The Analytical Landscape in mRNA Therapeutics: *Characterisation and Batch Release Strategies*

Messenger-RNA (mRNA) drug therapeutics have recently emerged as promising and increasingly viable treatment modalities for cancer, cardiovascular diseases, rare genetic disorders and autoimmune diseases. Furthermore, the potential of using mRNA technology during mass vaccination campaigns was successfully demonstrated during the COVID-19 pandemic.

With an increased number of RNA therapies currently in various stages of clinical trial around the world, the requirements for reliable analytical methods to ensure the quality and safety of this new class of therapeutics are high.

Here, we present an overview of the latest advancements in analytical platforms for the characterisation and batch release testing of mRNA-based therapeutics, focusing on those typically available in the biopharmaceutical industry.

Following the success of mRNA vaccines in response to the COVID-19 pandemic, mRNA-based therapeutics have demonstrated significant potential in treating various diseases, including cancer, cardiovascular diseases, infectious diseases, rare genetic disorders and autoimmune conditions. Currently, over 3000 mRNA therapeutics are in various stages of clinical trials worldwide, substantially increasing the demand for robust analytical platforms to monitor drug quality, safety and efficacy throughout development and for batch release testing.

Linear mRNA is the conventional form used in many mRNA-based therapies. It consists of: 1) a 5' cap (typically a modified guanine nucleotide) necessary for ribosome binding and translational initiation, to increase stability and reduce immune-response; 2) a single, linear strand of nucleotides with a sugar-phosphate backbone encoding the desired protein; and 3) a 3' poly(A) tail to improve mRNA stability.

Despite the incorporation of chemically modified nucleosides to enhance mRNA stability, a key challenge in producing reproducible, high-quality mRNA therapeutics is their inherent instability. This, coupled with the high heterogeneity, large size and negative charge of mRNA molecules, makes developing analytical workflows for mRNA characterisation and batch release testing to meet regulatory requirements a complex task.

In general, the ICH Q6B principles for biotechnological products are applicable to mRNA therapeutics. The European Medicines Agency (EMA) also recently published a guideline addressing specific aspects regarding the manufacturing process, characterisation, specifications and analytical control of mRNA vaccines. While the scope of this guideline is limited to mRNA vaccines against infectious diseases, certain sections and analytical considerations may be applicable to other mRNA therapeutics [EMA/CHMP/BWP/82416/2025].

mRNA possesses unique critical quality attributes (CQAs) that require thorough characterisation. These include identity, integrity, quality (including capping efficiency and poly(A) tail) and impurity control /purity profile. The development and validation of functionality assays based on *in vitro* translation and cell-based assays are also a critical regulatory requirement. In addition, well-known USP monographs are utilised to address appearance (USP 790), residual solvents (USP 467), pH (USP 791) and safety (USP 85, 61, 62, and 1115).

