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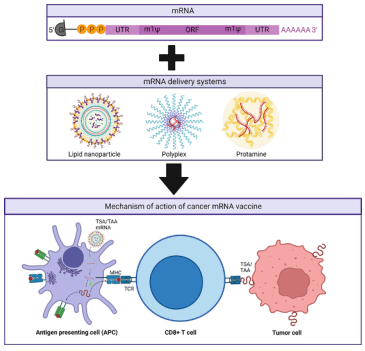
mRNA delivery systems for cancer immunotherapy: Lipid nanoparticles and beyond

Mariona Estapé Senti, Lucía García del Valle, Raymond M. Schiffelers *

CDL Research, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, the Netherlands

GRAPHICAL ABSTRACT



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ABSTRACT

mRNA-based vaccines are emerging as a promising alternative to standard cancer treatments and the conventional vaccines. Moreover, the FDA-approval of three nucleic acid based therapeutics (Onpattro, BNT162b2 and mRNA-1273) has further increased the interest and trust on this type of therapeutics. In order to achieve a significant therapeutic efficacy, the mRNA needs from a drug delivery system. In the last years, several delivery platforms have been explored, being the lipid nanoparticles (LNPs) the most well characterized and studied. A better understanding on how mRNA-based therapeutics operate (both the mRNA itself and the drug delivery system) will help to further improve their efficacy and safety.

In this review, we will provide an overview of what mRNA cancer vaccines are and their mode of action and we will highlight the advantages and challenges of the different delivery platforms that are under investigation.

1. Introduction

Cancer is a major cause of death globally [1,2]. Immunotherapies are novel and promising therapeutic strategies that aim to activate or boost the immune system in order to eliminate cancer cells [3,4,5]. They intend to overcome the limitations of chemo and radiotherapy, the standard procedures used against cancer together with surgery. These limitations include associated off-target effects and the resistance that

* Corresponding author.
E-mail address: r.schiffelers@umcutrecht.nl (R.M. Schiffelers).

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some tumor cells build up [5,6]. The entry into the clinics of checkpoint inhibitors (CPIs) [7] and also of chimeric antigen receptor (CAR) T cells [8,9,10] are examples of how immunotherapies can improve cancer treatment.

This review focuses on cancer vaccines as immunotherapy (Table 1). The administration of a cancer vaccine triggers an endogenous immune response which can either potentially eradicate existing tumors or induce long-term immunologic memory which might play prophylactic roles, preventing cancer formation, metastasis and relapses [11,12,13]. Most efforts and clinical trials have focused on developing therapeutic vaccines. However, they have shown a limited clinical success that may have been hindered by the immunosuppressive tumor microenvironment (TME). Therefore, preventive vaccines, which do its function before the establishment of the immunosuppressive TME, could be a promising option against cancer. Nonetheless, their development has been partially stopped by the difficulty to find shared antigens [5,14].

Vaccine-induced immunity against cancer is the result of the activation of cellular immune responses. These responses are characterized by the induction of activated CD8 + cytotoxic T lymphocytes (CTLs), which have the potential to eliminate altered cells, playing a key role on cancer treatment. CTLs are elicited against antigens loaded onto the major histocompatibility class I (MHC-I) complexes of antigen-presenting cells (APCs) (Fig. 1 and Fig. 2). To specifically target CTLs against cancer cells, cancer vaccines produce APCs bearing tumor-associated or tumor-specific antigens (TAA and TSA, respectively) [5,13,15–18].

TAAs are proteins that can be also found expressed in lower levels in other cell types, meaning that they would have a certain degree of immune tolerance. Oppositely, TSAs, also known as neoantigens, are antigens that are usually the result of genomic mutations, being exclusively expressed in tumor cells. TSAs are hence more specific for cancer treatment [13,19,20]. Nonetheless, similar as genomic mutations, the majority of neoantigens are specific to each patient. Neoantigens can therefore be used for generating personalized vaccines. For example, mutations detected in biopsies by next-generation sequencing are used to obtain neoantigens, which have shown better outcomes than TAAs in

Table 1
This table provides a comprehensive overview of key considerations when designing an mRNA cancer vaccine.

Principles of vaccine-induced immunity against cancer	Vaccine-induced immunity against cancer is the result of the activation of cellular immune responses. These responses are characterized by the induction of activated CD8+ cytotoxic T lymphocytes (CTLs), which have the potential to eliminate altered cells. CTLs are elicited against antigens loaded onto the MHC-I complexes of antigen presenting cells (APCs). To specifically target CTLs against cancer cells, cancer vaccines produce APCs bearing tumor-associated or tumor-specific antigens (TAA and TSA).
mRNA structure	 1. 5'cap with a 7-methylaguanosine (m^7G) 2. 5' untranslated region (UTR) 3. Open reading frame (ORF) 4. 3' untranslated region (UTR) 5. Poly(A) tail
mRNA synthesis	1. *In vitro* transcription (IVT) with a phage RNA polymerase (Sp6 or T7). 2. Treatment with DNase to get rid of the DNA template. 3. Enzymatic or chemical capping of the mRNA. 4. mRNA purification to get rid of reaction components, abortive and aberrant RNAs. There are different purification methods: a. Lithium chloride precipitation (used at laboratory scale) b. Size exclusion chromatography c. High-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) → lead to higher purity levels of mRNA 5. Sterile filtration 6. Storage in a storage buffer
Strategies to improve mRNA pharmacology	1. Use of synthetic cap analogues (CleanCap®) 2. Addition of regulatory sequences to the 5' and 3'-UTRs (e.g. 3'-UTRs of β- and α-globin mRNAs, 5'-UTR of the internal ribosomal entry sites) 3. Codon optimization 4. G:C content enrichment 5. A poly(A) tail of between 100 and 300 nucleotides 6. Use of modified nucleosides 7. Use of highly pure mRNAs, obtained by FPLC or HPLC 8. Use of adjuvants like the TLR 4 agonist monophosphoryl lipid A (MPLA) or the α-galactosylceramide (α-GalCer) to enhance the immunological response needed for DC maturation 9. RNA circularization 10. Fusion of the antigen mRNA with the C3d mRNA
Drug delivery systems	1. Lipid-based a. Ionizable lipid i. Crucial for driving cellular intake, endosomal escape and tolerability ii. Most of the ionizable lipid optimizations for cancer vaccines have focused in maximizing antigen-specific CTL responses b. Non-cationic (phospho)lipids i. Contribute to the LNP stability and fusogenicity c. Cholesterol as neutral helper lipid i. Contributes to the LNP stability d. Polyethylene glycol (PEG)-lipids or other stabilizing polymer-lipid conjugate i. Increase the LNP colloidal stability, reducing particle aggregation during both circulation and storage ii. Reduce the protein interaction with the particles, protecting them against opsonization and against clearance by the reticuloendothelial system (RES) iii. Downsides of PEGylated nanoparticles: 1. Accelerated blood clearance (ABC) upon repeated administrations Several strategies are being proposed to reduce it 2. Complement activation-related pseudoallergy (CARPA) reactions 2. Polymeric nanoparticles → PBAE, PLGA, HTA and charge-altering releasable transporters (CARTs) made of degradable oligo(carbonate-b-α-amino esters) among others 3. Peptide-based nanoparticles → cell-penetrating peptides (CPPs) and protamines 4. Inorganic nanoparticles → gold nanoparticles and mesoporous silica nanoparticles among others 5. Exosomes

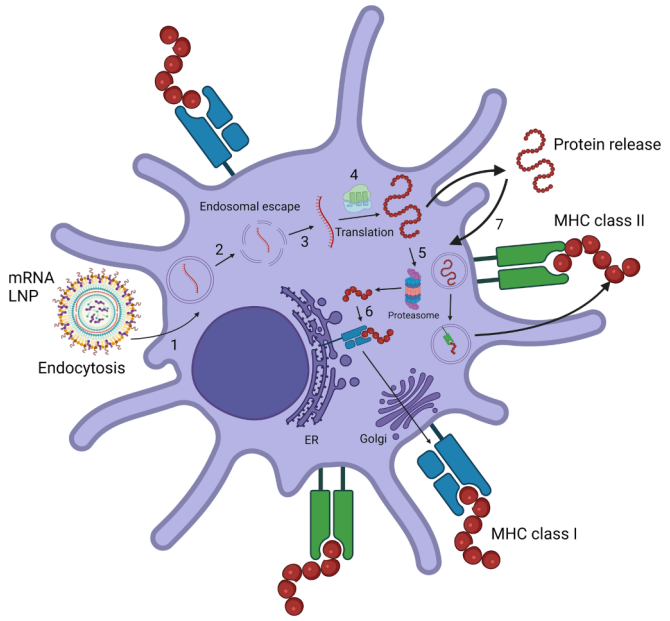

Fig. 1. Intracellular trafficking of antigen-encoding mRNAs. Lipid nanoparticle (LNP)-encapsulated mRNAs are endocytosed (1) and afterwards, trafficked through the endosomal pathways (2). Some mRNA molecules escape the endosome (3) and are then translated in the cytosol by the protein synthesis machinery of the cell (4). Subsequently, the translated protein can undergo post-translational modifications and act via autocrine, endocrine or paracrine mechanisms. For immunotherapeutic purposes, the protein has to be degraded into antigenic peptide epitopes. These peptides then have to be loaded onto major histocompatibility complexes (MHC) in order to be presented to the immune effector cells. There are two types of MHC molecules: class I and class II. While MHC-I molecules are expressed by almost all cells, MHC-II molecules are only expressed by antigen presenting cells. If the protein is degraded by the proteasome in the cytoplasm (5), the resulting peptides will be routed to the endoplasmic reticulum (ER) where they will be loaded on MHC-I molecules (6). Usually, intracellular proteins do not get loaded on MHC-II molecules. Nonetheless, to direct them to the MHC-II processing pathway, a routing signal to the extracellular space can be introduced into the sequence (7).

cancer applications [19–21]. Some personalized vaccines targeting neoantigens are already being evaluated in clinical trials [20].

