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Characterisation and analysis of mRNA critical quality attributes using liquid chromatography based methods

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ABSTRACT

mRNA technology has been successfully deployed to rapidly develop and mass-manufacture vaccines. Beyond vaccines, RNA-based therapeutics have potential for treatments for infectious diseases, cancer, metabolic disorders, cardiovascular conditions and autoimmune diseases. mRNA based vaccines and therapeutics work by translating exogenous mRNA into the target protein. Analytical methods for mRNA characterisation, lot release and stability testing of mRNA drug substance and drug product must be developed and performed to monitor critical quality attributes (CQAs). mRNA is a highly polar molecule due to its extensive negatively charged phosphodiester backbone. Its single stranded nature forms dynamic alternative secondary structures that can generate potential sample heterogeneity, creating challenges for the analysis and characterisation of this large biomolecule. In this review, we describe current analytical methods, focussing on high performance liquid chromatography in conjunction with both UV detection and mass spectrometry for the analysis and characterisation of mRNA. In particular, we describe recent developments covering a wide range of methods centred on liquid chromatography for the analysis of important CQAs including mRNA identity, mRNA integrity, 5′ capping efficiency and poly(A) tail length and heterogeneity.

1. Introduction

1.1. mRNA vaccines/therapeutics

mRNA has recently emerged as a new class of medicines, as demonstrated by the development and approval of two highly efficacious vaccines based on mRNA sequences encoding for a modified version of the SARS-CoV-2 spike protein [1,2]. The global use of the two pioneering mRNA vaccines, Comirnaty (Pfizer/BioNTech) and Spikevax (Moderna), has fuelled the development of further mRNA vaccine candidates [3]. Beyond vaccines, RNA-based approaches have potential for treatments for infectious diseases, cancer, metabolic disorders, cardiovascular conditions, and autoimmune diseases [4–7]. mRNA based therapeutics work by translating exogenous mRNA into the target protein, which was first demonstrated *in vivo* in 1990 by Wolff et al., where functional protein expression was demonstrated after direct injection of mRNA [7].

1.2. mRNA structure

mRNA therapeutics/vaccines contain five key essential components including; 1) 5' cap, which is required for ribosome initiation, translation, and stability [8]; 2) 5' untranslated region (UTR), that helps drive high levels of translation from the correct start codon and mRNA stability; 3) Coding sequence region, encoding for the gene of interest; 4) 3' UTR, required for translation and stability and finally; 5) 3′ polyadenylated (poly[A]) tail, required for ribosome initiation, translation, and mRNA stability (see Fig. 1) [9–11]. In addition, various chemical modifications (e.g. N1 methylpseudouridine) have been introduced into the mRNA to reduce immunogenicity and enhance stability and protein expression [12–14].

1.3. mRNA manufacturing

The mRNA manufacturing process centres on the synthesis of the mRNA drug substance (DS). Manufacturing of mRNA DS can be broken down into two essential steps: upstream enzymatic synthesis and downstream purification, typically utilising chromatography and

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ultrafiltration-based purification methods. The large size of typical mRNA therapeutics limits manufacturing via chemical synthesis methods. Enzymatic in vitro transcription (IVT) is considered a simple and inexpensive procedure for mRNA synthesis, which can yield products of variable sizes in gram quantities [15,16].

Plasmid DNA (pDNA) is commonly used as the DNA template for IVT reactions. The DNA is isolated, purified from bacterial cells and linearized with a suitable restriction enzyme. These plasmid vectors typically contain a T7 promoter sequence upstream of multiple cloning sites. The DNA template design also includes a poly(A) tail sequence, as well as 5′ and 3′ untranslated regions [17,18]. DNA template can also be produced from PCR products to include a T7 promoter sequence at the 5′ end. Usually, the 3′ poly(A) tail is incorporated into the initial plasmid DNA for transcription. However, mRNA can also be synthesised without a 3′ poly(A) tail by using a "tailless" pDNA template, followed by a post-transcriptional poly(A) tailing step [19,20]. In this case, poly(A) polymerase is used to add poly(A) tails of approximately 80–160 nucleotides.

An established cost-effective and scalable method of mRNA manufacturing is to perform IVT reactions using highly processive, single-subunit, bacteriophage DNA dependent RNA polymerases [21]. The IVT reaction incubates linearised pDNA template, containing an RNA polymerase promoter sequence, with the DNA dependent RNA polymerase, nucleotide triphosphates (NTPs), RNase inhibitor and inorganic pyrophosphatase (see Fig. 2). Bacteriophage T3, T7, and SP6 DNA-dependent RNA polymerases are single polypeptide chains that require only Mg^{2+} as a cofactor and have been widely employed for the transcription of RNA including large-scale mRNA production [15,22]. In addition, various mutant DNA-dependent RNA polymerases have been employed to incorporate modified nucleotides and reduce the synthesis of immunogenic double-stranded RNA (dsRNA) impurities [23,24]. Optimisation of IVT reactions and the mRNA production process has led to significant improvements in both mRNA yield and quality, reducing impurities and enhancing the integrity of the mRNA product. As a result, large quantities of mRNA can now be produced in just a few hours [23,24].

The mRNA DS is then purified and formulated to make the mRNA drug product (DP). mRNA-based therapeutics and vaccines require a delivery system such as polymers, polymer-based nanoparticles, lipids, or lipid nanoparticles (LNPs) for entry into recipient cells [25,26]. A variety of alternative vehicles are available to deliver mRNA however, LNPs are currently the most advanced system that has been demonstrated to be safe and effective. LNPs protect mRNA from degradation and enable cell entry through endocytosis [26–28]. Once in the endosome, mRNA molecules are released into the cytoplasm, delivering the message to the ribosome to produce multiple copies of the expressed protein. In an mRNA-based vaccine, the expressed protein serves as an antigen to stimulate an immunological response, which is the desired outcome of vaccination. When mRNA is used as a vaccine or therapeutic, modifications can be introduced into the mRNA molecule to enhance functionality including translation efficiency. This is achieved through RNA modifications, which can increase nuclease resistance, and decrease immunogenicity of the mRNA [11,12,14,29,30].

1.4. mRNA drug substance and drug product critical quality attributes

The purity of the mRNA can affect its translational efficiency, stability and safety. During the enzymatic manufacturing process of mRNA vaccines/therapeutics, incomplete mRNA products are generated in conjunction with other potential impurities, such as dsRNA. Furthermore, during manufacturing and storage, mRNA therapeutics can be degraded by exposure to heat, hydrolysis, oxidation, light and ribonucleases. The 5′ cap and 3′ poly(A) tail can be added to the mRNA either co-transcriptionally or post-transcriptionally during the enzymatic IVT process [31,32]. Both the 5' cap and 3′ poly(A) tail affect binding to the ribosomal machinery and mRNA stability [10]. Therefore, the percentage of 5′ capped mRNA (5′ capping efficiency) and the 3′ poly(A) tail length and heterogeneity are critical quality attributes (CQAs) that will impact the translational efficiency and *in vivo* stability of the mRNA [9,19,23,33]. Hence, analytical methods for characterisation of mRNA DS and DP must be developed and performed to monitor these CQAs and other product attributes in addition to stability studies. A summary of the CQAs for characterisation and release testing of mRNA DS are highlighted in Table 1 [34]. The CQAs currently analysed using high performance liquid chromatography (HPLC) based methods are highlighted in bold.

Current analytical methods for characterising large mRNA therapeutics (>1000 nucleotides) are limited, leading to significant demand to develop new and improved analytical methods to characterise mRNA. The large, negatively charged phosphodiester backbone of mRNA makes

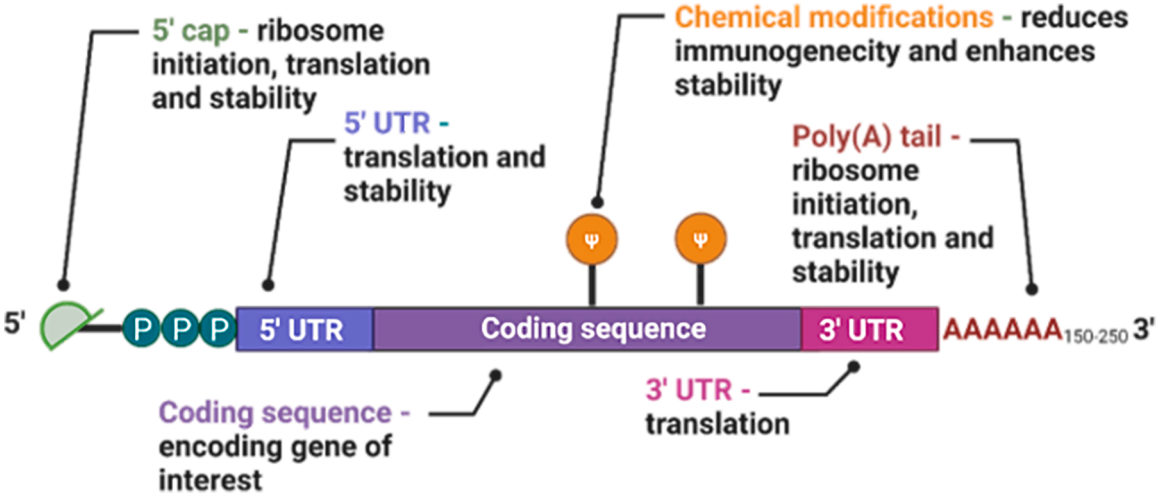

Fig. 1. Schematic illustration of the structure of synthetic mRNA. The key features of mRNA therapeutics/vaccines are shown with their functional roles highlighted. The 5′ cap reduces RNA degradation and facilitates ribosome initiation during translation. The 5′ UTR influences translation efficiency and stability. Coding sequence is essential for encoding the protein of the gene of interest. The coding sequence can be chemically modified using modified nucleoside triphosphates (NTPs) in the enzymatic reaction, which can reduce immunogenicity and enhance stability. The 3′ UTR regulates translation efficiency. Poly(A) tail enhances mRNA stability and also facilitates ribosomal initiation and translation efficiency. Created in BioRender. Dickman, M. (2024) https://BioRender.com/c49v683 .

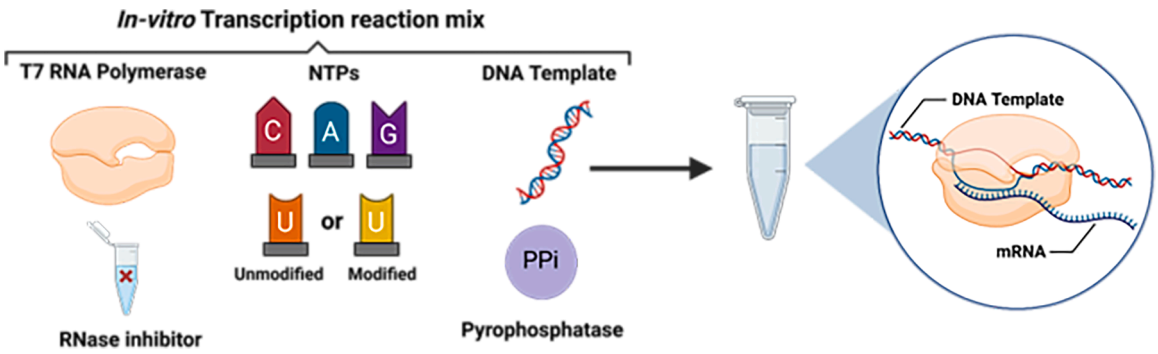

Fig. 2. Synthesis of mRNA using *in vitro* transcription. Synthetic mRNA is synthesized from NTPs using T7 RNA polymerase incubated with a linearised plasmid DNA template. Created in BioRender. Dickman, M. (2024) https://BioRender.com/f29f724.

