Nano Research 2018, 11(10): 5338–5354
https://doi.org/10.1007/s12274-018-2091-z
Review Article
ISSN 1998-0124
CN 11-5974/O4

Recent advances in mRNA vaccine delivery

Lu Tan and Xun Sun (✉)

Key Laboratory of Drug Targeting and Novel Drug Delivery System, Ministry of Education, West China School of Pharmacy, Sichuan University, Chengdu 610041, China

Received: 14 March 2018
Revised: 30 April 2018
Accepted: 4 May 2018

KEYWORDS
messenger RNA (mRNA) vaccines,
delivery systems,
polymer,
lipid

ABSTRACT

In recent years, messenger RNA (mRNA) vaccines have been intensively studied in the fields of cancer immunotherapy and infectious diseases because of their excellent efficacy and safety profile. Despite significant progress in the rational design of mRNA vaccines and elucidation of their mechanism of action, their widespread application is limited by the development of safe and effective delivery systems that protect them from ubiquitous ribonucleases (RNases), facilitate their entry into cells and subsequent escape from endosomes, and target them to lymphoid organs or particular cells. Some mRNA vaccines based on lipid carriers have entered clinical trials. Vaccines based on polymers, while not as clinically advanced as lipid vectors, show considerable potentials. In this review, we discuss the necessity of formulating mRNA vaccines with delivery systems, and we provide an overview of reported delivery systems.

1 Introduction

Messenger RNA (mRNA) vaccines carry transcripts encoding antigens, and use the host cell translational machinery to produce the antigens, which then stimulates an immune response [1]. In 1993, liposome-delivered mRNA encoding influenza virus nucleoprotein was shown to induce a virus-specific T cell response in mice [2], and since then these vaccines have been the subject of intense research. Their popularity reflects their numerous advantages over other vaccine platforms. Live attenuated vaccines carry the risk that the attenuated organism will revert to a virulent form, and their complex composition can trigger adverse effects [3]. In contrast, mRNA vaccines express well-defined antigens that induce focused immune responses specifically against the encoded antigens [4]. Inactivated pathogens or subunit vaccines are generally safer than live attenuated vaccines, but they are considered to elicit primarily humoral immunity. In contrast, mRNA vaccines can induce both humoral and cellular immune responses, including responses mediated by cytotoxic T lymphocytes (CTLs), which are essential for cancer immunotherapy and for eradicating pathogens "hidden" within host cells [5]. In addition, these vaccines can be constructed simply and inex-

Address correspondence to sunxun@scu.edu.cn

pensively in a similar way with high purity and stability [4, 6].

These advantages of mRNA vaccines mentioned above are shared by DNA vaccines and viral vectors. However, mRNA vaccines have advantages over these other nucleic acid vaccines. DNA vaccines must be delivered into the nucleus, where antigen transcripts are generated for subsequent translation in the cytoplasm. Ensuring entry of DNA vaccines into the nucleus brings technical challenges and carries risk of insertional mutagenesis [3, 7]. The application of viral vectors can be severely limited by anti-vector immunity resulting from live infection or previous immunization [3]. In contrast, mRNA vaccines are usually delivered by non-viral systems and are required only to reach the cytoplasm, eliminating risk of integration into the host genome [5]. In addition, mRNA vaccines can transfect cells that divide slowly or not at all, such as dendritic cells (DCs) [5]. DNA vaccines cross the nuclear membranes of such cells much less efficiently than they cross the nuclear membranes of rapidly dividing cells [8]. The fact that mRNA does not replicate and is metabolically degraded within a few hours [9] means that the expression of mRNA is rapid and transient. As a result, exposure to antigen is more controlled, minimizing the risk of tolerance induction [10]. Moreover, mRNA is not categorized by the US Food and Drug Administration as a genetically modified organism, whereas plasmid DNA is [11].

Despite all these advantages of mRNA vaccines, their development has long been surpassed by that of DNA vaccines because of uncertainties about RNA stability and large-scale manufacturing [3]. Technical advances in RNA biology and chemistry have eliminated these issues, but challenges remain, including how to protect mRNA from degradation, how to ensure its efficient arrival into the cytoplasm, how to target it to desired cells and tissues *in vivo*, and how to balance intrinsic adjuvant activity with translation inhibition. In the present review, we will discuss these challenges and describe the rational design of mRNA structure to ensure its stability and efficient translation. We will discuss the necessity of formulating mRNA vaccines with delivery systems, and review lipid- and polymer-based as well as hybrid delivery systems that have been reported in the literature. Finally, we will provide an overview of preclinical and clinical applications of mRNA vaccines.

