

Lipid nanoparticles employed in mRNA-based COVID-19 vaccines: an overview of materials and processes used for development and production

Ivana Pantelić*, Tanja Ilić, Ines Nikolić, Snežana Savić

University of Belgrade – Faculty of Pharmacy, Department of Pharmaceutical Technology and Cosmetology, Vojvode Stepe 450, 11221 Belgrade, Serbia

*Corresponding author: Ivana Pantelić, e-mail: ivana.pantelic@pharmacy.bg.ac.rs

Abstract

In the light of the recommended application of the third dose, both public and professional community would benefit from a detailed report on the technological advances behind the developed messenger ribonucleic acid (mRNA) based COVID-19 vaccines. Although many vaccine developers are yet to reveal their precise formulations, it is apparent they are founded on nanotechnology platforms similar to the one successfully used for registered drug Onpattro™ (INN: patisiran). Optimal encapsulation of mRNA requires the presence of four lipids: an ionizable cationic lipid, a polyethylene-glycol (PEG)-lipid, a neutral phospholipid and cholesterol. Together with other excipients (mainly buffers, osmolytes and cryoprotectives), they enable the formation of lipid nanoparticles (LNPs) using rapid-mixing microfluidic or T-junction systems. However, some limitations of thermostability testing protocols, coupled with the companies' more or less cautious approach to predicting vaccine stability, led to rigorous storage conditions: -15° to -25°C or even -60° to -80°C. Nevertheless, some inventors recently announced their mRNA-LNP based vaccine candidates to be stable at both 25° and 37°C for a week. Within the formulation design space, further optimization of the ionizable lipids should be expected, especially in the direction of increasing their branching and optimizing pKa values, ultimately leading to the second generation of mRNA-LNP COVID-19 vaccines.

Keywords: lipid nanoparticles, ionizable lipid, PEG-lipid, microfluidic device, storage conditions

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Introduction

According to the latest WHO COVID-19 vaccine tracker, compiling and reporting relevant information on twice-per-week basis of each COVID-19 vaccine in the pipeline, 183 and 112 vaccine candidates are in the preclinical and clinical development stage, respectively (1). Although all significant platforms are being investigated (namely, protein subunit (34% share), inactivated virus (14%), non-replicating (15%) and replicating (2%) viral vector, DNA (10%), virus like particle (4%), live attenuated virus (2%), etc.), ribonucleic acid (RNA) based vaccines (16% share) attracted special interest in both the professional and public domain. Their apparently rapid development brought mixed feelings - promising immunity against SARS-CoV-2 virus on one hand and safety concerns usually related to their composition, potentially interfering with innate genetic material, on the other. In less than a year, due to decades of scientific efforts in several disciplines, two messenger RNA (mRNA) vaccines were granted emergency use authorization, after demonstrating their safety and efficacy, leading to FDA's full approval of the BioNTech/Pfizer vaccine in August 2021. The ongoing pandemic gathered scientists across borders and disciplines, engaging the pre-existing knowledge in virology, vaccinology, pharmaceutical technology, and material science, to name a few (2, 3).

This paper reviews the technological advances that enabled the fast development of mRNA COVID-19 vaccines, explaining in detail the components present in the nanoparticulate carrier platforms used. Special attention is given to the applied manufacturing methods and the challenging storage conditions. Finally, critical opinion is offered on the future prospects of the next-generation mRNA COVID-19 vaccine formulations.

In the light of the recommended application of the third dose, both public and professional community would benefit from such a detailed report on the technologies and processes behind the available mRNA-based COVID-19 vaccines.

mRNA vaccines – structure and stability considerations

Although many aspects of mRNA structure, transcription and translation are beyond the authors' field of expertise, some fundamental facts on mRNA as a pharmaceutical modality need to be stated here. The technology readiness of the RNA-based therapeutics is based on the knowledge that mRNA only needs access to cytoplasm, i.e. ribosomes, without entering the nucleus and risking integration with the genome (4). After entering the ribosomal machinery, mRNA is theoretically capable of encoding any given protein (5). However, the mRNA itself (so-called "naked mRNA") is highly unstable and rapidly prone to degradation (simultaneous physical, chemical and enzymatic degradation reduces its serum half-life to less than 5 min) (6). Additionally, being a large, anionic macromolecule due to the presence of phosphate groups, the inevitable electrostatic repulsion forces additionally limit its cellular uptake (5, 7).

