RNA Engineering for Public Health: Innovations in RNA-Based Diagnostics and Therapeutics

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Abstract
RNA is essential for cellular function: From sensing intra- and extracellular signals to controlling gene expression, RNA mediates a diverse and expansive list of molecular processes. A long-standing goal of synthetic biology has been to develop RNA engineering principles that can be used to harness and reprogram these RNA-mediated processes to engineer biological systems to solve pressing global challenges. Recent advances in the field of RNA engineering are bringing this to fruition, enabling the creation of RNA-based tools to combat some of the most urgent public health crises. Specifically, new diagnostics using engineered RNAs are able to detect both pathogens and chemicals while generating an easily detectable fluorescent signal as an indicator. New classes of vaccines and therapeutics are also using engineered RNAs to target a wide range of genetic and pathogenic diseases. Here, we discuss the recent breakthroughs in RNA engineering enabling these innovations and examine how advances in RNA design promise to accelerate the impact of engineered RNA systems.
INTRODUCTION

RNA-based biotechnologies have a long history, beginning with the use of enzymatically synthesized RNA to decipher the genetic code (1) and continuing through the development of messenger RNA (mRNA) vaccines to combat the global coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2), with many milestones in between (Figure 1). This is due in large part to RNAs’ versatility, which makes it ubiquitous in all forms of life. It was first appreciated for its role as an intermediate in the transfer of DNA-encoded instructions for protein synthesis as mRNA, in reading the genetic code as transfer RNA (tRNA), and much later as the catalyst for synthesizing peptide bonds of proteins as ribosomal RNA (rRNA) (3). tRNA and rRNA are particularly interesting because they are some of the best-known examples of noncoding RNAs (ncRNAs)—RNAs that do not contain protein-coding sequences but instead perform critical cellular functions. The advent of RNA sequencing has uncovered a plethora of natural ncRNA classes that enact or regulate a wide range of cellular processes beyond protein synthesis (4, 5). Highlights of the various classes include bacterial small RNAs that regulate gene expression, small nucleolar RNAs that drive RNA modifications, eukaryotic microRNAs that regulate protein expression, and long ncRNAs that influence processes throughout the cell. This broad range of natural RNA functions makes it an ideal substrate for engineering, particularly in applications related to human health.

This diversity of natural RNA function is enabled by RNA’s ability to fold into sophisticated molecular structures. As a single-stranded nucleic acid polymer, RNA can fold back on itself into structures using interactions similar to DNA base pairing. At the secondary structure, or base pairing, level, these structures are superficially simple and consist of helices, loops, and bulges that form hairpin structures. Despite this apparent simplicity, these hairpin structures can have large effects on RNA function; for example, a hairpin structure can occlude protein–RNA or RNA–RNA interactions to control basic aspects of gene expression including transcription, translation, and RNA degradation. Furthermore, not all regions of an RNA’s structure participate in base pairing, and these unpaired regions can serve as targets for other RNA or DNA interactions that change the structure, and thus the function, of the RNA. The tertiary structure level is more sophisticated, with the 3D orientation of helices and intramolecular interactions combining with RNAs ability to form pseudoknots and noncanonical base pairing interactions to form the molecule’s overall structure. These structures can then give rise to emergent properties such as catalytic active sites (6) and exquisitely specific ligand-binding pockets (7). These structures can also be enhanced even further through the incorporation of chemically modified nucleotides (8).

Because an RNA molecule is encoded as a sequence of four nucleotides, we can design RNA sequences to fold into desired structures and perform specific functions—i.e., engineer synthetic RNAs. For example, synthetic RNAs have been designed to act as gene expression switches by folding into hairpins that block protein translation then unfolding to allow gene expression when bound to target RNAs (9). When these switches bind to target RNAs from pathogen genomes, they can then form the basis of a new class of pathogen diagnostics (10). Our knowledge of the sequence–structure–function relationship of RNA guides this design process, supported by computational algorithms that can predict RNA folds and even design RNA sequences that fold in specific configurations (11, 12). For example, engineered tertiary-level RNA structures can form specific binding pockets for intercellular signaling molecules such as neurotransmitters (13, 14). This combination of the diversity of RNA functions and our growing knowledge of the sequence–structure–function relationship has led to increased interest and ability in engineering RNA.

There have recently been significant advances in the engineering of RNA systems for applications in public health. For example, new diagnostic innovations offer RNA technologies that can detect emerging pathogens such as the Ebola (15) and Zika (16) viruses, clinically
Figure 1 (Figure appears on preceding page)
Timeline of select milestones in RNA engineering building toward applications in diagnostics and therapeutics. Abbreviations: DETECTR, DNA endonuclease-targeted CRISPR trans reporter; FDA, US Food and Drug Administration; mRNA, messenger RNA; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SHERLOCK, specific high-sensitivity enzymatic reporter unlocking.

relevant microRNAs (17, 18), and a range of chemical contaminants of drinking water (19, 20). Engineered RNAs also play a key role in enhancing the function of CRISPR (clustered regularly interspaced short palindromic repeat) systems (21, 22), a family of RNA-mediated programmable nucleases used in viral diagnostics (23, 24). The advent of CRISPR systems also provides new therapeutic applications for engineered RNAs, with RNA guiding genome- and base-editing technologies to treat disease (25) alongside previously validated RNA oligonucleotide drugs (26). Furthermore, the recently demonstrated efficacy of RNA-based vaccines highlights the potential of protein-coding RNA for rapid, large-scale public health intervention (27, 28). As a whole, the impact and accelerating pace of these innovations signals the beginning of a new era of RNA engineering in diagnostics and personalized medicine.

