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Review | REV

The storage and in-use stability of mRNA vaccines and therapeutics: Not a cold case

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Abstract

The remarkable impact of mRNA vaccines on mitigating disease and improving public health has been amply demonstrated during the COVID-19 pandemic. Many new mRNA-based vaccine and therapeutic candidates are in development, yet the current reality of their stability limitations requires their frozen storage. Numerous challenges remain to improve formulated mRNA stability and enable refrigerator storage, and this review provides an update on developments to tackle this multi-faceted stability challenge. We describe the chemistry underlying mRNA degradation during storage and highlight how lipid nanoparticle (LNP) formulations are a double-edged sword: while LNPs protect mRNA against enzymatic degradation, interactions with and between LNP excipients introduce additional risks for mRNA degradation. We also discuss strategies to improve mRNA stability both as a drug substance (DS) and a drug product (DP) including the (1) design of the mRNA molecule (nucleotide selection, primary and secondary structures), (2) physical state of the mRNA-LNP complexes, (3) formulation

composition and purity of the components, and (4) DS and DP manufacturing processes. Finally, we summarize analytical control strategies to monitor and assure the stability of mRNA-based candidates, and advocate for an integrated analytical and formulation development approach to further improve their storage, transport, and in-use stability profiles.

Keywords: mRNA structure, stability, degradation mechanisms, shelf life, physical and chemical analysis, formulation, lipid nanoparticles, LNP

Introduction

In the wake of the dramatic success of mRNA vaccines to protect against severe disease caused by SARS-CoV-2 infection, mRNA-based medicines are now becoming widely studied as pharmaceutical product candidates. At present, Comirnaty® (BioNTech/Pfizer) and SpikeVax® (Moderna) are the two key mRNA vaccines approved by FDA, EMA and many other regulatory authorities worldwide. These two mRNA vaccines have reached billions of recipients globally as transformative tools in the fight against the COVID-19 pandemic. Even as SARS-CoV-2 virus variants evolve, these vaccines continue to reduce the severity of COVID-19 disease. Moreover, sequence alterations to the *in vitro* transcribed (IVT) mRNA molecules employed in these vaccines were recently prepared, clinically studied and approved to better protect against new variants of the SARS-

CoV-2 pathogen[1,2]. This adaptability illustrates the potential of the IVT mRNA technology for use in future vaccines against emerging infectious diseases. Beyond vaccines for infectious diseases, many clinical programs are in progress for cancer immunotherapy or with mRNA-based medicines acting as a therapeutic modality, including mRNA as a template for gene editing enzymes[3–5].

All these clinical and commercial products based on mRNA technology share the same characteristic of limited stability from a pharmaceutical perspective. Multiple measures must be taken to preserve mRNA (and mRNA-LNP particle) integrity during manufacturing, storage, transport and at the time of injection. Long-term storage integrity of Comirnaty® and SpikeVax® is only achieved at subzero temperatures. Upon thawing the frozen drug product, the labelled shelf life is measured in weeks (refrigerator conditions) and in hours/days at room temperature. In previous publications, we (and others) have touched upon the reasons for the choice of these storage and in-use protocols especially in the context of use as a COVID-19 vaccine [6–8]. In this review, we dive deeper into the reasons for this instability with an emphasis on both drug substance (i.e., mRNA itself) and the formulated drug product (e.g., mRNA-LNP complexes). We discuss strategies that are currently used, and are emerging, to improve mRNA stability under non-frozen conditions, including examination of the mRNA molecule (nucleotide selection, primary and secondary structure), the physical state of drug product complex,

formulation composition (i.e., lipid type and excipient selection) and the purity of these components, as well as optimizing preparation/manufacturing protocols for DS and DP (Table 1). We also report on 'real life' experience of the stability of marketed mRNA vaccines published in the public domain so far. A brief discussion of packaging is also included, mainly due to the lack of published data on this important aspect to date.

Table 1: Summary of different strategies to stabilize pharmaceutical mRNA-formulations

Stabilizing pharmaceutical mRNA-formulations by:
• Design and production of the mRNA molecule
○ Selecting modified nucleotides
○ Increase GC content
○ Increase secondary structure, minimizing 'average unpaired probability', AUP
○ Shorten chain length
○ Purity levels of the mRNA drug substance
• Choice and purity of pharmaceutical excipients: buffers, lyo/cryoprotectants, osmolytes, etc.

Choice and purity of lipid excipients used to prepare LNPs
Optimization of manufacturing conditions to produce mRNA and mRNA-LNP formulations
Storage at lower temperatures; the removal of water by lyophilization or spray drying
'At- the point-of use' product assembly', using a 'kit-based approach'

Sensitive and selective analytical techniques are required to monitor the integrity of mRNA and mRNA-LNP complexes. Therefore, a section of this review is devoted to a discussion of the state-of-the-art analytical toolbox especially in the context of stability-indicating methods. Finally, there are two topics that are not covered in this review. First, designing improvements to mRNA-LNPs for better *in vivo* performance is not discussed since a large number of articles (including reviews) have recently appeared [9–12]. In contrast, the focus of this review is a topic that has not gained the same attention: the long-term and in-use stability issues with mRNA formulated as drug product. Second, only few references to small interfering RNA (siRNA) formulation studies are provided due to their limited applicability to mRNA formulations. Compared to mRNA, siRNA is a short and uniformly structured, and it frequently contains replacements at the 2'-OH on ribose to provide a more chemically stable analog, e.g., 2'-F or 2'-OMe). As a

result, marketed siRNA products can be stored as liquid formulations in a refrigerator, e.g., Onpattro®[13].

The mRNA molecule

Chemical components of the mRNA structure

Natural and IVT-transcribed mRNAs are delicate, high molecular weight (MW), macromolecules that are rich with specific information required for *in vivo* performance that can be easily lost by chemical degradation along the mRNA strand. The primary units of mRNA are ribonucleotides, introduced in the IVT reaction as 5'-triphosphate substrates (Fig. 1) for the T7 polymerase. As illustrated in Fig. 1, the nucleotides used in IVT mRNA include canonical (A, G, C, U, see Fig. 1) as well as naturally occurring, modified uridines, which reduce the innate immune stimulation associated with exogenous mRNA delivery[14]. The N1-Me-pseudouridine modification is used in the Pfizer and Moderna COVID-19 vaccines and may play an important role in their efficacy as well.

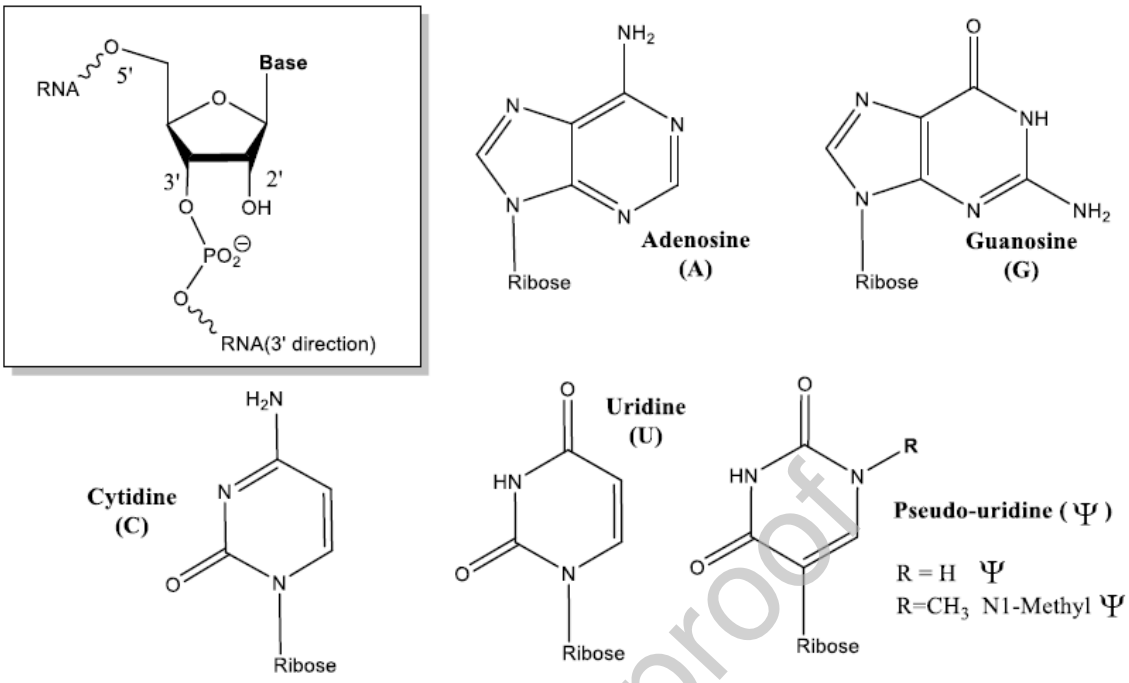

Fig. 1. Overview of the nucleotides found in natural and *in vitro* transcribed (IVT) mRNA molecules. While natural mRNA contains uridine, IVT mRNA instead may contain modified pseudouridine to increase the safety and efficacy of mRNA vaccines.

In addition to the antigen coding sequence and untranslated regions of the mRNA primary structure, a 5'-cap structure is also included as an essential termination element that is responsible for binding to ribosomal initiation factors (Fig. 2). This 3'-5'-reversed triphosphate bridged dinucleotide is only present on the 5'-end of the mRNA strand; all other components are 5'-3'-linked ribonucleotides incorporated by the polymerase based on a linearized DNA template. While enzymatic cleavage of the 5'-cap of mRNA is catalyzed by exonucleases, there is

no indication that loss of the 5'-cap is a particular vulnerability in regard to bulk drug substance (active pharmaceutical ingredient: API) or final drug product stability [15].

Finally, on the opposite side of the 5' terminal cap structure of mRNA transcripts is a poly-adenylation segment (Fig. 2). The so-called poly-A tail on the 3' end is involved in binding to the ribosomal machinery, along with the 5' cap on the opposite end of the mRNA, thus creating a closed loop track for ribosomes to traverse during translation of the polypeptide. Exonucleases cleave the poly-A tail, which consequently acts as a fuse for translational duration of the mRNA. Once the poly-A has been clipped to a substantial degree, the mRNA is released from the ribosomes and decays. Notwithstanding this enzymatic poly-A degradation pathway, there is currently no evidence published that the poly-A is a particular challenge for the chemical stability of mRNA transcripts in the absence of exonuclease [15,16]. Interestingly, circular RNA has recently been advanced as a concept to reduce the exonuclease impact on *in vivo* stability of mRNA transcripts. Stability data with circular RNAs in pharmaceutical dosage forms have yet to be published [17], so we focus in this review on the stability of linear mRNA transcripts.

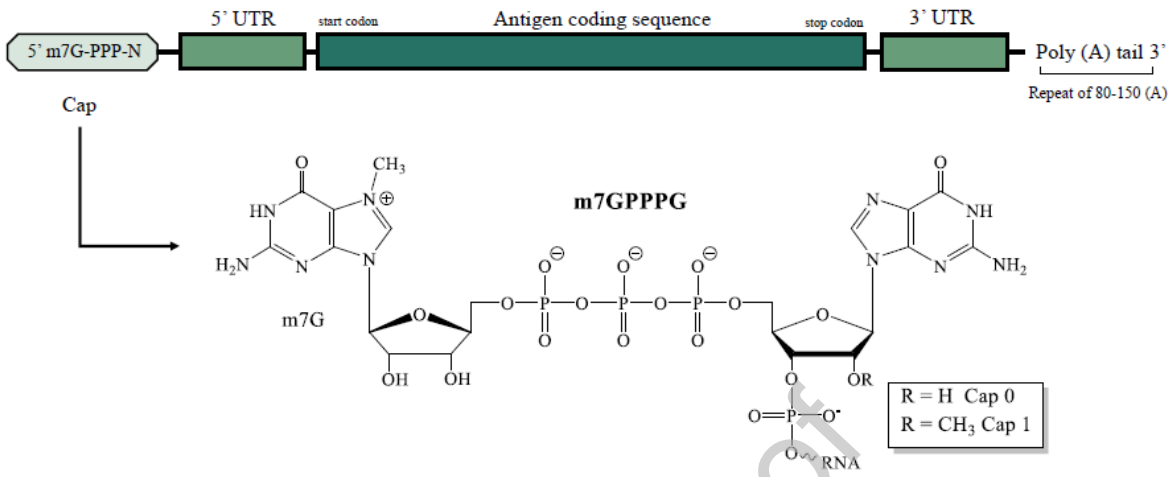

Fig. 2. Schematic diagram of IVT mRNA primary structure (5' terminal cap, untranslated regions, antigen coding region, 3' poly-A tail) including chemical structure of the 5'-cap dinucleotide caps. (UTR, untranslated regions)

The simple schematic structure of an mRNA strand displayed in Fig. 2 belies the pharmaceutical complexity of the API produced from the IVT processes. Firstly, the mRNA strands are very long, commonly in the range of 1000 nucleotides, and often considerably longer. During *in vivo* translation, protein encoding information in the mRNA molecule is in the coding sequence as a triplet of nucleotides each specifying a single amino acid in the resulting protein. In this regard, the "atom efficiency" of mRNA is low, meaning that for about 900 units of MW of mRNA (i.e., 3 nucleotides of ~ 330 g/mol each), an amino acid of ~110 MW is inserted into the growing polypeptide chain. Given that the untranslated

regions (UTRs) do not contribute to the decoding activity, the mRNA required to encode a given protein has an order of magnitude larger MW than the resulting translation product. Secondly, and most importantly, the expression of protein and fidelity of the primary structure of the intended protein product are highly dependent on the integrity of the primary structure of the mRNA strand from which it was translated. In summary, the susceptibility of large mRNA strands to chemical degradation can be directly linked to loss of *in vivo* performance.

The presence of the 2'-OH on the sugar unit in mRNA (in contrast to DNA) invites the intramolecular catalysis of strand breakage, generally *via* a transesterification reaction (Fig. 3). The 2'-OH is also essential to proper decoding in the ribosomes. As mentioned in the Introduction, this requirement for 2'-OH is a major difference between mRNA and the siRNA therapies, wherein the latter the 2'-position can be modified to fluorine or O-methyl with retention of binding affinity and driving major improvements in inter-residue linkage stability relative to native RNA residues. As summarized in Fig. 1, mRNA from IVT reaction can be produced using either uridine or pseudouridine substrates. Although it might be tempting to equate "modified uridine" mRNA with stabilization, this is in fact, not necessarily the case. The result of including N1-Me-pseudouridine in place of U is retention of the ribose ring with 2'-OH and an increase in molecular weight (by adding one methyl per U replacement), as well as altering the mRNA hydrophobicity. While

the effects of base modification on mRNA stability have yet to be directly compared in the published literature, retention of ribose in modified mRNA preserves the hydrolytic instability of the polymer chain.

RNA dinucleotides show subtle differences in intrinsic stability depending on the nucleobases in the dinucleotide [18]. The mRNA molecule, however, is substantially more complex than the dinucleotide setting, given the presence of significant secondary and tertiary structures. In the following sections, we will discuss the effects of environment, including sequence context and chemical modifications, as well as the presence of various formulations, on mRNA stability.