Therefore, mRNA needs to be encapsulated in a suitable delivery system, providing its stability and facilitating cell internalization (3).

Apart from the application of nanocarriers whose characteristics will be subsequently outlined in detail, current leaders in mRNA COVID-19 vaccines performed additional modifications to their mRNAs. BioNTech/Pfizer and Moderna applied nucleoside modifications, while CureVac resorted to sequence-engineering (codon optimization and uridine depletion) (4, 8). Besides these “conventional” mRNA approaches, self-amplifying mRNA (*syn.* self-replicating or replicon mRNA; samRNA) vaccines are also being tested, sponsored for example by Arcturus Therapeutics (USA) and Imperial College London (UK). samRNAs represent longer sequences (approx. 10 kilobases) due to additionally encoded genes – primarily RNA-dependent RNA polymerase capable of cellular self-replication without producing infectious particles (5, 9). This amplification process promotes vaccine efficacy, leading to considerable dose reductions (typical investigated samRNA doses are 1-10 µg compared to 30-100 µg for mRNA). Nevertheless, each of the aforementioned mRNA structure optimization approaches needs to be supported by a suitable nanostructured delivery system (8).

Although spike (S), nucleocapsid (N), membrane (M) and envelope (E) glycoproteins were discerned as SARS-CoV-2 immunogenic proteins, the majority of the developed mRNA COVID-19 vaccines encode the transmembrane anchored S glycoprotein, thus targeting similar antigens (8, 10, 11). The S glycoprotein was shown to be crucial for receptor recognition and subsequent cell fusion. It is important to note that they all utilize engineered mRNAs. These synthetic mRNAs behave similarly to the naturally occurring ones, initiating translation and encoding formation of a stabilized form of the S glycoprotein (12).

Lipid nanoparticles (LNPs) as mRNA carriers

All mRNA and samRNA COVID-19 vaccine candidates rely on lipid nanoparticles as delivery systems. While only some of the vaccine developers, such as BioNTech/Pfizer and Moderna, publicly disclosed exact compositions of their nanocarriers, it is presumed they are all founded on the experience behind the successful Onpattro™ story (Alnylam Pharmaceuticals, USA). This regulatory approved LNP-based short interfering RNA (siRNA) drug named patisiran obtained FDA approval in August 2018 for the treatment of polyneuropathies induced by hereditary transthyretin amyloidosis (13, 14). Naturally, certain optimizations of this LNP platform were in need, e.g., the accommodation of a much larger mRNA and shift from intravenous (i.v.) to intramuscular (i.m.) administration (14). However, a special benefit of LNP-based vaccines is that, due to inherent adjuvanticity of LNPs, no additional adjuvants are needed (8, 11).

Although LNPs are multicomponent delivery systems, their suitability for mRNA COVID-19 vaccine formulations depends on the careful optimization of 4 lipidic components: an ionizable cationic lipid, a polyethylene-glycol (PEG)-lipid, a neutral phospholipid and cholesterol (Table I). Although each of these lipids plays an important part in LNP stabilization, encapsulation efficacy and cell internalization, the mRNA-LNP

design space mainly evolved around optimizing ionizable and PEG-lipids, and defining satisfactory lipid ratio (4). As reported, this required screening of several hundreds of lipids, leading to thousands of preliminary formulations. Due to this overall carrier lipophilicity, LNP-based vaccines additionally provide sustained release of the cargo (10).

Table I Published or unofficially revealed components of mRNA COVID-19 nanovaccines (4, 11, 14).

Tabela I (Ne)zvanično objavljeni podaci o sastojcima iRNK nanovakcina za prevenciju COVID-19 (4, 11, 14).