In this review, we describe recent innovations in diagnostic and therapeutic applications of RNA engineering. We focus specifically on five broad application areas: pathogen diagnostics, chemical contaminant diagnostics, gene editing techniques, mRNA vaccines, and RNA-targeting drugs. We then conclude with a discussion of recent fundamental advances in the development of RNA design tools that promise to enhance the impact of engineered RNA systems. This work complements another excellent review on the applications of synthetic biology to public health that was published earlier this year (29).

ADVANCES IN RNA-BASED DIAGNOSTIC TECHNOLOGIES

Meaningful intervention in public health crises necessitates large-scale diagnostic testing. The most prominent example of this is the COVID-19 pandemic, which highlighted the need for high-throughput, scalable nucleic acid testing to guide mitigation efforts. However, this general need also holds true for a broad range of public health issues. For example, global-scale water contamination is another significant burden on public health, and its effects could be more effectively mitigated by high-resolution water sampling to identify contaminated sources. Unfortunately, existing gold-standard methods, such as quantitative polymerase chain reaction for pathogen detection and mass spectrometry for chemical contaminant detection, rely on centralized laboratory equipment and are thus difficult to scale up in response to increased demand. Portable and easy-to-use biochemical reactions using engineered RNAs as biosensors offer a powerful alternative to help address these challenges; the programmability of RNA enables the highly specific detection of targeted pathogen sequences, and its evolvable ligand-binding properties enable small molecule detection and fluorescent signal generation. These biochemical biosensing reactions can be put in test tubes or lateral flow strips and easily operated by applying a processed sample to the tube or strip and waiting for signal generation. Furthermore, these reactions can be mass produced and freeze-dried, allowing easy on-site distribution and deployment to accommodate surges in demand. Here, we discuss recent RNA engineering efforts toward detecting both pathogens and waterborne chemical contaminants of interest, highlighting recent successes with several high-impact targets.

Pathogen Diagnostics

Pathogen diagnostics are a critical aspect of public health approaches to combating disease (30). The scale of this challenge is enormous, with an estimated 5.5 million people dying of infectious
Figure 2

Summary of RNA-based pathogen biosensors. (a) General overview of RNA-based molecular diagnostics for pathogens. (b) Toehold switches control translation to modulate signal generation. They have been integrated into a paper-based sensor for Ebola detection. Panel adapted with permission from Pardee et al. (15). (c) STARs leverage transcriptional regulation to detect pathogenic nucleic acids. They have been used in a colorimetric assay to detect plant viruses. Panel adapted with permission from Veroslof et al. (36); https://pubs.acs.org/doi/10.1021/acssynbio.8b00526. Further permissions should be directed to the American Chemical Society. (d) SHERLOCK and (e) DETECTR leverage collateral cleavage activity of Cas proteins to detect viral RNA. Panels adapted with permission from (d) Zhang et al. (39) and (e) Broughton et al. (40). SHERLOCK and DETECTR have been used to detect SARS-CoV-2. Abbreviations: crRNA, CRISPR RNA; DETECTR, DNA endonuclease-targeted CRISPR trans reporter; gRNA, guide RNA; PAM, protospacer adjacent motif; RBS, ribosome binding site; RT, reverse transcriptase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SHERLOCK, specific high-sensitivity enzymatic reporter unlocking; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; STARs, small transcription activating RNA.

and parasitic diseases in 2016 (31) and more than 100 million confirmed cases and millions of deaths in the COVID-19 pandemic (32). Engineered RNA systems offer a flexible starting point for designing diagnostic systems that identify pathogens by detecting specific sequences in their genome (Figure 2a). These systems all use the same broad strategy of designing an RNA to bind to a target nucleic acid sequence, accomplished by simply changing the engineered RNA’s sequence. Diagnostic tools can then be made by using this binding interaction to generate a detectable signal, typically in the form of fluorescence or a color change. If needed, pathogen target sequences can be amplified before detection to enhance the diagnostic’s sensitivity (33).

Some engineered RNAs can detect specific sequences by using the programmability of RNA sequence and structure to design RNA–RNA interactions. In both the toehold switch (9) and small transcription activating RNA (STAR) (34) mechanisms, specific interactions drive a change
in RNA structure leading to the activation and detectable expression of a reporter gene. Toehold switches consist of a hairpin structure designed to sequester a ribosome binding site and start codon that govern the translation of a downstream reporter gene. This hairpin also includes a toehold sequence that is complementary to the desired target. When the target sequence is present in the reaction, its binding to the toehold switch initiates a structural rearrangement that unfurls the hairpin, exposing the ribosome binding site to initiate translation of the downstream reporter gene (Figure 2b). The STAR mechanism works in a similar fashion, though with target binding preventing formation of a terminating hairpin and enabling transcription of the downstream reporter gene (Figure 2c).

Both toehold switches and STARs have been implemented in viral diagnostics. The first application for toehold switches came near the beginning of the recent West African Ebola virus epidemic, where lyophilized reactions containing Ebola-specific toehold switches detected nanomolar concentrations of viral RNA via coupling to an isothermal amplification step (15). The subsequent South American Zika virus epidemic saw the expansion of this work, streamlining the sensor development and testing pipeline to generate toehold switches that functioned as viral sensors within a week of identifying a target sequence (16). Importantly, these sensors could detect viral RNA in infected plasma samples and distinguish between Zika and the clinically and genetically similar Dengue virus. In addition to these epidemic virus applications, toeholds have been used for the strain-specific screening of gut flora from stool samples (35), and STARs have been used to detect plant viruses (36).

