

Lipid Nanoparticles to Harness the Therapeutic Potential of mRNA for Cancer Treatment



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Abstract In recent years, mRNA has become an appealing platform for the development of therapeutic agents both for the prevention and treatment of cancer. Efficient delivery of mRNA into target cells is crucial for fully harnessing its therapeutic potential. However, mRNA possesses structural limitations, including its net negative charge and hydrophilicity, that impede its efficient cellular uptake. Likewise, mRNA is characterized by an intrinsic fragility, resulting in it being a highly instable molecule. Lipid nanoparticles (LNPs) have been successfully used for protecting and delivering mRNA encoding for various therapeutic proteins. This chapter is intended to give a comprehensive overview of the current approaches for mRNA synthesis and LNPs manufacturing. We provide an in-depth analysis of how mRNA technology is revolutionizing the area of cancer immunotherapy, critically reviewing the

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major fields of application of nanoformulated-mRNA medications and addressing the advantages and drawbacks of each one. Finally, we offer a wide landscape of future possibilities and remaining issues of current mRNA-based therapies.

Keywords mRNA · Immunotherapy · Lipid nanoparticles · Vaccines · Cancer

1 Introduction

Messenger RNA (mRNA) is a subtype of RNA containing the genetic information necessary to produce a specific protein. The synthesis of mRNA occurs in the cellular nucleus through a process called transcription whereby genomic DNA serves as a template (Bentley 2014; Cramer 2019). Mature mRNA that has been subjected to splicing and post-transcriptional modifications is then exported to the cytosolic compartment where its translation begins upon binding with the ribosomal subunits (Cramer 2019).

Since its discovery in 1960, different attempts have been made to exploit mRNA as a therapeutic platform for the development of gene-based therapies (Persano et al. 2017; Stadler et al. 2017; Thran et al. 2017; Guevara 2019a; Kong et al. 2019; Rybakova et al. 2019; Wei et al. 2020). mRNA-mediated transfection represents an appealing alternative to more conventional plasmid DNA (pDNA)-based strategies for inducing exogenous gene expression. This is because mRNA technology offers relevant advantages including superior transfection efficiency, especially in non-dividing and hard to transfect cells, without the risks of insertional mutagenesis (Leonhardt et al. 2014; Guevara 2019b). Indeed, unlike pDNA, mRNA does not need to enter the nucleus of the target cells to exert its function; it is sufficient that it reaches the cytosolic compartment for translation to occur (Leonhardt et al. 2014; Andreev et al. 2016). Once mRNA has completed its function, it is rapidly degraded thus promoting only a transient expression of the protein of interest, which is convenient for safer and efficient therapeutic approaches (Huch and Nissan 2014).

However, mRNA's application as a therapeutic molecule was limited until the second half of the last decade due to the several limitations inherent to its high fragility, poor ability to enter cells because of its net negative charge, and high immunogenicity (Karikó et al. 2008; Guevara 2019b; Bidram et al. 2021). Although the intrinsic adjuvanticity of mRNA, ascribable to the interaction with innate immune receptors, has been exploited to enhance the efficacy of mRNA-based vaccines (Kranz et al. 2016), extensive efforts have been dedicated to reducing the immunogenicity and improving the stability of mRNA molecules by incorporating chemically modified nucleotides and regulatory elements (Karikó et al. 2008; Anderson et al. 2010; Nance and Meier 2021).

Despite modified nucleotides were demonstrated to minimize the susceptibility to degradation by ribonucleases and improve the translatability of "naked" mRNA, effective delivery remained the principal obstacle for ensuring adequate production of exogenous proteins upon systemic or local mRNA administration (Pardi et al.

2018). Recent advances in non-viral mRNA delivery technologies have broadened the application of the mRNA technology in preclinical and clinical settings (Persano et al. 2017; Stadler et al. 2017; Thran et al. 2017; Guevara 2019a; Kong et al. 2019; Rybakova et al. 2019; Wei et al. 2020). A wide range of nanosized platforms has been investigated as mRNA delivery systems, such as polymeric, peptide-based, and lipid-based nanoparticles (McKinlay et al. 2017; Lou et al. 2019; Qiu et al. 2019; Kaczmarek et al. 2016; Sago et al. 2018; Veiga et al. 2018). Among these non-viral vehicles, lipid-based formulations represent the most advanced platform that has been successfully employed for in vivo mRNA delivery (Guevara et al. 2020; Pilkington et al. 2021; Schoenmaker et al. 2021).

The ongoing COVID-19 pandemic has further accelerated the development of mRNA technology, with two mRNA-based vaccines, BNT162b2 (BioNTech) and mRNA-1273 (Moderna), granted with the first historic authorization for clinical use by the Food and Drug Administration (FDA) and European Medicines Agency (EMA), while another mRNA vaccine, CVnCoV (CureVac), is currently in phase 3 clinical trials (Risma et al. 2021; Uddin and Roni 2021). This success has drawn the interest of several pharmaceutical companies and research groups in acquiring the necessary capabilities to set up the manufacturing of nanoformulated mRNA-based therapies not only for cancer immunotherapy but also for other purposes (Martin and Lowery 2020; Dolgin 2021a, b). Currently, dozens of mRNA-based therapeutics are in preclinical and clinical phases of development, with promising outcomes in diverse types of cancers (Table 1).

In this chapter, we summarize the current methods utilized for mRNA preparation and synthesis, and the progress that has been made to increase the structural stability and translation efficiency of synthetic mRNA. We describe the preparation of lipid-based nanoparticles by standard nanoprecipitation technique for mRNA encapsulation, and the major formulation parameters that can affect its ability to induce transgene expression. Finally, we critically evaluate the different forms of RNA-based therapies that have been proposed.

2 Structure, Synthesis, and Purification of in Vitro Transcribed (IVT) mRNA

2.1 Structural Organization of IVT-mRNA

Synthetic mRNAs can be classified mainly in two types, the non-replicating (or non-amplifying) and the virus-derived self-amplifying RNA (saRNA) (or replicon).

The minimum structure of conventional non-replicating in vitro transcribed (IVT) mRNA consists of all those elements present in mature eukaryotic mRNA, including an open reading frame (ORF) region that encodes the desired protein, 5'- and 3'-untranslated regions (UTRs), and 5' cap and 3' poly(A) tail (Fig. 1) (Chaudhary et al. 2021).

Table 1 Clinical trials investigating lipid-based nanoparticles for mRNA-based cancer therapeutics

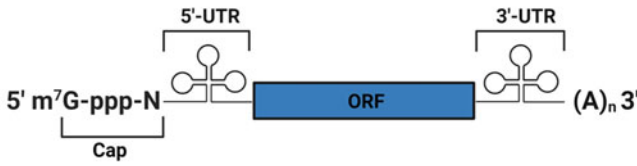
National clinical trial number	Disease	Status	Phases	Encoded proteins	Route	Sponsor
NCT03323398	Relapsed/refractory solid tumor malignancies or lymphoma	Recruiting	Phase 1/2	mRNA-2416 (mRNA encoding OX40L)	Intratumoral	Moderna
NCT03739931	Relapsed/refractory solid tumor or lymphoma	Recruiting	Phase 1	mRNA-2752 (mRNA encoding OX40L, IL-23, IL-36Y)	Intratumoral	Moderna, AstraZeneca
NCT02410733	Advanced melanoma	Active, not yet recruiting	Phase 1	NY-ESO-1, MAGE-C3, tyrosinase, gp100	Intravenous	BioNTech
NCT02316457	Triple negative breast cancer	Active, not yet recruiting	Phase 1	3 TAAs selected	Intravenous	BioNTech
NCT03289962	Melanoma, NSCLC, bladder cancer, CRC, breast cancer, etc.	Recruiting	Phase 1	Neo-Ag (mRNA)	Intravenous	BioNTech, Genentech
NCT04267237	NSCLC	Recruiting	Phase 2	Neo-Ag (mRNA)	Intravenous	Hoffmann-La Roche
NCT03815058	Advanced Melanoma	Recruiting	Phase 2	Neo-Ag (mRNA)	Intravenous	BioNTech, Genentech
NCT04486378	Stage II and III CRC (surgically resected)	Recruiting	Phase 2	Neo-Ag (mRNA)	Intravenous	BioNTech
NCT04161755	Pancreatic cancer (surgically resected)	Recruiting	Phase 1	Neo-Ag (mRNA)	Intravenous	Memorial Sloan Kettering Cancer Center, Genentech
NCT03313778	Resected/unresectable solid tumors	Recruiting	Phase 1	Neo-Ag (mRNA)	Intramuscular	Moderna, Merck