Vaccine identifier (Developer)	mRNA type	Ionizable lipid	Helper lipid	PEG-lipid	Lipid molar ratio (ionizable: helper: cholesterol: PEG: mol%)	Other excipients
BNT162b2 (BioNTech/ Pfizer)	nucleoside-modified	ALC-0315	DSPC	ALC-0159	46.3:9.4:42.7:1.6	Potassium dihydrogen phosphate Disodium hydrogen phosphate dehydrate Potassium chloride Sodium chloride Sucrose Water for injection
mRNA-1273 (Moderna)	nucleoside-modified	SM-102 (Lipid H)	DSPC	PEG2000-DMG	50:10:38.5:1.5	Tris (tromethamine) Sodium acetate Sucrose Water for injection
CVnCoV (CureVac)	unmodified	Acuitas lipid (ND)	DSPC		50:10:38.5:1.5	
MRT5500 (Sanofi/ TranslateBio)	unmodified	C12-200 or from ICE- or cysteine-based ionizable lipid families				
LUNAR®-COV19 (ARCT-021) (Arcturus Therapeutics)	self-amplifying	STARR™ lipid-mediated delivery system (speculated to contain Lipid 10a or Lipid 2,2 (8,8) 4C CH ₃)				
COVAC1 (LNP-nCoVsaRNA) Imperial College London	self-amplifying	Acuitas A9				
ChulaCoV19 (Chulalongkorn University)	nucleoside-modified	Genevant CL1				

Although the type and the ratio of the lipids determine the precise LNP structure, most researchers agree that mRNA-loaded LNP resemble the core-shell model of nanoparticles, consisting of a surface layer and amorphous, electron-dense, isotropic core (8). The core may be either constituted of water pores surrounded by exclusively cationic lipids, or various lipids may be homogeneously distributed and interrupted with small water domains. These notions were supported by cryogenic transmission electron microscopy (cryo-TEM), NMR spectroscopy, small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) (15-17). However, the mentioned techniques failed to precisely characterize the outer shell of mRNA-LNP, leading some authors to believe in a monolayer (17, 18), and others in one or several bilayers (15, 19, 20). As for the exact localization of the mRNA within the nanocarrier, the RiboGreen assay using a fluorescent dye implies its predominant positioning inside the LNPs (8, 19).

Naturally, size is one of the critical attributes of nanocarriers. Since the naked mRNA strands tend to have a size of around 50 nm according to dynamic light scattering (DLS) (4), the size of mRNA-loaded LNPs is usually reported to be < 200 nm (5, 16). A recent study favored mRNA delivery mediated by intermediate size LNPs (around 70 nm diameter), rather than larger (100 nm) or smaller (50 nm) ones (17). Smaller nanoparticles are believed to be easily flushed from the injection site, while larger ones remain there, so while considering the optimal LNP size, a 20% increase should be expected upon their administration, due to the formation of a biomacromolecular corona (5). Miao et al. (21) suggest an important role of ionizable lipids in this corona formation.

Charge is another important aspect of mRNA-LNP vaccines, starting from inherent anionic nature of mRNA to negatively charged biological barriers they will encounter *in vivo* (5). Although cationic delivery systems appear to be the obvious solution, they show unacceptable cytotoxicity due to generalized, off-target association with negatively charged substrates (22). Therefore, mRNA-delivery systems are developed to have a relatively neutral surface charge (5). This was enabled by the inclusion of specially designed ionizable lipids.

Lipid components of mRNA-LNP COVID-19 vaccines

Ionizable lipids

Ionizable lipids are considered to be the second generation of cationic lipids and are sometimes also referred to as pH-sensitive lipids, since they possess negligible charge at physiological pH but become positively charged in the acidic milieu (5, 23). The most prominent feature of the endo-lysosomal pathway is the gradient change of pH. Therefore, nanocarriers for mRNA needed to be responsive to the acidification of endosomes (5). Hence, a central role in LNP development was given to the selection of the ionizable lipid which needs to be neutral at physiological pH in order to prevent cationic charge *in vivo*, but protonated in the endosome at pH around 6.5 to enable endosomal release (4). This allows for not only electrostatic binding of mRNA, but also subsequent fusion with cell

membrane by formation of a membrane-destabilizing ion pair with an endosomal phospholipid (4, 24).

Hence, one of the major features of ionizable lipids suitable for mRNA-LNPs is their pKa. The pKa represents the pH at which 50% of the ionizable lipid within the LNP is protonated, and is believed to reflect the efficiency of these delivery systems (25). Experiments showed that pKa values measured by the TNS (2-p-toluidinylnaphthylene-6-sulfonate) dye-binding assay should preferably be in the range of 6-7. For example, the reported pKa of the Lipid H, used by Moderna, is 6.75, and Acuitas ALC-0315 lipid used by BioNTech/Pfizer is 6.09 (4). It is worth mentioning that, in this case, *in silico* pKa calculations proved to be unsuitable, always implying values at least 2-3 units higher than the measured ones, possibly due to high energy of solvation within the LNP lipid phase (26).