Table 1
Analytical methods for the characterisation of mRNA quality attributes. Liquid chromatography associated techniques are highlighted in bold. Adapted from USP Guidelines (June 2024) [34].

Quality	Attribute	Method
Identity	mRNA sequence confirmation	High throughput sequencing (HTS) Sanger sequencing Reverse Transcription Polymerase Chain Reaction (RT -PCR)
Content	RNA concentration	Quantitative reverse-transcription PCR (RT -qPCR) Reverse transcription digital PCR (RT–dPCR) Ultraviolet (UV) Spectrophotometry
Integrity	**mRNA purity**	**Ion-pair reversed-phase high performance liquid chromatography (IP-RP HPLC)**
Purity	**5′ capping efficiency**	**Reversed-phase liquid chromatography mass spectroscopy (RP–LC-MS/MS)** **IP-RP HPLC** **LC-MS/MS**
	Poly(A) tail	**LC-MS/MS** **IP-RP HPLC**
	dsRNA impurities	Immunoblot Enzyme-linked immunosorbent assay (ELISA)
	Aggregate quantitation	**Size exclusion-high performance liquid chromatography (SEC HPLC)**
	Percentage of fragment mRNA	**RP HPLC**
	Residual DNA template	quantitative PCR (qPCR)
	Quantitation of free/non-incorporated nucleosides	**RP–LC-MS/MS**
	Residual NTP and capping agent	**Anion exchange (AEX) HPLC**
	Residual T7 RNA polymerase content	ELISA
Potency	Expression of target protein	Cell-based assay
Safety	Endotoxin	USP <85>
	Bioburden	USP ⟨61⟩, ⟨62⟩, <1115>
Other	Appearance	USP <790>
	Residual solvents	USP <467>
	pH	USP <791>

it highly polar, whilst its single stranded nature leads to dynamic alternative secondary structures, resulting in sample heterogeneity. Additionally, mRNA manufactured using IVT often contains impurities, similar in size to the pure full-length mRNA, making separation and characterisation difficult. CQAs, like the 5′ cap and 3′ poly(A) tail, must also be thoroughly characterised to ensure the integrity and functionality of the mRNA product [24,35,36].

2. Analysis of mRNA purity/impurities

Analytical methods are required to monitor the level of impurities resulting from the synthesis of the active mRNA DS as well as the degradation products that are formed during the manufacturing and storage of the mRNA DP. During the manufacturing of mRNA, incomplete mRNA transcripts are generated due to early termination during IVT or degradation of the mRNA. Furthermore, impurities also include potential mRNA aggregates that may be associated with different types of dsRNA byproducts. These impurities can potentially compromise drug efficacy and safety. Therefore, analytical methods are required to measure the purity and monitor the stability of the mRNA. Suitable methods must demonstrate a high degree of selectivity for the intact mRNA transcript and be able to distinguish it from abortive transcripts, potential degradation products and other manufacturing impurities such as dsRNA.

Capillary Electrophoresis (CE) enables the high resolution separation of large RNAs (>1000 nt) in conjunction with the ability to achieve some selectivity of intact mRNA from abortive transcripts and potential degradation products [37–41]. Separations are largely performed under denaturing conditions and enable separations based predominantly on the size of the mRNA. Lu et al., successfully separated a 200 - 6000 nt RNA ladder using non-aqueous, formamide based background electrolyte in conjunction with polyethylene oxide polymer gels [37]. Conventional CE methods often require long run times, however the application of microchip CE (mCE) demonstrated the ability to separate large RNAs with rapid run times [38]. The applicability of microcapillary electrophoresis for high-throughput analysis of mRNA impurities has also been demonstrated [42]. Comparative analysis of mRNA separations on commercial capillary gel electrophoresis instruments demonstrated higher resolution was achieved on the Sciex PA800 Plus, while other commercial systems such as the Fragment Analyzer were more appropriate for high-throughput analysis [41]. The use of CE for the analysis of mRNA and other nucleic acid therapeutics has been reviewed further by Wei et al., [39].

CE based technology suffers from lower repeatability and robustness in comparison to HPLC, and it is more challenging to couple with MS and downstream fractionation/purification of mRNA related impurities for further characterisation. In addition to CE, HPLC methods are emerging as alternative orthogonal methods for the analysis of mRNA purity. Several modes of HPLC can be employed, each offering different advantages, while also having their own analytical limitations for the analysis of large polar mRNA molecules, which are discussed below.

2.1. Analysis of mRNA purity using ion-pair reversed phase HPLC (IP-RP HPLC)

2.1.1. Optimisation of IP-RP HPLC for the analysis of RNA

The high resolution separation of large RNA molecules (>1000 nt) in

length using HPLC is challenging. The development of alternative stationary phases and optimisation of mobile phases in conjunction with IP-RP HPLC has enabled high resolution separations of large RNAs, including mRNAs, in short analysis times. The application of IP-RP HPLC for the high resolution separation of larger RNA molecules, comparable to that of larger double-stranded DNA molecules, was first demonstrated during the development of an assay for group I intron ribozyme activity [43]. Further studies have employed the rapid, high resolution separation of RNA to analyse various large RNA transcripts and biological RNAs, including ribosomal RNA and mRNA [44]. IP-RP HPLC has been applied to separate larger RNA molecules, such as mRNA, with high resolution. The availability of alternative ion-pair agents and additives that can interact with the charged phosphate backbone of RNA has enabled the use of a wide range of alternative mobile phases [44–48]. Developments in hydrophobic stationary phases also allow for greater resolution of mRNA, which are discussed below.

The introduction of 2 μm, C18-surface, non-porous polystyrene-divinylbenzene (PS-DVB) columns, developed by Bonn et al. [49,50], and later commercialised as the DNASep column by Transgenomic, demonstrated high-resolution separation of large double-stranded and single-stranded nucleic acids in under 10 mins [51]. Rapid, high resolution separation of large nucleic acids has been achieved using these non-porous polymeric media. The highly mono-disperse nature of the particles results in the minimisation of the diffusion pathways and therefore provides high resolution with rapid analysis times. Effective stationary phases for the analysis of mRNA must be compatible with their large size and structure, ensuring high rates of mass transfer are achieved between the stationary phase and mobile phase. In addition to the non-porous polymeric columns, macro-porous PS-DVB resins and PS-DVB monoliths have been used for the analysis of large RNA and mRNA. Macro-porous PS-DVB particles with relatively large pore sizes, including spherical macro-porous 4 μm polymer resins (DNAPac RP) with a wide range of pore sizes (50 Å) to ultra-wide (2500 Å) have been widely used for the analysis of mRNA [45,52–56]. Previous studies using PS-DVB columns with pore sizes of 300 Å, 1000 Å, and 4000 Å, demonstrated that the largest pore size was most suited for mRNA analysis. Yamauchi et al., showed that non-porous, alkylated PS-DVB and monolithic PS-DVB columns gave optimum resolution for smaller RNA <1000 nt. The macro-porous PS-DVB resin performed better for mRNAs, demonstrating resolution for mRNA up to 8000 nt [57]. In addition to polymeric columns, silica-based particles have also been used. The optimum resolution for larger dsDNA/RNA molecules was achieved using superficially porous silica particles with pore sizes of 400 Å [58]. Further investigations have utilised 5 μm octadecyl spherical silica particles with wide pores >30 nm (COSMOSIL RNA-RP1) for the analysis of large RNA (500 - 5000 nt) and mRNA demonstrating advantages over the smaller pore sized particles [59].

Further studies have also been performed to evaluate alternative ion-pairing agents to assess their impact on the separation of large RNA fragments and the analysis of modified mRNA [45]. The investigation compared the use of the ion-pair reagents 100 mM triethylammonium acetate (TEAA), 25 mM dibutylammonium acetate (DBAA), and 15 mM hexylammonium acetate (HAA), in conjunction with macro-porous (PS-DVB) columns as the stationary phase. Their results concluded that the highest resolution of large RNA fragments, ranging from 100 to 1000 nt, was achieved when using 100 mM TEAA [45]. Ozaki et al., also demonstrated increased resolution of RNA (500–1000 nt peaks) using TEAA in comparison to triethylamine – 1,1,1,3,3,3 hexafluoro isopropanol (TEA-HFIP) mobile phases on the COSMOSIL RNA-RP1 column [55]. Maurer et al., systematically compared thirteen alkylamine ion-pair reagents for the separation of RNA of varying size (10– 6000 nt). The combination of 100 mM butylamine and 50 mM tripropylamine was determined to provide the best separation of larger RNAs (200–6000 nt) [60]. A similar systematic assessment of ion-pairing agents for the separations of much smaller oligonucleotides also established separation selectivity was based on hydrophobicity of the ion-pair agent [61].

Despite the advancements in macro-porous and super-wide pore particles, the ability to separate mRNAs >5000 nt remains a challenge. Although separations of larger ssRNA can be achieved using IP-RP HPLC, this required shallow gradients (7–10% ACN over 20 mins] and was unable to resolve 7000 nt and 9000 nt RNAs [59]. Furthermore, employing IP reagents such as TEAA results in separations dependent on both the sequence and size of the RNA, which can result in co-elution of RNAs of different sizes and potentially result in the order of elution that does not correspond to the length of the mRNA [44,47,60]. Therefore, the ability to separate intact mRNA from abortive transcripts and potential degradation products will be influenced not only by the length of the mRNA, but also the sequence composition. With the emergence of new mRNA vaccines/therapeutics, including longer mRNAs and large self-amplifying RNAs (saRNAs), this will require further optimisation of particle pore size, alternative column chemistries and novel mobile phases to enable high resolution separations of such large RNAs in conjunction with the ability to accurately measure mRNA integrity and accurately determine their size [59].

2.1.2. Development and application of IP-RP HPLC for the analysis of mRNA purity

IP-RP HPLC methods for mRNA integrity analysis have been described, with the aim of separating full-length mRNA from partial-length products, including degraded mRNA. These partial-length products elute prior to the full-length mRNA product. Other potential impurities, such as dsRNA, typically elute later than the full-length mRNA (under non-denaturing conditions). Analysis of pre- and post-main peaks, for multiple mRNAs, has enabled the development of methods to characterize mRNA integrity [42,45,53–55], with the aim of ultimately improving its stability. An example of a typical mRNA separation using IP-RP HPLC is shown in Fig. 3. The chromatogram shows the early elution of mRNA species that are 5′ capped, but do not contain the 3′ poly(A) tail. These species are the result of mRNA degradation or early termination during synthesis. Peaks eluting after the full-length mRNA are proposed to be dsRNA impurities.