(continued)

Table 1 (continued)

National clinical trial number	Disease	Status	Phases	Encoded proteins	Route	Sponsor
NCT03897881	Complete resection of high-risk Melanoma	Recruiting	Phase 2	Neo-Ag (mRNA)	Intramuscular	Moderna, Merck
NCT03948763	CRC, NSCLC, pancreatic cancer	Recruiting	Phase 1	Mutated KRAS: G12D, G12V, G13D, G12C	Intramuscular	Moderna, Merck
NCT03468244	Advanced esophageal squamous carcinoma; gastric adenocarcinoma; pancreatic adenocarcinoma; colorectal adenocarcinoma	Recruiting	Phase 1	Neo-Ag (mRNA)	Subcutaneous	Changhai Hospital, Stemima Therapeutics

a) Non-replicating mRNA



b) Self-amplifying RNA

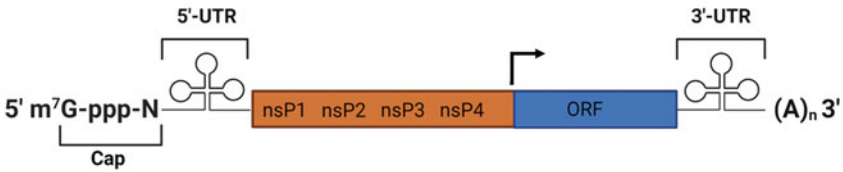


Fig. 1 Schematic illustration of the structural organization of non-replicating mRNA (**a**) and self-amplifying RNA platforms (**b**). Non-replicating mRNA is composed of a cap structure (m⁷GpppN, where N can be any nucleotide), the 5'-UTR, an open ORF encoding a gene of interest, the 3'-UTR, and a tail of 30–120 adenosine residues (poly(A) tail) (**a**). Self-amplifying RNA derives from an alphavirus genome and includes a 5' cap, nonstructural genes (nsP1–4), 26S subgenomic promoter (open arrow), the ORF encoding the desired protein, the 3'-UTR, and a poly(A) (**b**)

In contrast, saRNA derives from the genome backbone of an alphavirus, like the venezuelan equine encephalitis (VEE) virus, in which structural genes have been replaced by a transgene encoding the therapeutic protein of interest, whereas the genes responsible for RNA replication are maintained to preserve the auto-replicative capacity of the virus (Fig. 1) (Blakney et al. 2021). SaRNA is advantageous compared to conventional IVT-mRNA as it retains all the benefits of mRNA technology, such as rapid synthesis, transient activity, and suitability for customization, but the therapeutic effect can be achieved with a lower dose of RNA thanks to its self-replicative capacity (Vogel et al. 2018).

In the following sections, we will describe the role of the different regulatory elements in IVT-mRNA, how its sequence organization and composition can be optimized to maximize its therapeutic performances, and provide an overview of the preparation of synthetic mRNA by *in vitro* transcription.

2.1.1 5' and 3' Untranslated Regions (UTRs)

The UTR regions are non-coding elements located at the 5' and 3' ends of a mature mRNA (Leppek et al. 2018; Mignone et al. 2002). UTR sequences play a critical role in multiple processes conducting to mRNA translation into protein, including transport of mRNA from the nucleus to the cytosol, the assembly of the translation machinery, and mRNA decay (Leppek et al. 2018; Rabani et al. 2017; Mignone et al.

2002; Schuster and Hsieh 2019). Secondary structures (hairpins) in the UTRs are the major determinants of its regulatory function (Mignone et al. 2002). In UTRs of mRNA encoding proteins poorly expressed under normal conditions, hairpin structures are particularly abundant and form stable interactions with an average free energy of less than -50 kcal/mol (Mignone et al. 2002; Babendure et al. 2006). Secondary structures positioned in the proximity of the cap structure are more effective at inhibiting translation initiation; indeed, free energy of -30 kcal/mol is sufficient to impede the access of the 43S preinitiation complex, composed of the small ribosomal subunit (40S) bound by the initiation factors eIF1, eIF1A, eIF3, and the eIF2-Met-tRNA^{iMet}-GTP ternary complex (eIF2-TC) (Mignone et al. 2002; Babendure et al. 2006; Pestova et al. 2007). On the other hand, secondary structures situated closer to the starting codon require a free energy higher than -50 kcal/mol to be able to inhibit translation (Mignone et al. 2002; Babendure et al. 2006). The preinitiation complex scans along the 5'-UTR until it encounters the AUG start codon; afterward, the larger 60S subunit joins the 40S to form an 80S initiation complex, and protein synthesis begins. This mechanism is known as cap-dependent translation and describes the initiation of mRNA translation in most organisms (Hinnebusch and Lorsch 2012). Some viral RNAs use a cap-independent mechanism for initiating translation, which involves an internal ribosomal entry site (IRES) able to attract ribosomal subunits independently of the cap structure (Martinez-Salas et al. 2018; Zhao et al. 2020).

The UTR sequences employed in therapeutic IVT-mRNA are retrieved from specific databases (i.e., <http://utrdb.ba.itb.cnr.it/>) or from coding sequences (CDS) available in sequence banks (e.g., <https://www.ncbi.nlm.nih.gov/nucleotide/>). Those from human α - and β -globin probably represent the most studied and characterized UTRs (Babendure et al. 2006).

Length is a critical parameter for the 5'-UTR, and an optimized sequence should not exceed 70 nt and could include the Kozak consensus sequence (GCC-(A/G)-CCAUGG) immediately upstream of the translation start codon (AUG) to enhance translation from the correct initiation codon (Asrani et al. 2018). Importantly, the start codon AUG within 5' UTR is excluded to prevent alternative translation initiation and mutation of the amino acid sequence. In addition, secondary structure elements in the 5' UTR region of the mRNA should be minimized to reduce the energy barrier for the scanning ribosome to reach the start codon.

The 3'-UTR has a great effect on mRNA's stability; indeed, its optimization results in increased mRNA half-life (Holtkamp et al. 2006; Wang et al. 1999). Accordingly, the duration of mRNA expression can be regulated by varying its composition. The introduction of AU-rich elements in the 3'-UTR causes mRNA destabilization, leading to rapid mRNA decay thus shortening protein expression, while mRNAs with enriched CG-content in the 3'-UTR sequence exhibit increased stability and translation efficiency. Likewise, the proper combination of 5'- and 3'-UTR sequences can enhance the translation efficiency (Ferizi et al. 2016).

2.1.2 The Function of the Cap Structure

The introduction of a 5' end cap is a conserved post-transcriptional modification of eukaryotic mRNAs (Ramanathan et al. 2016). mRNA molecules are capped with a 7-methylguanosine (m^7G) connected by a 5'-to-5' triphosphate bridge to the first nucleotide to form a cap 0 structure (m^7GpppN). Cap 0 regulates the translation of mRNA by preventing its degradation and facilitating the assembly of the translation machinery (Ramanathan et al. 2016). In mammals, the first transcribed nucleotide is methylated in the 2' ribose position to form a cap 1 structure ($m^7GpppN_{2'O_m}$) and, in approximately 50% of transcripts, also the second transcribed nucleotide is 2' O-methylated in the 2' ribose position to form cap 2 ($m^7GpppN_{2'O_m}N_{2'O_m}$) (Ramanathan et al. 2016).

While cap 1 structure is ubiquitously expressed in humans, the expression of cap 2 is restricted to specific tissue types, such as in striated muscles and at lower levels in brain, testes, lung, liver, and skin tissues. The function of cap structures remains largely unknown, but they are known to be involved in modulating nuclear export, splicing, turnover, translation efficiency, and decapping of mRNAs (Galloway and Cowling 2019). Cap 1 is important for self/non-self-discrimination, by preventing the recognition by interferon (IFN)-induced proteins with tetratricopeptide repeats (IFITs) or pattern recognition receptors (PRRs) (Galloway and Cowling 2019). IFIT proteins recognize non-methylated cap structures, like cap 0, and mRNA molecules with 5'-triphosphate or 5'-monophosphate ends. IFIT-1 sequesters non-methylated mRNA from the translational machinery by competing with EIF4E proteins for the binding to the cap structure (Galloway and Cowling 2019).