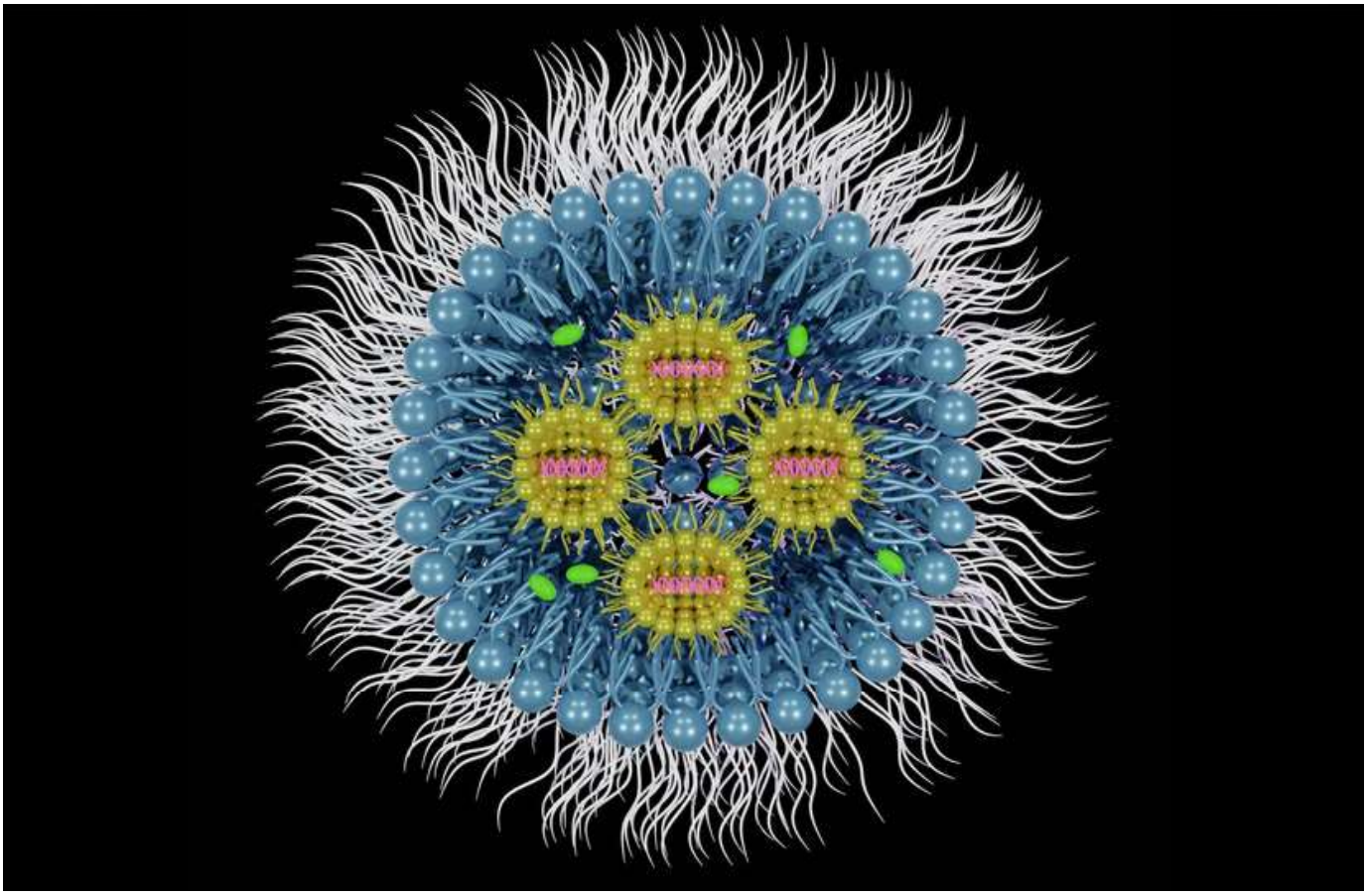
Identity

Confirming mRNA sequence identity is a regulatory requirement for both characterisation and batch release testing. Traditionally, identity testing has relied on molecular methods such as quantitative polymerase chain reaction (qPCR) and restriction enzyme analysis. While useful for batch release, these approaches target only specific regions of the mRNA, necessitating additional data for comprehensive sequence identity confirmation. Sanger sequencing offers an alternative to qPCR, but its limited resolution and high costs make it less suitable for stringent regulatory demands. Next-generation sequencing (NGS) presents a valuable alternative to Sanger sequencing and qPCR, as it is well-suited for thorough genomic characterisation, including the identification of nucleotide variants and sub-populations. This involves aligning the sequence data set against defined reference sequences. While NGS provides high throughput and detects sequence variations effectively, mass spectrometry is increasingly favoured for its ability to identify and locate post-translational modifications, as well as directly detect smaller mRNA fragments and dimers. Recent advancements have refined RNase mass mapping for characterisation through partial digestion using RNase enzymes like RNase T1, often combined with alternative enzymes such as MazF to enhance sequence coverage. Data are then processed using commercially available software or custom solutions. A key advantage of mass spectrometry is its capacity to detect chemically synthesised nucleotides and post-translational modifications (PTMs), generating data that regulators are already familiar with, as it parallels peptide mapping data for protein therapeutics. Implementing mRNA sequencing by mass spectrometry within a Good Manufacturing Practice (GMP) environment remains a significant challenge.

The higher-order structure of mRNA should also be assessed during characterisation and development, as it may impact its stability and translational efficacy. Techniques such as circular dichroism (CD), differential scanning calorimetry (DSC), or differential scanning fluorimetry (DSF) are generally utilised for this purpose

Capping Efficiency and Poly(A) Tail

Capping efficiency and poly(A) tail length must be monitored for characterisation and batch release purposes, as it's essential for efficient translation and increasing stability. However, due to the structural complexity of mRNA, those measurements can



be challenging. These attributes are commonly assessed using high-performance liquid chromatography (HPLC) coupled with either ultraviolet (UV) or mass spectrometry (MS) detection. Mass spectrometry based methods are particularly regarded for monitoring capping efficiency and Poly (A) tail because of its capacity to resolve and quantify heterogeneous forms with high resolution and concurrently monitor different critical quality attributes. Furthermore, LC-MS based methods are suitable for high-throughput analysis, making them valuable for quality control of mRNA therapeutics.

Purity and Integrity

The purity and integrity of mRNA therapeutics must be assessed throughout drug development and for batch release testing. Fragmentation of mRNA is very common and may result from the synthesis of the active mRNA or degradation during manufacturing and storage. Analysis of the molecule's integrity and its fragments is a mandatory requirement. Ion-pair reversed-phase liquid chromatography methods coupled with UV or mass spectrometry are generally used for the identification and quantification of mRNA fragments. Size exclusion chromatography (SEC) and capillary electrophoresis-based methods are also extremely valuable and provide orthogonal options.