Beyond these synthetic RNA structures, existing RNA-based detection mechanisms have been repurposed for pathogen diagnostics. In particular, the discovery of CRISPR-Cas systems has enabled the detection of viral sequences by leveraging the Cas proteins’ sequence-dependent nuclease activity. In nature, CRISPR-Cas systems serve as a bacterial immune system, cleaving recognized viral sequences to prevent reinfection. They function via a Cas (CRISPR-associated) protein bound to a guide RNA (gRNA) that binds to a target nucleic acid sequence for recognition. In this RNA–protein complex, the Cas protein facilitates gRNA binding to its intended target through canonical base-pairing interactions, enabling single-nucleotide specificity (21). These systems’ flexibility makes them high-value targets for engineering; a range of natural CRISPR-Cas systems are available, broadly divided into six types based on their structure, editing mechanism, and nucleic acid target.

One Cas protein used for pathogen sensing is the type VI Cas protein Cas13a, which detects RNA and acts as an indiscriminate ribonuclease after detection. This indiscriminate ribonuclease activity can form the basis for a diagnostic reaction through the use of an RNA-linked fluorophore–quencher pair: If the Cas13a–gRNA complex recognizes its target, it can then cleave the fluorophore–quencher pair and generate a detectable signal (Figure 2d). When combined with target amplification strategies, this technique allows for the sensitive detection of a range of pathogens. This was first demonstrated with SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) (23), which was reported to detect attomolar levels of viral RNA. Combined with a sample processing protocol to eliminate nucleases from body fluids (37), this method can be used to detect viral RNA directly from body fluids at titers as low as one copy per microliter. This system can also be massively multiplexed through the use of carefully designed gRNA sequences and automated liquid-handling methods, with simultaneous detection demonstrated for a panel of 169 unique pathogen targets (38).

By changing the Cas protein used to the type V Cas protein Cas12a, a similar scheme can be used to detect DNA targets. Cas12a acts as an indiscriminate single-stranded DNAse after binding its double-stranded DNA target, which can be used to cleave a single-stranded DNA-linked fluorophore–quencher pair upon detection (Figure 2e). This was demonstrated with DETECTR
(DNA endonuclease-targeted CRISPR trans reporter), which was used to detect low levels of human papillomavirus from patient samples (24). Further discovery and application of Cas proteins may yield an expanded toolbox of these sensors for diagnostic use.

These biosensors are currently transitioning from the lab into industry with the incorporation of the SHERLOCK and DETECTR technologies into Sherlock Biosciences and Mammoth Biosciences, respectively. The ongoing COVID-19 pandemic highlights the importance of this transition, with both companies developing coronavirus sensors using their respective technologies (39, 40). These engineered RNA systems are some of the most promising cutting-edge viral diagnostics; because they leverage RNA programmability to detect pathogenic sequences, they can be rapidly reprogrammed to detect novel pathogens as they emerge. This adaptability expedites development and deployment of viral sensors, potentially mitigating or preventing future public health crises.

**CHEMICAL DIAGNOSTICS**

Small-molecule water contaminants are one of the biggest contributors of disease burden to public health, with one in three people globally lacking reliable access to clean drinking water (41). The severity of this problem is underscored by the United Nations’ Sixth Sustainable Development Goal for 2030, one of a set of 17 humanitarian grand challenges (42). Specifically, this goal seeks to provide universal access to clean and safely managed drinking water, with progress determined by the proportion of the global population with access. Tracking this metric and guiding water policy will require the high-throughput testing of individual water sources, often in remote or resource-limited areas that restrict existing testing methods. This emphasizes the need for a new generation of field-deployable chemical sensors.

Fortunately, RNA sequences harvested from nature can be directly used for chemical contaminant detection. Riboswitches are a class of ncRNA that gate gene expression in response to the detection of their cognate ligands. Several classes of characterized bacterial riboswitches sense ligands of environmental interest, most notably fluoride (43) and manganese (44, 45). The recent use of the Bacillus cereus_crcB fluoride riboswitch as a point-of-use biosensor serves as an encouraging proof of concept that these ligand-binding RNAs can be used as portable small-molecule detection tools (19) (Figure 3a). These tools use the riboswitch to control the production of a colorimetric enzymatic reporter; if fluoride is present, the enzyme is produced and converts its colorless substrate into a readily visible yellow compound. In a sensor, this riboswitch-reporter system is embedded in a gene expression reaction then lyophilized for storage and distribution. These lyophilized reactions can detect fluoride onsite by simply rehydrating with a small volume (20 μL) of water, followed by incubation and readout. With this strategy, this system was used to detect below–2 parts per million aqueous fluoride in the field with no supplementary equipment. Significantly, this enables detection at the Environmental Protection Agency’s most stringent regulatory standard, highlighting the ability of these sensors to generate meaningful and accessible data on water quality at the testing site.

There are currently tens of characterized riboswitch classes responsive to ions, cellular metabolites, and enzymatic cofactors (46). Vastly dwarfing this number, however, is the set of uncharacterized orphan riboswitches, which are predicted small-molecule-sensing RNA sequences with unknown cognate ligands (47). Moving forward, identifying cognate ligands for these switches could yield an array of natural small-molecule-responsive riboregulators. Furthermore, RNA selection strategies can alter riboswitch specificity and enable the detection of novel targets of interest (14).