The importance of cap structures in preventing mRNA recognition by the innate immune system has been highlighted by the observation that cytoplasmic viruses often possess cap 1 structures and that the deletion of the viral methyltransferase responsible for the conversion of cap 0 into cap 1 resulted in viral attenuation (Bouvet et al. 2010). Retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are responsible for the cytoplasmic recognition of double-stranded RNA (dsRNA) with 5'ppp and cap 0 ends, and their activation induce the expression of type I IFNs (IFN-I) (IFN- α and IFN- β) and other pro-inflammatory cytokines (Ramanathan et al. 2016; Galloway and Cowling 2019). Cap 1 modification abrogates both RIG-I and MDA5 recognition of dsRNA, preventing innate immune activation. Therefore, methylation of the first transcribed nucleotide is thought to be a molecular signature that discriminates self and non-self mRNA.

Several synthetic cap analogs have been developed for the capping of IVT-mRNA (Jemielity et al. 2010; Tang et al. 2019). The anti-reverse cap analog (ARCA) is widely used for the preparation of synthetic capped mRNA. ARCA possess a cap 0 structure with a 3'-O-methyl group on the sugar adjacent to the m^7G ($m^{7,3'-O}GpppG$), which prevents it from incorporating in the incorrect orientation (Tang et al. 2019; Warminski et al. 2017). The variety of cap structures has been recently expanded following the observations that up to 30% of caps in animals and viral mRNAs are also methylated at the first encoded nucleotide adjacent to the 7-mG cap to obtain N6-methyladenosine (m^6A) or N6,2'-O-dimethyladenosine (m^6Am). In addition,

multiple methylations also occur in the 5' G cap (e.g., m^{2,2,7}GpppN) in viral RNAs and a subset of RNAP II-transcribed cellular RNAs (Warminski et al. 2017).

2.1.3 Role of the Poly(A) Tail

The poly(A) tail is a structure characteristic of mature mRNAs that plays a significant role in mRNA translation (Weill et al. 2012). It has been shown that a gradual increase in the poly(A) tail length of IVT-mRNA to 120 bases leads to increased translation, whereas shortening of the poly(A) sequence results in faster mRNA decay (patent WO 2017/059902 A1). A further increase in the poly(A) tail size beyond 120 residues does not enhance the translation efficiency.

The poly(A) tail is bound with high affinity by the poly(A) RNA binding proteins (PABPs), which interact with eIF4G and eIF4B to promote the circularization of the mRNA molecule and ribosomal recruitment to form a polyribosome complex (Weill et al. 2012). A sufficiently long poly(A) tail is necessary to ensure the circularization of mRNA via binding of PABPs to the poly(A) tail and the cap. The minimal length of poly(A) tail required for mRNA's stability has been determined to be 30 nt, which corresponds to the reported 25–30 nt footprint for a single PABP (Lima et al. 2017).

2.1.4 Modified Nucleotides

mRNA suffers from several limitations which impeded its use for a long time. In particular, limited stability and high immunogenicity were the most relevant issues limiting the therapeutic application of IVT-mRNA.

Chemically modified nucleotides are known to be present at low abundance in non-synthetic mRNAs (McCown et al. 2020). IVT-mRNAs incorporating modified nucleotides, commonly uridine, are termed modified mRNAs (modRNAs), while unmodified mRNAs (unmodRNA) do not contain chemically modified nucleotides. The most frequent naturally modified nucleotides are pseudouridine (Ψ), 5-methylcytidine (m⁵C), N⁶-methyladenosine (m⁶A), 5-methyluridine (m⁵U), and 2-thiouridine (S²U). The presence of modified nucleotides in the mRNA prevents its recognition as a foreign molecule by endosomal sensors, such as Toll-like receptors (TLRs) 3, TLR7, and TLR8 or cytoplasmic sensors, such as RIG-I and MDA5, responsible for the induction of type I IFNs, typically associated with antiviral responses (Karikó et al. 2005; Nelson et al. 2020). The activation of type I IFN signaling pathways causes the suppression of mRNA translation and its degradation, and it can even induce host cell death via apoptosis (Nelson et al. 2020; Palchetti et al. 2015).

It has been reported that the incorporation of modified bases in the mRNA sequence reduces innate immune activation, thus improving its translation and activity (Karikó et al. 2005, 2008). The replacement of uridine with Ψ is the predominant modification employed in the preparation of synthetic mRNA. Its incorporation into mRNA has shown to increase the resistance to RNase degradation and to limit

TLR activation, with a consequent improvement of its translatability and transfection efficiency both in vitro and in vivo (Anderson et al. 2011; Svitkin 2017; Roy 2021).

Nowadays, many types of modified bases have been developed, including m⁵C, N1-methylpseudouridine (m¹Ψ), and Ψ, and the impact of these modified nucleotides on mRNA's activity can be sequence-dependent and cell-type-dependent. In this regard, particularly interesting has been a study in which transfection of THP-1 macrophages with m⁵C/Ψ-modified mRNA encoding firefly luciferase (Fluc mRNA) resulted in a higher translation rate compared to unmodified Fluc mRNA, while the incorporation of m⁵C/Ψ modified nucleotide into mRNA encoding enhanced green fluorescence protein (eGFP mRNA) caused a decrease in protein production (Li et al. 2016). The authors also showed that transfection with m⁵C/Ψ-modified mRNA generated a significantly higher expression of Fluc in THP-1 cells than in hepatocellular carcinoma Hep 3B cells.

2.1.5 Codon Optimization of the ORF

Codon optimization relies on the degeneracy of the genetic code, according to which different codons code for the same single amino acid (Mauro and Chappell 2014). The optimization of the ORF sequence is intended to replace codons with low levels of charged tRNAs with codons recognized by abundant tRNAs, so that the exogenous mRNA can be translated with higher efficiency without causing modifications to the amino acid sequence (Mauro and Chappell 2014). Using luciferase and erythropoietin coding mRNAs as model, it has been demonstrated that codon usage optimization of the ORF improves the translation rate and consequently the activity of IVT-mRNA (Thess et al. 2015). The authors found that unmodRNA incorporating codons rich in guanosine and cytosine induced higher systemic levels of erythropoietin and stronger physiological effects compared to Ψ-modified mRNA. However, in some cases high translation rate of mRNA is not desired since some proteins require a slower translation to correctly fold into biologically active forms, and the inclusion in the ORF of codons with low frequency ensures the generation of protein products of higher quality (Brule and Grayhack 2017).

In conclusion, specific codon optimization strategies should be applied depending on the type of protein encoding by the ORF sequence to improve mRNA translation rate and concomitantly ensure optimal protein expression levels.

2.2 Synthesis of IVT-mRNA

IVT-mRNA compared to more traditional gene therapy platforms, such as viral vectors and pDNA, presents the advantage that its production requires simple procedures that can be easily engineered to the required scale. Besides, once the manufacturing process is established, in principle it can be applied for any RNA sequence with essentially no size limitations. mRNA is synthesized in a cell-free system by