Dayeh et al., used IP-RP HPLC to separate full-length mRNA products from partial-length products, as well as identifying ‘longmers’, which eluted post-main peaks. By monitoring percentage peak area over time, it was determined that Cas9 mRNA (4521 nt) degraded at a faster rate than eGFP mRNA (996 nt) [54]. Larger mRNAs can be subject to faster degradation due to a longer phosphate backbone, which provides more sites for hydrolysis. This highlights the need for rigorous analysis of mRNA for degradation, especially larger species during production, processing and storage. Currie et al., also monitored mRNA degradation in a stability-indicating study, using TEAA in conjunction with a DNAPac RP column to analyse mRNA that had undergone forced degradations in multiple ways: acidic (pH 3.5) and basic (pH 11.0) conditions, heat treatment and digestion using RNase A [45]. By determining the comparative percentage peak area of the pre-main peaks and the main peak, this method can be used to monitor mRNA stability under degrading conditions, providing scope for stability assessments of different mRNAs under alternative conditions.

The importance of monitoring mRNA integrity is further highlighted by the work of Camperi et al., who employed IP-RP HPLC analysis to show that mRNA can be impacted by differences in downstream and upstream processing methods. Camperi et al., demonstrated mRNA peak broadening and heterogeneity in the 3′ structure of GFP mRNAs produced by different suppliers. Further mass spectrometry analysis confirmed an alternative length 3′ poly(A) tail for one supplier’s eGFP mRNA [42]. The establishment of this method enabled temperature degradation studies to determine that the poly(A) tail is susceptible to heat stress and that the pre-main, “tail-les” peak increased by ~24% after heat stress [42].

2.1.3. Analysis of mRNA lipid adducts using IP-RP HPLC

IP-RP HPLC has also been utilised to analyse mRNA-LNP delivery

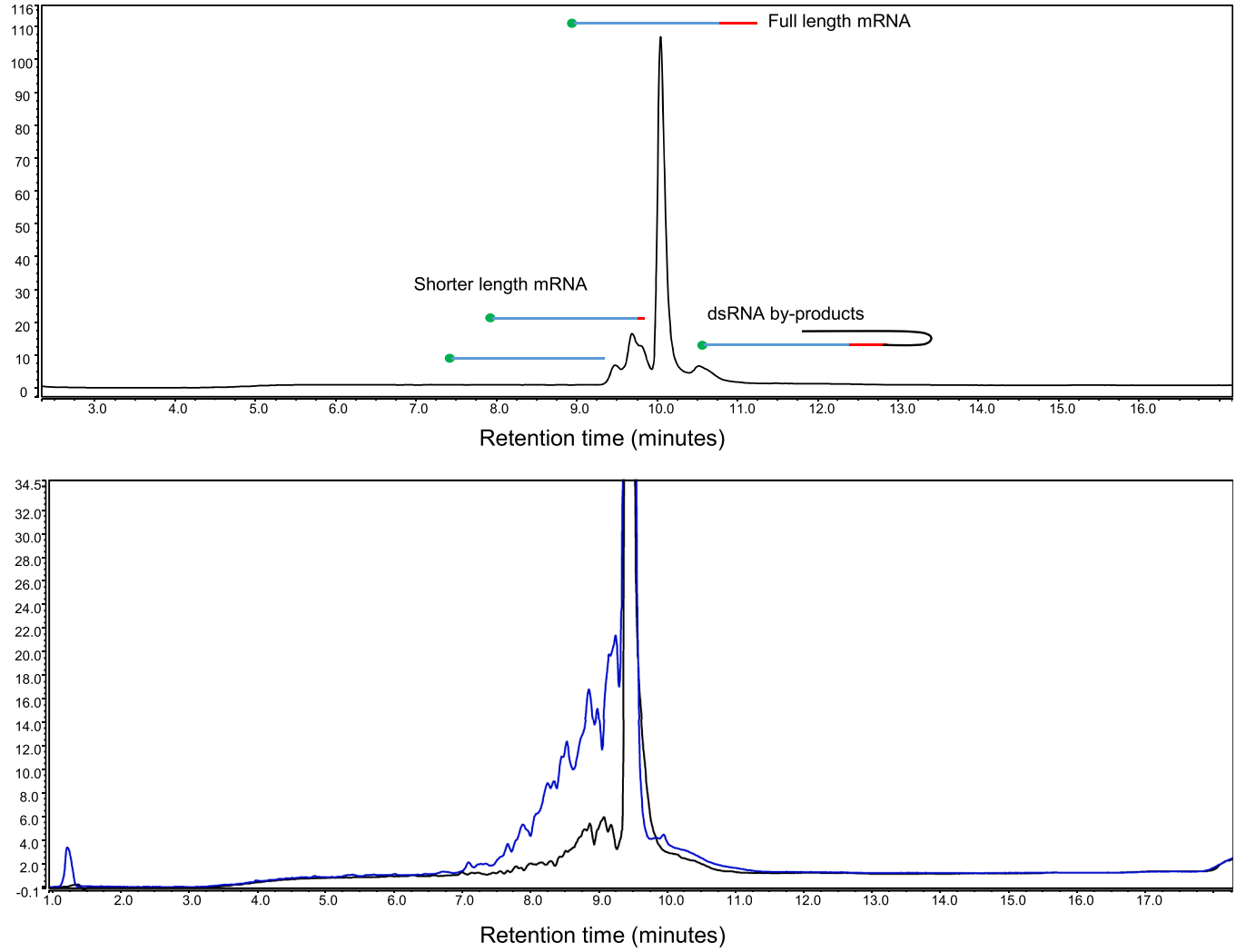

Fig. 3. IP-RP HPLC analysis of mRNA. A) IP-RP HPLC chromatogram of a typical mRNA. The chromatogram show the presence of peaks before and after the main peak. The corresponding peaks before the main full length mRNA product are typically generated from mRNA species with different poly(A) tail lengths and partial hydrolysis of the full length mRNA resulting in reduced hydrophobicity and earlier retention times. The corresponding peaks after the main peak are typically associated with dsRNA by-products resulting from 3′ loop back formation. **B)** IP-RP HPLC stability indicating analysis. eGFP mRNA was heat treated prior to IP-RP HPLC analysis. The chromatogram in black shows an early time point and blue a later time point. The increase in abundance of peaks prior to the main full-length mRNA peak demonstrate the increased abundance of shorter length hydrolysis products resulting in lower mRNA integrity. IP-RP HPLC analysis was performed using mobile phase A) 0.1 M TEAA; mobile phase B) 0.1 M TEAA, 25% ACN. Gradient elution starting at 35% B to 55% B over 15 mins on a DNAPac RP column (2.1 × 100 mm) with UV detection at 260 nm.

complexes, specifically the impact of the associated lipids on the mRNA [27]. IP-RP HPLC analysis of mRNA extracted from mRNA-LNPs using DBAA/TEAA on a macro-porous PS-DVB column showed there was a heterogeneous mixture of RNA-related species eluting after the full-length product mRNA. The species were due to lipid adducts, which increased the retention time of the corresponding mRNA. Moreover, the study also demonstrated that mRNA-lipid adducts led to reduced expression of the mRNA's encoded protein, highlighting the functional impact of these impurities. Mass spectrometry analysis confirmed that lipid adducts formed on cytosines and that these lipid adducts only occurred when mRNA was combined with the ionisable lipid component of the LNP. Packer et al., also discovered previously overlooked impurities formed by the N-oxide degradation of amino lipids into aldehydes. IP-RP HPLC not only identified aldehyde-modified mRNA, but also separated these modifications based on their length and branching [27]. The study therefore emphasised the importance of monitoring both impurities from upstream processing (synthesis) and downstream processing. It further highlighted the significance of IP-RP HPLC in identifying and purifying modified mRNA, ensuring product stability, purity, and potency.

These applications demonstrate the versatility of IP-RP HPLC for the analysis of mRNA. IP-RP HPLC provides scope for mRNA degradation monitoring, stability assessments, determining the impact of upstream and downstream mRNA processing methods, identifying structural features and analysing the impact of LNP encapsulation. In addition, IP-RP HPLC is readily interfaced with MS analysis of intact mRNAs (see Section 3). The use of alternative HPLC techniques such as anion exchange chromatography (AEX) and size exclusion chromatography (SEC) rely on other physicochemical properties in order to analyse the mRNA for attributes such as process related impurities and aggregation.

2.2. *Analysis of mRNA purity using AEX HPLC*

AEX HPLC methods are predominantly used for the purification of RNA [62,63] with analytical methods for the separation of larger RNA facing challenges, such as loss of resolution, potential carry-over and lack of quantitative recovery of the RNA from the stationary phase [46]. AEX HPLC methods have been employed for transcript purifications [64,

65]. AEX HPLC has been performed using denaturing conditions in an approach to remove secondary/tertiary structures in oligonucleotides and RNA [31,32,35,66]. Several approaches have been developed and applied using AEX for the analysis of mRNA molecules (reviewed in [67]). The use of strong denaturing conditions (high pH) in conjunction with a pellicular particle stationary phase (DNAPac PA200) was used to achieve high resolution separations [31]. Kanavaroti et al., analysed several mRNAs including eGFP and Cas9 with mobile phases at pH 12.0, demonstrating high resolution and reproducible mRNA separations, providing further information regarding mRNA quality. The strong denaturing conditions effectively remove the high degree of secondary structure present in the mRNA, in conjunction with minimal/negligible on column degradation, demonstrating the ability to effectively analyse mRNA and potential impurities [31].

Alternative AEX methods have recently been developed under milder conditions for the analysis of mRNA. Traditional AEX chromatography utilises salt gradients: typically 1–2 M NaCl or lower concentrations of NaBr or $NaClO_4$. Ion-pairing anion exchange "IPAX" has been developed, which utilises a gradient of weak ion-pair reagent cations (tetramethylammonium chloride) alone or in combination with an increasing salt concentration. The IPAX method was optimised using column temperatures between 30 and 45 °C [46], resulting in the elution of the mRNA under mild elution conditions. Furthermore, hydrophilically modified columns were used in AEX in conjunction with IPAX resulting in improved recovery and peak shape for the analysis of EPO and Cas9 mRNA [68]. Therefore, in addition to IP-RP HPLC, AEX can be used as an alternative, potentially orthogonal method for the characterisation and analysis of mRNA [67]. Different mechanisms of separation and manipulation of a wide range of mobile phases in AEX may provide an alternative method with the ability to selectively separate mRNA from impurities including short, degraded RNA and specific impurities, such as dsRNA. As AEX provides separations based predominantly on charge and ionic interactions, it can be used as an alternative and complementary system to IP-RP HPLC [46]. Although currently the typical resolution for the separation of large RNAs is lower than that obtained on CE, mCE and IP-RP HPLC, the ability to perform separations in the absence of ion-pair reagents and other potentially hazardous organic solvents, combined with the complementary mechanism of retention, highlights the potential advantages of AEX for the analysis of mRNA.