2 From production to function

Synthetic mRNA can be produced in a cell-free *in vitro* transcription system containing a DNA template encoding all the structural elements of a functional mRNA, ribonucleotides, bacteriophage RNA polymerase, and normally a synthetic cap analogue [7, 12]. In some cases, total RNA extracted from tumor cells can also be used as vaccines [13]. The antigen-encoding mRNA, whether *in vitro*-transcribed or extracted, is administered nakedly or complexed with an appropriate vector. In all cases, the mRNA induces an immune response via a similar process (Fig. 1).

At first, target cells internalize the mRNA vaccine; these target cells may be DCs in the case of intranodal injection [14] or non-immune cells at the administration site in the case of intramuscular injection [15]. Many cell types can take up naked mRNA spontaneously in a temperature- and dose-dependent manner [16]. Most mRNAs appear to enter cells via caveolae/lipid rafts and involve scavenger-receptor(s) [16, 17], but they enter DCs primarily via macropinocytosis [18, 19]. Uptake of mRNAs *in vivo* can be as efficient, or even more so, as uptake *in vitro* [7, 15, 20]. Internalized mRNA vaccines are usually trapped in endosomal vesicles, so they have to escape from the endosomes to reach the cytosol to be translated. The underlying mechanism remains unclear [5].

After escape into the cytoplasm, the mRNA is translated to produce the encoded antigens, which in DCs are degraded by cytosolic proteasomes, in the same way that endogenous proteins are degraded. The resulting epitope peptides are transported to the endoplasmic reticulum, where they bind with major histocompatibility complex (MHC) I molecules. MHC I/epitope complexes are presented on the cell surface and induce antigen-specific CD8+ T cell responses [5, 12, 21]. MHC II/antigen presentation, which leads to a CD4+ T helper cell response, is induced after DCs take up antigens released in the extracellular medium [21]. When an mRNA vaccine is internalized mainly by non-immune cells, a CD8+ T cell response is also