Another important feature of ionizable lipids is their geometry, depending on the characteristics of their head group, linker and tail (7). A cone shape is preferred (i.e. cross-section of the tails needs to be larger than that of the head) and believed to form inverted hexagonal phases that can disrupt membranes and facilitate endocytosis (4). Recently, Kim et al. (5) depicted structures of lipids potentially relevant for COVID-19 vaccine development.

Finally, since the lipid's biodegradability directly reflects its clearance, biodegradable ester-based ionizable lipids were used by Moderna and Acuitas (5).

PEG-lipids

It is generally accepted that PEG-lipids decorating the LNP surface ultimately determine particle size. They form a hydrophilic shell, a steric barrier controlling LNPs aggregation potential (7). Their properties are determined by the structure of both hydrophilic (PEG) and hydrophobic part (lipid anchor), as well as the applied molar percentage. A study varying the PEG-lipid's content in the range 0.25-5%, confirmed that PEG-increase tends to decrease the particle size, and singled out the formulation with 2.5% of the PEG-lipid with the particle size of 78 nm (27, 28). Hence, the ratio of this lipid type is kept rather low within the mRNA-LNP formulations (Table I) since excessive PEGylation may obstruct transfection (5).

It is important to note that PEG-lipid molecules are not firmly attached to the LNP surface and gradually dissociate from it. This so-called PEG-shedding process is considered important for the LNP transfection (controlling the kinetics of cellular uptake) but must not occur at a rate that would lead to the loss of ionizable and/or phospholipids (4, 5). Therefore, a new class of PEG-lipids named diffusible PEG-lipids is used, enabling LNP stability without compromising intracellular delivery. They are composed of 14C-long acyl chains and responsible for a satisfactory dissociation rate from the LNPs *in vivo* (7).

While BioNTech/Pfizer decided to use 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (PEG2000-DMA), Moderna resorted to 1,2-dimyristoyl-*rac*-

glycero-3-methoxy-poly(ethylene glycol)-2000 (PEG2000-DMG) (Table I). Unfortunately, precise lipid constituents of many mRNA-LNP COVID-19 vaccine candidates are yet to be revealed.

Finally, PEG-lipids are known to improve formulation retention in the lymph nodes and sometimes even reduce complement activation (4). However, although PEGs are generally considered as safe excipients, rare reports of anaphylaxis and other serious allergic reactions after mRNA COVID-19 vaccines' administration may be connected with a patient's pre-existing anti-PEG antibodies (11). This safety concern may also be diminished with controlled PEG-shedding (5).

Structural or helper lipids: phospholipids and cholesterol

When used in approximately 30-40 mol%, phospholipids and cholesterol provide LNPs with both stability and biocompatibility (5, 7). Table I shows that 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) is the chosen phospholipid in the majority of vaccine formulations, probably due to its bilayer-forming properties and high phase transition temperatures (7, 28). DSPC is responsible for formation of a stable bilayer, right underneath the PEG surface (4). Alternatively, some of the vaccine developers may have resorted to dioleoylphosphatidylethanolamine (DOPE) (23, 29).

Although it is a traditional excipient, in LNPs cholesterol is believed to be a multifunctional component, filling particle cavities, preventing certain LNP-protein interactions and even enhancing nanoparticles' fusogenic properties (4). This is not surprising, considering that cholesterol is a natural component of biological membranes, contributing to their fluidity (23). Therefore, while comparing cholesterol-containing and cholesterol-free LNPs as potential delivery systems for nucleic acids, Sato et al. (30) noted decreased formulation stability and potency in the absence of this helper lipid. However, cholesterol's detailed role in mRNA delivery still remains to be elucidated. It is interesting to note that a couple of studies favored the use of cholesterol in an amount exceeding its membrane solubility, resulting in the presence of cholesterol crystals as well (17, 28).

Ever since the EMA's guidance on minimizing the risk of Transmissible Spongiform Encephalopathies (TSEs) (31), interest in non-animal origin cholesterol increased. Several GMP-certified companies produce plant-derived cholesterol, suitable for parenteral administration: e.g. BotaniChol[®] (CordenPharma International, Germany) or PhytoChol[®] puriss (Wilshire Technologies, USA) (29, 32, 33).