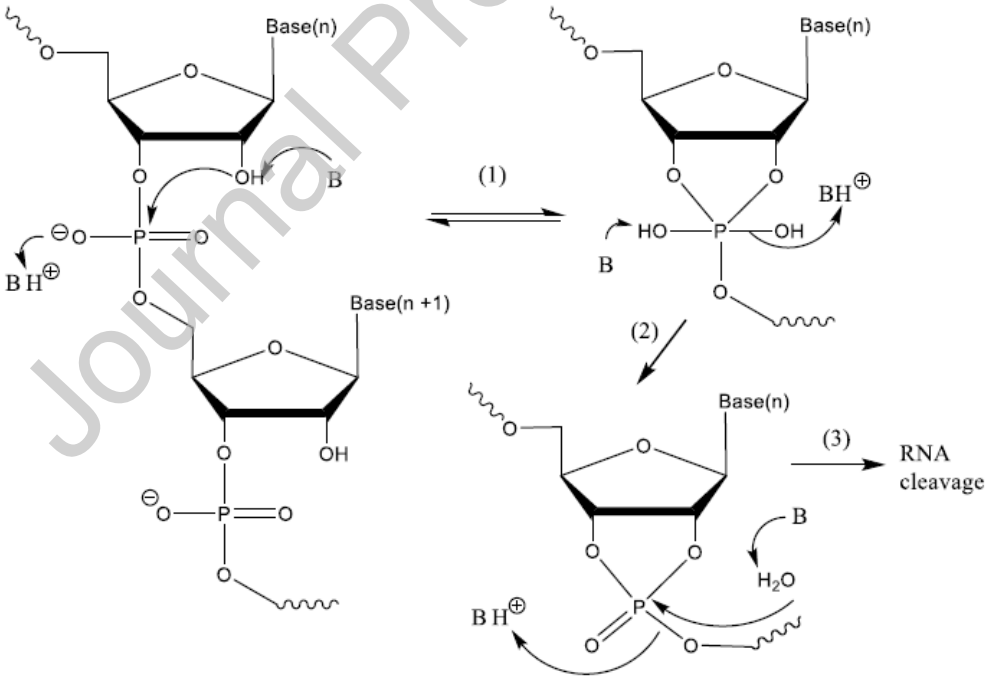

Fig. 3. Mechanism of the degradative transesterification reaction in mRNA strands leading to strand cleavage. B denotes a Brönsted base and BH^+ is the corresponding conjugate acid.

Effect of secondary structure on mRNA stability and degradation reactions

In aqueous solution (e.g., bulk drug substance) and in LNP formulations (i.e., drug product), mRNA degradation can proceed *via* hydrolysis, oxidation, or reaction with adventitious impurities, e.g., aldehydes, metal ions, peroxides. Generally, the degradation rate increases with the chain length of the mRNA [19]; this effect can be rationalized by a higher probability of mRNA modification as the number of nucleotide linkages available for reaction increases. Such statistical factors can only account for part of the mRNA reactivity, however, as the mRNA sequence and the secondary structure also have a large impact on degradation kinetics. Such effects were established experimentally years ago, for example, when kinetic data on the cleavage of several short poly(desoxy)ribonucleotides at pH 8.5 in cyclohexylaminoethanesulfonic acid (CHES) buffer showed greater than 100-fold differences between the pseudo-first order rate constants depending on the nature of cleavage sites and poly(desoxy)ribonucleotide sequences [20,21,22–24].

Critical for a successful phospho-diester cleavage and RNA strand break formation is a (near) in-line orientation of the attacking 2'-hydroxyl group (nucleophile) and

the 5'-oxyanion leaving group[22–24]. The transesterification mechanism summarized in Fig. 3 is ubiquitous and completely dependent on the presence of the 2'-OH on the sugar unit in mRNA (absent in DNA and siRNA-based products in clinical use). In addition, the most favorable conditions for this strand cleavage reaction involve puckering of the ribose to bring the 2'-OH into a 'reactive' alignment (in-line orientation) with the scissile phosphate on the 3'-oxygen. The availability of this preferred conformation to facilitate the strand cleavage reaction is expected to be greater when the nucleotides are unstructured and/or outside the context of base pairing arrangements, which may force relatively rigid and unreactive conformations. Calculations by Guo et al. indicate that while C2'-endo puckering of ribose in RNA is slightly disfavored energetically relative to C3'-endo, the lowering of overall transition state energies along the reaction path leads to transesterification in phospho-diesters with the intermediacy of the C2'-endo ribose conformation[25]. Flexibility to access the conformational space of ribose is therefore likely a key to enabling transesterification reactions of mRNA. Locked Nucleic Acids (LNAs) are chemically modified bases used for stabilization of oligonucleotide drugs, where the 2'-oxygen is permanently locked in the endo-position. However, in this chemical modification the oxygen is an ether, thereby protecting the base against hydrolysis. Because a hydroxyl group at the 2'-position

is essential for ribosome binding, modifications on this position are not compatible with mRNA.

Evidence for the importance of RNA structural features on phospho-diester cleavage is displayed in the crystal structure of the naturally occurring hammerhead ribozyme, an RNA motif that catalyzes reversible cleavage and ligation reactions at specific sites in RNA. It suggests an interaction between stem II loop and stem I bulge that control both the in-line orientation of nucleophile and leaving group as well as the positions of adjacent nucleotides involved in general base and general acid catalysis [24]. The loop/bulge interaction of the hammerhead ribozyme is described as "an intricate network of interhelical noncanonical base pairs and stacks". Nonetheless, the authors of this review are not aware of an "accidental" ribozyme-type structure in an IVT mRNA transcript, wherein the catalysis factor associated with the intricate folded sequence and reactive site would lead to degradation. If this were to occur, such an unfavorable interaction could likely be designed out by changing the mRNA primary sequence, leveraging the degeneracy of the genetic code. As a final word of caution, RNA structures derived from a crystal structure will not necessarily reflect the dynamics of an RNA molecule's structure in solution, where it may change conformations over various time scales [26]. The recent perspective on RNA structure by Vicens and Kieft discusses structural and conformational properties of RNA, offering advice

on the judicious use of algorithms for predicting secondary structure. Furthermore, the formulation of mRNA in LNPs may lead to environments and mRNA conformations, not observed in aqueous solution. In turn, that may affect the stability of RNA[27].

Contrary to the structural propensities promoting RNA cleavage in the hammerhead ribozyme described above, the design of mRNA molecules employed in vaccines or therapeutics could benefit from inclusion of secondary structure designs restricting the conformational mobility so that in-line orientation of nucleophile and leaving group is less probable[28]. Wayment-Steele et al. proposed to increase base-pairing to slow the rate of RNA cleavage, and employed various secondary structure prediction packages to compute two metrics reflecting base-pairing probability within RNA: the summed unpaired probability (SUP) and the average unpaired probability (AUP). The AUP is derived by normalization of the SUP on sequence length, and the value of AUP is located between 0 and 1, where lower values indicate a lower probability of being unpaired and thus correspond to a higher stability of RNA towards degradation. Through optimization of the AUP values for several small RNA sequences, a significant stabilization towards degradation was achieved. Of note, computational in-line probing will not predict the stability of mRNA towards other chemical degradation pathways, which may become more dominant as a result of formulation in LNPs. This will be further

addressed in the sections below on product-related impurities and mRNA-lipid adducts.

Effect of nucleoside selection on mRNA stability and degradation reactions

Interestingly, optimization based on the AUP values described above led to different nucleoside sequences compared to those obtained through codon optimization, maximizing the codon adaptation index (CAI), or optimization of minimum free energy (MFE). AUP optimization also worked for longer mRNA, e.g., the SARS CoV-2 spike protein RNA, where a two-fold stabilization towards degradation was achieved. However, as a potential drawback the authors recognized that AUP optimization would not predict mRNA function *in vivo* including effects on immunogenicity. Such biological properties are paramount and would likely be prioritized over chemical stability of an mRNA when the biology and chemistry requirements are not met in the same mRNA sequence. In a subsequent paper, Leppek et al. expanded computational approaches to enable the parallel evaluation of in-cell mRNA translation efficiency, in-cell mRNA stability, and in-solution stability (towards RNA cleavage) through integrated PERSIST-seq technology. Further, they developed a method referred to as In-line-seq for high-throughput in-line probing. This method revealed that “linkages 5’ of a uridine residue are particularly susceptible to degradation”, which can be mitigated

through substitution by pseudouridine or N_1-methyl-pseudouridine *in-cell* and potentially also *in vivo*[29].

An alternative approach to pseudouridine substitution is uridine depletion. This method, described by scientists at CureVac, comprises of sequence optimization to replace codons with an A or U at the third position, with codons with a G or C at that same position [30,31]. These codons, and their corresponding tRNAs are more abundant in human cells, and this is theorized to result in more efficient protein production. In addition, the uridine linkages are increasingly susceptible to hydrolysis, so GC replacement also reduces that probability[29]. This method is otherwise known as "G/C content increase", which also results in a higher thermodynamic stability of the mRNA molecule, which is thought to protect against chemical degradation, a result consistent with the computer simulations described above.

Because uridines in IVT-produced mRNA are known to be immunostimulatory [14], it was postulated that this uridine depletion strategy would abolish the need for chemically modified bases. Unfortunately, results from CureVac's phase 2b/3 clinical trial for the CVnCoV SARS-CoV-2 chemically unmodified mRNA-based vaccine candidate did not justify further development [32]. Instead, focus was placed on the second-generation vaccine candidate CV2CoV, that has additional sequence optimizations in the non-coding regions. Use of chemically unmodified

nucleosides was continued [33]. Self-amplifying mRNA (saRNA) vaccines also exclusively contain unmodified uridines. The amplification of the antigen coding sequence depends on the co-expression of an alphavirus-derived replicase. The currently used replicases are not compatible with modified bases, but because antigen coding RNA strands are *de novo* synthesized inside the cell, these will contain natural uridines regardless. The amplification/replication process is known to have an adjuvant effect and saRNA is therefore almost exclusively explored for vaccine applications [34,35].

Optimization of the mRNA secondary structure also results in extended protein expression [36]. Mauger et al. separate the effects of codon optimization from pseudouridine substitution and show the effect of Ψ, m1Ψ and mo5U (5-methoxy U) incorporation in secondary structural integrity by optical thermal melting experiments. Interestingly, although assumed to have a positive effect in terms of innate immune response evasion, mO5U incorporation had a negative effect on thermal stability compared to uridine. Pseudouridine (Ψ) and N1-methyl-pseudouridine (m1Ψ) form more stable base pairs than uridine, so the rank-ordering of the thermal stability of identical sequences with different uridine substitutions is mO5U < U < Ψ < m1Ψ. It was noted, however, that the effect may still be context-dependent, so the effect of base substitution may have a bigger impact on folding energies in one situation than the other. In addition, it was found

that a lower degree of secondary structure in the 5′ leader region (the 5′ UTR and first ~10 codons of the coding sequence) correlated with higher protein expression, probably because it allows easier access for the ribosome. Surprisingly, in the remainder of the mRNA coding sequence and 3' UTR, higher degrees of secondary structure correlated with increased expression, for reasons less well understood [36].

Taken together, these studies demonstrate that a fully intact mRNA molecule is a prerequisite for protein expression, and that a higher degree of secondary structure content is a more favorable feature for protein expression. At the same time, the effect of these structural factors on the pharmaceutical stability of mRNA products has not been addressed as yet. Interestingly, the same situation is described in a recently published overview on the effects of chemical modifications of mRNA molecules. It only considers in cell/in vivo performance as leading outcome parameter, and effects of these modifications on pharmaceutical properties of mRNA, such as storage stability or shelf life, have not been determined [37].

Stage-appropriate analytical characterization of mRNA Drug Products during development

In this section, we focus on the analytical methods used to assess the stability of mRNA and mRNA-LNP drug products during their clinical development (leading towards eventual market approval), rather than providing an exhaustive description

of all published methods for mRNA and LNP characterization [38]. The analytical toolbox for mRNA and mRNA-LNP characterization will evolve during the progression from pre-clinical development to clinical trials to regulatory approval and the purpose and requirements will be different for each stage of development. For example, pre-clinical work is focused on characterization of materials, understanding the processes, and development of the analytical methods themselves, especially for such new and complex drug products as mRNA-LNPs. During early clinical subsequent stages of development, the purpose of analysis can vary from in-depth characterization of impurities and degradation products, to monitoring the known and proposed critical quality attributes (CQAs) of batches derived from different processes during scale-up. In the late-stage development phase, this emphasis of analysis will shift to batch release, stability studies and establishment of shelf-lives[39,40]. Regulatory agencies also recognize that during different stages of development the depth of product knowledge varies, and thus different quality criteria (i.e., specifications) are appropriate for each analytical method. This is commonly referred to as ‘stage-appropriate characterization’ in which all the accumulated experience from the development stage work should ultimately be the basis for setting the quality criteria for ultimate market approval. The dynamics of this analytical development process are exemplified in the Comirnaty Public Assessment Report where it is mentioned that during the rolling

review of the data, additional characterization data and tests were requested before final approval[41].

In a recent review, a Quality-by-Design (QbD) framework for future development, design and control of manufacturing processes for mRNA and LNP products was proposed. In order to implement this approach, a thorough understanding of process parameters (Critical Process Parameters, CPPs), analytical methods, and Critical Quality Attributes (CQAs) is also required [42]. To ensure rapid regulatory convergence of the currently approved mRNA-based COVID-19 vaccines, they were developed under a more conventional Quality-by-Testing paradigm due to the unprecedented speed with which they were developed and the fact that these products were "first-in-class". The World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) hosted several meetings late 2020 to document regulatory considerations in the evaluation of the quality, safety and efficacy of mRNA-based prophylactic vaccines for infectious diseases[43]. Initial guidance for mRNA vaccines was based on previous guidelines for conventional vaccines, including criteria for the consistency of starting materials and production processes, and evidence for preclinical safety and efficacy. For regulatory considerations specific to this class, the WHO committee sought input from regulatory authorities from all WHO regions as well as from expert vaccine developers and manufacturers. At that time, mRNA vaccines against several other

viral pathogens had already been tested for safety in clinical trials[44,45]. Representatives from BioNTech, CureVac and Moderna shared their experiences with manufacturing, quality control as well as nonclinical and clinical aspects in the development of these clinical candidates. Interestingly, it was already flagged at this stage that the storage stability of these mRNA-based candidates is a major challenge, and that the required frozen vaccine cold chain may be a limitation for use in certain areas of the world. In addition, participants in these discussions acknowledged that early engagement with regulatory agencies and regulatory flexibility were key to the rapid development and approval of the COVID-19 vaccines[43,46]. Here, "regulatory flexibility" refers to the "Rolling Review" of the data. Rolling Review is a regulatory emergency tool that allows the applicant to submit sections of its Biologic License Application (BLA) or New Drug Application (NDA) for review as soon as data become available. In normal circumstances, the regulatory review does not begin until the entire application has been completed and submitted[47].

Because specific details of bulk and drug product production processes vary between manufacturers, and much of this information is currently kept confidential, the final criteria for regulatory approvals were left to be decided by each of the individual national regulatory authorities. For example, the criteria and acceptance ranges for specific manufacturing steps are not accessible in the public

domain and companies often decline to respond in public to such inquires[48]. Although the United States Pharmacopeia (USP) has recently released a Draft Chapter on Analytical Procedures for mRNA Vaccine Quality with methods to support the testing of quality attributes for mRNA-based vaccines, the document does not include any acceptance criteria. In Table 2, analytical methods for assessment of various quality attributes of the bulk purified mRNA drug substance, as proposed by the USP guidance, are listed[49]. A similar summary is provided for the formulated mRNA drug product in Table 3.

In this USP guidance, Drug Substance (DS) refers to the active pharmaceutical ingredient that is tested before being formulated into the Drug Product (DP). The DP is the final dosage form as it will be stored, shipped and presented to the patient or healthcare professional. In this case, the DP consists of a lipid nanoparticle formulation containing four lipid excipients, encapsulating the mRNA, dispersed in a buffered medium suitable for freezing and for injection. Several tests related to mRNA purity that are performed on DS are repeated after formulation in the DP, because the formulation process can have an impact on the quality of mRNA as well[27]. Additional tests are related to LNP quality and purity of the lipid materials. The attributes in the last rows include standard compendial tests for parenteral products, for example, pH, osmolality, visual appearance and viscosity. The two different analytical packages and moments of assessment also reflect the fact that

the DS and DP can be made in different facilities, which means that the DS will be shipped from one place to another and may be stored for an extended period of time. Re-testing of the DS therefore may also be required before it is formulated[50,51].

While different methods serve different purposes in different stages of development, ultimately analytical development should lead to a specification suitable for batch release and for stability testing of both the DS and DP. The EMA ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) definition of stability testing is to provide evidence on how the quality of the drug substance or drug product varies over time, under the influence of environmental factors such as storage temperature, humidity, and light. This should establish a re-test period for the drug substance or a shelf-life for the drug product at the recommended storage conditions. Stress testing of drug substances and/or drug products allows for the identification of degradation products and degradation pathways, and at the same time, can validate the stability indicating power of the analytical procedure[52]. To illustrate, endotoxin burden is an important quality attribute to ensure safety. As endotoxins are mostly introduced by the starting materials, endotoxin levels are not likely to change over time and measurements are not indicative of chemical stability of the mRNA. As another example, while RNA content is a critical attribute and is typically

measured by UV-spectroscopy, this method is not capable of distinguishing intact RNAs from degradation products, so additional assays are needed to monitor RNA stability. Below, we focus on the stability-indicating methods to monitor structural attributes of mRNA and mRNA-LNPs (highlighted in bold in Tables 2 and 3, respectively).