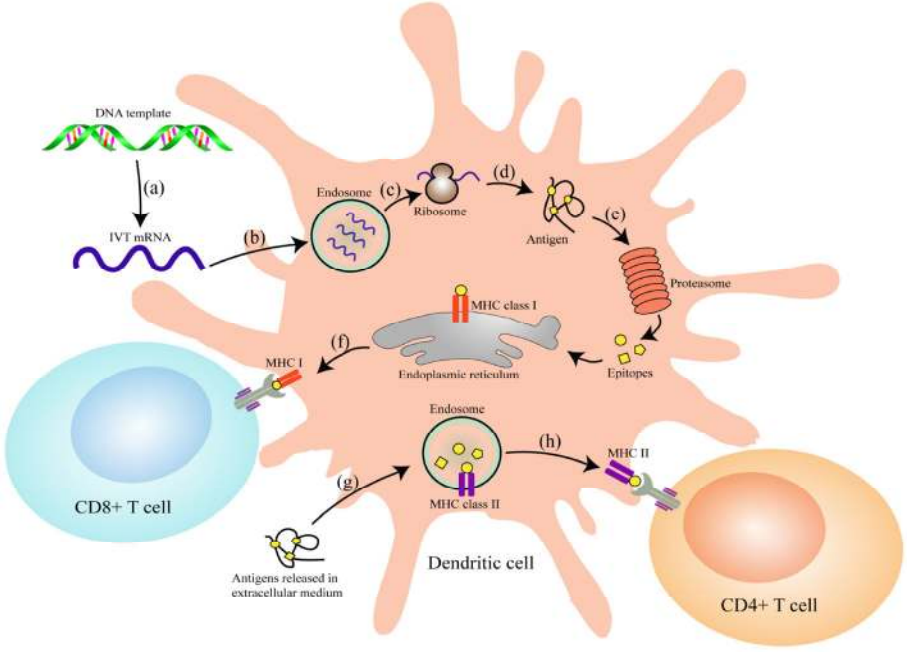

Figure 1 Major steps involved for mRNA vaccines from production to function. (a) *In vitro* transcription: mRNA is transcribed from a DNA template in a cell-free system. (b) Cellular uptake: mRNA vaccine is internalized by DCs and trapped in endosomal vesicles. (c) Endosome escape: Entrapped mRNA is released into the cytoplasm. (d) Translation: Antigen proteins are synthesized using translational machinery of host cells. (e) MHC class I epitope processing pathway: Antigen proteins are degraded by proteasomes in the cytoplasm and generated epitopes are transported into the endoplasmic reticulum to bind with MHC class I molecules. (f) Presentation of MHC I/epitope complexes: MHC I/epitope complexes are presented on cell surface, leading to the induction of the antigen-specific CD8+ T cell response. (g) MHC class II epitope processing pathway: Exogenous antigens are taken up and degraded in endosomes. Generated epitopes bind with MHC class II molecules. (h) Presentation of MHC II/epitope complexes: MHC II/epitope complexes are presented on the cell surface, leading to the induction of antigen-specific CD4+ T cell response.

observed, which might be partly obtained by cross-presentation of the translated antigens [22].

In addition, *in vitro*-transcribed mRNA possesses strong immunostimulatory effects and intrinsic adjuvant activity that may affect vaccine efficacy [23]. Like viral RNA, synthetic mRNA is recognized by specific pattern recognition receptors (PRRs) of the innate immune system, inducing the secretion of type I interferon (IFN) and establishing an antiviral response [5]. These PRRs are mainly Toll-like receptors (TLRs) localized in endosomal compartments, including TLR 3, 7, and 8, as well as the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family and the nuclear oligomerization domain-like receptor (NLR) family in the cytoplasm [5]. Each PRR recognizes various specific structural features of synthetic mRNA [24–33], and the PRRs involved differ between immune and non-immune cells [1, 12]. While this activation of the innate immune system is desirable for vaccine efficacy, it may actually reduce this efficacy by destabilizing the antigen-encoding mRNA and inhibiting its translation [1, 5, 12]. Therefore, one challenge in designing effective mRNA vaccines is how to deal with the "doubled-edged" sword of innate immune induction.

In order to arrive in the cytoplasm of target cells where it can be translated, naked mRNA faces several serious challenges. It is susceptible to ubiquitous extracellular ribonucleases (RNases) [34], and its large size, negative charge, and hydrophilic nature mean that it cannot easily diffuse passively across the cell membrane [12]. Many cells can internalize naked mRNA via caveolae/lipid rafts or macropinocytosis, but usually the uptake is inefficient and saturates at low doses [12]. Escape from endosomes is another issue. It has been reported that only small amounts of naked mRNA leak into the cytoplasm from lysosomes after scavenger-receptor mediated endocytosis [16].

Naked mRNAs also face challenges when they must be preferentially internalized by DCs in order to launch a CD8+ T cell response [5]. DCs can be pre-loaded *ex vivo* with antigen-encoding mRNAs [35], usually by electroporation, but the procedure is time-consuming and laborious, and it requires patient-specific cell preparations [7]. Another possibility is to administer mRNA vaccines intranodally [14], where they are taken up by resident DCs [5]; but this procedure usually requires surgery or guidance, making it complex and bringing potential risks [36].

These issues limit the ability of naked mRNA to induce potent antigen-specific immune responses. To achieve ideal vaccine potency, delivery systems are required, which protect mRNA from ubiquitous RNases, facilitate its entry into cells and escape from endosomes, and target lymphoid organs or particular cell types, especially DCs. Some carriers can even affect the immunostimulatory properties of mRNA [37]. Lipid-based vectors are the most frequently used nucleic acid carriers, and some have entered clinical trials [38]. Polymers can also efficiently deliver nucleic acids, and they offer greater flexibility than lipids. In this review, we will provide an overview of lipid- and polymer-based vectors as well as hybrid vectors that deliver mRNA vaccines *in vivo*.

3 Rational design of mRNA structure

The structural characteristics of mRNA have a strong impact on its intracellular stability and translation efficacy, so researchers have invested significant efforts into modifying the structural elements of synthetic mRNA. Normally, synthetic mRNA is designed based on the blueprint of eukaryotic mRNA [7], including adding a cap at the 5′ end, a poly(A) tail at the 3′ end, and 5′ and 3′ untranslated regions (Fig. 2). Coding regions are also optimized and base modifications are introduced as appropriate [39].