Other excipients

Although lipids are recognized as crucial components of mRNA-LNP vaccines, other excipients are also needed to assure their quality and efficacy. Apart from being injectable-grade, they also need to be RNase-free (5). Although these excipients are mainly included for buffering, osmotic and cryoprotective roles, some may cover several functions. For example, Tris-HCl buffer used by Moderna provides an additional stabilizing effect on mRNA, serving as a hydroxyl radical scavenger (8).

pH is obviously a critical quality attribute of a mRNA-LNP vaccine, from manufacturing to storage stages. Upon freezing, inadequate buffer selection may lead to a pH decrease of up to 3.5 units, while the inclusion of more pH-resistant buffers allows no more than 0.5 units drop (34). Despite using different buffering systems, BioNTech/Pfizer and Moderna disclosed identical, broad pH 7-8 range of their vaccine formulations (8). While Moderna opted for a combined use of Tris and acetate buffering, Pfizer/BioNTech used a somewhat conventional phosphate buffer possibly responsible for the dry ice storage temperatures described later (4).

Manufacturing methods of mRNA-LNP COVID-19 vaccines

Nowadays, the synthesis of clinical-grade mRNAs relies on relatively robust, standardized and scalable *in vitro*-transcription (IVT) processes that use a DNA template and necessary enzymes in a cell-free system (5). In fact, this enabled vaccine developers to act promptly the moment SARS-CoV-2 viral sequence was made public. However, attention will here be given to the preparation methods of mRNA-LNP vaccines. Unsurprisingly, various collaborations were made in the hope of merging infrastructures and succeeding in manufacturing batches that could appease global demand (2, 10).

The preparation of mRNA-loaded LNPs starts with the separate preparation of ethanolic dispersion of lipids (usually with 25% V/V of ethanol) and an aqueous buffer containing mRNA (e.g. acetate buffer pH 4) and their subsequent mixing using microfluidic or T-junction mixing systems. These rapid-mixing methods enable LNP formation and mRNA entrapment in a single step (7). Microfluidic devices offer the advantage of efficiently mixing very small volumes (e.g. several μ L), thus enabling rapid screening of a number of formulations. Both mixing systems were shown to produce nanosystems of comparable size and morphology (28). One part of ethanolic lipids' dispersion is usually mixed with three parts of mRNA's buffered dispersion. This elevates the pH to approx. 5.5, protonating the ionizable lipid and binding mRNA to it. Excess of aqueous phase makes the lipids insoluble, finally leading to the formation of a suspension of lipid nanoparticles. Further pH elevation to pH 7.4 is accomplished either by dilution, dialysis or tangential flow filtration, in order to neutralize the ionizable lipid (4). Stepwise preparation scheme of mRNA-loaded LNPs is given in Figure 1.