APCs play a central role in immunity, orchestrating innate and adaptive immune responses through several processes such as antigen presentation. Through MHC-I, APCs can induce CTL responses, whereas through MHC-II, CD4 + T cell responses are regulated, initiating also humoral responses [5,13,19,22] (Fig. 2). Among all APCs, dendritic cells (DCs) are the most relevant ones for cancer vaccinology, as they are the most potent inducers of CTLs [16,23,24]. Apart from uptake of the antigen that will be presented, DCs need to undergo a maturation process to facilitate T cell priming; without such activation, DCs induce tolerance. Activation naturally occurs in response to pathogen-associated molecular patterns (PAMPs), local damage or strong ongoing inflammation. DC activation results in DC migration to the lymph nodes, where they will encounter the native T cells. Moreover, overexpression of costimulatory molecules such as CD80 and CD86 in mature DCs facilitates optimal T cell priming, ensuring CTL responses towards the specific antigen present on the mature DCs [24,25]. Another strategy that researchers are exploring is the use of pro-inflammatory immunomodulators (cytokines and co-stimulatory ligands) to switch the immunosuppressive tumor microenvironment to a pro-inflammatory state [5,26]. For instance, Hewitt and colleagues achieved a 85 % complete response in immunosuppressive colon carcinoma models when combining a triplet pro-inflammatory cocktail (IL-23, IL-36γ and OX40L) with a CPI such as anti-PD-L1 [27]. In another study, Hewitt et al. developed an LNP-encapsulated IL-12 mRNA therapy and administered it intratumorally. They reported that a single administration of this therapeutic leads to tumor regression in several syngeneic mouse models. This tumor regression was caused by a transformation of the tumor microenvironment to a proinflammatory state characterized by both interferon (IFN) γ and $CD8^+$ T cells. Moreover, the animals with a complete tumor regression showed immunity to rechallenge [28]. Sayouret et al. developed a nanoparticle-encapsulated mRNA cancer vaccine to enhance the intratumoral and systemic immune responses in an immune-resistant tumor model. This therapeutic consisted of DOTAP liposomes loaded with untargeted tumor derived RNA (derived from the entire transcriptome). When combined with an immune checkpoint inhibitor (anti-PD-L1 monoclonal antibody), it had a synergistic effect and it further increased the antitumor activity [29].

Success of cancer vaccines requires the delivery of the TSA or TAA into the cytoplasm of DCs, where it would be processed by the proteasome and attached to MHC-I [22]. To achieve this, several types of cancer vaccines have been studied: I) the administration of tumor cell lysates; II) DCs vaccines in which DCs are *ex-vivo* modified; or III) antigen *in vivo* delivery to DCs [5]. This last option of *in vivo* delivery is the most feasible approach to reach the clinics due to its easy and fast manufacture and off-the-shelf availability.

Protein antigens may either be delivered as proteins themselves or as a nucleic acid encoding for such protein. Nucleic acids are usually preferred over proteins as antigen delivery form. This is because proteins exhibit lower tissue penetration and a shorter therapeutic effect than nucleic acids. Moreover, proteins are likely to induce stronger immune reactions which entail safety issues. Finally, by using nucleic acids, several antigens can be readily expressed simultaneously, enhancing the potency of these vaccines [13].

The use of mRNA for vaccinology presents several advantages over DNA. The risk of genomic integration associated to DNA vaccines poses a safety problem for their further development. The use of antigen-encoding mRNA represents a safer alternative since it does not integrate into the host genome and is only transiently translated into the encoded antigen [22]. It has been reported that a more controlled antigen exposure reduces undesirable effects such as the induction of tolerance by T cell exhaustion [30,31]. Besides an improved safety, mRNA vaccines present other advantages when compared to DNA vaccines: I) RNA translation machinery is located in the cytoplasm, meaning that mRNA vaccines do not need to be translocated into the nucleus to be functional; II) expression of the desired antigen occurs in a faster and more controlled manner; and III) co-expression of several antigens is easy to perform, increasing the vaccine efficiency as multiple targets can be treated at once [11,13,22]. Given these reasons, vaccine development has increasingly been focused on mRNA-based cancer vaccines, which is also the focus of this review. Moreover, the successful clinical use of two mRNA-based vaccines against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [32–34], has boosted even more the interest on using mRNA as a therapy against other infectious diseases, such as the human immunodeficiency virus (HIV) [35,36], and cancer [37] among others.

Several attempts have been made in order to deliver naked mRNA. However, its low efficiency has driven the development of different delivery platforms, which are crucial to ensure that the mRNA gets delivered to the site of action with minimal toxicity and off-target effects. Different materials can be used and combined for delivering RNA, creating a complicated field that is under investigation [5,38–40].

Here, the factors that need to be considered when designing mRNA-cancer vaccines, such as the use of mRNA modifications or the role of type I interferon responses on the functionality of the vaccines, will be discussed. Moreover, the different delivery platforms that are being used to deliver mRNA will be reviewed. We will dig into their characteristics, advantages and limitations.

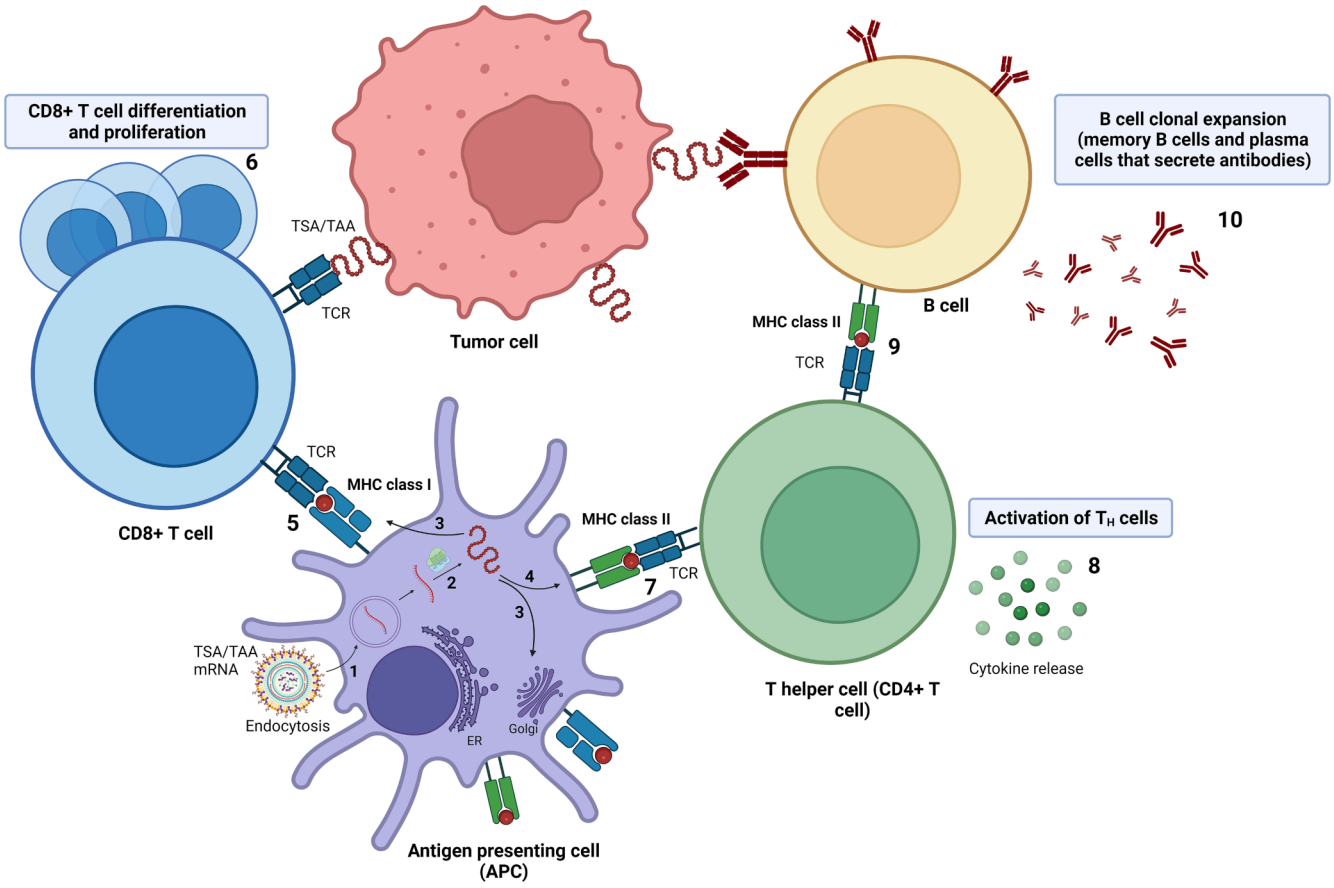

Fig. 2. Pharmacology of antigen-encoding mRNAs. The mRNA of a tumor-associated or tumor-specific antigen (TAA/TSA) is delivered to an antigen-presenting cell (APC) (1). The tumor antigen is then expressed in the APC (2) and it can either be loaded on an MHC-I (3) or an MHC-II molecule (4). If loaded on an MHC-I molecule, the epitopes are presented to CD8 + T cells (5) which then, proliferate and differentiate into CD8 + cytotoxic T lymphocytes (CTL) (6). If loaded on an MHC-II molecule, the epitopes are presented to CD4 + T cells (T helper (T_H) cells) (7) that then, activate, release cytokines (8) and activate B cells (9). The B cell activation results in the generation of memory B cells and plasma cells that produce and secrete antibodies against the TAA/TSA (10).

2. mRNA-based cancer vaccines

2.1. mRNA structure and synthesis

The study of mRNA as cancer vaccines started when Conry et al. found that intramuscular administration of carcinoembryonic antigen mRNA confers protective antitumor immunity [41]. Since then, several preclinical and clinical studies have shown the potential of mRNA-based cancer vaccines as an immunotherapy against cancer [37 42].

Two types of RNAs, self-amplifying RNA and non-replicating mRNA, have been developed into vaccines. In this review, we will focus on conventional non-replicating mRNA vaccines, where the antigen encoding sequence, also known as open reading frame (ORF), is flanked by 5′ and 3′ untranslated regions (UTRs), capped with a 5′ cap and polyadenylated. The mRNA cap is a 7-methylaguanosine (m^7G), that is linked to the first nucleotide via a 5′, 5′-triphosphate bridge [22]. This structure binds to both the translation initiation factor 4E [43] and the decapping enzymes (DCP1/DCP2/DCPS) [44]. Therefore, it is essential for both an efficient translation and to regulate the mRNA degradation. The UTRs, 5′ cap and poly(A) tail give stability to the mRNA and increase protein translation (Fig. 3). Once in the cytoplasm, the cellular translation machinery will translate the mRNA into a protein that lately, will go through post-translational modifications until becoming a functional protein. The mRNA will eventually be degraded using the regular cellular processes, what lowers the metabolite toxicity risk [22,45].

