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The International Society of RNA Nanotechnology and Nanomedicine (ISRNN): The Present and Future of the Burgeoning Field

Morgan Chandler, Brittany Johnson, Emil Khisamutdinov, Marina A. Dobrovolskaia, Joanna Sztuba-Solinska, Aliasger K. Salem, Koen Breyne, Roger Chammas, Nils G. Walter, Lydia M. Contreras, Peixuan Guo,* and Kirill A. Afonin*

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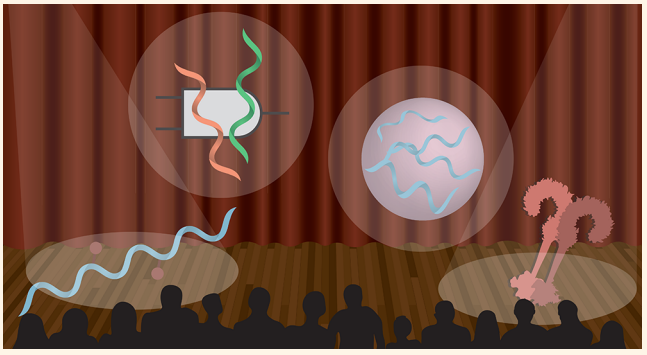
ABSTRACT: **The International Society of RNA Nanotechnology and Nanomedicine (ISRNN) hosts an annual meeting series focused on presenting the latest research achievements involving RNA-based therapeutics and strategies, aiming to expand their current biomedical applications while overcoming the remaining challenges of the burgeoning field of RNA nanotechnology. The most recent online meeting hosted a series of engaging talks and discussions from an international cohort of leading nanotechnologists that focused on RNA modifications and modulation, dynamic RNA structures, overcoming delivery limitations using a variety of innovative platforms and approaches, and addressing the newly explored potential for immunomodulation with programmable nucleic acid nanoparticles. In this Nano Focus, we summarize the main discussion points, conclusions, and future directions identified during this two-day webinar as well as more recent advances to highlight and to accelerate this exciting field.**



Nucleic acids have become established materials in the assembly of nanoscale structures, with their programmable dimensionality and direct biological applications making them amenable to a number of research objectives and biomedical applications. Progress in the field of RNA nanotechnology, in particular, is evident by the recent establishment of two FDA-approved RNA interference (RNAi)-based drugs,[1,2] the pipeline of several additional RNA-based therapies currently in clinical trials,[3] and the variety of therapeutic nucleic acids with diverse mechanisms of action being continuously developed and led by RNA-focused biotechnology companies.[4] Of these, the versatility of RNA is particularly exemplified by the rapid development of COVID-19 mRNA vaccines.[5−11] Overall, what was once referred to as an emerging field[12] has seen multifaceted advances in structural computation and design, therapeutic activation, and targeted delivery toward a variety of clinical opportunities.[13−17] While DNA nanotechnology has, to date, been explored more deeply,[18] RNA nanotechnology offers complementary opportunities. While some translational barriers, which are unavoidable for any innovative technology, need to be overcome to bridge academic work all the way to its clinical potential, the active communication and collaborations within the expanding research community continue to drive multidisciplinary work between medical, biological, chemical, physical, and computational areas of expertise.[19]

Annual meetings held by the International Society of RNA Nanotechnology and Nanomedicine (ISRNN) focus on the latest achievements in RNA technologies and nanomedicine, enabling groups from diverse research areas to gather to discuss the latest findings and to strategize the best approaches

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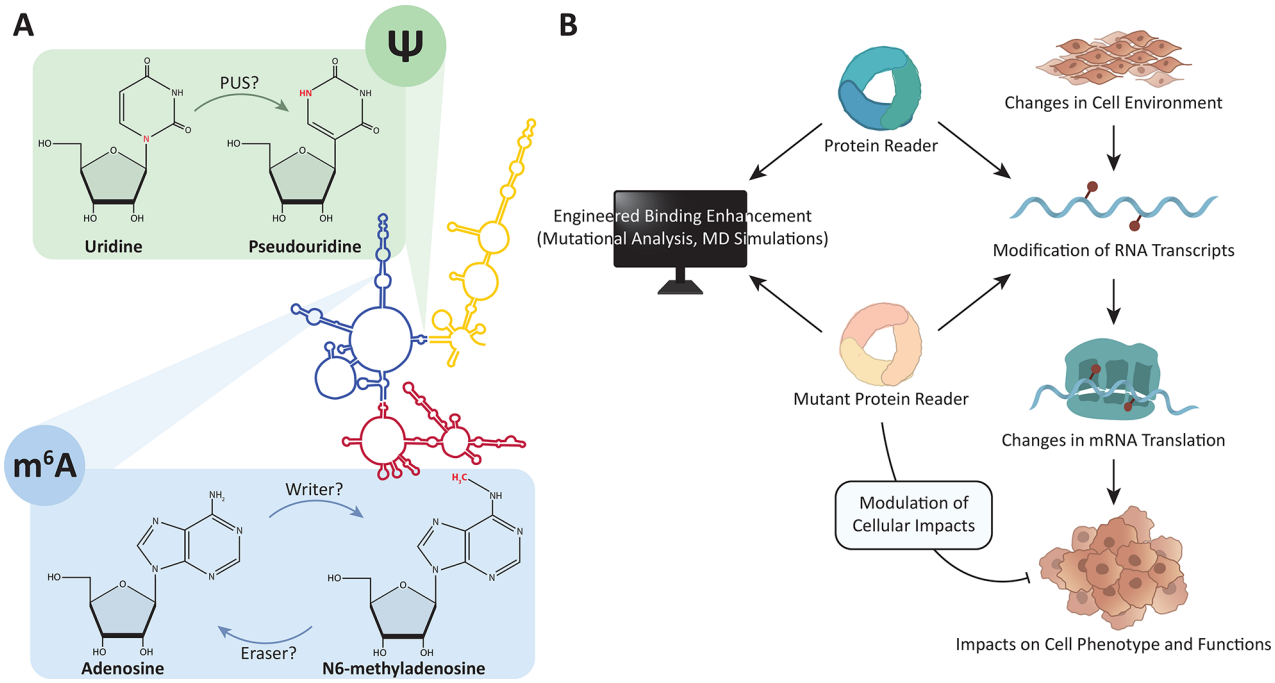

Figure 1. (A) Polyadenylated nuclear (PAN) RNA encoded by Kaposi's sarcoma-associated herpesvirus with roles in viral infectivity and implications for structural RNA biology. (B) Operating at the interface between computational and experimental RNA biology: characterization of RNA oxidation effects through engineered binding enhancement. Deposition of RNA modifications (*i.e.*, 8-oxoG) in mRNA due to changes in the cell environment (*i.e.*, oxidative stress) leads to a change in translation, leading to different phenotypic effects. Using mutational analysis and molecular dynamics simulations on the modification-specific protein readers (*i.e.*, 8-oxoG reader PNPase), engineered species with increased affinity and selectivity for modified RNA can modulate the effects of environmental change (*i.e.*, increased resistance to oxidative stress).

to expand the potential of this technology.[20] This productive mode of collaboration was paused during the COVID-19 pandemic, which postponed all traditional face-to-face meetings and travel plans.[21] In order not to lose the momentum of recent accomplishments, the ISRNN pivoted from previous meeting styles to host an online webinar series July 29−30, 2020. The meeting was organized by ISRNN to be held on an online platform *via* the University of North Carolina at Charlotte, which greatly increased the accessibility of the webinar series for students and international scholars who could view the talks for free in real time and could actively participate in engaging discussions. As a result, the webinar series drew a large virtual crowd over the two-day span, with hundreds of guests joining as their schedules allowed. The audience joined from at least 10 different countries across four continents, including 15 U.S. states. Individuals hailing from over 28 different academic higher institutions were in attendance along with representatives from the U.S. government (*i.e.*, National Institutes of Health) and industry representatives from around the globe.