2.2.1. Analysis of process related impurities using AEX

Welbourne et al., (2024) highlighted the benefit of using AEX chromatography for analysis of mRNA, notably for monitoring the production of mRNA via IVT reactions [32]. Optimisation of their high throughput AEX HPLC method achieved separation and quantification of the key IVT reactants and products, including NTPs, Cap analogue, plasmid DNA and mRNA product [32]. Separation of these components was achieved within six minutes using high pH mobile phases (10 mM NaOH in mobile phase A and 10 mM NaOH, 2 M NaCl in mobile phase B) in conjunction with a DNAPac PA200 column. Notably, their AEX method was able to perform baseline separation of the NTPs and quantitative elution of the mRNA. Therefore, this approach enabled quantification of each NTP and NTP consumption during IVT reactions in conjunction with mRNA production. The AEX HPLC method was also used to measure residual process related impurities (including residual NTPs and Cap analogue) in mRNA samples that had been purified using downstream purification methods, such as oligo-dT affinity chromatography and tangential flow filtration (TFF) [32].

Pregelic et al., utilised a fed-batch IVT system combined with at-line HPLC monitoring to optimise the production of mRNA. HPLC analysis was performed using a CIMac PrimaS™ column, mobile phase A (50 mM HEPES, pH 7.0) and mobile phase B (50 mM HEPES, 200 mM sodium pyrophosphate, pH 8.5). The ability to monitor the IVT reactions using this approach enabled optimisation of the fed-batch conditions to significantly increase the yield of mRNA, reliably achieving concentrations of up to 10 mg/ml within three hours [66].

The ability to separate key IVT reactants and products, including NTPs, Cap analogue, and mRNA product all in a single chromatographic analysis using AEX HPLC demonstrates advantages over alternative modes of separation such as SEC, which has limited resolution for the separation of individual NTPs. While IP-RP HPLC methods can perform separations of individual NTPs [69,70], oligonucleotides [49,58,61] and mRNAs [45,54], methods for separation of all components in a single analysis are limited. The combination of two columns in series (DNAPac RP column/ACQUITY BEH C18 300 Å column) was used to separate NTP standards, oligonucleotides (10–100 nt) and larger RNAs (500–6000 nt) in a single analysis, highlighting the potential to measure RNA integrity in conjunction with monitoring IVT reactions [60].

2.3. Analysis of mRNA purity using size exclusion chromatography

As an alternative to IP-RP HPLC and AEX HPLC, SEC is emerging as an alternative chromatographic mode for the analysis of mRNA and its product related impurities. SEC separations are based on size or hydrodynamic radius and the technique provides scope for the separation of DNA templates, enzymes and RNA, as well as for the separation of mRNA aggregates [56,71,72].

2.3.1. Optimisation of particle pore size for the analysis of mRNA using SEC

The particle size and column porosity have significant effects on SEC separations. Determining the appropriate pore size of the SEC column for mRNA analysis is dependent on the size of the mRNA. Due to the large, dynamically folded nature of mRNA, this can prove challenging to determine. The hydrodynamic diameter of the mRNA-LNPs in the COVID-19 vaccines have been measured at 92.89 ± 2.53 nm for Pfizer (Comirnaty™ COVID-19 Vaccine, mRNA) and 93.00 ± 3.00 nm for Moderna (Spikevax™ COVID-19 Vaccine, mRNA), although mRNA-LNPs have been reported to range between 60 and 250 nm [73,74]. It is therefore recommended that the particle size for packing material for SEC analysis of mRNA and mRNA-LNPs should not be below 2 μm [71].

Several studies have been performed comparing and optimising particle pore size for the analysis of mRNA. For SEC analysis, the selection of particle pore size is crucial for optimising resolution, typically using pores three times larger than the hydrodynamic radius of the analyte [75]. The analysis of fLuc mRNA has previously been performed using pore sizes of 200–450 Å [76]. Further studies by Goyon et al., used SEC columns with ultra-wide pores (pore size 1360 and 1275 Å) to separate mRNA and LNP aggregates. SEC effectively separated free mRNA from intact LNPs, with the ability to resolve differences in size and aggregation state. This separation is crucial for quality control in mRNA-LNP formulations, ensuring product consistency and stability. The study also established a correlation between SEC elution times and LNP sizes determined by dynamic light scattering, validating the effectiveness of SEC in characterising LNP size distributions [77].

De Vos et al., investigated SEC column particle pore size. Columns with pore size 1000–2000 Å were found to provide resolution for RNA between 500 and 10,000 nt in length. This scale could not be achieved using a 300 Å pore size, which was found to be limited to RNAs <500 nt. This limits the use of SEC columns with smaller pore sizes for analysis of full length mRNA, but does allow scope for use in the analysis of shorter fragments, including poly(A) tail characterisation [72]. D'Arti et al., investigated columns with ultra-wide pore sizes of 1000 Å (3 μm particles) and 2500 Å (5 μm particles), where it was shown that the 1000 Å pore size was optimal for the analysis of mRNA [56]. Furthermore, it was found that addition of 10 mM $MgCl_2$ to the mobile phase (50 mM Tris- buffer at pH 7.5, 200 mM KCl) resulted in improved resolution and recovery of some larger species of mRNA.

2.3.2. Analysis of mRNA aggregates using size exclusion chromatography

In addition, SEC was used to examine eGFP and Cas9 mRNA samples from alternative suppliers. The results showed differences in the amount

of aggregation, indicating that alternative manufacturing processes can promote aggregation, which can be analysed using SEC [56]. Recent developments in SEC column formats have also enabled the characterisation of poly(A) tail length [72]. SEC was also used to assess the presence of aggregates from two different commercial sources of eGFP mRNA. Further characterisation of the nature of the aggregates (covalent vs non-covalent) was studied using heat treatment of the mRNA prior to SEC analysis, demonstrating the non-covalent nature of the mRNA aggregates in one of the mRNA samples [77].

De Vos et al., evaluated size exclusion methods for characterising mRNA with different hydrodynamic sizes (1200 and 4200 nt). The impact of mobile phase composition, ionic strength, organic modifiers, pH and column temperature were assessed using the 5 µm Agilent Bio SEC-5 column (pore size 1000 Å). Neither altering the pH of mobile phases between pH 6 and pH 8 nor increasing the percentage of organic solvent in the mobile phase affected separation. However, altering the ionic strength of the mobile phase significantly affects the elution time of mRNA [72]. This is due to secondary structure motifs of the mRNA adopting lower melting temperatures in buffers with lower ionic strength. This consequently impacts mRNA folding, its hydrodynamic radius and elution time [78].

SEC analysis of mRNA can also be performed using multi-angle light scattering (MALS) detection. MALS is an advanced analytical technique used to determine the molecular weight, size and structure of biomolecules, such as mRNAs in solution. SEC-MALS can therefore measure biological features, such as molecular weight, size (radius of gyration), aggregation and provide further insight into mRNA conformation [79]. SEC-MALS analysis of mRNAs including eGFP, OVA, fLuc and Cas9 was used to determine a molecular weight of the mRNA monomers and corresponding multimers. Degradation was also monitored as SEC-MALS determined low molecular weight species after six days of thermal stress, indicating the presence of shorter mRNA fragments [54].

The application of SEC columns with ultra-wide pores and optimisation of alternative mobile phases, has improved the separation of large RNA molecules (>1000 nt). However, in comparison to IP-RP HPLC, CE and mCE lower resolution separations of large RNAs are typically achieved, therefore limiting such approaches for analysis of mRNA integrity and the ability to separate similar sized abortive transcript or degradation products.

In comparison to AEX and IP-RP HPLC, SEC analysis of mRNA predominantly focuses on mRNA-LNP interactions and mRNA aggregation. It provides valuable insight into the hydrodynamic radius of mRNA species and can be readily coupled to MALS detection. However, there are limitations to SEC analysis. The use of ultra-wide pore SEC columns poses challenges regarding column stability and lifetime, particularly under the high-pressure conditions required for the separation of large biomolecules. While SEC provides detailed aggregate analysis, it should be noted there are potential limitations in detecting smaller aggregates or distinguishing between closely related species.

2.4. Summary

A wide range of alternative modes of HPLC have been developed and applied to measure the purity/impurities and monitor the stability of mRNA. Measurement of the mRNA purity and integrity is dependent on the resolution and selectivity of the analytical method as it is dependent on the ability to resolve abortive transcripts and potential degradation products from the intact mRNA. CE and mCE approaches are typically employed for the analysis of mRNA integrity, achieving high resolution separations of large RNAs under denaturing conditions. Furthermore, the advantage of potential standardised assays using commercial CE instruments provides benefits for the analysis of mRNA integrity. IP-RP HPLC also enables high resolution separations of large RNA in short run times, although separation of fragments >5000 nt is currently challenging. Alternative stationary phases and mobile phases are often employed for the analysis of mRNA integrity, which can make comparative assessments of reported values of % mRNA integrity challenging across different studies. However, the development and application of a range of orthogonal HPLC modes to measure mRNA integrity and impurities has a number of advantages. These include the ability to manipulate the conditions employedby performing the analysis under non-denaturing or denaturing conditions, utilising alternative mobile phases, e.g. alternative IP reagents to manipulate sequence or size dependent separations, combined with the ability to purify or fractionate the wide range impurities present for further downstream characterisation.

3. Analysis of mRNA purity using intact mass analysis

The analysis of 5′ capping efficiency, 3′ poly(A) tail length and heterogeneity, and direct mRNA sequencing (identity) predominantly use "bottom-up" approaches whereby the large mRNA is digested into smaller oligoribonucleotides prior to LC-MS and MS/MS analysis (see Sections 4-5). Currently, the intact mass analysis of large mRNAs is challenging due to their large polyanionic nature, the propensity to form metal ion/ion-pair adducts and the heterogeneity arising from the 3′ poly(A) tail. IP-RP interfaced with MS has been performed using diisopropylethylamine with HFIP for intact mass analysis of EPO mRNA (858 nt) including its 3′ poly(A) tail [80]. In addition, native MS has also been used to measure the intact mass of shorter mRNAs with (783 nt) and without (683 nt) a poly(A) tail [42]. The authors show that increased heterogeneity was observed in presence of the poly(A) tail and used the intact mass analysis to further characterise mRNA impurities obtained after fractionation from IP-RP HPLC. The intact mass analysis provided important further information on the mRNA integrity and heterogeneity. However, due to the heterogeneity of the mRNA, this makes native MS analysis of larger mRNAs (>1000 nt) increasingly difficult as the charge state distribution can become unresolvable. Alternative approaches such as charge detection MS (CDMS), which simultaneously measures the m/z ratio and charge of single particles, may enable the intact mass analysis of larger mRNAs. CDMS has previously been applied for the characterisation of large nucleic acids including plasmid DNA [81–83]. The ability to perform high throughput intact mass analysis of mRNA using approaches such as CDMS may provide important further insight into mRNA heterogeneity and aid in the characterisation of impurities without the requirement for processing of the mRNA prior to analysis. These methods may provide important orthogonal information on the mRNA integrity and heterogeneity compared to bottom-up approaches. However, such methods may require specialist instrumentation not routinely available and may not provide the accurate quantitative measurements of 5′ capping efficiency and poly(A) tail heterogeneity that can be achieved using "bottom-up" LC-MS methods.