In vitro transcribed (IVT) mRNAs are produced using a linear DNA as template that is transcribed into mRNA using a phage RNA polymerase, such as Sp6 or T7. Afterwards, in order to get rid of the template DNA, an incubation with DNase is performed. Eventually, the mRNA is chemically or enzymatically capped. After the synthesis procedure, the mRNA is purified in order to get rid of the reaction components (residual DNA, enzymes or free nucleotides), abortive (shorter) or aberrant (longer) RNAs [46]. There are different RNA purification procedures that lead to different RNA purities. Lithium chloride precipitation is a purification method that is widely used at laboratory scale. At a clinical stage, to ensure good manufacturing practices (GMP), size-exclusion chromatographic methods, that separate mRNAs according to size, are preferred. Methods, such as high-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC), are the ones that lead to higher purity levels of the mRNA since they can efficiently eliminate double-stranded RNAs (dsRNAs) [47 11]. Once purified, the mRNA is sterile-filtered and stored in a storage buffer. The relative simplicity, rapidity, scalability and high yields of IVT reactions, together with the possibility to use GMP grade materials, make this type of therapeutics suitable for a clinic application [48 11].

Different strategies are employed to improve the mRNA pharmacology: use of capping enzymes and synthetic cap analogues, optimization of the poly(A) tail length, codon optimization, G:C content enrichment, use of modified nucleosides and use of highly pure mRNAs, obtained by FPLC or HPLC. The size of the poly(A) tail has been found to be critical to balance the synthetic capability of mRNAs. A poly(A) tail of between 100 and 300 nucleotides has been reported to be optimal [49–51]. Furthermore, it has been previously reported that the triphosphate group at the 5′ end of uncapped mRNA can activate the

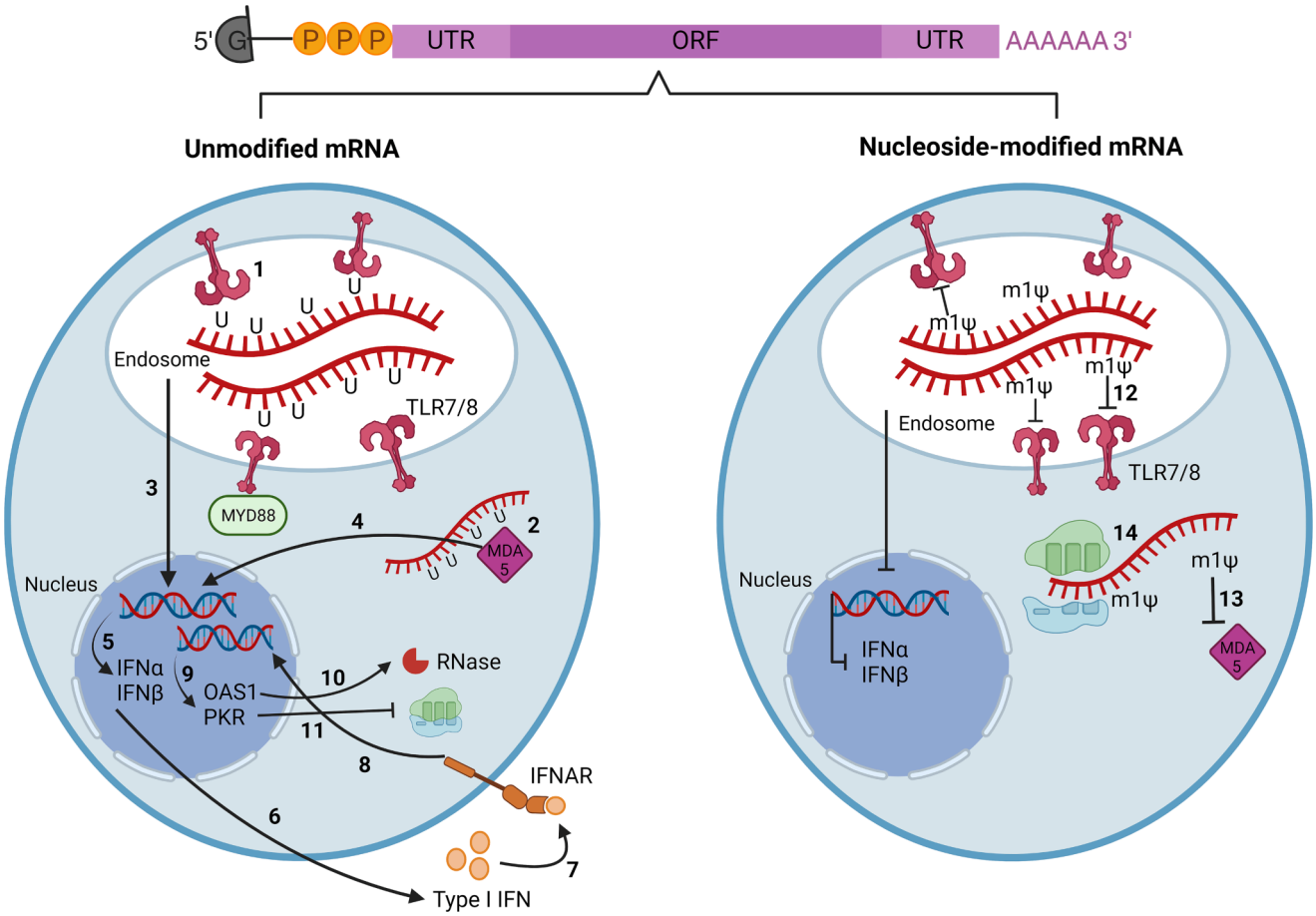

Fig. 3. Immune sensing of mRNA. The *in vitro* transcribed mRNA consists of a 5′cap, 5′ and 3′ untranslated regions (UTRs), an open reading frame (ORF) and a poly (A) tail. Unmodified mRNA is sensed by either the toll-like receptors (TLRs) in the endosome (1) or by cytosolic sensors such as Ror MDA-5 (interferon-induced helicase C domain-containing protein 1) (2). This recognition elicits a type I interferon (IFN) response (3, 4, 5, 6 and 7) that upregulates the expression and activation of 2′-5′-oligoadenylate synthetase (OAS) and protein kinase R (PKR) (8 and 9) among others, what leads to the mRNA degradation (10) and the inhibition of translation respectively (11). With modified nucleosides (such as 1-methylpseudouridine (m1Ψ)), the RNA is not recognised by the sensors (12 and 13) and it can be successfully translated into the encoded protein (14).

immune system through cytosolic RNA sensors [52,53]. Capping the triphosphate group at the 5′ end can reduce this immune activation. Two mRNA capping strategies have been reported: through a two-step multi-enzymatic reaction or co-transcriptionally, in which the cap analogue is added into the *in vitro* transcription reaction together with the DNA plasmid, the nucleotides and the RNA polymerase. The use of cap analogues reduces the amount of manufacture and purification steps and the number of enzymes needed, reducing therefore, the complexity and cost of the procedure [54]. The most popular cap analogues are the anti-reverse cap analogues (ARCAs) modified within the ribose moiety of the m^7G [55]. In the last couple of years, a 5′ cap analogue from TriLink BioTechnologies, named CleanCap®, was also developed. This analogue leads to a better capping efficacy, of around a 95 %, than the first-generation cap analogues, such as ARCA or mCap. Since the capping efficiency is not absolute, a phosphatase treatment can be performed to eliminate the remaining 5′ triphosphates [46]. Moreover, certain regulatory sequences can also be added to the 5′ and 3′-UTRs in order to regulate the stability and translation of mRNA. An example of this are the 3′-UTRs of β- and α-globin mRNAs, which have proven to be crucial to have an mRNA half-life longer than 1 day [56,57]. Apart from the globin UTRs, other UTRs, such as the 5′-UTR of the internal ribosomal entry sites and the human heat shock protein 70, have also been found to increase the stability and translation of the mRNA [58,59]. It has also been reported that a single codon substitution could have a great effect on protein folding and expression. Mauger et al. observed that the secondary structure of mRNA can modulate protein expression by modifying the half-life of mRNA translation [60]. These approaches stabilize the mRNA and/or decrease the immunogenicity, increasing protein translation.

2.2. *Immunogenicity of IVT mRNA*

Exogenous mRNA triggers an immune response in a similar manner as viral RNA does [22]. It occurs through toll-like receptors (TLRs) 7 and 8 – expressed in the endosomal compartment of APCs [61,62] – and other cytosolic sensors such the melanoma differentiation-associated protein 5 (MDA5) [63,64]. The activation of these receptors induces an immunological cascade that ultimately leads to the expression of type I interferon genes, particularly IFN-α and IFN-β, together with certain pro-inflammatory cytokines (e.g. TNF-α, IL6 and IL12) [65 22]. Type I IFN genes activate antiviral effectors such as, RNA endonuclease L (RNAseL), ADAR, protein kinase R (PKR) or 2′-5′-oligoadenylate synthetase (OAS), which either degrade the mRNA or block its translation [22,66,67] (Fig. 3).

This cocktail of immune effects could either be positive or negative in the context of generating antitumor immunity. It limits the longevity and expression of the mRNA but creates proinflammatory cytokines. Several authors have also observed that the type I IFN responses generated upon intravenous administration of mRNA lipoplexes are essential to elicit a robust CTL activation and antitumor effector

functions [68,69]. Furthermore, the entry into the clinics of the RNactive platform, the therapeutic effect of which is based on the induction of type I IFN responses, reinforces the possible beneficial role of type I IFN responses [70–72]. Contrarily to these findings, De Beuckelaer et al. reported that, the type I IFN responses generated upon intranodal, intradermal and subcutaneous administration of mRNA lipoplexes hampered the development of strong cytolytic T cell responses and the further clearance of B16 melanoma tumors. Importantly, the blockage at the site of administration of the type I IFN responses with an IFNAR (IFN α/β receptors) blocking antibody increased CTL responses and the antitumor efficacy against B16 tumors [73]. This discrepancy in the results could be due to the different routes of administration and whether the activation of IFN α/β receptors (INFAR) and T cell receptors (TCR) happens in a simultaneous or non-simultaneous manner. Coordinated activation of both receptors is associated with an induction on T cells of proliferation, differentiation and activation signals. In contrast, when INFAR activation occurs earlier than TCR stimulation, an antiproliferative and proapoptotic state is induced, resulting in a negative outcome for cancer vaccines [74].