In this Nano Focus, we summarize the key themes arising from the 32 talks hosted over the two-day period and provide a current snapshot of the field of RNA nanotechnology transcending the hiatus of traditional conferences.

FUNDAMENTAL PROPERTIES OF RNA NANOPARTICLES

To begin the conference, Peixuan Guo of The Ohio State University introduced the latest milestones in RNA nanotechnology, which he attributed to the many advantages of RNA stemming from its biocompatibility and programmability. Guo described the elastic and rubbery properties of RNA nanoparticles (NPs)[14] and reported his lab's recent discovery of how the unusual rubbery property of RNA contributes to both vessel extravasation to enhance tumor targeting and fast renal excretion to reduce toxicity.[22] Although the elastic and rubbery properties are similar in some respects, the former refers to the physical property of RNA whereas the latter describes the phenotype of the elasticity in cancer targeting and renal excretion. The concept of RNA elasticity is not new, but the finding that RNA's rubbery property contributes to cancer targeting is novel. Rubber is ubiquitous and elastomeric materials in nanotechnology are in high demand due to their technological potential. By virtue of their distinctive physicochemical properties, both RNA and DNA have been extensively investigated for a variety of applications. Guo reported that the phi29 DNA packaging motor contains a three-way junction (3WJ) with three angles. His lab was able to stretch and to compress the 97° interior angle into 60, 90, and 108° to construct two-dimensional (2D) RNA structures, including triangles, squares, and pentagons, and three-dimensional (3D) structures, including cubes, tetrahedra, and prisms. He also reported that RNA nanoarchitectures are stretchable and shrinkable in multiple repeats by disturbing the structure, as demonstrated by the use of optical tweezers that were built in his lab. Thus, RNA NPs are like rubber. Compared to Au and Fe NPs, RNA NPs show stronger cancer-targeting effects but less accumulation in organs. Generally, the upper limit of renal filtration is 5−6 nm, but RNA NPs larger than 6 nm, or even 20 nm, can pass through renal filtration and resume their original structures upon appearing in the urine. The results explain two previous findings: RNA NPs have unusually high tumor-targeting efficiencies because they are similar to rubber

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or an amoeba that can squeeze out of a leaky blood vessel.[23] In addition, RNA NPs are nontoxic because they are rapidly cleared from the body by the kidney into urine.

RNA CHEMISTRY, MODIFICATIONS, AND MODULATION

The structure of RNA as it is used as a nanoscale building material is derived from its original roles in biological systems: cellular fate is primarily dictated by the dynamics of the transcriptome. Every time a cell changes from one state to another (*i.e.*, during cellular division or under viral infection), the RNA in it must change and adapt, as well. This change needs to be carefully tailored to cellular needs, which can be accomplished by dynamic chemical modifications to RNA. In the context of cellular transcripts, these modifications are referred to as the epitranscriptome, and they include over 140 types of chemical signatures.[24] The methylation of adenine at position 6 (m^6A) and the isomerization of uridine to pseudouridine (Ψ) are the most abundant modifications that have been shown to hold profound effects over RNA biology. Although their presence in the genomes of RNA viruses and virus-encoded RNAs has been known for decades, elucidation of their biological significance remains lacking, especially in the context of a specific viral transcript.

The Sztuba-Solinska group leveraged the study of the polyadenylated nuclear (PAN) RNA encoded by Kaposi's sarcoma-associated herpesvirus (KSHV), a critical modulator of the viral lytic reactivation (Figure 1A). They previously resolved the secondary structure and protein-binding profiles of PAN RNA inside living cells and within viral particles by applying a high-throughput deep-sequencing probing technique, 2′-hydroxyl acylation analyzed by primer extension, and mutational profiling (SHAPE-MaP).[25] Their analyses indicated that PAN RNA's secondary structure is dynamic and likely to accommodate the multitude of its functions. Nevertheless, insight into the regulation of this crucial viral transcript is lacking. Polyadenylated nuclear RNA shares characteristics with some cellular long noncoding RNAs (lncRNAs): the stabilizing 3′ triple helix, similar to MALAT1; *β*-MEN lncRNAs; the association with chromatin-modifying complexes; and, as Sztuba-Solinska and co-workers discovered, epitranscriptomic regulation. In addition, PAN RNA offers a beneficial system for the mechanistic studies that are otherwise challenging in scarcely expressed or labile lncRNAs. At the same time, PAN RNA is unique in terms of its involvement in viral replication or virus–host interactions. This distinctiveness may provide insight into the regulation and functionality of viral lncRNAs in the realm of infected cells.

By combining next-generation sequencing epitranscriptomic mapping with direct RNA sequencing (using nanopores) and a quantitative reverse transcription and ligation-assisted PCR analysis, Sztuba-Solinska's group was able not only to dissect the exact position of m^6A and Ψ on PAN RNA but also to quantify the fraction of PAN transcripts that carry these marks.[26,27] They noted that the PAN RNA epitranscriptomic patterns for both signatures vary and depend on the stage of viral replication. Using RNA affinity capture, mass spectrometry, and immunoblotting, they also delineated the cellular epitranscriptomic machinery involved in the installation of m^6A and Ψ on PAN RNA, which include specific m^6A writers and erasers and pseudouridine synthase. The researchers also dissected the structural influence of m^6A in the context of full-length PAN RNA by performing SHAPE-MaP on cells that were ablated for the regulatory enzymes. Future studies will focus on elucidating the m^6A and Ψ functions in the context of PAN RNA biology (*i.e.*, stability, structure, interactome network, and KSHV infectivity cycle).

The Contreras lab shared their recent progress on developing methods to detect RNA–protein complexes and RNA accessible structures *in vivo* to improve understanding of RNA assemblies.[28−31] In addition, by combining experimental and computational methods in the study of the epitranscriptome, the Contreras lab has gained valuable mechanistic insight into the effects of RNA oxidation on cellular health, as well as into the molecular specificity of protein readers that recognize modified RNA, particularly in response to oxidative stress.

In a recent collaboration with Texas A&M University, the Contreras lab characterized the reader activity of polynucleotide phosphorylase (PNPase), a protein "reader" for 8-oxoG, and developed a platform to enhance its affinity and specificity for oxidized RNA transcripts (Figure 1B).[32,33] Of the expanding pool of known chemical modifications to RNA, 8-oxo-7,8-dihydroguanosine (8-oxoG) has been found at higher levels in cells exposed to oxidative stress.[34,35] The Contreras lab characterized levels of 8-oxoG found in human lung epithelial cells in response to pollution exposure, also linking the oxidation of specific mRNA transcripts to phenotypic effects. Specifically, they found the transcript encoding for squalene synthetase (FDFT1) to be significantly enriched in 8-oxoG[34] in model lung epithelium cells after exposure to a mixture of volatile organic compounds (VOCs) and ozone. This result might explain the oxidative effects of VOCs on cholesterol accumulation and altered cytoskeletal properties through the downregulation of FDFT1 synthesis. The techniques used in these works can be applied to studies of other RNA modifications and show promise as powerful tools for understanding the epitranscriptome.