4. Characterisation of 5′ caps and 5′ capping efficiency of mRNA

4.1. 5′ cap

The mammalian 5′ cap structure consists of a guanosine nucleotide methylated at its N7 position linked to the 5′ end of the mRNA strand via a 5′−5′ triphosphate linkage (see Fig. 4). Manufacturing of mRNA vaccines requires inclusion of a 5′ cap structure to prevent degradation and evade host immune response [11]. In nature, m7G, forms the Cap 0 structure m7GpppN, where N is any ribonucleotide, representing a non-sequence encoded chemical modification. The Cap 0 structure can be converted to Cap 1 by methylation of position R1 (2′-OMe) in the first transcribed nucleotide (see Fig. 4). Methylation of 5′ the cap structure is a marker of "self" mRNA and enables evasion of the host innate immune response [84].

4.2. 5′ capping analysis using LC-MS or LC-MS/MS methods

The importance of the 5′ cap to mRNA function therefore requires

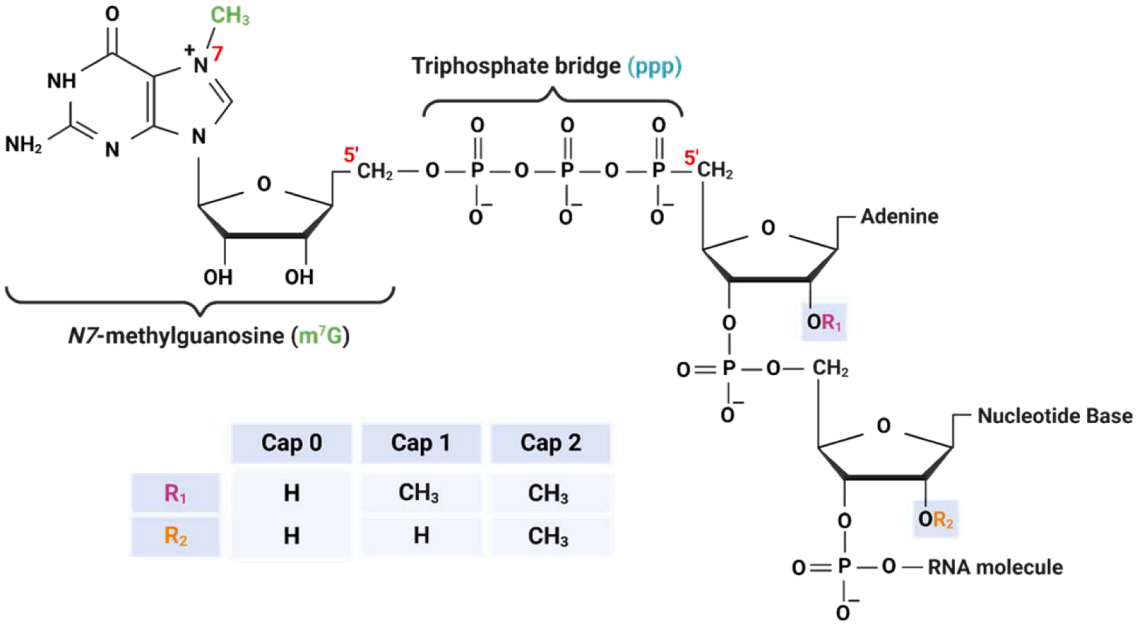

Fig. 4. mRNA cap structures. Cap 0 is a N7-methyl guanosine connected to the 5′ nucleotide through a 5′ to 5′ triphosphate linkage. The presence of 2′-O-methyl groups at either R1 only or both R1 and R2 determine whether a cap structure is Cap 1 or Cap 2 respectively. Created in BioRender. Dickman, M. (2025) https://BioRender.com/a44e767.

methods to confirm success of the 5′ capping reaction. 5′ capping of mRNA vaccines can be affected by a range of factors, including the purity of the components of the reaction, template DNA quality, and design. Co-transcriptional 5′ capping enables "one pot" synthesis of 5′ capped mRNA and typically generates Cap 1 structures. Cap 1 is commonly used for capping mRNA vaccines. It is also possible to add the 5′ cap post-IVT employing an additional enzymatic processing step to generate 5′ Cap 0 or Cap 1 using Vaccinia virus enzymes.

Methods to evaluate 5′ capping use LC-MS or LC-MS/MS methods. A 'bottom-up' approach generates fragments for intact mass measurement (LC-MS) or data-dependent fragmentation (LC-MS/MS). A key requirement is the generation of 5′ cap and related analytes as single digest fragments for quantitative accuracy. Samples are analysed by either LC-MS or LC-MS/MS, depending on the context of information required. A general limitation of LC-MS based methods for quantifying the 5′ cap is the lack of confirmatory sequence information. The structure of 5′ cap species can be verified through fragmentation of selected ions (LC-MS/MS). Both LC-MS and LC-MS/MS methods can be used for quantitative evaluation of 5′ capping efficiency. This efficiency is calculated as a percentage of a total of 5′ capped mRNA and process-related 5′ species present in the sample [85]. Additionally, 5′ species may occur with metal adducts [85], which should be minimised by prior cleaning and selection of appropriate buffers and reagents to minimise sodium adducts [86].

The different cleavage strategies used for generating 5′ capped and uncapped species are discussed below. Methods employing directed cleavage strategies including RNA/DNA chimeric probes, DNA probes or use of ribozymes require prior knowledge of the mRNA sequence under test. Of these, RNase H is recommended by draft 3 of the USP guidelines [34]. Enzymatic digestion with RNase T1 or nuclease P1 are also recommended [34] (see Fig. 5, Table 1). These alternative methods offer an advantage of universal application, since they do not require the design of sequence specific probes.

4.3. RNA/DNA hybrid cleavage by RNase H

RNase H was the first method reported for analysing the 5′ cap, demonstrating the proof of principle for RNA/DNA chimeric probes that facilitate RNase H cleavage [85]. RNase H endonuclease cleaves phosphodiester bonds of RNA and DNA hybrids only. It will not cleave dsDNA, dsRNA or ss nucleic acids. This specificity was exploited to direct cleavage to short sequences from the 5′ end of the mRNA at a specific site. The resulting uncapped and 5′ capped fragments differ by one nucleotide in length, with the mass difference corresponding to incorporation of the 5′ cap. The method employed biotin-tagged DNA sequences, complementary to the 5′ end of the mRNA, designed to generate oligonucleotides of either 40 nt or 50 nt in length, which are purified by streptavidin beads to capture the 5′ fragments. LC-MS analysis was performed utilising a C18 column with TEA/HFIP mobile phases online and a quadrupole-Orbitrap mass analyser. The unique masses of cleavage fragments derived from LC-MS/MS analysis with a 5′ cap were compared to those containing the corresponding uncapped diphosphate, triphosphate, or unmethylated 5′ cap (GpppG). Linearity of detection of uncapped mRNA species was demonstrated, enabling quantitative assessment of 5′ capping efficiency. This label-free approach was shown to be compatible with mRNA containing modified nucleotides pseudouridine and 5-methylcytidine, demonstrating the general utility of the technique to study mRNA capping. However, it has also been demonstrated that pseudouridine substitution can alter the cleavage specificity of RNase H, potentially due to base-pairing with the target oligonucleotide and/or binding of RNase H [87], which is likely dependent on the sequence of the mRNA.

Challenges and limitations of the RNAse H method include low recovery and the generation of additional minor cleavage species after RNase H cleavage [88,89]. Further optimisation of this approach has been achieved using thermostable RNase H and silica based sample clean up [88]. Improving the specificity of RNase H cleavage was addressed by implementation of a screening framework designed to test RNase H specificity on an empirical basis. The outcome was minimisation of additional, multiple cleavages that can occur close (−2 to +2 nucleotides) to the target site of cleavage and thus improvement of cleavage specificity [87].

A rapid five minute method that is applicable to the RNase H digest protocol, using intact mass measurement, was developed by Nguyen et al., [90]. This LC-MS approach was tested using a synthetic 25 nt RNA for LC-MS and achieved quantitation of m7GpppGm (Cap 1) and four

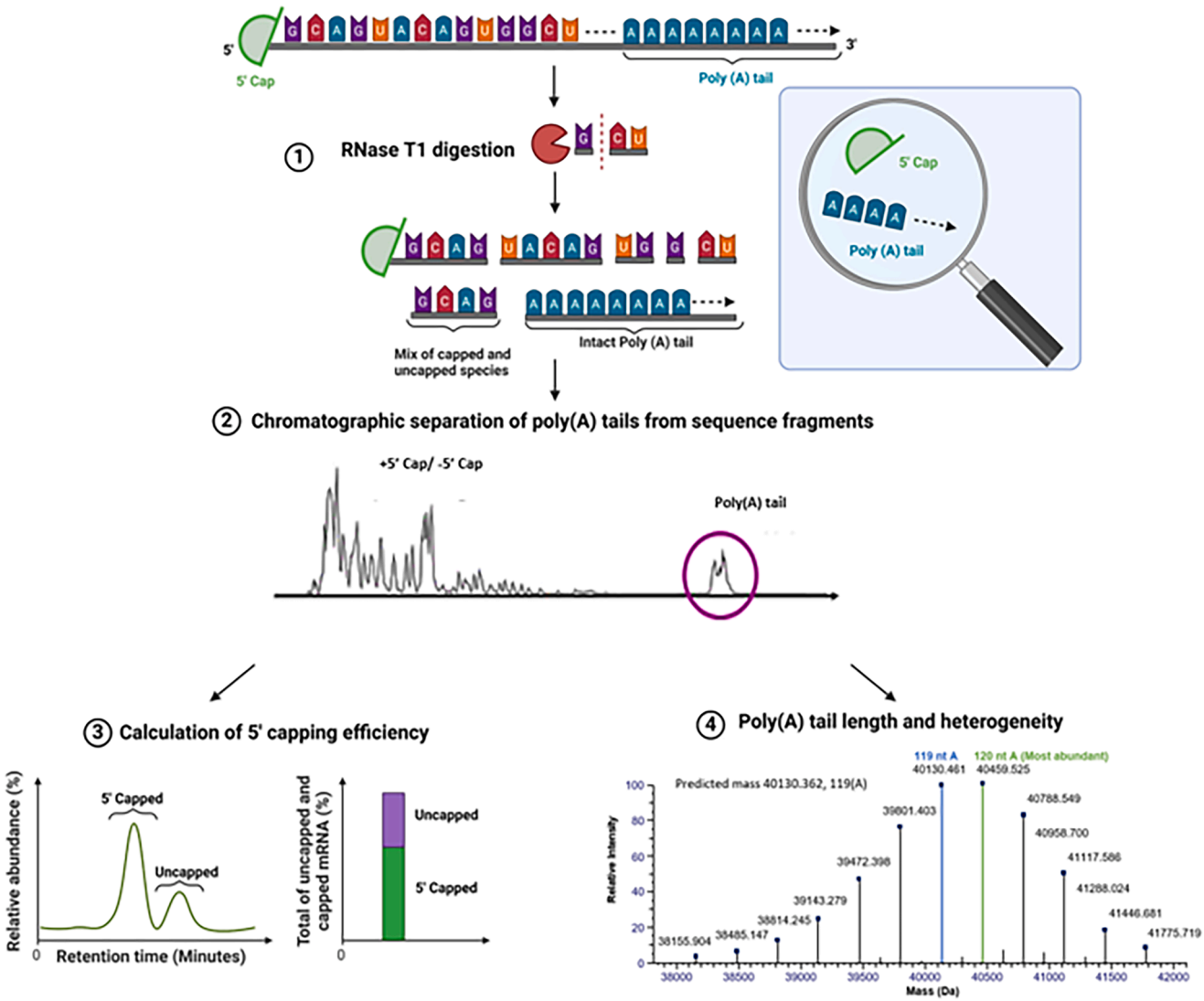

Fig. 5. RNase T1 based LC-MS workflow for parallel analysis of 5′ cap and 3′ poly(A) tail. There are four key steps; 1) Complete digestion of mRNA with RNase T1 yields 5′ capped and uncapped oligonucleotides in addition to fragments containing the poly(A) tail. 2) IP-RP HPLC separations are interfaced with LC-MS and LC-MS/MS. 3) 5′ capping efficiency is determined based on the abundance of the 5′ capped and uncapped fragments 4) 3′ poly(A) tail analysis is performed using intact mass deconvolution. An example is shown from a typical poly(A) tail, where each of the individual masses identified correspond to an additional A residue. Created in BioRender. Dickman, M. (2024) https://BioRender.com/f93c835.