Table 2. Critical Quality Attributes and methods of assessment for mRNA Drug Substance as outlined in draft USP guidance (ref. 47). Stability-indicating attributes for DS are highlighted in bold.

Quality	**Attribute**	**Method(s)**
Identity	*mRNA sequence confirmation*	*Next generation sequencing (NGS), Sanger sequencing, Reverse Transcriptase–PCR*
Content	***mRNA content***	*RT-qPCR and RT–dPCR, Ultraviolet Spectroscopy*
Purity and Impurities	***Percentage of mRNA main peak, pre- and post -main peaks***	*IP-RP-HPLC, Capillary gel electrophoresis*

	5' cap	*IP–RP–HPLC*
	3' poly(A)	*IP-RP–HPLC*
	dsRNA	*Immunoblot, dot blot, ELISA, analytical LC*
	Residual DNA template	*qPCR*
	Residual solvents	*GC*
Safety	*Endotoxin*	*USP <85>*
	Bioburden	*USP <61>, <62>, <1115>*
	Sterility	*USP <71>*
Other	***Appearance***	*USP <790>*
	pH	*USP <791>*

Abbreviations: RT-qPCR, Reverse Transcriptase, quantitative Polymerase Chain Reaction; dPCR, digital PCR; IP-RP-HPLC, Ion Pairing Reverse Phase High Performance Liquid Chromatography; dsRNA, double-stranded RNA; ELISA, enzyme linked immunosorbent assay; LC, liquid chromatography; GC, Gas Chromatography

Table 3. Critical Quality Attributes and methods of their assessment for mRNA-LNP Drug Product as outlined in draft USP guidance (47). Stability-indicating attributes for DP are highlighted in bold.

Quality	**Attribute**	**Method**
Identity	*mRNA Sequence confirmation*	*Next generation sequencing (NGS), Sanger sequencing, Reverse Transcriptase–PCR*
Content	***mRNA content***	*RT-qPCR and RT–dPCR, Ultraviolet Spectroscopy, Ribogreen assay*
	mRNA Encapsulation	*Ribogreen assay, IEX chromatography*
Potency	***In vitro expression***	*Bioassay*
Purity and Impurities	***Percentage of mRNA main peak, pre- and post -main peaks***	*IP-RP-HPLC, Capillary gel electrophoresis*
	5' cap	*IP–RP–HPLC*
	3' poly(A)	*IP-RP–HPLC*
DP specific attributes	***Lipid content***	*LC-CAD, LC-MS, FFF-*

		MALS-UV-dRI
	Lipid identity	*LC-CAD, LC-MS, FFF-MALS-UV-dRI*
	LNP size	*Dynamic light scattering*
	LNP polydispersity	*Dynamic light scattering*
	LNP surface charge	*Electrophoretic light scattering, PALS, Capillary Electrophoresis*
Purity and Impurities	***Individual and total lipid impurities***	*LC-CAD, LC-MS, FFF-MALS-UV-dRI*
	RNA-lipid adducts	*IP–RP–HPLC*
	Residual solvents	*Gas Chromatography*
Safety	*Endotoxin*	*USP <85>*
	Bioburden	*USP <61>, <62>, <1115>*
	Sterility	*USP <71>*
Other	***Appearance***	*USP <790>*
	pH	*USP <791>*

	Particulate Matter	*USP <787>, USP <788>*
	Osmolality	*USP <785>*
	LNP morphology	*CryoEM, SANS, SAXS, FFF-MALS-UV-dRI, DSC*

Abbreviations: RT-qPCR, reverse transcriptase, quantitative polymerase chain reaction; dPCR, digital PCR; IEX, ion-exchange; IP-RP-HPLC, ion pairing reverse phase high performance liquid chromatography; LC, liquid chromatography; CAD, charged aerosol detector; MS, mass spectrometry; FFF, field-flow fractionation; MALS, multi-angle light scattering; UV, ultraviolet; dRI differential refractive index; CryoEM, cryo electron microscopy; SANS, small angle neutron scattering; SAXS small angle X-ray scattering; DSC, differential scanning calorimetry

RNA purity and degradation products

Fragments, extended strands, and dsRNA. A key aspect of Chemistry, Manufacturing and Controls (CMC) development for mRNA drug products is to implement analytical quality control protocols to monitor the level of impurities resulting from the synthesis of the active substance as well as the degradation products that are formed during the manufacturing and storage of the drug product. The mRNA synthesis process (the IVT reaction) will inherently produce many

impurities that are very similar in structure to the correct, full-length mRNA[53]. The levels and nature of impurities were also found to vary greatly from batch to batch, especially when produced at different scales and different processes[39]. As shown in the schematic outline of an mRNA sequence in Fig. 2, the antigen coding region makes up the largest part of the mRNA strand and is the part of the sequence that is translated to the desired protein. Therefore, the coding region needs to be intact and correct, otherwise the desired protein will not be produced. Impurities from the synthesis are typically shorter fragments that arise from premature termination in the IVT reaction. Occasionally, the produced strands can be extended on the 3' end by a mechanism called self-priming, resulting in strands that are longer than the expected mRNA sequence[54]. Another unwanted byproduct of the IVT reaction is double-stranded RNA (dsRNA) that inside a cell can be recognized as a sign of viral invasion and is therefore highly immunogenic[55]. The levels of these impurities are critical quality attributes (CQAs) for the mRNA and hence closely monitored during the clinical release process and while testing the stability, as they may affect drug product quality. In Tables 2 and 3, analytical tests to monitor these CQAs are referred to as mRNA sequence identity, mRNA sequence confirmation and mRNA purity tests for the DS and DP, respectively.

Capless and tail-less mRNA. Other full-length impurities that arise from the synthesis are the cap-less and tailless mRNA; these altered mRNAs are also

potential degradation products (Fig. 2). The cap-less impurity refers to an mRNA without a 5’ cap or an analog of the cap and tailless refers to an mRNA without the 3'-polyadenosine (poly-A) tail. Both the 5’cap and the poly-A tail are essential for the mRNA to be translated into a protein. Consequently, an mRNA chain without cap and /or tail will be less active or inactive, meaning both the amount of capped mRNA and amount of Poly-A tail need to be controlled. In addition, the 5’ cap is important for the mRNA stability, as it protects the mRNA from exonuclease degradation[56]. During the capping process, the cap part is incorporated into the mRNA. The 5′-5′ m7GpppG cap, which is referred to as cap 0, is then further methylated to produce the cap 1 analog m7GpppG(2’-OMe) as shown in Fig 2. The efficiency of the 5’ capping process as well as the level of each cap analog should be included in the analytical control strategy for both DS and DP (Tables 2 and 3).

RNA degradation pathways during manufacturing and storage

In addition to impurities formed during synthesis, degradation products formed during manufacturing and storage (of the DS and the DP) need to be assessed. While impurities coming from the DS production process (the IVT reaction) will not likely increase over time, impurities formed by degradation *are* likely to increase over time. Table 4 summarizes the theoretical degradation pathways for mRNA and Fig. 4 shows some reaction prone sites on the mRNA molecule[57–60].

Table 4. Summary of different types of mRNA degradation pathways and their potential sources

Degradation products	Source
RNA Fragments	Hydrolysis (base) Heat Peroxides, H_2O_2 RNase enzymes
Depurination (abasic site)	Hydrolysis (acid) Oxidation
Deamination-hydrolyzed bases	Hydrolysis (acid)
Oxidation of bases	Auto oxidation Metal residues Light

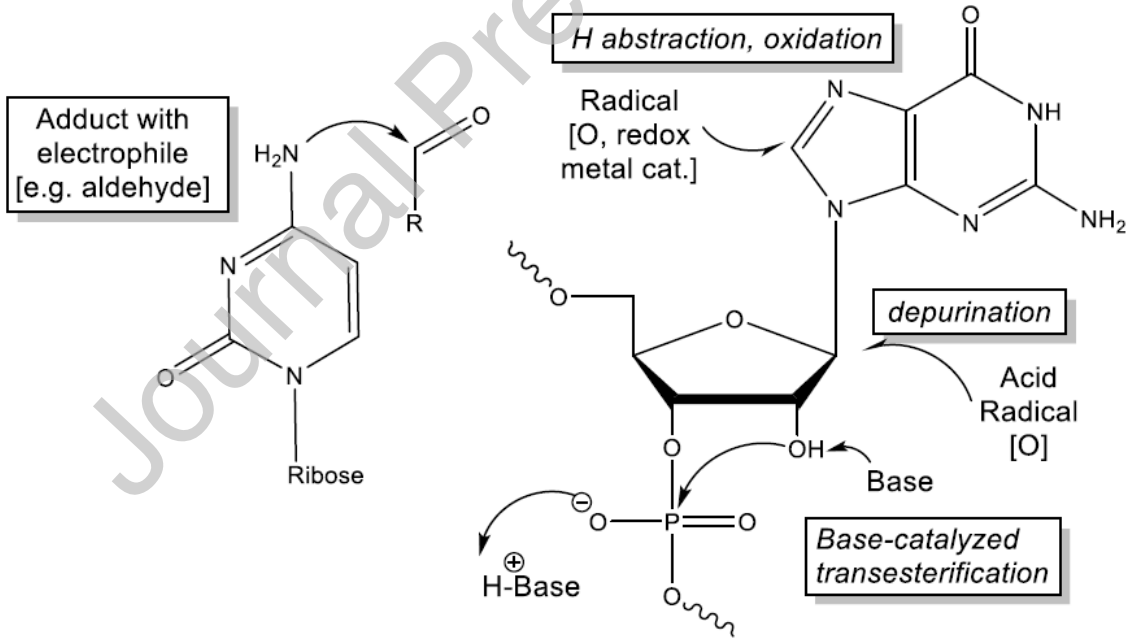

Fig. 4. Schematic of theoretical mRNA transesterification mechanisms and their sites on the mRNA molecule. [O] : oxidant. The R (lipid)-aldehyde forming the lipid-mRNA adduct is the result of lipid oxidation reactions of the ionizable lipid.

Shorter RNA fragments are the most probable mRNA degradation product formed by hydrolysis of the 3'- 5'-phosphate diester bond in the backbone, resulting in cleavage of the chain[61]. The kinetics of the transesterification reaction are accelerated by alkaline pH and thermal stress. Thus, around pH 7 and through storage in a freezer (-20°C or -70°C), the mRNA degradation by chain breakage will be minimized. The formation of cap-less and tailless mRNA follows the same chemical degradation pattern as for the formation of any otherfragment throughout the mRNA chain. Strand breakage can also be caused by other mechanisms including oxidation (in presence of peroxides or by auto-oxidation[62]) or by enzymatic degradation (RNases). To mitigate such mRNA degradation and prevent cleavage by RNases, a drug delivery system to encapsulate the mRNA is almost always needed. There is evidence that 'naked mRNA' intradermally injected in human patients leads to translation and in the case of a intranodally injected vaccine, to the desired immune response[63,64]. But nowadays, almost all mRNA trials use a delivery system to protect the mRNA cargo *in vivo* and facilitate uptake in the target cells following more practical injection routes. Currently, the most commonly used delivery system is a lipid nanoparticle (LNPs). However, the lipids

used to prepare LNPs can be a source of reactive impurities such as peroxides and aldehydes, that could also degrade the mRNA[65].

Two other chemical degradation pathways include depurination and deamination (Table 4; Figure 4). Depurinated or abasic sites of the mRNA are degradation products where the backbone and sugar are intact, but the base is lost. These products are formed by hydrolysis at low pH or by oxidation [60]. This reaction is commonly referred to as depurination because loss of guanine and adenine bases occurs at a much higher rate than depyrimidination. Deamination is degradation through hydrolysis of the base, which can happen in acidic conditions. The sugar and base will remain attached, but the reaction will transform cytidine, adenosine and guanosine into uridine, inosine and xanthosine, respectively[66]. Therefore, this degradation pathway can theoretically change the coding sequence of the mRNA, which in turn would lead to amino acid mutations in the produced protein.

When the mRNA drug substance or drug product is stored at neutral pH and frozen, degradation caused by depurination and deamination has been found to be of low risk with degradation rates being extremely low; therefore, no control strategy is required[67,68]. In contrast, oxidation of the bases in the mRNA, as caused by oxidation reactions often catalyzed by redox-active metal residues (iron, copper, etc.) or promoted by light exposure, are more likely to occur (Table 4; Figure 4). These impurities, often associate with the lipids in the LNPs, are a potential risk

for degradation of the mRNA and require analytical control strategies as outlined below.

Potency/ In vitro expression / Biological activity

The chemical degradation pathways of the mRNA described above can generally be monitored with physicochemical methods. However, bioassays will tell the effect of such alterations on biological potency and when the correlation between chemical alterations and biological performance is not yet fully established, such potency tests are expected by most regulatory authorities as described by Knezevic et al.[44]. Methods to consider for monitoring *in vitro* potency include cell-free translation of mRNA extracted from the DP or transfection of cells in a culture with the DP to confirm the mRNA encodes the desired protein. Currently, there is no regulatory convergence yet on how these bioassays are best performed and whether they can ever be fully replaced by physicochemical analytical methods.

mRNA Purity and degradation products: mRNA-lipid adducts

As recently pointed out by Packer et al., amino lipids, such as those used in the Moderna and Pfizer/BioNTech mRNA-LNP COVID-19 vaccines, are a potential source of reactive electrophilic impurities that can inactivate the mRNA[65]. These reactive impurities are generated through N-oxidation of the tertiary amine and subsequent hydrolysis leads to generation of secondary amines and reactive

aldehydes. It was already known that aldehydes can react with DNA bases[69,70]. Subsequently, it was shown that aldehydes from the N-oxide degradation pathway of a representative ionizable lipid can also form covalent mRNA-lipid adducts (Fig. 4) that correlated negatively with biological performance[65]. Of note, any amino lipid with a tertiary amine is susceptible to N-oxidation. Quaternary amines are not susceptible to N-oxidation due to lack of available electron pairs, but their permanent charge may introduce safety risks

It is therefore of great importance to control the conditions in which these electrophilic impurities are formed. This means that all lipid components in an LNP (including the helper lipids) have to be evaluated for the risk of inducing reactive lipid impurities that result in mRNA-lipid adducts. A test for the amount of mRNA-lipid adducts is now expected to be part of the quality control protocol. This assumption is based on questions to Moderna and Pfizer by EMA about "*lipid-related impurities originating from the degradation of the LNP*" and the data presented in the Packer publication[41]. The amount of mRNA lipid adducts is also expected to increase during long-term storage and handling of the drug product, and therefore, should be tested in all stability studies for LNP containing drug products.

LNP (lipid) purity and degradation products: Product-related impurities

In addition to the active drug substance (mRNA), LNPs consist of several components that can interact with each other and thus can influence the overall stability of the drug product. For the lipids, the major degradation pathway is oxidation due to exposure to light, oxygen, metal residues and high temperatures. The two chemical groups that are well-known as potential substrates for oxidation on the lipids are unsaturated alkyl chains and polyethylene or polypropylene oxide groups[71,72]. The LNP formulations used in the Moderna and Pfizer mRNA vaccines do not contain such unsaturated lipids, but the ionizable amino lipid found in the siRNA/LNP product Onpattro (Dlin-MC3-DMA) does contain allylic bonds in the tail region, similar to linoleic acid tails. In fact, it was reported that oxidation of this structure does indeed occur[73].

As an example of detrimental interactions between the lipid components, the PEG-lipid (PEG = polyethylene glycol) is a potential source of hydroperoxides. Hydroperoxides are frequently unstable and the decomposition of hydroperoxides can lead lead to chain reactions that may also catalyze degradation of the ionizable lipid. Some of these lipidic degradation products may also react with the mRNA, where some lead to formation of covalent mRNA-lipid adducts, as described above, or induce other reactions that lead to degradation of the mRNA. As such, peroxides are a suspected source of degradation that can damage the mRNA

directly or indirectly. It is therefore of great importance to source high-quality raw materials that are purified from hydroperoxides and metals[74].

