Manufacturing Process of mRNA

Subjects: Biotechnology & Applied Microbiology

Contributor: Maryam Youssef , Cynthia Hitti , Julia Puppin Chaves Fulber , Amine A. Kamen

Advances and discoveries in the structure and role of mRNA as well as novel lipid-based delivery modalities have enabled the advancement of mRNA therapeutics into the clinical trial space. The manufacturing of these products is relatively simple and eliminates many of the challenges associated with cell culture production of viral delivery systems for gene and cell therapy applications, allowing rapid production of mRNA for personalized treatments, cancer therapies, protein replacement and gene editing.

mRNA therapeutics mRNA manufacturing in vitro transcription

1. Upstream Process: DNA Template Sequence Design

The production process of mRNA therapeutics begins with the design of a DNA template for subsequent IVT. Transcription templates for mRNA synthesis can be in the form of plasmid DNA (pDNA), PCR products or synthetic double-stranded oligonucleotides [1][2]. Typically, the DNA template should include the following elements [3]: promoter sequence, gene of interest (GOI), 5′ and 3′ untranslated regions (UTRs), poly(A) tail. Each element can be modified or selected accordingly to improve the stability and translation of the mRNA, as has been thoroughly explored in other publications [4][5].

Most commonly, the T7 promoter sequence (5′-TAATACGACTCACTATA-3′) is used for recognition by the T7 RNA polymerase during IVT, considered the standard polymerase for manufacturing purposes [6]. If using the cap analog CleanCap AG, an additional A is required at the 3′ end of the promoter sequence for an AGG initiator sequence [7]. If the manufacturer aims to use a different RNA polymerase, such as from T3 or SP6, the corresponding promoter must be present in the DNA template. Additionally, in the case of a plasmid DNA construct, an antibiotic resistance marker sequence for bacterial selection and restriction sites for DNA template linearization are required.

In applications in which the gene of interest encodes for a therapeutic protein, optimization of the coding sequence can be performed to reduce protein immunogenicity and increase protein expression [8]. For instance, codon optimization can lead to more controllable translation and increased mRNA half-life [4][8][9][10]. It has been reported that high GC content increases mRNA stability, ribosome association, and thus translation efficiency [4][11][12]. Optimization of the GC content in the GOI, with concurrent uridine depletion in therapeutic mRNA design, not only improves the elongation rate and translation efficiency, but can also alter RNA secondary structures that can interfere with gene expression [9].

Both the 5′- and 3′-UTRs are indispensable for the stability and translation initiation of the therapeutic mRNA molecule being delivered [13]. The β-globin UTRs have been widely used in both clinical trials and research contexts [14][15]. 5′-UTR features such as the length, sequence elements and secondary structures play an important role in translation initiation during scanning [16], with the average length of 5′-UTR in eukaryotes ranging from ~100 to ~200 nucleotides (nt) in mammals [4]. However, it has been proposed that a shorter 5′-UTR with at least 20 nt minimizes the scanning process and thus maximizes protein expression [4]. Moreover, highly stable secondary structures near the 5′-end should be avoided as they can disrupt ribosome loading and scanning [17][18], and potential upstream start codons should be eliminated to avoid leaky scanning [4]. Selective translation can be achieved by introducing additional sequence elements to the 5′-UTR depending on the therapeutic purpose. In the context of cancer therapy, special 5′-UTR elements capable of translation under nutrient restriction may be needed for intratumor mRNA injection [4].

The 3′-UTR, similarly to the 5′-UTR, contains regulatory elements that affect translation efficiency and mRNA stability. It is generally believed that a shorter 3′-UTR increases the stability of the mRNA due to the loss of microRNA binding sites, thus escaping mRNA degradation [4]. Additionally, the use of two sequential β-globin 3′UTRs resulted in significantly higher maximum protein levels and prolonged persistence of the protein [19]. Lastly, high throughput techniques have been developed for 3′-UTR optimizations, including a novel cell-based selection process to identify 3′-UTRs that increase protein expression encoded by synthetic mRNA [20] and a massive parallel functional assay for optimization [21].

The poly(A) tail plays a key role in mRNA translation and stability, as it protects the mRNA from nuclease degradation [22]. The poly(A) tail can be added to the mRNA either by using a poly(A) polymerase after transcription or by already having a poly(A) sequence in the DNA template [23]. The latter is the standard practice for clinical applications, as it allows for a consistent, predetermined length of the poly(A) tail [24]. The extension of the poly(A) tail up to 120 nt has been demonstrated to improve translation efficiency, showing that the length is an important aspect to consider [25][26].

2. Upstream Process: DNA Template Production

2.1. Bacterial Fermentation Approach

Plasmid DNA generation is generally performed through fermentation of *Escherichia coli* (*E. coli*). Several different strains have been reported for pDNA production such as DH5α [27], DH5 [28], DH10B [29], DH1 [30], JM108 [31], and SCS1-L [32]. Among them, DH5α remains the standard strain used in laboratory and industry practices due to the existence of effective widespread protocols that have been previously established [31]. For this methodology, *E. coli* competent cells are transformed with the designed DNA plasmids. At the industrial scale, bacterial culture expansion follows three main steps: inoculation for the creation of a master cell bank, shake flask fermentation, and large-scale bioreactor fermentation. To improve pDNA production yields using this production platform, several strategies can be adopted such as selecting a high-producing bacterial strain and/or combining varying medium composition and culture strategies such as batch or fed-batch mode [33].

Vector engineering has contributed to higher yields such as the use of pUC-based plasmids and R1-based plasmids [33]. A study conducted by Lopes et al. demonstrated that a fed-batch mode culture leads to higher plasmid volumetric yields compared to a batch-mode culture [33]. Another study by Carnes et al. demonstrated that using standard high-copy pUC origin-containing plasmids and novel control parameters for fed-batch fermentation resulted in increased specific pDNA yield with respect to cell mass (up to 1500 mg/L of culture medium) compared to 100–250 mg/L for typical plasmid fermentation media and processes [34]. An *E. coli* DH5α culture in the fed-batch mode, with glucose and glycerol as initial carbon sources in the batch phase, showed a 2.2-fold increase compared to similar feeding phases but with no glycerol [33]. In fact, glycerol is considered a complementary carbon source of glucose because of its high specific plasmid DNA productivity and can be used to increase plasmid yield up to 70.6% [35]. The most used growth medium for *E. coli* is Luria Broth (LB) with yeast extract as the nitrogen source, although other medium compositions have successfully been used, including a modified MBL medium [35].

2.2. Synthetic DNA Approach

To avoid the cloning and preparation steps involved in pDNA production through bacterial fermentation, which are both costly and time consuming, several publications and industrial production platforms have adopted alternative template production methods. The fermentation process can take several days or, at times, several weeks, and it involves expensive reagents, including bacteria and antibiotics [36]. The associated risk of biocontamination in the final product has also become a concern for the good manufacturing practice (GMP) of these therapeutics in the clinical industry [1]. As such, synthetic DNA approaches such as PCR, which allow for time-effective manufacturing, have been used to generate the DNA template for mRNA synthesis [23].