precursor fragments (pppG, ppG, GpppG, and m7GpppG, or Cap 0). The method employed IP-RP HPLC separation using DIPEA/HFIP buffers and a novel hybrid surface technology BEH Amide C18 LC column. The method was demonstrated as suitable for general application to 5′ capping analysis of Cap 1 mRNA vaccines [90].

4.4. DNA probe directed cleavage using RNases

Directing site specific cleavage can be simplified by replacing the DNA/RNA hybrid with DNA probes. Use of DNA primers (probes) protects sites from ribonuclease cleavage, and thus directs cleavage downstream of the probe-hybridized region, generating fragments of a target size range. The first use of this approach employed 5′ end probes to direct RNase T1 cleavage, to enable compatibility with polyacrylamide gel electrophoresis analysis [91]. While this method was proposed as an affordable alternative to 5′ capping assessment by mass spectrometry, it can be utilised in conjunction with LC-MS as demonstrated by Wolf et al., [92]. In this study DNA probes were designed to direct cleavage by RNase T1, RNase MC1 and hRNAse 4. Conjugation of the DNA probe to either biotin or desthiobiotin at the 5′ or 3′ end enabled affinity purification of hybridized fragments by streptavidin prior to LC–MS/MS analysis. The finding that hRNase 4 reduced cleavage product heterogeneity, independent of the identity of the DNA probe, is of particular interest, since this use of hRNase 4 is complementary to a method from the same group evaluating hRNase 4 for identity analysis, including sequence mapping and 5′ cap identification [93]. The inclusion of 3′ end-repair enzyme, in this case, T4 PNK, in combination with hRNase 4 generates oligonucleotides with dephosphorylated 3′ termini, thereby increasing confidence in the assignment of 5′ modifications to isobaric fragments, such as 5′-pppN vs 5′-ppNp. The use of DNA probe directed cleavage represents a simplified, versatile workflow [92].

4.5. Ribozyme directed cleavage

Generation of predefined short fragments from the 5′ end by trans-acting ribozymes has been shown to be an effective strategy. Proof of concept was exemplified by use of hammerhead ribozymes: small catalytic RNA capable of endonucleolytic (self-) cleavage [94]. A set of hammerhead ribozyme assays were designed for specific cleavage of mRNA at a unique position to generate 5′-end capped and the

corresponding uncapped cleavage products by cleavage in a structurally accessible region between position 10 and 30 nt from the 5′-end of the mRNA. The use of silica-based purification of the 5′ cleavage fragments was beneficial as it reduced mRNA input amount. The method was applied to calculation of 5′ capping efficiency of 5′ enzymatically capped without 2′-O-methylation (Cap 0), enzymatically capped and 2′-O-methylated (Cap 1) or ARCA co-transcriptionally capped (Cap 1). IP-RP HPLC was performed using a C18 column with TEA/HFIP mobile phases in conjunction with a tandem quadrupole mass spectrometer. A key benefit of the ribozyme assay is the single cleavage site and absence of non-specific cleavage products [94].

4.6. RNase T1 cleavage

Complete digestion with RNase T1, generates a 5′ terminal capped dinucleotide, typically a G terminated dinucleotide, and the corresponding 5′ uncapped species: uncapped diphosphate, triphosphate, or unmethylated fragments (NpppG). High resolution accurate mass (HRAM) approaches allow unambiguous detection of trace-level uncapped species (5′ppp-AG) relative to capped form (5′ cap-AG] for determination of 5′ capping efficiency. This was demonstrated for the Pfizer-BioNTech COVID-19 vaccine (BNT162b2) and variant constructs Original, Delta, and Omicron. This analysis employed a C18 column, TEA/HFIP buffers and an Orbitrap Tribrid mass spectrometer [95].

Combination of RNase T1 digest with stable isotope labelling of a reference mRNA standard, a variant of Stable Isotope-Labeled riboNucleic Acid as an internal Standard (SILNAS), enables parallel analysis of 5′ cap, coding sequence and poly(A) tail [96]. A stable isotope labelled standard (sRNA) was generated by IVT using “heavy” GTP ($^{13}C_{10}$) to generate uncapped mRNA. The test 5′ capped mRNA (p-RNA) was synthesised using the “light” GTP in the IVT reaction. The test p-RNA and reference s-RNA were mixed 1:1 prior to RNase T1 digestion and IP-RP LC-MS analysis. Quantification was achieved by comparison of the stable isotope-labelled (uncapped) reference standard, with an identical sequence to the target RNA. 5′ capping efficiency was calculated based on the relative intensity of the 5′ triphosphorylated fragments between the s- and p-RNAs, i.e. loss of the 5′ triphosphorylated form by 5′ capping. 5′ capping analysis requires a complete digest to generate a single, unique 5′ capped fragment, typically a G terminated dinucleotide, and the corresponding 5′ uncapped species: uncapped diphosphate, triphosphate, or unmethylated G cap (NpppG).

4.7. Nuclease P1

Methods employing nuclease P1 were first developed for the analysis of 5′ caps *in vivo*. The Cap Analysis Protocol with Minimal Analyte Processing (CAP-MAP) [97] and the related CAP-Quant method have been developed [98,99]. Nuclease P1 digestion generated intact 5′ cap structures and nucleoside monophosphates (NMPs), which were resolved by LC followed by triple quadrupole (QqQ) mass spectrometry analysis in conjunction with Multiple Reaction Monitoring (MRM). The MRM method is directed to monitoring of predefined precursor masses and their fragment ions. The method was thus targeted to detection and quantification of selected 5′ cap species. Method development was based on screening of specified precursor *m/z* values for precursor → product ion transitions to identify transitions that, as far as possible, were unique to each species of interest. The comprehensive lists of mass transitions and optimised collision energies derived from these foundational studies serve as a vital resource, enhancing the application of this method for assessing 5′ capping efficiency as a CQA of synthetic mRNA. Muthmann et al., developed an MRM based protocol complete with a detailed workflow from *in vitro* mRNA production and enzymatic modification to LC-MS/MS analysis [100]. The study analysed addition of 5′ GpppG and the transfer of methyl groups from S-Adenosyl-L-Methionine (AdoMet) to form m7GpppG (Cap 0), specifically N7-methylation of GpppG-FLuc-RNA. It was noted that quantification of m7GpppG relative to GpppG is more accurate than m7 G against G, since G is highly abundant and present in the coding sequence at multiple sites in the transcript. m^7GpppA_mG addition was also monitored in this study indicating applicability of the method to the analysis of synthetic mRNA.

Nuclease P1 digestion can either be used alone to generate intact 5′ cap structures and NMPs or combined with additional enzymatic steps to generate the corresponding nucleosides [100,101]. The use of additional processing to nucleosides can only be applied for 5′ cap quantification if the derived cap nucleoside is unique in terms of mass relative to internal sequence derived nucleosides [101]. Both methods utilised C18 columns in conjunction with ammonium acetate buffer (pH) 6 with a gradient elution formed with acetonitrile to separate the NMPs/nucleosides by LC prior to mass spectrometry analysis on a triple quadrupole instrument [100,101]. The inclusion of a nuclease P1 cleavage step, post RNase T1 digest, forms part of a proposed Multi Attribute Monitoring method for 5′ cap analysis of CleanCap AG mRNA [102]. The SILNAS approach of Nakayama et al., for 5′ capping analysis can also be applied to nuclease P1 digest fragments for quantification of 5′ capping efficiency [96].

4.8. 5′ cap orientation

The forward and reverse orientations of the 5′ cap are isobaric and co-elute in LC-MS [85]. An example is m7 G first generation dinucleotide cap analogues that can be incorporated in the reverse orientation, i.e. GpppGm7 rather than m7GpppG. Selective cleavage of the 5′ cap can be used to confirm the correct orientation of the 5′ cap and quantify the reverse orientation. 5′ RNA pyrophosphohydrolase generates pGp if the cap is in the forward/correct orientation or pm7Gp if in the reverse orientation, these characteristic species can be resolved by LC-MS [85]. The development of second generation Anti-Reverse Cap Analogue (ARCA) [103], plus third generation reagents that generate Cap 1 structures that mimic natural structures, minimises the requirement to monitor correct orientation [104]. LC-MS methods have also been used to verify the mass and structures of novel capping reagents, such as the light-sensitive FlashCap, for photoreactive activation of mRNA translation *in vitro* [105]. These methods also have value in the assessment of structure and homogeneity of the 5′ cap using di- and trinucleotide reagents for co-transcriptional capping [106,107].

5′ cap chemistries have proven potential for mechanistic analysis of cellular proteins interacting with exogenous therapeutic mRNAs. Current mRNA vaccines utilise Cap 1 structures since these discriminate the mRNA as "non-self". However, recent publications demonstrate that Cap 2 structures reduce activation of the innate immune response *in vivo* [108] and enhance translation efficiency relative to Cap 1. Development of novel third generation 5′ caps is occurring at a rapid pace including development of Cap 2 reagents [107,109,110].

