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## Review

## Challenges and emerging trends in liquid chromatography-based analyses of mRNA pharmaceuticals

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## ABSTRACT

Lipid encapsulated messenger RNA (LNP mRNA) has garnered a significant amount of interest from the pharmaceutical industry and general public alike. This attention has been catalyzed by the clinical success of LNP mRNA for SARS-CoV-2 vaccination as well as future promises that might be fulfilled by the biotechnology pipeline, such as the in vivo delivery of a CRISPR/Cas9 complex that can edit patient cells to reduce levels of low-density lipoprotein. LNP mRNAs are comprised of various chemically diverse molecules brought together in a sophisticated intermolecular complex. This can make it challenging to achieve thorough analytical characterization. Nevertheless, liquid chromatography is becoming an increasingly relied upon technique for LNP mRNA analyses. Although there have been significant advances in all types of LNP mRNA analyses, this review focuses on recent developments and the possibilities of applying anion exchange (AEX) and ion pairing reversed phase (IP-RP) liquid chromatography for intact mRNAs as well as techniques for oligo mapping analysis, 5' endcap testing and lipid compositional assays.

## 1. Introduction

Synthetic nucleic acid is providing new forms of vaccination and new means of modulating protein expression. Most recently, it has been in vitro transcribed mRNA that has garnered widespread attention [1,2]. An mRNA drug substance is a marvel of recent breakthroughs in biotechnology. mRNA can be produced by cell free synthesis, made to contain novel nucleobases, and still be manufactured to contain important post transcriptional modifications. To be properly recognized by a human ribosome, mRNA must be endcapped on its 5'-end with a triphosphate bridged guanosine-based moiety and modified on its 3'-end to contain a poly adenosine tail. Moreover, Karikó and Weismann have found it to be highly advantageous for mRNA pharmaceuticals to contain pseudouridine as it can increase the resulting molecule's biological stability and significantly increase levels of in vivo translation [3]. These and other structural features of an mRNA molecule are shown in Fig. 1. Another major breakthrough that has helped unlock the potential of these molecules has been the formulation of lipid components to effectively encapsulate and thereby provide a vehicle for their cellular

endocytosis [4–7]. Cationic lipids are at the heart of this technology, and they serve as ion pairs and counter ions to the negatively charged backbones of the nucleic acid therapeutics. With these stabilizing intermolecular interactions, the nucleic acids adopt structures that can be further built upon with cholesterol and zwitterionic phosphatidyl choline lipids and ultimately some amphipathic hydrophilic counterparts to yield solid lipid nanoparticles and corresponding drug product. Various lipid molecules, both naturally occurring and man-made, are being explored to achieve more ideal structure-function and stability properties. As one might predict, the stoichiometries of each one of the drug product components in an LNP mRNA must be robustly optimized and carefully measured. A schematic representation of a four-component lipid nanoparticle is provided in Fig. 2.

The use of LNP mRNA has proven to be highly effective for vaccination against SARS-CoV-2, but therapeutic applications expand well beyond just vaccines [8]. In another promising application, Verve Therapeutics is exploring the use of LNP mRNA to deliver a CRISPR/Cas9 complex to patient cells that will then edit a protein in the liver and correspondingly reduce levels of low-density lipoprotein [9]. Given that

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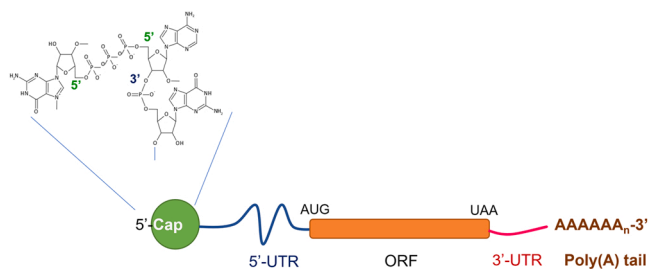
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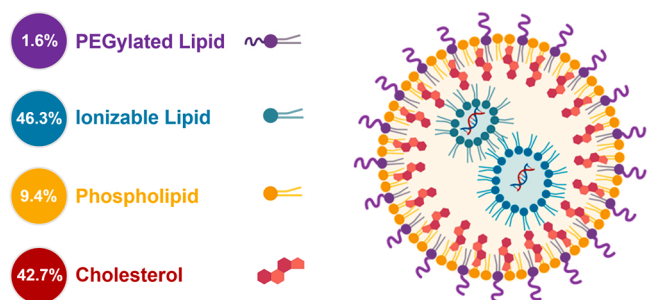
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**Fig. 1.** Anatomy of mRNA: A typical mRNA therapeutic contains 5'-cap, open reading frame (ORF) flanked by untranslated regions (UTR at 5'- and 3'- side), and a poly(A) tail. The 5'-cap contains 7-methylguanosine attached to 2'-O-methyladenosine through a 5'->5' linkage as shown in the inset in contrast to the rest of the nucleotide building blocks, which are linked in a 5'->3' fashion. The open reading frame contains the nucleotide sequence flanked by start codon (AUG) and stop codon (UAA or UAG or UGA).



**Fig. 2.** Anatomy of a lipid nanoparticle (LNP). Lipids are color coded and schematically represented in an illustration of an LNP. Molar percent lipid composition values are provided for the Comirnaty™ vaccine. Double stranded nucleic acid is shown as the encapsulated drug substance. Created with Bio-Render.com.

an LNP encapsulated mRNA drug is comprised of various chemically diverse molecules brought together in a sophisticated intermolecular complex, there is a major analytical burden on developers to demonstrate that drug substances and products are pure, stable, safe, effective, robustly designed and reproducibly manufactured. Considerations for mRNA testing are quite different than those previously made for other nucleic acid therapeutics, too. Different than chemically synthesized oligonucleotides, the impurity profile of *in vitro* transcribed mRNA can include nucleotide triphosphates, DNA plasmids and their residual components, as well as various enzymes [10]. To this end, much like liquid chromatography (LC) has been a workhorse for the analytical testing of protein therapeutics, it is also becoming a relied upon technique for LNP mRNA analyses.

In one case, LC techniques can be applied to analyze the intact mRNA drug substance. Separations by anion exchange (AEX), ion-pair reversed phase (IP-RP) or size exclusion (SEC) chromatography are typically applied and are used to detect heterogeneity or check for molecular integrity. AEX and SEC can also be applied to the LNP encapsulated mRNA. With nuclease digested nucleic acid, it is possible to map the digested components while confirming sequence information, detecting post/co-transcriptional modifications, and investigating nucleobase modifications. It has also become common practice to test lipid and nucleotide raw materials by LC to check for impurities prior to their use in manufacturing. In addition, it has become important to study all of these molecular components even after they have become part of the LNP mRNA drug. Lipids can be dissolved from an LNP using a solvent based extraction so that they can then be individually analyzed by reversed phase chromatography. In addition to LC, capillary electrophoresis is also well established for assessing mRNA purity and integrity [11,12].

Though there have been a considerable number of developments in the analysis of LNP mRNA, this review focuses on AEX and IP-RP liquid chromatography for intact mRNAs, oligo mapping analysis, 5' endcap testing and lipid compositional assays. With new insights emerging from SEC, supercritical fluid chromatography (SFC) and hydrophilic interaction chromatography (HILIC), we are sure more topics will come to be covered in future reviews.

## 2. Analysis of intact mRNAs by anion exchange chromatography (AEX)

### 2.1. Introduction; non-denaturing and denaturing AEX

In most instances, oligonucleotide purifications have been performed with either AEX or IP-RP chromatography [13]. Due to the phosphate groups in their sugar-phosphate backbone, nucleic acids are negatively charged ( $pK_a1 = 2.14$ ). Accordingly, AEX would seem perfectly suited to separating a sample comprising a nucleic acid, such as mRNA. Indeed, AEX has been regularly used to purify mRNA [14]. However, it is interesting that only a few example analytical separations have been published. It is our belief that if methods were to be improved (e.g. by using low adsorption column hardware and chromatographic systems, and applying new elution modes such as "ion-pairing AEX"), there might be potential for AEX to become a common analytical approach for intact RNA samples. AEX can be applied with less denaturing conditions than IP-RP, and it can thus preserve mRNA structure and potentially be used to analyze their intact native heterogeneity.