Beyond using RNAs ligand-binding properties to directly sense contaminants, RNA engineering has also yielded tools that can supplement protein-mediated strategies for chemical
RNA can serve as both a sensor and a reporter. (a) Schematic for detection of aqueous fluoride via the *crcB* fluoride riboswitch. The fluoride aptamer in the *crcB* riboswitch binds fluoride, with ligand binding changing the RNA switch’s final conformation to allow production of a downstream enzymatic reporter. The enzyme converts a colorless compound into a yellow pigment. Without fluoride, transcription of the reporter gene is prohibited. Panel adapted with permission from Thavarajah et al. (19); https://pubs.acs.org/doi/full/10.1021/acssynbio.9b00347. Further permissions should be directed to the American Chemical Society. (b) Schematic for ROSALIND (RNA output sensors activated by ligand induction). Contaminants are detected via a contaminant-specific transcription factor, with ligand binding enabling the production of a fluorescent RNA aptamer. In the absence of the target compound, transcription is blocked. Panel adapted with permission from Jung et al. (20).

contaminant detection. Chief among these are fluorescent aptamers—RNA-based analogs to fluorescent proteins created by evolving RNA sequences to bind fluorescent dyes. This was first reported with the creation of an RNA motif binding to the malachite green dye (48). More recently, the selection of the Spinach aptamer demonstrated the utility of these fluorescent aptamers as reporters, offering a genetically encoded means of producing a robust, easily measurable output (49). Because these outputs operate on transcriptional, rather than translational, timescales, these fluorescent aptamers provide a faster alternative to fluorescent proteins without meaningfully compromising signal intensity (20). Improving our skills with this selection process will continue to yield brighter, more diverse, and further optimized aptamers for future sensing applications.

RNA design strategies can also be extended to produce systems that go beyond simply switching gene expression on or off and instead add layers of genetic logic and feedback to improve the performance of diagnostics. This is accomplished by programming the pairing and unpairing of RNA under certain conditions to control RNA-mediated reactions and enable more advanced functions. For example, conditional structural changes can allow a fluorescent RNA aptamer to form only upon the binding of a small molecule (50) or another RNA sequence (17). Alternatively, the splitting of a fluorescent aptamer to generate a signal upon the dimerization of its two halves shows the potential for RNA pairing to encode Boolean logic (51). Additional logical operations can be performed using synthetic RNA sequences called kleptamers, which can displace and inhibit ligand-binding RNAs such as fluorescent aptamers (52).

These strategies have been synthetized with the recent development of ROSALIND (RNA output sensors activated by ligand induction), a modular platform consisting of protein-based small-molecule biosensors that use the three-way junction dimeric Broccoli RNA aptamer as a fluorescent output (20) (Figure 3b). Gating production of the fluorescent aptamer behind
a modular, transcription factor–based system allows detection of several high-impact water contaminants, including lead, copper, zinc, and antibiotics. Furthermore, this work demonstrates the ability of RNA engineering to tune both the sensitivity and specificity of protein-based small-molecule biosensors. For example, a promiscuous sensor detecting both copper and zinc was made copper specific by using a kleptamer to inhibit the fluorescent aptamer upon zinc detection. Additionally, an RNA evolved to bind to one of the transcription factors within a feedback circuit enhanced the system’s sensitivity without any protein engineering. By gating production of both the fluorescent RNA output and a tetracycline repressor–inhibiting RNA behind the tetracycline repressor, the presence of even low titers of tetracycline results in a positive-feedback loop of tetracycline derepression and production of a robust fluorescent output.

Future development of RNA selection strategies could yield binders and regulators to develop sensors for a wide range of targets (53–55). Furthermore, there is a growing effort to develop computational tools capable of rapidly generating synthetic riboswitches against targets of interest (56, 57). Combined with advances in fluorescent aptamer engineering, further development of this suite of tools could enable the rapid detection of a library of high-impact chemical targets.

ADVANCES IN RNA-BASED THERAPEUTIC TECHNOLOGIES

Complementing its diagnostic applications, RNA engineering is being increasingly applied to developing the next generation of vaccines and precision therapeutics. Modified RNAs can be used to improve editing rate and specificity for CRISPR systems, and carefully designed mRNA coding sequences can be combined with delivery strategies to act as vaccines by producing pathogen proteins to provoke an immune response. Furthermore, small molecules and engineered RNAs are being designed to target endogenous RNAs for disease therapies and treatments. Taken as a whole, this suite of therapeutic applications allows for treatment of previously intractable genetic diseases and prevention of pathogen infection. Here, we discuss key advances in RNA engineering as applied to CRISPR-Cas gene editing systems, mRNA vaccines, and RNA-targeting drugs, highlighting recent successes and discussing the potential for future applications.

Gene Editing

The discovery and engineering of CRISPR systems offers a means to cure genetic diseases instead of just managing their symptoms. Of particular interest are diseases caused by single-nucleotide polymorphisms, such as sickle-cell anemia (58), cystic fibrosis (59), and β-thalassemia (60). Here the potential for CRISPR systems to edit a single nucleotide has sparked a wave of engineering to develop therapies restoring normal biological function.