5. Direct mRNA sequence mapping using LC-MS/MS

mRNA identity is a CQA of mRNA vaccines and therapeutics as it is inherent to drug efficacy. Determining the mRNA sequence, and thus confirming that the target drug has been manufactured correctly, must be completed during the development and production of mRNA-based drugs. One method that has emerged as a highly productive alternative to the more conventional Sanger or next-generation sequencing methods is direct mRNA sequence mapping by LC-MS/MS. This bottom-up sequencing method, as outlined in Fig. 6, utilises endonuclease digests in conjunction with powerful mass spectrometry-based analysis to enable the rapid identification and characterisation of large mRNA constructs. Mass spectrometry analysis is performed using instruments with the capability for accurate mass analysis as well as MS/MS, which is used to effectively sequence and identify oligoribonucleotides. This sequencing method is a direct approach, providing an unbiased and accurate evaluation of the mRNA primary sequence and its modifications without the need for conversion to cDNA or amplification. Therefore, it can be employed as an orthogonal method to Sanger or

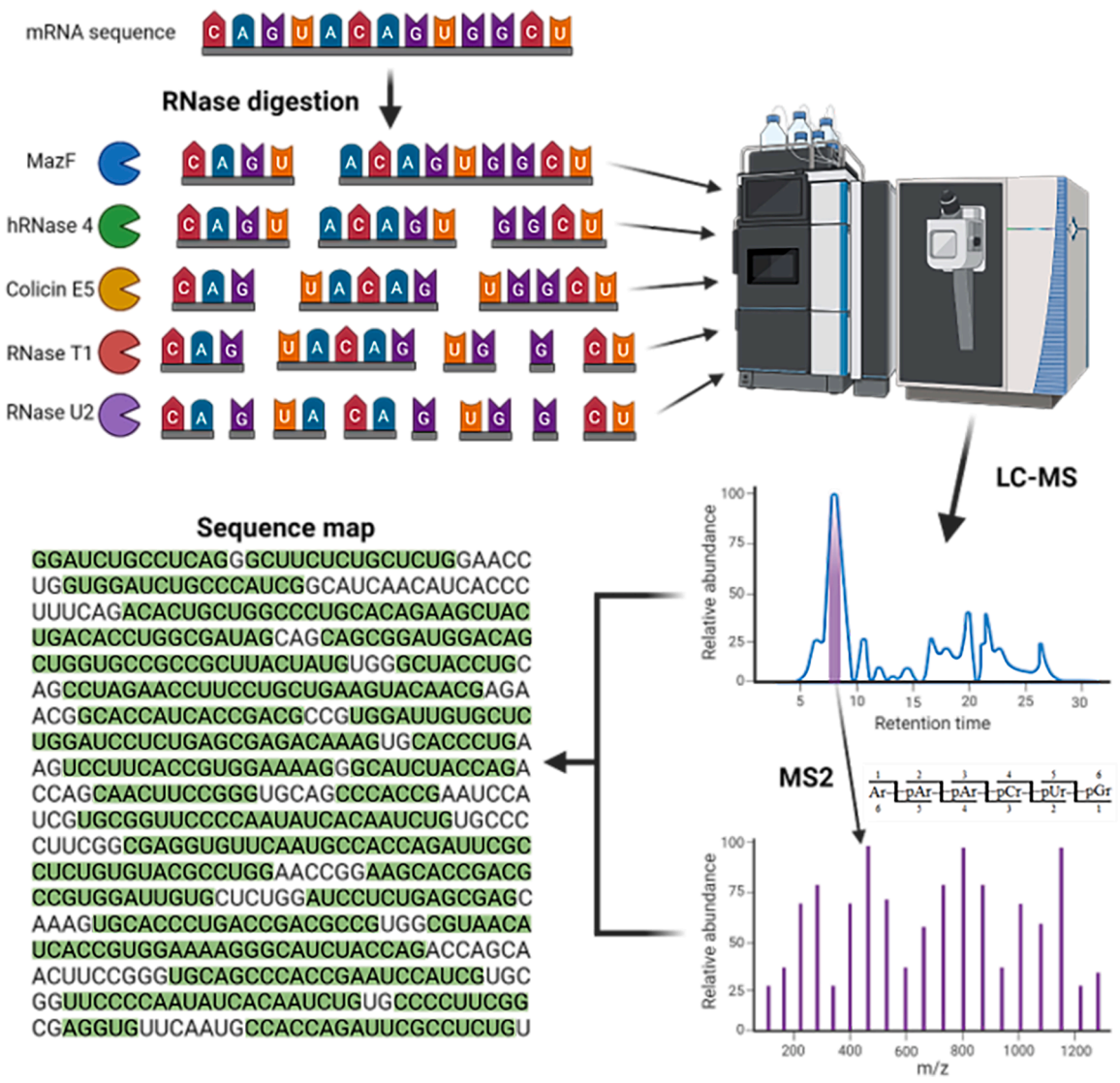

Fig. 6. Schematic representation of direct mRNA sequencing using LC MS/MS workflows. RNase(s) are used to digest the mRNA into smaller oligoribonucleotide fragments. These fragments are separated using IP-RP HPLC interfaced with high resolution tandem mass spectrometry (LC-MS/MS). Data analysis and visualisation tools are then employed to match experimentally identified oligoribonucleotides with the theoretical mRNA sequence. Created in BioRender. Dickman, M. (2024) https://BioRender.com/c73t543.

next-generation sequencing, as well as providing high-throughput sequence identification and sensitive impurity detection. To achieve efficient and effective sequence mapping via LC-MS/MS, it is necessary for the mRNA of interest to be cleaved in a way such that the resulting oligoribonucleotides are of both the ideal length for LC-MS/MS analysis (<50 nt) and are unique sequences that map only once to the mRNA sequence. This poses a challenge for designing direct sequencing workflows, which has been addressed through the use of parallel RNase digests, partial RNase digests and the use of lower frequency enzymes.

LC-MS/MS was first implemented for mRNA sequence mapping using parallel multiple endonucleases prior to LC-MS/MS analysis [111]. The endonucleases employed in this study were RNase T1 (cleavage 3′ to G), colicin E5 (major cleavage within GU) and interferase MazF (major cleavage 5′ to ACA). Chromatographic separation of digestion products was performed via IP-RP HPLC, using an ACQUITY BEH C18 column in conjunction with diisopropylethylamine (DIEA)/HFIP mobile phases. MS/MS analysis was performed with a quadrupole time-of-flight instrument. Due to their variation in cleavage sites, and thus frequencies, the parallel endonuclease digests provided complementary sequence coverages and enabled mapping of large mRNAs to >70% sequence coverage. This led to positive identification of a single correct sequence from hundreds of similarly sized ones. In addition, Jiang et al., demonstrated the ability to detect minor sequence impurities with a sensitivity of <1%.

Further research focussed on simplifying the digestion element of the workflow, without compromising sequence coverage, through the use of single, controlled RNase T1 digests [112]. This led to the development of a more automated, high-throughput workflow for the rapid characterisation of large mRNAs. Here, immobilisation of the RNase T1 on magnetic beads allowed for programmed, partial digestion of mRNA, which consistently generated oligoribonucleotide fragments that were of an ideal size for LC-MS/MS analysis and uniquely mapped onto the target mRNA sequences. Similarly to Jiang et al., [111] and Gau et al., [95], IP-RP HPLC was used for chromatographic separation. In this case, a DNAPac RP column and TEA/HFIP mobile phases were used, in conjunction with a quadrupole-Orbitrap mass spectrometer for high resolution MS/MS analysis. This method demonstrated high sequence coverage (>80%) for a range of large RNAs and mRNA therapeutics, alongside low or no matching against random control RNA sequences. Additionally, automation of data analysis (using BioPharma Finder)

enabled rapid verification of the long RNA sequences from complex LC-MS/MS data sets. The entire workflow, from mRNA to generating the mRNA sequence map and mRNA sequence validation was performed in <90 mins. The relatively high sequence coverages, through unique identifications, in conjunction with the elements of workflow automation, make this method more appropriate for mRNA sequencing in quality control testing.

Direct mRNA sequence mapping using LC-MS/MS was implemented for the identity testing of Comirnaty (Pfizer/BioNTech) [95]. In this work, mRNA was fully digested by RNase T1. Separation was performed by IP-RP HPLC using an ACQUITY BEH C18 column, with TEA/HFIP buffers. MS/MS analysis was performed using an Orbitrap-tribrid instrument. The use of a complete T1 digest for the preparation of the mRNA sample prior to LC-MS/MS analysis is simple, however, due to the frequency of the cleavage of RNase T1, this method produces an abundance of non-unique mRNA fragments. In this study 55.5% sequence coverage from unique fragments was obtained and demonstrated highly reproducible and completely annotated UV chromatograms from the RNase T1 digest. However, the sequence coverage based on unique fragments was lower than that obtained using parallel endonuclease digests [111] and partial RNase T1 digests [112]. The simplicity of this workflow would make it appropriate for incorporation into quality control analysis of mRNA manufacturing.

An alternative enzyme was explored by Wolf et al., in 2022: human RNase 4 was demonstrated as a more optimal enzyme for the digestion of mRNA in a LC-MS/MS sequence mapping workflow [93]. Due to its cleavage specificity (downstream of U, upstream of purines), RNase 4 produces a large population of cleavage products that are both uniquely mappable and of the optimal length for LC-MS/MS analysis. In this work, a similar method to Vanhinsbergh et al., [112], was employed for LC-MS analysis: IP-RP HPLC with a DNAPac RP column, in this case with DIEA/HFIP buffers, followed by a quadrupole-Orbitrap instrument for MS/MS analysis. It was shown that they could achieve similar sequence coverage to the combination of RNase T1, colicin E5 and mazF used by Jiang et al., [111] with RNase 4 alone. Furthermore, despite the cleavage specificity, the incorporation of specific modified uridine nucleotides did not affect the efficacy of the method. It was suggested that further enzyme screening could be beneficial for the development of LC-MS/MS based mRNA sequencing, as this could yield novel endonucleases with similarly useful cleavage specificities, as well as those that might operate preferentially at high temperatures, thus facilitating denaturing of the RNA structure.

In a novel approach to improving mRNA sequence mapping by LC-MS/MS, Goyon et al., recently developed a method for online RNase digests for a more streamlined and efficient platform [113]. They implemented immobilised RNase cartridges in conjunction with two-dimensional LC (2D-LC) and MS/MS for automated digestion-analysis of mRNA. This system consisted of parallel RNase digests (via the RNase cartridges) on the first dimension and chromatographic separation of the digest products on the second dimension. Hydrophilic interaction liquid chromatography (HILIC) was employed for the chromatography using ammonium acetate mobile phases, as previously developed by this group for sequence mapping of single guide RNA [114]. Using this method, sequence coverages of 5.8–51.5% and 3.5–19.3% from unique digestion products of five model mRNAs (996–4521 nt) were achieved using RNase T1 and RNase A respectively. These results are comparable to previous conventional nucleotide mapping completed by Jiang et al., [111] however, the method by Goyon et al., reduced the digest time required and increased signal intensity by >10x. This demonstrates the online RNase digest workflow as a more efficient method for LC-MS sequence mapping of mRNAs; the platform outlined by Goyon et al., has the potential to reduce time, labour and resources, whilst improving sequence coverage through further optimisation.

LC-MS sequence mapping employing stable isotope-dilution LC-MS has also been developed for the analysis of mRNA [96]. This type of method had previously been established for the analysis of post-transcriptional RNA modifications in ribosomal RNA [115]. Nakayama et al., used RNA standards that had identical sequences to that of the target product, but were transcribed using "heavy" GTP ($^{13}C_{10}$) and combined in an equimolar mixture with the target. The workflow comprises a full RNase T1 or A digest of the RNA, followed by IP-RP HPLC of the digestion products using an in-house packed C30-UG column and TEAA buffers, completed with MS/MS analysis via a quadrupole-Orbitrap instrument. Through automated database processing with Ariadne software, this method allowed for the quantification of long, modified mRNAs (200–4300 nt), achieving high sequence coverage and the identification of sequence defects. This study additionally used this workflow to analyse 5′ capping and characterise the poly(A) tails [96] (see sections 3.6 and 5.1), which opens up the potential for combining multiple quality control tests in one.

