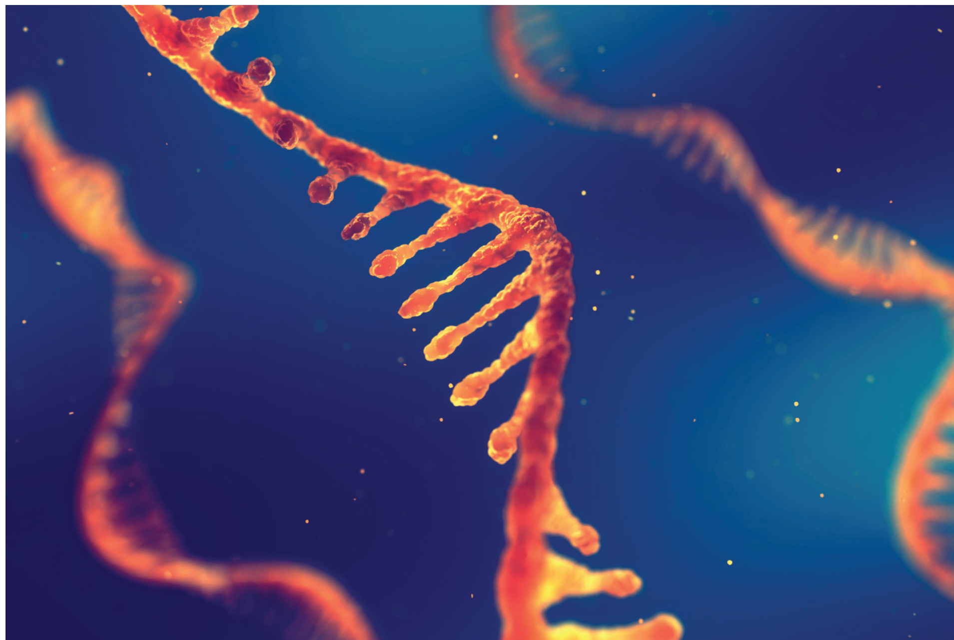

The Marriage of RNA and Mass Spectrometry

Considerable efforts have been made over the years to resolve the key issues of stability and delivery of RNA-based therapeutics.

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For several years after Crick (1) first described the genetic role of RNA, this molecular entity was perceived simply as an "inert" carrier between DNA and protein. Today, however, this view has almost entirely changed, and RNA-based molecules have been implicated in a broad range of functions including the activation/deactivation of genes, the excision of genetic material, and the transport of intercellular components. Indeed, it is expected that in the coming years, further discoveries will uncover even greater biochemical significance to this molecular type.

Given the ubiquity and variety of roles associated with RNA, it was inevitable that it would become a focus for investigators involved in the development of therapeutics. As early as 1978, Zamecnik (2) described the therapeutic use of an RNA-based oligonucleotide to inhibit replication of the Rous sarcoma virus, and, today, there are approximately 16 FDA-approved RNA therapies, 28 in clinical development, and many more expected in the near future (3).

Currently, RNA-based medicines can be segregated by their functionality and structure and include species, such as messenger RNA (mRNA), antisense oligonucleotides (ASO), small interfering RNA (siRNA), and microRNA (miRNA). Other types of RNA include aptamers which are single-stranded and form higher-order structures, and more recently described, circular RNA (circRNA or oRNA), which appears to have multiple functions prior to and following the transcription process (4). Additionally, mature clustered, regularly interspaced, short palindromic repeats RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) constitute components of the recently developed CRISPR technology.

CHALLENGES TO DEVELOPMENT

While not unique, the use of RNA in therapeutic applications is generally incumbered by two key issues: stability and delivery. Over many years, considerable efforts have been directed at attempts to resolve both these issues, leading to some creative solutions which, in turn, has necessitated the application of a broad range of analytics. However, one technique, namely mass spectrometry (MS), is particularly notable for: its ability to

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provide analysis of all RNA types, regardless of size and modification; the fact that it can be used for qualitative as well as quantitate investigations; the technology's ability to be applied in both research and quality control (QC); and its applicability in the area of delivery systems.

Since the late 1970s, when both therapeutic biopolymers and analytical MS began a decades-long connection, there have been some considerable advancements in both therapeutic development and analytics. Most early biopolymer MS was applied to peptides and proteins, and, today, this technique has become arguably the single most important analytical tool in this field. Indeed, several laboratories within SGS, for example, were founded by one of the early innovators, H.R. Morris, whose contributions included the first combination of high-field magnet technology with soft ionization (5) and some of the first protein-mapping studies (6). These laboratories and others continue to encourage the adoption of such technological innovations, which have resulted in the application of advanced analytical services in support of the most challenging biopolymer studies.

ADAPTING ADVANCES IN MS

Many of the advances made in protein MS have been adapted to make the technique appliable to the study of oligonucleotides, where attributes such as sequence, modification, and quantitation are critical.