Elution order and selectivity in AEX depends mostly on the size of the nucleic acid molecule. With each nucleotide added to a growing chain, a nucleic acid takes on an extra negative charge. As its size increases so does its overall charge, which directly contributes to increased AEX retention [13]. A full-length *in vitro* transcription product thus elutes as a more strongly retained peak while shorter chain lengths are expected to elute with earlier retention times. This elution behavior has been observed in several publications concerning separations of synthetic DNA, RNA, natural products, and supercoiled versus linear plasmids [15–17]. Most published reports on oligonucleotide AEX have seemed to show sharp peaks only for 25 nt or shorter analytes. Larger molecules have often been seen to elute as broad peaks [17,18]. To date, one of the highest resolution AEX separations to have been reported was accomplished with a pellicular particle stationary phase and either a neutral pH sodium chloride or sodium perchlorate salt gradient combined with a 60 °C separation, or with a high pH (pH = 12) salt gradient separation performed at 10 °C [19]. These conditions may not be ideal for preserving mRNA and optimally profiling their intact heterogeneity.

The main reason for applying such relatively harsh (denaturing) conditions is that longer oligoribonucleotides (e.g., > 50 nt) exhibit secondary structure due to short intramolecular interactions and a wide array of accessible base-stacked conformations [20]. Their self-structure may lead to the presence of several conformers. Denaturing conditions, like high pH, elevated temperature, and the presence of organic cosolvent, can aid a separation by disrupting base-pairing and base-stacking interactions. Under such conditions, the intact RNA can thereby be linearized such that there is a chance to separate and elute sharper chromatographic peaks. Several conditions - either alone or in combination - might lead to linearization of the mRNA molecules. Most commonly, mobile phase temperature, mobile phase pH, organic modifier or denaturing agents are used to affect the shape/conformation of nucleic acid molecules and thus tune AEX selectivity. The use of elevated temperature to produce fully or partially denaturing conditions is a common technique, and it is known that retention increases with temperature [21,22]. The separation can also be modulated by controlling the eluent pH [21,22]. However, degradation of RNA molecules may occur at elevated pH [13]. In reality, pH induced degradation is a very slow process and normally does not arise during the time scale of a chromatographic analysis [19,22]. In the range of pH 8–10.5, thymine

(T) and guanine (G) are present in ionized forms. These extra negative charges will result in increased solute retention. Due to differences in the ratio of G + T (G + U in RNA) to the total number of bases, it is often feasible to separate nucleic acids of identical lengths but different sequence compositions [13]. The number of extra charges induced by mobile phase pH increase will vary according to the G + T ratio, thus enabling an extra means of controlling chromatographic selectivity. Another effect of high pH is the elimination of inter- and intra-strand hydrogen bonding, which can typically be overcome at pH values higher than 10. High pH separations are often combined with low temperature ( $T = 10\text{--}15^\circ\text{C}$ ) conditions to maintain acceptable retention. At both high pH and high temperature, elution of mRNA species might be challenging due to undesirably strong interactions and high retention. Organic solvents such as acetonitrile or methanol in low amounts (e.g. 10–15 % in mobile phase) might also lead to partial denaturation and in some cases can be used to adjust selectivity. The addition of 5–6 M urea or 10 % formamide can also be used to perform denaturing AEX [19]. Short RNAs elute in sharp peaks in non-denaturing conditions simply because they take on more homogenous adsorption/desorption and exhibit minimal secondary structure. On the contrary, RNA molecules longer than 100 nt often elute as broad, tailing peaks in AEX if non-denaturing conditions are applied.

## 2.2. AEX method considerations

Classical methods apply a linear or multi segmented salt gradient in buffered aqueous mobile phase. Typical mobile phase buffers are based on TRIS (Tris(hydroxymethyl)aminomethane) at pH 7–9, HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 6.8–8.2, sodium carbonate at pH 10–11 or sodium phosphate at pH 11–12. Most often, the mobile phase buffer concentration is 20–30 mM. When performing traditional salt gradient separations, it is common for 1–2 M NaCl, KCl, MgCl, NaBr or NaClO<sub>4</sub> to be used as additives in the eluent mobile phase. In most cases, UV detection is applied with a wavelength set to 260 nm.

Recently, the use of an ion pairing agent and relatively mild elution conditions have been proposed as the basis for new alternative analytical methods [23,24]. It was found that a gradient of weak ion-pairing cations (e.g. tetramethylammonium chloride, TMAC) can produce intriguing AEX separations of mRNAs. This approach has been referred to as ion pairing anion exchange “IPAX” and it has appeared to provide different recovery and selectivity effects compared to classical salt gradients [23]. The concept of ion pairing in AEX can also be used in combination with a sodium chloride gradient. TMAC can be used as a mobile phase additive to form partially ion-paired mRNA molecules (thus decreasing the number of accessible charges) and the solutes can be eluted with less NaCl in the salt gradient (for a so-called ‘ion-pair mediated salt gradient’). IPAX has thus far been performed with 25 mM TRIS and HEPES buffer (pH 7.5–8) and 1–3 M TMAC gradients. In a classical salt gradient separation, relatively low temperatures ( $T = 30\text{--}40^\circ\text{C}$ ) resulted in better recoveries while in the IPAX separation, higher temperatures up to  $60^\circ\text{C}$  seemed to be more beneficial. Fig. 3 shows representative examples of optimized ion-pairing mediated salt gradient separations.

A previous piece of work reported that mRNAs in AEX follow an on-off like elution mechanism [23]. Based on two linear gradient experiments, model parameters can be derived and the mobile phase composition ( $c^*$ ) to elute mRNA with retention of  $k = 1$  can be determined. From this, one can set a symmetrical %B window around the %B composition obtained for the  $c^*$  value. With that, one can expect the solute to elute in the middle of the gradient window (symmetrical elution window scaling approach). The gradient time and elution window (symmetry range) can then be arbitrarily set such that either very fast or long separations can be designed as desired. To separate closely eluting mRNA species, it might be most useful to apply a multi-isocratic elution method [25].

In addition to the optimization of gradient steepness, the column

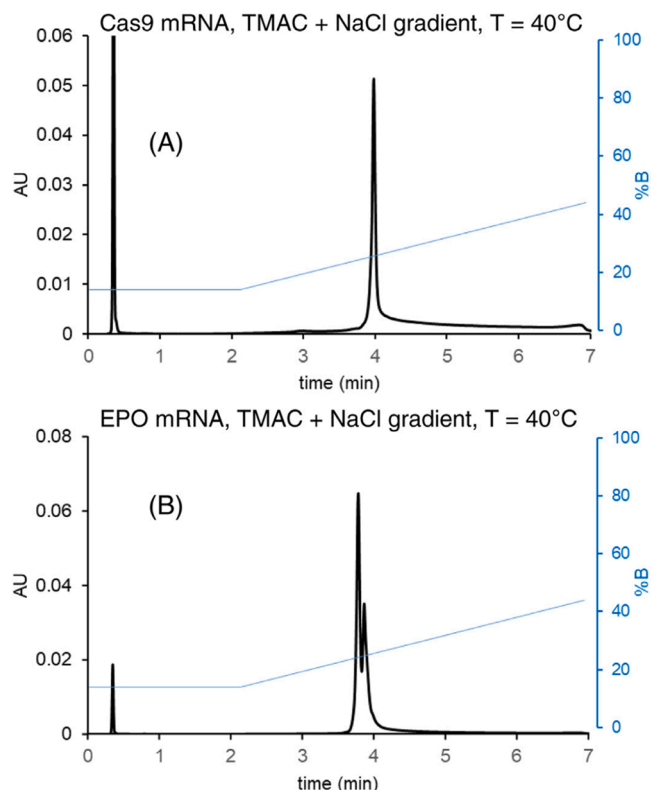


Fig. 3. Optimized separations with an ion-pairing mediated salt gradient technique. Samples: Cas9 mRNA (A) and EPO mRNA (B). (With permission from Ref [23]).

temperature has been found to be a very important factor affecting both mRNA recovery and carry-over [23,24]. Recovery as function of temperature was found to be sample and salt system dependent across a relatively broad range (e.g.  $30\text{--}70^\circ\text{C}$ ). An ion-pairing mediated salt gradient appeared to be best matched to temperatures between 30 and  $45^\circ\text{C}$ . Classical salt gradients seemed to be an entropy driven process, while the IPAX separations seemed to be more complex. It exhibits an entropy driven process at lower temperatures and then an enthalpy controlled one at higher temperatures.

Lastly, column hardware effects should be considered. Nucleic acids often suffer from poor recovery when analyzed by LC [26]. Undesirably strong adsorption can occur on the stationary phase as in the case of adsorptive interactions occurring with the surface of column hardware (e.g., frit, column inner wall). To decrease non-specific adsorption and undesired secondary interactions between column hardware and large biomolecules (e.g., mRNA), a new hydrophilically modified hybrid surface (h-HST) was recently developed for metal-based column hardware and applied to size exclusion chromatography and AEX. With h-HST, improved recovery and peak shape could be obtained in both EPO and Cas9 mRNA AEX separations [27]. This new surface incorporates additional oxygen and carbon atoms onto an ethylene bridge hybrid siloxane polymer. As a result, it exhibits reduced electrostatic properties and hydrophilicity, which in turn facilitates challenging aqueous separations. There is significant potential in the use of h-HST chromatographic hardware to facilitate more reproducible, robust, and sensitive analyses.

