassociation of the hybrids. Besides therapeutic functionalizations, additional incorporation of functional elements into the RNA nanoparticles allows for multiple simultaneous applications, such as molecular imaging and biosensing.[124] In some cases, RNA can be substituted by DNA to provide a DNA scaffold for the conditionally activated hybrid RNA/DNA arms. In the nanocube's case (Figure 2b.iii), comparative characterization studies indicated significantly lower interferon and proinflammatory cytokine responses for a DNA-based scaffold. These are important characteristics for the intended therapeutic use.[118]

■ CHALLENGES IN DELIVERING RNAS

There are several issues that need to be addressed when delivering drugs or nanoparticles to cancer and diseased cells. The therapeutic agents need to get in the vicinity of the targeted cells, and they have to enter these cells to be fully effective. The enhanced permeability and retention (EPR) effect in many cases increases drugs or particle accumulation in the vicinity of a tumor environment, normally due to the leaky vasculature surrounding the tumor.[125] This can usually be accomplished without targeting agents. Also, the addition of appropriate targeting moieties can facilitate the delivery of therapeutic agents.[126] Such targeting moieties can be designed to specifically target tissues, whole cells,[127] or particular proteins or membrane receptors.[128,44] Various kinetic and thermodynamic hindrances can also impede the RNA NPs cellular delivery to their target. These challenges exist at various levels of the delivery process, and include not only cellular uptake, but also extracellular barriers, that is, RNA's stability and degradation in blood serum, its organ uptake and biodistribution, the hepato-renal clearance, as well as immunogenic response, and intracellular barriers, including endosomal escape and off-target effects. These challenges are discussed more in detail below.

Degradation.

RNA's half-life at physiological conditions is fairly short, ranging from a few minutes to an hour, leaving little time for the systemically administered RNA to reach an intracellular target.[129] Several chemical modifications have been explored in an attempt to increase the resistance of siRNAs to nuclease digestion. The chemical versatility of RNA allows for easy modifications of the ribose 2′-position, as well as the 3′-position at the oligonucleotide terminus.[129,130] At the strand's 3′-end, the phosphate group can be replaced with phosphothioate or boranophosphate.[131] Although the latter modification prolongs siRNA's half-life, it might result in decreased interference activity and toxicity caused by metabolites. As the 2′-OH within a siRNA is not required for silencing, 2′-ribose modifications have become a common method to increase siRNA stability without sacrificing potency. Several 2′modifications can be used to protect RNA from nuclease degradation, such as 2′-fluorination, 2′-oxymethilation, and 2′amination of pyrimidines.[12,132−134] In some cases, the substitutions can be made without negatively affecting siRNA efficiency.[135] In fact, in some instances, 2′-modified siRNAs have been observed to display enhanced potencies, such as in vitro increased target silencing, reduced off-target effects, and decreased innate immune response, as compared to unmodified siRNAs.[136] Locked nucleic acids (LNAs) with methyl linkages between the ribose's 2′- and 4′-positions, are another methodology used to increase RNA nuclease resistance.[136] This modification does not affect its

compatibility with the RNAi machinery, preserves or increases hybridization affinity with mRNA and can decrease off-target effects, sometimes with improved efficiency of the siLNA.[136,137]

Previously, the synthesis of RNA molecules containing 2′ modified nucleotides required the use of chemical synthesis or in vitro transcription using a mutant T7 polymerase to achieve significant RNA yields. However, recent work has revealed that the addition of Mn^{2+} during in vitro transcription allows wildtype T7 RNA polymerase to incorporate 2′-fluoro dNTPs and produces transcription yields comparable to a standard transcription reaction using unmodified NTPs.[12]

Stability in Blood.

Interactions of RNA with the bloodstream are a concern, as retention and stability in blood are necessary to achieve cellular uptake following systemic delivery. The ribose-phosphate RNA backbone of RNA is susceptible to enzymatic hydrolysis. Nanoparticle surface modifications with hydrophilic polymers can increase the circulation time and delay nanoparticle clearance, allowing the nanoparticles to reach their targeted tissue.[138,139] Thus, characterization of nanoparticle interactions with protein components of the blood is essential, as nanocarriers have unique properties and mechanisms of interaction. Interaction between nanoparticles and coagulation factors could result in deficient, prolonged, or unwanted coagulation. Formation of larger aggregates in the bloodstream can lead to thrombosis and presents a major concern when considering nanoparticle injection. Even in cases in which the same basic material and overarching methodology are used to create nanoparticles that may differ only slightly from one another (e.g., differing liposome, dendrimers, quantum dots, etc.), these nanoparticles may result in very different interactions with various components found in the blood.[140]

Hepato-Renal Clearance.

Clearance via kidneys impacts very small particles (below approximately 8 nm or about 40 kDa), while very large nanoparticles (greater than 200 nm) can be cleared by the spleen or get trapped in the lungs.[35,141] “Naked” siRNAs, for example, are subject to renal clearance minutes after systemic administration.[142] siRNAs with chemically modified nucleotides or conjugated with cholesterol withstand the renal clearance up to half an hour. Furthermore, siLNAs (locked nucleic acids) and siRNAs complexated with lipid-based formulations, such as chitosan, liposomes, or JetPEI, impede the renal clearance. Biodistribution studies show accumulation of these formulations in various organs after 24 h. Conversely, the potential problem of rapid clearance of small molecules presents an opportunity for nanotechnology: increasing the particle size above 40 kDa may increase the half-life for renal clearance.[35]

Toxicity and Immunogenicity.

Exogenous RNA delivered to cells can activate an immune response through the recognition of the delivered RNA by pattern recognition receptors (PRRs) via Toll-like-receptor (TLR)-dependent pathways and TLR-independent pathways.[143] Toll-like receptors are membrane receptors that are activated via different structural properties of RNAs. Toll-like receptors TLR7 and TLR8 are bound to membranes of intracellular vesicles and recognize single-

stranded RNA. TLR3 is activated by binding double-stranded RNA.[144] Recognition of double-stranded RNA by TLR3 seems to be due to at least two different binding conformations (a stable conformation attainable by RNA duplexes with at least 46 base pairs and a less stable conformation for RNA duplexes with lengths between 21 and 30 base pairs).[145]

In contrast, some pattern recognition receptors are not membrane-bound but cytoplasmic, and are activators of TLR-independent pathways. Examples are Protein Kinase R (PKR) as well as RIG-I (retinoic acid-inducible gene 1). It was shown that RNA duplexes with lengths of at least 33 base pairs lead to PKR activation and that the response is maximal for duplexes that are 80 base pairs in length.[146] Another pathway that is activated by double-stranded RNA is the 2′, 5′-AS/RNase L pathway. Double-stranded RNA of at least 70 base pairs activates 2′, 5′-oligoadenylate synthetase (2′, 5′-AS) that then leads to the activation of RNase L.[147] RNase L is a cytoplasmic endoribonuclease, whose activation can trigger the degradation of cellular and viral RNA (thus leading to the reduction of protein synthesis). Lastly, the protein RIG-I mediates an immune response by recognizing uncapped 5′-phosphorylated RNAs−another molecular pattern that is indicative of RNA originating from an invading pathogen.[148]