Further investigations into the structure, folding, and activity of RNAs have been carried out by the Lilley/Norman Lab at the University of Dundee, where recent studies have looked at the structural mechanisms of dynamic systems such as the pistol and hammerhead ribozymes[36] and the *S*-adenosylmethionine (SAM)/*S*-adenosylhomocysteine (SAH) riboswitch.[37] They have also characterized noncoding Box C/D RNA–protein complexes, which function as independent motifs.[38]

The final talk on structural studies was from the Shi-Jie Chen research group at the University of Missouri, Columbia, where computational work has led to the recent development of a Web server, the VfoldLA server, for the prediction of RNA 3D structures.[39] Through their computational approach to structural determination, they have shown that folding kinetics predictions are consistent with experimental SHAPE[40] results and have even been used to rebuild some SHAPE profiles.[41] Insight into the structures of naturally occurring RNAs enables a better understanding of their functions but also expands the repertoire of design motifs that can be utilized in RNA NPs. Future work in structural prediction, especially in combination with RNA chemical modifications, is essential to the further downstream development of nucleic-acid-based nanoplatforms.

ON DYNAMIC STRUCTURES AND LOGIC GATING

Nanoscale RNA structures are highly dynamic, with many examples from nature rapidly emerging and driving the design of synthetic nucleic acid circuits. To uncover the potentials of nucleic acid computing fully, it is important that we first gain

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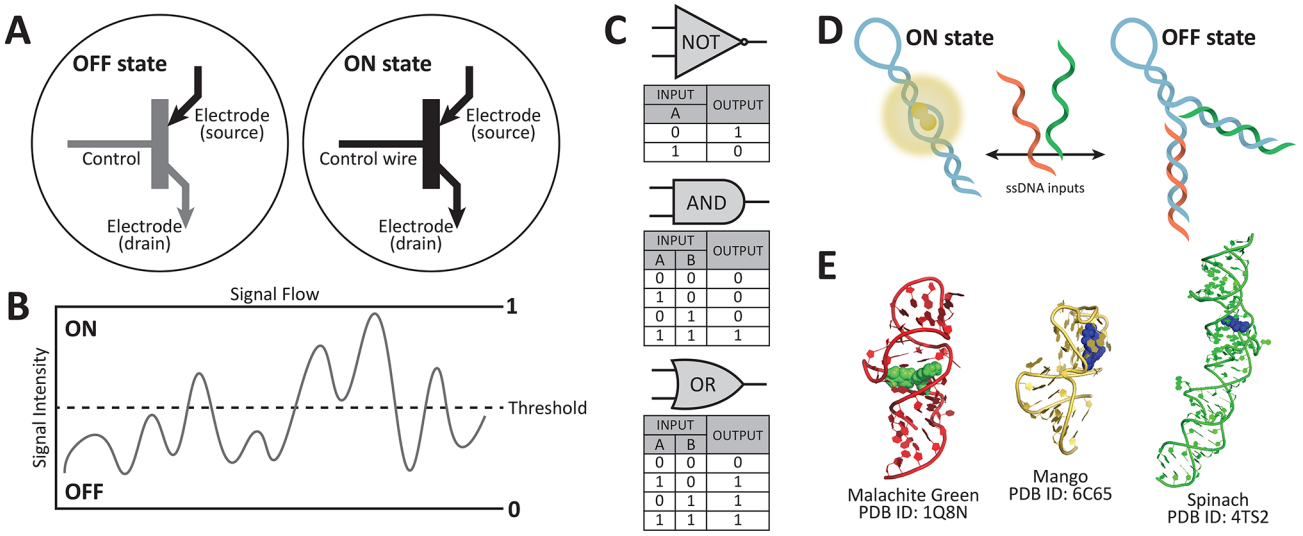

Figure 2. (A) Schematic diagram of an electronic transistor. (B) Exemplified two-state (binary system) electrical output. (C) Example of truth tables for NOT, AND, and OR operations. (D) Exemplified view of the working principle light-up RNA aptamer acting as a switch ON and OFF. (E) Three-dimensional structures of some commonly used RNA light-up aptamers.

insight into transistors, the key active components of electronics. Transistors embedded into computer chips can turn the flow of electricity "ON" and "OFF": important and complex information are represented with just these two states of electricity. Electronic transistors are simply electrically controlled switches that contain three components: two electrodes (for current to flow in and out) and a control wire. Once electricity is applied to the control wire, electrical current can flow from one electrode to another. The input role serves the control wire, and the output is the electrode, as shown in Figure 2A. The flow of electricity is represented by 1, and the state of electricity not flowing is represented by 0: this setup is referred to as binary, meaning "of two states". Although computer systems can be ternary (three state) or even quinary (five state), having additional states can create challenges. The more intermediate states there are, the harder it is to keep distinct signals. Even minor electrical noise could ruin readouts. Therefore, it is advantageous to set up a threshold with only two signals that provide distinct ON and OFF signals (Figure 2B).

To uncover the potentials of nucleic acid computing fully, it is important that we first gain insight into transistors, the key active components of electronics.

So, how can the binary system be used to perform simple tasks such as adding two numbers? This challenge requires the implementation of Boolean algebra, named after English mathematician George Boole. His approach enabled TRUTH to be systematically and empirically proven through logic equations (refer to Boole's original work, *The Mathematical Analysis of Logic*, 1847).[42] For example, in everyday algebra, the values of variables are numbers, and operations of those numbers include subtraction, addition, and multiplication, to name a few. In Boolean algebra, the values of variables are TRUE and FALSE, and the operations are the logical constitution of three fundamental operations: NOT, AND, and OR. A NOT logic takes one input and has a single output by taking a single Boolean value (either true or false) and inverting it by flipping true to false or false to true. The AND and OR logic operations take two inputs and have a single output. For the AND operation, the output is true if both inputs are true, whereas for the OR operation, only one of the inputs must be true to reach the true output (Figure 2C). This single transistor has one input and one output; turning ON the input turns ON the output and turning input OFF turns the output also OFF. This is an example of a YES gate. The term gate is commonly used because it controls the path of the current. Theoretically, to form a complete set of all possible logic gates, four basic YES, NOT, AND, and OR gates are required. Logic gates play a role as the elementary building blocks of digital circuits. Depending on the complexity of the operations and tasks, some circuits may have only a few logic gates, whereas others, such as microprocessor chips, have complex combinatorial integrated circuits (IC) embedding millions of logic gates or transistors. The number of transistors in a computer chip is defined by their counts, and to date, the highest count contains two trillion 4-bit transistors in an IC 3D chip of a 1 terabyte universal flash storage V-NAND model developed by Samsung.[43]

In accordance with Moore's observation, stating that transistors' count in a dense IC area doubles about every 2 years, microchip-developing companies have almost reached limits in the fabrication of miniaturizing chips.[44,45] Thus, major goals of the 21st century include the development of new technologies that enable the storage of a vast amount of data in a very small compartment, increasing the rates of performance to millions of operations simultaneously and operating in parallel processing.