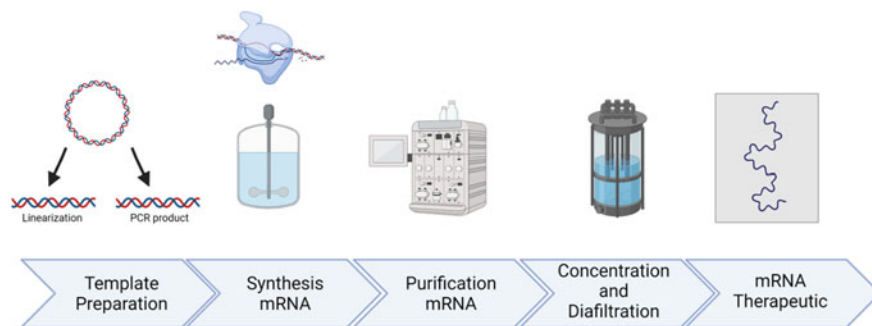


Fig. 2 Schematic illustration of the mRNA production process. The manufacturing of mRNA involves the preparation of a DNA template by PCR or pDNA linearization followed by a cell-free enzymatic *in vitro* transcription reaction. After synthesis, the mRNA is purified, concentrated, and diafiltered

in vitro transcription (Henderson et al. 2021), which does not foresee the use of hosts like bacteria, yeast, or mammalian cells, thus preventing the associated quality and safety concerns in the production (Fig. 2). Additionally, this host-free system allows to avoid complicated downstream purification procedures, consenting a rapid scale-up and cost-effective manufacturing. *In vitro* transcription utilizes a linearized pDNA or a PCR product containing a bacteriophage promoter (i.e., T7, T3 or SP6) as template for a bacteriophage DNA-dependent RNA polymerase, which recognizes the promoter within the DNA template and catalyzes the *de novo* synthesis of mRNA in the presence of ribonucleoside triphosphates (rNTPs) (Henderson et al. 2021). The mRNA sequence can be tailored to meet specific needs, concerning stability and translation efficiency, which can be modulated or enhanced by including further *cis*-acting elements such as 5'-cap structure and signal peptide (SP) or GSG linker, internal ribosome entry site (IRES) and 2A peptide sequences in multicistronic mRNAs (Mignone et al. 2002; Chng et al. 2015). Furthermore, the sequence of IVT-mRNAs can be optimized by changing the codon composition, and if needed, modified nucleotides can be inserted to improve its translatability and stability (Li et al. 2016; Thess et al. 2015). *In vitro* transcription has been commonly used for synthesizing both non-replicating mRNA and saRNA (Henderson et al. 2021; McKay et al. 2020).

The first step of *in vitro* RNA synthesis consists in the design of the DNA template containing a protein-encoding open reading frame (ORF) flanked by regulatory sequences (Henderson et al. 2021). Both pDNA and PCR products can be employed as a template for transcription. In a minimum composition, a pDNA must contain typical elements, such as a promoter sequence, an ORF sequence, 5'/3'-UTR sequences, a poly(A) tail, unique restriction endonuclease sites, a bacterial origin of replication (*ori*), and an antibiotic resistance gene (Avci-Adali et al. 2014). The *ori* and selectable marker in the vector backbone allow replication and selection of the plasmid in bacteria, potentially facilitating the establishment of a pDNA template bank.

In the case pDNA is utilized as template, purification, normally by chloroform/phenol extraction followed by ethanol precipitation (Dowhan 2012), and DNA linearization by digestion with a restriction enzyme producing blunt or 5'-overhang ends upstream the promoter sequence, are required steps (Henderson et al. 2021). PCR products for in vitro transcription are generated using a primer containing the desired bacteriophage promoter sequence (Henderson et al. 2021).

The RNA polymerase initiates transcription of the DNA template in the presence of natural rNTPs or with chemically modified rNTPs (5-methoxy-UTP, pseudo-UTP, etc.) to produce several copies of RNA molecules. The DNA template can be removed from the mRNA preparation by treatment with DNase (Henderson et al. 2021).

Functional RNA molecules require a cap structure at the 5' end and a poly(A) tail at the 3' end. Regarding the capping of the IVT-mRNA, it can be achieved by two different approaches, enzymatic capping, using vaccinia virus-derived enzymes, or a co-transcriptional method (Henderson et al. 2021; Muttach et al. 2017).

The enzymatic method utilizes a 2'-O-methyltransferase, which consents the generation of a cap 1 structure with a potential capping yield of 100%. However, the enzymatic method has exhibited some drawbacks that have recently limited its use, including a high variation in the capping efficiency, the requirement of an unstable temperature-labile cofactor (S-Adenosylmethionine), and high scale-up costs. For all these reasons, co-transcriptional methods are currently preferred for mRNA capping (Henderson et al. 2021).

In the co-transcriptional method, cap analogs are incorporated directly at the 5'-end of the IVT-mRNA by RNA polymerases, and erroneous internal incorporation of cap analogs during mRNA polymerization cannot occur since cap analogs lack a free 5'-triphosphate. The cap structure m⁷GpppG represents the most largely employed cap analog (Muttach et al. 2017; Kocmik et al. 2018), but many other alternative cap analogs have also demonstrated good compatibility with commonly employed T7 bacteriophage RNA polymerase. A major limitation of this first generation co-transcriptional capping is that cap analogs compete with GTP as initiator nucleotide and, therefore, the method exhibits variable capping efficiency and reproducibility (Muttach et al. 2017; Kocmik et al. 2018). Additionally, the use of this first generation of cap analogs as nucleotide initiators can cause cap incorporation in reverse directions, due to the presence of a 3'-OH group on m⁷G; thus, up to one half of the mRNA contains the cap in the wrong orientation and it is not translatable (Muttach et al. 2017; Kocmik et al. 2018).

This problem was solved by developing a second generation of cap structures named anti-reverse cap analogs (ARCA) with a methylated or deoxygenated 3'-OH group at the N7-methylguanosine ribose (m^{7,3'-O}GpppG or m^{7,3'-d}GpppG) (Kocmik et al. 2018). This prevents elongation at the "wrong" 3'-OH, avoiding the incorporation of ARCA analogs in the reverse orientation. Nevertheless, ARCA capping has the disadvantage that only cap 0 mRNAs can be prepared, and the cap structure is characterized by the presence of an unnatural 3'-O-methyl group that could be promptly recognized by innate immune receptors. The introduction of the ARCA cap analog is ensured by conducting the transcription reaction using an excess of ARCA over GTP (ARCA:GTP ratio of 4:1) (Kocmik et al. 2018; Liu et al. 2019). However, even if

working at optimized conditions, the capping efficiency rarely exceeds 80%, which means that at least 20% of IVT-mRNA possesses uncapped 5'-triphosphate ends, thus requiring additional purification steps. Due to the strict preference of bacteriophage RNA polymerases for G or A, depending on the promotor, artificial mRNAs starting with a U or C at the 5'-end cannot be prepared using in vitro transcription (Henderson et al. 2021).

Trilink BioTechnologies, a US biotech company, has recently developed CleanCap® mRNA, a third generation of co-transcriptional capping technology (Henderson et al. 2021). CleanCap® results in the incorporation of N⁶-methyladenosine methylated cap (m⁶A or m⁶Am) at the 5'-end. This method offers important technical advantages compared to previously proposed cap analogs, since it displays superior efficiency and reproducibility (94–99% of complete capping), yields a natural unmodified cap structure (cap 1) with reduced immunogenicity, possesses a greater cost-effectiveness than enzymatic capping, and is easily scalable for large-scale manufacturing (Henderson et al. 2021).

The addition of a poly(A) tail to the synthetic mRNA can be performed either post-transcriptionally using a poly(A) polymerase or co-transcriptionally by including a poly-A tail in the DNA template. While a poly(A) tail with a maximum length of 80 nt can be incorporated into the pDNA, since longer poly(A) sequences can give stability problems due to recombination events that may occur in the host bacteria, by using the poly(A) polymerase no inherent length limitation of poly(A) tail synthesis has been found (Chaudhary et al. 2021). However, the enzymatic incorporation of poly(A) does not consent a high reproducibility, and consequently, the poly(A) products display a larger and more variable size distribution compared to those obtained using a co-transcriptional technique. A solution to address this stability issue is to include a short linker sequence in the poly(A) tail (Trepotec et al. 2019). In addition, enzymatic polyadenylation is less affordable and inadequate for scaled-up manufacturing.

Thanks to the enormous progress in the field, 5'-capped and 3'-poly-adenylated mRNAs can be easily produced in “one-pot” reaction, achieving cap and poly(A) tail additions concomitantly during the in vitro transcription of synthetic mRNA (Henderson et al. 2021).

2.3 Purification of Synthetic mRNA

IVT-mRNA purification is required to ensure the removal of contaminants that may affect the therapeutic performance of the mRNA molecules. This includes the residual DNA template, unincorporated rNTPs and cap analogs, enzymes employed in the reaction, truncated mRNA products, and double-stranded mRNA. The method chosen for purification depends on the length and abundance of the IVT-mRNA, the type of impurities (nucleic acids and/or proteins), and the type of downstream application.