Activation of these pattern recognition receptors can lead to an activation of an immune response in a variety of ways. For example, activation of interferons can lead to the expression of interferon-induced proteins (the mentioned pattern recognition receptors like PKR and RNase L are themselves interferon-inducible).[147] Induced interferons can be secreted by a cell and trigger inflammatory responses in neighboring cells. Another response is the activation of eIF2α (a translation initiation factor) that in turn leads to inhibition of translation.[147] Such responses of the innate immune system can have a cumulative effect of increasing the prevalence of apoptosis. The working of the innate immune system was summarized by the following statement: “Similar to virologists, the innate immune system may therefore have learned to classify viruses by their genomes.”[148]

Importantly, utilizing modified nucleotides can reduce the activation of innate immune response.[149] For example, exchanging the 2′-hydroxyl of uridines with 2′-fluoro, 2′-deoxy, or 2′-O-methyl dramatically reduces the recognition of exogenous RNA by Toll-like receptors.[150]

It should, however, also be remembered that the majority of delivered material in nucleic-acid based therapeutics can be attributed to the delivery agent or to the formulation as opposed to the active molecules. In other words, the toxicity and immunogenicity of the combined system depends to a substantial amount on the properties of the delivery agent. Because there is such a wide variety of delivery agents with vastly different properties, reviewing corresponding cellular responses is beyond the scope of this review. The reader is encouraged to consult the literature for ascertaining the properties of the considered delivery agents.

Cellular Uptake.

Cells have developed distinct pathways for cellular uptake of materials including endocytosis, pinocytosis and macropinocytosis. These processes can be dependent on active energy and cytoskeletal elements. Cellular uptake can be viewed as molecules mastering the challenge of passing a formidable barrier in the form of the lipid bilayer cellular membrane. Cellular uptake is mediated by a variety of different mechanisms: single molecules can diffuse through the cellular membrane, provided that their physicochemical properties are within certain ranges. The “Lipinski Rule of 5” summarizes these constraints by stating that molecules have “drug-like” properties if they have limited water-solubility, are “small” (molecular weight less than 500 Da) and contain less than 5 hydrogen bond donor sites and less than 10 hydrogen bond acceptors.[151] Lipinski himself noticed exceptions to these observations and attributed this to molecular transport mechanisms that go beyond diffusion across the membrane.

Efficient cellular uptake is also possible for larger complexes and nanoparticles: In a recent report, several classes of antisense oligonucleotides (ASOs) were analyzed for uptake mechanism and uptake efficiency.[152] It was found, that scavenger receptors (SCARAs) contributed to receptormediated cellular uptake of the ASOs. Moreover, their incorporation into nanoparticles was correlated with improved cellular uptake, resulting in a “second window” of efficient uptake for particle sizes ranging from 22 to 60 nm. Scavenger receptors are involved in the cellular uptake not only of ASOs, but of a wide range of molecules such as cell penetrating peptides (CPPs).[153,154] Combined with the fact that some CPPs self-assemble into nanoparticles,[155] one can view this as emerging evidence for a general preference for receptormediated cellular uptake for self-assembling nanoparticles.[152]

Because of their high molecular weight, size, and charge, RNAs cannot readily diffuse through cellular membranes. Diffusion of charged molecules, such as RNA, which is negatively charged, is thermodynamically unfavorable. Depending on whether RNAs are delivered “naked” or assisted by carriers, and on the resulting size of RNA/carrier complexes, RNAs can enter the cell through different pathways. Particles of 200 nm or less seem to be internalized by clathrin-mediated endocytosis, with more than half passing through the membrane in the first half of hour. Particles bigger than 200 nm internalize seemingly by caveolae-mediated endocytosis, and they take hours to accumulate. No uptake has been detected for particles >1000 nm in size.[156]

Endosomal Escape.

Since endocytosis is the main entry route for delivered RNAs, endosomal escape is crucial for their functioning. One can distinguish “early” and “late” stages of endosomes. These stages are characterized by increasing acidity of the endosomal compartment.[35] The most common endosomal escape mechanisms are membrane destabilization[157] or rupture due to the “proton-sponge effect”.[158] New delivery systems exploit the change in the environment’s pH when the nucleic acid-delivery agent complex enters the endosome. pH-dependent polymers, dendrimers, peptides, fusogenic lipids, and chemical agents have been used to aid in the particle’s release from the endosome.[159] The efficiency of therapeutic interfering

RNAs can be evaluated through biodistribution, cellular and subcellular localization studies, endosomal escape efficiency and RISC-loading efficacy studies.[160]

Off-Target Effects.

The efficacy of the siRNAs silencing can vary as a function of the targeted mRNA sequence. Of concern are off-target effects, where siRNA binds in a miRNA-like manner to the message, with only partial complementarity to mRNA, thus repressing the message of nontargeted genes and possibly leading to undesired effects. Sequence searches ensuring the uniqueness of the siRNA target site can alleviate off-target effects to some extent. Off-target effects can occur in an analogous manner for other types of RNA therapeutics that rely on sequence-complementarity to the transcript or genomic regions.

■ DELIVERY METHODS FOR RNA THERAPEUTICS AND RNA NPS

In the remainder of this Review, we present a variety of strategies for cellular delivery of RNA nanostructures. It should be understood, that there may be a certain amount of variability in the precise structural composition of the shown assemblies.

“Naked” Delivery.

Nucleic acid delivery methods can be roughly classified into physical or chemical methods. Nonmodified naked RNAs can be delivered directly to the target site through a variety of physical means, frequently aided by high concentration or a mechanical, magnetic or electric force. Nuclear or cytoplasmic microinjections (mechanical force),[161,162] electroporation,[163] electrical mediation,[164] cell squeezing (physical force),[165] sonoporation (ultrasound),[166,167] and optical transfection (lasers),[168−170] deliver nucleic acids directly to the targeted site with limited inhibiting effects due to poor uptake and degradation.[171] Nonetheless, there is currently a variety of clinical trials of siRNA-based therapeutics that are based on naked delivery.[35]

In certain cases, “hybrid” methods employing a combination of physical and chemical means are used to deliver nucleic acids (NA) to cells. For example, nucleic acids, such as siRNA, can be chemically conjugated with nanoparticles, and the nanoparticles are delivered to cells through physical means. The nanoparticles also present specific physical and chemical properties that assist nucleic acids in entering cells. Such hybrid delivery methods include the gene gun (inert solid (gold) NP + NA),[172] magnetofection (magnetic NP + NA),[173] impalefection (nanostructure NP + NA),[174] and bombardment (microprojectiles + NA).[175,176] Hybrid methods also include nucleofection, where electroporation is used in conjunction with cell specific reagents that aid in membrane disruption.[177] Physical methods offer the advantage of direct therapeutic delivery to the targeted site or tissue, increased availability because of proximity and avoidance of systemically induced side effects, such as immune system stimulation.[178] The biggest drawback is that not all types of cancers and diseases are topical or localized, and thus amenable to physical siRNA delivery methods.