As nature's form of data storage, the programmable structures of nucleic acids offer the potential for complex logic gates to be designed that also yield biologically functional outputs. Interactions between specifically designed sequences can drive strand displacement reactions and conformational changes. The concentration of the inputs is an important factor, as this can be fine-tuned for desirable limits of detection and define the distinct readouts between 1 and 0 states. Single-

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stranded DNAs used as input strands are relatively inexpensive, stable in aqueous solutions, and can be hybridized with RNA to form RNA−DNA duplexes. For instance, as presented by Lorena Parlea of the National Cancer Institute, single-stranded RNA toeholds can be used to drive the reassociation of RNA−DNA hybrids to transition from inert to functional products in cells.[46−48] The same working principles can be implemented for the controlled organization of inorganic materials (*e.g.*, quantum dots) that is synchronized with the activation of therapeutic responses inside diseased cells.[49] The Kolpashchikov lab at the University of Central Florida uses strand displacement for the design of multistrand DNA nanodevices programmed to recognize single nucleotide variations,[50] including in cancer marker RNAs.[51] In addition, Marc Van Der Hofstadt at Laboratoire Jean Perrin, Sorbonne Université, has utilized DNA switches in a programmable extracellular medium that are active at 37 °C and can thus function in the presence of living cells.[52] Nucleic acids' programmable organization, which was demonstrated by Oleg Gang's group at Columbia University and Brookhaven National Laboratory, has produced precise nucleic acid architectures[53] in the form of DNA nanochambers for organized arrays.[54] This research has also led to enhancing the stability of DNA origami for biomedical applications,[55,56] which can additionally be conjugated with fluorophores and antibodies.[57] Thomas LaBean at North Carolina State University and co-workers at iNano in Aarhus, Denmark, have explored using RNA origami as an anticoagulant.[58] Thomas Hermann's lab at the University of California, San Diego, has developed a library of RNA−DNA hybrid nanoshapes that self-assemble from modules[59,60] and can be programmed to do so only in the presence of a target ligand.[61]

As a functional output, RNA is advantageous not only as a therapeutic moiety, but also as a biosensing component. A growing library of RNA fluorogenic light-up aptamers are being employed as potential candidates for binary logic system development. Various aptamers now can be routinely engineered through repeated rounds of *in vitro* selection, also known as systematic evolution of ligands by exponential enrichment (SELEX), the powerful technique that was developed in the early 1990s.[62,63] Since then, numerous different types of aptamers against different target molecules have been selected in academic research laboratories and have found applications in various biotechnological fields.[64−68] In particular, the fluorogenic RNA aptamers are known for their specificity in binding otherwise nonfluorescent dyes; the fluorescent emission of the dye is activated only upon binding to its RNA aptamer. This binding specificity enabled the design not only of label-free nucleic-acid-based sensors *in vitro*[69−72] but also the attractive development of binary 1 and 0 systems, as shown in Figure 2D,E.[73,74] The input signal induces the conformational change of the fluorophore-binding pocket (analogous to a "gate"), dictating the overall affinity strength of the RNA−fluorophore complex. Various factors can potentially serve as inputs, including single-stranded oligonucleotides and nonfluorescent molecules mimicking structures of the ligand dye. Once correctly designed, the overall fluorogenic aptamer structure can mimic a computer's function by toggling between fluorescent (1) and nonfluorescent (0) states. Over the past two decades, several RNA fluorogenic aptamers were implemented in logic gate constructions, including AND,[74,75] NOR,[76] and combinatorial AND and XOR logic gates[73] for applications in biosensing and data processing.[77] Emil Khisamutdinov's research group at Ball State University demonstrated these combinatorial gates as corners in the design of an RNA tetragon containing both Malachite Green and Broccoli RNA aptamers.[73] The design of these RNA-based complexes is cost-effective and does not require covalent labeling of dye or quencher molecules to the oligonucleotides. Fluorogenic RNA aptamers can be designed to function as a simple circuit within individual binary logic gates. The Tenenbaum Lab at SUNY Polytechnic Institute has introduced the rational design of structurally interacting RNAs (sxRNAs), which form *trans*-3WJs as an additional level of functional regulation.[78,79] Sensors built from sxRNA switches have utilized embedded fluorescent aptamers for demonstrating rapid ON and OFF states.[80]

A number of other sensor designs are well-suited to the use of aptamers as highly selective and specific recognition elements.[81−83] Aptamers as recognition elements serve as part of stimuli-responsive assemblies for control within cells, as recently demonstrated by Weihong Tan at Hunan University and Shanghai Jiao Tong University.[84]

As naturally occurring RNA circuits, Nils Walter shared his group's work on riboswitches, which are structured RNA motifs frequently found embedded in the 5′ untranslated regions of bacterial mRNAs that utilize aptamers to affect gene regulation.[85−89] Numerous classes of transcriptional and translational riboswitches regulate gene expression in response to metabolic or environmental cues such as small molecules, cations, anions, or other RNA molecules, interfering with the function of the much larger transcription or translation machineries, respectively. By assembling an entire transcription elongation complex and interrogating it using a combination of transcription assays, single-molecule fluorescence probing, and molecular modeling, the Walter lab was able to show that, in the case of a $preQ_1$ riboswitch, pausing at a site immediately downstream of the riboswitch depends on a ligand-free pseudoknot in the nascent RNA transcript, a consensus pause sequence in the DNA template at a precisely spaced distance, and direct interactions of the RNA with the exit channel of the transcribing RNA polymerase.[86] Binding of the small metabolite ligand $preQ_1$ to the riboswitch then triggers release of the polymerase from its pause so that it presumably runs into a terminator. In the case of the larger Mn-sensing riboswitch, an RNA four-way junction structure is needed to discriminate the divalent transition metal ion Mn^{2+} from the closely related, much more abundant Mg^{2+} ion to regulate bacterial Mn^{2+} homeostasis genes. Combining X-ray crystallography, molecular dynamics simulations, and single-molecule Förster resonance energy transfer enabled the Walter lab to discover an extended conformation that samples transient docked states in the presence of millimolar Mg concentrations but becomes stably docked in the presence of submillimolar Mn.[89] Taken together, these observations illustrate how even subtle local binding events can lead to conformational dynamics that cascade into large, biologically relevant conformational and functional changes in natural RNA nanomachines,[88] an inherent capacity that can be leveraged to inform the design of future RNA nanotechnology and nanomedicine devices.

Another advantage of dynamic RNA structures is the ability to utilize their ensuing interactions with enzymes and RNA-binding proteins for an additional tier of functional output. Jong Bum Lee's lab at the University of Seoul has utilized RNA for a biomaterial method-based approach to cell reprogram-

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ming.[90] Their work utilized RNA's enzymatic self-assembly to include short hairpin RNA nanoparticles[91] as well as rolling circle transcription, which was shown for the assembly of RNA nanosponges.[92] Dr. Elisa Franco's lab at the University of California, Los Angeles, has shown both the enzyme-driven assembly and disassembly of DNA/RNA nanotubes[93] as well as the self-assembly of these hybrid structures in water-in-oil emulsion droplets.[94] In addition, Dr. Friedrich Simmel's lab at the Technical University of Munich has applied nucleic acid dynamics to CRISPR complexes, enabling additional layers of programmability for Cas12a, a CRISPR effector protein, for use in strand displacement circuits,[95] as well as the development of low-cost biosensors using Cas13a.[96] To visualize these dynamic processes, Yuri Lyubchenko's lab at the University of Nebraska Medical Center has developed a robust set of methods for using atomic force microscopy (AFM) and high-speed AFM imaging to visualize the dynamic behavior of biomolecules over time.[97] They have demonstrated this visualization not only with DNAs and DNA−protein complexes[98] but most recently with the viral infectivity factor protein, which is essential for the replication of HIV-1.[99]

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In summary, the advantages of RNA nanotechnology and aptamer technology make it possible to construct *de novo* nanoassemblies possessing intermediate roles between electronic computers and biological systems. The structural features of RNA light-up aptamers, switches, and complexes with other biomolecules can be manipulated for conditionally activated function. These advantages hold promise for the development of an alternative RNA-based computing field, which is currently in its infancy. There are various problems yet to be addressed. For example, processing straightforward logical tasks will require finite amounts of time. Currently, the individual RNA gates or combinatorial RNA gates are too simplistic for applications involving complicated calculations and decision-making processes: they also need to consider interactions with the gene expression machinery, as illustrated by the work on riboswitches. Although further research and development are urgently needed to overcome these current challenges, this research has the potential to shed light on computing applications in a wide variety of fields.