Interestingly and contrarily to what happens with lipoplexes, according to Van der Jeught et al., with lipopolyplexes (LPP), a hybrid nanocarrier consisting of a polymeric mRNA nanoparticle covered with a lipidic shell, the type I IFN responses were not essential to elicit an effective T-cell response. Therefore, it seems that lipoplexes and lipopolyplexes have different ways of eliciting T-cell immunity. That allowed to load these nanoparticles with nucleoside-modified mRNA, what lead to enhanced inflammatory safety and mRNA expression without hindering the T cell response [75]. The nucleoside modifications will be further discussed in the next section.

Therefore, depending on the goal, this intrinsic immunogenicity of the mRNA can be beneficial or detrimental. If the purpose is to develop an immunotherapy, this property can be beneficial or even essential since it can significantly increase the vaccine activity. Nonetheless, the toxicity associated to the potent pro-inflammatory activity of type I interferons can limit the dosing. Additionally, if the goal is to develop a therapeutic, this property can severely endanger the protein expression.

2.3. Avoiding the immunogenicity: Nucleoside-modified mRNA and other strategies

In 2005, an important finding changed the history of synthetic mRNA. The group of Katalin Karikó described that the mRNA immunogenicity could be reduced, without compromising the translation capacity, by introducing naturally occurring modifications (N6-methyladenosine (m6A), 5-methylcytidine (m5C), pseudouridine, 2-thiouridine (s2U) and 5-methyluridine (m5U)) [76]. These modifications prevent the recognition by innate immune sensors of exogenously derived mRNA, what decreases the type I IFN response and yields increased levels of protein production compared to unmodified mRNA [77] (Fig. 3). Dendritic cells and other cell types expressing toll-like receptors are heavily activated by mitochondrial and bacterial RNA but not by mammalian RNA, which contains a lot of modified nucleosides. Therefore, the immune system may specifically detect unmodified RNAs as a response to either necrotic tissue or bacteria [76 77]. The mechanism by which for instance, pseudouridines enhance translation of IVT mRNA, is by lowering the degree of activation from the protein kinase R that phosphorylates the translation initiation factor 2-alpha (eIF-2α). It is known that this phosphorylation of eIF-2α represses the mRNA translation [78].

Among all the analysed modifications, the substitution of uridine by 1-methylpseudouridine (m1ψ) was one of the most successful [79]. Pardi et al. reported that, upon LNP-mRNA intradermal administration, the incorporation of m1ψ yields a 20-fold increase in mRNA translation compared to unmodified mRNA [80].

Interestingly, opposed findings have also been reported. Kauffman et al. described no improvement in protein expression or mRNA immunogenicity when LNPs containing pseudouridine modified RNA (modRNA) were intravenously administered to mice and compared to LNPs containing unmodified mRNA [81]. Also note that the modRNA used by Kauffman et al. was not purified by HPLC. There are multiple reasons that could explain the contradictory results between authors: the route of administration, the type of modification used, the lack of standardised procedures for mRNA synthesis and purification, differences in the RNA sequence optimization or the innate immune sensing level of the target cell. For instance, Karikó et al. have showed that the use of highly pure modRNA, obtained by HPLC purification, leads to increased protein expression compared to non-purified modRNA [47]. This is due to the fact that some purification methods are not able to efficiently eliminate contaminants resulting from the *in vitro* transcription, such as dsRNAs, abortive transcripts and aberrant RNAs. dsRNAs are a PAMP since they are the typical shape of viral genomes. dsRNA impurities lead to a type I IFN response that upregulates the PKR and OAS expression, resulting in the inhibition of translation and the mRNA degradation [22]. Therefore, the incorporation of nucleoside modifications and the purification method used to purify the IVT mRNA could be crucial when maximizing protein expression and circumventing an undesired activation of the innate immune system.

There is also no agreement about the type I IFN responses elicited by modRNA and whether they are necessary for the maturation of DC and to elicit robust CTL activation. On one hand, Verbeke et al. reported that, upon intravenous injection, ψ/m5C modRNA fails to activate DCs due to its incapability in triggering a strong type I IFN response, needed for DC maturation. However, this activation can be possible in the presence of an adjuvant [82]. On the other hand, the efficacy of m1ψ modRNA-LNP vaccines against Zika suggests that modRNA could be successfully used in cancer vaccines [83,84]. These successful results with modRNA might indicate that either type I IFNs are not as crucial as observed in some studies or that modRNA does not suppress the type I IFN response as hypothesized.

Researchers are investigating other ways to tweak the RNA immunogenicity and translation. One of these is the co-delivery of modRNA with adjuvants like the TLR 4 agonist monophosphoryl lipid A (MPLA) or α-galactosylceramide (α-GalCer) [82,85]. This strategy may allow both, enhanced antigen expression and the immunological response needed for DC maturation. Another approach that has been found to be effective to increase the *in vivo* protein expression is RNA circularization. Generally, RNA degradation commences by the tail. Since circular RNAs do not have an open end, it allows them to last longer than their linear counterparts. Wesselhoeft et al. discovered that unmodified circular RNA is not recognized by Toll-like receptors in mice, what leads to decreased immunogenicity and prolonged expression [86]. Qu et al. also used circular RNA to develop a vaccine against SARS-CoV-2 that was able to elicit strong T cell responses and neutralizing antibodies that conferenced protection against SARS-CoV-2. Compared to an 1mΨ-modified mRNA vaccine, the circular mRNA led to increased and more sustained antigen expression and antibody production [87]. In a recent study, Yang et al. injected intratumorally a naked circular mRNA that was successfully expressed in cancer cells 6 h after injection. Furthermore, when administering a circular mRNA encoding for a cytokine cocktail, they successfully triggered anti-tumor immune responses [88]. Another tactic that was recently employed by Li et al. to tune the immunogenicity of LNP mRNA vaccines, was the fusion of the antigen mRNA with the C3d mRNA, a protein fragment that originates from the activation of a complement protein known as C3. The C3d fragment, when bound to the C3d receptor on follicular dendritic cells and B cells, increases the antigen presentation on follicular dendritic cells and elicits a potent B cell activation. This strategy was used to developed a SARS-CoV-2 vaccine in which the antigen mRNAs were fused to the C3d mRNA; that led to 10 times more antibodies than its counterpart without the C3d mRNA [89].

mRNA vaccines have a great potential over the classical approaches used against cancer, mainly due to their high potency and safeness

together with an straightforward and rapid manufacture. However, a better understating, of the mRNA innate immune sensing and how to modulate it, is needed to further improve mRNA-based cancer vaccines and take them a step forward from basic research to extended therapeutics against cancer. Moreover, in order to ensure an efficient *in vivo* delivery, the field needs from delivery platforms that will be further discussed.

3. Delivery tools for mRNA cancer vaccines

3.1. General characteristics of delivery platforms

mRNA vaccines are promising therapeutic agents for cancer immunotherapy. Nevertheless, limitations owing to their instability and inefficient delivery have halted their clinical progress. The inherent physicochemical properties of mRNAs hinder its direct administration to patients: they are large, hydrophilic, polyanionic molecules that rarely cross cell membranes. Moreover, mRNAs are rapidly degraded upon delivery due to the abundance of RNases in the extracellular space and in physiological fluids [90].

Delivery systems have been designed to overcome these limitations. The optimal delivery tool should accomplish certain objectives, such as efficiently encapsulate the mRNA, guarantee cargo protection avoiding degradation prior to reaching its target site, and facilitate cellular internalization and once inside the cell, endosomal escape. Ideally, it should also provide spatiotemporal control in order to reduce off-target effects and show minimal toxicity. Finally, the desired delivery platform should have an easy and scalable manufacturing process [38 40 39].

Over the past decades, different delivery platforms, including both viral and non-viral systems, have been developed and evaluated for mRNA cancer vaccinology. In this review, the most prominent non-viral delivery platforms will be discussed (Fig. 4).

3.2. Lipid-based mRNA platforms in cancer vaccinology

Lipid-based nucleic acid delivery tools, including lipoplexes and lipid nanoparticles (LNPs), are the most clinically advanced platforms for mRNA delivery. Historically, lipoplexes and LNPs were developed for DNA and siRNA delivery, respectively [91,92]. Due to an increasing interest in the therapeutic use of mRNA, lipid-based delivery technologies were also optimized as a delivery platform for mRNA [93].

Lipid-based mRNA systems are typically composed of: I) a cationic (lipoplex) or ionizable lipid; II) a non-cationic (phospho)lipid; III) a cholesterol derivate and IV) a lipid that prevents aggregation (stabilizing agent, e.g. PEG-lipid conjugate) [90]. Rapid mixing of an organic (ethanol) phase, which contains the lipidic compounds, and an aqueous phase, which contains the mRNA, enables efficient complexation of mRNA with cationic or ionizable lipids, yielding high mRNA encapsulation efficiencies [38,94]. Resulting lipoplexes and LNPs are characterized by concentric multilamellar or electro-dense solid core structures, respectively [95].

Several mRNA LNPs/lipoplexes are currently under investigation in clinical trials, with the goal of achieving either intratumoral expression of immunostimulatory cytokines or a vaccination effect (either therapeutic or prophylactic). Some examples are mRNA-4157 [96], BNT112 [97], BNT113 [98], mRNA-2752 [99] and MEDI1191 [100]. The clinical trial evaluating the combination of mRNA-4157 and anti-programmed cell death protein 1 (PD-1) has demonstrated remarkable success in reducing the distant metastases and death by 65 % compared to treatment with PD-1 alone [101]. Another successful approach involving LNPs are CAR antigen vaccines (CARVac) [102]. In a clinical trial conducted by BioNTech, a combination of CAR-T therapy targeting the CLDN6 antigen and an mRNA vaccine (CARVac) encoding for the CLDN6 antigen was utilized for the treatment of solid tumors. The findings of the study revealed that the mRNA vaccine significantly enhanced the antitumor efficacy of CAR-T cells. Additionally, the combined treatment demonstrated a favourable safety profile [102]. The ongoing clinical trials on mRNA vaccines for cancer treatment have been extensively reviewed elsewhere [37].