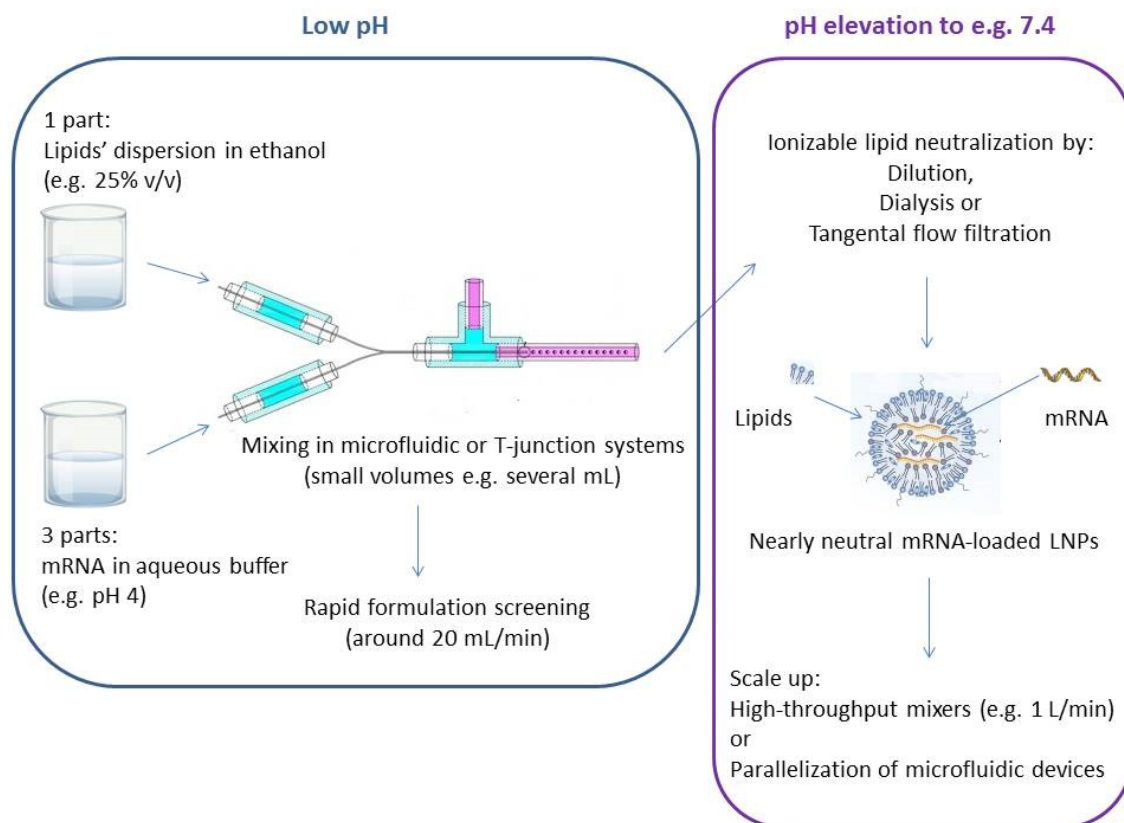


Figure 1. Schematic presentation of a manufacturing approach to obtaining mRNA-encapsulated lipid nanoparticles as potent vaccines for COVID-19 prevention.

Slika 1. Šematski prikaz proizvodnog procesa vakcina za prevenciju COVID-19 koje se zasnivaju na iRNK inkapsuliranoj u lipidne nanočestice.

The majority of mRNA-LNP COVID-19 vaccines were produced through microfluidic mixing, a scalable system suitable for GMP facilities offering highly controlled and reproducible mixing forces (5, 11). During the mixing, the pH of the formulation is kept low at first in order to protonate the ionizable lipid's functional groups (e.g. in aqueous buffer pH 4), thus enabling electrostatic complexation of the now positively charged lipid with the negatively charged mRNA. A dialysis or ultrafiltration is usually the next step, in order to neutralize the pH (switching to isotonic buffer approximately pH 7), resulting in nearly uncharged LNPs encapsulating the mRNA (7, 11).

However, the size of the obtained LNPs may easily change after mixing, usually when mRNA-LNP suspension is diluted in or dialyzed against the aqueous buffer to raise the pH and completely eliminate ethanol (4). Final LNPs are believed to stem from the merging of smaller lipid vessels, through a kind of nucleation-growth-ripening model. Due to the fact that both hydrophobic and electrostatic interactions are responsible for

particle growth, the entire process is sometimes referred to as bottom-up self-assembly of LNPs (5, 28).

Since unsuitable shear stress may cause changes in the mRNA's tertiary structure and compromise its effect (6), it is not surprising that vaccine developers opted for the presented fast but controlled mixing methods to produce homogeneous LNPs with entrapment efficiency of over 90% (7).

However, scale up is largely determined by the type of the mixing system used during the development phase. Widespread clinical application of mRNA-LNP vaccines required high-throughput but rigorously controlled manufacturing, high entrapment efficiency and reproducibility (28). The dynamics of the selected microfluidic mixer (e.g. local mixing environment with speeds of approx. 20 mL/min) must be coupled with equally high-throughput mixers (e.g. 1 L/min) in later stages, since prolonging the mixing step may compromise the homogeneity of the obtained LNPs (7). Clinically relevant volumes may also be obtained with the parallelization of suitable mixers (28).

Storage considerations of mRNA-based COVID-19 vaccines

According to the information disclosed so far, it appears that the delicate mRNA structure, rather than LNP instability, is primarily responsible for stringent storage conditions of pertaining vaccines (5, 35). With temperature increase, sulfur substitution occurs over the mRNA backbone, making it more prone to RNase degradation (6). Therefore, maintaining the cold chain during vaccine distribution and storage seems unavoidable so far, and may account for as much as 80% of its final price (10).