The most well-known and best-characterized Cas protein is the type II Cas protein Cas9, which uses a gRNA to recognize and cleave a specific double-stranded DNA target (61), excising it from the genome (Figure 4a). Manipulation of the subsequent repair process enables CRISPR-mediated genome editing—the cellular recombination machinery used to repair the break will incorporate supplied exogenous DNA, provided it has regions homologous to the break site. Thus, an excised sequence can be replaced with a sequence of choice when the break is repaired.

Unfortunately, although Cas9 is touted to function at single-nucleotide resolutions, it has well-documented off-target activity and can remain highly active in human cell lines even with small mismatches between the target and gRNA (62–64). A more recent study in mice has demonstrated the full effect of this activity by identifying multiple off-target editing sites distributed throughout the genome (64). Fortunately, these off-target effects can be mitigated by carefully selecting target sites, along with engineering the gRNA for increased fidelity (63). These RNA engineering
Figure 4

Enhancement of Cas9 editing rate and specificity via guide RNA (gRNA) engineering.

(a) Schematic of Cas9 editing mechanism. Cas9 mediates the sequence-specific binding of its gRNA spacer sequence to a corresponding site on the target DNA. Recognition initiates site-specific DNA cleavage. Cellular recombination machinery then uses externally supplied synthetic DNA containing homology regions to the desired editing site to repair the break, adding the desired donor sequence to the DNA.

(b) Example gRNA modifications (bolded) and functions. Engineering the gRNA with additional sequences and chemical modifications can increase cleavage efficiency and gRNA stability, reduce off-target editing, and confer novel functions.

(c) Schematic of prime editing mechanism. An RNA-guided nickase/reverse transcriptase (RT) fusion mediates the writing of an edit encoded in the gRNA. Cellular recombination machinery then seals the nick, incorporating the edit into the genome.
strategies are effective because a gRNA's structure plays a key role in determining its editing activity, so structural modifications can tune its editing behavior (Figure 4b). For example, previous work showed that strong base pairing in the gRNA's stem regions, nexus, and hairpins decreases editing activity (65), whereas the extension of the gRNA's upper stem significantly increases editing activity by disrupting a thymine-rich sequence of nucleotides (66). Designed structures can also be used to increase specificity: The addition of a hairpin to the gRNA spacer sequence reduces off-target activity by disfavoring off-target binding (67). This occurs because the hairpin must be displaced by the target DNA sequence to enable gRNA binding and cleavage, which is more thermodynamically favorable for an entirely complementary sequence than for a partially mismatched one.

Moving beyond what is possible with nature's biological tools offers a means to further tune CRISPR-Cas systems—chemically synthesizing gRNAs enables the modification of their nucleotides with functional groups to improve editing rate and specificity. For example, 2′-O-methylation and related RNA modifications increase gRNA stability, resulting in a corresponding increase in editing rate (68). Similar modifications have also been used to reduce off-target cleavage without sacrificing on-target activity (69). Notably, although modifying portions of the gRNA that directly interface with Cas9 has been shown to reduce its activity, the remainder of the gRNA sequence tolerates heavy modification (70). Combining these chemical modifications with optimized gRNA structures stands to significantly increase the fidelity and efficiency of our gene editing toolkit.

Moving beyond these simple but effective gRNA engineering strategies, researchers have begun to use more advanced RNA engineering approaches to confer new functions to the gRNA itself. Among the more compelling of these is the addition of triggers to activate the Cas protein and induce cleavage, enabling finer temporal control over editing activity. There have been two approaches to this, both of which focus on adding functional modules to the gRNA. Expanding the applications of the toehold switch, the addition of a toehold immediately after the gRNA's spacer enables Cas protein activation by the toehold's cognate trigger sequence (71, 72). Alternatively, the addition of an aptamer to the upper stem of the gRNA enables activation by the addition of the aptamer's cognate ligand (73). This is particularly promising for the future clinical application of CRISPR systems—the ability to activate a Cas protein with a small molecule paves the way for control of editing time, duration, and location with external drug-like triggers.

Recently, this suite of gRNA engineering strategies has been combined with protein engineering tools to impart entirely novel editing mechanisms to CRISPR systems. This is exemplified by the recent development of prime editing (74), which allows for genome editing with combined DNA cleavage and repair by fusing an RNA-guided DNA-nicking domain to a reverse transcriptase (Figure 4c). This editing strategy differs from conventional CRISPR-Cas9 editing because it does not use supplied exogenous DNA encoding the desired edit to be incorporated upon repair; instead, the desired edits are written into the gRNA itself on the strand’s 3’ end. Upon binding to the target DNA, the complex nicks it, creating a flap that can bind to the edit-encoding 3’ end. This flap then serves as a primer for reverse transcription, reading the gRNA and incorporating the edits into the DNA sequence. Endogenous repair mechanisms then seal the flap, yielding edited double-stranded DNA. This strategy has been used in vitro for the targeted repair of mutations causing sickle-cell anemia and Tay-Sachs disease in a human cell line (74), along with the precise addition of affinity tags and epitopes to targeted protein coding sequences. We are even progressing beyond DNA editing toward editing RNA itself; by fusing a catalytically inactive Cas13 mutant to a base editor, we can directly target full-length RNA transcripts for modification (75).
Many of these advances in CRISPR-mediated gene editing have enabled therapeutic applications in a series of recent clinical trials. A partnership between CRISPR Therapeutics and Vertex Pharmaceuticals launched trials for sickle-cell and β-thalassemia treatments (76), with another between Allergan and Editas Medicine seeking to remediate a genetic cause for blindness by editing cells within the eye (77). These early-stage clinical efforts highlight the maturation of engineered CRISPR systems as they begin the move from the bench to the clinic. CRISPR-mediated gene editing has also inspired the development of minimally immunogenic RNA-targeting proteins to address anticipated challenges around CRISPR-based therapeutics, such as delivery of and immunological responses against the Cas proteins (78). Moving forward, further increasing the fidelity of RNA-guided gene editing systems through advances in RNA and protein engineering could enable their application to a larger set of genetic diseases.