Another form of delivery is the escape of the RNA cargo from a repository placed in proximity to the target site. In a recent study a siRNA-releasing polymer pellet (called LODER) was surgically implanted in close proximity to an otherwise difficult to treat

pancreatic cancer. The synthetic siRNAs were programmed to target the mRNA of the oncogenic KRAS gene. The authors demonstrated favorable delivery properties of their approach and a clinical benefit of their RNAi-based cancer therapy.[179] The biggest disadvantage of the physical delivery methods is nonspecific targeting, limiting their use to topical or local tissues.180−182

A more general approach to evade nuclease degradation is incorporation of modified nucleotides. pRNA NPs with fluorinated Us and Cs (2′-fluorine-deoxy-CTP and 2′fluorine-deoxy-UTP) have a much longer half-life and are more resistant to nucleases.[183] Such pRNA NPs, incorporating modified pyrimidines into the RNA strands, were functionalized with siRNAs. The tetramer pRNA construct augmented with distinct siRNAs successfully targeted firefly luciferase.[184] A 3WJ-based pRNA NP functionalized with folic acid for epithelial cancer targeting and decorated with fluorescent dies was used for imaging in vitro.[185] Systemically delivered X-core pRNA incorporating folic acid targeting moieties and Alexa 647 displayed preferential accumulation in targeted tissues in vivo. Other pRNA NPs incorporating aptamers, such as malachite green, STV-binding, prostate cancer aptamer, endothelial growth factor aptamer, or other functional elements such as fluorescent image markers, the HBV ribozyme, and various siRNAs were studied and characterized in vitro and in vivo.[97,98,186−190]

Mediated Delivery.

Mediated delivery refers to cases where RNAs cross biological barriers while forming noncovalent complexes with other molecules that act as delivery agents.

Polymers.—RNAs can form complexes with various cationic polymers through electrostatic interactions potentially leading to efficient delivery in vitro and in vivo. Potential advantages of current polymer-based carriers include tunable chemical properties, chemical stability, and biocompatibility. The properties of RNA−polymer complexes depend on the type of chosen polymer. For example, delivery agents complexated with the cancer drug paclitaxel (Taxol) can decrease or increase its toxicity.[179] Polysaccharide polymers, such as chitosan, have received attention as nanocarriers because of their high biocompatibility and low toxicity.[191] Several current synthetic polymers are used for delivery of nucleic acids, including polylactic acid, *N*-(2-hydroxypropyl)-methacrylamide copolymer, and dendrimers. Delivery of nucleic acids in vivo has been achieved as well with a commercially available linear polymer, “in vivo JetPEI”.[192,193]

Hydrogels.—Nanohydrogels are yet another promising type of nanocarrier for siRNA delivery, synthesized by polymerization of pentafluorophenyl methacrylate and tri(ethylene glycol)-methyl ether methacrylate.[194] They are polymer-based cationic carriers which can complexate with and deliver siRNA in vivo. Both the hydrogels and the siRNA delivered separately aggregate with the proteins in blood, but the hydrogel−siRNA complex does not aggregate. Complexing siRNA with nanohydrogel particles shows strong stability while minimizing aggregation, providing an effective method for in vivo siRNA delivery.[195]

Peptides.—An alternative cross-membrane delivery mechanism can be achieved with cell penetrating peptides (CPPs) that are capable of crossing the cellular membrane directly into the cytoplasm.[196] The mechanism of entry may depend on the type and composition of peptide and is the subject of ongoing research. The possible entry mechanisms include endocytosis, pore-formation, or receptor mediated uptake.

Dendrimers.—Cellular delivery of functional RNAs has been accomplished using small molecule dendrimers (i.e., molecules that self-assemble in a tree-like fashion).[197] For example, positively charged ammonium-terminated carbosilane dendrimers have been used for the cellular delivery of siRNAs that target HIV genes.[198] Poly(amidoamine) (PAMAM) and polypropylenimine (PPI) dendrimers are frequently utilized for drug delivery. The delivered RNAs form a complex with the dendrimer (called dendriplex) based on electrostatic interactions. Advantages of dendrimer-based delivery systems are their favorable protection of the RNA cargo from degradation, their ability to deliver a wide variety of compounds and their biocompatibility. Potential disadvantages are nonspecific cytotoxicity and liver accumulation because of their high charge density, as well as rapid clearance and limited control over drug release.[197]

Conjugated Delivery.

RNAs can be covalently conjugated to various carrier platforms to aid their intracellular delivery. Possible RNA-carrier conjugate systems range from the relatively small and “simple”, such as aptamers or small drug molecules, to lipids, peptides and proteins, polymers, and composite nanoparticles.

Aptamers.—Nucleic acids aptamers can be evolved through SELEX and SELEX-adjacent methods against particular cell types based on the differential expression of certain membrane proteins in the normal versus pathogenic cells. Consequently, aptamers binding membrane receptors have been selected against whole cells. Thus, aptamers can be used to facilitate targeted delivery of therapeutic molecules to a specific cell type. Note that aptamers were mentioned earlier in the section on Functional RNAs. This quality of aptamers reflects their dual role: aptamers can be used as therapeutic agents or as delivery agents. In the first case it is sufficient for aptamers to bind and block or activate a protein; in the later it is necessary for the aptamers to be internalized inside the cell along with their cargo. Thus, aptamers can facilitate targeted delivery, as well as have the role of an RNA nanoparticle cargo that carries out a molecular function related to, for example, protein inhibition or imaging.[199]

There are several well established aptamers targeting oncogenic or pathogenic cells, such as lymphoma or thrombosis, to name a few. Thus, aptamers can be used for cell specific targeting, such as tumor cell recognition.[200] Whole-cell aptamers have been selected against bacteria, parasites, viruses, and cells,[201−203] and some were proven to internalize into the cell.[199] Moreover, aptamers have been shown to have low, if any, toxicity or immunogenicity.[200,204] Aptamers have been used both as direct carriers for drugs or interfering RNAs, or incorporated in nanoparticles containing therapeutic molecules. Aptamers conjugated with drugs were used to target various types of cancers, such as breast,

colorectal, prostate, pancreatic, liver, and lymphoma, and certain inflammatory diseases, as for example Crohn's, rheumatoid arthritis, psoriasis, and systemic sclerosis, age-related macular degeneration, and HIV-targeting therapies.[205] Aptamers-interfering RNA Chimeras were designed to deliver si, mi, antimiR, sh RNAs for tissue specific[206,207] or HIV[208] targeting and therapeutics. Aptamers have also been coupled with inorganic nanomaterials, such as gold nanoparticles, through modified chemistry on the nanoparticle surface.[209] A DNA aptamer, Sgc8, selected against T-cell acute lymphoblastic leukemia cells (CCRF-CEM cell line),[210] was covalently coupled with the drug doxorubicin and used in targeted chemical therapies.[209] The same aptamer has been attached to a gold nanoparticle incorporating doxorubicin to target specifically the CCRF-CEM cell line.[211] A prostate-specific membrane antigen targeting aptamer was conjugated to siRNA with streptavidin −biotin interactions and shown to uptake even without the aid of delivery agents.[212]

As with other RNA entities, incorporation of modified nucleotides can be used to avoid the degradation of aptamers by nucleases. To this end, SELEX methods can be performed using 2′-modified nucleotides to produce active, high affinity aptamers with increased resistance to nucleases.