Standard purification methods include lithium chloride (LiCl) precipitation, alcohol-based precipitation (i.e., ethanol precipitation), and techniques based on

silica membranes (i.e., spin columns) (Henderson et al. 2021; Walker and Lorsch 2013). LiCl precipitation is employed to remove the majority of the unincorporated rNTPs and enzymes used for the synthesis of IVT-mRNA. Instead, ethanol precipitation can ensure the complete removal of free rNTPs, salts, and proteins (Walker and Lorsch 2013; Rio et al. 2010). Silica membranes selectively bind nucleic acids, allowing the elimination of salts, free rNTPs, and proteins (Baronti et al. 2018). Such membranes will retain the intact DNA template, and its removal requires pre-treatment with DNase after *in vitro* transcription.

None of these techniques are effective in the removal of truncated RNA and dsRNA impurities generated from abortive initiation of *in vitro* mRNA synthesis. Previous studies have identified dsRNA and truncated RNA as the contaminants that mostly affect IVT-mRNA's translation efficiency, as they trigger innate immunity through their recognition by RNA sensors. To date, the most common method employed to eliminate nucleic acid contaminants from IVT-mRNAs is reversed-phase high-performance liquid chromatography (HPLC) (Karikó et al. 2011). However, this procedure requires extremely toxic solvents and is not suitable for the large-scale production of mRNA. Moreover, reversed-phase HPLC is less efficient at purifying very large molecules of mRNA.

Alternative methods have been proposed for the purification of synthetic mRNA, including ion-exchange chromatography, oligo(dT) affinity chromatography, and other separation techniques that rely on differences in size, such as size exclusion chromatography (SEC) and cross-flow filtration (CFF) (Baierdörfer et al. 2019).

Recently, a simple, fast, and cost-effective way to eliminate dsRNA contaminants from IVT-mRNA has been reported (Baierdörfer et al. 2019). This method is based on the selective binding of dsRNA to cellulose in an ethanol-containing buffer. The authors showed that at least 90% of the dsRNA impurities can be removed with a good recovery rate (>65%), independently from the length and nucleoside composition of the IVT-mRNA. The different purification methods can also be combined to improve the purity of IVT-mRNA.

3 Lipid Nanoparticles for Therapeutic mRNA Delivery

The inability of naked mRNA to cross the outer membranes of cells thus to reach the cytoplasmic compartment, together with its lack of stability under physiological conditions, represent the major barriers impeding the complete exploitation of mRNA-based therapies (Guevara et al. 2020).

Various strategies have been proposed for enabling efficient delivery of mRNA into target cells, including chemical modification of mRNA (Zangi et al. 2013), ionic complexation with cationic polymers, physical methods (i.e., electroporation) (Van Tendeloo et al. 2001), and viral vectors (Segel et al. 2021), but so far, lipid nanoparticles (LNPs) have demonstrated the most encouraging results (Persano et al. 2017; Stadler et al. 2017; Thran et al. 2017; Guevara 2019a; Kong et al. 2019; Rybakova et al. 2019; Wei et al. 2020; Kranz et al. 2016). The intense research

efforts dedicated toward the development of LNP as mRNA delivery systems recently culminated in the approval of two mRNA-based vaccines for clinical use (Dolgin 2021a, b), and the development of a growing number of clinical trials currently ongoing (Table 1).

LNPs have shown to efficiently protect mRNA from hydrolysis by RNases and at the same time allow endosomal escape so to achieve mRNA delivery into the cytosol of specific cells (Sago et al. 2018; Cheng et al. 2020). Once in the cytosol, it can be sequestered by the translation machinery for initiating protein synthesis.

Compared to the most popular viral vectors, LNPs are less immunogenic, they can carry larger genetic material payloads and are easier to manufacture. Therefore, even if usually LNPs exhibit lower transfection efficiencies than viral vectors, they are becoming the preferred tool for mRNA transfection.

LNPs are self-assembled nanostructures with a size of approximately 100 nm, consisting of different lipid components that can be grouped in three major types: ionizable or permanently cationic lipids, helper lipids, and stealth lipids (i.e., PEGylated lipids) (Fig. 3) (Guevara 2019b; Guevara et al. 2020). Ionizable cationic lipids are usually preferred over permanently charged cationic lipids, as they exhibit higher biocompatibility and efficiency. A great number of screening studies, testing a variety of ionizable lipids composed of different combinations of hydrophilic head groups and non-polar lipid tails, have allowed to increase the number of available lipids (Billingsley et al. 2020; Miao et al. 2020; Guimaraes et al. 2019; Carrasco et al. 2021). These studies showed that the performance of ionizable lipids is controlled by the chemical and structural characteristics of both the head group and lipid tail region. The main feature of ionizable lipids is their ability to respond to an acidic pH, which is usually defined by the pKa value. A single or a mixture of ionizable

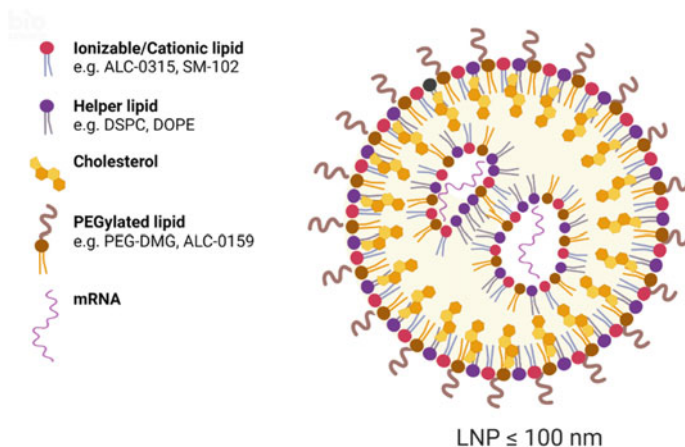


Fig. 3 Schematic of mRNA LNPs. LNPs are composed of four components, such as ionizable lipid (e.g., ALC-0315, SM-102), helper lipid (e.g., DSPC, DOPE), cholesterol, and PEGylated lipid (e.g., PEG-DMG, ALC-0159). The molecules of mRNA within the LNP are confined into aqueous regions

lipids determines the overall pKa of the LNPs that needs to be around 6.5, as established using the anionic fluorescent dye 2-(p-toluidino)-6-naphthalenesulfonic acid (TNS) binding assay (Guevara et al. 2020; Carrasco et al. 2021). At neutral pH, these lipids are in a zwitterionic form or do not possess any charged functional groups, and only upon internalization in the endosomal compartment of the cell, where the pH is near 5, the head group of the lipids is protonated and assumes a net cationic charge that promotes its interaction with the anionic endogenous endosomal phospholipids. Ionizable lipids with a conical shape are more desirable since this conformation is incompatible with a lipid bilayer organization, thus favoring the destabilization of the endosomal membrane and allowing the mRNA payload to be released into the cytosol (Carrasco et al. 2021). Regarding the lipid tail portion, a series of features like length of the hydrophobic tail, level of unsaturation, and presence of branches have been found to affect dramatically the transfection capability of LNPs (Carrasco et al. 2021).

Other components, like helper lipids, are included in the formulation to enhance the stability and delivery efficiency of LNPs, whereas PEGylated lipids are essential to reduce the opsonization of LNPs by serum proteins, which drive their undesired high accumulation in off-target organs (e.g., liver), and rapid clearance from bloodstream (Guevara et al. 2020; Patel et al. 2020). As recently demonstrated, the interaction of LNPs with serum proteins is dictated by mechanisms that are more complicated than it was initially thought and that do not rely only on the net charge of LNPs but involve other factors that still need to be determined and that deserve further attention (Miao et al. 2020).

The relative abundance of ionizable lipid, helper lipid, and stealth lipid critically determines the efficacy of LNPs and therefore needs to be opportunely optimized depending on the application and according to the administration route that is intended to be used (Guevara et al. 2020; Hassett et al. 2019; Ryals et al. 2020; Ndeupen et al. 2021). The number of possible options in the design of LNPs has been further increased due to the recent evidence that points out that different ionizable lipids can synergize in boosting mRNA transfection (Miao et al. 2020). Moreover, even if this has not been proven yet, it is highly plausible that the same synergism might be observed between helper lipids that are considered chemically and functionally equivalent.