Initiated by McLuckey *et al.*'s report in 1992 (7), there has been increased attention given to the application of MS-based technologies in the analysis of RNA, including top-down methods that provide data from intact RNA species. In 2012, Taucher and Breuker (8) were amongst the first to report sequence coverage of full-length transfer RNA (tRNA) using Fourier transform ion cycloctron resonance (FT-ICR), combining data from electron detachment dissociation (EDD) and collision-activated dissociation (CAD) experiments. However, while such MS-based studies continue to be explored, they remain constrained by several somewhat related factors, such as the large molecular size, the inability to distinguish different species with the same mass, the high degree of purity required, and the limited availability of software to support top-down RNA analysis.

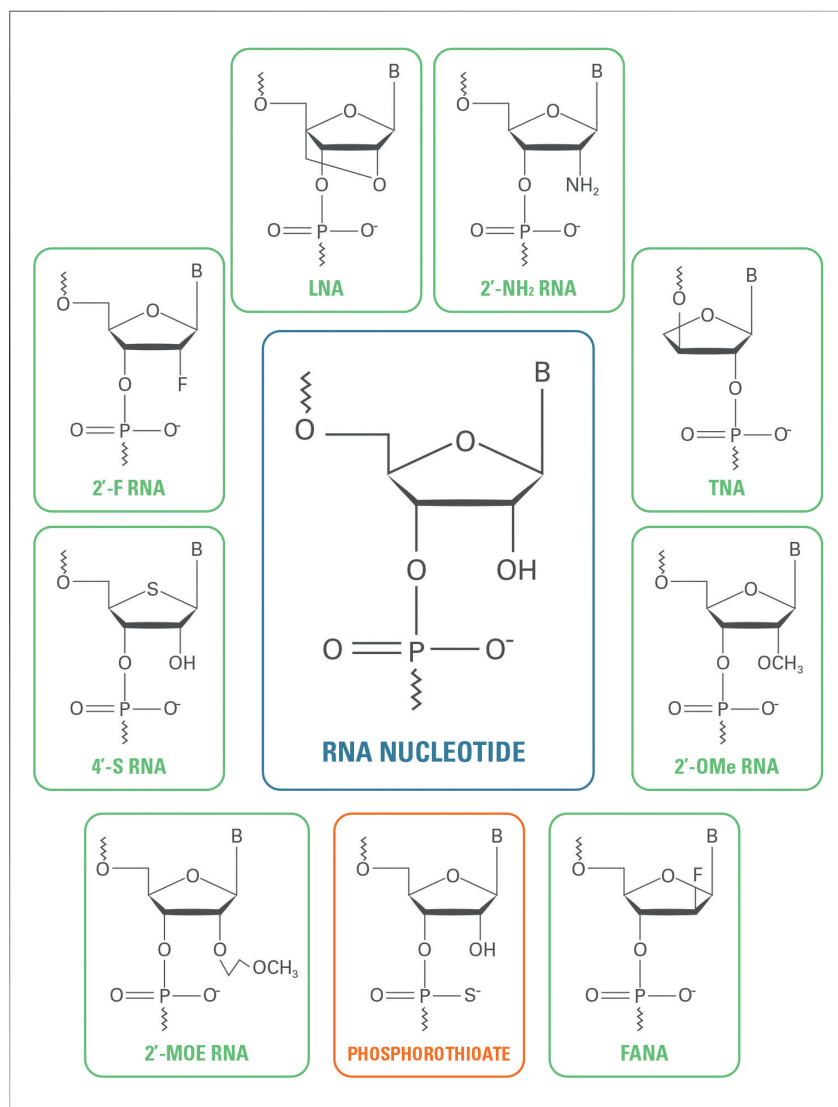
Perhaps to a lesser extent, these limitations also apply to top-down protein studies, although in this field these constraints have largely been overcome by mapping techniques. This approach involves controlled and specific digestion of the protein to yield a peptide mixture that can then be analyzed by a variety of MS-related methodologies, the most familiar being liquid chromatography–mass spectrometry (LC–MS). It was inevitable that such an approach would evolve in the RNA field, and this has now been successfully applied to species such as the mRNA coding for the SARS-Cov-2 spike protein (9). Such methods rely on digestion (partial and complete) with RNAses such as T1, RNAse A, and MazF prior to analysis of the products by LC–MS/MS. This is a very effective strategy because it can be automated with the use of immobilized enzymes (9), provides sequence confirmation as well as the ability to conduct de novo investigations, is able to identify and locate post-transcriptional and process modifications, can be used qualitatively and quantitatively, and may be applied to bioanalytical applications.

In contrast to polymerase chain reaction (PCR) methodologies, MS has the ability to detect and locate the more than 150 post-transcriptional RNA modifications that have been described to date. Importantly, MS is also able to identify the presence of unexpected nucleotide alterations. For many years, mass spectroscopists have taken advantage of the method's ability to detect stable isotopic forms of elements such as hydrogen, nitrogen, carbon, oxygen, and sulfur. Nucleic acid isotope labeling combined with MS (NAIL–MS) has been used in a variety of applications to study the dynamics of RNA modification (10).