Inorganic Nanoparticles.—Gold nanoscale spheres and rods have a variety of applications in biomedicine. For example, gold nanorods have been utilized for the cellular delivery of RNA/DNA nanoparticles.[213] Gold is a frequently used moiety for the in vivo delivery of compounds because of its ease of conjugation, high stability, and biocompatibility. Gold nanoparticles have electronic and optical properties tunable through their shape and size. Flow cytometry and atomic absorption spectroscopy showed selective targeting for cancer cells and the effective killing of the targeted cells. Gold nanorods with the AS1411 aptamer were used to construct a particle with a dimeric G-quadruplex.[214] A gold nanoparticle functionalized with the Sgc8c aptamer was used to deliver doxorubicin.[211]

Microsponges.—RNA's instability in vitro and in vivo limits the amount of siRNA that can reach its intracellular target. Traditionally, this is mitigated through incorporation of modified nucleotides in the RNA or the use of additional carriers that may result in increased cytotoxicity or immunogenicity. A novel approach to therapeutics that allows for delivery of more than half million siRNA copies in a particle presents itself in RNAi-microsponges. DNA encoding for both sense and antisense siRNA strands and the T7 promoter sequence were nicked in a circular DNA. The rolling circle transcription principle was used to produce long RNA strands containing tandem copies of hairpined siRNAs. The resulting RNA folds into secondary structures and self-assembles into a compact tertiary structure. The final structure is comprised of repetitive copies of Dicer-cleavable RNA strands that self-assemble into pleated sheets and ultimately form sponge-like microspheres. The microsponges' structure was visualized by scanning electron microscopy.[215,216]

Microsponges circumvent several cellular barriers by encompassing the properties of both a carrier with its cargo. Additional complexation with cationic polymers like polyethylenimine (PEI) generates a net positive surface for enhanced uptake without altering the siRNA conformation. The microsponge's design principles allows for incorporation of multiple siRNA species intended for combination therapies. The efficiency of RNAi-microsponges

for delivery and carrier volume provides an effective new tool in RNA nanoparticle therapeutics.[217] The disadvantage of this technology is that the RNAi machinery can be overpowered by the presence of a high dosage of small interfering RNA molecules, at any given step, from Exportin-5 to Ago/RISC overloading.[218,219]

Encapsulated Delivery.

Some drug delivery vehicles function by encapsulating the molecular cargo in a particle, so the delivery vehicle rather than cargo predominantly determines the delivery properties. A particle that consists of a lipid-phase membrane and an aqueous phase core is called a vesicle; a particle consisting only of a hydrophobic phase without an aqueous phase is called a micelle. These different molecular entities can lead to encapsulation of molecular cargo.

Cationic Lipids.—Some lipids can form micelles or vesicles, thus being potential nanocarriers for drugs and pharmaceuticals.[220−222] Several features of lipids make them an attractive candidate for drug delivery, such as their biocompatibility, ability to assemble into discrete structures to accommodate a payload of drugs,[223] ability to manipulate the particle size distribution[224−226] and modification of the nanoparticle surface for ligand attachment.[222,227] These basic traits of lipid molecules have been exploited further for the delivery of nucleic acids including DNA and RNA.[228,229]

An important consideration for using lipids for nucleic acid delivery relies on the presence of one or more positive charges in the molecules of cationic lipids that can electrostatically interact with negatively charged nucleic acids. Needless to say, the choice of lipid with desired hydrophobic, hydrophilic, and linker domains will dictate the resulting interactions with nucleic acids and overall particle design. In this Review, we will mention the lipoplexes (liposomes), in the context of siRNA delivery.[230−233]

Historically, in the field of nucleic acid (including siRNA) delivery, the cationic lipids DOTAP and DOTMA have been extensively utilized and studied for cell culture-based systems as well as for small animal studies (Figure 3.a). As expected, electrostatic interactions between the positive charges associated with the head groups of the lipid molecules and the negative charges on the siRNA are critical for the formation and stability of the resulting lipoplexes. It may be noted that the stereochemistry of the cationic lipids as well as their self-assembly characteristics also contribute to the overall efficiency of siRNA transfection.[234]

Noncationic lipids such as cholesterol or DOPE are often included to provide stability of the self-assembled particles that contain the cationic lipids. These lipids are known to facilitate the complexation with RNA, as well as improve transfection efficiency.[235] Therefore, such lipids are dubbed as “helper lipids”. A lipid mixture containing DOTAP and DOPE is a frequently used formulation for RNA delivery and additional lipid molecules, such as DSPC and cholesterol have also been included in the DOTAP/DOPE mixture to enhance the efficiency of intracellular delivery of siRNA.[230]

Lipofectamine 2000 and RNAimax are commercially available cationic lipids frequently utilized for transfection of nucleic acids (including siRNAs or RNA nanostructures).[236] The

electrostatic interactions between the positively charged lipid and the negatively charged nucleic acids lead to the formation of lipoplexes.[35] Nanorings functionalized with DSRNAs and complexed with the commercially available transfection agent Lipofectamine 2000 (Invitrogen) display silencing of eGFP in human breast cancer cells (MDA MB-231) that stably express the fluorescent protein.[12]

Sood and colleagues reported studies using neutral liposomes for siRNA delivery.[237] A neutral phospholipid, DOPC, was utilized to encapsulate a fluorescent siRNA targeted to downregulate an oncoprotein EphA2, overexpressed in bladder cancer. DOPC formulations also contain low levels of a detergent Tween20 that presumably assists in maintaining the liposome integrity during the reconstitution step.

Stable nucleic acid-lipid particles (SNALPs) are composed of cationic and fusogenic lipids and contain a polyethylenglycol (PEG)-lipid.[238] The combination of these lipids in SNALPs renders them amenable to efficient intracellular uptake with an endosomal release potential. As expected the PEG-lipid modulates the surface charge from positive to neutral, while contributing to stability in circulation. The SNALP design also relies on uncoating of the PEG-lipid near diseased tissue resulting in the presentation of the positively charged siRNAlipoplexes for intracellular uptake. PLK1 SNALP (TKM-PLK1, TKM-080301) is a formulation that contains small interfering RNA (siRNA) directed against the serine/threonine kinase PLK1 (an enzyme involved in cell cycle progression) and is currently undergoing clinical trials (NCT01262235).