Traditional PCR has successfully been used to produce a DNA template for IVT [37]. In 2022, de Mey et al. introduced a novel approach for the production of mRNA based on a synthetic DNA template generated using assembly PCR with synthetic oligonucleotides [1]. It was reported that using this method, the DNA template amplification can go up to several micrograms, allowing for a fast transition from the DNA production step to the mRNA synthesis in only a few hours [1].

Other synthetic DNA production methods have also been described. For example, Touchlight Genetics Ltd. developed a synthetic DNA manufacturing platform using an in vitro dual enzyme process [36]. This proprietary enzymatic platform enables multi-gram DNA production in weeks, allowing for rapid and large-scale production [36]. However, despite the potential of synthetic DNA template production, these approaches have yet to be established for longer DNA strands. It is crucial to consider not only yield but also the error rate, as mutations should be avoided in the sequence. To produce RNA for therapeutic applications, DNA templates are required to be several kilobases long, and thus bacterial fermentation currently remains most appropriate for these productions.

3. Upstream Process: DNA Template Purification

For use in the IVT reaction, the DNA template must be purified and linearized to ensure the quality of the subsequently produced mRNA. The purification process is most extensive in the case of bacterial fermentation.

Purification of pDNA from bacterial cells typically begins with an alkaline lysis step after cell harvesting [38][39], in which a detergent solution such as sodium dodecyl sulfate (SDS) and sodium hydroxide are used to disrupt the cell membrane [2]. Next, the lysate is neutralized before clarification [2][38][40]. However, due to the viscous nature of the resulting precipitate, separation of cellular components can only be performed by pre-filtration or centrifugation followed by clearing filtration, which can be time-consuming and expensive [41]. Moreover, DNA sensitivity to shear stress requires low shear stress techniques to gently mix the cell lysate and the neutralizing agent, such as a flotation-based method described in a patent by PlasmidFactory [40][41]. Another method that has been explored for pDNA purification is boiling cell lysis [42], which has been successfully scaled up using a streamlined method of plasmid DNA extraction by continual thermal lysis [43].

In many traditional plasmid DNA purification processes, RNAse enzymes are used to degrade the RNA prior to proceeding with chromatography steps for pDNA isolation [44]. However, RNAse A is purified from bovine components and is of concern in large scale manufacturing [44]. Furthermore, for a process ultimately aiming to produce RNA, the addition of RNAse should be avoided. To this end, RNAse-free purification methods have been proposed: Duval et al. implemented calcium chloride precipitation, followed by tangential flow filtration (TFF) for the removal of high molecular weight RNA and low molecular weight RNA species, respectively [44][45]. These two steps also contribute to the reduction in microbial proteins and chromosomal DNA and to concentration of the product. In addition, performing concentration prior to chromatography reduces column loading time, accelerating the overall process [45].

For pDNA isolation, chromatography steps based on three different principles result in high-purity plasmid free of host DNA, RNA, proteins, and endotoxins: size-exclusion chromatography, ion-exchange chromatography, and hydrophobic interaction chromatography [38][46][47][48][49]. The plasmids can be collected in several isoforms: supercoiled circular isoforms, open circular, and linear. A hydrophobic interaction chromatography method has been proposed to select for supercoiled pDNA, as it is the most stable isoform [49][50].

Finally, the DNA template must be linearized to prepare for mRNA transcription. For the use of the SP6 and T7 RNA polymerase during IVT, a 5′overhang is known to be preferable to ensure the stability of the polymerase and to reduce artifacts [51]. To achieve this overhang after linearization, endonucleases such as HindIII, SpeI, SapI, NotI, EcoRI may be used [52]. Removal of this enzyme as well as of the un-linearized DNA is required to isolate the final DNA template and proceed with transcription. In both laboratory- and industry-scale productions, phenol chloroform extraction has been established as the gold standard technique for this step. However, this technique requires the management of highly hazardous materials and is no longer preferred for large-scale clinical operations [53]. As such, Cui et al. demonstrated a method using positively charged resins for strong anion exchange chromatography, which was deemed comparable to phenol chloroform extraction in terms of the quality of the resulting mRNA [53]. Subsequent TFF allows for removal of smaller impurities while filtering the DNA template into an appropriate solvent for the subsequent IVT [52].

4. Upstream Process: mRNA Synthesis

4.1. Enzymatic Synthesis

mRNA is produced by IVT, a relatively rapid and simple process in which an RNA polymerase consumes NTPs (nucleotide triphosphates) to catalyse the synthesis of the mRNA from the corresponding DNA linear template. The required components include RNA Polymerase, NTPs, Magnesium ($MgCl_2$), and a reaction buffer. Various bacteriophage polymerases have been used in the field, such as T7, T3, or SP6 RNA polymerases [54]. The T7 RNA polymerase (T7 RNAP) is the most used RNAP in both research and industry, owing to its ability to produce full-length RNA transcripts (longer than 20 kb) with high fidelity [54][55].

Despite its high fidelity and tolerance for incorporation of non-natural NTPs [55], T7 RNAP can also generate immunostimulatory by-products such as double-stranded RNA (dsRNA) which may affect protein expression and render the downstream purification process more difficult [56][57]. Double-stranded RNA molecules are innate immune response activators and should therefore be avoided in therapeutic applications in which immunotolerance to the treatment is important [57][58][59]. The generation of dsRNA by-products can be significantly decreased by engineering a mutant T7 RNAP using computational, structural, mechanistic and laboratory screening approaches. For example, a double-mutant T7 RNAP (G47A + 884G) successfully reduced dsRNA content while maintaining RNA yield and purity [56]. Other advances include the development of thermostable RNA polymerases, such as Hi-T7 RNA Polymerase M0658 by BioLabs, which has been engineered to withstand IVT performed at high temperatures, preventing loopback transcription [60]. However, some experts have noted that high temperatures (≥48 °C) are difficult to scale up and may lead to RNA degradation [56]. Lastly, the addition of urea at a concentration of 1 M during IVT was shown to be an effective method to reduce the undesired nucleobase pairing that causes dsRNA formation [61].

Moreover, magnesium ions are required as a cofactor for the T7 polymerase. Kern et al. found that below 5 mM of free Mg^{2+}, both the transcription rate and IVT efficiency are greatly reduced [62]. However, there is a lack of consensus on the ideal conditions for free Mg^{2+} concentration. While Sartorius claims that 12–20 mM of $MgCl_2$ per reaction increases mRNA yield [63], Young et al. claim an ideal range between 50 and 60 mM of free Mg^{2+} [64]. Magnesium counter-ions also have an impact on mRNA yield, with both magnesium acetate and magnesium chloride having been successfully used for IVT [37][65]. A study showed that magnesium acetate is preferred over chloride [65], which was corroborated by Samnuan et al. [66].

The use of modified NTPs such as N1-methylpseudouridine (m1ψ) has been found to reduce the immunogenicity of synthetic mRNA and to drive high levels of protein production, which is in part attributed to its ability to blunt TLR3 activation [55]. The incorporation of other modified nucleotides such as pseudouridine (ψ), 5-methylcytidine (m5C), N6-methyladenosine (m6A), 5-methyluridine (m5U), or 2-thiouridine (s2U) have also shown reduced immunostimulatory effect of the delivered RNA and enhanced translation [55][67][68][69]. The reduction in immune stimulation and increase in stability of the mRNA molecule are especially important in the context of mRNA therapeutics and protein replacement therapies where degradation and lack of translation pose a direct obstacle to the function of the product.