## 3. Analysis of intact mRNA by ion-pairing reversed-phase (IP-RP) liquid chromatography

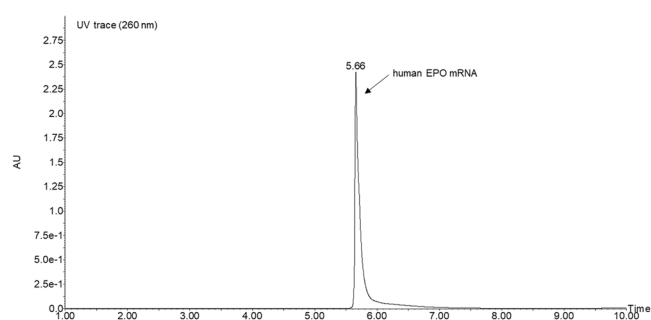
Ion-pairing reversed-phase (IP-RP) liquid chromatography is a well-established separation technique for the analysis of short synthetic oligonucleotides (20–100 nucleotides long), but its application to larger



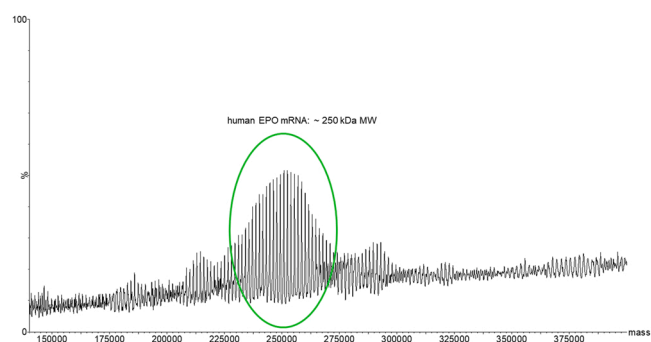
oligonucleotides, including intact RNAs, is much more limited. The first publication using commercially available HPLC columns for the separation of large RNA oligonucleotides appeared in 1982 [28]. The authors used two 10  $\mu\text{m}$  125 Å C18 particle columns connected in series to partially resolve 80-nucleotide long transfer RNA (tRNA) from rabbit globin mRNA (~650 nt). For this, an ion pairing mobile phase containing 5 mM TBA (tetrabutylammonium) phosphate was applied. Almost a decade later, a second IP-RP publication showed that methylmercuric hydroxide could be used as a denaturing mobile phase reagent to disrupt the secondary structure of mRNAs and act as an RNAase inhibitor to minimize sample degradation [29]. The ion pairing reagent used in this publication was 5 mM TBA hydroxide and separations were performed on two reversed phase columns containing particles with shorter C4 and C8 alkyl chain bondings. Further work in IP-RP turned to the use of an alkylated non-porous polystyrene-divinylbenzene (PS-DVB) stationary phase, through which it became possible to separate DNA fragments up to 500 bases [30] and then 2000 base pairs [31]. RNA separations were attempted when this column was made commercially available in 1999 [32,33]. The initial experimental conditions for the functionalized PS-DVB particle columns specified the use of 100 mM TEAA (triethylammonium acetate) as the ion pairing reagent. In the early 2000s, two publications reported intact RNA separations on the aforementioned non-porous PS-DVB column. Georgopoulos et al. used 4.6  $\times$  50 mm columns with a 100 mM TEAA mobile phase for the separation of single stranded RNA standards (ladders) and other RNAs up to 1 kb in size, and they noticed that retention times increased according to RNA size [34]. Azarani et al. used similar experimental conditions to demonstrate separations of a variety of RNA molecules including RNA ladders (200–2000 nt), RNA transcripts (750–3000 nt), a digoxigenin (DIG)-labeled RNA transcript (588-mer), and mixtures of ribosomal RNA (rRNA) and messenger RNA (mRNA) [35]. In addition, the same publication found that their IP-RP assay could be used to quantify intact RNA over two orders of magnitude (8–1000 nanograms RNA loaded on-column). These PS-DVB columns were later applied by Karikó and Weissman for the purification of in vitro transcribed mRNA [36,37]. Shortly after, in 2013, RNA separations for molecules up to 8000 nt were reported on a large pore (4000 Å) non-alkylated PS-DVB particle column [38].

In more recent years, the on-column stability of long RNA molecules has come to be questioned. Kanavarioti reported in 2018 that IP-RP was less suitable compared to AEX chromatography for purity evaluations due to incomplete linearization of the mRNA molecules [19]. However, Currie et al. proposed an IP-RP method over AEX separations for studying the degradation products of modified mRNA [39]. As one might anticipate, the value of AEX versus IP-RP continues to be investigated and contemplated.

The coupling of IP-RP separations with intact electrospray ionization mass spectrometry (ESI-MS) has received significant interest over the last two years. A step change in LC-MS compatibility was first achieved with the switch from acetic acid counter ion mobile phases to solutions containing hexafluoroisopropanol [40,41]. Schneeberger and Jiang have continued in this vein of work to expand LC-MS capabilities and have recently described the analysis of a 2000 nt intact mRNA following HILIC separations with ion-pairing reagents added just before MS detection [42]. Intact mass confirmation of several large mRNAs (~ up to 750 kDa) was performed with mass errors under 50 ppm after removal of their Poly A tails. Another example of intact mRNA LC-MS analysis was just recently presented by Brophy and co-workers, and their work is represented here again in Figs. 4 and 5 [43]. An intact human erythropoietin (EPO)-encoding mRNA (858 nt) was separated by IP-RP using a 1.8  $\mu\text{m}$  100 Å silica particle bonded with trifunctional C18. With such conditions, it is known that the mRNA is totally excluded from the intraparticle porous network. As such, high resolution separations were achieved despite the application of a conventional pore size C18 column. To ensure MS compatibility, the mobile phase used in this separation was based on diisopropylethylamine titrated with



**Fig. 4.** LC-UV chromatogram (260 nm) for the IP-RP separation of intact Tri-Link EPO (erythropoietin) mRNA. Mobile phase composition was: 8 mM DIPEA (N,N-diisopropylethylamine), 60 mM HFIP (hexafluoroisopropanol) in 18.2 MΩ water for Eluent A; and 3 mM DIPEA, 4.5 mM HFIP in 75 % ACN for Eluent B. Separation was performed on a 1.0  $\times$  100 mm ACQUITY UPLC HSS T3 C18 column, packed with 1.8  $\mu\text{m}$  particles with 100 Å pore size. The column was kept at 60 °C and operated at a flow rate of 100  $\mu\text{L}/\text{min}$  using a 5 min gradient from 10 % to 50 % Eluent B.



**Fig. 5.** Deconvoluted ESI-MS spectrum of the TriLink EPO mRNA (858 nucleotides length) recorded on a SYNAPT<sup>TM</sup> XS QTOF Instrument (Waters). Significant mRNA heterogeneity is observed due to the wide distribution of the Poly A Tail oligonucleotide region located at the 3'-end of the molecule.

hexafluoroisopropanol (HFIP). Fig. 4 displays the UV chromatograms recorded at 260 nm from this analysis, and it indicates the presence of a single mRNA species. The deconvoluted time of flight mass spectrum of the intact mRNA (including the Poly A tail) is shown in Fig. 5. Ion signal was observed at an average molecular weight of ~ 250 kDa and detected with significant heterogeneity that is thought to be attributable to the molecule's Poly A tail.

Another MS based technique has emerged that is called charge detection mass spectrometry and it seems to be better suited for the analysis of large RNA molecules (5–30 kb) [43]. However, it remains a challenge to interface the technique with on-line chromatographic separations.

In all, IP-RP has been and is likely to continue to be a valuable technique for the analysis of mRNA. Ion pairing conditions continue to be explored along with more advanced stationary phase technologies. To its credit, IP-RP has been relatively simple to couple with mass spectrometry, yet there is still promise for AEX methods to emerge with comparable MS compatibility. At the same time, new forms of mass spectrometry, like charge detection MS, are going to be needed to successfully take on the LC-MS analysis of megadalton (MDa) analytes. More progress is needed in this area of work.