Lipid-based nanoparticles (LNP) have been employed to deliver both in vitro and in vivo and are yet another class of therapeutic RNAs: mRNAs for protein supplementing or protein replacement therapies.[76] Moreover, mRNA-based vaccines successfully induce immune response with prophylactic resolutions. These vaccines are delivered with smart designed lipid nanoparticles, which incorporate targeting moieties on their surface and endosomal escape adjuvants in their composition, and are in various stages of clinical trials.[239] LNP formulation for mRNA delivery can be specifically optimized for structure and stability, protection from aggregation and nonspecific endocytosis, and enhanced endosomal escape.[240]

Oxime Ether Lipids.—Oxime ether lipids (OELs) are a relatively new class of cationic agents explored as efficient transfection tools (Figure 3a).[241,242] In contrast to commonly used transfection lipids, OELs contain oxime ether bonds and can be synthesized by a simple and efficient click chemistry approach. Oxime ether linkages are relatively stable at neutral pH but can be cleaved at low pH values, thus providing a builtin nucleic acid release mechanism. By synthesizing a variety of oxime ether lipids and examining their siRNA delivery potential, it was demonstrated that an interplay between the hydrophobicity, degree of unsaturation of the fatty acyl chains and headgroup polarities play a role in their electrostatic interaction with RNA, protection from nucleases, their uptake, and gene silencing in a cell culture system.[243]

Bolaamphiphile Surfactants.—Amphiphilic molecules contain both hydrophilic, as well as hydrophobic regions. Aggregates of amphiphilic molecules can lead to the situation

that regions with mostly hydrophilic groups try to maximize their contact with water, while the hydrophobic groups try to minimize their contact with water. Bolaamphiphiles consist of a hydrophobic aliphatic chain that is terminated by a hydrophilic headgroup or groups at each of the two ends (Figure 3b). They are able to form either micelles or vesicles. The name originates from “bola”, a type of weapon consisting of a cord with weights at each end.[244]

Bolas have been shown to be capable not only of crossing the cell membrane, but also of delivering cargo across the blood-brain barrier.[193] The types of cargos tested with bola carriers include RNAs, plasmid DNAs, proteins and peptides.[245–247] In one of the studies aimed at the characterization of the biophysical properties of different bola variants it was demonstrated in vitro and in vivo that siRNAs formulated with bolas GLH-19 and GLH-20 (see Figure 3b) can be delivered efficiently and with low toxicity.[248] These two bola variants have a hydrophobic core derived from vernonia oil and acetylcholine-based head groups (AChHG). In the GLH-20 variant, AChHG can be hydrolyzed by acetylcholine esterase, thus helping to free the cargo from the carrier in the brain. Molecular dynamics (MD) simulations of the GLH-19 and GLH-20 micelle variants predicted higher binding affinity to RNA and thus better protection against degradation for GLH-19. In vitro results agreed with these predictions, showing nearly no degradation by nucleases and a slightly better cellular uptake of the RNA with the GLH-19 carrier, while the silencing of the green fluorescent protein (GFP) expression in human breast cancer cells (MDA-MB-231) by siRNAs formulated with GLH-19 and GLH-20 was equally effective. In vivo biodistribution studies in athymic nude mice with MDA-MB-231-based xenograft tumor demonstrated differentially higher uptake of the siRNA formulated with GLH-19 into the tumor following a systemic tail-vein injection.[248] Finally, independently of the previous result, hexameric ring nanoparticles with siRNA Dicer-substrate arms, using bolas GLH-19 and 20 as carriers and delivered by intratumoral injections demonstrated silencing of the GFP expression.[14] The computational and in vitro characterization of bolas GLH-19 and 20 illustrate the balance between the binding affinity affecting transfection efficiency and the ease of RNA release inside the cell that effectively may compensate for the lower transfection rate. Similar effects were observed in a more recent study in which two other bola micelle variants GLH-58 and GLH-60 were characterized both computationally and experimentally (in vitro).[243] GLH-58 and GLH-60 have hydrophobic cores derived from jojoba oil, and two (GLH-58) or four (GLH-60) GLH-20-like ACh head groups. 3D structure modeling and MD simulations (Figure 4), combined with experimental results indicated that the design differences between GLH-58 and GLH-60 influence the balance between the RNA protection against degradation, efficiency of delivery, and ease of RNA release. In MD simulations, the GLH-58 bolas formed more stable nanosize micelles, while the GLH-60s-based micelle models tended to fall apart quickly, indicating the repulsive forces of these four-headed bolas as disruptive to their stability and aggregation. MD results showed similar amounts of solvent-accessible surface areas of RNA helices complexed with both bolas, implying similar levels of direct contact with solvent (and nucleases). These results are consistent with the experimental data showing comparable protection against nucleases in DNA helices formulated with GLH-58 and GLH-60. The four-headed GLH-60 showed a tendency to bind more strongly by bringing more head groups in the proximity of RNA, but it reached

saturation levels at lower concentration, with one layer of the bolas repulsing additional bola molecules present in solvent. By contrast, the two-headed GLH-58 can coat RNA in larger numbers and potentially multiple layers, yet its binding, measured by the number of head-groups in the proximity of RNA, is weaker, thus enabling easier release of its cargo (see Figure 4). All these characteristics combined are consistent with the experimentally observed better silencing of the GFP gene in human breast cancer cells by siRNAs using GLH-58 as carrier, relative to the GLH-60. All of the computational, in vitro and in vivo results consistently indicate that bolas can be practical nucleic acid carriers with adjustable protection and release qualities.

RNA NPs can be complexated with delivery agents that assist their uptake into cells. The presumed mechanism of cellular entry for such formulations is endosomal uptake, regardless of the type of interaction between the delivery agent and the RNA NPs (conjugation, mediation, or encapsulation). Upon endosomal release, the NP is available to perform its intended role. Such a role may simply be the delivery of a payload to the cytoplasm, where it can be acted upon by cellular components, and ultimately result in a predetermined response. Delivery of DS RNA by RNA nanoparticles allows for the generation of siRNA once processed by Dicer, resulting in decreased levels of target protein expression due the resulting RNAi response (Figure 5).