In addition, degradation by hydrolysis of lipids containing ester bonds needs to be considered. The key factors that have an effect on hydrolysis are lipid chemical structure (sterics and electronics of the ester function), pH, temperature and buffer composition. The first stage degradation by-products of hydrolyzed phospholipids are single lipid tails with a carboxylic acid head and single tail residues of the phosphate head called lysophospholipids[75]. The ionizable lipids in the mRNA-LNP products contain carboxylic ester bonds and therefore, in theory, are also susceptible to hydrolysis during storage. This degradation pathway is less relevant for freezer-storage temperatures, but may become a bigger risk when non-frozen storage conditions are used. Lipid hydrolysis is expected to have an impact on the structural integrity of the LNP which could in theory lead to a lower potency of the DP. An *in vitro* expression assay, or bioassay, should be able to detect such changes in potency if these lipid hydrolysis reactions occur.

Tests and methods typically used for stability assessment of mRNA

The following section covers the analytical principles used to assess the purity and the stability of the mRNA, both in aqueous solution as a drug substance as well as formulated in (or extracted from) the LNP as a drug product. Molecular size,

physical and chemical properties of the mRNA pose a significant analytical challenge. Due to the large size of the mRNA, the number of negative charges, and the structural similarity of degradation products (compared to the main component), several powerful separation techniques are required to assess the stability. Intact mRNA is too large for MS detection, therefore, any analytical method using MS for characterization requires a digestion step prior to any analysis. Consequently, bioinformatics requirements for interpreting MS data increase.

A common method to characterize the integrity of the mRNA, i.e., to measure the purity and impurity profile, is IP-RPLC-UV (ion-pair reverse-phase liquid chromatography coupled to UV detection) (USP vaccine chapter[76].[49] A reversed-phase stationary phase together with a mobile phase containing ion-pair reagent enables retention and separation of the negatively charged mRNA. The separation of fragments with different sizes is based on hydrophobic interaction with the column material. UV detection at 260 nm is employed, as the aforementioned size restrictions preclude the use of MS detection. The IP-RPLC-UV method needs to be optimized to detect the main degradation products, i.e. the shorter fragments, to ensure the method is stability-indicating.

Capillary electrophoresis is an orthogonal method to IP-RPLC-UV, where the separation is based on size. The separation medium is a low viscosity “separation

gel", consisting of polymers such as polyvinylpyrrolidone (PVP) and additives, typically glycerol, as viscosity enhancers[49,77,78]. The polymers form a three-dimensional network that allows the separation based on size. Larger molecules tangle up in the polymer network to a greater extent than smaller fragments and thus migrate slower during the electrophoresis process. The quantification of the fragments and intact mRNA can be either by UV 260 or fluorescence detection. In the case of using fluorescence detection, an intercalating dye is required to be included in the gel to enable detection[49].

An alternative technique for purity measurements for mRNA suggested by Kanavarioti[76], is Ion Exchange Chromatography (IEX). In the case of mRNA, anion exchange is the chromatographic mode. Here the stationary phase consists of a cationic resin that separates the molecule based on charge. This technique involves high risk for on-column degradation of the mRNA analyte, however, since extreme conditions such as high pH and high temperature are required for elution of the mRNA from the column.

For quantitative assessment of the 5´capping efficiency and potential degradation of capped mRNA to uncapped mRNA, the suggested chromatographic mode is IP-RPLC coupled to MS. Several publications demonstrate the possibility to isolate the cap part from the mRNA chain by using RNase H digestion[56,79]. This approach requires a corresponding cap-binding complex that will form a DNA-RNA hybrid.

RNase H then specifically cleaves the DNA-RNA hybrid from the rest of the mRNA chain. Typically, the size of the cap fragment is around 25 nucleotides, which can be characterised with LC-MS to distinguish between different cap variants and quantify the level of capped mRNA[49,56,79].

To control the amount of tailless mRNA, IP-RPLC-UV can be used. Compared to the method for fragment detection described above, where the separation is size dependent, the tailless impurity method needs to be sequence specific. Adding chemical additives to the mobile phase enables separation of chains that are tailless from chains that have an intact Poly-A tail, and a quantitative assessment of the tailless impurity is possible[80]. To characterise the Poly-A tail length and distribution, Beverly et al. propose IP-RPLC coupled to MS using RNAse T digestion. In this case, RNase T cleaves the tails from the mRNA chain and after isolation by dT magnetic beads, the extracted poly-A tail materials can be analysed further [79,81].

Finally, the total content of the RNA needs to be controlled. For the drug substance this can be measured with UV absorbance readings at 260 nm. In addition, for LNP-based drug products, the encapsulation efficiency is a critical quality attribute. For the RNA quantification in LNPs, the Quant-it RiboGreen reagent (Thermofisher) characterization is widely used, because it can differentiate

between RNA inside and outside the LNP to calculate a percentage encapsulated RNA, which is not straightforward with other methods[82].

Methods for analysis of lipids, lipid impurities and lipid-mRNA adducts

The FDA has published a 'Guidance for industry for liposome drug products' in 2018[83] but a similar document that is directly applicable to RNA-LNP drug products is not yet available. Prior to the approval of the mRNA vaccines, the only documented example was the registration of Onpattro™, the first LNP-siRNA product approved[84]. The major differences between liposomal drug products and RNA-LNPs (both siRNA and mRNA) are the internal structure of the particle and the complexity of the API. As discussed before, for the mRNA-LNP products, analytical methods related to the possible interaction of the ionizable lipid and the API were requested. In addition, FDA and EMA guidelines state that for the characterization of novel excipients such as ionizable lipids or novel PEG-lipids, the level of detail provided in the submission should be comparable to that for a drug substance[85,86].

For determination of the purity and stability of lipids in the LNP, methods based on LC with CAD (charged aerosol detection) are most common, as the lipid components tend to lack a UV chromophore. Chromatographic conditions can be chosen such that the four main lipid components and their degradation products are

separated[87]. The work by Kinsey et al. also includes an example of how stress testing, or a forced degradation study could be conducted for the lipid components. For detailed characterization of lipid impurities and lipid degradation products, additional analytical detection techniques based on mass spectrometry are required[88]. The identification and quantification of secondary oxidation degradation products of the lipids by LC-MS methods has been described by Abeyrathne et al.[89]. For measurement of hydroperoxide levels in the lipids, methods based on iodometric titration, UV spectroscopy, chromatography, among other techniques, have been summarized[90].

Methods for the detailed characterization of the proprietary lipid components and their oxidation/degradation products are still emerging. Recently, an application note was published describing the use of electron-activated dissociation (EAD) to distinguish the oxidative impurities from ionizable lipids with great resolution and sensitivity[73].

The amount of mRNA-lipid adducts in LNP drug products that result from reactive impurities in the ionizable lipid can be measured by RP-IP HPLC [65]. These impurities cannot be measured using commonly-used kit based capillary electrophoresis methods with fluorescence detection. This may be due to interference with the fluorescence derivatization by the lipid covalently bonded to the mRNA, or the different nature of the separation mechanism of the mRNA-lipid

adducts from that of RNA fragments and full-length mRNA. As described above, conventional RP-HPLC is based on hydrophobic interactions with the stationary phase, but the phosphodiester backbone of the mRNA is highly charged, necessitating use of high concentrations of ion pairing salts to neutralize the negative charges. Alkylammonium salts were selected to retain selectivity to differences in hydrophobicity due to sequence variation or chemical modifications. When IP-RP-HPLC was used to analyze mRNA extracted from LNPs, an additional peak was detected that eluted later than the full-length mRNA. This late peak could not be detected by capillary electrophoresis. It was found to be caused by the lipid-mRNA adducts and their presence correlated negatively with the amount of protein produced from the mRNA[65]. As mentioned earlier, a test for these lipid-mRNA adducts should now be included in every stability assessment.

Attributes specific to the LNP formulation

This review has largely focused on the stability of the mRNA, but as shown in Table 3, there are several quality attributes to be monitored related to the drug product nanoparticle formulation. First, there is the chemical identity and stability of the lipid excipients which is described above. Secondly, there is the physical stability of the nanoparticle that can be monitored by measuring particle size, size distribution (polydispersity) and surface charge, using light scattering techniques. Changes to these attributes indicate that there is a disintegration or redistribution of

the LNP components. The Onpattro siRNA-LNP product has a shelf-life of 36 months as a liquid formulation stored refrigerated[13]. This profile indicates that particle physical stability is not a major risk when a chemically stable API is used. However, the internal structure of mRNA containing particles is different from those containing siRNA[7] and mRNA-LNP particles are more likely to disintegrate upon mechanical stress, such as shaking and dropping of vials[91,92] . Another example is the formation of layers of aggregates on filter surfaces during sterile filtration[93]. A more in-depth description of characterization of the particles can be found in these four publications[27,94–96]

Can we get rid of the sub-zero cold chain for long term storage of mRNA-products? What are the options?

Stabilizing mRNA and mRNA formulations in aqueous milieu

From the discussion above, it is clear that the major determinants for the stability of the mRNA-LNP drug product includes (1) length of the mRNA chain, (2) the secondary/tertiary structure of the mRNA and the mRNA-LNP complex, (3) selection of nucleotides, and (4) drug product formulation composition including associated impurities (e.g., lipid type and source, excipient type and source, solution pH) . Alishetty et al.[97] calculated the half-lives of free FLuc mRNA at room temperature with an equation published by Li and Breaker[98]. This equation

doesn't take secondary structural effects into account. The outcome of the calculation was a lower degradation rate for a free mRNA molecule compared to the mRNA in two LNP formulations. Moreover, a clear degradation rate dependence on the type of ionizable lipid was observed[97]. These effects could potentially be explained by the formation of stabilizing secondary/tertiary structures of the free mRNA, which are disturbed in the complexation with LNP, or by local upward pH shifts inside the LNP because of the presence of closely packed cationic lipids or the higher 'proton solvation energy' [97,99,100].

Shortening the mRNA chain length reduces the chance of chain breakage resulting in the inactivation of the mRNA. Zhao et al. employed a shortened mRNA chain length of 1100 nt (nucleotides) compared to 4000+ nt for the Pfizer BNT162b2 and Moderna mRNA-1273 vaccines, and showed 76% intact mRNA, no change in particle size-polydispersity index or encapsulation efficiency, and preserved immunogenicity in mice after six months of storage at 2-8 ^{o}C[101].

The choice of the cationic lipid influences LNP-mRNA stability as reported by Ripoll et al. who used imidazole cationic lipids with a flexible connection between lipid and headgroup, and they found enhanced stability compared to 'reference' cationic lipids[102]. Multiprotic cationic lipids reportedly have a similar stabilizing effect[97]. Moreover, the sourcing and associated impurity profile of excipients in the formulation has been shown to be critical to ensure mRNA integrity on storage.

For example, as discussed in detail above- cationic lipids used in the mRNA products are sensitive to oxidation reactions, followed by hydrolysis and aldehyde formation[65]. Since cholesterol, phospholipids and PEG-derivatives may all contain peroxide impurities, attention should be paid to the quality of those LNP constituents[103,104]. usp

Comirnaty®, the Pfizer/BioNTech COVID-19 vaccine, was originally formulated in a phosphate buffer containing KCl/NaCl ("purple cap formulation"), which presents major challenges in the freezer due to potential pH shifts during freezing of phosphate buffers in the presence of sodium ions. The formulation was changed to a second-generation formulation containing Tris buffer at the same solution pH ("gray cap formulation")[105]. A prolonged storage time in the refrigerator was granted by regulatory agencies, from up to 1 month to 10 weeks at refrigerator conditions with retention of the long-storage condition of -80 ^{o}C before refrigeration[106,107]. Interestingly, Tris buffer was used in Moderna's SpikeVax® formulation from the beginning, but it still has a shelf life of 30 days in the refrigerator. This is explained by the difference in long-term storage conditions. For SpikeVax® the long-term storage has always been -20 ^{o}C, to facilitate widespread distribution during the pandemic. Under these conditions some level of degradation is seen over long periods (relative to -80 ^{o}C). Therefore the storage period in the refrigerator is not as long as for Comirnaty®, which continues to use

the -80 ^{o}C infrastructure for distribution and long-term storage. Another advantage of Tris buffer, apart from being more suitable for freezing of liquid formulations compared with phosphate-buffered saline, is that it reacts with aldehyde impurities and acts as an 'aldehyde sink' and by that reduces mRNA-lipid adduct formation (Figure 5)[19]. This is an excellent example that the choice of excipients can have a significant impact on mRNA shelf life. The conclusion of this section is clear on the basis of the present insights: long-term storage of mRNA-LNP drug products in non-frozen condition is not feasible unless the product can be commercialized with a very short shelf-life.

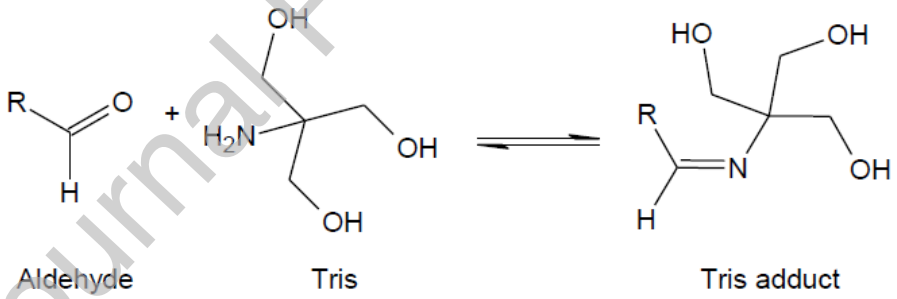

Fig 5. Tris buffer acting as an "aldehyde sink" by formation of an imine adduct (adapted from Moderna Science and Technology day, May 2022, slide 76)[19]

Stabilizing mRNA and mRNA formulations by drying

Freeze-drying of mRNA Drug Products: Given the poor long-term stability profile of mRNA or mRNA-LNP in aqueous solution, and the limited success to improve shelf-life under non-frozen conditions as described above, the pharmaceutical formulation scientist would turn to drying the drug product, and lyophilization is then the logical drying technique to begin with due its wide use in the pharmaceutical industry. In India, GEMCOVAC™-19, a freeze-dried form of an mRNA vaccine encoding for the S-protein of SARS-CoV-2, is authorized for use[108]. In addition to the mRNA, this formulation consists of DOTAP (dioleoyl-3-trimethylammonium propane) and squalene stabilized with polysorbates, sucrose as lyoprotectant-osmolyte, and citrate buffer. This product can be stored in a refrigerator, but no further details regarding its stability profile could be found in the public domain.

The patent literature is a rich source of information for freeze-drying of mRNA formulated in various delivery systems. For example, mRNA–protamine complexes were freeze-dried with lyoprotectants such as trehalose. Under optimized process conditions, mRNA chain integrity and *in vivo* preservation of activity after prolonged (12 months) storage at 25 ^{o}C was claimed[109–112]. Most subsequent reports of freezing-drying mRNA formulations have focused on mRNA-lipid complexes, and the number of publications is growing. For example, Zhao et al. prepared mRNA-LNP with a cationic lipid different from the marketed

mRNA-COVID-19 vaccines[113,114]. They compared physicochemical parameters, and *in vitro* and *in vivo* translation of luciferase-encoding mRNA-LNP before and after different processing, including: freeze- thaw cycles, freezing in liquid nitrogen, and freeze-drying using trehalose, sucrose and mannitol as cryo-/lyoprotectants. Interestingly, they found a discrepancy between *in vitro* and *in vivo* performance of the freeze-dried formulation: while no detectable loss of activity was seen in the *in vitro* read out, a significantly lower translation was observed *in vivo*. Importantly, no such discrepancy was observed with the formulations after freezing and storage of the product in liquid nitrogen.