The addition of spermidine to the reaction mixture at a concentration of 1 to 3 mM has been shown to enhance transcription while having an inhibitory effect at higher concentrations [37]. A design of experiment (DoE) performed by Samnuan et al. found that spermidine enhances transcription when present at a concentration of 0.2 to 2 mM [66], further supporting its use.

Mature mRNA requires the 5′ cap structure for mRNA stability and gene expression. A Cap 1 (m^7GpppN_1mp) structure is preferred for optimal mRNA stability and expression, as it is recognized as self by the immune system [70]. Cap 0 ($m^7GpppNp$), on the other hand, can activate an innate immune response, impairing stability and expression levels [71]. There are two main methods for capping: (1) post-translational capping, where the transcribed mRNA is capped in an additional step using enzymes, or (2) co-transcriptional capping, a one-step process where a Cap analog is incorporated in the IVT reaction.

The vaccinia virus capping enzyme (VCE) is commonly used for enzymatic capping and results in a Cap 0 structure [72]. An additional step using 2′-O-methyltransferase (2′-O-MTase) modifies Cap 0 into a Cap 1 structure [73], reaching a capping efficiency of up to 100% [71]. Moderna has successfully employed this capping strategy in their mRNA-1273 vaccine against SARS-CoV-2 [74]. It is important to note, however, that the addition of several enzymatic steps, including added purification and buffer exchange between the steps, can make the process more difficult to streamline and control [71].

The second capping method uses cap analogs co-transcriptionally, thus reducing production steps. Early iterations of cap analogs had the risk of mRNA elongation in the reverse direction, reducing translation efficiency [75][76]. To avoid this, the anti-reverse cap analog (ARCA) emerged; however, it could only be used to generate constructs with a Cap 0 structure and lower capping efficiency (60–80%) [71][77]. Later, CleanCap technology revolutionized the field as this cap analog allowed for a co-transcriptional addition of the naturally occurring Cap 1 structure at 90–99% efficiency [71], which was successfully implemented in the Pfizer-BioNTech BNT162b2 vaccine against SARS-CoV-2 for emergency use [78]. Cap analogs can lead to a simpler and faster process compared to enzymatic capping, but the use of patented technology can come at a high cost, which should be carefully analyzed in comparison to the cost of enzymatic capping [79].

The IVT reaction may be conducted in both batch and fed-batch modes. The fed-batch mode involves the addition of NTPs and Mg feed during the reaction, as these components have the highest impact on the rate of the reaction as well as the yield [80]. This approach was first demonstrated in 1999 by Kern et al. to produce short RNA molecules [81]. Fed-batch IVT has since been employed on larger mRNA molecules to achieve increased yields [82]. The consumption of NTPs may be monitored using HPLC throughout the duration of the reaction, and they can be supplemented accordingly [80]. Both exceedingly low and high NTP concentrations have been shown to be limiting to the production of RNA, thus supporting the approach to control NTP levels throughout production [66]. This method has previously led to mRNA production with yields of up to 12 g/L [80] compared to the usual 5 g/L [83], showing the immense potential in process intensification.

4.2. Towards Automated Production of mRNA

Although production of mRNA therapeutics in a continuous mode has yet to be implemented, several publications have explored this perspective [79][84][85][86]. The intensification of mRNA manufacturing by integrating production and purification in a continuous manner could decrease the hold times and freeze–thaw cycles during the process, potentially increasing the quality and yield of the final product. This could address the challenge in obtaining a higher amount of product with low immunogenicity required for the repeated dosing of mRNA therapeutics. Self-amplifying RNA (saRNA) has been proposed as a modality to reduce manufacturing burden and costs, as a lower dose is required to reach the same level of protein expression as conventional mRNA [83]. However, it is important to note that saRNA can have immunogenic effects [87] that would make it undesirable for chronically dosed treatments.

Continuous processes require the implementation of controls throughout the production to validate the product and to facilitate their automation. Well-defined process models are vital in the transition to continuous automated production. To this end, Helgers et al. produced in silico models for the continuous production of mRNA in plug flow and continuously stirred tank reactors and determined a theoretical improvement factor of 56 times for the space-time yield in comparison to batch production in a continuously stirred tank reactor [84]. Vetter et al. also suggested that the use of control loops, such as proportional integral derivative (PID) control, can be key to improving productivity, robustness and compliance with critical quality attributes (CQA) [85]. Rosa et al. and Ouranidis et al. produced digital designs and conceptual designs for the continuous end-to-end manufacturing of mRNA therapeutics, establishing the initial frameworks for future experimental work to build upon these initial approaches [79][86].

4.3. Solid-Phase Synthesis

First established in 1963 by Merrifield for the production of peptides [88], the chemical synthesis method was adapted to the production of short oligonucleotides and advanced by Beaucage and Caruthers [89][90]. Once scaled up to the industrial scale, this method allowed for the production of oligonucleotides up to the kilogram scale, and it can be fully automated [91][92]. The method relies on phosphoramidite chemistry and involves the cyclical addition of nucleosides in a sequence-specific manner on a solid support [93][94]. However, this method for RNA production is only suitable for short oligomers, with some claiming its ability to form chains of up to 40 nucleotides and others using this method extending up to approximately 100–150 nucleotides [73][95][96]. This renders this method currently unsuitable for mRNA production, but rather, it is appropriate for the production of siRNA, miRNA and anti-sense oligonucleotide (ASO) molecules as a straightforward chemical synthesis. It is possible to generate longer RNA molecules through the synthesis of two separate strands and joining them together through ligation strategies, namely T4 ligase [97][98], but this has not yet reached the efficiency and productivity that is established with enzymatic synthesis of mRNA.

5. Downstream Process: mRNA Purification

Isolation of complete mRNA transcripts from reagents and reaction by-products is critical for both product functionality and regulatory considerations. Process-related impurities include residual reagents (DNA template,

enzymes, unincorporated NTPs) and by-products (immunogenic dsRNA and aborted mRNA products). DNA, RNA (around 300 kDa per kb [99]) and T7 polymerase (99 kDa [53]) are the larger components, while NTPs and cap analogs are much smaller (less than 1 kDa). The DNA template is typically larger than the mRNA produced, as the linearized plasmid includes a backbone sequence that is not transcribed. The purification of mRNA is essential to ensure immunotolerance and to achieve biologically active and therapeutically administrable mRNA [100].