Exosomes.—Exosomes are vesicles of cellular origin. The vesicles do not possess cellular organelles but contain cellular molecular content like proteins, RNAs, and even DNA fragments. These vesicles can be repurposed for drug-delivery by loading them with the desired cargo. Cellular siRNAs delivery using exosomes has been accomplished.[249]

Viral Delivery.—Cellular delivery of RNA or DNA can be performed with viral delivery, where viral vectors transfect the exogenous cargo. Cellular delivery of exogenous RNA has been accomplished with attenuated Adenovirus, Adeno-associated virus, Lentivirus, Baculovirus and others.[250] Most applications are based on replication-defective viral vectors. A potential use of replication-competent viral vectors is to deliver drugs to otherwise difficult-to-reach solid tumor-tissue.[250] An advantage of viral vectors are their efficient transfection of molecular cargo. A potential disadvantage is immunogenicity due to a potential immune system response.[35]

■ PROSPECTS IN RNA NANOBIOLOGY

Nucleic acids are a suitable nanotechnological material for in vivo applications: their biological origin provides natural solutions to toxicity, biocompatibility and biodegradability concerns. Their inexpensive, simple and accurate synthesis, both chemically and enzymatically, is an additional advantage for utilizing them in nanomedical applications. RNA-based nanoparticles are fully programmable, with precise control over the dimension, geometry, and composition. Concurrent selfassembly with cotranscriptional synthesis allows for their in vivo production, another great advantage over other materials. The ease of designing and fabricating an “antidote” based on sequence complementarity confers RNA NPs rapid reversal of drug activity. Various RNA nanoconstructs have been assembled, yet only few have been functionalized and tested in cell culture and in vivo. The size and shape

of RNA NPs influence their efficacy and transfectability. Thus, an optimization of functional construct dimension and form would be imperative for RNA therapeutic applications. Other challenges left in RNA nanotechnology pertain to the scaling up of production and the purity of the nanoconstructs.

Systemic, intravenous delivery of therapeutic RNA NPs requires consideration to be given to all cellular barriers. The enzymatic stability of RNA in the bloodstream is one of the primary concerns. To increase their resistance to nucleases, one option is to chemically modify (at least) the most susceptible nucleotides in the single stranded-elements of the nanoparticles. Moreover, chemical modifications provide a way to tune the efficiency, enzymatic and thermal stability, biodistribution, uptake and toxicity of RNAs, especially siRNAs. Silencing lasts a few days to a week, and readministration of siRNAs might be necessary for prolonging their effect. An optimized sequence design can aid in the matter as well, further minimizing siRNA off-target effects. Therapeutic usage of RNA NPs with modified nucleotides incorporated permits functionalization for direct targeting and minimal immune response, allowing for repeated treatment required by cancer and chronic diseases.

An alternative protective method is to couple the RNA NPs with carrier platforms that can aid in their cellular delivery. Such compounds can act as transfection agents and give the RNA NPs additional protection against aggregation with serum proteins, which may interfere with the designed NP’s function. Both the RNA NP and the carrier have to avoid undesirable protein interactions and stimulation of an innate immune response. Current viral delivery platforms are used to deliver plasmid DNA and permit higher nucleic acid packing capacity, but present side effects, such as insertional mutagenesis, and intrinsic immunogenicity. Nonviral delivery carriers are generally less immunogenic and are easily appended with other functional moieties for multivalent purposes or direct targeting capabilities but have less packing ability. In the case of using a delivery vehicle to supply the RNA NPs to the cells, the overall size of the complex should be 10−60 nm to avoid renal clearance and yet be able to be internalized by the cells.

Addition of cell-specific targeting elements to the RNA NPs themselves or to the carriers can further enhance the strength and the selectivity of the initial contact between the NPs and cells. Ideally, the distribution of the particles should be specific to and uniform within the tumor, with little or no accumulation in organs, and no immune reaction.

Since endocytosis is the typical mechanism responsible for the uptake of nanoparticles into cells, late endosomal escape is a crucial aspect to be considered in the delivery platform design. Furthermore, the release of the functional components of the nanoparticle in the cellular environment has to be achieved in order to activate the designed functionalities. Recently reported designs of nucleic acid delivery platforms avoid their endosomal entrapment and lysosomal degradation. Incorporating protonabsorbing elements in cationic polyethylenimine (PEI) polymers and poly(amidoamine) (PAMAM) dendrimers, or hemolytic, pH-dependent elements in anionic polymers enhances endosomal escape. Acidification-dependent, membrane disruptive peptides or proteins make use as well of the change in the environment’s pH, ensuring the release of their cargo. Natural or nature-imitating pore-forming toxins have been employed to aid in phagosomes or phagolysosomes

escape. Lipoplexes resulting from fusogenic lipid incorporated liposomes, such as 1,2-dioleoyl-snglycero-3-phosphoethanolamine (DOPE), also promote endosomal escape. Likewise, chemical compounds, such as amphotericin B, chloroquine, or chloroquine analogs, with a lower cytotoxicity, have been successfully used as endosome-disruptive agents.

Plasmid DNA has to reach the nucleus, while miRNAs and siRNAs have to reach the cytoplasm. Several delivery platforms are suitable for in vitro use, yet they prove to be toxic in vivo. The fine balance between their transfection efficiency and their cellular toxicity is still a work in progress. Accordingly, the process of designing the RNA NPs and their delivery platforms has to balance protection, cellular delivery, and release characteristics. Taken together, one finds that the cellular delivery of RNA nanoparticles is achievable via many routes as evidenced by a growing number of publications as well as ongoing clinical trials.

■ ACKNOWLEDGMENTS

This work has been funded in whole or in part with Federal funds from the Frederick National Laboratory for Cancer Research, National Institutes of Health, under Contract No. HHSN261200800001E. This research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

■ ABBREVIATIONS

3D	three-dimensional
kDa	kilodalton
MD	molecular dynamics
NP	nanoparticles
nt	nucleotide
OEL	oxime ether lipids
PEG	polyethylenglycol
miRNA	micro-RNA
piRNA	piwi-RNA
siRNA	small interfering RNA
RNAi	RNA interference
DS RNA	dices substrate RNA
RISC	RNA-induced silencing complex