Cap structure at the 5′ end of mRNA is recognized by cap-binding factor eIF4E, and facilitates the recruitment of 43S pre-initiation complex to the 5′ end of mRNA [40]. As a consequence, capping of synthetic mRNA improves translation initiation. One approach to capping mRNA is to add a synthetic cap analogue to the *in vitro* transcription reaction [12]. Conventional cap analogues (m7GpppG) are added in both forward and reverse orientations, but only the forward-added caps are functional [41]. The use of anti-reverse cap analogs (ARCAs), such as 7-methyl(3′-O-methyl)GpppG and 7-methyl(3′-deoxy)GpppG, ensures that caps are incorporated in the correct orientation [42]. The ARCAs can be further modified with phosphorothioate substitutions [43]. This cap analogue binds tightly to eIF4E and is resistant to the decapping pyrophosphatase (DCPS). Another approach to capping mRNA is to perform a second step with recombinant vaccinia virus-derived capping enzymes after the initial *in vitro* transcription [44]. The poly(A) tail prevents mRNA degradation and facilitates binding of poly(A) binding protein, increasing protein expression [45]. One method of adding a poly(A) tail is to transcribe

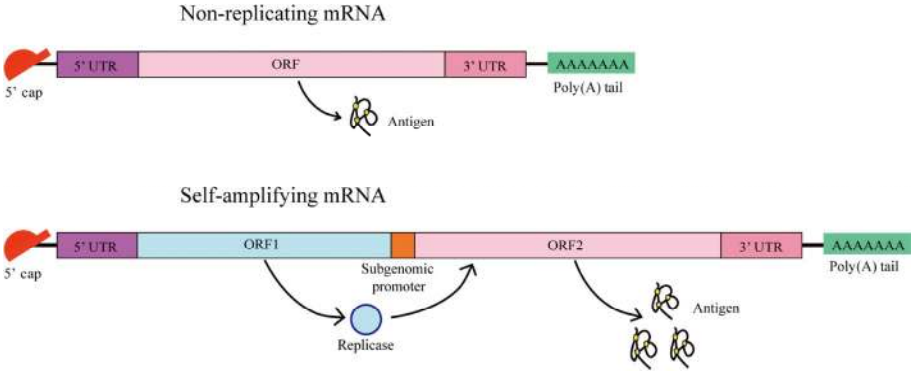

Figure 2 Structure of non-replicating mRNA and self-amplifying mRNA. Non-replicating mRNA is designed based on the blueprint of eukaryotic mRNA, consisting of the 5′ cap, the 5′ and 3′ untranslated regions (UTRs), the open reading frame (ORF) encoding the antigens, and the 3′ poly(A) tail. Self-amplifying mRNA is derived from alphavirus genomic sequences, in which structural genes have been replaced with antigen-encoding sequences (ORF2) while genes encoding replication machinery (ORF1) remain intact.

the mRNA from a DNA template containing the poly(A) stretch, which yields mRNA with a poly(A) tail of defined length [12]. Another method of adding a poly(A) tail is enzymatic polyadenylation, which generates a mixture of mRNAs differing in the length of the poly(A) tails [12]. In this enzymatic approach, modified nucleotides can be incorporated into the poly(A) tail to inhibit deadenylation by poly(A)-specific nucleases [12, 46]. Extending the poly(A) tail with a nucleotide other than A significantly reduces expression efficiency [45].

The stability and translation efficacy of mRNA can be further improved by adding 5′ and 3′ untranslated regions containing multiple regulatory sequence elements [12], such as those of the Xenopus β-globin gene [47]. Production of the antigens encoded by the mRNA can also be affected by codon context (that is, neighboring nucleotides and codons) [48] and nucleotide content [49]; replacing rare codons with synonymous frequent codons improves translational yield [50].

Substituting uridine and cytidine with their modified counterparts can also enhance mRNA translation efficacy [51–53]. They increase protein expression mainly by modulating mRNA's interaction with innate immune systems. Cells have various pathways to dispose foreign RNAs, involving TLRs, RNA helicase RIG-I, and protein kinase R, which will result in innate immunity system activation and the cell's translational machinery to be shut down [54]. In contrast, base-modified mRNA can evade such recognition. These base modifications can also protect mRNA molecules from degradation by RNases [55].

Other strategies have been described to produce more effective mRNA vaccines. For example, incorporating the coding sequence for a viral replicase into the mRNA turns it into a self-amplifying "replicon" [54], which can reduce the dose needed to elicit an immune response [56]. An mRNA encoding a tumor antigen together with the MHC II-targeting sequence from an endosomal or lysosomal protein can elicit CD8+ as well as CD4+ T cell responses [57], which is essential for a potent immune response. The same mRNA encoding only tumor antigen does not effectively elicit CD4+ T cell responses.