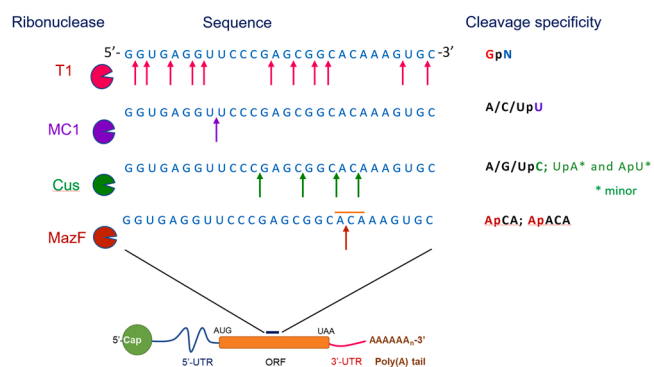
## 4. Oligo mapping with ribonuclease enzymes and fragment matching MS software

### 4.1. Oligo mapping, enzymatic cleavage

mRNA is constructed from four canonical ribonucleosides (rA, rC, rG, and rU) or from chemically modified and isomerized ribonucleosides. The integrity of mRNA is evaluated by analyzing it in its intact form as previously mentioned. Such an analysis, however, cannot reveal where modifications exist and where specific adducts might have formed during storage with the lipid molecules of the LNP [44]. Such characterization requires two types of analysis. In one case, mRNA can be completely hydrolyzed into its individual building blocks and the resulting residues can be analyzed by LC-MS to reveal the extent to which chemical modifications and adducts are present in the molecule [45]. Location specific information is lost in such an analysis. To determine where things are occurring in an mRNA sequence, a different type of approach called ‘oligo mapping’ is required. In this approach, an mRNA molecule is partially hydrolyzed into digestion products, and the obtained sample is then separated by either IP-RP or HILIC chromatography and analyzed with tandem mass spectrometry. Fragment masses and their sequences can thereby be determined. In this way, the sequence of an mRNA can be confirmed and modifications can be detected and tracked with residue specific sequence resolution. This capability is different than what is possible with other assays. For instance, new next-generation sequencing (NGS) platform technologies may also allow direct RNA sequencing [46]. MS reveals such information through mass shifts, and sequence context can be derived from gas phase fragmentation and analysis of fragment ions derived from backbone dissociation. In practice, tandem mass spectrometry and gas phase fragmentation techniques are most effectively applied to oligonucleotides species < 100 nt in size [47,48]. Therefore, intact mRNA is cleaved into smaller pieces by either enzymatic or non-enzymatic methods. Nonenzymatic methods include alkaline [49] or acid [50] hydrolysis.

By their nature, these non-enzymatic techniques lack specificity and do not yield predictable, consistent reaction products. Nucleobase-specific or sequence-specific ribonucleases, on the other hand, cleave RNA in a predictable fashion. For example, RNase T1 cleaves at the 3'-end of guanosine, and a list of theoretically expected digestion products can be predicted and matched to large RNA sequences [51,52]. An enzyme-based strategy enables sample-to-sample comparisons and more straightforward data interpretation. The usefulness of this information depends on the nucleotide composition of the mRNA. If the sequence is rich in guanines, smaller digestion products will be obtained that cannot be uniquely placed against the mRNA sequence, leading to ambiguous, unusable information. One way to mitigate this situation is to slow down the enzymatic reaction in such a way that partial or undercut digestion products are obtained. This leads to longer digestion products that can be unambiguously matched to specific mRNA sequences. Unfortunately, sample preparation reproducibility can be compromised while attempting partial digestion reactions.

Another option is to digest RNA with enzymes that exhibit complementary nucleobase specificity to generate a new pattern of digestion products (Fig. 6). Unique digestion products in combination with some areas of overlapped mapping can help build confidence in the data. This complementary enzyme approach assists in filling in sequence gaps that are generated when using a single enzyme by itself while increasing confidence in data interpretation. A uridine-specific ribonuclease like RNase 4 [53] or MC1 [54] can generate complementary sequence information to RNase T1. Similarly, a cytidine-specific enzyme, called cusativin, can be used. It cleaves RNA at the 3'-end of cytidine [55], but not sequences wherein there are tandem cytidines [56]. Further, these enzymes cleave RNA at certain dinucleotide motifs with lower efficiency despite the presence of a cytidine [57,58]. This produces skipped cleavage sites and longer digestion products unique to specific segments of mRNA to allow more confident sequence interpretations. Two other



**Fig. 6.** Nucleobase/sequence specific cleavage of mRNA by endoribonucleases. Specificity is illustrated by depicting the sites of phosphodiester bond cleavage by each endoribonuclease through an upward pointing arrow on a portion of mRNA sequence. RNase T1 cleaves RNA backbone at 3'-end of every guanosine leading to smaller digestion products. MC1 cleaves at 5' end of uridine, unless it is preceded by guanosine, making -GU- non-substrate, leading to longer digestion products. Cusativin cleaves RNA at 3'-end of cytidine; however, it does not cleave RNA when cytidine residues are present in tandem. This enzyme also cleaves UpA and ApU bonds at lower efficiency. The sequence specific MazF recognizes ACA sequence and cleaves the bond between A and C or ApA of AACA with an outcome of longer digestion products. Thus, the digestion product output of a given endoribonuclease digest depends on the nucleotide composition and sequence structure.

nucleases worth mentioning include colicin E5 [59], which cleaves the bond between G and U nucleotides, and Maz F, which is a sequence-specific mRNA interferase that cleaves at ACA sequence motifs. Both generate longer digestion products to obtain enhanced sequence coverage while complementing the T1 digestion products through parallel ribonuclease digestions and LC-MS analysis [51]. Usage of these enzyme tools through these bottom-up analytical approaches are expected to increase in the near future not only to decipher the entire sequence of biopharmaceutical mRNA [51] but also to monitor for lipid adduction [44].

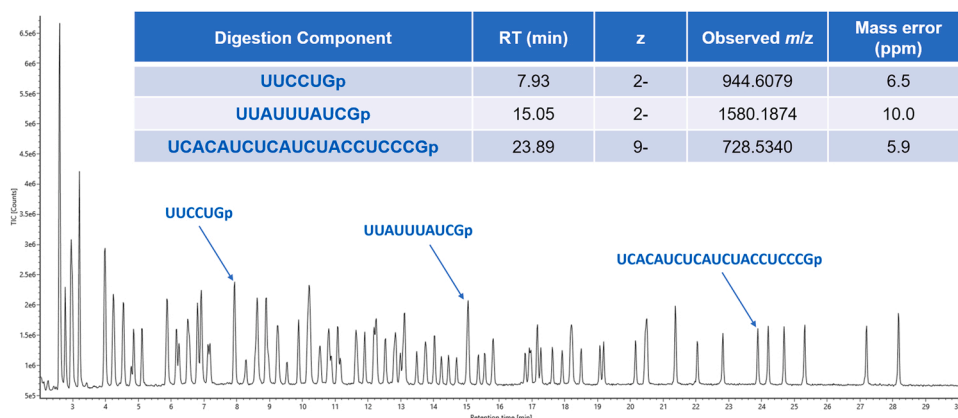
Within the oligo mapping analysis, it is also critical that oligonucleotide sequencing by MS proceed efficiently. For this, a digestion component must be efficiently dissociated across the phosphodiester backbone. Gas phase dissociation yields a particular type of information-rich product ions. When the ions share a 5' terminus, they are referred to as a-B<sub>n</sub> and c<sub>n</sub> ion series. Those sharing a 3' terminus are referred to as w<sub>n</sub> and y<sub>n</sub> ion series [60]. Computation of the mass differences in each of these ion series allows for the sequence to be interpreted from both directions (5' and 3' termini) of the digestion product. Similarly, location-specific information of a modification is indicated by the observation of certain mass shifts and where they occur in the fragment ion series. This process is extended to all of the oligonucleotide digestion products observed across the entire oligo map LC run. Matched ions and their sequences can be mapped back to the mRNA template to compute sequence coverage. The sheer complexity and number of LC-MS/MS spectra in each datafile poses a significant challenge to high throughput data processing.

### 4.2. Software tools

Software solutions for this task have been developed starting with simple oligonucleotide sequencer (SOS) [61] and its ab initio sequencing of individual oligonucleotides. Subsequently, several more algorithms have been introduced, including a web-based computational platform built with a database search engine called 'Ariadne' [62]. Another piece of software was also made that provides a platform-independent oligonucleotide mass assembler (OMA) and oligonucleotide peak analyzer (OPA) tools that calculate the theoretical fragment ions of an input sequence and annotates them onto an experimental spectrum [63].