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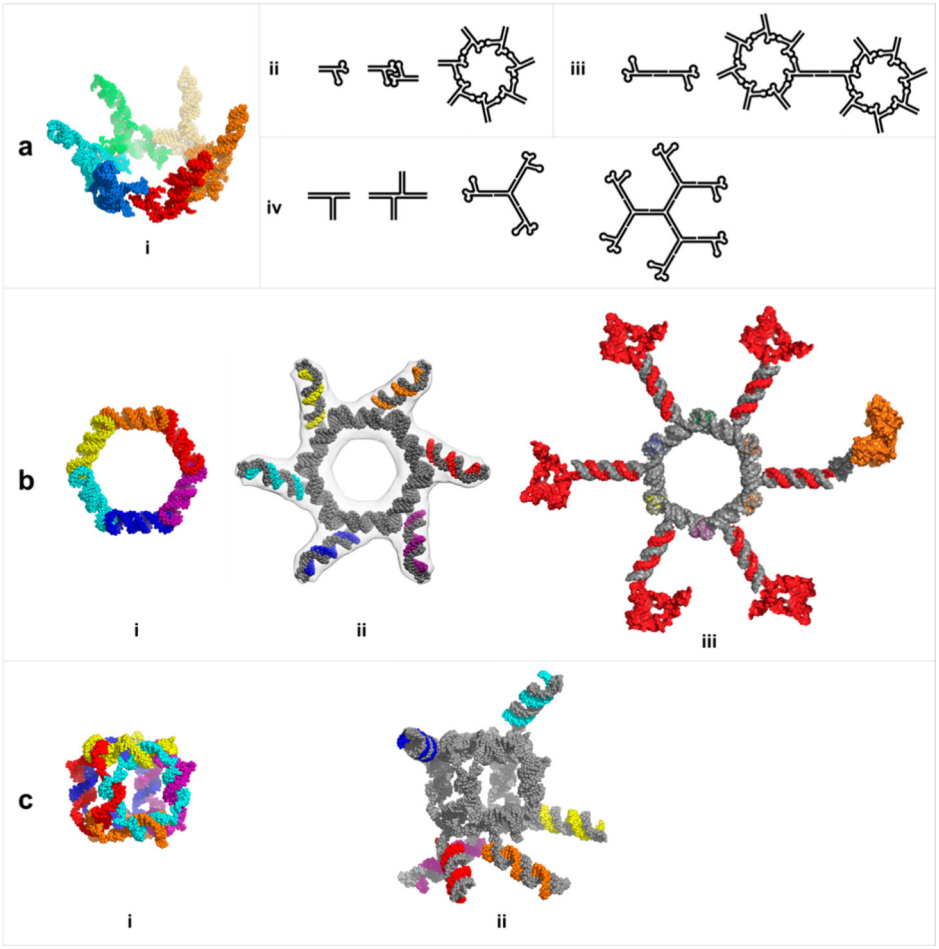

Figure 1.

RNA nanoconstructs and functionalized RNA nanoparticles. (a) Bacteriophage phi29 packaging RNA (pRNA). (i) 3D computer model of the hexameric pRNA, modeled from PDB ID 1L4O (ref 94) colored by monomeric units. (ii) Schematic representations of monomer and hand-to-hand assemblies of dimer and heptamer pRNAs. Monomers can also assemble in hand-in hand trimers, tetramers, pentamers and hexamers, not represented in this figure. (iii) Schematic representations of foot-to-foot assemblies of dimers and heptamers. Foot-to-foot assemblies include trimers, tetramers, pentamers and hexamers, not represented here. (iv) Three-way junction core, x-shape junction core, and branch-extended trimer and hexamers. (b) RNA nanoring: (i) 3D model of the hexameric nanoring, colored by monomer units; (ii) cryo-EM map superimposed on the 3D model of the nanoring functionalized with six DS RNAs; and (iii) nanoring functionalized with six DS RNAs five of which are capped with human epidermal growth factor receptor aptamers (for direct cellular targeting) and one with the red pigment phycoerythrin (for in vivo visualization). (c) RNA nanocube: The nancube was in-silico designed to assemble through intermolecular interactions between six strands. (i) 3D model of the hexameric nancube, colored by monomer units and (ii) nanocube functionalized with six DS RNAs. (a) Adapted with permission from ref 97. Copyright 2011 Springer Nature. (b) Reprinted with permission

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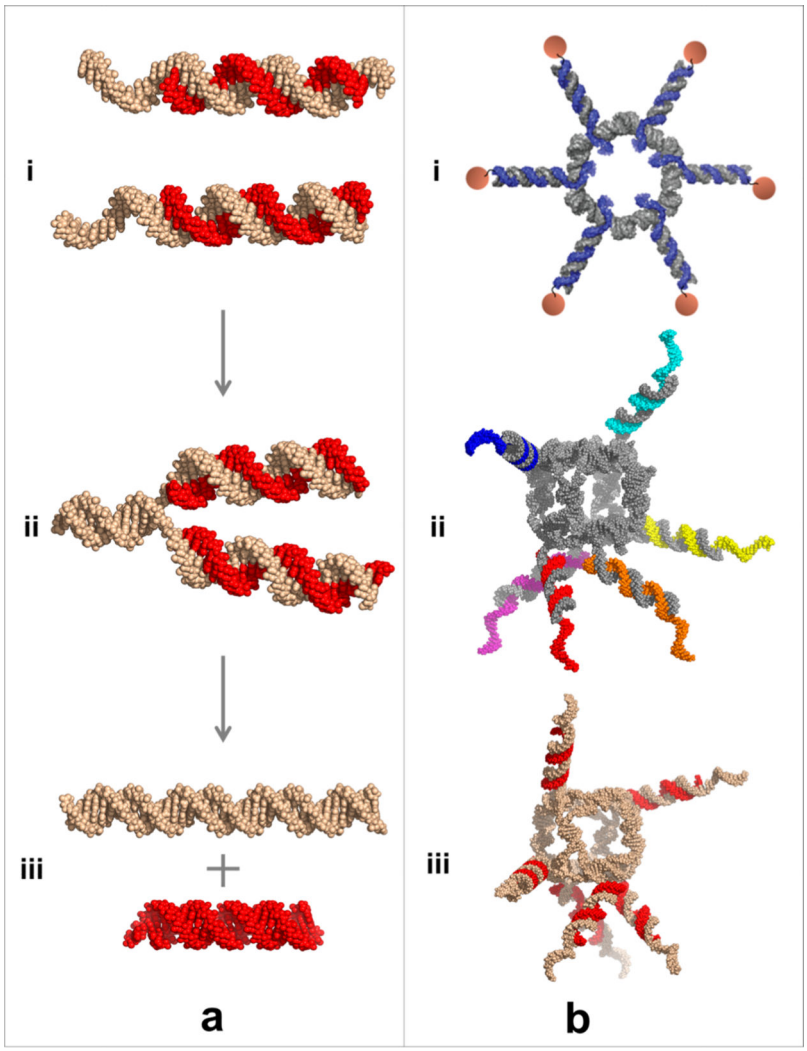

Figure 2.

DNA/RNA hybrid nanoconstructs. (a) DNA/RNA hybrids: (i) two DNA/RNA hybrids, with 12 nucleotide toeholds, containing split functionality for a siRNA; (ii) strand exchange between hybrid duplexes is initiated through base pairing of the complementary toeholds; (iii) the DNA duplex and the DS-RNA resulting from reassociation of DNA/RNA hybrids. (b) RNA nanoparticles functionalized with RNA/DNA hybrid arms. To restore its functionality, a nanoparticle needs to reassociate with six complementary DNA/RNA hybrids in order to produce functional nanoparticle and six DNA duplexes. (i) RNA nanoring with six hybrid RNA/DNA arms and Alexa 546 dyes (for FRET studies). (ii) RNA cube with six hybrid RNA/DNA arms, with DNA toeholds. iii. DNA cube with DNA/RNA arms, with DNA toeholds. (a) Adapted with permission from ref 16. Copyright 2014 Oxford University Press. (b.i) Reprinted with permission from ref 14. Copyright 2014 American Chemical Society. (b.ii, iii) Adapted with permission from ref 118. Copyright 2015 American Chemical Society.