The removal of the DNA template is typically accomplished via enzymatic digestion with DNAse I prior to other purification steps [79][101][102], followed by inactivation with EDTA. However, industry experts have previously indicated that the use of DNAse I may lead to the small DNA template fragments hybridizing to the final mRNA product [52]. Alternatively, chromatography capture methods have been implemented in some processes to remove the DNA template without digestion. Oligo-dT purification is of particular interest due to its ability to bind the polyA tail of the complete mRNA transcripts, without binding the DNA template, truncated mRNA transcripts, unused nucleotides, and the enzyme [52][103]. Cui et al. demonstrated that Oligo-dT chromatography purification may lead to mRNA recoveries of over 90% [53]. However, Oligo-dT purification may not be sufficient to separate dsRNA from the product and must be followed by polishing chromatography steps in the cases of high dsRNA content [79]. Several other chromatography techniques have been demonstrated for mRNA purification, but they were typically preceded by a TFF step, while the Oligo-dT method was used directly as a capture step for a more streamlined process [53].

For therapeutic applications, an emphasis has been placed on the elimination of dsRNA from the IVT mixture to improve translation efficacy and limit induction of cytokines. Several strategies have been described in the literature to ease the burden on purification by avoiding dsRNA formation throughout the reaction, including the addition of urea or use of modified T7 RNAP, as discussed in the mRNA synthesis section. The gold standard purification method for the removal of dsRNA is high-performance liquid chromatography (HPLC) using an alkylated non-porous polystyrene-divinylbenzene copolymer matrix [104]. A simple method using cellulose has proven successful to selectively remove dsRNA at the small scale [105]. Other methods, such as hydroxyapatite chromatography, core bead chromatography, and anion exchange chromatography, have been explored to improve the scalability of the platform [53]. For example, GSK has previously detailed an RNA purification method using DNAse, TFF, and CaptoCore resins, a core bead chromatography resin [106]. These purification methods may be product dependent; however, it has previously been demonstrated that mRNA recovery using core bead chromatography varies based on the length of the sequence [53].

6. Downstream Process: mRNA Delivery

6.1. Microfluidic Manufacturing of mRNA-LNPs

The lipid concentration, the mRNA concentration, the lipid to mRNA ratio as well as the encapsulation technique impact the particle size, polydispersity, surface charge and RNA encapsulation efficiency, all of which are critical quality attributes of the product and impact the activity of the product [107]. As such, the encapsulation protocol should be carefully optimized to meet these standards.

mRNA LNPs are most commonly formulated using T-junction mixing and microfluidic mixing, with work ongoing to improve the scalability of these processes for clinical applications. At a laboratory scale, sonication and bulk mixing have been used, but they present limitations in reproducibility and scalability [108]. Benchtop instruments such as Precision Nanosystems Spark and Ignite systems have been adopted for mRNA LNP formation, both in laboratories and in the industry. There are, however, a number of publications which have designed custom microfluidic devices for this formulation step.

6.2. mRNA-LNP Formulation

Thermostability and physical stability of mRNA-LNPs remains a challenge in the translation of mRNA therapeutics to the market. In non-urgent applications, as in the case of therapeutic use rather than the case of pandemic use, the product may require long-term storage prior to its use. Furthermore, to improve treatment accessibility and transport, refrigerated or room temperature storage are preferable. Both lyophilized and liquid formulations have been considered for the improvement of mRNA-LNP storage. It has been demonstrated that the choice of buffer and cryopreservatives impact the stability of the LNPs both in liquid and solid formulations. Henderson et al. found that, for example, the Hepes buffer better maintains the morphology of the LNPs after freeze–thaw compared to Tris and PBS [109]. Furthermore, their study found that LNPs stored in Tris buffer lead to improved expression of the delivered gene compared to those stored in PBS and Hepes [109]. Zhao et al. evaluated cryopreservatives in the long-term storage of lipid-like nanoparticles under aqueous, freezing and lyophilized conditions [110]. Their results suggested that, when stored at 4 °C, the particles lose the majority of their delivery efficiency within 5 months of storage. Additionally, they found that sucrose and trehalose outperformed mannitol maintaining the delivery efficiency when freezing in liquid nitrogen [110]. Similar results were obtained concerning the efficacy of both sucrose and trehalose at preserving LNPs throughout freezing for LNPs encapsulating siRNA [111]. Similarly, Kim et al. found that LNPs stored in 10% *w*/*v* sucrose in PBS did not lose potency after 1 month of storage. Despite most of the current publications presenting data in support of freezing of mRNA-LNPs rather than aqueous conditions, Zhang et al. previously presented a thermostable aqueous vaccine which remains stable for 7 days at 4 °C and 25 °C [112].

Furthermore, formulations undoubtedly vary based on the target application and administration method as well. For example, for lung delivery through inhalation, the formulation requires nebulization. This process can easily destabilize the mRNA-LNPs, impacting size, encapsulation efficiency and subsequent mRNA expression. While the lipid composition of the LNPs impacts their stability throughout the aerosolization process [113], formulation excipients can also be used to decrease changes in these critical quality attributes throughout the process. A patent from Moderna, for example, has previously described mRNA-LNP formulation variations appropriate for nebulization which include the addition of P188 and sucrose to a Tris buffer [114]. Their formulations were also able to maintain both size and encapsulation efficiency over the course of 19 freeze–thaw cycles [114].

Several studies involving the lyophilization of mRNA-LNPs have also been completed since the evolution of the lipid nanoparticle [115][116][117]. Lyophilization promises the ability to store mRNA-LNP formulations at room temperature without the effects of hydrolysis, as is the case of aqueous formulations [115]. Lamoot et al. found that

the addition of 20% sucrose (*w*/*v*) in a Tris-based buffer allowed for successful lyophilization without major impacts on LNP size, zeta potential or in vitro expression of the encoded gene [115]. Additionally, Muramatsu et al. found that lyophilization of mRNA LNPs in the presence of sucrose (10% *w*/*v*) and maltose (10% *w*/*v*) provided long-term stability (for at least 12 weeks) of the formulation both at room temperature and at 4 °C [115]. These results emphasize the need for the evaluation of cryoprotectants and lypoprotectants throughout the manufacturing process of lipid-based mRNA therapeutics.

References

1. de Mey, W.; De Schrijver, P.; Autaers, D.; Pfitzer, L.; Fant, B.; Locy, H.; Esprit, A.; Lybaert, L.; Bogaert, C.; Verdonck, M. A synthetic DNA template for fast manufacturing of versatile single epitope mRNA. Mol. Ther.-Nucleic Acids 2022, 29, 943–954.
2. Prather, K.J.; Sagar, S.; Murphy, J.; Chartrain, M. Industrial scale production of plasmid DNA for vaccine and gene therapy: Plasmid design, production, and purification. Enzym. Microb. Technol. 2003, 33, 865–883.
3. Deng, Z.; Tian, Y.; Song, J.; An, G.; Yang, P. mRNA vaccines: The dawn of a new era of cancer immunotherapy. Front. Immunol. 2022, 13, 887125.
4. Jia, L.; Qian, S.-B. Therapeutic mRNA Engineering from Head to Tail. Acc. Chem. Res. 2021, 54, 4272–4282.
5. Xia, X. Detailed Dissection and Critical Evaluation of the Pfizer/BioNTech and Moderna mRNA Vaccines. Vaccines 2021, 9, 734.
6. Trepotec, Z.; Aneja, M.K.; Geiger, J.; Hasenpusch, G.; Plank, C.; Rudolph, C. Maximizing the translational yield of mRNA therapeutics by minimizing 5′-UTRs. Tissue Eng. Part A 2019, 25, 69–79.
7. TriLink Biotechnologies. CleanCap Reagent AG Product Insert (Catalog No. N-7113 Version v3). Available online: https://www.trilinkbiotech.com/media/folio3/productattachments/product_insert/n7113_insert_v3.pdf (accessed on 5 October 2023).
8. Weng, Y.; Li, C.; Yang, T.; Hu, B.; Zhang, M.; Guo, S.; Xiao, H.; Liang, X.-J.; Huang, Y. The challenge and prospect of mRNA therapeutics landscape. Biotechnol. Adv. 2020, 40, 107534.
9. Mauger, D.M.; Cabral, B.J.; Presnyak, V.; Su, S.V.; Reid, D.W.; Goodman, B.; Link, K.; Khatwani, N.; Reynders, J.; Moore, M.J. mRNA structure regulates protein expression through changes in functional half-life. Proc. Natl. Acad. Sci. USA 2019, 116, 24075–24083.