In addition to the lipid composition, other features such as size and surface charge are known to have an enormous impact on the behavior of LNPs *in vivo*, and hence, it is pivotal to appropriately tune these parameters to achieve the desired therapeutic outcomes (Cheng et al. 2020; Ryals et al. 2020; Ndeupen et al. 2021; Nakamura et al. 2020; Hassett et al. 2021). The most common helper lipids that have been tested are 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol. DSPC is a phosphatidylcholine with saturated hydrophobic tails and a cylindrical shape that allows DSPC molecules to organize in a lamellar phase, which stabilizes the structure of LNPs (Guevara et al. 2020; Hou et al. 2021). DOPE is a phosphoethanolamine with a *cis*-unsaturated double bond in the two oleyl fatty acid chains. It exhibits a conical shape and

adopts an inverted hexagonal H(II) phase at acidic pH, which destabilizes endosomal membranes and facilitates the endosomal escape of LNPs (Guevara et al. 2020).

Nowadays, solvent injection, also known as nanoprecipitation (or antisolvent precipitation), is the most common technique used for the preparation of mRNA-loaded LNPs (Guevara et al. 2020; Hou 2021). In the last years, the implementation of microfluidic devices for LNP manufacturing has improved the reproducibility and scalability of the method. In these systems, the mixing of an organic solution, containing a mixture of lipids dissolved in an organic solvent (i.e., ethanol), with an aqueous solution, in which the mRNA molecules are dissolved, is realized in microchannels molded on a chip. The channels are designed in a way that the liquids are forced to flow in two separate microchannels and then come into contact at the crossing of channels, thus promoting the self-assembly of lipids into LNPs at the interfacial layer where the lipids are exposed to an environment with increased polarity (Fig. 4) (Guevara et al. 2020).

Microfluidic chips can be prepared with different mixing patterns, such as standard T-junction, microfluidic hydrodynamic flow focusing (HFF), microfluidic micromixer (MM), and staggered herringbone micromixer (SHM) (Riewe et al. 2020). The widely utilized NanoAssemblr™ platform employs a Y-shaped architecture incorporating SHM pattern. The channel configuration together with the flow rate are important parameters affecting dramatically the physicochemical characteristics of LNPs (Guevara et al. 2020; Riewe et al. 2020; Roces et al. 2020). LNPs with

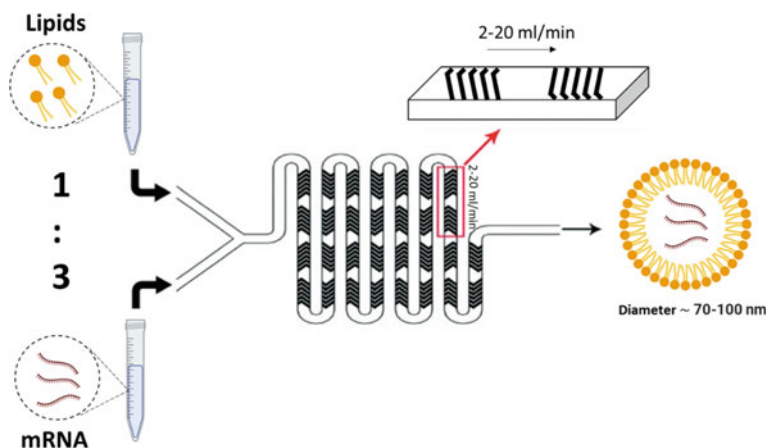


Fig. 4 Schematic of mRNA-loaded LNPs preparation by a microfluidic system with a SHM configuration. One volume of ethanol solution containing a mixture of lipids and three volumes of mRNA dissolved in an acidic aqueous solution are pumped separately into two distinct inlets of the SHM chip by a syringe pump at a flow rate of 2–20 ml/minute. The SHM design favors a rapid mixing of the ethanol and aqueous phases with consequent increases of the net polarity of the lipid solution. Above a certain threshold of polarity, the lipids precipitate as form of LNPs with a size usually ranging from 70 to 100 nm. The ionic interactions taking place between the negative charges of the phosphate groups (P) in mRNA molecules and the positive charges of the amino groups (N) in cationic/ionizable lipids are the force that drives the encapsulation of the mRNA into LNPs

a smaller size and narrow size distribution are typically produced with a higher flow rate (Roces et al. 2020).

4 mRNA-Based Cancer Immunotherapy: Antitumor Vaccines

Cancer immunotherapy represents the major area of research where mRNA has found application as therapeutic agent. It has been demonstrated that the mRNA technology can be utilized to create different immunotherapeutic products, such as vaccines, monoclonal antibodies, chimeric antigen receptor (CAR) cells, and immunomodulatory proteins (Persano et al. 2017; Stadler et al. 2017; Thran et al. 2017; Guevara 2019a; Kong et al. 2019; Rybakova et al. 2019; Wei et al. 2020; Lai et al. 2018).

Cancer vaccines are the most advanced application of mRNA, with both prophylactic and therapeutic potentials and relying on the capability of mRNA to simultaneously deliver genetic information and act as immunoadjuvant by interacting with innate immune receptors (Persano et al. 2017; Stadler et al. 2017; Kranz et al. 2016; Guimaraes et al. 2019). The immunomodulatory properties of mRNA can be particularly relevant for the development of antitumor vaccines that require overcoming of immune tolerance, a characteristic of many malignancies (Kranz 2016). On the other hand, upon recognition of the RNA by innate immune receptors, IFN-I triggers the expression of interferon-stimulated genes (ISGs), such as IFN-inducible double-stranded RNA-activated protein kinase (PKR), and 2',5'-oligoadenylate synthetase (OAS), with consequent decreased translation and increased decay of mRNA (De Beuckelaer et al. 2016; Yang and Shah 2020). This could be extremely deleterious for other applications of mRNA, including chimeric antigen receptor (CAR) cell therapy and gene therapy, for which maximization of the expression might be essential for ensuring a therapeutic effect. In these cases, the use of modified nucleosides, such as pseudouridine, N1-methylpseudouridine, and 2-thiouridine, is highly desirable in order to increase the translation efficiency of synthetic mRNAs (Karikó et al. 2008; Svitkin et al. 2017).

Vaccines can be designed to target tumor-associated antigens (TAAs) (overexpressed antigens, tissue differentiation antigens, and tumor germline antigens) or tumor-specific antigens (TSAs) (oncoviral antigens and neoantigens), thus promoting an antitumor response that specifically attacks and destroys cancer cells and achieves a prolonged response and prevention of relapse due to the generation of an immunological memory (Kranz et al. 2016; Hollingsworth and Jansen 2019).

Recently, several studies have pointed out the importance of neoantigens as targets for immunotherapy (Sahin et al. 2017; Cafri et al. 2020; Blass and Ott 2021). The considerable progresses made in sequencing technologies and bioinformatic tools have permitted to reveal that neoantigen-specific CD8⁺ and CD4⁺ T cells are detectable in most tumors, independently if they have a viral etiology (Hollingsworth

and Jansen 2019; Sahin et al. 2017; Cafri et al. 2020; Blass and Ott 2021). Neoantigens are the consequence of somatic mutations that occur in malignant cells during cancer progression due to the high genomic instability typical of tumors. This class of TSAs is particularly appealing because, unlike TAAs, they are detectable only in cancer cells, therefore not subjected to central tolerance, and are characterized by a high immunogenicity, high affinity toward the MHC, and individual specificity (Hollingsworth and Jansen 2019; Blass and Ott 2021).

The first step in neoantigen identification is the comparison of whole exome sequencing (WES) or mRNA sequencing (mRNA-Seq) data from tumor and normal tissues obtained using high-throughput sequencing techniques (i.e., next-generation sequencing (NGS)) (Blass and Ott 2021; Esprit et al. 2020). Then, the data from the sequencing analysis are analyzed with bioinformatic tools to predict whether the identified mutations can generate tumor neoantigens (Esprit et al. 2020). Most of these bioinformatic softwares are based on HLA binding affinity and tend to ignore other important factors that have an impact on the antigen presentation process, such as the C-terminal cleavage by proteasome, efficiency of transporter associated with antigen processing (TAP)-mediated transport of peptides, expression abundance of neoantigens, tumor heterogeneity, heterogeneity and clonality of neoantigens, and loss of heterozygosity of HLA.