3.2.1. Cationic or ionizable lipids or lipid-like materials

The amino lipid has been shown crucial for driving cellular intake, endosomal escape and tolerability. By changing the molecular structure of this component, the efficiency and tolerability of the LNP is modulated. Optimization approaches have therefore focused mostly on modifying this component.

Initially, permanently positively charged lipids, such as 1,2-di-O-octadecenyl-3-trimethylammonium-propane (DOTMA) or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), were used. These lipids contain alkylated quaternary ammonium groups, constantly retaining their cationic nature. The electrostatic interactions of the phosphate mRNA backbone groups with the positively charged lipids facilitates the mRNA encapsulation. Moreover, the cationic lipids facilitate the efficient delivery and endosomal escape via the electrostatic interactions with the negatively charged cell and endosomal membranes [103]. However, these lipids have also been associated with toxic effects like the disruption of the cellular and nuclear membranes [104] and the generation of reactive oxygen species [105].

With the purpose of overcoming the cytotoxicity associated with permanently cationic lipids, ionizable lipids were developed. These lipids are characterized by the presence of tertiary amines on the head groups that are sensitive to pH, showing a pH-dependent protonation [90,94,103]. Under physiological conditions, the amine groups remain neutral. When the pH becomes acidic, the amine groups become protonated, acquiring a positive net charge. This characteristic facilitates mRNA encapsulation in acidic environments and reduces the toxicity associated to a cationic state during circulation as lipids remain uncharged [90,94,103]. Additionally, once inside the acidic endosomal compartment, protonation of the ionisable lipids is thought to contribute to endosomal membrane destabilization and disruption [90,103].

Onpattro, from Alnylam Pharmaceuticals, is an siRNA-carrying LNP that was approved in 2018 for the treatment of transthyretin induced amyloidosis (hATTR) [92]. (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA), the ionizable lipid used in the Onpattro formulation, was identified from a library of 56 ionizable lipids in which they systematically varied the head group and kept constant the hydrophobic dilinoleyl tail. In this study, they identified the pK_a value as a crucial characteristic of ionizable lipids for achieving a high transfection efficiency. The optimal apparent pK_a was found to be between 6.2 and 6.5 [106]. In a similar study, Whitehead et al. synthesized 1400 degradable lipidoids and used them to deliver siRNA *in vivo* upon intravenous administration. Based on the knockdown *in vivo* efficacy data, they looked for correlations between structure and *in vivo* transfection potency. They could identify four different parameters that strongly predict *in vivo* siRNA delivery potency: there should be at least 3 alkyl chains of 13 carbons, one tertiary amine and the pKa value of the LNP should be at least 5.4. From these four parameters, pKa remained to be the most crucial. It was the sole criteria that, when not met, lead to inefficient gene silencing [107].

Regarding mRNA, the most clinically advanced ionisable lipids are ALC-0315 and SM-102, from the BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna) COVID-19 vaccines respectively [108]. The lipid SM-102 came from a screening performed at Moderna in which they screened 30 different biodegradable ionizable lipids for expression, immunogenicity and tolerability upon intramuscular administration [109]. With the purpose of identifying an LNP formulation for chronic dosing, Moderna also developed and screened several amino lipids aimed to improve the efficiency and tolerability when compared to MC3. As a result of this screening, they identified “lipid 5”, which showed reduced toxicity and enhanced protein expression in comparison to MC3, when injected intravenously to rodents and non-human primates. The enhanced protein levels in both liver and spleen might be a

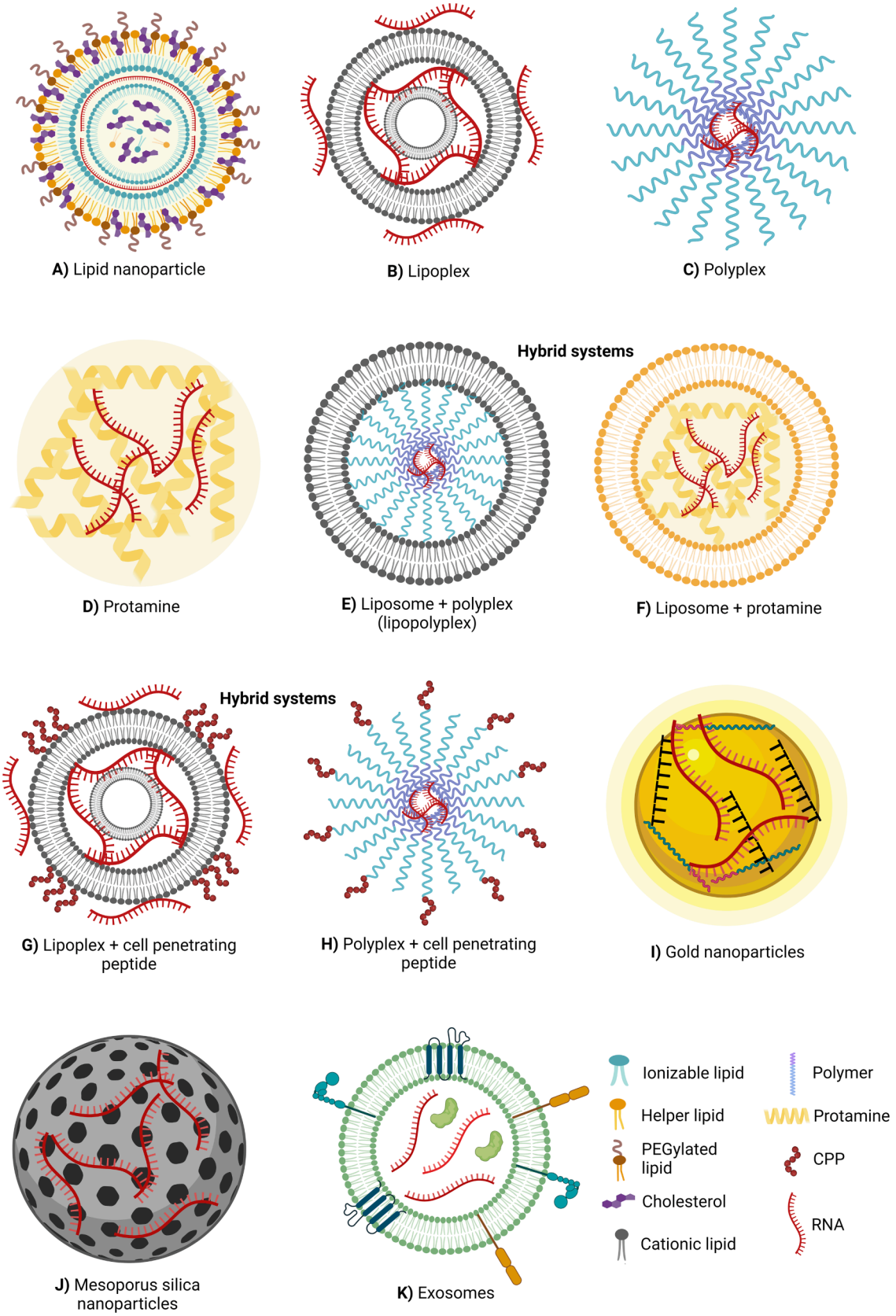

Fig. 4. Main mRNA delivery systems for cancer immunotherapy. There are lipid-based (A and B), polymer-based (C), peptide-based (D and cell-penetrating peptides (CPPs)), inorganic (I and J) and naturally-derived mRNA delivery systems (K). Moreover, there are also multiple hybrid systems (E, F, G and H).

consequence of the replacement of the linoleic tail with a primary ester-containing lipid tail, that enhances endosomal escape [110]. Moreover, lipid 5/DSPC/chol/C14-PEG2000 (50:10:38.5:1.5) LNPs could efficiently co-deliver OX40L, IL-23 and IL-26γ m1ψ modRNAs. When administered intratumorally in combination with checkpoint inhibitors (like anti-PD-L1), it achieved great anti-tumor responses in MC38 carcinoma models with an immunosuppressive TME [27].

Kauffman et al. optimized a C12-200 based formulation, that had previously been used to deliver siRNA, to efficiently deliver mRNA. They tunned the C12-200:mRNA weight ratio, the lipid ratios and the helper lipid used. They could achieve 7 times higher potency than the formulation previously used to deliver siRNA [111]. Oberli and colleagues optimized an LNP formulation in order to maximize antigen-specific CTL responses upon subcutaneous injections. They loaded the LNPs with the mRNAs of two tumor-associated antigens (gp100 and TRP2) and they achieved great anti-tumor efficacy in the B16F10 melanoma model [112].

Later, Miao et al. designed a library of ionizable lipid-like materials in order to deliver mRNA vaccines against different *in vivo* tumor models. From the 1080 tested lipids, LNPs containing the A18 heterocyclic lipid showed higher levels of antigen translation than MC3. Moreover, the heterocyclic head group of A18 induced robust CTLs via STING-dependent type I IFN responses, what lead to reduced tumor size and improved overall survival when compared to other delivery tools, such as MC3 LNPs or RNactive [18].

The exact mechanism by which LNPs mediate uptake is not yet fully understood. There have been studies showing that the surface charge of the LNPs in blood and therefore, the surface composition, affects the protein corona composition and consequently, the uptake mechanism of the nanoparticles [113]. Akinc et al. unravelled the mechanism by which siRNA-loaded Dlin-KC2-DMA LNPs mediate the efficient delivery of siRNA to hepatocytes *in vivo*. They found out that *in vivo,* the apolipoprotein E (ApoE) found in the plasma binds to this type of LNPs, acting as an endogenous targeting ligand for the low-density lipoprotein receptor (LDLR) found in hepatocytes [114]. In order to achieve extrahepatic delivery upon intravenous administration, Cheng et al. developed a strategy named selective organ targeting (SORT) in which to the classic four LNP constituents, they added an additional component (called SORT molecule). The classic 4-component LNP, which had an almost neutral surface charge, led to almost an exclusive delivery to the liver. To achieve lung delivery, the SORT molecule of choice was DOTAP, a permanently positively charged lipid. For splenic delivery, they incorporated to the LNPs 1,2-dioleoyl-*sn*-glycero-3-phosphate (18PA), a negatively charged lipid [115]. In a later paper, they tried to unravel the mechanism by which SORT LNPs can achieve extrahepatic delivery. Similarly to what other authors had previously found for Dlin-KC2-DMA LNPs, they discovered that the charge of the added SORT molecule, regulates the apparent pKa and therefore, the interaction with serum proteins and ultimately, the biodistribution [116]. The mechanism by which LNPs mediate endosomal escape is also not completely understood. The proposed mode of action is that upon endosomal maturation, the pH drops below the pKa of the ionizable lipid, what leads to its protonation and the disruption of the endosome upon the interaction with the negatively charged lipids of the endosomal membrane [117]. According to Hafez et al., when charged, ionizable lipids interact with the anionic lipids of the endosomal membrane, promoting an hexagonal inverted phase and the formation of non-bilayer structures that lead to the disruption of the endosomal membrane [118].