10. McClellan, D.A. The codon-degeneracy model of molecular evolution. J. Mol. Evol. 2000, 50, 131–140.
11. Mordstein, C.; Savisaar, R.; Young, R.S.; Bazile, J.; Talmane, L.; Luft, J.; Liss, M.; Taylor, M.S.; Hurst, L.D.; Kudla, G. Codon usage and splicing jointly influence mRNA localization. Cell Syst. 2020, 10, 351–362.e358.
12. Thess, A.; Grund, S.; Mui, B.L.; Hope, M.J.; Baumhof, P.; Fotin-Mleczek, M.; Schlake, T. Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals. Mol. Ther. 2015, 23, 1456–1464.
13. Qin, S.; Tang, X.; Chen, Y.; Chen, K.; Fan, N.; Xiao, W.; Zheng, Q.; Li, G.; Teng, Y.; Wu, M. mRNA-based therapeutics: Powerful and versatile tools to combat diseases. Signal Transduct. Target. Ther. 2022, 7, 166.
14. Kariko, K.; Kuo, A.; Barnathan, E. Overexpression of urokinase receptor in mammalian cells following administration of the in vitro transcribed encoding mRNA. Gene Ther. 1999, 6, 1092–1100.
15. Benteyn, D.; Anguille, S.; Van Lint, S.; Heirman, C.; Van Nuffel, A.M.; Corthals, J.; Ochsenreither, S.; Waelput, W.; Van Beneden, K.; Breckpot, K. Design of an optimized Wilms' tumor 1 (WT1) mRNA construct for enhanced WT1 expression and improved immunogenicity in vitro and in vivo. Mol. Ther. Nucleic Acids 2013, 2, e134.
16. Leppek, K.; Das, R.; Barna, M. Author Correction: Functional 5′ UTR mRNA structures in eukaryotic translation regulation and how to find them. Nat. Rev. Mol. Cell Biol. 2018, 19, 673.
17. Ding, Y.; Tang, Y.; Kwok, C.K.; Zhang, Y.; Bevilacqua, P.C.; Assmann, S.M. In vivo genome-wide profiling of RNA secondary structure reveals novel regulatory features. Nature 2014, 505, 696–700.
18. Wan, Y.; Qu, K.; Zhang, Q.C.; Flynn, R.A.; Manor, O.; Ouyang, Z.; Zhang, J.; Spitale, R.C.; Snyder, M.P.; Segal, E. Landscape and variation of RNA secondary structure across the human transcriptome. Nature 2014, 505, 706–709.
19. Holtkamp, S.; Kreiter, S.; Selmi, A.; Simon, P.; Koslowski, M.; Huber, C.; Tureci, O.z.; Sahin, U. Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood 2006, 108, 4009–4017.
20. von Niessen, A.G.O.; Poleganov, M.A.; Rechner, C.; Plaschke, A.; Kranz, L.M.; Fesser, S.; Diken, M.; Löwer, M.; Vallazza, B.; Beissert, T. Improving mRNA-based therapeutic gene delivery by expression-augmenting 3′ UTRs identified by cellular library screening. Mol. Ther. 2019, 27, 824–836.
21. Zhao, W.; Pollack, J.L.; Blagev, D.P.; Zaitlen, N.; McManus, M.T.; Erle, D.J. Massively parallel functional annotation of 3′ untranslated regions. Nat. Biotechnol. 2014, 32, 387–391.

22. Kwon, H.; Kim, M.; Seo, Y.; Moon, Y.S.; Lee, H.J.; Lee, K.; Lee, H. Emergence of synthetic mRNA: In vitro synthesis of mRNA and its applications in regenerative medicine. Biomaterials 2018, 156, 172–193.
23. Sahin, U.; Karikó, K.; Türeci, Ö. mRNA-based therapeutics—Developing a new class of drugs. Nat. Rev. Drug Discov. 2014, 13, 759–780.
24. Kim, S.C.; Sekhon, S.S.; Shin, W.-R.; Ahn, G.; Cho, B.-K.; Ahn, J.-Y.; Kim, Y.-H. Modifications of mRNA vaccine structural elements for improving mRNA stability and translation efficiency. Mol. Cell. Toxicol. 2021, 18, 1–8.
25. Kormann, M.S.; Hasenpusch, G.; Aneja, M.K.; Nica, G.; Flemmer, A.W.; Herber-Jonat, S.; Huppmann, M.; Mays, L.E.; Illenyi, M.; Schams, A. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. Nat. Biotechnol. 2011, 29, 154–157.
26. Jalkanen, A.L.; Coleman, S.J.; Wilusz, J. Determinants and implications of mRNA poly (A) tail size–does this protein make my tail look big? Semin. Cell Dev. Biol. 2014, 34, 24–32.
27. Lara, A.R.; Knabben, I.; Regestein, L.; Sassi, J.; Caspeta, L.; Ramírez, O.T.; Büchs, J. Comparison of oxygen enriched air vs. pressure cultivations to increase oxygen transfer and to scale-up plasmid DNA production fermentations. Eng. Life Sci. 2011, 11, 382–386.
28. Listner, K.; Bentley, L.; Okonkowski, J.; Kistler, C.; Wnek, R.; Caparoni, A.; Junker, B.; Robinson, D.; Salmon, P.; Chartrain, M. Development of a highly productive and scalable plasmid DNA production platform. Biotechnol. Prog. 2006, 22, 1335–1345.
29. Lahijani, R.; Hulley, G.; Soriano, G.; Horn, N.A.; Marquet, M. High-yield production of pBR322-derived plasmids intended for human gene therapy by employing a temperature-controllable point mutation. Hum. Gene Ther. 1996, 7, 1971–1980.
30. Cooke, J.R.; McKie, E.A.; Ward, J.M.; Keshavarz-Moore, E. Impact of intrinsic DNA structure on processing of plasmids for gene therapy and DNA vaccines. J. Biotechnol. 2004, 114, 239–254.
31. Selas Castiñeiras, T.; Williams, S.G.; Hitchcock, A.G.; Smith, D.C. E. coli strain engineering for the production of advanced biopharmaceutical products. FEMS Microbiol. Lett. 2018, 365, fny162.
32. Singer, A.; Eiteman, M.A.; Altman, E. DNA plasmid production in different host strains of Escherichia coli. J. Ind. Microbiol. Biotechnol. 2009, 36, 521–530.
33. Lopes, M.B.; Gonçalves, G.A.; Felício-Silva, D.; Prather, K.L.; Monteiro, G.A.; Prazeres, D.M.; Calado, C.R. In situ NIR spectroscopy monitoring of plasmid production processes: Effect of producing strain, medium composition and the cultivation strategy. J. Chem. Technol. Biotechnol. 2015, 90, 255–261.
34. Carnes, A.E.; Hodgson, C.P.; Williams, J.A. Inducible Escherichia coli fermentation for increased plasmid DNA production. Biotechnol. Appl. Biochem. 2006, 45, 155–166.