Automated *de novo* sequencing of modified nucleic acids has even been made possible with the introduction of Robooligo software [64]. Furthermore, data interpretation for modified RNA analysis has received attention. RNAModMapper is software that has proven to be beneficial in its ability to interpret and annotate LC-MS/MS data sets of modified RNA using sequence database and genome searches [65,66]. With this software, user-defined scoring thresholds can be also applied to ensure high confidence data interpretation. Even newer software tools have been published on in the last several years. NucleicAcidSearchEngine (NASE) was developed and introduced in 2020 [67]. This software provides a scalable database-matching tool that processes tandem MS/MS spectra within the OpenMS framework, and it features false discovery rate (FDR) estimation, precursor mass correction and salt adduct support. Even more recently, another program known as Pytheas was introduced that uses an empirical scoring function to match theoretical spectra generated from *in silico* digestion of target RNA with the experimental MS/MS data [68]. It also computes false discovery rates based on target-decoy approaches and it supports custom nucleoside chemistries. In summary, data interpretation software is advancing and likely to receive even more attention in the future.

When oligo mapping an mRNA molecule, it is the mass spectrometer that provides the analytical data to confirm sequences and track modifications. However, the quality of the MS data is highly dependent on chromatographic performance of the separation used during data acquisition. If the employed LC separation has low peak capacity, the mass spectrometer will be overwhelmed with coeluting species, and the resulting spectra will be crowded with convoluted, undecipherable information. For this reason, a high peak capacity LC separation is sought. UHPLC separations are being adopted, and ion pairing methods with a high pH stable stationary phase are becoming the relied upon techniques. Nevertheless, HILIC is equally promising because it offers reasonable resolution without requiring the use of an ion pairing additive [69]. For IP-RP, C18 bondings can be taken advantage of, and there is often a preference to use pH resistant organosilica or organosilica coated particles to achieve long-lived column lifetimes [70]. On top of this, the use of low adsorption column hardware has been explored and is providing new means of obtaining sharper peaks and higher recoveries while minimizing the need to passivate new columns [69]. In addition to C18 columns, PS-DVB phases have also been used for oligo mapping analyses [71]. Fig. 7 provides a state-of-the-art IP-RP separation as applied to oligo mapping. With this chromatography, a 4-sigma peak capacity of approximately 600 was obtained such that many RNase T1 generated peaks were separated with baseline resolution.



wherein a 6-mer, 10-mer and 21-mer T1 cleavage products were identified as 3' phosphate species with mass errors of  $\leq 10$  ppm. Note that this is a previously unpublished result. Additional information can be obtained through correspondence with the authors.

## 5. 5' Endcap analysis

In addition to intact molecular integrity, sequence, and nucleobase modifications, it is critical for the 5' endcap modification of mRNA to be thoroughly analyzed. The 5' endcap is an essential element of mRNA that confers resistance to exonuclease degradation and allows it to be recognized by proteins for translation [72]. Based on regulatory guidelines [73,74], the capping efficiency of the Cap-1 structure must be quantified and any impurities, such as precursor cap structures, must be identified.

In eukaryotes, the cap structure consists of a 7-methyl guanosine that is connected to the first nucleotide of the mRNA through a 5' to 5' triphosphate bridge. For mRNA produced via *in vitro* transcription, there are typically two ways of adding the 5'-end cap. Post-transcriptional capping involves the use of enzymes, usually from the vaccinia virus, to add a guanosine to the triphosphate 5' terminus that can later be methylated to form Cap-0 (m7GpppG cap) and Cap-1 (m7GpppGm cap) structures. An alternative, co-transcriptional capping method has been invented, and it provides a dinucleotide cap reagent as an initiation point for *in vitro* transcription. This replaces the guanosine triphosphate but has historically been less efficient due to the competition and generation of reverse caps, or cap structures in the reverse orientation [75]. However, recent advances in "anti-reverse" cap analogs and co-transcriptional capping based on alternative T7 promoters have led to highly efficient capping and the implementation of this new technology in the manufacturing of COVID-19 vaccines [76].

Historically, methods for the analysis of mRNA cap structure have relied on enzymatic digestion approaches. For instance, the use of RNase T2 is commonly used to estimate capping efficiency and cap orientation with the use of tobacco acid pyrophosphatase, where the two cap structures can be differentiated with liquid chromatography. In combination with a radioactive labeled guanosine triphosphate, digestion of the resulting mRNA with RNase T2 can generate a radioactive cap structure for analysis [75]. Estimates on capping efficiency can be made through measurements with anion exchange chromatography or detecting changes in radioactivity. This method requires the use of radioactive materials and longer process times.

Affinity-based approaches can be used to elucidate cap structures. By utilizing a number of ligation assays for phosphate enumeration and electrophoresis as accomplished by Northern Blotting, it has been shown to be possible to determine capping efficiency [77]. Sequencing-based assays can also be used to study mRNA capping. Blewett and co-authors have demonstrated a quantitative decapping approach in conjunction with splinted ligation using RT-PCR [78], and work by

**Fig. 7.** Oligo mapping IP-RPLC chromatography example. Total ion chromatogram (TIC) of firefly luciferase beta mRNA digested with RNase T1 and analyzed using a hybrid surface technology (HST) BEH C18 300 Å, 1.7  $\mu$ m particle  $2.1 \times 150$  mm column. Eluting digestion components were detected by negative ion mode ESI-ToF MS with a BioAccord™ System with ACQUITY™ RDa Detector. The LC separation employed the use of ion pairing mobile phases comprised of 0.1 % (v/v) N,N-diisopropylethylamine (DIPEA) and 1 % 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) in deionized water (mobile phase A) and 0.0375 % DIPEA and 0.075 % HFIP in 65:35 acetonitrile/water (mobile phase B). A 60 min gradient from 3 % to 30 % mobile phase B was applied along with a column temperature of 70 °C and flow rate of 0.4 mL/min. Three example peak identifications are provided

Moya-Ramirez and co-authors has demonstrated the potential of biosensors to qualitatively assess mRNA capping [79].

Separations by gel analysis and liquid chromatography can also be paired with enzymatic digestions to characterize the 5' terminus of an mRNA. Here, short strands of uncapped RNA or even mononucleotides need to be detected. USP analytical guidelines advise that IP-RP should be employed as the analytical technique. IP-RP is a well-established technique and detection can be performed by 260 nm UV absorbance [80]. IP-RP in combination with MS have also shown promise in analyzing the capping efficiency of in vitro transcribed RNA, where identification of the 5' cap can be differentiated through both mass and retention time as well as quantified after cleavage from the mRNA. Beverly and co-workers utilized a biotinylated probe complementary to the 5'-end of mRNA to direct RNase H to cleave pre-determined cap structures. They then purified and enriched the 5' termini species through streptavidin-coated beads and analyzed them by LC-MS. Vlatkovic and coworkers described a similar technique but used ribozymes to cleave fragments from the 5'-end. In a different approach, Muthmann and co-authors digested and dephosphorylated their entire RNA transcript based on an incubation with snake venom nuclease P1. From this, they obtained single nucleosides and were able to use gel electrophoresis and MS to quantify different cap species [81,82]. For industry laboratories, it might remain desirable to apply an RNase H based sample preparation because reagents are readily available. Lastly, it has been shown that 5' endcap fragments can be quantified by a rapid IP-RP technique and the use of extracted ion chromatogram analysis. With this method, rapid throughput can be achieved without the need for LC method development to baseline resolve individual components [83]. Example data from this type of rapid LC-MS analysis are shown in Fig. 8.

## 6. Poly-A tail analysis

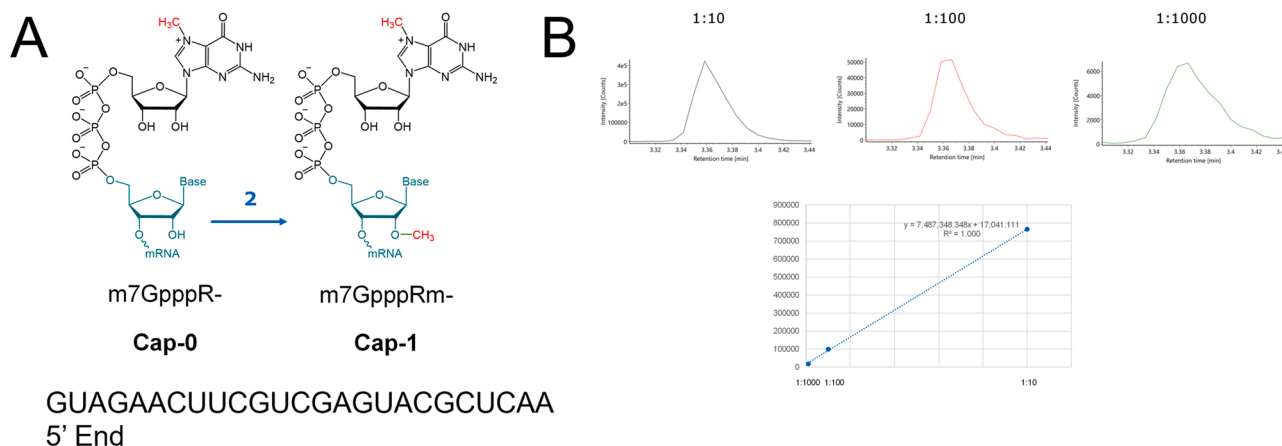
In addition to the analysis of 5' end cap species, it is critically important to have robustly established techniques for the measurement of poly-A tail length and heterogeneity. This is a significant assay because the poly-A tail is involved in the initiation of translation and because its length correlates with stability and translation efficiency [84]. The 3' end of IVT synthesized mRNA is often designed to have a 120 nucleotide long poly-A tail, whether it be added by polyadenylation or through the use of a DNA template and polyT-nucleotides [85]. Measurements of poly-A tail heterogeneity is generally limited if attempted at the intact level. Consequently, researchers have taken to