4 Lipid-based vectors

Various synthetic and naturally derived lipids are commonly used for nucleic acid delivery [21]. Lipids can be prepared as liposomes (the complexes of liposomes and nucleic acids are called lipoplexes) or lipid nanoparticles (LNPs) (Fig. 3(a)), both of which have been reported to efficiently deliver mRNA vaccines.

4.1 Lipoplexes

Liposomes have long been used as drug carriers because of their easy preparation, minimal toxicity, and biodegradability profiles [58]. Several liposome formulations carrying small chemical drugs have been approved by the FDA [59]. Safety and efficacy of small interfering RNA-liposomal formulations have been demonstrated in human trials [59]. In fact, diverse liposomes have been designed to efficiently deliver genes *in vivo* [60, 61], and some have been evaluated for mRNA vaccine delivery, showing promise for cancer immunotherapy [38] and infectious diseases [2]. Especially, cationic liposomes can complex with RNAs via electrostatic interaction, and the resulting liposome-based formulations are called lipoplexes [21]. These lipoplexes form in a self-assembly process, comprising a topological transition from liposomes into compact RNA-lipoplex nanoparticles with a distinct internal molecular organization [62]. Cationic lipids, such as 1,2-di-O-octadecenyl-3-trimethylammonium-propane (DOTMA) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and zwitterionic lipids, such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), have been used for mRNA vaccine delivery [38, 63]. Neutral lipids are also incorporated into cationic liposomes to decrease toxicity and attain high transfection levels *in vivo* [64].

Several factors can affect the physicochemical characteristics and biological activity of lipoplexes, such as lipid components, ratio of cationic lipid to mRNA, and ionic conditions [62]. One study varied lipid:RNA ratios of a intravenously administered lipoplex formulation to evaluate the effect of overall particle charge on *in vivo* targeting of DCs, and found that gradual decrease of the cationic lipid content shifted encoded protein expression from the lungs

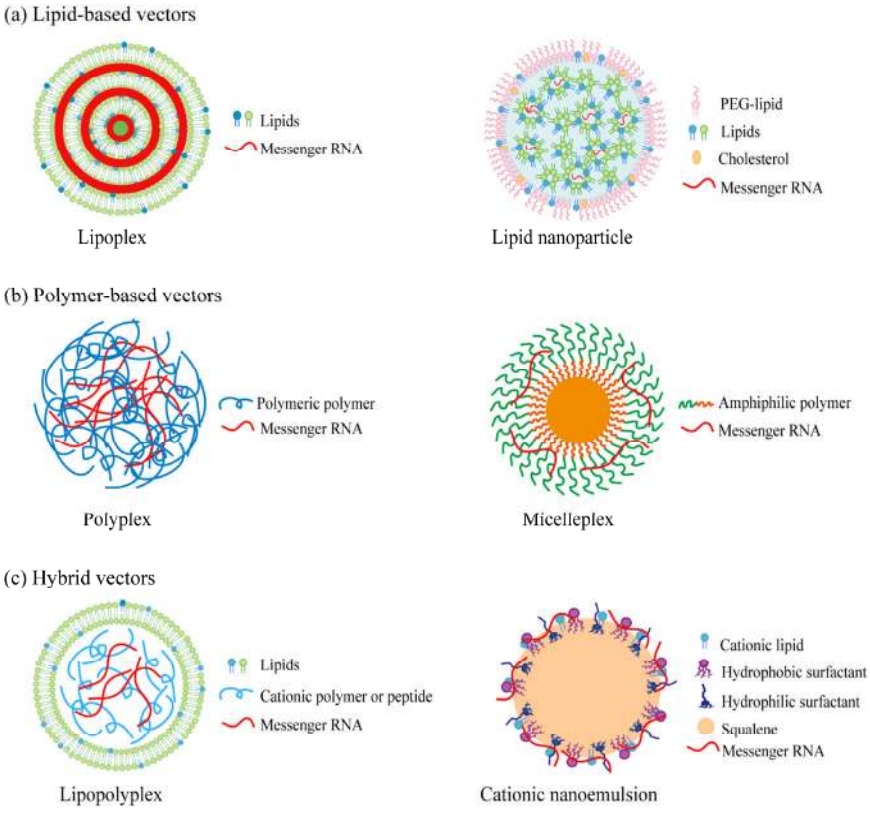

Figure 3 Vectors for mRNA vaccines. (a) Lipid-based vectors, including lipoplexes and lipid nanoparticles. (b) Polymer-based vectors, including polyplexes and micelleplexes. (c) Hybrid vectors, including lipopolyplexes and cationic nanoemulsions.