mRNA Vaccines

The human cost and societal disruption caused by recent epidemic and pandemic diseases—swine flu, Ebola, Zika, and most pressingly COVID-19—emphasize the role that rapid vaccine development and manufacturing can play in mitigating or preventing future public health crises (79, 80). In response to the 2010 swine flu pandemic, the US government released a report identifying inefficiencies in the vaccine development process and suggesting four research focus areas to remedy them, which included support for developing genetic engineering tools (81). This particular research thrust has recently skyrocketed in importance, with the rapid response to the COVID-19 pandemic by vaccine developers CureVac, ModernaTX, and BioNTech demonstrating the ability for mRNA vaccines to quickly combat public health crises.

Broadly speaking, mRNA vaccines are engineered RNA sequences encoding a pathogen protein to be expressed within the patient’s cells to provoke an immune response. This strategy removes obstacles that occur with protein vaccine manufacturing, such as maintaining native protein structure and incorporating posttranslational modifications that can be critical for protein-based vaccine efficacy (82). Because they eliminate the protein production aspect from the vaccine development process, mRNA vaccine manufacturing is rapid, scalable, and cell-free (83). In addition, mRNA vaccines can be quickly redesigned for emerging threats by simply changing the RNA sequence. They can also be put toward purposes outside of infectious disease prevention, such as personalized medicine for cancer treatment (84). Thus, the combination of RNA programmability and the scalable, relatively inexpensive manufacturing process has significant potential to transform medicine.

The versatility of mRNA vaccines partially stems from their modular components (Figure 5a), with decades of work establishing a set of principles for designing each of their features (85). For example, codon optimization of the pathogenic sequence in the open reading frame (ORF) is a delicate design balance of maximizing translation speed without causing mRNA-degrading ribosome collision or hindering protein folding. The coding RNA must also be minimally structured to increase translational initiation and prevent recognition from the innate immune system (85). Supporting the coding sequence, the 5′ cap, 5′ untranslated region (UTR), 3′ UTR, and poly-A tail allow the cell to recognize the RNA and express its protein while avoiding natural degradation pathways. The 5′ cap causes the host cell to recognize the mRNA molecule as self to prevent its immediate destruction by the innate immune system. The UTRs are important determinants of mRNA stability. The 5′ UTR should have little secondary structure, and the 3′ UTR should avoid coding for microRNA binding sites, the number of which correlates negatively with mRNA half-life (85). Alternatively, the introduction of tissue-specific microRNA binding sites can be used for programmed degradation in designated parts of the body (86). The poly-A tail influences translation efficiency, with longer tails increasing mRNA stability. However, shorter tails and 5′ cap
modifications increase translation efficiency by promoting RNA looping, a process in which the mRNA is circularized to enable quick ribosome reentry and prevent enzymatic degradation (85). mRNA vaccines can also be designed as self-amplifying mRNAs (SAMs), which include a sequence based on viral RNA-dependent RNA polymerases to allow for the message to be replicated in the host cell (83). SAM vaccines have shown protection levels comparable to those of non-SAM vaccines with much smaller dosages (87). Additionally, ncRNA messages that lack an ORF and cap may be able to act as vaccines, as shown by a potential cancer vaccine (88).

To serve as an effective vaccine, these engineered mRNA templates must be minimally immunogenic; RNA can activate the innate immune response through recognition by Toll-like receptors, resulting in transcript degradation. Early studies demonstrated that incorporating pseudouridine and 1-methylpseudouridine nucleoside modifications throughout the molecule could prevent this immune response (82, 89), with a pseudouridine modification additionally
shown to increase mRNA stability and translation capacity (90). The efficacy of these strategies was demonstrated when a 1-methylpseduouridine-modified mRNA vaccine outperformed a chemically modified protein vaccine against herpes simplex virus type 2 (91). This was countered by later work suggesting that mRNA sequence optimization was the key determinant of transcript longevity (92). Then, other types of chemical modifications include those on the 2′-hydroxyl or phosphate backbone (93). The many different options for chemical modification demonstrate the importance of carefully designing an mRNA molecule through structure, sequence, and nucleotide modification to optimize the vaccine candidate.

mRNA vaccines have previously demonstrated high potential efficacy with minimal side effects (94), but before the COVID-19 pandemic no vaccine candidate had passed phase 3 clinical trials to move on to mass distribution. In the intervening time, the rapid development and deployment of mRNA vaccines have validated RNA technologies as a tool for safeguarding public health. The SARS-CoV-2 viral genome was first uploaded online on January 11, 2020, and within 48 hours ModernaTX had used it to design vaccine candidates. Phase 1 clinical trials began two months later, on March 16. This is an order-of-magnitude improvement from the 2007 response to SARS-CoV-1, where 20 months passed between the publication of the virus’ genome and the commencement of clinical trials. By November 16, 2020, ModernaTX’s mRNA vaccine cleared phase 3 trials with a 94.1% efficacy rate (27), and a second mRNA vaccine developed by BioNTech and Pfizer reported 95% efficacy (28). The first doses of these vaccines were administered in December 2020, less than a year after the first cases reported outside of China. The role that mRNA vaccines have played in combating the COVID-19 pandemic is the ultimate proof of their potential and may, in the coming years, be seen as one of the most impactful scientific achievements of the twenty-first century.