Looking forward, there are significant challenges and opportunities for direct mRNA sequence mapping in conjunction with LC-MS/MS. The further development and application of additional enzymes with complementary cleavage specificity that can provide higher sequence coverage than RNase T1 or A is required. The application of lower frequency enzymes, such as interferase MazF, that offer a much higher level of cleavage specificity come with the caveat of the production of digest fragments that are not amenable for sequence identification based on their MS/MS spectra. Human RNase 4 appears to offer a compromise, generating mRNA fragments that are in a more optimal size range for satisfying both LC-MS/MS analysis and unique identity.

In addition, further developments and applications of alternative modes of fragmentation to HCD/CID for sequencing oligoribonucleotides generated from RNase digests will enable confident sequence identification of larger oligoribonucleotides (>50 nt) and therefore provide additional sequence coverage. Achieving high sequence coverage using direct sequence mapping of large mRNAs such as saRNAs is challenging. Combining multiple RNase digests and partial RNase digests will be required to achieve high sequence coverages of these large mRNAs. Moreover, the likely complex mixture of oligoribonucleotides generated from the analysis of large mRNAs and saRNAs will also require high resolution chromatographic separations or the application of 2D-LC methods interfaced with MS/MS. Finally, further developments in LC-MS/MS data analysis and visualisation tools in conjunction with standardised platforms and data processing methods are required to ensure accurate and reproducible direct sequencing of mRNA using LC-MS/MS.

6. 3′ poly(A) tail length and heterogeneity

Poly(A) tails can be DNA template encoded for direct synthesis during IVT or added post-transcriptionally with the use of poly(A) polymerases (PAPs) [116]. The length of the poly(A) tail affects the stability and translation efficiency of the mRNA. Assessment of the poly(A) tail involves measuring its length, which typically ranges from 100 to 150 nt, as well as evaluating its heterogeneity. In the context of 3′ heterogeneity, this variation can arise from transcriptional slippage, particularly in regions containing repeat mononucleotide sequences. This phenomenon can lead to the incorporation of additional nucleotides, resulting in heterogeneity [117]. Poly(A) tails can be intact sequences of A or segmented, i.e. "split" by spacer elements, designed to mitigate technical challenges associated with plasmid DNA encoded templates for IVT [118].

6.1. Characterisation of 3′ poly(A) tails length and heterogeneity

In common with sequence mapping analysis of mRNA, current methods are based on a ‘bottom-up’ approach. Assessment of the length of the poly(A) tail requires cleavage of the mRNA to generate fragments of appropriate size with the poly(A) sequence left intact. Fragments are typically generated based on enzymatic cleavage of mRNAs, typically by

RNase T1, which cleaves single-stranded RNA 3′ of G residues. RNase T1 can be either employed in a solution digest format [86,95,119–121] or immobilised on magnetic beads [93,112]. RNase T1 digestion does not result in cleavage of the poly(A) sequence and thus the 3′ fragments are longer than other sequences and 5′ sequences present in a sample. The poly(A) tail fragment can either be directly resolved by LC from smaller fragments [95] or by analysis post enrichment by oligo dT purification, which is biased to tails >40 nucleotides in length [119]. RNase T1 digestion must achieve completion, to ensure that each poly(A) tail species is present as a single fragment, therefore enabling accurate relative quantification. LC-MS based methods to assess poly(A) tail length and heterogeneity are based on measurement of intact mass. This requires deconvolution of complex data sets associated with multiple charged state series and spectral complexity. Analysis requires accurate mass data at single nucleotide ($n/n+1$) resolution. LC-MS methods for assessment of poly(A) tail length and heterogeneity provide valuable insights into poly(A) tail populations derived from IVT protocols [96,119–121].

IP-RP LC-MS methods were first developed by Beverly et al., to characterise IVT-synthesised mRNA of 2100 nt with poly(A) tails of 27, 64, 100, or 117 nucleotides in length [119]. RNase T1 digests were performed prior to enrichment of the poly(A) tail fragments using oligo dT-coated magnetic beads. Intact mass measurement was performed on poly(A) tails separated by IP-RP HPLC using TEA/HFIP buffers and a quadrupole-Orbitrap mass spectrometer. The key features of this method were assignment of poly(A) tail length to single nucleotide resolution even for co-eluting species, by direct measurement and assignment of intact mass. Complete chromatographic separation is not required, and is assessed by UV monitoring at 260 nm. Equivalent poly(A) tail length distributions were generated by DNA template encoded poly(A) tail using T3, T7, SP6 polymerases. Detailed evaluation of a range of poly(A) tail lengths was achieved by reference to the sequence of the DNA templates obtained by Sanger sequencing. Poly(A) tails resulting from minor variant species of template were detected [119]. The presence of longer than template poly(A) tails was attributed to transcriptional slippage [117], consistent with the finding of extension via extra nucleotide addition to the 3′ end of RNA [122].

Use of LC-MS employing RNAse T1 digestion has been applied to poly(A) tail analysis of the Pfizer-BioNTech's (BNT162b2) Covid-19 mRNA vaccine [53,95]. This approach also forms the basis of a proposed Multi Attribute Monitoring method for 5′ cap and poly(A) tail workflow [102]. An additional parameter, the integrity of poly(A) tails, was also analysed via similar workflows using the SILNAS approach. This method involves comparing a stable isotope-labelled reference mRNA with an identical sequence of the target mRNA medicine [96]. In this workflow the mRNA was engineered such that a guanosine was present on the 3′ side of the poly(A) sequence.

In addition to the LC-MS methods described above, LC-UV and similar methods, offer an alternative approach for laboratories without access to MS instrumentation for the analysis of 3′ poly(A) tails. However, the ability to achieve baseline resolution and determine the accurate size of the poly(A) tails is challenging using LC-UV approaches. SEC has been developed for the analysis of the 3′ poly(A) tail length and heterogeneity, and compared to IP-RP HPLC methods, using RNase T1 digests of mRNA [120]. SEC columns with a range of pore sizes (125 Å, 250 Å and 400 Å) were evaluated with optimum resolution of typical poly(A) tails achieved on the 250 Å and 400 Å columns. The SEC mobile phase consisted of aqueous 0.1 M phosphate buffer, pH 8. Accurate sizing required use of RNA standards (rA) due to altered retention time relative to DNA standards (dA). IP-RP HPLC with UV detection was also performed using octylammonium acetate (OAA) mobile phases. For both methods, sizing was performed based on size calibration using rA standards [120]. Assignments of poly(A) tail lengths by UV based measurement were verified using IP-RP LC-MS. The development of alternative SEC and IP-RP HPLC UV methods are attractive for checking process performance of a pre-defined mRNA, since it is easier to implement compared to LC-MS for routine analysis. It does however require prior knowledge of the mRNA sequence and sequence of the RNase T1 fragment encoding the poly(A) tail.

6.2. Analysis of chemically modified poly(A) tails

Chemical modification of 3′ poly(A) tails provides a strategy to reduce or prevent their degradation [123–125]. LC-MS/MS based methods have also been used to monitor incorporation of phosphodiester modifications in mRNA poly(A) tails to provide further insights into structure-function relationships. The methods were developed to simultaneously quantify the number of modified phosphodiester bonds and measurement of the average poly(A) tail lengths [123].

The incorporation of phosphate-modified ATP analogues (ATPαS and ATPαBH_3) were used to generate phosphorothioate and boranophosphate modified poly(A) tails respectively [123]. A two-enzyme strategy was optimised using snake venom phosphodiesterase and human CNOT7 deadenylase to generate 5′-mononucleotides (NMP and phosphate-modified analogues) from both modified and unmodified mRNA. A key factor during method optimisation was the adjustment of degradation conditions to ensure complete RNA degradation without desulfurisation of AMPS to AMP. IP-RP HPLC separations were performed on an Eclipse XDB-C18 column using N,N-dimethylhexylamine as a mobile phase. A MRM based method on unique precursor to product mass transitions was performed using a QTRAP 3200 triple quadrupole/linear ion trap mass spectrometer to quantify AMPS and selected NMPs of interest. Synthetic phosphate (^{18}O) labelled isotopologues were included as internal standards for accurate quantification. The average poly(A) tail length was calculated using concentrations of unmodified and modified AMP since the mRNA sequence was engineered to only include A in the poly(A) tail sequence [123].

Click chemistry approaches for "modular" addition of chemical modifications to the 3′ poly(A) tail, including the addition of chemically modified synthetic oligonucleotides [124] or trimers of modified poly(A) tail via a branched topology, have been developed [125]. Therefore, methods for 3′ poly(A) tail analysis will need to be adapted or methods developed to characterise these novel structures.

7. Conclusions

mRNA vaccines/therapeutics have emerged as a powerful new class of medicines. However, due to the physicochemical properties of these large RNA molecules, this provides significant challenges for their analysis and characterisation. mRNA is a highly polar molecule due to its extensive negatively charged phosphodiester backbone. Its single stranded nature leads to dynamic alternative secondary structures, resulting in potential sample heterogeneity. Moreover, mRNA manufactured using IVT often contains impurities, many of which are similar in size and nature to the full-length mRNA, making separation and characterisation difficult. Furthermore, CQAs, such as the 5′ cap and 3′ poly(A) tail, must also be extensively characterised to ensure the safety and efficacy of mRNA vaccines/therapeutics.

Liquid chromatography-based methods interfaced with UV and mass spectrometry are powerful tools for the analysis and characterisation of mRNA vaccines and therapeutics. A wide range of modes of liquid chromatography have been developed and utilised to characterise mRNA with recent work focussing on the development and application of novel stationary phases and mobile phases to improve chromatographic resolution. These methods have provided further insight into the purity and impurities present that are generated from the manufacturing of mRNA. Furthermore, novel LC-MS/MS methods have been developed and applied to characterise important CQAs of the mRNA including 5′ capping efficiency, mRNA identity (via direct mRNA sequencing), and the 3′ poly(A) tail length and heterogeneity. The majority of these LC-MS methods focus on "bottom-up" approaches whereby the large mRNA is digested into smaller oligoribonucleotides prior to LC-MS and MS/MS

analysis to measure the 5′ capping efficiency, 3′ poly(A) tail length and heterogeneity, and direct mRNA sequencing (identity). LC-MS methods for intact mass analysis of mRNAs is challenging due to their large size and heterogeneity. However, the potential for alternative specialist methods such as CDMS may overcome some of the current limitations, enabling intact mass analysis of large mRNAs. Looking forward, further improvements in speed, resolution, sensitivity and selectivity of the current analytical methods, combined with the emergence of new analytical methods such as mass photometry will provide important new tools to analyse mRNA and provide further insight and understanding of mRNA CQAs.

CRediT authorship contribution statement

Alexandra L.J. Webb: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Emma N. Welbourne:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Caroline A. Evans:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Mark J. Dickman:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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