towards the spleen [38]. Near-neutral and slightly negative particles provided an exclusively splenic signal. Further research indicated that DCs are the main source of encoded protein expression in the spleen. In this way, DCs were targeted precisely and effectively *in vivo* by optimally adjusting the net charge of the lipoplex formulation, irrespective of the lipids used (for example, DOTAM, DOPE, DOTAP, cholesterol), without the need for functionalization of particles with molecular ligands. The study showed that lipoplexes encoding viral or neo-antigens or endogenous self-antigens induce strong effector and memory T-cell responses, and mediate potent IFNα-dependent rejection of progressive tumors. To translate this lipoplex formulation from bench to bedside, lipolpex melanoma RNA immunotherapy ("Lipo-MERIT"), an investigational medicinal product for application to patients, was developed [62]. Briefly speaking, this product consists of two kits: Kit A contains RNA drug products. Kit B contains the liposomes and isotonic saline solution as diluent. The injectable product is obtained by addition of the diluent and the liposomes to the RNA. This ready-to-use product allows for the flexibility in the use of different RNA components, paving the way for personalized cancer immunotherapy.

As cationic lipids are associated with rapid clearance and toxicity concerns, ionizable lipids can be an alternative [65]. Ionizable lipids are positively charged at low pH but are neutral at physiological pH, retaining advantageous transfection efficacy while reducing the toxicity induced by positive charge. However, ionizable lipids are not broadly used in lipoplex formulation, but are used in LNPs, which will be discussed in the next section.

4.2 LNPs

LNPs are one the most frequently used vectors for *in vivo* RNA delivery [66]. They typically consist of four components, including a cationic or ionizable lipid, cholesterol, a helper phospholipid, and a polyethylene glycol (PEG) lipid [65]. Cationic lipids can efficiently

complex with anionic RNA molecules, but their permanent positive charge makes them more toxic as mentioned earlier. As a result, ionizable lipids are more typically used. Ionizable lipids allow RNA to be encapsulated under acidic conditions and maintain a neutral or mildly cationic surface charge at physiological pH, consequently reducing non-specific lipid-protein interactions and facilitating RNA release in the cytoplasm [67]. The ability for ionizable lipids to ionize as the pH drops is crucial for endosomal escape as well. It is thought that the lipids' positive charge facilitates their electrostatic interaction and fusion with the negatively charged endosomal membrane [68]. Apart from ionizable lipid, cholesterol is incorporated for particle stability and helper phospholipid is incorporated to preserve the lipid bilayer structure. In addition, PEG lipid is incorporated to reduce interactions with plasma proteins *in vivo* and prolong circulation time [69]. LNPs are typically formulated by mixing an aqueous solution of RNA at low pH with lipids in ethanol, while lipoplexes are formulated by mixing RNA with the finished liposomes in a second step.

The extensive research of LNPs for siRNA delivery facilitates the development of LNPs for mRNA delivery. An LNP formulation, which was originally used to deliver short interfering RNA [70–72], has been used to deliver mRNA vaccines against the Zika virus [73, 74]. These vaccines used mRNA encoding the premembrane and envelope (prM-E) glycoproteins of the Zika virus. In one study, a single immunization of prM-E mRNA-LNPs via the intradermal route elicited a potent and durable neutralizing antibody response in wild-type C57BL6 and BALB/c mice and rhesus macaques, and the vaccinated animals were highly protected from subsequent Zika challenge [73]. In a second study, two intramuscular immunizations of similar prM-E mRNA-LNPs with lower total dose led to high neutralizing antibody titers that protected against Zika infection and conferred sterilizing immunity in immunocompromised mice and immunocompetent mice [74]. To diminish production of antibodies that might cross-react with the related dengue virus (DENV), the study designed modified prM-E mRNA encoding mutations destroying the conserved fusion-loop epitope in the E protein. The modified mRNA vaccine can reduce the risk of sensitizing individuals to subsequent exposure to DENV.

LNPs have also been used for cancer immunotherapy, such as against B16F10 melanoma [75]. In that study, different lipids for the individual components and the molar compositions of the components were optimized to induce a potent T cell response *in vivo*. As a result, treatment of B16F10 melanoma tumors with the optimized LNP formulation containing mRNA encoding tumor-associated antigens led to a decrease in tumor volume and longer overall survival. Incorporating adjuvant lipopolysaccharide into the LNPs may further enhance the immune response.