Efficient worldwide vaccine distribution requires a vaccine to have a sufficiently long shelf life, preferably at room or refrigerator temperatures (2-8°C). As shown in Table II, currently available mRNA-based vaccines against COVID-19 necessitate even lower temperatures. Unfortunately, the developing companies did not disclose the precise stability issues lying behind such stringent storage conditions (8). Apparent similarities in Moderna and BioNTech/Pfizer vaccines (e.g. similar sucrose concentration as a cryoprotectant) bring into question the difference in required storage conditions (4). It remains to be seen if the phosphate buffer is indeed the culprit for ultra-cold storage at -80° to -60°C, due to its tendency to precipitate and lead to rapid pH change (4). Therefore, it is entirely feasible that these vaccines share similar stability issues, and that the extreme storage conditions required by certain developers are due to some limitations of thermostability testing protocols coupled with the companies' more or less cautious approach to predicting vaccine stability (8).

Table II Currently disclosed details on storage and administration instructions for mRNA-based COVID-19 vaccines (4, 5, 12, 39).

Tabela II Trenutno dostupni podaci o uslovima čuvanja i primene vakcina za prevenciju COVID-19 zasnovanih na iRNK (4, 5, 12, 39).

Vaccine identifier (Developer)	Route of administration	Dose (dosage volume)	Storage requirements	Directions for use
BNT162b2 (BioNTech/Pfizer)	i.m.	30 µg (0.3 mL) Supplied as a 5-dose vial.	At -60° to -80°C (protected from light): 6 months. At 2° to 8°C (protected from light): 5 days. At room temperature: 2 hours.	The frozen suspension must be thawed and diluted with 1.8 mL of sterile 0.9% saline (preservative free). After dilution, the vaccine must be used within 6 h (stored at 2-25° C).
mRNA-1273 (Moderna)	i.m.	100 µg (0.5 mL) Supplied as a 10-dose vial.	At -15° to -25°C (protected from light): 6 months. At 2° to 8°C (protected from light): 30 days. At 8° to 25°C: 12 hours.	The frozen suspension must be thawed before use. The thawed vaccine may be administered as is, but must be used within 6 hours (stored at 2-25°C).
CVnCoV (CureVac)	i.m.	12 µg	At 2°-8°: 3 months At room temperature: 24 hours	
MRT5500 (Sanofi/TranslateBio)	i.m.		Targeting a -20°C storage temperature for late-stage clinical trials and at launch	

While pros and cons of lyophilization as stability-enhancing step towards mRNA-LNP vaccines' more acceptable storage conditions are outlined in the next section, a thermostable mRNA COVID-19 vaccine called ARCoV is allegedly being developed by Walvax Biotechnology and collaborators, also based on a LNP platform and so far found stable at both 25° and 37°C for at least 7 days (10).

Future prospects in the mRNA-LNP vaccines' development

The final section of this review will cover certain underexplored aspects of mRNA-LNP vaccines' formulation, manufacture and characterization.

Within the formulation design space, further optimization of the ionizable lipids should be expected, especially in the direction of increasing their branching (e.g. the lipid used by Moderna has 3, while Acuitas lipids have 4 or 5 branched chains). It is currently believed that a degree of branching is directly responsible for the prominent cone-shape structure and its improved cell membrane disrupting potential (4). Optimization of the lipid components may even improve the LNP's inherent adjuvanticity.

Further, mRNA-LNP vaccines need to be additionally stabilized in order to store and distribute them in higher temperatures. Lyophilization may be a logical step in

improving long-term stability, especially after a review of positive examples in clinical trials (e.g. lyophilized mRNA-based cytomegalovirus vaccine mRNA-1647 with a claimed shelf life of 18 months at 5°C) (36). On the other hand, lyophilization of LNPs is sometimes known to increase their particle size, sometimes as much as fourfold (5). Recently, Zhao et al. (37) screened the impact of cryoprotectant type and concentration (namely trehalose, glucose and mannitol) and several physical conditions (aqueous, frozen, lyophilized) on mRNA-loaded nanoparticles. They noted a significant change in mRNA-LNP nanostructure during lyophilization and reconstitution processes, possibly influencing the properties of their biomolecular corona upon administration and, hence, overall *in vivo* results.

As a relatively time-consuming stage in any drug development, the required speed of launching safe and effective COVID-19 vaccines obviously prevented their developers from including the lyophilization step, which could possibly lead to less demanding storage conditions. Advanced lyophilization techniques such as SMART freeze-drying involving manometric temperature measurements and process analytical technologies (PAT) for in line monitoring of critical process parameters (38) may lead to better storage requirements of mRNA-based vaccines, among other biopharmaceuticals. Apart from lyophilization, some alternative, supercritical drying techniques could also upgrade the long-term stability, but possibly affect the product cost as well (8).