Another aspect that is being debated is the adjuvant activity of the ionizable lipid. From the vaccine point of view, the potential adjuvant effect of the ionizable lipid could enhance the vaccine efficacy. Nonetheless, from the protein replacement point of view, this could be less favourable especially if several doses are needed. For instance, Alameh et al., by comparing LNPs with and without ionizable lipid, found out that a proprietary ionizable lipid from Acuitas Therapeutics, has an intrinsic adjuvant activity. It induces robust cellular and humoral immune responses that lead to the production of protective and durable antibodies against the antigen in the vaccine. They also claim that the adjuvant activity is not mediated by toll-like receptors but by the production of IL-6. Moreover, when using DOTAP LNPs instead of the proprietary ionizable lipid, this adjuvant activity was lost, indicating that it is most likely lipid related [119]. With the purpose of enhancing the immunogenicity of SARS-CoV-2 mRNA vaccines, Han et al. partially substituted the ionizable lipidoid by an adjuvant lipidoid based on a TLR7/8 agonist. With this modification, they achieved increased transfection as well as an enhanced innate immunity that led to improved cellular and humoral responses [120]. In a recent paper, Li et al. also improved the immunogenicity of SARS-CoV-2 mRNA LNP vaccines by adjuvating the ionizable lipid with cyclic amines [89]. Nonetheless, for therapeutic nucleic acid treatments, the immunogenicity of the ionizable lipid may not be as interesting. For instance, in a paper of Kenjo et al., they aimed to develop an LNP formulation with sufficient low immunogenicity to repeatedly deliver the CRISPR-Cas9 mRNA/sgRNA system into the skeletal muscle tissue to do genome editing therapy for Duchenne muscular dystrophy [121].

Furthermore, lipid nanoparticles can be combined with cell–specific surface ligands or adjuvants in order to enhance the activity of cancer vaccines. For instance, Markov et al. created mannosylated liposomes, complexed with RNA from B16 melanoma cells, to effectively target mannose receptors, highly expressed in DCs. They observed that when injecting them intravenously in mice bearing B16 tumors, they induced a B16-specific cytotoxic response that was two-fold more potent than the liposome control group [122]. Goswami et al. adopted a similar strategy when developing an mRNA influenza vaccine with the influenza hemagglutinin as antigen. By mannosylating the LNPs, upon intradermal injection, they achieved increased antigen-specific T cell responses when compared to unmannosylated LNPs [123].

To sum up, ionizable lipids are crucial for the *in vivo* behaviour of LNPs. Depending on the ionizable lipid used, different biodistribution and delivery efficiencies can be observed. Despite all the optimizations made in the field, further research needs to be conducted to asses optimal ionizable lipids for delivering mRNA and achieving antitumoral effects.

3.2.2. Non-cationic (phospho)lipids

It is hypothesized that the helper lipids (non-cationic (phospho)lipids and cholesterol) of an LNP contribute to its stability. Kulkarni et al. showed that in empty LNPs, cholesterol and DSPC reside in the outer layers. Contrarily, in siRNA-loaded LNPs, a fraction of cholesterol and DSPC are found on the inside together with the siRNA. They also showed that at least to a certain extent, helper lipids are essential to achieve a high encapsulation efficiency of the siRNA payload [124]. Moreover, the spatial structure of the non-cationic (phospho)lipids defines the fusogenicity of the LNP by its ability to form a non-bilayer hexagonal H_{II} phase, essential for bilayer disruption. Therefore, they determine, at least partially, the endosomal scape efficiency [90,125]. Typically, 10 to 20 % of the LNP composition consists of neutral phospholipids, being 10 % the most common one [90].

When it comes to the non-cationic phospholipids, the most commonly used are 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) [90,125]. DOPE has a small phosphoetanolamine head group and two unsaturated oleoyl chains, resulting in a conical lipid structure. This leads to a high fusogenicity, which is beneficial for the endosomal scape [125–127]. Nevertheless, one of its limitations is the low colloidal stability of DOPE-based LNPs, leading to particle aggregation [125,128]. On the other hand, DSPC has a large head group and saturated acyl chains, resulting in a cylindrical lipid structure. This leads to highly stable LNPs, with a better uptake profile than the LNPs with PE phospholipids but lower endosomal escape and intracellular delivery [125,127]. With intravenously injected C12-200 mRNA LNPs, Kauffman et al. found out that when changing DSPC for DOPE, there is an increase

in both uptake and intracellular delivery. Nonetheless, this improvement was not seen for LNPs loaded with siRNA [111]. This seems to indicate that optimization done for one type of nucleic acid cannot be extrapolated to all the other types.

Zhang et al. formulated 96 different C12-200 LNPs, with either DSPC or DOPE, and injected them intravenously. They observed that frequently, the DOPE formulations accumulated more in the liver and the DSPC formulations had a preference for the spleen. They argue that this is due to DOPE binding to ApoE more strongly than DSPC [129].

3.2.3. Cholesterol as neutral helper lipid

Cholesterol can modulate the physicochemical properties of any lipid membrane. Moreover, it plays an important role in the stability of nanoparticles by occupying the empty spaces between the other lipidic components, making the particles more stable and less prone to drug leakage [125,130,131]. Even if this effect may not be important for big payloads such as nucleic acids, Kulkarni et al. showed that at least to a certain extent, helper lipids are essential to achieve a high encapsulation efficiency [124]. Moreover, high molar ratios of cholesterol are included in the LNPs in order to prevent particle destabilization due to lipid exchange with biological membranes. In circulation, cholesterol-deficient particles could sequester cholesterol and therefore, lead to particle destabilization [94,132]. The molar percentage used oscillates between 35 and 50 %, being 38.5 the most used concentration [90].

Recently, Patel et al. have proven that the replacement of cholesterol with cholesterol analogues (C-24 alkyl phytosterols), can improve gene transfection *in vitro* [133]. This transfection enhancement could potentially be explained by the polymorphic shape, multilamellarity and lipid partitioning of these LNPs, that facilitate endosomal escape [95]. Additionally, Paunovska et al. reported that LNPs incorporating oxidized cholesterols, when injected intravenously, were preferentially delivered into hepatic endothelial cells and Kupffer cells instead of hepatocytes [134].

3.2.4. Stabilizing polymer-lipid conjugate

To further optimize drug delivery systems, polyethylene glycol (PEG)-lipids were developed. These combinational molecules are based on the conjugation of PEG molecules, a hydrophilic polymer that has been reported to be safe for humans, to lipids that are easily anchored into lipidic membranes [135]. Both, the lipid tail structure of the PEG lipid and the molecular mass of the PEG molecule influence the biological activity of the LNPs.

The addition of PEG-linked lipids in LNPs increases their colloidal stability, reducing particle aggregation during both circulation and storage [94,136,137]. Moreover, the presence of PEG-lipids reduces the protein interaction with the particles, protecting them against opsonization and against clearance by the reticuloendothelial system (RES), what enhances the circulation half-life [103,138].

In LNPs, since they do not have an aqueous core, most of the PEG lipid is found on the LNP surface, influencing the size and delivery efficiency [139]. By means of cryo-transmission electron microscopy, Kulkarni et al. showed how changing the PEG percentage influences the structure and size of the LNPs [124]. High PEG concentrations, of above 2.5 %, are associated with smaller particles but also decreased cell uptake, what reduces the overall performance of the LNPs [140]. Generally, molar percentages ranging from 1 to 3 % are used [90].

Two downsides of PEGylated nanoparticles are: 1) the accelerated blood clearance (ABC) upon repeated administrations [141] and 2) the complement activation-related pseudoallergy (CARPA) reactions [142 143]. Previous research has shown that this is caused by an anti-PEG antibody response that happens after a first exposing dose. The following dose is then, quickly cleared from the circulation. It has been proposed that anti-PEG antibodies are induced by a type 2 T-cell independent mechanism. When the nanoparticles reach the marginal zone of the spleen, they bind to surface immunoglobulins found on B cells, that then start producing anti-PEG antibodies in a T cell independent manner. Upon a second immunization with PEGylated nanoparticles, the antibodies that remain in circulation, bind to PEG and activate the complement system, what results in opsonization by C3 fragments, enhancing phagocytosis by Kupffer cells and ultimately, clearance [141]. The complement activation triggered by the anti-PEG antibodies results in the formation of several anaphylatoxins (C3a, C4a and C5a) that activate immune cells to generate secondary inflammation intermediaries. These anaphylatoxins also activate mast cells, which are known to trigger the anaphylaxis symptoms [144 143].

A more recent study, researched the ABC mechanism for mRNA containing LNPs. They claim that upon a first injection, the LNPs get opsonized by pre-existing anti-phosphatidylcholine IgM Abs (that bind the PC epitope from DSPC). Once in the spleen, the opsonized LNPs get phagocytosed by APC cells that present the PEG lipids to B-2 lymphocytes, which start producing antibodies against PEG. Upon a second injection, due to the existence of anti-PEG antibodies, more LNPs get opsonized and phagocytosed, what turns into lower protein expression at the target site [145]. Different factors, such as the length of the PEG chain, the type of anchor lipid together with the size of its acyl chains, or the PEG percentage in the LNP, are believed to influence the ABC response [146]. Pharmacological parameters such as the administered dose, the regimen and type of administration can also affect the activity and adverse effects of the LNPS [146].

The development of diffusible PEG lipids allowed the particle stabilization while enabling intracellular delivery. These lipids contain acyl chains with a length of 14 carbons. When in circulation, this PEG lipid rapidly dissociates from the LNP. Two hours after administration, only a 20 % of the injected C-14 PEG-lipid is on the LNP surface [114]. These characteristics of C-14 PEG-lipids minimize the ABC effect, offering an advantage for RNA delivery [145,147,148]. For Onpattro LNPs, which are intended to deliver an siRNA into hepatocytes, this desorption of the PEG lipid from the surface allows the binding of apolipoprotein E (ApoE), that mediates the internalization of the LNPs via the low-density lipoprotein receptor (LDLR) found in hepatocytes [114]. Therefore, for this specific application, having too much PEG lipid could hamper the cellular uptake and consequently, the efficient siRNA delivery [149].