MODES OF DELIVERY FOR THERAPEUTIC NUCLEIC ACIDS AND RNA NANOSTRUCTURES

To overcome the challenges associated with delivering nucleic-acid-based nanomedicines and nanoparticles into cells, an assortment of carriers is being developed to offer tunable and targeted approaches. Although they vary in composition from inorganic to organic and may even be biologically derived in nature, the overall goal of these modes of delivery is to be compatible with nucleic acids as cargo and for an overall profile that is highly biocompatible and nontoxic at functional concentrations. With many such candidates available, talks at the ISRNN Webinar Series focused on several innovative approaches, briefly described below.

As an inorganic nanoparticle-mediated delivery approach for functional RNA nanoassemblies, the Vivero lab at the University of North Carolina at Charlotte has developed mesoporous silica nanoparticles as a platform for the delivery of various compositions and shapes of functional nucleic acid nanoparticles (NANPs)[100] and has demonstrated that these can be used with the addition of other small molecules and drugs for broad applications as combinatorial therapies.[101] These nontoxic silica-based vehicles loaded with NANPs demonstrate efficient uptake into various cancer cells for silencing *via* RNA interference.[102]

The use of lipid-based carriers was introduced by Anu Puri of the National Cancer Institute.[103] Hydrophobic-headed oxime ether lipids[104] and double-headed bolaamphiphiles[105,106] have each been investigated for the optimal delivery of siRNA. Yuanyu Huang's research group at the Beijing Institute of Technology has also demonstrated the formation of ionizable lipid nanoparticles which were used for the hepatic delivery of mRNAs.[107] They have also expanded their delivery candidates to include polyplex carriers which are activatable by reactive oxygen species (ROS) for synergistic cancer treatment[108] and a microneedle array, which is entirely carrier-free.[109] Cárdenas and co-workers at Malmö University have explored the escape of mRNA from lipid nanoparticles upon binding apolipoproteinE (ApoE).[110] Much remains to be done in this important area.

Various biologically derived carriers were discussed, beginning with an introduction of extracellular vesicles (EVs) from Koen Breyne of Massachusetts General Hospital and Harvard Medical School. Although little is known about their function, EVs have been increasingly appreciated as omnipresent natural communicative modalities between both prokaryotic[111] and/or eukaryotic cells.[112] Extracellular vesicles have the ability to protect and to transport biological cargos and have heretofore not been reported as a causative agent for disease.[113] In recent years, the pharmaceutical industry has shown marked interest in EVs because they are well-tolerated by patients, as indicated by an increasing number of reported clinical studies.[114]

Analogous to what has been pursued during viral vector development in the 1970s, current efforts are focused on customizing naturally occurring EVs to suit our therapeutic delivery needs.[115] Extracellular vesicles share many similarities with enveloped viruses: They are nanoscale, enclosed membranes with a lumen that is able to carry and to stabilize many important biomolecules that are useful for both research and medicine.[116,117] Despite their close relationship, which primarily arises from their corresponding intracellular biogenesis pathways, EVs lack many of the viral functional effectors. Tackling these bottlenecks will be essential for using EVs for nonviral delivery. In short, this battle occurs on two fronts: first, loading the desired cargo into an EV.[118] Endogenous EV loading tries to add tags to biomolecules and to place them into EVs.[119] Depending on the types of molecules to be loaded, different approaches need to be considered. Approaches have been successfully established for membrane-associated EV proteins but less so for luminal EV proteins, miRNA, *etc.* Exogenous EV loading does not require EV-producing cells and uses purified EV solutions. This strategy has the advantage that nonbiologicals can be used, such as synthetic stabilized RNAs.[120] Methods have been adopted from other fields such as electroporation or the use of transfection agents to pack payloads into EVs; recently, more

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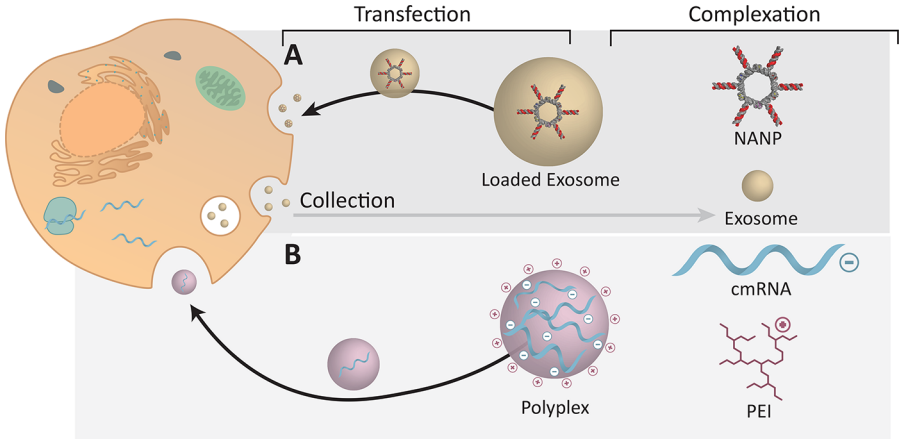

Figure 3. Strategies for the delivery of RNA into cells. (A) Exosomes collected from cells are loaded with nucleic acid nanoparticles (NANPs) for their cellular delivery. (B) Chemically modified ribonucleic acid (cmRNA) encoding BMP-2 which is then complexed with polyethylenimine (PEI) enables polyplex formation and subsequent transfection.

EV-specific strategies have been used, such as association to the outer surfaces of EV proteins.[121] The second front is the effective and sufficient delivery of EV cargo into a desired cellular compartment for functionality. In contrast to viral delivery vectors, EVs do not have distinct fusogens or other mechanisms to escape the endosomal entrapment postuptake of an EV by a cell. Although this topic remains the subject of ongoing debate, incorporating viral fusogens has been reported to aid in this aspect.[122] Extracellular vesicles may be the next-generation nonviral vector for biodegradable biomolecules and thereby find a place next to their viral counterparts, focused on genetic material delivery.

Stemming from this larger group of EVs is a subset of smaller membrane-bound vesicles known as exosomes, which were also extensively discussed for their delivery advances. Several recent studies coming from the Guo lab have utilized EVs[123] or exosomes for the efficient delivery of therapeutic siRNAs in a targeted fashion with the use of folate.[124] The researchers have also demonstrated uptake using exosome-like nanovesicles derived from ginger.[125] Victoria Portnoy of System Biosciences presented research findings from a recent collaboration with the Afonin lab (at the University of North Carolina at Charlotte); they used a combined approach to deliver several different functional NANPs by utilizing freshly isolated exosomes from cell culture (Figure 3A).[126,127]