Some reports have addressed the use of MS for the investigation of dimer formation and higher-order structures, but here great care should be taken in data interpretation and extrapolation given the tendency for non-covalent molecular association within the mass spectrometer and the fundamental differences between the solution-phase environment and the environment within the mass spectrometric process. Ion-mobility MS has been used to investigate the gas-phase structures of nucleic acids such as duplexes, triplexes, and quadruplexes to try to determine if these structures are indeed similar to the structures in solution (11). Today, the use of MS for the analysis of RNA-based therapeutics has become widespread due largely to the technique's agnostic ability to manage a wide diversity of molecular structures. From the development of Macugen (pegaptanib sodium) in the early 2000s, laboratories such as SGS and others have been highly active in analytical support for nucleic acid-based therapeutics, including today's complex, multi-faceted structures.

Issues of stability, delivery, and binding have resulted in the development of RNA therapeutics that often involve significant modification(s) to the basic oligoribonucleic acid structure. **Figure 1** provides several examples of these modified RNA structures. Changes to the phosphodiester linkage have included replacement of the free oxygen with sulfur (producing the more stable phosphorothioate linkage) and substitution with amide and boronophosphate linkages (12). Similarly, multiple, diverse structural alterations to both sugar and base moieties have been described, including methylation, methoxyethylation, fluorination, and cyclization of the 2' sugar hydroxyl, resulting in so called "locked nucleic acid" (LNA) (10). Often, several of these modifications may be incorporated within the same therapeutic. In addition, linking of these RNA-based therapeutics

Figure 1. Examples of modified RNA structures.



with a wide range of conjugates has been exploited as a means to significantly enhance and direct their delivery and greatly improve their effectiveness. A notable example of such modification is the conjugation of siRNAs with N-acetylgalactosamine providing specific binding to asialolycoprotien receptors in hepatocytes. This structural concept has led to the successful development of the drug, givosiran, which treats hepatic porphyria by silencing the expression of aminolevulinate synthase 1 mRNA in the liver (13). Many other conjugates have been described such as lipids, peptides, antibodies (complete or fragments), vitamins, and a range of other molecular classes (14).

Currently, the most widely used hyphenated analytical technique is LC–MS. Multiple separation methodologies have been reported including ion-pair reversed-phase chromatography, hydrophilic interaction liquid chromatography, ion-exchange chromatography, and size exclusion chromatography. A report by Santos and Brodbelt (15) provides a comprehensive review of these and other related techniques as applied to nucleic acids. Two-dimensional LC has also been described as a means to overcome incompatibility between mobile-phase composition and MS detection (16), while 3D-LC–MS—incorporating in-line digestion and hydrophilic interaction liquid chromatography (HILIC) separations—has been used for the sequencing of CRISPR-guide RNAs (17).

The majority of mass spectrometric applications involving nucleic acids have utilized either laser desorption (matrix or surface assisted) or electrospray ionization (ESI) techniques. Both are considered "soft," thereby reducing in-source fragmentation of these large biomolecules and providing a relatively high ion current for the intact molecular species. Almost all analyzer types have been employed, including magnetic sector, time of flight, quadrupole, ion trap, and combinations of each. Today, a technology that has gained considerable acceptance both for quantitative and qualitative investigations is the so-called Orbitrap that was introduced commercially in 2005 and is shown in **Figure 2**. Combining ion trap and/or quadrupole mass filters, orbitrap-based mass spectrometers provide high sensitivity with high resolution, the latter feature rising in importance as the complexity and heterogeneity of materials under examination become more acute. The implementation of multiple fragmentation functions (e.g., collision-induced dissociation, higher energy collisional dissociation, electron-transfer dissociation, ultraviolet photodissociation) on the orbitrap-based mass spectrometers also enables researchers to tackle a wide range of challenging applications.

CONCLUSION

The use of MS in the study of oligonucleotides has become almost as significant as applications in the peptide/protein area despite the historical impact of techniques such as PCR. It is highly likely that novel RNA structures are yet to be discovered, leading to greater understanding of the function and role of this molecular type and potentially the development of more effective

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therapeutic agents. As with protein chemistry, higher-order structure governs the function of many RNA species. It is likely that MS will play an important role in defining these large RNA structures that currently remain unresolved (18). Mass spectrometric techniques are just beginning to provide insights in some critical therapeutic areas where, for example, the quantitative identification of RNA modifications (genetic and epigenic) appears to provide a source of potential biomarkers and treatment monitoring in areas such as oncology (19). MS has also become important in the development and understanding of therapeutic products themselves as illustrated by the work of Packer *et al.* (20), who used LC–MS/MS to study mRNA modifications as a result of reaction with certain lipids used in lipid nanoparticle-vector vaccines.

There is little doubt that the past decade has seen significant advances in the understanding of ribonucleic acid functionality leading to the development of some novel and highly effective therapeutics. The growing need for sophisticated analytics to support both the investigative and routine analytical requirements of these complex therapeutics is, in large part, being met by the progression of mass spectrometric technologies. It is certain that MS will continue to maintain this close "marriage" with RNA becuase many aspects of this nucleic acid's biochemistry are yet to be uncovered.

Figure 2. SGS uses liquid chromatography tandem mass spectrometry (LCMSMS) Orbitrap technology to solve complex analytical problems.

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