WHITE PAPER

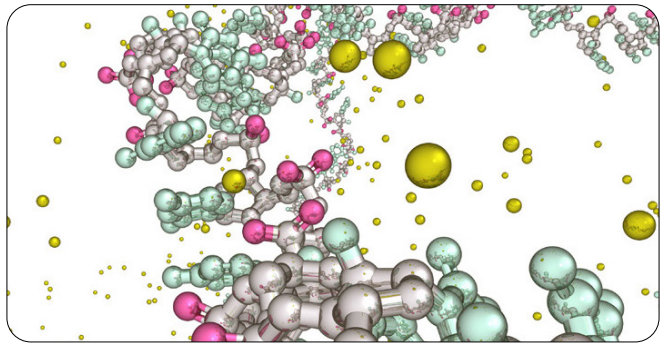
Deconstructing and leveraging 3D structures of RNAs for novel therapeutics

Developing a new technology to forestall RNA degradation in cells

The COVID-19 pandemic has brought into the spotlight the therapeutic potential of ribonucleic acid (RNA), with some of the first COVID-19 vaccines authorized and approved for use being based on messenger RNA (mRNA). While there is now a clamor of excitement surrounding mRNA, its first reported existence dates back to the 1960s. Since this time, decades of research have been conducted on these versatile macromolecules to further understand their structure and diverse functions.

RNAs are single-stranded molecules that are utilized by cells for a broad range of biological functions, including the regulation and expression of genes, and their subsequent translation into proteins. For many years it was thought that there were just three types of RNA: mRNA, transfer RNA (tRNA), and ribosomal RNA (rRNA). However, in recent years, researchers have begun to realize that there are many other types which play key roles in cellular processes. These include small interfering RNA (siRNA), microRNA (miRNA), and long non-coding RNA (ncRNA), to name but a few.

Interestingly, deficiencies in mRNA or ncRNA have been linked to certain neurodegenerative and neurodevelopmental disorders. For example, if there is too little of a specific mRNA or ncRNA in the transcriptome – the totality of mRNA an organism expresses – certain cellular functions may be degraded or disabled. RNA is therefore now seen as a potentially powerful research tool and perhaps even the foundation for certain neurological therapies in the not-so-distant future.



Three-dimensional structures

RNAs can fold into complex 3D structures that range from simple helical elements to complex tertiary and quaternary structures. These structures, and how they fold in response to changing cellular conditions, are key determinants of RNA function and have been studied extensively by structural biologists. Understanding how these 3D structures form and the subsequent biological processes they regulate is an area of interest for Dr. Jeffrey Kieft, who is based at the University of Colorado School of Medicine. His lab has spent almost two decades researching the structure and role of viral RNAs, and how this knowledge might be exploited to improve human health. “You can think of viruses as molecular innovators – they are constantly evolving new ways to use RNA and RNA structures as part of their infection process,” explained Dr. Kieft. “They are a treasure chest of biologically-active RNAs that we can study and from which we can learn basic foundational lessons.”

One of the key technologies Dr. Kieft uses in his lab is X-ray crystallography, which is one of the most widely used techniques to characterize RNA-protein complexes. They also use cell culture-based experiments and novel tools like CRISPR to gain further insights. “Any technology that allows

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us to understand the three-dimensional structure of RNA and provide us with information about its role and function is potentially useful and important, "he said. "One technology that will have a huge impact in my lab is cryo-electron microscopy (cryoEM), which has emerged as an extremely powerful structural technique. This will allow us to assess not just three-dimensional structures, but also molecular movement, which is a whole other layer understanding how structure creates function."

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Degradation-resistant mRNAs

The level of specific mRNAs in cells often correlates with levels of their respective proteins, and cells have many ways to control and maintain these levels. For example, mRNAs undergo extensive processing steps during their lifecycle, including splicing, polyadenylation, editing, transport, translation, and degradation. Dr. Kieft and colleagues hypothesized that by slowing the degradation step independent of increasing transcription rates, they could potentially increase the corresponding protein expression levels of specific mRNAs. "Our thinking was that if we could boost the cells natural pool of mRNAs in a very specific way, that might have applications for diseases that are caused by haploinsufficiency, where there's not enough of a certain mRNA," he said. This approach would apply for neurological disorders such as spinal muscular atrophy (SMA), where there is insufficient expression of *SMN1*; this results in too little expression of a protein survival motor neuron (SMN), which is critical to the proper functioning and maintenance of motor neurons.