The final delivery platform was one targeted to its application and specific RNAs of interest. Tissue engineering strategies such as protein therapy, gene therapy, cell therapy, and their combinations are currently being explored for oral- and craniofacial regeneration and repair.[128−130] Bone tissue engineering strategies typically involve the use of recombinant proteins such as rhBMP-2 (protein therapy), cells (cell therapy), gene-encoding growth factors and morphogens, biomimetic scaffolds, and their combinations.[131−134] For example, the Salem lab successfully demonstrated the application of a nonviral gene-based delivery system to enhance bone regeneration.[132,135−137] They employed a gene-activated matrix (GAM) consisting of collagen containing nanoplexes of polyethylenimine (PEI) complexed with plasmid DNA encoding platelet-derived growth factor-BB. They demonstrated superior bone regeneration capacity of GAM in calvarial defects in rats. *In vivo* studies showed significantly higher new bone volume/total volume (BV/TV)% in calvarial defects treated with the complex-activated scaffolds following 4 weeks of implantation (14- and 44-fold higher) when compared to empty defects or empty scaffolds, respectively.[138] Protein, plasmid DNA, and cell therapy have some significant drawbacks: they are expensive and exhibit some associated safety issues. Using RNA (encoding therapeutic growth factors), however, offers several advantages that have the potential to overcome these limitations. Chemically modifying the RNA improves its stability and reduces immunogenicity, thereby facilitating the potential of RNA to become an alternative to protein and DNA-based therapies.[139,140] The Salem lab tested the *in vivo* efficacy of chemically modified RNA (cmRNA) encoding BMP-2 for bone regeneration applications in a rat calvarial defect model (Figure 3B).[140] In bone marrow stromal cells (BMSCs), they evaluated the transfection efficiency, cytotoxicity, osteogenicity and *in vivo* bone regenerative capacity of cmRNA encoding BMP-2. They also compared cmRNA with plasmid DNA. Additionally, they assessed the expression of bone-specific genes, osteocalcin, and alkaline phosphatase in transfected BMSCs and evaluated bone matrix deposition to validate the functionality of transfection. In all of the above assessments, they demonstrated the enhanced performance of cmRNA (BMP-2) when compared to the pDNA equivalent. Using a calvarial bone defect model in rats, they demonstrated stronger bone regeneration capacity of cmRNA (encoding BMP-2)-activated matrices compared to pDNA (BMP-2)-activated matrices. These results demonstrate that collagen scaffolds loaded with nonviral vectors complexed to cmRNA encoding BMP-2 are an effective strategy for local bone regeneration.[140] The researchers have since shown that cmRNA encoding BMP-9 can also effectively regenerate bone and that cmRNA-loaded scaffolds can be capped with salicylic-acid-based polymers to guide the direction of bone regeneration.[141]

RNA delivery to plants also offers many opportunities for agriculture.[142] This area is growing rapidly and a number of delivery strategies have been explored. Markita Landry at the University of California, Berkeley, has tested carbon nanotubes, DNA, and metal nanoparticles as possible delivery vehicles to get RNA to plants.[143−146] Jason White at the Connecticut Agricultural Experiment Station and Yukui Rui at China Agricultural University and their co-workers explore nanodelivery to address pathogenic plant viruses.[147] Nicole

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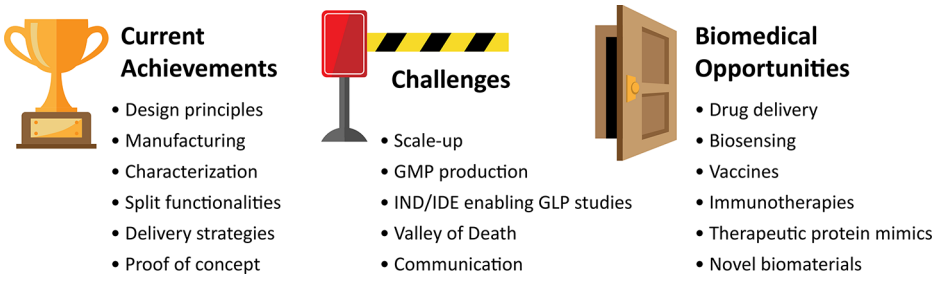

Figure 4. Current achievements, challenges, and biomedical opportunities for therapeutic nucleic acid nanoparticle (NANP) technologies. Achievements and opportunities are summarized along with barriers hindering NANPs' translation from bench to clinic. IND, investigational new drug; IDE, investigational device exemption; GMP, good manufacturing practice; GLP, good laboratory practice; VLP, virus-like particle; APC, antigen-presenting cell.

Steinmetz, now at the University of California, San Diego, explores nanodelivery to plants[148] and also using plant viruses and parts of plant viruses to treat diseases in humans.[149,150]

IMMUNOMODULATION AND IMMUNOTHERAPY WITH NUCLEIC ACID NANOPARTICLES

Experimental evidence is growing for the use of NANPs in medical applications, including but not limited to gene deliveries and controlled immunomodulation. Achievements in design principles, manufacturing, creating split functionalities, and delivery strategies of NANPs have created opportunities to use these materials in vaccines, immunotherapies, and other therapeutic applications. However, the lack of harmonized procedures and reference standards for scaled-up, good manufacturing practice (GMP)-compatible manufacturing and good laboratory practice (GLP)-compatible translational studies enabling investigational new drug (IND) and investigational device exemption (IDE) filing with regulatory agencies such as the U.S. Food and Drug Administration (FDA) creates barriers that are slowing down the translation of NANPs from bench to clinic (Figure 4). Overcoming these challenges requires consolidated efforts from NANPs' stakeholders, including academic researchers, industry, and government agencies, among others. Some strategies for overcoming the translational "valley of death" include unifying efforts between stakeholders, developing and standardizing protocols for NANPs' characterization, and expanding *in vivo* studies for both NANPs' efficacy and safety.

Experimental evidence is growing for the use of nucleic acid nanoparticles in medical applications, including but not limited to gene deliveries and controlled immunomodulation.

Several years ago, the Drug Information Association (DIA) established a working group that focused on the safety of traditional therapeutic nucleic acids (TNAs) such as antisense DNA oligonucleotides, siRNA, miRNA, and CpG DNA oligonucleotides. After comparing the main properties of TNAs to those of biologics (*e.g.*, therapeutic proteins, monoclonal antibodies) and small molecule drugs, the working group concluded that despite some features that TNAs share with both small molecules and biologics, they have unique properties justifying a placement of these materials into an independent drug category.[151] Following the same strategy, in 2016, analyses of NANPs suggested that these materials are more complex than TNAs and could also qualify for placement into a separate group of products.[152] Gaps in NANP characterization include a lack of systematic investigations of their absorption, distribution, metabolism, excretion, and toxicological properties (ADME/Tox), pharmacokinetics (PK), and pharmacodynamics (PD)[153] and overlap with gaps pertaining to the characterization of similar categories of complex nucleic-acid-based materials such as DNA origami.[154] Lessons learned from investigating the toxicity of TNAs and their delivery vehicles could be used to inform the design of similar studies of NANPs.[153,155−157]

As noted above, NANPs are a versatile platform that can be applied to broad clinical applications. However, the recognition of NANPs by host immune systems can trigger unwanted and damaging inflammatory responses, which limits NANPs' successful use in the clinic.[157,158] Pattern recognition receptors (PRRs) that identify nucleic acids also initiate production of immune mediators in response to NANPs.[159−166] As such, significant research efforts are underway to characterize the immunostimulatory properties of NANPs. Previous studies have indicated that NANP molecular weight, melting temperature, and half-life strongly correlate with NANP immunostimulation.[167] Importantly, both nucleic acid structure and composition determine the physicochemical properties of NANPs.[167,168] A comprehensive *in vitro* study analyzing a library of NANPs with different compositions, sizes, shapes, and connectivities in primary human blood cells concluded that naked NANPs are invisible to blood immune cells.[159,169] Once complexed with the delivery carrier, NANPs are taken up by the cells *via* scavenger receptor-mediated endocytosis and induce a type I and type III interferon (IFN) response by triggering the activation of endosomal Toll-like receptors (TLRs). The structure and composition of NANPs determine their recognition by immune cells: RNA-based NANPs are more potent at inducing IFNs than their DNA-based analogues; globular NANPs are more potent than planar and fibrous particles; larger NANPs are more potent than their smaller counterparts; and single-stranded stretches of uracils present in RNA NANPs may contribute to their recognition by TLRs, whereas the sequences of double-stranded regions of NANPs do not play a role.[159] Also, as was recently demonstrated, the quality and quantity of cytokine induction triggered by NANPs can be additionally regulated with the carriers used for NANPs' intracellular delivery.[170] One of the most remarkable findings of the study investigating the role of individual TLRs in NANPs' immunorecognition was the discovery that TLR7,