A vaccination platform capable of targeting multiple patient-specific antigens is highly desirable for developing personalized neoantigen vaccines with enhanced immunogenicity. In this regard, synthetic mRNA offers the possibility to easily incorporate multiple neoantigens in a single molecule that can be manufactured with a cost-effective and scalable approach.

Preliminary studies on mRNA-based neoantigen vaccines were conducted using mRNA-electroporated dendritic cells (DCs) (Wilgenhof et al. 2013). However, previous studies have reported that several factors may intervene in limiting the efficacy of DC vaccines including optimal maturation, subset of cells employed, antigen-loading efficiency, and the ability of DCs to migrate to vaccine-draining lymph nodes (Santos and Butterfield 2018). Therefore, currently most of the groups working in the area of mRNA vaccines are switching from DC platforms toward strategies that involve the delivery of neoantigen mRNAs into APCs directly in vivo, thus avoiding DC isolation and manipulation ex vivo.

The first proof of concept that in vivo delivery of tumor antigen-encoding mRNA into APCs is an achievable path for successful antitumor vaccination was reported only few years ago. For the first time, it was shown that DCs can be passively targeted in vivo upon systemic administration of mRNA-carrying lipoplexes displaying a negative net charge (Kranz et al. 2016). Vaccination with mRNA lipoplexes encoding tumor-specific or tumor-associated antigens stimulated strong type I IFN-dependent effector and memory T cell responses resulting in tumor rejection and protection from tumor rechallenge.

In situ vaccination is an alternative form of vaccine effective at eliciting antigen-specific T cell responses. This is achieved using a cytotoxic agent, alone or in combination with an immunoadjuvant, able to induce immunogenic cell death (ICD), which not only directly kills tumor cells, but also promotes the release of tumor antigens

and molecular signals that promote the activation of immune cells that are able to reach even distant cancer cells.

The use of mRNA-based therapeutics has also been proposed to induce ICD in tumor cells. For this scope, it has been utilized a mRNA encoding proapoptotic proteins, caspase or PUMA, and including in the 3'-UTR microRNA (miRNA) target sites to minimize the expression of the proteins in healthy hepatocytes, thus preventing side effects due to off-target expression (Jain et al. 2018).

4.1 mRNA-Loaded LNP-Mediated Monoclonal Antibody Delivery

Monoclonal antibodies are emerging as one of the most promising classes of cancer immunotherapy, so much so that several antibodies have received approval for clinical use for the treatment of several forms of cancers (Boyiadzis and Foon 2018; Mullard 2021). Antibodies have been designed to target specific proteins expressed on tumor cells and immune cells or released into the tumor microenvironment. The use of monoclonal antibodies for targeting immune checkpoints and inhibiting their functions (immune checkpoint inhibitors, ICIs) represents one of the most investigated areas of application.

Several types of ICIs have been or are currently under investigation, and many of them have received clinical approval for the treatment of different tumors (Gravbrot et al. 2019). Despite the encouraging outcomes from preclinical and clinical studies, and the fact that many patients have already benefited from the use of monoclonal antibodies, there are still concerns regarding this type of therapeutic agents that limit their wider application in the clinic (de Miguel and Calvo 2020; Palmieri and Carlino 2018; Chames et al. 2009; Hernandez et al. 2018). Major concerns are mainly related to the complex and expensive procedures required for their manufacturing and purification (Chames et al. 2009; Hernandez et al. 2018). Therapeutic antibodies are typically full-size immunoglobulins (Ig), mostly of the IgG type, which require a wide variety of post-translational modifications, including glycosylation, disulfide bond formation, and many other modifications that cannot be introduced synthetically (Jank et al. 2019; Yang and Li 2020; Lu et al. 2020). For this reason, their preparation is commonly realized in mammalian cell lines (Lu et al. 2020; Dangi et al. 2018). Then, a purification step is required to have an injectable antibody therapeutic free from any potential harmful contaminants. Given that these modifications can directly affect the functionality of monoclonal antibodies, it is of essential importance to implement analytical assays that can ensure the quality of the product. All these aspects contribute to the elevated costs of antibody-based treatments and make these therapies poorly affordable. To achieve the synthesis of functional antibodies in prokaryotic expression systems, like *E. coli*, that can enable faster and cheaper production, different types of antibody fragments, such as single-chain variable fragments (scFv), heavy-chain-only VH (VHH) domains, and nanobodies, have

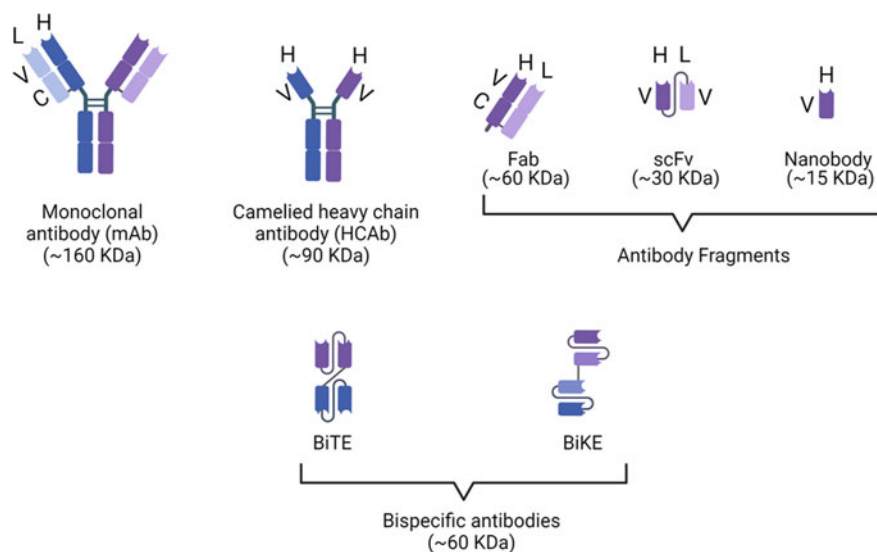


Fig. 5 Illustration of the different types of antibodies: monoclonal antibody, camelid heavy-chain antibody (HCAb), antigen-binding fragment (Fab), single-chain fragment variable (scFv), nanobody, bispecific T cell engagers (BiTE), and bispecific killer cells engagers (BiKE)

been developed (Fig. 5) (Jank et al. 2019). These are smaller than conventional antibodies and lack glycosylation. Another advantage of antibody fragments is that the small size can improve their penetration ability into tissues that are not reachable by conventional full-size antibodies, which is advantageous for many therapeutic applications. The antibody fragment technology has been also used to generate bispecific antibodies, and chimeric antigen receptors (CARs) usually consisting of scFv linked to intracellular signaling molecules, capable of triggering T cell effector activities (Hernandez et al. 2018; Jank e al. 2019; Yang and Li 2020). Yet, antibody fragments are cleared from circulation much more rapidly than conventional full-size antibodies, and since they do not have the Fc domain, fragments are unable to elicit Fc-mediated cytotoxicity (Jank et al. 2019; Yang and Li 2020).

The use of mRNA-loaded LNPs has recently emerged as a promising approach to overcome current limitations of monoclonal antibodies by consenting the production of a specific antibody directly in the body of the patient, circumventing the complicated and expensive purification steps and thereby avoiding the batch-to-batch variation that can be found when using antibodies (Van Hoecke and Roose 2019). Since IVT-mRNA contains all the instructions for appropriate folding and assembly, and for post-translational modifications, the antibodies generated from exogenous mRNA are perfectly functional. A potential limitation of this strategy is that only antibodies with natural modifications can be produced, and it cannot be employed for delivering antibodies conjugated to synthetic molecules, such as polyethylene glycol (PEG), which can extend the blood circulation half-life of antibody fragments. However, recent studies demonstrated that mRNA-mediated delivery can enhance the serum

half-life of both full-size and fragment antibodies which can improve the therapeutic efficacy of these treatments (Rybakova et al. 2019; Tiwari et al. 2018).