Another report on findings with freeze-dried mRNA-LNPs was published by Ai et al. 2022[114]. Freeze-drying did not affect mRNA integrity, led to small increases in particle size, and slight reductions in encapsulation efficiency. These physicochemical quality attributes remained stable over 2 weeks. Under the chosen conditions, the performance of the freeze-dried/reconstituted material in a number of *in vivo* preclinical tests showed no difference with the 'fresh' mRNA-LNP material. No details were provided on the freeze-drying conditions, nor on the chosen formulation such as pH, buffer and lyoprotectant. In contrast, a recent publication by Muramutsu et al. [115]provides detailed information on the formulation (Tris buffer, pH 8 and sucrose plus maltose), the freeze-drying protocols, and the stability-indicating assays (for mRNA and for the LNP

constituents). After freeze-drying and reconstitution, full integrity and activity of the mRNA-LNP were retained. Stability during storage was dependent on the storage temperature over the wide temperatures range chosen i.e., -80^{o} C, -20 ^{o}C, 4^{o} C, 25 ^{o}C, 42 ^{o}C. These dried formulations showed a significant improvement in stability over time compared to the Moderna and Pfizer/BioNTech COVID-19 vaccines, i.e., weeks compared to hours, respectively, and freeze-drying conditions and lyoprotectants may be further studied to optimize the outcome. [115] So far, no publication has reported on freeze-drying of mRNA-LNPs after the first mixing step with ethanol still in the mRNA-LNP dispersion. This so-called 'alcohol-dilution-lyophilization method', showed success with siRNA[116].

A study of mRNA complexed with DOTAP-squalene (cf., GEMCOVAC™-19 vaccine mentioned above) using preformed lipid particles (so-called nanostructured lipid carriers, NLC) were freeze-dried and showed preservation of mRNA activity after storage in the dried state for 21 months at 4 ^{o}C. This is another example demonstrating freeze-drying as an option for prolonging shelf life of formulated mRNA under refrigerator conditions[117]. Similar results were reported for different mRNA products with the same technology platform by Voigt et al.[118] DOTAP-based lipoplexes with mRNA were freeze-dried in sucrose and translational activity in terms of protein translation was observed *in vitro* and *in*

vivo. No long-term storage data were provided, however, to support the authors' claim for a prolonged shelf-life at refrigerator temperatures[119].

Alternatives to freeze-drying of mRNA Drug Product-

Large-scale vaccine freeze-drying operations to fight pandemics are limited in terms of worldwide production capacity, and moreover, require time, energy input and expensive equipment. Therefore, other alternative drying technologies have been suggested for mRNA formulations. Recent reviews provide a comprehensive overview of alternative drying techniques for vaccines, not specifically mRNA-vaccines[120,121]. Most attention was paid to spray-drying and spray freeze-drying. Examples in the patent literature report on spray-drying of mRNA [122–125] or spray-drying or spray freeze-drying[122] , but so far, no products have been approved using these stabilization approaches. Interestingly, although no publications are yet available, there is interest in the future use of microneedle platforms for intradermal (ID) delivery of mRNA vaccines for future pandemic responses and such dosage forms are often manufactured using evaporative drying[126].

Assembling formulated mRNA drug products 'at the point of use' using a kit approach

There are several other approaches to shorten the supply chain or to circumvent extended contact of the fragile mRNA with the formulation excipients. One way to

avoid long distance frozen shipment is to move the entire factory closer to the place where the vaccines will be used. A turn-key example of this approach is BioNTech's "BioNTainer", a portable factory, consisting of two modules, one for bulk manufacturing of the DS and one for the DP. Fill-and-finish will be supported by a local external partner. Each module is built up from six standard-size shipping containers that will be assembled in the country where the factory will be operated, the first ones to be deployed in Africa[127]. It is expected that from a regulatory perspective, products from this facility will be treated the same as those coming from a conventional factory.

An alternative solution with a lower technological bar, is to assemble the drug product itself at "the point of use". To this end, mRNA drug products can also be considered modular, consisting of the mRNA DS and the delivery system for *in vivo* delivery — the latter being either a lipid-based nanoparticle such as LNPs or NLCs or a polymer / protein such as protamine. If one can stabilize the mRNA molecule, e.g., by freeze-drying (see below) and design stable delivery systems in aqueous solution, then assembling the active drug product 'at the point of use' may address the stability problem and avoid subzero storage conditions (see Fig. 6). This strategy has been explored by a number of groups and their experience is discussed in the following paragraphs. First, stabilizing the mRNA molecule at

non-freezing temperatures and then experiences with assembling the product 'at the point of use' are discussed.

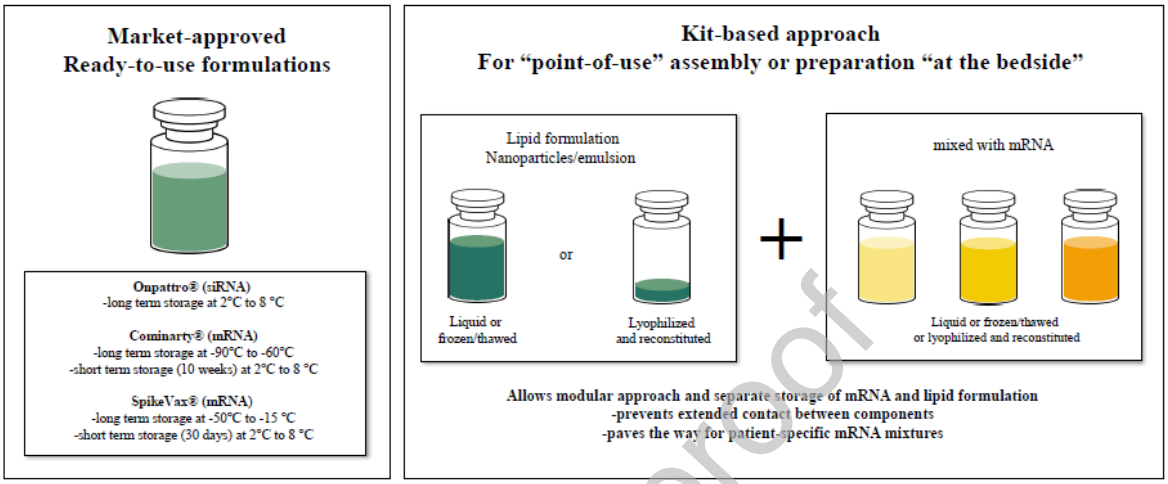

Fig 6: Schematic of 'ready-to-use' mRNA drug product formulations currently marketed versus an approach using 'at point of use' mRNA drug product formulation by employing a 'kit-based approach'.

Drying and stabilizing of the mRNA drug substance

A dried mRNA molecule stable during long-term 4^{o}C storage would be an important step in developing 'at-the-point-of-use' mRNA vaccine formulations with reconstitution using, for example, LNP, emulsion or protamine solutions. What is generally considered the first publication on freeze-drying of mRNA (as naked nucleic acid polymer) appeared in 2007 [128]. One freeze-drying protocol was employed and only two mRNA solutions were compared: with water for injection

(WFI) and with a solution of the lyoprotectant trehalose. Dried samples were stored at -70^{o} C, -20 ^{o}C, 4 ^{o}C, room temperature and 37 ^{o}C. While trehalose clearly performed better than WFI in stabilizing the mRNA during drying and storage, there was clearly room for further improvement. In a 2012 patent ('background of the invention'), Mutzke discusses the state-of-the-art of long-term stabilization of nucleic acid-based pharmaceuticals, including mRNA. Lyophilization is the preferred drying process, and they conclude that among the tested lyoprotectants mannose stood out as most stabilizing [111]. In a patent published around the same time, doubts are raised about the possibility to use sugars as lyoprotectants at high nucleic acid concentrations and reports enhanced stability with lactate when compared to water for injection[110]. In another patent, mannose was not recommended to be used in production operations because of its low -unpractical- glass transition temperature value (compared to disaccharides) of -47^{o}C[109]. It should be pointed out that mannose, a reducing sugar, is a rich source of aldehyde reactants for the unpaired RNA bases, and as such this sugar (and any other reducing sugar, such as lactose) should be avoided as an obvious incompatibility with mRNA.

As an alternative to freeze-drying, spray-drying of mRNA has been described. For example, Karve et al. demonstrated the stability of spray dried mRNA-polymer (not in LNP) combinations both after the drying process and after storage at 4^{o}C

for 5 months[129]. In another approach, Fabre and co-workers looked at the stability of several mRNA molecules in the dried state- using an undisclosed stabilization solution for the vacuum drying process at temperatures above 0 °C. They predict very low chain break degradation rates based on extrapolation of observed chain break rates at high temperatures using an Arrhenius model analysis, a result implying that drying would be a realistic option to improve the stability of mRNA products at non-freezing temperatures. Absolute protection from air/humidity is a prerequisite[130]. In a proof-of-concept study capillary-mediated vitrification of mRNA solutions resulted in dried mRNA with improved storage stability. No information on the underlying stabilization mechanism has been published yet[131,132].

"mRNA printing" for point of use production of the drug substance

An emerging technology that could circumvent frozen storage and shipment of the mRNA vaccines is called "mRNA printing". This term is used for miniaturized, highly automated mRNA manufacturing suites, with the main goal to reduce the footprint of mRNA production, so it can be deployed in remote locations or within the walls of a hospital pharmacy[133]. In a hospital setting, this technique could further pave the way for personalized medicines, for example, to treat (ultra)rare disease or to produce individualized mRNA-cancer vaccines[134]. Moreover, such an

approach could also enable local, larger-scale production of a mRNA DS without the need for shipment or extended cold storage in locations where maintaining cold chain operations could be challenging. The company Greenlight Biosciences is currently preparing for a Phase 1 trial in Africa, with the ambition to develop a vaccine product that could cost $1 per dose. This is a highly innovative field that is exploring very different manufacturing technologies to reduce costs, summarized well by Sheridan. For example, CureVac has partnered with Tesla-Grohmann Automation to develop a device that is fully automated and uses a DNA template that is suspended on magnetic beads, which can be handled by robots and allows multiple production rounds using the same starting material to reduce costs[133,135]. Greenlight Biosciences uses cell-free microbial lysates as a source for the nucleoside monomers and a series of enzymatic reactions to turn them into nucleotides and full-length mRNA. Time will tell which of these technologies will prove to be competitive from an economic and product quality standpoint, and most importantly, how they will be viewed by regulators for wide-spread use. Regardless of the success of such approaches, they will all ultimately require a formulated mRNA drug product to be produced at the point of use, and thus will still need to be assembled into a delivery system prior to administration.

Examples of approaches to assemble mRNA-delivery systems 'at the point of use'.

Currently, large-scale formulation of LNPs containing ionizable lipids requires multiple steps where the lipids in ethanol are mixed with the RNA in an acidic aqueous buffer and where the pH is neutralized and the organic solvent is removed. The RNA-LNPs precipitate into colloidally stable assemblies with the RNA incorporated into the LNPs. This DP manufacturing process can be performed at large scale, but requires specific equipment and multiple down-stream processing steps. This complexity is the reason why, up until now, mRNA-LNP vaccines are presented as ready-to-use formulations, that are manufactured in a few dedicated factories[39,42,50].

A number of groups have explored different approaches to assemble mRNA-delivery systems 'at the point of use' or 'at the bedside' (Figure 6). Some of these studies utilize permanently cationic lipids, which allow for mixing with the mRNA at a pH and in a buffer that is also suitable for injection. For example, in 2014, Brito et al. published results from preclinical immunization studies where mRNA binds to positively charged particles of a pre-manufactured DOTAP/squalene emulsion that enhances the immunological response a 1000-fold compared to 'free' mRNA[136]. In 2018, Erasmus et al. published a two-vial approach where they followed a similar route: a replicating mRNA encoding Zika virus antigens interacts with the surface of pre-manufactured cationic lipid emulsions containing squalene. This study demonstrated complete protection against a Zika virus

challenge in mice[137]. When opting to use aqueous lipid dispersions, mixing mRNA with preformed cationic particles may lead to undesired aggregate formation. To overcome this issue, Kurimoto et al. designed short PEG-oligoRNA strands to hybridize with the therapeutic mRNA molecule. Formation of aggregates upon mixing the mRNA with preformulated LNPs can be mitigated with this approach[138].

Point-of-use assembly can also be done with formulations resembling the marketed mRNA-LNP vaccines more closely, if the formulation parameters are changed. Most importantly, somewhere during the process, the charge on the ionizable lipid needs to be introduced again. For example, Kulkarni et al. reported that mixing 'empty' LNPs in acidic aqueous medium and (primarily) siRNA, leads to formation of complexes that are capable of transfection *in vitro*. Characterization of the morphological changes occurring during the interaction between the siRNA and 'empty' preformed vesicles using cryoTEM demonstrated that particles with similar internal structure as regular LNPs can be produced in absence of ethanol and without the need of specialized mixing devices[139,140]. A similar approach using mRNA was described, where empty LNP formulations were dialyzed to neutral pH for storage, and mRNA or saRNA was later added in acidic buffer for complexation. Most probably due to the larger size of the cargoes, here the nucleic acids were referred to as being on the outside of the LNP, contrary to the siRNA

formulations described earlier. But a comparison of formulations with either externally or internally located saRNA resulted in similar protein expression *in vivo* (mice). A later study with human skin explants showed that externally complexed RNA is a viable approach for this application[141,142].

Finally, an example of 'point of use mixing' actually being applied in the clinic is in BioNTech's Lipo-MERIT study (NCT02410733). This study was one of the first to explore mRNA-based cancer immunotherapies that are specific to a patient's tumor antigens. The drug product consists of four different mRNAs that are complexed to cationic liposomes. To manufacture and supply GMP grade materials, a kit-based approach was selected, where the mRNA and liposomes are manufactured and packed individually as outlined schematically in Fig 6. Immediately before injection, mRNA is diluted and added to the liposomes. Extensive prior optimization and characterization experiments ensure the formation of consistent DP products suitable for intravenous administration[143]. This "kit approach" allows for separate storage of the components at different conditions and for different lengths of time, in case of varying shelf lives. It also allows for a modular approach, where liposome and mRNA kits can be combined at request for different studies. And finally, it opens the door for truly individualized therapies, where an 'off-the-shelf' lipid formulation can be combined with a mRNA cocktail that is specifically produced for one single

patient. Here, mRNA "printers" might be of use as well, so the technologies described in this section will have additional advantages other than shortening the supply chain and relaxed storage conditions.

Real-life experience with in-use stability of mRNA-LNPs

Two studies on real-life in-use stability of mRNA-LNP DP have been published. First, the stability of mRNA-LNPs during storage between being drawn into of syringes and being injected was monitored [92]. The second study looked at the stability of left-over material in vials of Comirnaty[91]. Both publications concluded that — within the limits of the analytical techniques at their disposal — only mechanical shear stress caused immediate loss of mRNA integrity and aggregation of the LNP-mRNA complexes. This means that the mRNA-LNPs are stable when stored and handled as instructed, but this sensitivity to mechanical stress could have implications for transport as a liquid product. Another published example is the technical failure of a freezer in a hospital, which caused a temperature excursion to higher, but still subzero, temperatures for hours for SpikeVax®. No detectable loss of mRNA integrity was observed in an electrophoretic assay[144]. In a following publication, the authors intentionally thawed and re-froze vials of Comirnaty® and Spikevax® and found that mRNA integrity (by electrophoresis) and immunogenicity in mice were preserved after 1 month of storage at -80 °C and -20 °C[145]. Re-freezing of reconstituted vials could in theory minimize waste, but it

should be noted that this currently goes against manufacturer's instructions, since once the vial seal is punctured the sterility barrier has been broken[106]. Therefore, the product should be used within 12 hours (per label) and then discarded to prevent microbial contamination. Thus, reformulation of mRNA-LNPs products with antimicrobial preservatives to allow for more prolonged storage after opening the multidose vial (e.g., for up to 28 days) is a potential future option. To date, no systematic studies of mRNA-LNP products with antimicrobial preservatives have been published. Development of such multidose formulations will require ensuring the chemical and physical stability of the mRNA-LNP components as well as maintaining antimicrobial effectiveness during handling conditions.