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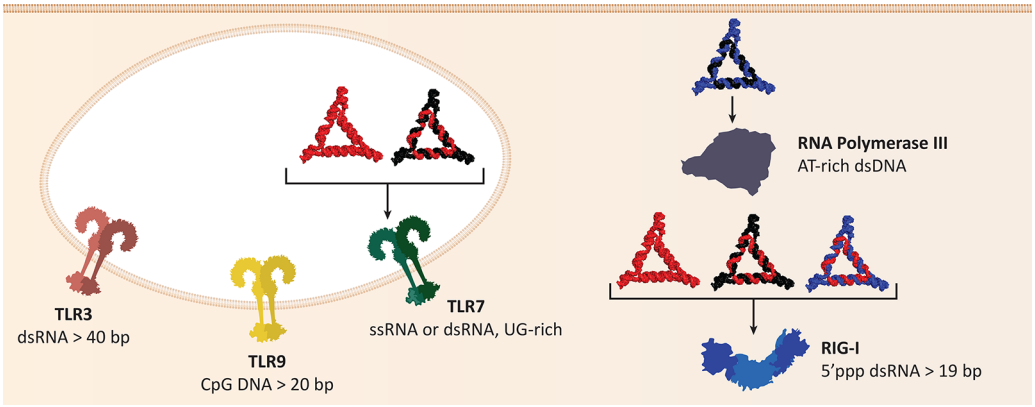

Figure 5. Schematic of the endosomal and cytosolic nucleic acid immune sensors that identify a targeted panel of triangular nucleic acid nanoparticles (NANPs) that differ in chemical composition but have the same connectivity, shape, size, charge, and sequence. NANPs are represented as triangles and the strands are color coded according to chemical composition (red = RNA, black = DNA, blue = 2′F-modified strands).

rather than TLR3, plays a key role in the NANP-mediated IFN response.[171] These recent studies suggested the potential for NANPs to be used for vaccines and immunotherapies, which has been extensively discussed elsewhere.[153] Nucleic acid nanoparticle composition can be optimized to achieve activation or avoidance of PRR-triggered immune responses. However, more research is needed to understand the role of various complexation agents and delivery carriers on the recognition of NANPs by immune cells.

In a recent study introduced by Brittany Johnson of the University of North Carolina at Charlotte, a follow-up investigation covered a panel of six NANPs each composed of a central strand and three outer strands that self-assemble into a triangle conformation due to Watson−Crick complementary base pairing. The six NANPs were constructed as follows: all RNA (RcR), RNA central strand with DNA outer strands (RcD), RNA central strand with 2′F-modified outer strands (Rc2′F), all DNA (DcD), DNA central strand with RNA outer strands (DcR), and DNA central strand with 2′F-modified outer strands (Dc2′F). The resulting assembled NANPs form triangles with 22 base pairs per side. Consistent with previous studies, the researchers observed activation of the transcription factors NF-κB and IRF, corresponding production of the inflammatory cytokine IL-6, and type I IFN-β in response to NANPs with a central RNA strand (RcR, RcD, Rc2′F) with minimal immunostimulation in response to NANPs with a central DNA strand (DcD, DcR). The researchers further examined the endosomal and cytosolic PRRs required for identification of NANPs and stimulation of immune mediator release (Figure 5). This panel of NANPs failed to elicit TLR3 or TLR9 responses, which may in part be due to length restrictions of TLR ligands because TLR3 and TLR9 require a minimum of 40 and 20 base pairs, respectively.[172,173] This finding suggests that NANP strand length could be optimized in future studies to avoid or to target PRR activation. Notably, the data demonstrates that TLR7, which can identify both ssRNA and short dsRNA,[174,175] is activated in response to RcR and RcD NANPs. In agreement with previous findings, Rc2′F NANPs do not stimulate TLR7 activation, demonstrating that chemical modification can be applied to abrogate TLR activation.[176,177]

The researchers also examined the cytosolic RNA sensor, the retinoic acid inducible gene-I (RIG-I), that binds 5′-triphosphorylated RNA.[178] All NANPs with a central RNA strand (RcR, RcD, and Rc2′F) possess a 5′-triphosphorylated motif and, as such, activated RIG-I-dependent responses. Importantly, DcR NANPs did not activate RIG-I-dependent responses due to an absence of a 5′-triphosphorylated group, demonstrating the critical role of ligand motifs in determining PRR activation. Interestingly, Dc2′F NANPs also stimulate RIG-I-dependent responses. RNA polymerase III is known to transcribe AT-rich dsDNA into a 5′-triphosphorylated RNA ligand that activates RIG-I.[179−181] Consistent with this model, the researchers observed that knockdown of RNA polymerase III significantly reduced the release of immune mediators, indicating that RIG-I recognition of Dc2′F is dependent on RNA polymerase III. This finding suggests that NANP sequence could be optimized in future studies to avoid or to target PRR activation because RNA polymerase III preferentially binds AT-rich DNA.[179−182] Collectively, the data indicate that through understanding PRR ligand characteristics, the chemical composition of NANPs can be rationally engineered to avoid or to activate PRR-driven immune responses.

Approaches that take advantage of these tailorable immune responses include their adaptation in immune therapies. Ionizing radiation acts not only as a potent DNA damaging agent, thus inducing death of targeted cells, but also as a strong immunoadjuvant through the release of cytokines, such as type I IFNs. Increasing evidence indicates that the therapeutic response to ionizing radiation depends on the individual response to type I IFNs.[183] Applications of radiation therapy have recently been suggested as a strategy to increase tumor immunogenicity. Integration of radiotherapy with immune checkpoint inhibitors are treatment regimens recently tested both preclinically and clinically.[184] Nucleic acid nanoparticles trigger similar mechanisms to those of ionizing radiation, inducing both IFN responses and activation of TLRs and other PRRs, as discussed previously.[158] At the mechanistic level, therefore, the studies using radiation therapy open a wide niche for the application of NANPs.

SCAFFOLDED DELIVERY OF MULTIPLE THERAPEUTICS WITH NUCLEIC ACID NANOPARTICLES

The versatility of rationally designed nanostructures enables a number of innovative approaches because the NANPs can carry specific information for silencing gene products, tailoring

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cellular response. This strategy has been used to silence genes whose products lead to cell survival. Many of these gene products are members of large gene families, which display redundant and compensatory expression profiles. The groups led by Chammas and Afonin presented data on the concomitant silencing of different forms of lysophosphorylcholine acetyltransferases, a family of four distinct phospholipid remodeling enzymes that are involved in the synthesis of lipid mediators such as platelet activating factor (whose synthesis is dependent on the activity of LPCAT1 and LPCAT2) and other phosphatidylcholine derivatives that are dependent on the activity of LPCAT3, which plays a role in cell death through ferroptosis.[185−187] Concomitant silencing of all members of the LPCAT gene family led to an increase in sensitivity of human melanoma cells to ionizing radiation.[188] A future challenge will involve the vectorialization of these designed nanostructures to exploit the possibility of adding specific homing peptides to the tailored NANP, which could then be delivered to either tumor cells or tumor microenvironmental cells, such as tumor-associated macrophages. Nucleic acid nanoparticles could then help in the fine-tuning of tumor and microenvironmental responses to ionizing radiation, optimizing its immunogenicity.