5 Polymer-based vectors

Polymer vectors, although not as clinically advanced as lipid vectors, show considerable potential for nucleic acid delivery. The complexation of anionic mRNA molecules with cationic polymers occurs spontaneously through electrostatic interaction, forming polyplexes or micelleplexes (Fig. 3(b)), which are usually not differentiated. However, there are differences between them. Micelleplexes are produced by cationic and amphiphilic copolymers that can aggregate to form micelles, whereas the polyplexes do not form micelles [76]. They normally show higher stability than lipoplexes [77]. Modifications to polymers such as molecular weight, geometry (linear vs. branched), and ligand attachment can be easily achieved as well [78].

5.1 Polyplex nanoparticles

Polyethylenimine (PEI) is one of the most frequently used cationic polymers for gene and oligonucleotide delivery [79, 80]. It has been prepared into polyplex nanoparticles for mRNA vaccines. In one study [81], a self-amplifying mRNA encoding influenza virus hemagglutinin and nucleocapsid was incorporated into nanoparticles by using linear PEI (molecular weight, 22 kDa) or histidylated PEI (molecular weight, 34.5 kDa) to improve the efficacy of mRNA vaccines. The resulting polyplex formulations successfully delivered the mRNA to DCs and facilitated its translocation to the cytosol, eliciting both humoral

and cellular immune responses.

PEIs with large molecular weights may be appropriate for the delivery of self-amplifying mRNAs because of self-amplifying mRNAs' great size (12–14 kb) and complexity. However, the high molecular weight of PEI is associated with higher toxicity [64], and in the case of non-replicative mRNA, large PEI-polyplex delivery vehicles usually are too stable to release mRNA in the cytoplasm [8]. However, the transfection activity of smaller PEIs is poor. To mitigate these disadvantages, our group developed a CP/mRNA nanocomplex formulation for nasal delivery against HIV [37, 82]. CP is a polymer synthesized with β-cyclodextrin (molecular weight, 1,135 Da) and either branched PEI2k or PEI600. This PEI modification helps the mRNA vaccine to safely cross epithelial barriers and reach the nasal-associated lymphoid tissue [37], while retaining the potent mucosal adjuvant activity of PEI [82]. Screening molecular weights of branched PEI molecules as well as N/P ratios of PEI to mRNA showed that a CP2k/mRNA nanocomplex prepared at N/P 16 transfected cells most efficiently *in vitro* and elicited the strongest immune responses *in vivo*. Intranasal inoculation with this formulation led to strong systemic and mucosal immune responses involving a balanced Th1/Th2/Th17 profile. The fact that CP2k/mRNA nanocomplexes triggered lower production of type I IFN than naked mRNA suggested that the immunostimulatory effects of mRNA delivered in CP2k/mRNA nanocomplexes were weakened. Nevertheless, CP2k/mRNA complex triggered moderately higher production of type I IFN than untreated mice, indicating that the nanocomplex formulation still triggered a slight innate immune response. The CP2k/mRNA nanocomplex may achieve a balance between antigen-specific immune response and innate immunity.

Apart from PEI and its derivatives, other polymers that have been used as gene delivery vectors were demonstrated to form polyplex nanoparticles with mRNA and facilitate expression of encoded proteins, such as poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) [83]. Charge-altering releasable transporters (CARTs), which serve initially as oligo(α-amino ester) cations that complex mRNA and then change physical properties through a degradative, charge-neutralizing intramolecular rearrangement to release mRNA in the cytoplasm, have been used to promote systemic mRNA delivery and subsequent translation *in vivo* [84, 85]. These polyplex vectors may prove powerful for delivering mRNA vaccines, but further research is necessary.

5.2 Micelleplex nanoparticles

Micelleplexes have the same characteristics of polymeric micelles, including possessing a spherical inner core constituted by hydrophobic blocks and an outer shell constituted by hydrophilic units [86]. In addition, they are positively charged, facilitating their interactions with nucleic acids. Micelleplex formulation offers the possibility to obtain a combined therapy (drug and nucleic acid delivery), using the same system [87].