Ultimately, for their modularity and clinical success, mRNA vaccines offer hope for a more direct and rapid approach for drug development. In the future, these technologies can be further developed through the implementation of higher-order control schemes. For example, SAMs can be evolved for more efficient self-amplification (95), or RNA circuits enable control through small-molecule regulators and RNA-binding proteins (96, 97). An engineered CRISPR-Cas9 system loaded in an mRNA vaccine can also be used to immunize cells against viruses that reverse transcribe into double-stranded DNA (98). mRNA vaccines have come a long way since their first idealization; have proven themselves during the COVID-19 pandemic; and hold even greater potential for future therapeautic development.

RNA-Targeting Drugs

RNA engineering has also paved the way for the development of new classes of therapeutic agents, both RNA based and RNA targeting. These drugs offer a means to circumvent one of the key limitations of traditional, protein-targeting drugs, which is the comparably small number of potential targets. Specifically, only 1.5% of the human genome encodes proteins (99), emphasized by the fact that as of 2018, only 0.05% of the human genome has been used for protein-targeting drug development. In contrast, 70% of the human genome is transcribed into ncRNAs (99), drastically widening options for drug development.

Beyond widening the target possibilities, RNA targeting defines a new drug development strategy. Like proteins, small molecules can target specific RNA structures and processes (100). Furthermore, drugs can be designed solely around RNA sequences, dramatically simplifying RNA-targeted drug development. Such sequence-dependent drugs fall under two categories: antisense oligonucleotides (ASOs) and RNA interference (RNAi). Both are similar to mRNA vaccines in that they have modular optimization components and a designated target sequence.
ASOs specifically are short nucleic acid sequences designed to target endogenous mRNAs through direct base pairing (101). Once bound to their target, they can effect a response by promoting increased translation (102), alternative splicing, or degradation of an mRNA target (103) (Figure 5b). The current therapeutic excitement for ASOs began after 2016, when the US Food and Drug Administration (FDA) fast-tracked the ASO drug nusinersen, marketed as Spinraza®, which treats spinal muscular atrophy by inhibiting a splicing mutation that leads to muscle weakness and atrophy (104, 105). ASO-based gene therapies are now being developed for other splicing diseases, such as cystic fibrosis (106), and even viral infections, such as Ebola (107). They also show promise for treating neurological conditions (108), with ongoing clinical trials for Huntington’s disease (109), amyotrophic lateral sclerosis (110, 111), and Alzheimer’s disease (112). A patent has also been filed for an ASO against a mitochondrial ncRNA to treat cancerous stem cells (113). Furthermore, ASOs also have important implications for the future of personalized medicine, with the ease of RNA design and manufacturing enabling patient-specific therapy (114). A proof-of-principle for these tailor-made medicines is currently being tested through the Dutch Center for RNA Therapeutics. Despite outstanding challenges with cell-specific delivery and cargo release after delivery (115), ASOs are widening the druggable targets within the human genome to treat a wide array of diseases and, with further development, may transform the medical field.

Complementing ASOs in RNA targeting is RNAi, or the delivery of RNA that silences the expression of targeted sequences in the cell. Once this RNA is in the cell, it gets processed into short RNA strands called small interfering RNAs, which hijack the cellular translation inhibition machinery to cause translational delays or mRNA degradation (116) (Figure 5c). Much like ASOs, RNAi uses sequence complementarity to recognize specific targets. RNAi’s therapeutic potential was first realized in 2001, with its successful targeting of genes in a mammalian cell line (117). In 2018, this technology moved to the clinic when the FDA and European Commission approved patisiran, marketed as Onpattro®, an RNAi–lipid complex that treats hereditary transthyretin-mediated amyloidosis (118). Expanding on this, ongoing clinical studies are using RNAi to treat diseases from ophthalmological, cardio-metabolic, and endocrine conditions, along with infectious diseases and cancer (119). As researchers learn more about RNAi, they are establishing design guidelines for its use (117) and developing computational models to design small interfering RNAs to combat future viral outbreaks (120, 121).

Delivery optimization adds another level of design to these RNA-targeting drugs. There are two broad strategies for their transport into the cell: They can be either carried in by a lipid nanoparticle or conjugated to a targeting molecule capable of entering the cell. In the latter case, the linker used can either cleave upon entry to assist in cargo release or remain uncleaved for increased stability. Furthermore, these drugs are brought into the cell as double- rather than single-stranded RNA. The two strands can be either of the same length, as conventionally designed, or mismatched to possibly assist with loading the drug into RNA machinery or cellular localization (122). This breadth of potential delivery mechanisms combined with their versatility in target choice showcases our ability to carefully tailor ASOs and RNAi to treat a wide range of diseases.

There is also an interest in targeting cellular RNAs using traditional small-molecule drugs. For example, small molecules interacting with RNA (SMIRNAs) have been shown to bind their target and induce RNA cleavage or processing disruption (100) (Figure 5d). There is a strong historical precedent for using small molecules to target RNA, beginning with the 1944 discovery of the antibiotic streptomycin, which binds to the 16S rRNA and S12 ribosomal protein (123). Like streptomycin, there are SMIRNAs that affect the RNA–protein interface, as well as those that bind RNA structures such as multi-helix junctions, pseudoknots, and bulged helix motifs (99). In fact, one SMIRNA addresses the same splicing malfunction in spinal muscular atrophy that nusinersen targets (124–126) by targeting a tertiary RNA structure in the precursor mRNA.
responsible for binding splicing machinery (127). Notably, this drug can cross the blood–brain barrier, overcoming one of the key limitations of ASOs and highlighting the therapeutic potential of SMIRNAs.