> The versatility of rationally designed nanostructures enables a number of innovative approaches because the nucleic acid nanoparticles can carry specific information for silencing gene products, tailoring the cellular response.

Other RNA-based approaches to cancer therapy enable the precise targeting of specific hallmarks. Xiaoting Zhang's laboratory at the University of Cincinnati College of Medicine has developed multifunctional pRNA NANPs for the efficient targeting of breast cancer *via* a HER2 aptamer, enabling their subsequent treatment using siRNAs against Mediator Subunit 1 (MED1).[189] Previously, MED1 silencing has been shown to increase breast cancer sensitization for cells that have developed fulvestrant drug resistance.[190] Dr. Handan Acar's lab at the University of Oklahoma has developed therapeutic peptides as materials for immunoengineering[191] with peptide amphiphiles acting as intracellular delivery agents.[192] Using this strategy, peptide mimics complementary to BCL-2 proteins were used to trigger dose-dependent apoptosis.[193]

Furthermore, mechanistic studies of immune cell activation enable a broader overall understanding of these systems beyond a solely RNA basis. Mark Bathe's laboratory recently utilized DNA origami to study the effects on B-cell activation of presented antigens by varying their spacing and scaffolding. As a proof-of-concept, the eOD-GT8 HIV immunogen was implemented into the DNA scaffolds, enabling precise programmability over the stoichiometry, interantigen distance, and dimensionality of larger origami self-assemblies.[194] Erdem Tabdanov presented a recent approach to define the microstructural dynamics of T cell migration,[195] which is heavily affected by the dense mechanical cues found in tumor microenvironments.[196,197] Understanding these migrations stemming from different mechanisms of motility offers the potential to enhance T cell motility.

With an eye toward future studies, the Nanotechnology Characterization Laboratory (NCL) was funded by the National Cancer Institute to improve the translation of promising nanotechnology concepts to the clinic, and it provides a unique and free resource for the nanotechnology community. In addition to the comprehensive portfolio of both *in vitro* and *in vivo* assays, protocols for which are freely available to researchers online,[198] the laboratory supports researchers by conducting preclinical studies. The NCL assay cascade services are free of charge for qualified investigators.[199] Although NCL is dedicated to cancer nanotechnology, lessons learned from its experience with cancer nanomedicines and protocols inform the translation of nanotechnology concepts intended for noncancer indications.

CURRENT STATE, LIMITATIONS, AND FUTURE DIRECTIONS

Paul Weiss of the University of California, Los Angeles, highlighted the current state of nanotechnology at large and its expanding role in biology and biomedical sciences. He noted that the nanoscale is the scale of function of biology and that presents opportunities for both fields. Weiss highlighted the multidisciplinary work that has enabled rapid advances in nanomedicine and encouraged such collaborations to continue. He noted that the development of the field of nanotechnology led to interdisciplinary communication skills that nanotechnology can use to advance related fields.[200] For RNA research, in particular, which has advantages highlighted by recent achievements in the clinical setting, these multidisciplinary collaborations between computational, dynamic, delivery, and immunostimulatory approaches are essential.

Many limitations discussed in each of these themes also pose associated challenges. For example, there remain several unknown variables surrounding the immune stimulation of nucleic acids and how recognition may be affected by the mode of delivery. Discoveries in naturally occurring structural RNA biology can be used to inform and to inspire the development of dynamic systems, such as those seen in riboswitches and ribozymes.[201] The continuous interplay between all aspects is therefore essential to drive the field forward toward optimal platforms for nanotechnology and nanomedicine.

In addition, the opportunity to host an online webinar series called for adaptations to the traditional conference style. A moderator, Morgan Chandler (University of North Carolina at Charlotte), was in place to introduce and to transition between speakers, to maintain the schedule, and to facilitate discussion questions, which were asked in the chatbox by the audience and vocalized by the moderator after each talk. Lunch breaks were built into the schedule with flexible transition times to account for delays. There were limitations to this style of meeting, including minor technical difficulties transitioning between talks. Although the published schedule allowed any audience member to drop-in for talks at their convenience, the scheduled speakers and audience members included many international scholars who were joining from different time zones, which made some of the talks inconvenient to attend. Furthermore, although audience members could contact a speaker directly to discuss their work, most questions were passed from the audience through the moderator, which led to a lack of interpersonal discussions to which regular conferencegoers are accustomed. To aid in making the meeting more personable, there were group photos on both days with

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audience members joining in from their webcams. In future iterations, we recommend the use of breakout groups or smaller discussion panels to encourage additional interactions.

AUTHOR INFORMATION

Corresponding Authors

Peixuan Guo − *Center for RNA Nanobiotechnology and Nanomedicine, College of Pharmacy, Division of Pharmaceutics and Pharmaceutical Chemistry, College of Medicine, Dorothy M. Davis Heart and Lung Research Institute, James Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210, United States;* orcid.org/0000-0001-5706-2833; Email: guo.1091@osu.edu

Kirill A. Afonin − *Nanoscale Science Program, Department of Chemistry, University of North Carolina at Charlotte, Charlotte, North Carolina 28223, United States;* orcid.org/0000-0002-6917-3183; Email: kafonin@uncc.edu

Authors

Morgan Chandler − *Nanoscale Science Program, Department of Chemistry, University of North Carolina at Charlotte, Charlotte, North Carolina 28223, United States;* orcid.org/0000-0003-3078-6000

Brittany Johnson − *Department of Biological Sciences, University of North Carolina at Charlotte, Charlotte, North Carolina 28223, United States*

Emil Khisamutdinov − *Department of Chemistry, Ball State University, Muncie, Indiana 47304, United States*

Marina A. Dobrovolskaia − *Nanotechnology Characterization Lab, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, Frederick, Maryland 21702, United States;* orcid.org/0000-0002-4233-9227

Joanna Sztuba-Solinska − *Department of Biological Sciences, Auburn University, Auburn, Alabama 36849, United States;* orcid.org/0000-0002-5227-6629

Aliasger K. Salem − *Department of Pharmaceutical Sciences and Experimental Therapeutics, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242, United States;* orcid.org/0000-0002-1923-6633

Koen Breyne − *Molecular Neurogenetics Unit, Department of Neurology and Center for Molecular Imaging Research, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachussets 02114, United States*

Roger Chammas − *Nanoscale Science Program, Department of Chemistry, University of North Carolina at Charlotte, Charlotte, North Carolina 28223, United States; Centro de Investigação Translacional em Oncologia, Departamento de Radiologia e Oncologia, Instituto do Cancer do Estado de São Paulo - ICESP, Faculdade de Medicina da Universidade de São Paulo - FMUSP, São Paulo 01246-000, São Paulo, Brazil*

Nils G. Walter − *Single Molecule Analysis Group, Department of Chemistry and Center for RNA Biomedicine, University of Michigan, Ann Arbor, Michigan 48109, United States;* orcid.org/0000-0002-7301-1275

Lydia M. Contreras − *McKetta Department of Chemical Engineering and Department of Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas 78714, United States;* orcid.org/0000-0001-5010-5511

Complete contact information is available at: https://pubs.acs.org/10.1021/acsnano.0c10240

Notes

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