the use of endonucleases to digest away the upstream nucleotide sequence of the transcript [86]. Digestion approaches have generally relied upon RNase T1 and at times the combined use of RNase A. The resulting poly-A tail fragment can thereafter be analyzed by an LC-based method. With an LC analysis, Beverly and co-workers have found success in coupling MS detection such that tail lengths can be directly measured [87]. While powerful, it should be noted that separation-based analyses are not the only means to measuring poly-A tail heterogeneity. Indeed, both NGS sequencing and CE-based sequencer approaches have proven to be viable. Measurements with nanopore sequencing are also possible [88,89].

## 7. Lipid compositional analysis

Lipid nanoparticles as a delivery mechanism for mRNA-based therapies present their own challenges in terms of characterization and analysis. Fundamentally, the lipid nanoparticle is comprised of four lipids (e.g., cationic/ionizable lipids, phospholipids, pegylated lipids, and cholesterol) at a specific ratio, each of which play a role in the stability, delivery, and efficacy of the nanoparticle. While elegant in design, similarities in the physicochemical properties of the lipids, as well as their relative abundance, require consideration when developing characterization and stability indicating methods.

The first analytical consideration to make in this regard is one about sample preparation. Ideas for sample preparation can be drawn from understanding how LNPs are formed. Ganesan and Narayanasamy have comprehensively reviewed a wide array of LNP preparation approaches [90]. Most commonly, two solutions are prepared for mixing - one is an ethanol solution of lipids and the other is an aqueous solution of mRNA. The aqueous solution is ratiometrically introduced to the ethanolic lipid solution using mixing techniques ranging from pipette aspiration to microfluidics. The assembly of the LNP can be reversed for the sake of compositional analysis. A preferred approach for this involves dissolving the particle by the addition of organic solvent. An RPLC analysis with either evaporative light scattering, charged aerosol, or mass spectrometric detection can thereafter be performed. Methanol is a commonly used organic solvent for liposome disruption where it can alter bilayer membrane structures and release the encapsulated drugs. Methanol and other organic solvents have proven to be equally effective at dissolving solid lipid nanoparticles. In fact, mixtures of chloroform and methanol and solutions containing methanol or isopropanol have also been used with success [91]. Li et al. prepared individual samples for siRNA and



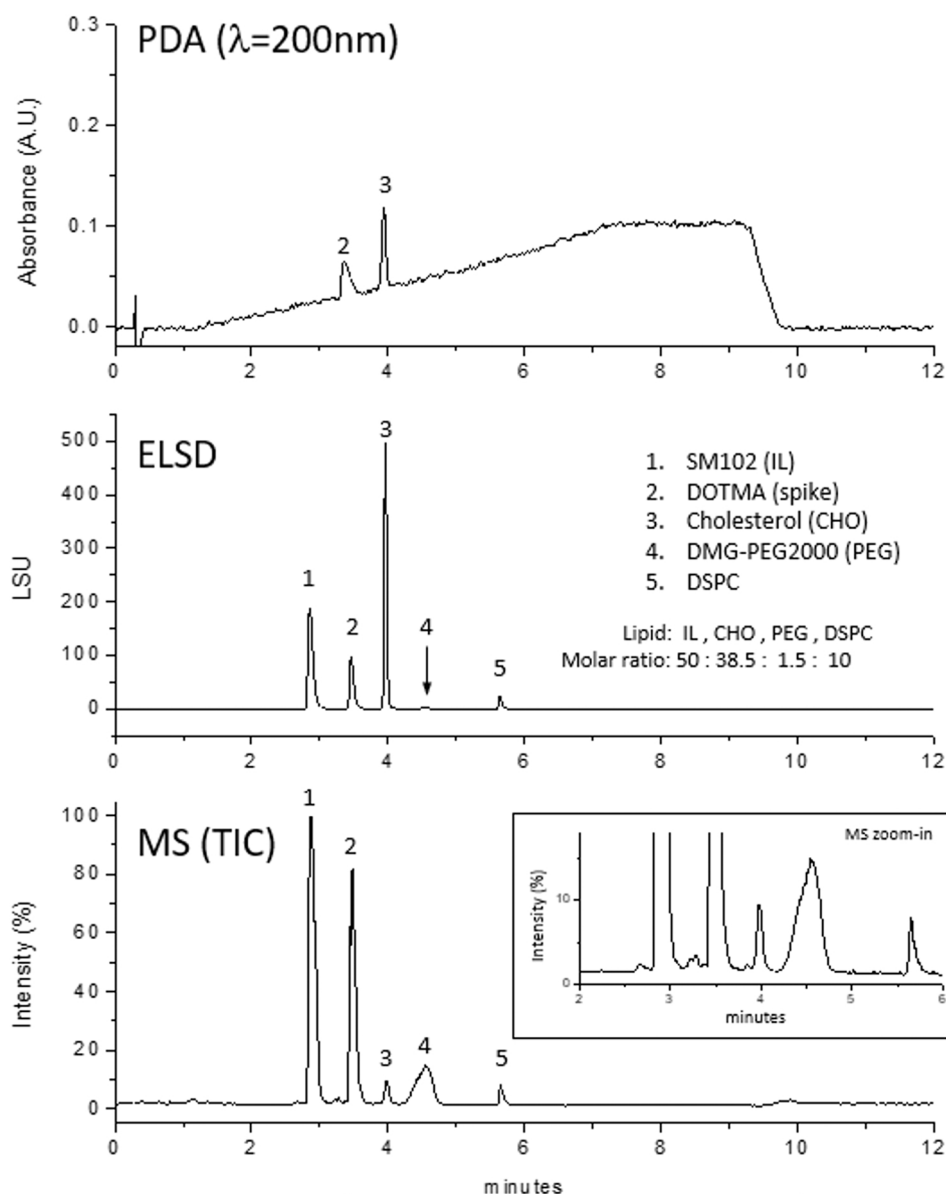
**Fig. 8.** Use of LC-MS to quantify a Cap-0 5'-end terminus species in the presence of its Cap-1 analog. Extracted ion chromatograms of Cap-0 modified GUAGAACUUCGUCGAGUACGCUCAA as obtained from samples containing 1:10, 1:100, and 1:1000 ratios of Cap-0 to Cap-1 species (with Cap-1 species fixed at a 12.5 pmol/ $\mu$ L concentration). This is an oligonucleotide that can be prepared by RNase H based DNA/RNA duplex directed cleavage. XICs were prepared from the [M-10H]<sup>+</sup>10-charge state. Separations were performed on a 2.1  $\times$  50 mm hybrid surface technology column packed with BEH<sup>TM</sup> C18 130 Å, 1.7  $\mu$ m Stationary Phase, a flow rate of 0.3 mL/min, column temperature of 60 °C, and a 5 min gradient from 5 % to 25 % B (using DIPEA-HFIP modified mobile phases noted in Fig. 6). (With permission from Ref [83]).



lipid analysis using the addition of ethanol along with sample inversion and vortexing. For their lipid analysis, 1 mL of ethanol was added to each 100  $\mu$ L of lipid nanoparticle suspension [92]. Packer et al. described the discovery of a lipid-modified mRNA component in an LNP-mRNA formulation and the steps taken to identify it [93]. To characterize the LNP-mRNA sample, mRNA was extracted using an isopropanol precipitation. A 100  $\mu$ L volume of mRNA-LNP suspension was diluted 10-fold with 60 mM ammonium acetate in isopropanol, vortexed, and centrifuged. The supernatant was discarded, and the mRNA pellet was washed, dried, and resuspended in RNase-free water for subsequent analysis. As in a previous reference [92], the lipids were prepared from the sample by a dilution in ethanol and the supernatant was analyzed using RP-LC and charged aerosol detection [93]. While simple short chained alcohols like methanol and ethanol are used frequently to dissolve LNPs, future LNP-drug product formulations may require other types of solutions. For off-line RNA quantitation in size exclusion fractions, Jia et al. used a surfactant solution to disrupt their LNPs. A 200  $\mu$ L aliquot of an LNP suspension was disrupted using 10  $\mu$ L of a 2 % solution of a non-ionic surfactant [94]. In another study, Fan and co-workers disrupted LNPs with a 0.75 % solution of non-ionic surfactant [95]. In all cases, it is preferred that the applied sample

preparation techniques provide quantitative, unbiased dissolution of each lipid. Further studies on disruption mechanisms might lead to new insights on preferred approaches.