Additionally, some authors put faith in the WHO's extended controlled temperature chain (ECT) initiative that aims to allow a vaccine to be stored at conditions outside the conventionally controlled 2°-8°C for a limited period of time (39).

Finally, quality specifications for mRNA-LNP COVID-19 vaccines are still not publicly available. Specific regulatory guidelines are also lacking. Apart from the FDA's Guidance for the development and licensure of vaccines to prevent COVID-19, published in June 2020 (40), more specific documents, preferably focusing on mRNA-based vaccines have yet to be developed. Characterization techniques used in the stability tests would thus be more refined in terms of sensitivity and defined acceptance criteria (8). Defining specific critical quality attributes and their acceptance criteria could further quiet down the anti-vaccination part of the (professional) public. Meanwhile, both developers and regulators must rely on available guidelines for parenteral biological products on one, and nanomaterials on the other hand (6, 9, 38, 41).

Conclusion

Although the design space for mRNA-LNP vaccines against COVID-19 appears infinite, the nanoparticle platform used by the Onpattro™ developers was successfully optimized to accommodate mRNA as a much larger cargo. Careful selection of four lipids was crucial for successful mRNA encapsulation and subsequent cell internalization. Although nucleic acids currently constitute only around 1% of the approved therapeutic modalities, in case of mRNA-based COVID-19 vaccines rapid launch of several candidates was enabled by decades of research in the nanotechnology field. Nevertheless, leading vaccine developers are undoubtedly continuously focused on the outlined

challenges and will soon enough come out with the next generation of mRNA-based COVID-19 vaccines.

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Lipidne nanočestice u vakcinama za prevenciju COVID-19 zasnovanih na iRNK: pregled materijala i procesa primenjenih tokom razvoja i proizvodnje

Ivana Pantelić*, Tanja Ilić, Ines Nikolić, Snežana Savić

Univerzitet u Beogradu – Farmaceutski fakultet, Katedra za farmaceutsku tehnologiju i kozmetologiju, Vojvode Stepe 450, 11221 Beograd, Srbija

*Autor za korespondenciju: Ivana Pantelić, e-mail: ivana.pantelic@pharmacy.bg.ac.rs

Kratak sadržaj

U susret preporučenoj trećoj dozi, razumevanje tehnološkog napretka koji je doveo do razvoja vakcina na bazi informacione ribonukleinske kiseline (iRNK) od interesa je kako stručne tako i šire javnosti. Iako mnoge kompanije koje stoje iza razvoja ovih vakcina još uvek nisu učinile dostupnim podatke o formulaciji, očigledno je da se dominantno oslanjaju na nanotehnološke platforme slične onoj uspešno primenjenoj za registrovani lek Onpattro™ (INN: patisiran). Zadovoljavajuća inkapsulacija iRNK zahteva prisustvo četiri lipida: jonizujućeg katjenskog lipida, PEGilovanog lipida, neutralnog fosfolipida i holesterola. Zajedno sa ostalim ekscipijensima (puferima, sredstvima za podešavanje toničnosti i krioprotektantima), pažljivo odabrani lipidi omogućavaju obrazovanje lipidnih nanočestica (LNPs) mikrofluidnim ili T-junction tehnikama mešanja velike brzine. Međutim, određena ograničenja u primenjenim protokolima procene stabilnosti vakcina, zajedno sa povećanim oprezom kompanija od kojih se željno očekivalo plasiranje vakcina na tržište, dovelo je do rigoroznih uslova čuvanja: -15° do -25°C ili čak -60° do -80°C. Ipak, pojedine kompanije izveštavaju o zadovoljavajućoj stabilnosti svojih iRNK-LNP vakcina kako na 25°, tako i na 37°C tokom nedelju dana. Dalji formulacioni razvoj će svakako obuhvatiti optimizaciju jonizujućih lipida, naročito u smeru povećanja broja bočnih lanaca i podešavanja pKa vrednosti, te dovesti do pojave tzv. druge generacije iRNK-LNP vakcina za prevenciju COVID-19.

Ključne reči: lipidne nanočestice, jonizujući lipid, PEG-lipid, mikrofluidni sistem, uslovi čuvanja