An algorithm to model the status of critical quality attributes over time based on temperature monitoring devices during transport and storage would help in stock management and reduce vaccine wastage, a major problem on a global scale — not specifically for mRNA COVID-19 vaccines[146,147]. Since mRNA-LNP product degradation includes many different pathways, Kis discusses the complexity of designing such multivariable algorithms[61]. In the Assessment report of the EMA-CHMP for the Moderna COVID-19 vaccine the following text can be found: 'In relation to mRNA purity and stability, data on the degradation rate was provided and shown to demonstrate Arrhenius behavior, with first-order kinetics. The stability profiles were demonstrated to be predictable and amenable to modelling,

enabling a good understanding of the chemical degradation process' [148]. Unfortunately, neither experimental data nor details to support this claim can be found in the public domain so far. Moreover, it remains to be fully established if the chemical degradation process of mRNA is the only critical quality attribute for vaccine performance as is indicated by observations e.g., by Kudsiova et al., and Selmin et al. on mRNA-LNP particle size and aggregation[91,92]. Such considerations play a key role in determining the selection of primary containers and secondary packaging, as well as the need for temperature monitoring devices during shipping and transport, for example, as described for COVID vaccines by Ramakanth et al. [149]

Concluding remarks

When will guidance be available for mRNA and mRNA-LNP product testing?

In earlier publications, we made the point that there is clearly a need for regulatory guidance documents — preferably globally harmonized – that describe the critical quality attributes and acceptance criteria for mRNA-based products as defined by regulatory authorities[6]. Several articles in scientific journals recently described the WHO-led efforts that aimed to facilitate regulatory convergence in the assessment of the COVID vaccines. But at the time of these discussions, alignment on critical

quality attributes was not yet achieved, and details or specifications were not disclosed[43,44,48].

When comparing to the field of oligonucleotide-based drugs, authors from various drug companies worked together to publish the analytical methodology for characterization of the drug molecule and its impurities in a series of papers[150,151]. However, they deliberately avoided discussion of acceptance criteria: "Like those of any API, acceptance criteria for oligonucleotide APIs are based on preclinical and clinical data, manufacturing process variability, and analytical control strategies. However, these factors are likely to vary dramatically among oligonucleotide APIs, rendering it impossible to provide even general suggestions on acceptance criteria" [150]. In other words, acceptance criteria are data-driven and may vary between products and manufacturing processes.

For the mRNA vaccines, WHO committees took the initiative during the pandemic, but we now see mRNA-LNP vaccine manufacturing experts starting to share quality attributes, analytical methods and some data in the scientific literature[39,42,50,51,152]. Moreover, the US Pharmacopeia has recently released their first draft chapter on Analytical Procedures for mRNA Vaccine Quality as discussed in detail above (see Tables 2 and 3)[49]. Recently, the European Pharmacopeia (PhEur) announced that a new working party called mRNAVAC (working party for mRNA Vaccines) will be tasked with developing a consolidated

strategy on future standards for mRNA vaccines and their components[153]. When these standards are in place and more data are published, it will become more clear what ranges are achievable and which limits may be acceptable. While suggested specifications for the currently approved vaccines have been summarized by Daniel et al. [42], it should be noted that the mRNA technology is advancing rapidly and the currently used analytical methodologies, and their associated specifications, will be further developed to keep pace with the progress.

Which changes to the formulation could be made to improve stability of mRNA drug products?

As described above, changing the product presentation could improve the stability or enable storage of mRNA products under non-frozen conditions, for example, by presenting the mRNA in a freeze-dried form as part of a kit for "at-the-bedside" mixing with the drug delivery system. But to improve the shelf-life of the canonical, centrally produced mRNA-LNP vaccine that could be stored as a liquid formulation in a refrigerator, several lessons-learned can be summarized from the topics discussed in this review. Naturally, these strategies can be combined and are generally not mutually exclusive.

The first lesson is the selection and design of excipients: LNP excipients can contain and generate impurities that react with other excipients and the mRNA.

The most important and abundant excipient of the LNP is the ionizable amino lipid. While oxidation of the amine headgroup is unlikely to be completely avoided by selecting a different structure, the acyl tails of the lipid can be designed to prevent oxidation. The ionizable lipid DLin-MC3-DMA in the Onpattro® product contains unsaturated acyl chains that can oxidize. The ionizable lipids in the approved mRNA vaccine products both contain branched saturated tail structures that are not susceptible to oxidation. It is likely that this design choice was made to improve the product stability and compatibility with the vulnerable mRNA cargo, at least as one of the considerations. The other LNP excipient that is a well-known source of peroxides is the stabilizing PEG-lipid. As suggested before by Schoenmaker et al. alternative excipients such as a lipid-polysarcosine could have a similar stabilizing effect on the formulation, without the risk of introducing peroxides inherent to PEG units[7,154]. An example of how other excipients such as buffer ingredients can impact stability is the choice of Tris instead of phosphate buffer[105]. In addition, other pharmaceutical products contain anti-oxidants and metal chelators to improve the stability[72] , and the use of both together has been shown to greatly stabilize plasmid DNA[58]. These additives are not used in any of the current mRNA-LNP products so if they are found to be compatible and useful, this is another step that could be made to potentially enable extended storage under non-frozen conditions.

Another important point is the selection of high-quality, high-purity excipients. If PEG-lipids are used, they can be vacuum dried to remove peroxides but the content will increase again when stored at higher temperatures, unless covered with inert air and protected from light[74]. This demonstrates that proper handling and storage is also very important for the raw materials, as it will greatly improve the quality of the final product. Similarly, excipients that have been properly purified from metal ions will enhance the storage stability. These are key examples of how the shelf-life of a mRNA drug product can be improved by changing the quality of the excipients.

Finally, the quality and stability of the mRNA drug product can be improved by carefully controlling all process parameters during manufacturing. As there are so many different steps in both the DS and DP production processes, and so many different ways the mRNA or excipients could degrade, much can be gained from optimizing the conditions to minimize their occurrence. For example, one challenge with mRNA products is their sensitivity to degradation by RNases, so proper protection against the introduction of adventitious RNases should be ensured. In addition, mixing of the components at acidic pH is a fundamental step in the standard LNP formulation process that cannot be circumvented, because a charge on the ionizable lipid is required for complexation of the cargo. However, changing parameters such as pH, ionic strength, holding time at low pH,

temperature and light exposure during processing can still have a big impact on the formation of impurities even before storage.

What challenges remain en route to thermostable mRNA drug products?

With few exceptions, vaccines and therapeutic candidates based on IVT-transcribed mRNA are, at present, complex assemblies of the nucleic acid and lipid compounds that form nanoparticles. As such, the stability of these mRNA-based drug products on storage remains a challenge for three main reasons: 1) mRNA is not an intrinsically stable molecule, 2) lipid components required for delivery must be essentially free of impurities that can induce mRNA degradation, and 3) the physical stability of mRNA-LNP complexes is precarious, and sensitive to handling and thermal effects.

One of the key stabilization strategies in active development for formulated mRNA drug products to be stored in the refrigerator is lyophilization. This is logical, given the success of this dehydration approach with other biological products including other vaccines[155 156]. Overcoming the cryo- and lyo-induced particle degradation effects is a key goal to be tackled to make freeze-dried mRNAs formulated in LNPs successful product candidates. If these hurdles can be addressed, then the prospects for leaving the freezer for long-term storage look promising. An example of an mRNA-LNP product currently in a Phase 3 trial as a lyophilized product is

Moderna's CMV vaccine[157]. However, the worldwide capacity for production of lyophilized products is limited. Therefore, such an approach has limited utility for certain therapeutic applications, for example, mRNA vaccines that require billions of doses to be produced per year for global use.

Packaging approaches including selection of the primary container to preserve the formulated mRNA DP integrity and increase ease of use are future targets for improvements. Currently, vial products are the main option feasible for frozen liquids. Freezing pre-filled syringes is a challenge, because of the volume expansion of frozen water and the reduction in trapped air pressure during the freezing process, but recently technical solutions have been proposed[158]. Other packaging approaches are being explored as well. For example, systems using dual-chamber cartridges where the lyo cake (preformed in the syringe barrel) is separated by a stopper from the liquid for reconstitution, are being actively tested [159]. Pre-filled syringe options may be attractive for future therapeutic uses of mRNA, but are not currently compatible with the high volume, multi-dose usage that has been required for the COVID-19 vaccines for fighting the ongoing pandemic. We may therefore see the continued use of glass vials during pandemics or other large-scale uses of mRNA vaccines rather than a migration to prefilled syringes as product presentation.

Thermal stability improvements of IVT mRNA and formulated mRNA-LNP assemblies remains an active effort across vaccine industry and academia, and the understanding of ways to improve their stability profiles is increasing. The capture of "wins" can occur in stages. The improved storage conditions that were granted for Pfizer/BioNTech's Comirnaty® are an example of this approach: the liquid product can be frozen through depot storage, after which the product can be stored refrigerated until use for a period of 10 weeks; previously, it was limited to 30 days[105]. A lyophilized product could offer the same possibility of relaxed temperature storage conditions: for example, a refrigerated lyophilized product through depot storage, with the "last mile" storage being at room temperature.

In summary, for the mRNA-based vaccine and therapeutic candidates in development, improvements in product quality will benefit from enhanced integration of fundamental teachings in pharmaceutical, (bio)chemistry and manufacturing sciences. The mRNA formulation literature has been expanding in the past two years and industry has been increasingly sharing key findings that can support development of a wide variety of mRNA products in the future. The urgency of fighting the COVID-19 pandemic by employing mRNA vaccines has enabled tremendous learnings, and unprecedented focus on applying them, to commercialize mRNA—based products. This should lead to the development, regulatory approval and distribution of future mRNA-based products with

improved storage profiles to ensure safety and effectiveness in an increasing array of unmet medical needs. Thus, the quest for improved storage and in-use stability of formulated mRNA vaccines and therapeutics continues and is definitely *not* a cold case.

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.

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Reference

1. FDA. Coronavirus (COVID-19) Update: FDA Authorizes Moderna, Pfizer-BioNTech Bivalent COVID-19 Vaccines for Use as a Booster Dose. Published 2022. Accessed October 2, 2022. https://www.fda.gov/news-events/press-announcements/coronavirus-

covid-19-update-fda-authorizes-moderna-pfizer-biontech-bivalent-covid-19-vaccines-use

2. EMA. Adapted vaccine targeting BA.4 and BA.5 Omicron variants and original SARS-CoV-2 recommended for approval. Published 2022. Accessed October 2, 2022. https://www.ema.europa.eu/en/news/adapted-vaccine-targeting-ba4-ba5-omicron-variants-original-sars-cov-2-recommended-approval

3. Barbier AJ, Yujie Jiang A, Zhang P, Wooster R, Anderson DG. The clinical progress of mRNA vaccines and immunotherapies. doi:10.1038/s41587-022-01294-2

4. Garber K. mRNA pioneers refocus on therapeutics. *Nat Rev Drug Discov*. 2022;21:699-701.

5. Lorentzen CL, Haanen JB, Met Ö, Svane IM. Clinical advances and ongoing trials on mRNA vaccines for cancer treatment. *Lancet Oncol*. 2022;23(10):e450-e458. doi:10.1016/s1470-2045(22)00372-2

6. Crommelin DJA, Anchordoquy TJ, Volkin DB, Jiskoot W, Mastrobattista E. Addressing the Cold Reality of mRNA Vaccine Stability. *J Pharm Sci*. 2021;110(3):997-1001. doi:10.1016/j.xphs.2020.12.006

7. Schoenmaker L, Witzigmann D, Kulkarni JA, et al. mRNA-lipid nanoparticle COVID-19 vaccines: structure and stability. *Int J Pharm*. Published online April 2021:120586. doi:10.1016/j.ijpharm.2021.120586

8. Uddin MN, Roni MA. Challenges of storage and stability of mrna-based covid-19 vaccines. *Vaccines (Basel)*. 2021;9(9). doi:10.3390/vaccines9091033

9. Buschmann MD, Carrasco MJ, Alishetty S, Paige M, Alameh MG, Weissman D. Nanomaterial Delivery Systems for mRNA Vaccines. Published online 2021. doi:10.3390/vaccines

10. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines-a new era in vaccinology. *Nat Rev Drug Discov*. 2018;17(4):261-279. doi:10.1038/nrd.2017.243

11. Verbeke R, Lentacker I, de Smedt SC, Dewitte H. The dawn of mRNA vaccines: The COVID-19 case. *Journal of Controlled Release*. 2021;333:511-520. doi:10.1016/j.jconrel.2021.03.043

12. Wang Y, Zhang Z, Luo J, Han X, Wei Y, Wei X. mRNA vaccine: a potential therapeutic strategy. *Mol Cancer*. 2021;20(1). doi:10.1186/S12943-021-01311-Z

13. EMA. *Onpattro Summary of Product Characteristics*.; 2018. Accessed May 19, 2022. https://www.ema.europa.eu/en/documents/product-information/onpattro-epar-product-information_en.pdf

14. Nelson J, Sorensen EW, Mintri S, et al. Impact of mRNA chemistry and manufacturing process on innate immune activation. *Sci Adv*. 2020;6:1-13. doi:https://www.science.org/doi/10.1126/sciadv.aaz6893

15. Mugridge JS, Coller J, Gross JD. Structural and molecular mechanisms for the control of eukaryotic 5′–3′ mRNA decay. doi:10.1038/s41594-018-0164-z

16. Nanjappa DP, Babu N, Khanna-Gupta A, O'Donohue MF, Sips P, Chakraborty A. Poly (A)-specific ribonuclease (PARN): More than just "mRNA stock clearing." *Life Sci*. 2021;285. doi:10.1016/j.lfs.2021.119953

17. Wesselhoeft RA, Kowalski PS, Parker-Hale FC, Huang Y, Bisaria N, Anderson DG. RNA Circularization Diminishes Immunogenicity and Can Extend Translation Duration In Vivo. *Mol Cell*. 2019;74(3):508-520.e4. doi:10.1016/j.molcel.2019.02.015

18. Soukup GA, Breaker RR. *Relationship between Internucleotide Linkage Geometry and the Stability of RNA*.; 1999.

19. White P. Moderna Science and Technology Day . Published 2022. Accessed May 19, 2022. https://s29.q4cdn.com/435878511/files/doc_presentations/2022/05/Science-Day-2022-Master-Slides-FINAL-(05.17_7am).pdf

20. Kaukinen U, Lyytikäinen S, Mikkola S, Lönnberg H. The reactivity of phosphodiester bonds within linear single-stranded oligoribonucleotides is strongly dependent on the base sequence. *Nucleic Acids Res*. 2002;30(2):468-474. http://bioinfo.math.rpi.edu/

21. Mikkola S, Kaukinen U, Lonnberg H. The Effect of Secondary Structure on Cleavage of the Phosphodiester Bonds of RNA. *Cell Biochem Biophys*. 2001;34:95-119.

22. van Tol H, Buzayan JM, Feldstein PA, Eckstein' F, Bruening G. Two autolytic processing reactions of a satellite RNA proceed with inversion of configuration. *Nucleic Acids Res*. 1971;18(8):19711975.

23. Torres RA, Bruice TC. *Molecular Dynamics Study Displays near In-Line Attack Conformations in the Hammerhead Ribozyme Self-Cleavage Reaction*. Vol 95.; 1998. www.pnas.org.