Even if diffusible PEGs lead to decreased ABC effect and CARPA, the vaccination against COVID-19, with either mRNA-1273 or BNT162b2, led to some cases of hypersensitivity reactions. The rates of anaphylaxis were 2.5 cases per million doses for the BioNTech vaccine and 4.7 cases per million doses for the Moderna vaccine [146]. The allergic reactions against COVID-19 vaccines are very rare but greater than for conventional vaccines. Moreover, it has also been reported that these vaccines induce an increase in the titers of anti-PEG antibodies. Interestingly, the Moderna vaccine induces higher titers of anti-PEG antibodies than the Pfizer vaccine [150]. These differences could be due to either the slightly different chemical structure of the PEG lipid (the BNT162b2 vaccine uses the lipid ALC-0159 and the mRNA-12773 vaccine uses DMG-mPEG2000) or the higher dose that is administered with the Moderna vaccine (100 μg versus 30 μg) [144].

Oppositely to C-14 PEG lipids, C-18 PEG lipids do not dissociate from the particle in the circulation [151]. For some applications, such as tumor delivery upon intravenous injection, this may be beneficial. It has been previously reported that LNPs with 1.5 % DSG-PEG2000 show greater circulation times than 1.5 % DMG-PEG2000 LNPs [151], what could potentially lead to more tumor accumulation. Similarly, Lee et al. found out that increasing the percentage of DSG-PEG2000 lipid from 2.5 to 5 % leads to substantially enhanced circulation times (from 30 min to more than 8 h) and to increased tumor accumulation [152]. Bevers et al. employed a design-of-experiments approach to optimize the PEG lipid type (among DMG-PEG2000, DSG-PEG2000 and DSPE-PEG1000) and the lipid ratios between the different LNP lipid components. The objective of the optimization was to achieve high levels of tumor-specific CD8 T cells in a mouse TC-1 tumor model upon intravenous administration of LNPs loaded with mRNA encoding for papillomavirus 16 oncoprotein E7. Their findings revealed that formulations with high

percentages of SS-EC ionizable lipid and low percentages of DOPE and PEG lipid were more favourable for achieving this goal [153]. A better understanding on how the PEG lipid affects the *in vivo* properties of LNPs may help fine tuning them for different clinical needs.

In the last years, scientists have been proposing several strategies to avoid the accelerated clearance of nanoparticles. Initially, two of the strategies that were proposed were: 1) the blockage of the mononuclear phagocyte system (MPS) by using pre-dosed empty liposomes [154] and 2) the transient depletion of monocytes and macrophages by using inorganic and organic materials such as dextran sulfate or gadolinium chloride [155]. Nonetheless, since the effectiveness of these approaches is dependent on the use of high doses, which usually lead to a high toxicity, their translation into the clinics has not yet been possible [155]. Recently, Li et al. have suggested to inhibit the complement opsonization on nanoparticles as a strategy to increase the nanoparticle circulation times. More precisely, they propose to co-administer nanoparticles with a fusion construct that comprises the complement receptor 2 (that binds to de complement 3 protein deposited on the surface of nanoparticles) and the complement receptor 1 (that inhibits the C3 convertase activity). They reported that the *in vitro* co-incubation of this construct with different types of nanomaterials inhibits the deposition of complement proteins on the nanomaterial surface. In rats, the co-injection of superparamagnetic iron oxide nanoparticles with the CR2-CR1 construct, almost fully blocked the complement opsonization and the undesired monocyte/granulocyte uptake [156]. It would be interesting to test whether this construct could also block the binding of complement proteins to the surface of PEGylated LNPs in the presence of anti-PEG antibodies. Another potential approach is the one proposed by Nikitin et al. They reported that a transient and slight depletion of erythrocytes in mice (with an anti-erythrocyte antibody) can increase the liposome circulation. The anti-erythrocyte promotes clearance of intact red blood cells by the MPS, which in turn, causes a reduction on nanoparticle clearance [157]. The different strategies that have explored in order to increase the circulation times of nanoparticles in blood have been extensively reviewed elsewhere [158].

The drawbacks that the therapeutic use of PEG implies have stimulated the development of PEG alternatives such as pSarcosinylated lipids [159]. Polysarcosine is a polypeptoid consistent of sarcosine repetitions that showed stealth properties comparable to PEG. Nogueira et al. have reported that LNPs with pSarcosinylated lipids display a comparable transfection efficiency to PEG LNPs while improving their safety upon intravenous injection in mice [160]. Some authors have proposed the use of other water-soluble polymers as a PEG alternative. Kierstead et al. reported that HPMA (N-(2-Hydroxypropyl)methacrylamide) and PVP (Poly(N-vinylpyrrolidone)) polymers show increased circulation times and do not induce the ABC effect that PEGylated nanoparticles trigger [136].

3.3. Polymeric nanoparticles

Despite improvements on mRNA delivery with LNPs, other alternative delivery systems that overcome some of their limitations are being explored. The main LNP limitations to be overcome are the associated toxicity, what limits the administered dose, and their tendency to accumulate in the liver [90].

Cationic and ionisable synthetic polymers have been extensively researched to deliver nucleic acids and other cargoes such as proteins. Poly(β-amino esters) (PBAEs) and poly(lactic-co-glycolic acid) (PLGAs) have emerged as an option for mRNA delivery due to their reduced toxicity compared to previously explored polymers, such as polyethylenimine (PEI) [38,161].

For instance, Zhang et al. designed a polymeric nanoparticle consisting of PBAE, poly-glutamic acid (PGA) and di-mannose moieties that they loaded with the mRNAs encoding for the transcription factors necessary to reprogram tumor-associated macrophages, which usually display tumor-promoting functions, to express an anti-tumor phenotype. They proved the therapeutic efficacy of these nanoparticles in models of melanoma, glioblastoma and ovarian cancer [162]. Similarly, Huang and colleagues developed a nanoparticle containing an *ortho*-hydroxy tertiary amine (HTA) polymer and cholesterol. *In vivo*, these polymeric nanoparticles showed antitumor efficacy against the B16-OVA melanoma tumor model [163]. Liu et at. came up with a strategy to functionalize cationic polymers that were unable to deliver mRNA *in vivo* into zwitterionic phospholipidated polymers that can efficiently load and deliver mRNA to the lymph nodes and spleen upon systemic administration. Both the spleen and the lymph nodes are organs of great importance for cancer immunotherapy [164].

In order to combine the benefits of polymeric and lipidic drug delivery systems, some researchers developed hybrid systems. They saw that their combination could improve the delivery and stability in serum of the polymeric nanoparticles [165,166]. Persano et al. developed a lipopolyplex mRNA vaccine in which, the mRNA is complexed with PBAE and afterwards, encapsulated into a lipid shell. The authors reported that when treating mice bearing B16-OVA tumors with their LPPs, there was a 90 % reduction of the tumor nodules [167]. Guevara et al. loaded PBAE-LPPs incorporating α-GalCer, an immunoadjuvant, with a therapeutic mRNA. In the B16F10 model, the LPPs showed greater DC maturation, CTL responses and survival rates than when using LNPs, proving the potential of these delivery tools [85]. Moignic et al. developed a lipopolyplex by complexing the mRNA with PEGylated histidinylated polylisine and then, encapsulated this with several lipids that incorporated an α-D-mannopyranoside functionalization. When loaded with therapeutic mRNAs, these lipopolyplexes exerted a therapeutic effect in three different murine tumor models [168].

Another polymer that has been frequently used is PLGA. Nonetheless, at neutral pH, it cannot complex nucleic acids. Therefore, to do so, researchers have added cationic chemical groups [169]. Moreover, some others have lipid-coated PLGA nanoparticles. For example, Hasan et al. developed lipid-coated PLGA nanoparticles that could efficiently deliver different siRNAs against prostate cancer [170]. Fan et al. tested a combination of poly(ethylene glycol)-block-PLGA (PEG-b-PLGA) and cationic lipids to deliver mRNA against a lymphoma mouse model [171].

In order to avoid the toxicity associated to the permanently positively charged polymers, Mckinlay et al. developed what they name as the charge-altering releasable transporters (CARTs) [172], which are made of degradable oligo(carbonate-b-α-amino esters). These nanocarriers possess an interesting property: after entering the cells, they experience a rearrangement that changes the initial poly-cationic backbone into neutral and small amides, what enables the decomplexation and endosomal scape of the mRNA. They proved that CARTs can efficiently deliver mRNA and produce an antitumor response upon intravenous and subcutaneous administration [173,174]. With this same technology, they also sought to transfect resting natural killer (NK) cells in order to convert them into chimeric antigen receptor (CAR) NK cells, which hold great promise as a cell therapy against cancer. Nonetheless, they are known for being very difficult to transfect. This platform, compared to electroporation, which is the gold standard method for non-viral transfection of primary NK cells, they achieved a transfection that is 300 times higher and preserves better the cell viability [175]. More recently, they developed a second generation of CARTs that is based on oligo serine esters (Ser-CARTs). These ones have different advantages including a better biocompatibility (it degrades into serine peptides under physiological pH) and a smaller size [176].

3.4. Peptide-based nanoparticles

Lipid-based and polymeric nanoparticles are not the only platforms available for mRNA delivery. Peptides have also been explored as an option due to their high biocompatibility. The two main examples of peptide-based nanoparticles are cell-penetrating peptides (CPPs) and protamines [177].

Protamines were one of the first materials investigated for mRNA complexation. They are small (4 kDa), arginine-rich peptides that can be used to condense mRNA into nanoparticles of approximately 300 nm in diameter [38]. Nonetheless, these protamines activate the immune system via TLR7 and TLR8. Even though the immunogenicity of drug delivery systems is generally to be avoided, it can be of interest for certain applications such as cancer vaccinology [38,70,178,179]. By exploiting this property, CureVac developed RNActive™, in which modRNA and protamines were combined to treat prostate [72] and non-small cell lung cancer [71].