35. Xu, Z.-N.; Shen, W.-H.; Chen, H.; Cen, P.-L. Effects of medium composition on the production of plasmid DNA vector potentially for human gene therapy. J. Zhejiang Univ. SCIENCE B 2005, 6, 396–400.
36. Ohlson, J. Plasmid manufacture is the bottleneck of the genetic medicine revolution. Drug Discov. Today 2020, 25, 1891.
37. Rosa, S.S.; Nunes, D.; Antunes, L.; Prazeres, D.M.; Marques, M.P.; Azevedo, A.M. Maximizing mRNA vaccine production with Bayesian optimization. Biotechnol. Bioeng. 2022, 119, 3127–3139.
38. Sun, B.; Yu, X.; Yin, Y.; Liu, X.; Wu, Y.; Chen, Y.; Zhang, X.; Jiang, C.; Kong, W. Large-scale purification of pharmaceutical-grade plasmid DNA using tangential flow filtration and multi-step chromatography. J. Biosci. Bioeng. 2013, 116, 281–286.
39. Bimboim, H.C.; Doly, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 1979, 7, 1513–1523.
40. Voss, C.; Flaschel, E. Method for Producing Extra-Chromosomal Nucleic Acid Molecules. U.S. Patent US7842481B2, 30 November 2010.
41. Schmeer, M.; Schleef, M. Pharmaceutical grade large-scale plasmid DNA manufacturing process. In DNA Vaccines: Methods and Protocols; Humana: New York, NY, USA, 2014; pp. 219–240.
42. Holmes, D.S.; Quigley, M. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 1981, 114, 193–197.
43. Zhu, K.; Jin, H.; He, Z.; Zhu, Q.; Wang, B. A continuous method for the large-scale extraction of plasmid DNA by modified boiling lysis. Nat. Protoc. 2006, 1, 3088–3093.
44. Eon-Duval, A.; Gumbs, K.; Ellett, C. Precipitation of RNA impurities with high salt in a plasmid DNA purification process: Use of experimental design to determine reaction conditions. Biotechnol. Bioeng. 2003, 83, 544–553.
45. Eon-Duval, A.; MacDuff, R.H.; Fisher, C.A.; Harris, M.J.; Brook, C. Removal of RNA impurities by tangential flow filtration in an RNase-free plasmid DNA purification process. Anal. Biochem. 2003, 316, 66–73.
46. Latulippe, D.R.; Zydney, A.L. Size exclusion chromatography of plasmid DNA isoforms. J. Chromatogr. A 2009, 1216, 6295–6302.
47. Eon-Duval, A.; Burke, G. Purification of pharmaceutical-grade plasmid DNA by anion-exchange chromatography in an RNase-free process. J. Chromatogr. B 2004, 804, 327–335.
48. Bo, H.; Wang, J.; Chen, Q.; Shen, H.; Wu, F.; Shao, H.; Huang, S. Using a single hydrophobic-interaction chromatography to purify pharmaceutical-grade supercoiled plasmid DNA from other isoforms. Pharm. Biol. 2013, 51, 42–48.

49. Černigoj, U.; Štrancar, A. Scale-up of plasmid DNA downstream process based on chromatographic monoliths. In DNA Vaccines: Methods and Protocols; Humana: New York, NY, USA, 2021; pp. 167–192.
50. Parker, T.; Cherradi, Y.; Mishra, N. Scalable Purification of Plasmid DNA: Strategies and Considerations for Vaccine and Gene Therapy Manufacturing; Application Note MS-WP7159EN Ver; Millipore Sigma: Burlington, MA, USA, 2020; Volume 1.
51. Hornblower, B.; Robb, G.B.; Tzertzinis, G. Minding Your Caps and Tails—Considerations for Functional mRNA Synthesis. Available online: https://international.neb.com/tools-and-resources/feature-articles/minding-your-caps-and-tails (accessed on 5 October 2023).
52. Bancel, S.; Issa, W.J.; Aunins, J.G.; Chakraborty, T. Manufacturing Methods for Production of RNA Transcripts. U.S. Patent US10138507B2, 27 November 2018.
53. Cui, T.; Fakhfakh, K.; Turney, H.; Güler-Gane, G.; Toloczko, A.; Hulley, M.; Turner, R. Comprehensive studies on building a scalable downstream process for mRNAs to enable mRNA therapeutics. Biotechnol. Prog. 2023, 39, e3301.
54. Kwon, S.; Kwon, M.; Im, S.; Lee, K.; Lee, H. mRNA vaccines: The most recent clinical applications of synthetic mRNA. Arch. Pharmacal Res. 2022, 45, 245–262.
55. Nance, K.D.; Meier, J.L. Modifications in an emergency: The role of N1-methylpseudouridine in COVID-19 vaccines. ACS Cent. Sci. 2021, 7, 748–756.
56. Dousis, A.; Ravichandran, K.; Hobert, E.M.; Moore, M.J.; Rabideau, A.E. An engineered T7 RNA polymerase that produces mRNA free of immunostimulatory byproducts. Nat. Biotechnol. 2023, 41, 560–568.
57. Mu, X.; Greenwald, E.; Ahmad, S.; Hur, S. An origin of the immunogenicity of in vitro transcribed RNA. Nucleic Acids Res. 2018, 46, 5239–5249.
58. Durbin, A.F.; Wang, C.; Marcotrigiano, J.; Gehrke, L. RNAs containing modified nucleotides fail to trigger RIG-I conformational changes for innate immune signaling. mBio 2016, 7, e00833-16.
59. Peisley, A.; Jo, M.H.; Lin, C.; Wu, B.; Orme-Johnson, M.; Walz, T.; Hohng, S.; Hur, S. Kinetic mechanism for viral dsRNA length discrimination by MDA5 filaments. Proc. Natl. Acad. Sci. USA 2012, 109, E3340–E3349.
60. Wu, M.Z.; Asahara, H.; Tzertzinis, G.; Roy, B. Synthesis of low immunogenicity RNA with high-temperature in vitro transcription. RNA 2020, 26, 345–360.
61. Piao, X.; Yadav, V.; Wang, E.; Chang, W.; Tau, L.; Lindenmuth, B.E.; Wang, S.X. Double-stranded RNA reduction by chaotropic agents during in vitro transcription of messenger RNA. Mol. Ther. Nucleic Acids 2022, 29, 618–624.