24. Martick M, Scott WG. Tertiary Contacts Distant from the Active Site Prime a Ribozyme for Catalysis. *Cell*. 2006;126(2):309-320. doi:10.1016/J.CELL.2006.06.036

25. Guo F, Yue Z, Trajkovski M, et al. Effect of Ribose Conformation on RNA Cleavage via Internal Transesterification. *J Am Chem Soc*. 2018;140(38):11893-11897. doi:10.1021/jacs.8b06313

26. Vicens Q, Kieft JS. Thoughts on how to think (and talk) about RNA structure. Published online 2022. doi:10.1073/pnas.2112677119/-/DCSupplemental

27. Kloczewiak M, Banks JM, Jin L, Brader ML. A Biopharmaceutical Perspective on Higher-Order Structure and Thermal Stability of mRNA Vaccines. *Mol Pharm*. 2022;19(7):2022-2031. doi:10.1021/acs.molpharmaceut.2c00092

28. Wayment-Steele HK, Kim DS, Choe CA, et al. Theoretical basis for stabilizing messenger RNA through secondary structure design. *bioRxiv*. Published online August 24, 2020. doi:10.1101/2020.08.22.262931

29. Leppek K, Woo Byeon G, Kladwang W, et al. Combinatorial optimization of mRNA structure, stability, and translation for RNA-

based therapeutics. *Nat Commun*. Published online 2022. doi:10.1038/s41467-022-28776-w

30. Thess A, Grund S, Mui BL, et al. Sequence-engineered mRNA Without Chemical Nucleoside Modifications Enables an Effective Protein Therapy in Large Animals. *Molecular Therapy*. 2015;23(9):1456-1464. doi:10.1038/mt.2015.103

31. von der Mülbe F, Hoerr I, Pascolo S. PHARMACEUTICAL COMPOSITION CONTAINING A STABILISED MRNA OPTIMISED FOR TRANSLATION IN ITS CODING REGIONS. *Trends Genet*. 2019;12(5). Accessed September 23, 2022. https://patentimages.storage.googleapis.com/6d/8f/d6/f86f4d877ae3c1/US10188748.pdf

32. Kremsner PG, Ahuad Guerrero RA, Arana-Arri E, et al. Efficacy and safety of the CVnCoV SARS-CoV-2 mRNA vaccine candidate in ten countries in Europe and Latin America (HERALD): a randomised, observer-blinded, placebo-controlled, phase 2b/3 trial. *Lancet Infect Dis*. 2022;22(3):329-340. doi:10.1016/S1473-3099(21)00677-0

33. Roth N, Schön J, Hoffmann D, et al. Optimised non-coding regions of mRNA SARS-CoV-2 vaccine CV2CoV 1 improves homogeneous and

heterogenous neutralising antibody responses 2. *bioRxiv*. Published online 2021. doi:10.1101/2021.05.13.443734

34. Blakney AK, Ip S, Geall AJ. An update on self-amplifying mRNA vaccine development. *Vaccines (Basel)*. 2021;9(2):1-26. doi:10.3390/vaccines9020097

35. Pollock KM, Cheeseman HM, Szubert AJ, et al. Safety and immunogenicity of a self-amplifying RNA vaccine against COVID-19: COVAC1, a phase I, dose-ranging trial. Published online 2022. doi:10.1016/j

36. Mauger DM, Joseph Cabral B, Presnyak V, et al. mRNA structure regulates protein expression through changes in functional half-life. *Proc Natl Acad Sci U S A*. 2019;116(48):24075-24083. doi:10.1073/pnas.1908052116

37. Liu A, Wang X. The Pivotal Role of Chemical Modifications in mRNA Therapeutics. *Front Cell Dev Biol*. 2022;10:901510. doi:10.3389/fcell.2022.901510

38. Cheung E, Xia Y, Caporini MA, Gilmore JL. Tools shaping drug discovery and development. *Biophys Rev*. 2022;3(3):031301. doi:10.1063/5.0087583

39. Whitley J, Zwolinski C, Denis C, et al. Development of mRNA manufacturing for vaccines and therapeutics: mRNA platform requirements and development of a scalable production process to support early phase clinical trials. *Translational Research*. 2022;242:38-55. doi:10.1016/j.trsl.2021.11.009

40. Guerrini G, Magrì D, Gioria S, Medaglini D, Calzolai L. Characterization of nanoparticles-based vaccines for COVID-19. *Nat Nanotechnol*. Published online June 16, 2022. doi:10.1038/S41565-022-01129-W

41. EMA. Assessment report Comirnaty Common name: COVID-19 mRNA vaccine (nucleoside-modified) Procedure No. EMEA/H/C/005735/0000. Published 2021. Accessed February 24, 2022. https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf

42. Daniel S, Kis Z, Kontoravdi C, Shah N. Quality by Design for enabling RNA platform production processes. *Trends Biotechnol*. Published online October 1, 2022. doi:10.1016/j.tibtech.2022.03.012

43. Liu MA, Zhou T, Sheets RL, Meyer H, Knezevic I. WHO informal consultation on regulatory considerations for evaluation of the quality,

safety and efficacy of RNA-based prophylactic vaccines for infectious diseases, 20-22 April 2021. *Emerg Microbes Infect*. 2022;11:384-391. doi:10.1080/22221751.2022.2026742

44. Knezevic I, Liu MA, Peden K, Zhou T, Kang HN. Development of mRNA Vaccines: Scientific and Regulatory Issues. *Vaccines (Basel)*. 2021;9(2). doi:10.3390/vaccines9020081

45. Jackson NAC, Kester KE, Casimiro D, Gurunathan S, Derosa F. The promise of mRNA vaccines: a biotech and industrial perspective. doi:10.1038/s41541-020-0159-8

46. Popkin ME, Goese M, Wilkinson D, et al. Chemistry Manufacturing and Controls Development, Industry Reflections on Manufacture, and Supply of Pandemic Therapies and Vaccines. *AAPS J*. 2022;24(6):101. doi:10.1208/s12248-022-00751-9

47. FDA. Fast Track _ FDA. Published 2018. Accessed September 24, 2022. https://www.fda.gov/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/fast-track

48. Tinari S. The EMA covid-19 data leak, and what it tells us about mRNA instability. *The BMJ*. 2021;372. doi:10.1136/bmj.n627

49. USP. *Analytical Procedures for MRNA Vaccine Quality*.; 2022. Accessed May 10, 2022. https://www.usp.org/mrna

50. Webb C, Ip S, Bathula N v., et al. Current Status and Future Perspectives on MRNA Drug Manufacturing. *Mol Pharm*. 2022;19(4):1047-1058. doi:10.1021/acs.molpharmaceut.2c00010

51. Thorn CR, Sharma D, Combs R, et al. The journey of a lifetime-Development of the Pfizer's COVID-19 vaccine. *Curr Opin Biotechnol*. Published online September 1, 2022:102803. doi:10.1016/J.COPBIO.2022.102803

52. *ICH Topic Q 1 A (R2) Stability Testing of New Drug Substances and Products Step 5 NOTE FOR GUIDANCE ON STABILITY TESTING: STABILITY TESTING OF NEW DRUG SUBSTANCES AND PRODUCTS*.; 2003. Accessed August 28, 2022. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-1-r2-stability-testing-new-drug-substances-products-step-5_en.pdf

53. Rosa SS, Prazeres DMF, Azevedo AM, Marques MPC. mRNA vaccines manufacturing: Challenges and bottlenecks. *Vaccine*. 2021;39(16):2190-2200. doi:10.1016/j.vaccine.2021.03.038

54. Gholamalipour Y, Karunanayake Mudiyanselage A, Martin CT. NAR breakthrough article 3 end additions by T7 RNA polymerase are RNA self-templated, distributive and diverse in character—RNA-Seq analyses. *Nucleic Acids Res*. 2018;46(18):9253-9263. doi:10.1093/nar/gky796

55. Baiersdörfer M, Boros G, Muramatsu H, et al. A Facile Method for the Removal of dsRNA Contaminant from In Vitro-Transcribed mRNA. *Mol Ther Nucleic Acids*. 2019;15:26-35. doi:10.1016/j.omtn.2019.02.018

56. Beverly M, Dell A, Parmar P, Houghton L. Label-free analysis of mRNA capping efficiency using RNase H probes and LC-MS. *Anal Bioanal Chem* . 2016;408:50215030. doi:10.1007/s00216-016-9605-x

57. Simms CL, Zaher HS. Quality control of chemically damaged RNA. *Cellular and Molecular Life Sciences*. 2016;73(19):3639-3653. doi:10.1007/s00018-016-2261-7

58. Evans RK, Xu Z, Bohannon KE, Wang B, Bruner MW, Volkin DB. Evaluation of degradation pathways for plasmid DNA in pharmaceutical formulations via accelerated stability studies. *J Pharm*

Sci. 2000;89(1):76-87. doi:10.1002/(SICI)1520-6017(200001)89:1<76::AID-JPS8>3.0.CO;2-U

59. Nunomura A, Lee HG, Zhu X, Perry G. Consequences of RNA oxidation on protein synthesis rate and fidelity: implications for the pathophysiology of neuropsychiatric disorders . *Biochem Soc Trans*. 2017;45(5):1053-1066.

60. An R, Jia Y, Wan B, et al. Non-Enzymatic Depurination of Nucleic Acids: Factors and Mechanisms. *PLoS One*. 2014;9(12):1-17. doi:10.1371/journal.pone

61. Kis Z. Stability Modelling of mRNA Vaccine Quality Based on Temperature Monitoring throughout the Distribution Chain. *Pharmaceutics*. 2022;14(430). doi:10.3390/pharmaceutics14020430

62. Bauer NC, Corbett AH, Doetsch PW. SURVEY AND SUMMARY The current state of eukaryotic DNA base damage and repair. *Nucleic Acids Res*. 2015;43:10083-10101. doi:10.1093/nar/gkv1136

63. Sahin U, Derhovanessian E, Miller M, et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature Publishing Group*. Published online 2017. doi:10.1038/nature23003

64. Gan LM, Lagerström-Fermér M, Carlsson LG, et al. Intradermal delivery of modified mRNA encoding VEGF-A in patients with type 2 diabetes. *Nat Commun*. 2019;10(1). doi:10.1038/s41467-019-08852-4

65. Packer M, Gyawali D, Yerabolu R, Schariter J, White P. A novel mechanism for the loss of mRNA activity in lipid nanoparticle delivery systems. *Nat Commun*. 2021;12(1). doi:10.1038/S41467-021-26926-0

66. Mair S, Erharter K, Renard E, et al. Towards a comprehensive understanding of RNA deamination: synthesis and properties of xanthosine-modified RNA. *Nucleic Acids Res*. 2022;50(11):6038-6051. doi:10.1093/nar/gkac477

67. Rentel C, DaCosta J, Roussis S, Chan J, Capaldi D, Mai B. Determination of oligonucleotide deamination by high resolution mass spectrometry. *J Pharm Biomed Anal*. 2019;173:56-61. doi:10.1016/J.JPBA.2019.05.012

68. Terui Y, Yoshida T, Sakamoto A, et al. Polyamines protect nucleic acids against depurination. *Int J Biochem Cell Biol*. 2018;99:147-153. doi:10.1016/J.BIOCEL.2018.04.008

69. Garaycoechea JI, Crossan GP, Langevin F, et al. Alcohol and endogenous aldehydes damage chromosomes and mutate stem cells. Published online 2018. doi:10.1038/nature25154

70. Shishodia S, Zhang D, El-Sagheer AH, et al. NMR analyses on N-hydroxymethylated nucleobases-implications for formaldehyde toxicity and nucleic acid demethylases. *Org Biomol Chem*. 2018;16(21):4021-4032. doi:10.1039/c8ob00734a

71. Zhang Y, Sun C, Wang C, Jankovic KE, Dong Y. Lipids and Lipid Derivatives for RNA Delivery. *Chem Rev*. 2021;121(20):12181-12277. doi:10.1021/ACS.CHEMREV.1C00244

72. Musakhanian J, Rodier JD, Dave M. Oxidative Stability in Lipid Formulations: a Review of the Mechanisms, Drivers, and Inhibitors of Oxidation. *AAPS PharmSciTech*. 2022;23(5). doi:10.1208/s12249-022-02282-0

73. Crowe A, Jain N, Higgins R, Proos R, Stone M, Pohl K. *Distinguishing Oxidative Impurities from Ionizable Lipids Used in LNP Formulations Using Electron Activated Dissociation Achieving Structural Elucidation and Distinction of Oxidative Isomer Impurities of Lipids with Electron-Activated Dissociation (EAD) and the ZenoTOF 7600*

System.; 2022. Accessed September 24, 2022. https://pages.precisionnanosystems.com/Sciex-Distinguishingoxidativeimpuritiesfromionizablelipids_Download.ht

74. Kumar V, Kalonia DS. Removal of Peroxides in Polyethylene Glycols by Vacuum Drying: Implications in the Stability of Biotech and Pharmaceutical Formulations. *AAPS PharmSciTech 2006*. 2006;7(3):E47-E53. doi:doi: 10.1208/pt070362

75. Grit M, Crommelin DJA. Chemical stability of liposomes: implications for their physical stability. *Chem Phys Lipids*. 1993;64(1-3):3-18. doi:10.1016/0009-3084(93)90053-6

76. Kanavarioti A. HPLC methods for purity evaluation of man-made single-stranded RNAs. *Sci Rep*. 2019;9(1). doi:10.1038/s41598-018-37642-z

77. Lu T, Klein LJ, Ha S, Rustandi RR. High-Resolution capillary electrophoresis separation of large RNA under non-aqueous conditions. *J Chromatogr A*. 2020;1618. doi:10.1016/j.chroma.2020.460875

78. de Scheerder L, Sparén A, Nilsson GA, Norrby PO, Örnskov E. Designing flexible low-viscous sieving media for capillary

electrophoresis analysis of ribonucleic acids. *J Chromatogr A*. 2018;1562:108-114. doi:10.1016/J.CHROMA.2018.05.048

79. Chan SH, Whipple JM, Dai N, et al. RNase H-based analysis of synthetic mRNA 5′ cap incorporation. *RNA (2022) 28:1144–1155*. 2022;28:1144-1155. doi:10.1261/rna

80. Moderna. METHODS FOR HPLC ANALYSIS. Published online 2017.

81. Beverly M, Hagen C, Slack O. Poly A tail length analysis of in vitro transcribed mRNA by LC-MS. *Anal Bioanal Chem*. 2018;410(6):1667-1677. doi:10.1007/s00216-017-0840-6

82. Jones LJ, Yue ST, Cheung CY, Singer VL. *RNA Quantitation by Fluorescence-Based Solution Assay: RiboGreen Reagent Characterization*.; 1998.

83. FDA. *Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation*.; 2018. Accessed September 24, 2022. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/liposome-drug-products-chemistry-manufacturing-and-controls-human-pharmacokinetics-and

84. FDA CDER. NDA 210922 ONPATTRO(patisiran) Lipid Complex Injection - Addendum to Drug Product Quality Review. Published online 2017. Accessed November 1, 2022. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210922Orig1s000ChemR.pdf

85. FDA. *Guidance for Industry Nonclinical Studies for the Safety Evaluation of Pharmaceutical Excipients*.; 2005. http://www.fda.gov/cder/guidance/index.htmorOfficehttp://www.fda.gov/cber/guidelines.htm

86. EMEA. EMEA 2007 guideline-excipients-dossier-application-marketing-authorisation-medicinal-product-revision-2_en. Published 2007. Accessed October 9, 2022. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-excipients-dossier-application-marketing-authorisation-medicinal-product-revision-2_en.pdf

87. Kinsey C, Lu T, Deiss A, et al. Determination of lipid content and stability in lipid nanoparticles using ultra high-performance liquid chromatography in combination with a Corona Charged Aerosol

Detector. *Electrophoresis*. 2022;43(9-10):1091-1100. doi:10.1002/elps.202100244

88. Fan Y, Marioli M, Zhang K. Analytical characterization of liposomes and other lipid nanoparticles for drug delivery. *J Pharm Biomed Anal*. 2021;192:113642. doi:10.1016/J.JPBA.2020.113642

89. Abeyrathne EDNS, Nam K, Ahn DU. Analytical methods for lipid oxidation and antioxidant capacity in food systems. *Antioxidants*. 2021;10(10). doi:10.3390/antiox10101587

90. Zhang N, Li Y, Wen S, et al. Analytical methods for determining the peroxide value of edible oils: A mini-review. *Food Chem*. 2021;358. doi:10.1016/j.foodchem.2021.129834

91. Kudsiova L, Lansley A, Scutt G, et al. Stability testing of the Pfizer-BioNTech BNT162b2 COVID-19 vaccine: A translational study in UK vaccination centres. *BMJ Open Science*. 2021;5(1). doi:10.1136/bmjos-2021-100203

92. Selmin F, Musazzi UM, Franzè S, et al. Pre-Drawn Syringes of Comirnaty for an Efficient COVID-19 Mass Vaccination: Demonstration of Stability. *Pharmaceutics*. 2021;13(7):1029. doi:10.3390/pharmaceutics13071029

93. Messerian KO, Zverev A, Kramarczyk JF, Zydney AL. Pressure-dependent fouling behavior during sterile filtration of mRNA-containing lipid nanoparticles. *Biotechnol Bioeng*. Published online 2022. doi:10.1002/bit.28200

94. Malburet C, Leclercq L, Cott JF, et al. Size and Charge Characterization of Lipid Nanoparticles for mRNA Vaccines. *Anal Chem*. 2022;94(11):4677-4685. doi:https://doi.org/10.1021/acs.analchem.1c04778

95. Brader ML, Williams SJ, Banks JM, Hui WH, Zhou ZH, Jin L. Encapsulation state of messenger RNA inside lipid nanoparticles. *Biophys J*. Published online 2021. doi:10.1016/j.bpj.2021.03.012

96. Leitão JH, Markova N, Cairns S, et al. Biophysical Characterization of Viral and Lipid-Based Vectors for Vaccines and Therapeutics with Light Scattering and Calorimetric Techniques. Published online 2021. doi:10.3390/vaccines10010049

97. Alishetty S, Carrasco M, Alameh MG, et al. Novel lipid nanoparticle provides potent SARS-CoV-2 mRNA vaccine at low dose with low local reactogenicity, high thermostability and limited systemic

biodistribution. *Res Sq*. Published online 2022. doi:10.21203/rs.3.rs-798453/v1

98. Li Y, Breaker RR. Kinetics of RNA degradation by specific base catalysis of transesterification involving the 2γ-hydroxyl group. *J Am Chem Soc*. 1999;121(23):5364-5372. doi:10.1021/ja990592p

99. Wayment-Steele HK, Kim DS, Choe CA, et al. Theoretical basis for stabilizing messenger RNA through secondary structure design. *Nucleic Acids Res*. 2021;49(18):10604-10617. doi:10.1093/nar/gkab764

100. Zuidam NJ, Crommelin DJA. Chemical Hydrolysis of Phospholipids. *Journal Pharmaceutical Sciences*. Published online 1995:1113-1119.