With this in mind, Dr. Kieft turned his attention to specific viral RNA structures that resist 5′ → 3′ degradation by Xrn1, the major eukaryotic exoribonuclease. These exonuclease-resistant RNAs (xrRNAs) can be found in the 3′ untranslated region (3′ UTR) of the positive-sense RNA genomes of Flaviviruses and do not require a bound protein factor for function. "Originally, we just wanted to understand how these xrRNAs worked and how they could form this discrete structure that blocks such a powerful cellular enzyme," explained Dr. Kieft. "We studied and solved the structure using X-ray crystallography, and this led me to wonder if we could engineer them so they would protect RNAs from decay in other systems."

The team set out to design mRNAs that were not only degradation resistant, but that retained their capability to be translated into functional proteins in host cells. "We soon realized this was going to take a fair amount of engineering and that we would have to stitch multiple structured RNAs together," said Dr. Kieft. The second RNA structure they decided to use was an internal ribosomal entry site (IRES) from Cricket Paralysis virus (CrPV), which allows initiation of translation in the absence of any host initiation factors.

For their experiment,[1] they transformed plasmids encoding xrRNA-modified reporters into a yeast strain and subsequently observed a buildup of specific 5′ → 3′ truncated, Xrn1-resistant RNA fragments (xrFrag) via radiolabeled primer extension assays. These initial results provided evidence to the researchers that their xrRNA structures could successfully be installed in mRNAs in yeast, fold properly in the cell, and function outside of their natural context within the 3′ UTR of the flaviviral genome. To determine if there was an increase in protein production related to this buildup, the team used a β-galactosidase assay to monitor the enzymatic activity of lysates obtained from yeast cells expressing xrRNA-modified reporters. Interestingly, they observed 30 times more protein production from xrRNA-protected reporters compared to unmodified or mutated controls. Additionally, by monitoring the translation of dual-luciferase reporters the researchers demonstrated that these xrRNA sequences did not interfere with the progression of an elongating ribosome.

The findings of their experiments not only highlighted the portability of viral RNA elements, but also the potential utility of combining functional RNAs from different viruses to engineer degradation-resistant mRNAs, which could prove useful for neuroscientists looking to develop therapeutics for neurodegenerative and neurodevelopmental disorders.

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Next steps

While these results show great promise for the use of viral RNA elements in therapeutic research, Dr. Kieft notes that there is still much more to discover. "Whether it's a new class of RNA or a new biological pathway, there's always something being uncovered that opens up new avenues for exploration," he said. "For example, there's a lot of interest in post-transcriptional modifications of RNA and how this adds another layer of regulation, which again feeds into the potential of new RNA therapeutics or diagnostics." The emergence of high-throughput next-generation sequencing (NGS) technologies has also opened up new possibilities in the field, producing vast amounts of data for researchers to analyze. This monumental task is now being simplified by the use of AI and machine learning, which allows researchers to extract more information, more efficiently, from their experiments. "I'm still amazed at the amount of data that NGS is creating, so the emergence of information technologies is going to be essential in extracting the information embedded in those data," he said. Collaborations will be critical to grow the field's ability to leverage these growing databases of sequences together with structural insights to gain a much more comprehensive view of RNAs and their expression, function, and downstream impacts on protein levels.

The next goal for Dr. Kieft is to explore how RNAs operate within a larger context. "I'm starting to think about more viral RNAs not just as discrete elements," he explained. "You often have multiple RNA structures which might be interacting or influencing one another, and that's been challenging to explore. These new technologies, such as cryo-EM, are making that information available to us and allowing us to think about the bigger picture."

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In addition to his academic work, Dr. Kieft has also been named Chair of the NIH Molecular Structure and Function A (MSFA) study section, and was elected as Director of the RNA Society, to serve for the two-year term of 2022-2023. "The RNA society is actively engaged in all areas of RNA research, but also in mentoring and enhancing diversity, equity, and inclusion. I'm pretty excited about that aspect of it, and there's a lot of young people involved. We want the next generation of scientists with fresh ideas, fresh energy, and fresh perspectives to develop into independent thinkers and leaders of the future."

Jeffrey S. Kieft, PhD

Dr. Kieft is Professor and Vice Chair at the University of Colorado Anschutz Medical Campus. He received his undergraduate degree at the United States Military Academy and then served as an active-duty Army Officer in Europe. He completed his PhD at the University of California, Berkeley and conducted postdoctoral research at Yale University. Jeff's research aims to understand how RNA forms complex 3D structures and how this controls diverse biological processes. His lab is particularly interested in how viral RNAs interact with, control, and manipulate cellular machines.

Reference

1. Franklin A, Macfadden A, Kieft J, Hesselberth J, Chapman E. Custom-designed, degradation-resistant messenger RNAs in yeast. bioRxiv 2020. doi: https://doi.org/10.1101/2020.06.25.169177

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