62. Kern, J.A.; Davis, R.H. Application of Solution Equilibrium Analysis to inVitro RNA Transcription. Biotechnol. Prog. 1997, 13, 747–756.

63. Nemec, K.S.; Livk, A.G.; Celjar, A.M.; Skok, J.; Sekirnik, R.; Kostelec, T.; Gagnon, P.; Štrancar, A. Effect of Mg2+ Ion Concentration on IVT Reaction Kinetics Determined by Novel Rapid Analytical HPLC Assay; Sartorius Company: Göttingen, Germany, 2021.

64. Young, J.S.; Ramirez, W.F.; Davis, R.H. Modeling and optimization of a batch process for in vitro RNA production. Biotechnol. Bioeng. 1997, 56, 210–220.

65. Nikolic, M.; Gasiūnienė, M.; Asa, D.; Šeputienė, V. Determination of the Optimal Buffer Conditions and Nucleotide Concentrations to Maximize mRNA Yield Using In Vitro Transcription; ThermoFisher Scientific: Waltham, MA, USA, 2022.

66. Samnuan, K.; Blakney, A.K.; McKay, P.F.; Shattock, R.J. Design-of-experiments in vitro transcription yield optimization of self-amplifying RNA. bioRxiv 2021.

67. Karikó, K.; Muramatsu, H.; Welsh, F.A.; Ludwig, J.; Kato, H.; Akira, S.; Weissman, D. Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol. Ther. 2008, 16, 1833–1840.

68. Fotin-Mleczek, M.; Duchardt, K.M.; Lorenz, C.; Pfeiffer, R.; Ojkic-Zrna, S.; Probst, J.; Kallen, K.-J. Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. J. Immunother. 2011, 34, 1–15.

69. Yang, L.; Tang, L.; Zhang, M.; Liu, C. Recent advances in the molecular design and delivery technology of mRNA for vaccination against infectious diseases. Front. Immunol. 2022, 13, 896958.

70. Borden, K.L. The eukaryotic translation initiation factor eIF4E wears a “cap” for many occasions. Translation 2016, 4, e1220899.

71. Linares-Fernández, S.; Lacroix, C.; Exposito, J.-Y.; Verrier, B. Tailoring mRNA vaccine to balance innate/adaptive immune response. Trends Mol. Med. 2020, 26, 311–323.

72. Fang, E.; Liu, X.; Li, M.; Zhang, Z.; Song, L.; Zhu, B.; Wu, X.; Liu, J.; Zhao, D.; Li, Y. Advances in COVID-19 mRNA vaccine development. Signal Transduct. Target. Ther. 2022, 7, 94.

73. Pradère, U.; Halloy, F.; Hall, J. Chemical synthesis of long RNAs with terminal 5′-phosphate groups. Chem. Eur. J. 2017, 23, 5210–5213.

74. Corbett, K.S.; Edwards, D.K.; Leist, S.R.; Abiona, O.M.; Boyoglu-Barnum, S.; Gillespie, R.A.; Himansu, S.; Schäfer, A.; Ziwawo, C.T.; Di Piazza, A.T. SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness. Nature 2020, 586, 567–571.

75. Jemielity, J.; Fowler, T.; Zuberek, J.; Stepinski, J.; Lewdorowicz, M.; Niedzwiecka, A.; Stolarski, R.; Darzynkiewicz, E.; Rhoads, R.E. Novel “anti-reverse” cap analogs with superior translational

properties. RNA 2003, 9, 1108–1122.

76. Stepinski, J.; Waddell, C.; Stolarski, R.; Darzynkiewicz, E.; Rhoads, R.E. Synthesis and properties of mRNAs containing the novel “anti-reverse” cap analogs 7-methyl (3′-O-methyl) GpppG and 7-methyl (3′-deoxy) GpppG. RNA 2001, 7, 1486–1495.
77. Kuhn, A.; Diken, M.; Kreiter, S.; Selmi, A.; Kowalska, J.; Jemielity, J.; Darzynkiewicz, E.; Huber, C.; Türeci, Ö.; Sahin, U. Phosphorothioate cap analogs increase stability and translational efficiency of RNA vaccines in immature dendritic cells and induce superior immune responses in vivo. Gene Ther. 2010, 17, 961–971.
78. Henderson, J.M.; Ujita, A.; Hill, E.; Yousif-Rosales, S.; Smith, C.; Ko, N.; McReynolds, T.; Cabral, C.R.; Escamilla-Powers, J.R.; Houston, M.E. Cap 1 messenger RNA synthesis with co-transcriptional cleancap® analog by in vitro transcription. Curr. Protoc. 2021, 1, e39.
79. Rosa, S.S.; Prazeres, D.M.; Azevedo, A.M.; Marques, M.P. mRNA vaccines manufacturing: Challenges and bottlenecks. Vaccine 2021, 39, 2190–2200.
80. Pregeljc, D.; Skok, J.; Vodopivec, T.; Mencin, N.; Krušič, A.; Ličen, J.; Nemec, K.Š.; Štrancar, A.; Sekirnik, R. Increasing yield of in vitro transcription reaction with at-line high pressure liquid chromatography monitoring. Biotechnol. Bioeng. 2023, 120, 737–747.
81. Kern, J.A.; Davis, R.H. Application of a fed-batch system to produce RNA by in vitro transcription. Biotechnol. Prog. 1999, 15, 174–184.
82. Skok, J.; Megušar, P.; Vodopivec, T.; Pregeljc, D.; Mencin, N.; Korenč, M.; Krušič, A.; Celjar, A.M.; Pavlin, N.; Krušič, J. Gram-Scale mRNA Production Using a 250-mL Single-Use Bioreactor. Chem. Ing. Tech. 2022, 94, 1928–1935.
83. Kis, Z.; Kontoravdi, C.; Shattock, R.; Shah, N. Resources, production scales and time required for producing RNA vaccines for the global pandemic demand. Vaccines 2020, 9, 3.
84. Helgers, H.; Hengelbrock, A.; Schmidt, A.; Strube, J. Digital twins for continuous mRNA production. Processes 2021, 9, 1967.
85. Vetter, F.L.; Zobel-Roos, S.; Mota, J.P.B.; Nilsson, B.; Schmidt, A.; Strube, J. Toward Autonomous Production of mRNA-Therapeutics in the Light of Advanced Process Control and Traditional Control Strategies for Chromatography. Processes 2022, 10, 1868.
86. Ouranidis, A.; Davidopoulou, C.; Tashi, R.-K.; Kachrimanis, K. Pharma 4.0 continuous mRNA drug products manufacturing. Pharmaceutics 2021, 13, 1371.
87. Liu, C.; Shi, Q.; Huang, X.; Koo, S.; Kong, N.; Tao, W. mRNA-based cancer therapeutics. Nat. Rev. Cancer 2023, 23, 526–543.
88. Merrifield, R.B. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc. 1963, 85, 2149–2154.