101. Zhao H, Wang TC, Li XF, et al. Long-term stability and protection efficacy of the RBD-targeting COVID-19 mRNA vaccine in nonhuman primates. *Signal Transduction and Targeted Therapy* . 2021;6:438. doi:10.1038/s41392-021-00861-4

102. Ripoll M, Bernard MC, Vaure C, et al. An imidazole modified lipid confers enhanced mRNA-LNP stability and strong immunization properties in mice and non-Human primates. *Biomaterials*. Published online May 7, 2022:121570. doi:10.1016/J.BIOMATERIALS.2022.121570

103. Lang JK, Vigo-Pelfrey C. Quality control of liposomal lipids with special emphasis on peroxidation of phospholipids and cholesterol. *Chem Phys Lipids*. 1993;64:19-29.

104. Torchilin V, Weissig V, eds. *Liposomes: A Practical Approach*. second edition. Oxford University Press; 2003. Accessed October 10, 2022. https://global.oup.com/academic/product/liposomes-a-practical-approach-9780199636549?cc=nl&lang=en&#

105. Lewis LM, Badkar A v, Cirelli D, Combs R, Lerch TF. The Race to Develop the Pfizer-BioNTech COVID-19 Vaccine: From the Pharmaceutical Scientists' Perspective. *J Pharm Sci*. Published online September 2022. doi:10.1016/j.xphs.2022.09.014

106. FDA CBER. Package Insert - COMIRNATY (gray cap). Published 2022. Accessed October 9, 2022. https://www.fda.gov/media/154834/download

107. Fda, Cber. *Package Insert - COMIRNATY (Purple Cap)*.; 2022. http://vaers.hhs.gov.

108. Gemcovac-19. GEMCOVAC-19 - SMPC-02 July 2022. Published 2022. Accessed September 14, 2022. https://img1.wsimg.com/blobby/go/0986aca2-5a8a-45d3-89a6-

8641924d4682/downloads/GEMCOVAC-19%20-%20SMPC-02%20July%202022.pdf?ver=1657276544539

109. Ketterer et al. Lyophilization of mRNA. Published online 2016. Accessed September 15, 2022. https://patentimages.storage.googleapis.com/1b/13/ef/9b040d082f5b6c/WO2016165831A1.pdf

110. Mutzke Thorsten. LYOPHILIZATION OF NUCLEIC ACIDS IN LACTATE-CONTAINING SOLUTIONS WO2011069587. Published online 2011. Accessed May 14, 2022. https://patentimages.storage.googleapis.com/f0/4d/af/5c1c24d8098d2c/WO2011069587A1.pdf

111. Mutzke T. Mannose-containing solution for lyophilization, transfection and/or injection of nucleic acids. Published online 2012. Accessed May 14, 2022. https://patentimages.storage.googleapis.com/6c/bf/ea/8f0cbce191a4b2/WO2011069529A1.pdf

112. Stitz L, Vogel A, Schnee M, et al. A thermostable messenger RNA based vaccine against rabies. *PLoS Negl Trop Dis*. 2017;11(12). doi:10.1371/journal.pntd.0006108

113. Zhao P, Hou X, Yan J, et al. Long-term storage of lipid-like nanoparticles for mRNA delivery. *Bioact Mater*. 2020;5(2):358-363. doi:10.1016/j.bioactmat.2020.03.001

114. Ai L, Li Y, Zhou L, et al. Lyophilized mRNA-lipid nanoparticle vaccines with long-term stability and high antigenicity against SARS-CoV-2. Published online 2022. doi:10.1101/2022.02.10.479867

115. Muramatsu H, Lam K, Bajusz C, et al. Lyophilization provides long-term stability for a lipid nanoparticle-formulated, nucleoside-modified mRNA vaccine. *Molecular Therapy*. Published online 2022. doi:10.1016/j.ymthe.2022.02.001

116. Shirane D, Tanaka H, Nakai Y, Yoshioka H, Akita H. Development of an Alcohol Dilution-Lyophilization Method for Preparing Lipid Nanoparticles Containing Encapsulated siRNA. *Biol Pharm Bull*. 2018;41(8):1291-1294.

117. Gerhardt A, Voigt E, Archer M, et al. A flexible, thermostable nanostructured lipid carrier platform for RNA vaccine delivery. *Mol Ther Methods Clin Dev*. 2022;25:205-214. doi:10.1016/j.omtm.2022.03.009

118. Voigt EA, Gerhardt A, Hanson D, et al. A self-amplifying RNA vaccine against COVID-19 with long-term room-temperature stability 1. Published online 2022. doi:10.1101/2022.03.22.485230

119. Christian Hong H, Sung Kim K, Ae Park S, et al. An mRNA vaccine against SARS-CoV-2: Lyophilized, liposome-based vaccine candidate EG-COVID induces high levels of virus neutralizing antibodies. *bioRxiv*. Published online 2021. doi:10.1101/2021.03.22.436375

120. Preston KB, Randolph TW. Stability of lyophilized and spray dried vaccine formulations. *Adv Drug Deliv Rev*. 2021;171:50-61. doi:10.1016/j.addr.2021.01.016

121. Ghaemmaghamian Z, Zarghami R, Walker G, O'Reilly E, Ziaee A. Stabilizing vaccines via drying: Quality by design considerations. *Adv Drug Deliv Rev*. 2022;187. doi:10.1016/j.addr.2022.114313

122. Panah et al. WO2016184577A2 Dry powder composition comprising long chain RNA. Published online 2016.

123. Eber F, Yazdan Panah B, Ketterer T, Wiggenhorn M, Kolland K. DRY POWDER COMPOSITION COMPRISING LONG-CHAIN RNA. Published online 2019.

124. Eber F, Yazdan Panah B, Sewing S, Ketterer T, Mutzke T, Wiggenhorn M. DRY POWDER COMPOSITION COMPRISING LONG - CHAIN RNA. Published online 2021.

125. Karve S, DeRosa F, Bhavsar Z, Heartlein M. US 2018 / 0153822 A1 PROCESS OF PREPARING MRNA - LOADED LIPID NANOPARTICLES. Published online 2018. Accessed April 22, 2022. https://patentimages.storage.googleapis.com/bc/f8/b1/ce992e0f0047e4/US20180153822A1.pdf

126. Kapnick SM. The Nanoparticle-Enabled Success of COVID-19 mRNA Vaccines and the Promise of Microneedle Platforms for Pandemic Vaccine Response. *DNA Cell Biol*. 2022;41(1):25-29. doi:10.1089/dna.2021.0538

127. Alatovic J. BioNTech introduces first modular mRNA manufacturing facility to promote scalable vaccine production in Africa. Published 2022. Accessed October 30, 2022. https://investors.biontech.de/node/11561/pdf

128. Jones KL, Drane D, Gowans EJ. Long-term storage of DNA-free RNA for use in vaccine studies. *Biotechniques*. 2007;43(5):675-681.

129. Karve S.D. DRY POWDER FORMULATIONS FOR MESSENGER RNA. Published online 2020. Accessed April 25, 2022. https://patentimages.storage.googleapis.com/11/29/12/567db77e8e8845/US20200022921A1.pdf

130. Fabre AL, Colotte M, Luis A, Tuffet S, Bonnet J. An efficient method for long-term room temperature storage of RNA. *European Journal of Human Genetics*. 2014;22(3):379-385. doi:10.1038/ejhg.2013.145

131. MOHANTY PS ;CHAKRABORTY, N. Capillary assisted vitrification processes and devices. Published online 2020.

132. Renu S, Shank-Retzlaff M, Sharpe J, Bronsart L, Mohanty · Pravansu. Capillary-Mediated Vitrification: Preservation of mRNA at Elevated Temperatures. *AAPS J*. 2022;24:75. doi:10.1208/s12248-022-00723-z

133. Sharidan C. mRNA printers kick-start personalized medicines for all. *Nat Biotechnol*. 2022;40:1160-1161. doi:https://doi.org/10.1038/s41587-022-01430-y

134. Sahin U, Karikó K, Türeci Ö. MRNA-based therapeutics-developing a new class of drugs. *Nat Rev Drug Discov*. 2014;13(10):759-780. doi:10.1038/nrd4278

135. CUREVAC. CureVac Establishes Fully-Owned Company Dedicated to Advancing The RNA Printer®. Published 2022. Accessed October 3, 2022. https://www.curevac.com/wp-content/uploads/2022/03/20220301_PR-CureVac_CureVac-establishes-fully-owned-company-dedicated-to-advancing-The-RNA-Printer%C2%AE.pdf

136. Brito LA, Chan M, Shaw CA, et al. A cationic nanoemulsion for the delivery of next-generation RNA vaccines. *Molecular Therapy*. 2014;22(12):2118-2129. doi:10.1038/mt.2014.133

137. Erasmus JH, Khandhar AP, Guderian J, et al. A Nanostructured Lipid Carrier for Delivery of a Replicating Viral RNA Provides Single, Low-Dose Protection against Zika. *Molecular Therapy*. 2018;26(10):2507-2522. doi:10.1016/j.ymthe.2018.07.010

138. Kurimoto S, Yoshinaga N, Igarashi K, Matsumoto Y, Cabral H, Uchida S. PEG-oligoRNA hybridization of mRNA for developing sterically stable lipid nanoparticles toward in vivo administration. *Molecules*. 2019;24(7). doi:10.3390/molecules24071303

139. Kulkarni J. Compositions and systems comprising transfection-competent vesicles free of organic-solvents and detergents and methods

related thereto. WO/2020/077007. Published online 2020. Accessed April 29, 2022. https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2020077007

140. Kulkarni JA, Thomson SB, Zaifman J, et al. Spontaneous, solvent-free entrapment of siRNA within lipid nanoparticles. *Nanoscale*. 2020;12(47):23959-23966. doi:10.1039/d0nr06816k

141. Blakney AK, McKay PF, Yus BI, Aldon Y, Shattock RJ. Inside out: optimization of lipid nanoparticle formulations for exterior complexation and in vivo delivery of saRNA. *Gene Ther*. 2019;26(9):363-372. doi:10.1038/s41434-019-0095-2

142. Blakney AK, Deletic P, McKay PF, et al. Effect of complexing lipids on cellular uptake and expression of messenger RNA in human skin explants. *Journal of Controlled Release*. 2021;330:1250-1261. doi:10.1016/J.JCONREL.2020.11.033

143. Grabbe S, Haas H, Diken M, Kranz LM, Langguth P, Sahin U. Translating nanoparticulate-personalized cancer vaccines into clinical applications: Case study with RNA-lipoplexes for the treatment of melanoma. *Nanomedicine*. 2016;11(20):2723-2734. doi:10.2217/nnm-2016-0275

144. Grau S, Ferrández O, Martín-García E, Maldonado R. Accidental interruption of the cold chain for the preservation of the Moderna covid-19 vaccine. *Vaccines (Basel)*. 2021;9(5). doi:10.3390/vaccines9050512

145. Grau S, Martín-García E, Ferrández O, et al. COVID-19 mRNA Vaccines Preserve Immunogenicity after Re-Freezing. *Vaccines (Basel)*. 2022;10(4). doi:10.3390/vaccines10040594

146. UNEP. *Why Optimized Cold-Chains Could Save a Billion COVID Vaccines*.; 2020.

147. WHO. *Monitoring Vaccine Wastage at Country Level*.; 2005.

148. CHMP. *COVID-19 Vaccine Moderna*.; 2021. Accessed May 15, 2022. https://www.ema.europa.eu/en/documents/assessment-report/spikevax-previously-covid-19-vaccine-moderna-epar-public-assessment-report_en.pdf

149. Ramakanth D, Singh S, Pradip ·, et al. Advanced packaging for distribution and storage of COVID-19 vaccines: a review. 2021;19:3597-3608. doi:10.1007/s10311-021-01256-1

150. Capaldi D, Ackley K, Brooks D, et al. Quality Aspects of Oligonucleotide Drug Development: Specifications for Active

Pharmaceutical Ingredients. *Drug Inf J*. 2012;46(5):611-626. doi:10.1177/0092861512445311

151. Capaldi D, Teasdale A, Henry S, et al. Impurities in Oligonucleotide Drug Substances and Drug Products. *Nucleic Acid Ther*. 2017;27(6):309-322. doi:10.1089/nat.2017.0691

152. Challener CA. Challener 2022 Analysis of mRNA Therapeutics and Vaccines. *BioPharm International 35 (2)(2022)*. 2022;35(2):10-15.

153. EDQM. Ph. Eur. Commission establishes a new working party on mRNA vaccines - European Directorate for the Quality of Medicines & HealthCare 2022. *Newsroom* . Published online 2022. Accessed September 25, 2022. https://www.edqm.eu/en/-/ph.-eur.-commission-establishes-a-new-working-party-on-mrna-vaccines?p_l_back_url=%2Fen%2Fsearch-edqm%3Fq%3Dmrnavac

154. Nogueira SS, Schlegel A, Maxeiner K, et al. Polysarcosine-Functionalized Lipid Nanoparticles for Therapeutic mRNA Delivery. *ACS Appl Nano Mater*. 2020;3(11):10634-10645. doi:10.1021/acsanm.0c01834

155. Kumru OS, Joshi SB, Smith DE, Middaugh CR, Prusik T, Volkin DB. Vaccine instability in the cold chain: Mechanisms, analysis and

formulation strategies. *Biologicals*. 2014;42(5):237-259. doi:10.1016/j.biologicals.2014.05.007

156. Crommelin DJA, Volkin DB, Hoogendoorn KH, Lubiniecki AS, Jiskoot W. The Science is There: Key Considerations for Stabilizing Viral Vector-Based Covid-19 Vaccines. *J Pharm Sci*. 2021;110(2):627-634. doi:10.1016/j.xphs.2020.11.015

157. Moderna. A Study to Evaluate the Efficacy, Safety, and Immunogenicity of mRNA-1647 Cytomegalovirus (CMV) Vaccine in Healthy Participants 16 to 40 Years of Age. *ClinicalTrials.gov Identifier: NCT05085366*. Published online 2021. Accessed November 1, 2022. https://clinicaltrials.gov/ct2/show/NCT05085366

158. Wei M. Pre-Filled Syringe Design For Frozen Formulation (Part 1&2). Published 2021. Accessed October 10, 2022. https://www.linkedin.com/pulse/pre-filled-syringe-design-frozen-formulation-part-1-min-wei/

159. LTI. Lyophilization Technology Inc. Published 2022. Accessed October 3, 2022. https://www.lyotechnology.com/cartridges.cfm