When it comes to cell-penetrating peptides, they usually have a short sequence, not longer than 40 amino acids, with either amphipathic or cationic regions thanks to which they can cross the cell membrane. Generally, cationic CPPs contain arginine, histidine and lysine, which due to their charges interact with the negatively charged cell membranes. The amphipathic CPPs contain lipophilic and hydrophilic amino acids that facilitate the peptide translocation though the cell membrane [180]. In several studies, researchers have used CPPs, either on its own or combined with other drug delivery systems, to deliver siRNAs against the tumor progression [181–185]. When it comes to mRNA, a few have attempted to deliver it by using CPPs. For instance, Chen et al. came up with a drug delivery system that was inspired in polymeric micelles and consisted of PEG, PNIPAM polymer and cyclic Arg-Gly-Asp peptides (cRGD). The addition of the cRGD peptides led to increased tumor accumulation and mRNA expression [186]. Van den Brand et al. used the CPP PepFect14 to prepare CPP-mRNA nanoparticles. They compared the *in vivo* transfection efficacy of these CPPs and Lipofectamine MessengerMAX (LipMM) when injecting intraperitoneally into an ovarian cancer mouse model [187]. Tateshita et al. combined a lipoplex-like mRNA nanocarrier with the KALA peptide to use as an *ex vivo* dendritic cell-based cancer vaccine [188].

Some have also developed combined already existing systems such as protamine and lipid-based nanoparticles. Protamine allows the mRNA condensation and the lipidic cover ensures the needed neutral charge [38]. For instance, Zhang et al. showed efficient anti-tumor responses in colon cancer mice models when survivin-T34A mRNA, an agonist of the endogenous survivin with anticancer effects, was delivered both intravenously and intratumorally with protamine-lipid complexes [189]. Mai et al. formulated a cationic liposome/protamine complex with cytokeratin 19 mRNA and delivered it intranasally into an aggressive Lewis lung cancer model. This led to a potent cellular immune response and slowed down the tumor progression [190]. Similarly, Lei et al. encapsulated IL-15 mRNA in a protamine/liposome system. Upon local and systemic administration, this therapeutic showed certain potential against colorectal cancer [191].

3.5. Inorganic nanoparticles

The most well studied inorganic nanoparticles to deliver nucleic acids are iron oxide nanoparticles, quantum dots, silica nanoparticles and gold nanoparticles. Generally, the most interesting aspects about inorganic nanoparticles as drug delivery systems are their tuneable geometry and size. Nonetheless, their lack of biodegradability and the potential toxicity still pose a great risk and limit their implantation into the clinics. Gold nanoparticles possess various advantages ranging from a small and tuneable size, a good biocompatibility or the potential for scalability [192,193]. These gold particles can easily be functionalized with cationic moieties that allow the complexation of the negatively charged nucleic acids. Poly-amidoamine (PAMAM) is an example of cationic polymer that is typically used to complex RNA to gold nanoparticles. The functionalization with PAMAM allows for an efficient mRNA binding and also to protect it from RNases [194]. Yeom et al. conjugated gold nanoparticles with DNA oligonucleotides to then, hybridize them with an mRNA encoding for an apoptotic factor. These gold nanoparticles could efficiently deliver the mRNA into tumor cells, which then produced pro-apoptotic factors and suppressed the tumor growth [195]. Similarly, Chan et al. functionalized gold nanoparticles with poly (T) sequences that could efficiently hybridize with the poly(A) tail of the mRNA. They found that these complexes could efficiently deliver mRNA into HeLa cells [196]. The use of mesoporous silica nanoparticles as a drug delivery system for mRNA has only been reported in the last few years. One of the first papers describing its use as an mRNA delivery agent was by Wang et al [197]. They reported that mesoporous silica nanoparticles could better deliver mRNA if they had a diameter of around 50 nm and a pore size bigger than 20 nm. In 2021, Zhang et al. developed some mesoporous silica nanoparticles that encapsulated and were able to deliver, upon subcutaneous administration, both mRNA and a protein kinase inhibitor that allowed to enhance the mRNA translation [198].

3.6. Exosomes

Exosomes are a type of extracellular vesicles (EVs) with a size that ranges between 40 and 120 nm. They originate when the endosomal membrane buds inwards, resulting in the formation of multivesicular bodies (MVBs) that are then released to the extracellular space. Exosomes can transport both nucleic acids and proteins between cells and tissues. Moreover, given their natural origin, the exosome lipid bilayer shows a great biocompatibility and low immunogenicity when compared to synthetic drug delivery systems. Furthermore, they have intrinsic targeting capabilities, they can cross physiological barriers and use natural intracellular trafficking pathways. For these reasons, exosomes and bioinspired drug delivery systems have been receiving a lot of attention in the last years [193,199,200].

Nonetheless, this is still a very exploratory field with a lot of challenges to overcome. One of these challenges is the loading large molecules like mRNA. Short nucleic acids, like siRNAs and miRNAs, have been successfully loaded by conventional bulk electroporation [201 202]. However, for mRNA, bulk electroporation is not an efficient method [201]. Alternative methods have been developed to load exosomes with mRNA. For instance, Yang et al. developed a novel type of electroporation named cellular-nanoporation. More precisely, they transfected cells with plasmid DNAs and stimulated them with a transient electrical pulse that triggers the release of exosomes loaded with the transcribed mRNAs. This new method of electroporation led to a 50-fold increase in the exosome production and an mRNA loading 100 times higher than the one achieved with bulk electroporation [203]. Another strategy is the one used by Tsai et al., in which they complexed the mRNA with cationic lipids and then, mixed and incubated these complexes with purified exosomes [204]. Others have engineered mammalian cells so that they produce exosomes containing an RNA packaging device that they named EXOsomal transfer into cells (EXOtic). This system allows to enhance the exosome production, to pack specific mRNAs and to deliver them to target cells [205]. Maugeri et al. used the intrinsic endocytic pathways of LNPs to substitute LNPs with exosomes as mRNA delivery system. When injected *in vivo*, these vesicles could efficiently deliver the mRNA and showed a lower expression of inflammatory cytokines than the LNPs [206]. Apart from the mRNA loading, exosomes come with other challenges such as the difficulty to produce them at a large scale.

Recently, Tsai et al. developed an exosome-mRNA vaccine against SARS-CoV-2. Upon intramuscular administration, this vaccine was able to trigger both cellular and humoral responses [204]. Even though these kind of studies show the great potential of exosomes as an mRNA delivery system, they are still far from being used in the clinics. To overcome the limitations of synthetic and exosome nanocarriers, some researchers are developing hybrid systems [207,208]. These extracellular vesicle-based hybrid systems have been extensively reviewed elsewhere [209].

4. Summary and outlook

This review describes what mRNA-cancer vaccines are and their mode of action. We also go over the mRNA structure, the *in vitro* transcription process and the mechanism by which *in vitro* transcribed mRNA activates the immune system. We discuss as well the different types of mRNA modifications that can be added to the mRNA sequence in order to increase its half-life, decrease its immunogenicity and increase the overall protein translation. Among all the modifications, we pay special attention to the nucleoside modifications discovered by Katalin Karikó and Drew Weissman, who have been just awarded with the Nobel Prize in medicine. Finally, we reviewed the different drug delivery systems that are being considered for mRNA cancer vaccines, including LNPs, which are the current state-of-the-art and have been well characterized and studied, and also more experimental nanomaterials such as the polymeric nanoparticles and the peptides-based nanoparticles. Since LNPs are the most advanced ones, we paid especial attention to them and described the role that each of the building blocks plays (cationic or ionizable lipids or lipid-like materials, non-cationic (phospho)lipids, cholesterol and stabilizing lipid-polymer conjugates). We also draw the attention to the immunogenicity problems that both ionizable and PEG lipids entail.

Even though the LNP-encapsulated mRNA vaccines against cancer have shown great progress, there is still a lot to be understood. Concerning the mRNA, there is a need to identify new tumor antigens and to come up with new screening methods that allow for a quick and inexpensive identification. Developing new ways in which we can fine tune the mRNA immunogenicity are as well of great interest for the field. Two examples that have been explained in this review are the use of circular mRNA or the fusion of the antigen mRNA with the C3d mRNA. Regarding the LNPs, we strongly believe that in order to exploit their full potential, we need to better understand their mode of action and their interactions in the human body. We need to invest more efforts and resources to unravel the mechanisms by which LNPs are taken up, how does the mRNA escape the endosome and the mechanisms by which the mRNA and the lipid components interact with the immune system. We also think that having more understanding, will help us to rationally tailor LNPs so that they can be directed beyond the liver, which is the natural destination for most nanomedicines, especially upon intravenous administration. Furthermore, a better standardization of the manufacture, characterization and *in vitro* and *in vivo* testing procedures will help researchers in the field compare their studies with previously published ones. Additionally, in order to avoid the translatability issues between preclinical models and humans, we also need to invest more energies into developing complex tumor models that can recapitulate the complex conditions that are seen in patients. Despite the great results that LNPs have yielded in the past years, we reckon that we should not only put our hopes and efforts on them; we should also explore alternative drug delivery systems, such as polymeric nanoparticles or peptide-based systems, that could help us avoid the issues that LNPs entail.

An additional challenge in the development of mRNA cancer vaccines is the route of administration, a crucial factor influencing vaccine efficacy and the distribution of mRNA. Each route of administration possesses different advantages and disadvantages. For instance, upon intradermal or subcutaneous administration, the mRNA is readily translated by local antigen-presenting cells, but this method often leads to significant local reactions at the injection site [210,37]. With intranodal administration, on the other hand, the mRNA directly reaches the lymphatic antigen-presenting cells [11] but similarly to what happens with intratumoral injections, the technical challenges associated with this method restrict the injection volume to small amounts [211,212]. Intratumoral administration is primarily employed when using mRNAs encoding for co-activating molecules to induce localized inflammation [211,212]. The intravenous administration allows the mRNA to reach several lymphoid organs. When compared to local injections, this method is capable of eliciting a strong CD8 + T-cell response, a crucial component in anti-tumor immune responses [68 37]. Nonetheless, the systemic administration usually leads to higher levels of systemic toxicity compared to local administration. Finally, the majority of mRNA cancer vaccines in development are therapeutic rather than prophylactic, requiring multiple doses to elicit a tumor response when administered as a monotherapy [37]. It is probable that these therapeutics will necessitate combination strategies with other immunotherapies, such as oncolytic viruses or immune checkpoint inhibitors, for optimal efficacy. We believe that addressing the aforementioned points will lead to a greater understanding of these therapeutics and the successful development of an mRNA vaccine against cancer.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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