As a class of compounds, lipids are predominantly hydrophobic which makes them a natural fit for RP-LC. However, their solubility in organic solvents also makes normal phase (NP), supercritical fluid chromatography (SFC) as well as hydrophilic interaction liquid chromatography (HILIC) viable as options for analysis. As mentioned, LNPs are often dissolved with alcohols [96]. This in turn requires that stationary phases be used that offer optimal retentivity. Otherwise, strong solvent effects and diminished chromatographic performance would come to be encountered. Reversed phase stationary phases including C8, C18, and C30 alkyl chains have been successfully applied to separate lipid species under gradient conditions. Both weak and strong organic solvents have been used for elution; solvent choices have ranged from methanol and acetonitrile to isopropanol and tetrahydrofuran [97–99]. Method optimization has included the use of ion-pairing agents such as trifluoroacetic acid or triethyl amine as well as acetate or formate buffers as a means to control pH and selectivity of lipid species for improved chromatographic resolution [98,100]. While successful, some method development considerations are necessary in these approaches.



**Fig. 9.** Comparison of detector response of a lipid nanoparticle mixture prepared in a molar ratio representative of a therapeutic product using UV (PDA), light scattering (ELSD), and mass spectrometry (MS) as end-point detectors. Separations were performed under reversed phase conditions using a phenyl-hexyl stationary phase. Mobile phase A:  $\text{H}_2\text{O}$ , 0.4 % FA v/v, MP B: 25:75 IPA:MeCN, 0.4 % FA v/v, gradient: 5 %B/min. Peaks 1–5 are SM102, DOTMA, cholesterol, DMG-PEG2000, and DSPC. SM102, cholesterol, DMG-PEG2000, and DSPC were prepared in a molar ratio of 50:38.5:1.5:10 representative of a formulated LNP sample. DOTMA was spiked-in at half the concentration relative to SM102. with sample concentration scaled for detector response based on SM102 concentration.

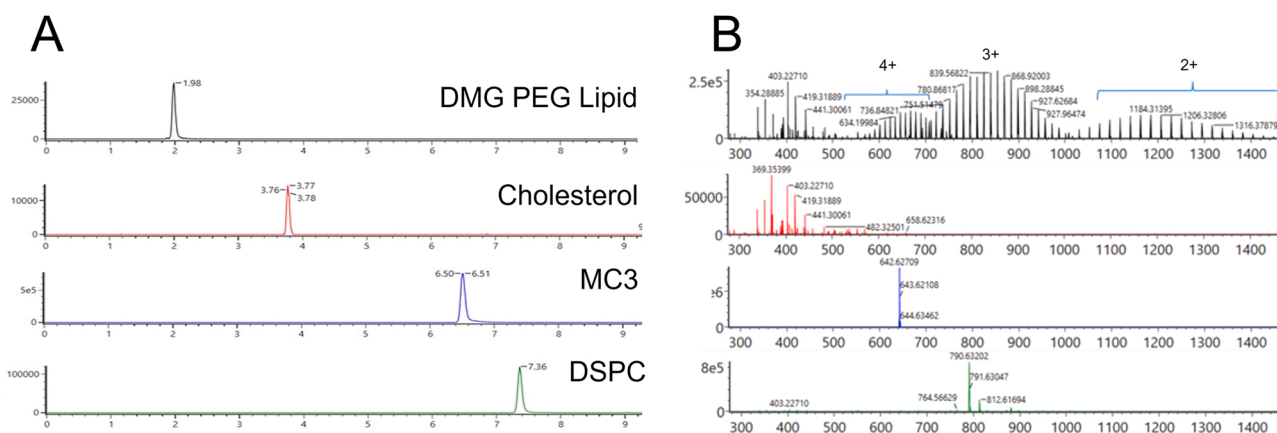
In one aspect, the use of isopropanol may preclude the use of high efficiency (sub-2  $\mu\text{m}$ ), longer LC column formats, because of its high viscosity and column backpressure effects. In another aspect, the use of strong solvents such as tetrahydrofuran can be problematic because it can cause PEEK (polyether ether ketone) material to swell and erode LC seals. A systematic review of LC system components is suggested before considering tetrahydrofuran mobile phases. Lastly, the use of strong ion-pairing agents such as TFA and high concentration ammonium buffers can impact the sensitivity and overall usefulness of certain detectors, such as mass spectrometers. More recently, less retentive bonded phases such as phenyl-hexyl have been shown to deliver acceptable performance in the separation of LNP species with modest throughput and performance using conventional water/acetonitrile mobile phase systems paired with additives such as formic acid. Stationary phase particles exhibiting a charged surface have also found favor for such an analysis and method condition. Particles designed to have a positive surface potential can help provide sharper peak shapes and unique selectivity for cationic, ionizable lipids. An example lipid separation that was achieved with a phenyl-hexyl bonded, positive surface potential stationary phase is shown in Fig. 9.

Due to their low UV-activity, alternative detection techniques such as Charged Aerosol Detection (CAD) and Evaporative Light Scattering Detection (ELSD) have been needed for lipid analysis [98–115]. From a method development perspective, both detectors offer user-controlled parameters to assist in the optimization process. Mobile phase composition has been shown to impact response factors in both detector types where an inverse gradient may be needed to facilitate uniform detector response [101,109–111]. Both detector types have been found to offer sufficient sensitivity and dynamic range for most analytical needs and are well suited to impurity profiling, compositional analysis and stability indicating assays [98,99,104,108,113].

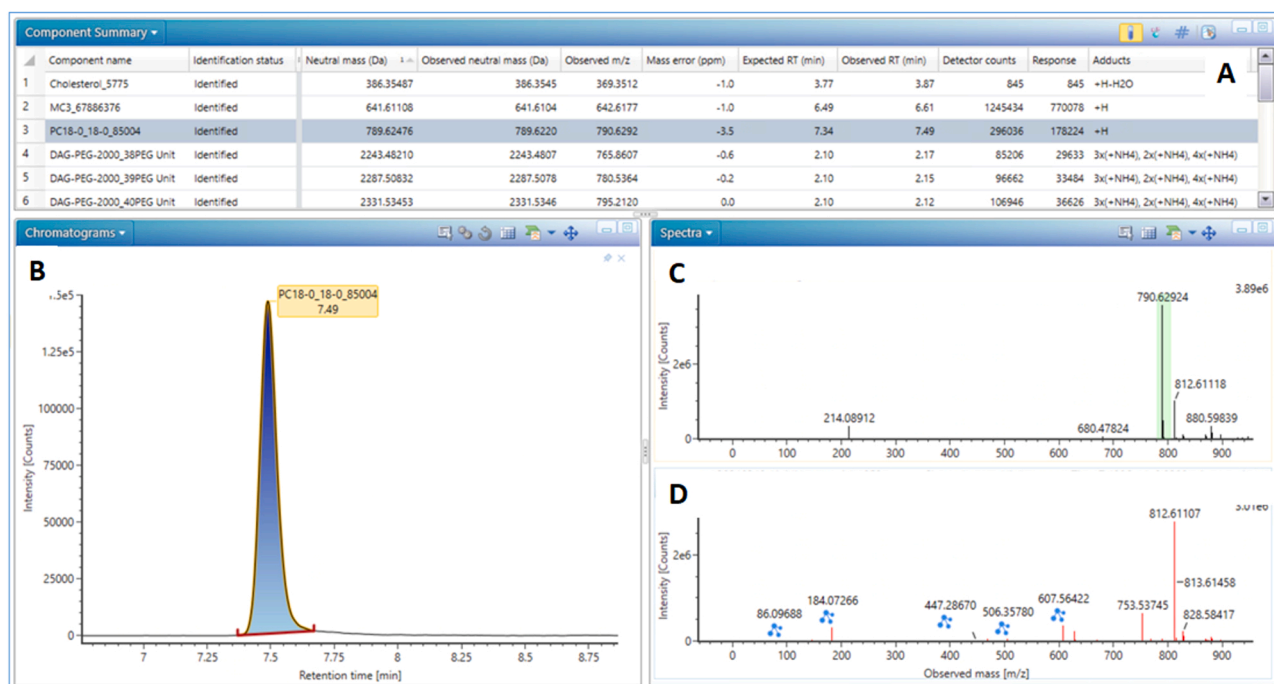
Lipids can also be detected by mass spectrometry. In particular, the use of high-resolution mass spectrometry (HRMS) as a detector provides confidence in structural characterization and identification, especially when it is important to learn more about low-level impurities and degradants. Unlike other methods, LC-HRMS offers high sensitivity and specificity for qualitative and quantitative analysis of lipids even from a complex sample extract [114–119]. Liquid chromatography coupled with mass spectrometry (LC-MS) has become increasingly important because it affords more capabilities for structural characterization, identification, and the quantitation of lipids. Many studies have been performed, even if most examples published to date focus on lipidomics [120–125].