As with most biologics, mRNA aggregates need to be measured to address any potential implications they may have on vaccine or therapeutic efficacy and safety. Size exclusion chromatography is one of the well-established HPLC methods for measuring aggregates of various types of molecules, including antibodies, and has been extensively applied to mRNA. When coupled with multi-angle light scattering (MALS) detection, it provides the additional of the molecular size and structure of mRNAs in solution, enabling a powerful elucidation of the mRNA molecule.

An increased interest in mass photometry-based methods has recently emerged, spurred by the introduction to the market of robust and qualified instrumentation for batch release testing and the significant advantages of this method. Mass photometry is based on the principles of interferometric scattering microscopy and uses light to measure the mass of single biomolecules in solution by directly measuring their native molecular mass, providing insights into the presence of fragments, aggregates and incomplete molecules (such as those lacking a poly(A) tail). Mass photometry does not require sample denaturation or complex preparation, enabling quick, picomolar-to-nanomolar level analysis in minutes and offering ideal solutions for batch quality control in mRNA production and research.

Alongside the product related impurities, process related impurities, residual solvents, residual plasmid and double stranded RNA (dsRNA) should be monitored.

Process-related impurities include residual DNA template and host cell DNA, residual proteins, any chemicals used during manufacturing and unincorporated nucleotides. Of particular importance is the requirement for orthogonal test methods to quantify and characterise residual DNA. Characterisation of the fragment size of residual DNA is expected to be performed to demonstrate the effectiveness of the enzymatic reaction and purification process. Residual DNA template is generally monitored by quantitative polymerase chain reaction (qPCR), the quantitation of free/non-incorporated nucleosides is

performed by RP–LC-MS/MS and residual NTP and capping agent are measured by anion-exchange chromatography. Finally, the content of T7 RNA polymerase is generally provided by ELISA measurements. Double-stranded (ds) RNA can potentially be formed during *in vitro* transcription and its presence should be monitored because of potential unwanted effects. Methods to monitor dsRNA include immunoblotting or ELISA.

Content

The total RNA concentration is an important critical quality attribute that must be monitored for both characterisation and batch release testing. Reverse transcriptase qPCR (RT-qPCR), as well as ultraviolet measurements, is generally used for this purpose. The data is then combined with information from the RNA integrity analysis to provide a more detailed picture of the product's quality and its consistency

Functionality

Beyond physicochemical techniques for characterising specific quality attributes, it is also critical to have assays that assess the overall functionality of mRNA. Functionality testing evaluates the combination of all the analytical characteristics of the mRNA, such as capping efficiency, poly(A) tail length, dsRNA content and other structural features, ensuring that the synthesised mRNA can be efficiently translated into a functional protein. These assays typically evaluate translation efficiency, often measured as the yield and/or quality of the encoded protein product. Overall, a wide variety of assays can measure mRNA translation efficiency, ranging from cell-free systems utilising recombinant proteins to more complex cell-based assays.

Conclusions

mRNA vaccines and therapeutics have emerged as a powerful new class of medicines. However, due to the physicochemical properties of these large RNA molecules, the development of robust analytical workflows is challenging. mRNA is a highly polar molecule characterised by high heterogeneity due to its extensively negatively charged phosphodiester backbone. Its single-stranded nature leads to dynamic alternative secondary structures, resulting in potential sample heterogeneity. Moreover, mRNA manufactured using *in vitro* transcription (IVT) often contains impurities, many of which are similar in size and nature to the full-length mRNA, making separation and characterisation difficult.

Several analytical technologies are currently available, each offering distinct advantages and limitations for characterising mRNA-based therapeutics and their batch release testing. Considerable effort has been invested in developing new analytical tools to characterise the diverse critical quality attributes of mRNA-based therapeutics. Capillary electrophoresis (CE), chromatography, mass spectrometry, mass photometry and new sequencing technologies being the analytical platforms mostly utilised.

Dr. Milena Quaglia

Dr. Milena Quaglia is a leader in biological measurements with over 20 years of industrial experience, holding scientific leadership and management roles for protein measurements in R&D and analytical regulatory environments (UKAS and GMP). After completing her PhD in analytical chemistry and four years of postdoctoral experience, Milena joined the National Measurement Laboratory at LGC in the UK, where she led nationally and internationally protein metrology issues, coordinated European Measurement Projects and was heavily involved in international organisations. Milena joined RSSL in 2022 as a Senior Associate Principal Scientist working in the Bioanalysis Department. A recent achievement was the successful application of a Knowledge Transfer Program with the University of Sheffield on mRNA characterisation by mass spectrometry.