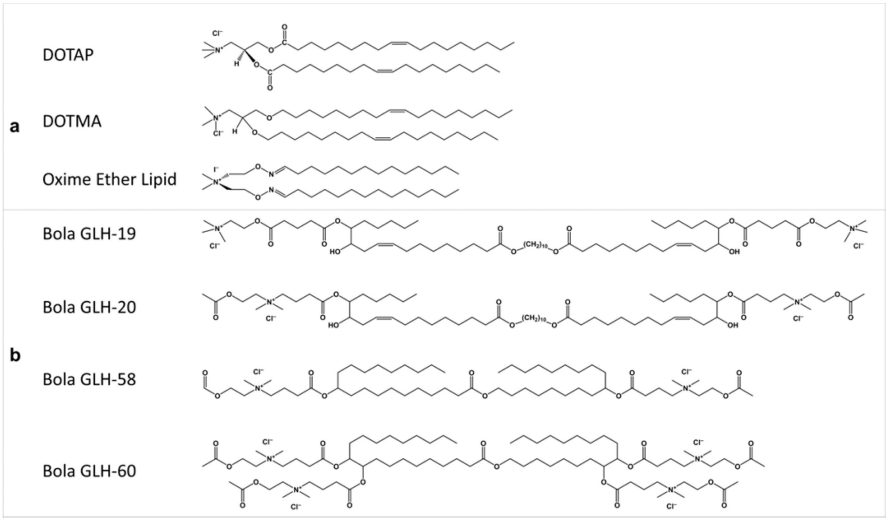

Figure 3.

Chemical structure of relevant lipids used as RNA delivery agents. (a) Single-headed lipids: DOTAP, DOTMA, and oxime ether lipid. DOTAP and DOTMA are some of the most commonly used lipids to deliver siRNAs. Both have hydrophilic heads and fatty acyl chains. Oxime either lipids incorporate in their hydrophilic head oxime ether bonds. (b) Double-headed lipids: Bola-amphiphiles (Bolas). Bolas with two single charge heads are derived from vernonia oil (Bola 19 and Bola 20) and Bolas with four single charge heads are derived from jojoba oil (Bola 58 and Bola 60).

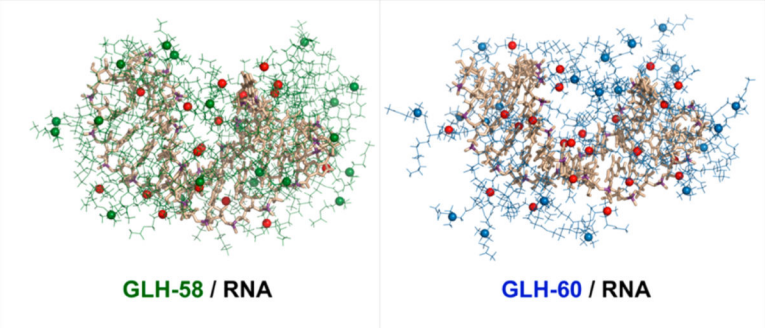

Figure 4.

Molecular dynamics (MD) simulation snapshots of complexes formed by bolaamphiphiles GLH-58 (green, left panel) and GLH-60 (blue, right panel) with RNA. Bola hydrophobic chains are colored green and blue, and the central Nitrogen atoms in their head groups are shown as spheres (two in each GLH-58 and four in each bola GLH-60). RNA is colored tan with its backbone P atoms shown in purple. MD simulations start with bolas placed randomly around the RNA fragments. At the end of simulations 19 GLH-58s form a two-layered micelle, bringing 17 head groups within 5 Å of the RNA (red spheres), while only 11 GLH-60s form a single-layered micelle around the RNA, with 24 head groups within 5 Å of the RNA (red). The predicted RNA exposure to solvent (and potential nucleases) is the same for both, but the electrostatic forces (and hydrogen bonds) are stronger in the GL-60/RNA complex. These predictions are consistent with the experimental results that showed equal protection against RNA digestion offered by both bolas, but diminished release of siRNA from the GLH-60 micelles inside the cell (and less efficient silencing of the target gene).

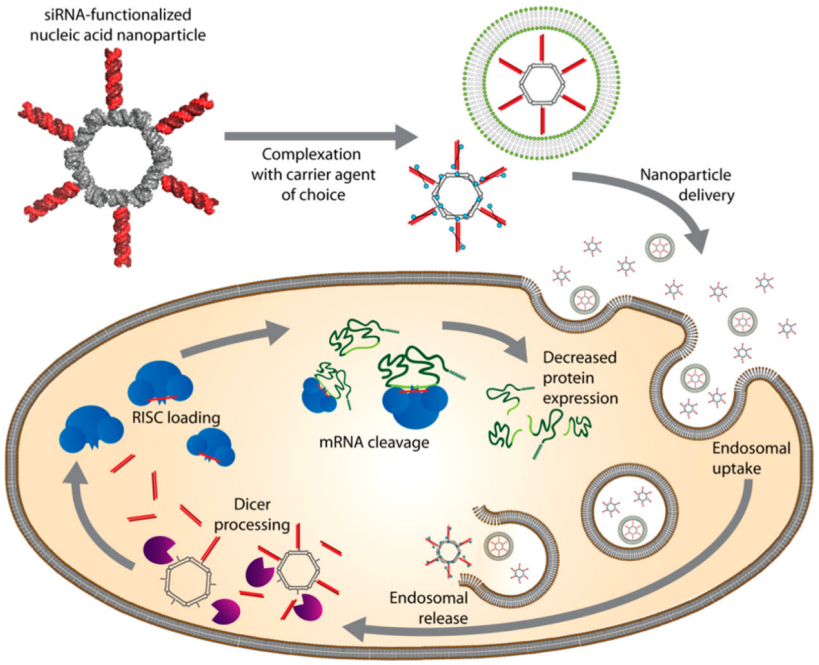

Figure 5.

Schematic representation of RNA nanoparticle delivery, uptake, and release of functional RNAs. An RNA nanoparticle, such as the RNA nanoring chosen here to present the concept, can be encapsulated or complexated with a delivery agent of choice to be delivered to cells. The presumed cellular uptake mechanism is endocytosis. Once the endosomal escape occurs, the RNA nanoparticle is exposed to cellular components, such as the Dicer enzyme (colored in purple). Following dicing of the DS RNAs into siRNAs, the guide RNA strand (colored in red) is loaded into the RISC (represented schematically by the blue complex) and guided to the mRNA. The mRNA is cleaved in the middle of the guide RNA-mRNA complementary paired region, and the protein synthesis is repressed, resulting in downregulation of the protein level of expression.