In previous work, a standard mixture of lipids was prepared and diluted in methanol so that it contained 0.5 ng/ $\mu\text{L}$  MC3, 0.5 ng/ $\mu\text{L}$  DSPC, 0.5 ng/ $\mu\text{L}$  DMG-PEG-2000 and 2 ng/ $\mu\text{L}$  cholesterol [126]. This sample was then analyzed using electrospray ionization with a benchtop time of flight mass spectrometer. Extracted ion chromatograms for each of the 4 lipids are shown in Fig. 10 A. The lipids were eluted in the order of DMG-PEG-2000, cholesterol, ionizable lipid MC3 and DSPC when separated on a charged surface, C18 bonded 1.7  $\mu\text{m}$  particle column using a ternary gradient with acetonitrile and isopropanol containing eluent. With this work, we also studied the serial dilution of each individual lipid in concentrations ranging from 0.1 to 500 pg/ $\mu\text{L}$ . The lower limit of detection (LOD) was determined to be 5 pg/ $\mu\text{L}$  (25 pg on column) for the ionizable lipid MC3, DSPC and DMG-PEG-2000. The LOD for cholesterol was determined to be 250 pg/ $\mu\text{L}$  (1.25 ng on column). Fig. 10 B shows corresponding spectra for the four representative lipids of DMG-PEG-2000, cholesterol ( $m/z$  369.352), ionizable lipid MC3 ( $m/z$  642.627) and DSPC ( $m/z$  790.632). The combined spectra for DMG-PEG-2000 is shown in Fig. 10 B (top panel). DMG-PEG-2000 was seen to form an ammonium adduct under the applied LC-MS conditions as well as 2+, 3+, and 4+ charge states. In addition, DMG-PEG-2000 ion signal showed evidence of a heterogeneous, polydisperse polymer, which can be attributed to uneven PEG chain lengths ranging from 38 to 50 ( $\text{CH}_2\text{CH}_2\text{O}$ ) units. A total of 13 different PEGylated lipids were identified based on observed polydispersity. For the identification of individual lipid components, a custom database containing the various lipid compounds and their potential degradation products and impurities was created. Fig. 11 A shows an example peak identification summary, wherein data in support of identified lipids are displayed. Fig. 11 B shows even more data in support of an identification, including an extracted ion chromatogram (Fig. 11 B), low energy parent ion spectrum (Fig. 11 C) and corresponding characteristic fragment ions (Fig. 11 D). An approach to lipid analysis, like this one, that incorporates high resolution, high mass accuracy MS is coming to be adopted, because it can strengthen the information known about incoming raw materials, aid the design of novel synthetic lipids, produce more insights during formulation stability studies, and give new tools for drug development and quality control.

LNPs are comprised of four known lipid components and quantitation can be performed using ELSD, CAD or mass spectrometry. Successful quantitation is based on a proper analytical method paired with the appropriate internal standards. The use of stable isotopic labeled internal standard (SILS) or chemically similar standard to the lipid components is recommended to compensate for variation in lipid



**Fig. 10.** (A) Positive ion mode extracted ion chromatograms and (B) Corresponding spectra of DMG-PEG-2000, cholesterol ( $m/z$  369.352), ionizable MC3 lipid ( $m/z$  642.627) and DSPC ( $m/z$  790.632). The PEGylated lipid exits as multiply charged series of + 2, + 3 and + 4 with 38–50 polyethylene glycol repeat units. The spectra are color coded to their corresponding chromatogram. Separations were performed with a hybrid surface technology (HST) CSH C18 130 Å, 1.7  $\mu\text{m}$  particle  $2.1 \times 150$  mm column and mobile phases comprised of 600/390/10 (ACN/Water/1 M aqueous ammonium formate) in 0.1 % formic acid and 900/90/10 (IPA/ACN/1 M aqueous ammonium formate) in 0.1 % formic acid. A flow rate of 0.4 mL/min and column temperature of 55 °C was applied. Mass spectra were acquired with a BioAccord LC-MS System containing and ACQUITY RDA Detector.



**Fig. 11.** Component summary plot showing (A) the identified lipid nanoparticles of cholesterol, cationic lipid MC3, DSPC and 14 different DMG-PEG-2000 (B) example extracted ion chromatogram of DSPC (C) Low energy exact mass of DSPC and (D) High energy spectrum and corresponding characteristic fragment ion spectrum of DSPC. The blue icon indicates matched predicted in silico and experimental fragment ions. Data analysis was performed using a custom-made UNIFI™ Scientific Library. SYNAPT, BioAccord, ACQUITY, BEH, and UNIFI are trademarks of Waters Technologies Corporation. Comirnaty is a trademark of BioNTech SE sociedades Europae. All other marks are the property of their respective owners.

extraction, matrix effects and ionization efficiency (for MS based quantitation). The SILS should be added before extraction to assess percent recovery [127,128].

## 8. Conclusion and perspectives

LNP mRNA pharmaceuticals are comprised of various chemically diverse molecules that are brought together in a sophisticated inter-molecular complex. Molecular and compositional analysis of them can thus be quite challenging. Nevertheless, analytical approaches are coming to be established and liquid chromatography is becoming increasingly relied upon.

Although there have been significant advances in all types of LNP mRNA analyses, we have focused here on the use of AEX, IP-RP, oligo mapping, 5' endcap testing and lipid compositional assays. Separations by AEX and IP-RP are predicted to be majorly important to the detection of heterogeneity and for checking molecular integrity. Various modes of elution can be applied for AEX including a classical salt gradient, ion-pairing gradient, and ion-pair mediated salt gradient. Both non-denaturing and denaturing conditions can be used, and each has its advantages and limitations. Beside the stationary phases, column hardware itself may have a huge impact on solute recovery and carry-over and thus on the reproducibility of the measurements. Therefore, PEEK or h-HST column hardware are preferred over common stainless-steel columns. When it comes to method optimization, mobile phase temperature, composition and pH are known have a huge impact on the quality of the separations and thus need to be optimized for each specific sample. Intact mRNA analysis via IP-RP assays has the potential to become an essential characterization tool for mRNA, but more progress is needed in order to extend the capabilities of this method for the detection of larger molecules up to 10,000 nt long. That IP-RP can uniquely resolve hydrophobic variants suggests that it may become a mainstay as an mRNA stability indicating assay.

Other analyzes of LNP mRNA drug substances and products, such as

5' endcap analysis, oligo mapping and lipid compositional assays, are equally important. The 5'-end of an mRNA can be selectively cleaved via a duplexing probe and RNase H, and the resulting digestion products can be quantified by IP-RP with UV and/or MS detection. Moreover, ribonucleases can be applied to partially digest mRNA so that it can be possible to map and deeply interrogate its molecular composition. Detailed analysis of mRNA for sequence alterations, adduct verification and full sequence confirmation will require ribonucleases of complementary specificity and ribonucleases capable of generating intermediate size digestion products that can be more optimally separated and sequenced by LC-MS. Further efforts on enzyme technology and data interpretation software are warranted. The composition of the lipid components in the LNP must also be analyzed. This can be achieved by disrupting or destabilizing the LNP-mRNA formulation. Oftentimes, a simple dilution with an alcohol is an effective starting point, but some fine tuning may be needed for specialized formulations. Lipids are amenable to reversed-phase chromatography and can be detected with universal detectors, such as CAD and ELSD modules. With this, it is possible to quantify percent composition and to monitor for degradants. LC coupled to HRMS is also important because it allows the simultaneous analysis of LNP components and impurities for a heightened level of information during drug development and/or quality control.

We hope each of the above topics have shed light on some of the very important ways in which LC-based methods have aided the development of the first generation of LNP mRNA pharmaceuticals. New progress on analytical tools for LNP mRNA are bound to be realized along with further clinical success, and the field of separation science appears to be at the heart of future efforts.

## Declaration of interests

The authors are employed by Waters Corporation, a manufacturer of UHPLC systems and columns.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors are employed by Waters Corporation, a manufacturer of UHPLC systems and columns.

## Data Availability

Data will be made available on request.

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