The first proof of the feasibility of mRNA-mediated delivery of therapeutic antibodies was reported in 2017 (Pardi et al. 2017). In that study, passive vaccination was achieved by systemic administration of LNPs carrying a modRNA encoding both light and heavy chains of an anti-HIV-1 neutralizing antibody. Similarly, the mRNA technology can be also exploited to obtain in vivo production of bispecific antibodies with two binding domains directed against a tumor antigen and CD3 marker, able to redirect and activate the antitumoral action of circulating T cells (Stadler et al. 2017). A single dose of 3–5 μg of formulated mRNA was sufficient to induce a rapid synthesis of bispecific antibodies and triggered complete eradication of advanced tumors. To obtain a comparable outcome with a recombinant bispecific antibody, it was necessary to give a dose three times higher than that of formulated mRNA. While most of the reported studies have achieved passive immunization through intravenous administration of mRNA-loaded nanoparticles, very recently it has been shown that intramuscular injection of formulated saRNA encoding an anti-Zika virus neutralizing human antibody (ZIKV-117) also induced high antibody titers and protected mice from Zika infection (Erasmus et al. 2018).

Taken together, these studies clearly show the potential of mRNA-loaded delivery systems for in vivo production of therapeutic antibodies, offering in this way a series of advantages compared recombinant antibodies, including reduced costs and prolonged serum half-life, thereby making antibody-based treatments more effective and accessible to a larger portion of patients.

4.2 mRNA-Loaded LNPs for CAR Immune Cell Engineering

CAR cell therapy is considered the most advanced modality of personalized immunotherapy in which immune cells, like T cells or NK cells, are isolated from a patient or a donor, genetically engineered ex vivo and ultimately infused into the patient (Sternier and Sternier 2021). The efficacy of adoptive T cell therapy has been demonstrated by numerous clinical trials showing remarkable outcomes in relapsed or refractory hematologic cancers. These clinical successes have led to the approval of CAR T cell products for children with acute lymphoblastic leukemia and adults with large B cell lymphoma by two of the major regulatory agencies, the FDA and the EMA (Sternier and Sternier 2021; Lin et al. 2021).

Despite these encouraging premises, CAR cell therapies suffer from considerable limitations concerning toxicity, primarily cytokine release syndrome (CRS) and neurologic adverse effects, and the high costs and complex procedures involved in the manufacturing of CAR cell-based treatments (Sternier and Sternier 2021; Lin et al. 2021). Interleukin 6 (IL-6) seems to be the major responsible for CRS since elevated levels of IL-6 have been observed in these patients and in murine models of the disease (Kishimoto 2021). While CRS may be alleviated through the administration

of tocilizumab, an anti-IL-6 receptor antibody, costs, and manufacturing challenges may be addressed with the advent of mRNA technology.

Viral transduction and electroporation are the most common techniques for CAR introduction into T cells (Van Hoecke and Roose 2019). However, both strategies present limitations. Viral vectors are associated with limited genetic cargo, safety concerns, and high costs, whereas electroporation can result in reduced viability, aberrant gene expression profile, and relative low transgene expression in the surviving transfected cells (Van Hoecke and Roose 2019). Therefore, alternative strategies for CAR delivery are highly desirable.

In recent years, ionizable lipid nanoparticles encapsulating mRNA encoding CAR have been extensively tested in preclinical studies for their ability to transfect immune effector cells either *in vivo* or *ex vivo*. A large screening study has allowed to identify seven distinct formulations capable of enhanced mRNA transfection of Jurkat T cells over lipofectamine (Billingsley et al. 2020). The best performing LNP formulation of these was tested with primary human T cells, displaying a CAR transfection efficiency equivalent to those observed with electroporation, but with a significantly inferior cytotoxicity. The potent killing activity of CAR T cells generated by transfection with mRNA LNPs was proven in a coculture assay with acute lymphoblastic leukemia cells.

Lately, *in vivo* targeting and transfection of lymphocytes have emerged as a fascinating viable route for simple and cost-effective generation of CAR T cells directly in the body of the patient. On this regard, an injectable LNP formulation functionalized on the surface with anti-CD3 antibody was developed for active targeting and mRNA transfection of circulating T cells, to induce transient expression of CAR or TCR recognizing disease-relevant targets (Smith et al. 2017).

5 Future Prospective and Conclusions

IVT-mRNA has the unprecedented potential to address major challenges of current immunotherapies and offers the basis for the development of innovative cancer therapies. The enormous progress in LNP formulations along with a better understanding of mRNA translation regulation has allowed the development of numerous mRNA-based treatments successfully tested in preclinical settings and currently under investigation in clinical trials.

Although considerable strides have been made in the design and manufacturing of LNP formulated mRNA-based therapies, to leverage the full potential of mRNA technology it is still needed to improve the transfection and targeting efficiency of LNPs and to increase the translatability of mRNA molecules by the engineering of the RNA sequence.

As discussed throughout this chapter, for its adequate activity, eukaryotic mRNA requires five structural elements, the cap structure, poly(A) tail, protein-coding sequence, and 5' and 3' UTRs. These elements are pivotal in regulating translation initiation, translation termination, stability, decapping, and post-transcriptional

modifications of mRNA. Thus, sequence optimization of IVT-mRNA can maximize the expression of the therapeutic protein *in vivo*.

In addition, alternative forms of RNA such as saRNA and most recently circular RNA (circRNA) have been proposed to enhance mRNA properties (Holdt et al. 2018; Wesselhoeft et al. 2019). circRNA is particularly appealing since, unlike linear RNA, it has no 5' cap structure and poly(A) tail, and IRES sequences are harnessed to ensure maximum protein synthesis. Given that circRNA lacks free 5' end cap and a 3' poly(A) tail, this kind of RNA resists to exonuclease digestion and, therefore, has a longer half-life than conventional linear mRNA. Thus, the use of circRNA may further revolutionize the mRNA field in the coming years.

From the formulation standpoint, despite the encouraging results from passive targeting approaches by modulating physicochemical properties of LNPs, like charge and size, the ability of LNP to reach certain sites in the body or specific cell populations within organs with not relevant or absent off-target accumulation needs to be significantly improved. Indeed, it is clear from studies performed in small animal models that current LNP formulations generally suffer from low specificity, with the tendency to be sequestered by the reticuloendothelial system (RES) of the liver and spleen, or accumulate in the first draining organs (e.g., lungs) after intravenous administration. In addition, if compared to their viral counterpart, LNP-based non-viral vectors usually exhibit much lower transfection efficiencies.

The transfection efficiency of LNPs could be potentially improved, for example, by the rational design of ionizable lipids with optimized head groups and hydrophobic tails so to increase their ability to promote endosomal escape upon internalization by target cells. The incorporation of well-defined helper lipids into the formulation can also play a crucial role in enhancing the overall transfection efficiency of LNPs. Furthermore, hybrid delivery systems including, for instance, pH-responsive polymers (e.g., β -amino ester), or molecules that are known to enhance mRNA delivery by altering the endocytic pathway, can further enhance the endosomal escape of mRNA.

Selectivity of LNP formulated mRNAs can be improved by modulating the structure of the single lipids included in the formulation and the overall lipid composition of LNPs. For instance, modification of the alkyl length of ionizable lipids leads selective accumulation of mRNA-loaded LNPs in the liver or spleen (Fenton et al. 2018). In another study, the impact of cholesterol derivatives on cell selectivity of LNPs was investigated. The results of this study demonstrated that the tropism of LNPs in liver endothelial cells, Kupffer cells, and hepatocytes strictly depends on cholesterol structures (Patel et al. 2020).

Finally, biodegradability and immunogenicity are important aspects that need to be considered throughout the design of novel lipid components. Indeed, biodegradability can promote fast elimination of the LNP components, thus minimizing any potential toxicity effect. mRNA-based medicines can either benefit from the intrinsic immunogenicity of lipids, especially in the case of anti-cancer vaccines, or this immunogenicity can be detrimental, by altering mRNA translation and/or causing undesirable adverse effects.

In summary, mRNA platforms are suitable for the treatment of a wide variety of pathologies since they allow the development of any protein-based therapy. However, despite the great therapeutic potential confirmed in a number of clinical trials with diverse applications, mRNA-loaded LNPs could still benefit from further studies aimed at improving the selectivity, transfection efficiency, and toxicity of LNP formulations and increase mRNA stability and translatability. All this together can ensure the development of next generation of mRNA-based therapies with superior therapeutic properties.

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