89. Beaucage, S.; Caruthers, M. Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Lett. 1981, 22, 1859–1862.
90. RL, L.; Mahadevan, V. Oligonucleotide synthesis on a polymer support. J. Am. Chem. Soc. 1965, 87, 3526–3527.
91. Li, N.-S.; Frederiksen, J.K.; Piccirilli, J.A. Automated solid-phase synthesis of RNA oligonucleotides containing a nonbridging phosphorodithioate linkage via phosphorothioamidites. J. Org. Chem. 2012, 77, 9889–9892.
92. Sanghvi, Y.S. Large-scale automated synthesis of therapeutic oligonucleotides: A status update. Adv. Nucleic Acid Ther. 2019, 68, 453–473.
93. Cedillo, I.; Chreng, D.; Engle, E.; Chen, L.; McPherson, A.K.; Rodriguez, A.A. Synthesis of 5′-GalNAc-conjugated oligonucleotides: A comparison of solid and solution-phase conjugation strategies. Molecules 2017, 22, 1356.
94. Kumar, R.K.; Guzaev, A.P.; Rentel, C.; Ravikumar, V.T. Efficient synthesis of antisense phosphorothioate oligonucleotides using a universal solid support. Tetrahedron 2006, 62, 4528–4534.
95. Ryczek, M.; Pluta, M.; Błaszczyk, L.; Kiliszek, A. Overview of Methods for Large-Scale RNA Synthesis. Appl. Sci. 2022, 12, 1543.
96. Flamme, M.; McKenzie, L.K.; Sarac, I.; Hollenstein, M. Chemical methods for the modification of RNA. Methods 2019, 161, 64–82.
97. Yu, C.-H.; Kabza, A.M.; Sczepanski, J.T. Assembly of long l-RNA by native RNA ligation. Chem. Commun. 2021, 57, 10508–10511.
98. Kershaw, C.J.; O'Keefe, R.T. Splint ligation of RNA with T4 DNA ligase. In Recombinant and In Vitro RNA Synthesis: Methods and Protocols; Springer Science + Business Media: Berlin/Heidelberg, Germany, 2012; pp. 257–269.
99. Kowalski, P.S.; Rudra, A.; Miao, L.; Anderson, D.G. Delivering the messenger: Advances in technologies for therapeutic mRNA delivery. Mol. Ther. 2019, 27, 710–728.
100. Webb, C.; Ip, S.; Bathula, N.V.; Popova, P.; Soriano, S.K.; Ly, H.H.; Eryilmaz, B.; Nguyen Huu, V.A.; Broadhead, R.; Rabel, M. Current status and future perspectives on MRNA drug manufacturing. Mol. Pharm. 2022, 19, 1047–1058.
101. Whitley, J.; Zwolinski, C.; Denis, C.; Maughan, M.; Hayles, L.; Clarke, D.; Snare, M.; Liao, H.; Chiou, S.; Marmura, T. Development of mRNA manufacturing for vaccines and therapeutics: mRNA platform requirements and development of a scalable production process to support early phase clinical trials. Transl. Res. 2022, 242, 38–55.

102. Ouranidis, A.; Vavilis, T.; Mandala, E.; Davidopoulou, C.; Stamoula, E.; Markopoulou, C.K.; Karagianni, A.; Kachrimanis, K. mRNA therapeutic modalities design, formulation and manufacturing under pharma 4.0 principles. Biomedicines 2021, 10, 50.

103. Von Der Mülbe, F.; Reidel, L.; Ketterer, T.; Gontcharova, L.; Bauer, S.; Pascolo, S.; Probst, J.; Schmid, A. Method for Producing RNA. U.S. Patent US1001 7826B2, 2018.

104. Karikó, K.; Muramatsu, H.; Ludwig, J.; Weissman, D. Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res. 2011, 39, e142.

105. Baiersdörfer, M.; Boros, G.; Muramatsu, H.; Mahiny, A.; Vlatkovic, I.; Sahin, U.; Karikó, K. A facile method for the removal of dsRNA contaminant from in vitro-transcribed mRNA. Mol. Ther.-Nucleic Acids 2019, 15, 26–35.

106. Scorza, F.B.; Wen, Y.; Geall, A.; Porter, F. RNA Purification Methods. U.S. Patent US20210214388A1, 2016.

107. Daniel, S.; Kis, Z.; Kontoravdi, C.; Shah, N. Quality by Design for enabling RNA platform production processes. Trends Biotechnol. 2022, 40, 1213–1228.

108. Wang, X.; Liu, S.; Sun, Y.; Yu, X.; Lee, S.M.; Cheng, Q.; Wei, T.; Gong, J.; Robinson, J.; Zhang, D. Preparation of selective organ-targeting (SORT) lipid nanoparticles (LNPs) using multiple technical methods for tissue-specific mRNA delivery. Nat. Protoc. 2023, 18, 265–291.

109. Henderson, M.I.; Eygeris, Y.; Jozic, A.; Herrera, M.; Sahay, G. Leveraging biological buffers for efficient messenger RNA delivery via lipid nanoparticles. Mol. Pharm. 2022, 19, 4275–4285.

110. Zhao, P.; Hou, X.; Yan, J.; Du, S.; Xue, Y.; Li, W.; Xiang, G.; Dong, Y. Long-term storage of lipid-like nanoparticles for mRNA delivery. Bioact. Mater. 2020, 5, 358–363.

111. Ball, R.L.; Bajaj, P.; Whitehead, K.A. Achieving long-term stability of lipid nanoparticles: Examining the effect of pH, temperature, and lyophilization. Int. J. Nanomed. 2017, 305–315.

112. Zhang, N.-N.; Li, X.-F.; Deng, Y.-Q.; Zhao, H.; Huang, Y.-J.; Yang, G.; Huang, W.-J.; Gao, P.; Zhou, C.; Zhang, R.-R. A thermostable mRNA vaccine against COVID-19. Cell 2020, 182, 1271–1283.e16.

113. Zhang, H.; Leal, J.; Soto, M.R.; Smyth, H.D.; Ghosh, D. Aerosolizable lipid nanoparticles for pulmonary delivery of mRNA through design of experiments. Pharmaceutics 2020, 12, 1042.

114. Mike Smith, O.A.; Brito, L. Stabilized Formulations of Lipid Nanoparticles. U.S. Patent US20200069599A1, 8 November 2017.

115. Lamoot, A.; Lammens, J.; De Lombaerde, E.; Zhong, Z.; Gontsarik, M.; Chen, Y.; De Beer, T.R.; De Geest, B.G. Successful batch and continuous lyophilization of mRNA LNP formulations depend on cryoprotectants and ionizable lipids. Biomater. Sci. 2023, 11, 4327–4334.

116. Meulewaeter, S.; Nuytten, G.; Cheng, M.H.; De Smedt, S.C.; Cullis, P.R.; De Beer, T.; Lentacker, I.; Verbeke, R. Continuous freeze-drying of messenger RNA lipid nanoparticles enables storage at higher temperatures. J. Control. Release 2023, 357, 149–160.

117. Muramatsu, H.; Lam, K.; Bajusz, C.; Laczkó, D.; Karikó, K.; Schreiner, P.; Martin, A.; Lutwyche, P.; Heyes, J.; Pardi, N. Lyophilization provides long-term stability for a lipid nanoparticle-formulated, nucleoside-modified mRNA vaccine. Mol. Ther. 2022, 30, 1941–1951.