RNA structures can also be targeted outside of eukaryotic systems. For example, viruses are encoded by a targetable RNA genome, as seen with a SMIRNA drug for hepatitis C virus (128) and SARS-CoV-2 (129). Furthermore, bacteria use an extensive network of ligand-binding riboswitches, which provide an array of bacteria-specific drug targets (130, 131). This approach’s clinical efficacy was demonstrated with the targeting of a riboswitch that regulates genes necessary for bacterial growth, showing inhibition of gram-positive bacteria including methicillin-resistant Staphylococcus aureus (132). To facilitate the development of these SMIRNA drugs, multiple experimental and computational approaches, such as PEARL-Seq (133) and AnnapuRNA (134), both predict and visualize small-molecule binding sites in an RNA sequence. Moving forward, further development of these tools will enable the targeting of more genomic regions for therapeutic applications.

The ability to harness RNAs as both designer drugs and targets has allowed for rapid design and development of new disease treatments. Historically, RNA-targeting drugs had to overcome various challenges, with ASOs and RNAi facing issues with molecule delivery, stability, and degradation by the immune system. Ultimately, understanding how to deliver these drugs into the correct cells will be the breakthrough needed to begin fulfilling the potential of RNA-targeting drugs outside the spinal cord (135) and liver (115). Furthermore, small molecules were initially thought to be inapplicable to RNA until it was learned that RNA formed complex structures capable of ligand binding (100, 136), offering a nucleic acid analog to protein antibodies (137). As the research addresses these challenges, computational tools are being developed to assist in drug development, such as computational SMIRNA discovery pipelines Inforna (138) and R-BIND (139), and PFRED (140), a computational pipeline for the design, analysis, and visualization of ASOs and RNAi. The potential of RNA-targeting drugs has led to significant corporate investment, withIonis Pharmaceuticals, Ribometrix, Arrakis Therapeutics, Novartis, Expansion Therapeutics, and others working to develop products. Owing to the unprecedented modularity and programmability of these drugs, this development must occur in parallel with conversations between industry, academia, and government to update regulatory processes. Many avenues remain for continued optimization of RNA-targeting drugs regarding drug delivery and stability, but the future is bright for this area of disease treatment.

FUTURE PERSPECTIVES

The progress in advancing RNA engineering has enabled the rapid and accurate detection of arbitrary pathogenic targets, along with a growing library of small-molecule chemical contaminants. Ongoing therapeutic research holds the promise for new classes of drugs for otherwise intractable genetic diseases, along with the rapid and scalable manufacturing of inexpensive custom medicines. Moving forward, further unlocking the potential of engineered RNA will require the development of more advanced computational tools for the rapid in silico design of functional RNA systems.

Computational methods generating 2D structure prediction currently do not accurately reflect the complex 3D folding patterns of RNA (141). Traversing RNA folding energy landscapes is made computationally difficult by backbone flexibility, long-range tertiary interactions, and local energy minima. Moreover, much of the thermodynamics of noncanonical base pairing and tertiary structure formation remains unknown (142, 143), as well as the influence of sequence and geometric context on these interactions. The RNA-Puzzles project, which regularly evaluates the state of the art in 3D structure prediction methods, has found that although secondary
Structure prediction has become quite accurate in recent years, prediction of noncanonical base pairs is generally poor (≤20%), even when global folds are generally correct (142, 143). Recent advances in prediction algorithms have taken advantage of stochastic sampling and divide-and-conquer strategies to traverse energy landscapes faster and more finely (144, 145). On top of these considerations, designed structures must also consider the complex dynamics of RNA folding, which naturally occurs across multiple timescales in diverse cellular conditions and plays a key role in determining cellular function (146).

Improving our ability to computationally design structures speeds the design–build–test cycle by providing more accurate predictions of RNA function. Crowdsourcing of structure prediction offers a method to overcome the limitations of existing computational tools to design more complex or dynamic structures (147–151). Furthermore, machine-learning tools can facilitate the high-throughput design of diagnostics against arbitrary sequence targets (152). These advances, combined with the design of modular RNA pieces and new thermodynamic data, have enabled the creation of progressively more complex RNA nanostructures (153–158) that could be loaded or functionalized for therapeutic applications (159, 160).

Ultimately, the combined efforts of bioinformaticians developing RNA structure prediction models; basic researchers uncovering ncRNA functions, new nucleic acid chemistries, and how biomolecular mechanisms occur; and engineers applying the rules of biology to enhance, expand, and ultimately program RNA function will forward the field of RNA engineering. The sum of these emerging therapeutic and diagnostic applications has the potential to conquer some of the longest-standing public health challenges. Continued development of these RNA engineering strategies will allow us to build more refined synthetic structures and mechanisms to provide rapid, accessible medical treatments while preventing and mitigating harm from large-scale humanitarian crises.

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J.B.L. is a cofounder of and has a financial interest in Stemloop, Inc. W.T. and J.B.L. have filed patents related to several technologies discussed in this article. These interests were reviewed and managed by Northwestern University in accordance with their conflict of interest policies. All other authors are unaware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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**Errata**

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