The first micelleplex system to deliver mRNA vaccines was developed in our group using branched PEI2k and stearic acid conjugates (PSA) [88]. Those so-called PSA/mRNA micelles were taken up quite efficiently by cells, and they effectively escaped endosomes. Subcutaneous immunization of PSA micelles containing HIV gag mRNA into mice induced production of anti-gag antibodies and a gag-specific T cell response. PSA/mRNA micelles were capable of stimulating DC maturation and their safety profile was better than that of PEI/mRNA complexes, indicating that this PSA/mRNA nanomicelle formulation had the potential to be an efficient and safe vaccine delivery system.

Most other reports of micelleplexes to deliver mRNA have focused on non-vaccine applications. Nanomicelles based on various PEG-polyaspartic acid (PAsp) block copolymers have been used to deliver mRNA for protein replacement [89], cellular reprogramming [90], and cancer therapy [91]. Variations of PEG-PAsp block copolymers used to deliver mRNA include PEG-PAsp(poly(N′-(N-(2-aminoethyl)-2-aminoethyl)aspartamide)) (DET), PEG-PAsp-(poly((N‴(N″(N′-(N-(2-aminoethyl)-2-amino-ethyl)2-aminoethyl)-2-aminoethyl)aspartamide)) (TEP), and PEG-PAsp(poly(N″(N′-(N-(2-aminoethyl)-2-aminoethyl)-2-aminoethyl)aspartamide)) (TET). These micelles have been used to drive expression of the encoded protein

in nasal neurons [92], livers [89], knee joints [90], and tumor sites [91]. It may not be possible to apply these nanomicelles directly to the delivery of mRNA vaccines, since such vaccines need to target immune organs and cells. Future work should examine the capability of nanomicelles to elicit strong immune responses.

6 Hybrid vectors

The vectors for mRNA vaccine delivery may consist of multiple materials sometimes, which can be categorized as "hybrid vectors". These hybrid formulations normally integrate potential advantages of their components and provide more flexibility compared with non-hybrid systems [93].

6.1 Lipopolyplexes

Lipopolyplexes are typical hybrid vectors (Fig. 3(c)). They consist of a preformed nucleic acid-polycation complex core and a lipid shell, forming ternary complexes [94–96]. On the basis of the polycationic component, they are divided into cationic peptide-based lipopolyplexes and cationic polymer-based lipopolyplexes [97]. They combine the advantages of both lipoplexes (high stability, low cytotoxicity, acceptable cellular uptake) and polyplexes (homogenous and small particle size, endosomal escape, high transfection activity) [97], and they can perfectly protect mRNA from degradation. One study found that lipopolyplexes consisting of histidylated cationic lipids, histidine-rich polymers, both of which were reported to be efficient gene delivery vectors [98–100], and mRNA encoding MART1, but not corresponding lipoplexes or polyplexes, significantly inhibited B16 melanoma tumor progression and reduced lung metastasis formation in mice after their intravenous administration [94]. This result indicates that lipopolyplexes are more potent nucleic acid vectors than lipolexes and polyplexes.

Another study used poly-(β-amino ester) (PBAE) to form a complex core with mRNA, which was then encapsulated into a double-layered lipid shell to form a lipopolyplex formulation. This core–shell structured mRNA vaccine displayed intrinsic adjuvant activity and enhanced the antigen-presenting ability of DCs. When mice bearing lung metastatic B16-OVA tumors were subcutaneously vaccinated with this mRNA vaccine, tumors shrank by over 90% [96]. PBAE used in this formulation is an ionizable, degradable polymer. As it is easy to synthesize chemically distinct PBAEs, they have been investigated for a range of gene delivery applications [101, 102]. However, PBAE-mRNA complexes just slightly induced IFN-γ secretion in mice for vaccine applications [10].

6.2 Cationic nanoemulsions

Components other than lipids and polymers can also be incorporated into hybrid vectors. Novartis has reported a cationic nanoemulsion for delivering self-amplifying mRNA vaccines (Fig. 3(c)) [103]. This nanoemulsion formulation was based on the company's proprietary adjuvant MF59, an oil-in-water emulsion consisting of squalene and surfactants. As a safe and potent adjuvant for use with human vaccines [104], MF59 is the second adjuvant, after aluminum, to become commercially available. Starting from MF59 as a base, the researchers added DOTAP to the oil phase to bind the mRNA electrostatically. This method of delivering self-amplifying mRNA elicited immune responses as potent as those triggered by viral vectors in mice, rabbits, and non-human primates with antigens against respiratory syncytial virus, human immunodeficiency virus, and human cytomegalovirus. In addition, the cationic nanoemulsion enhanced the local immune environment after intramuscular injection; it did so by recruiting immune cells, similar to subunit vaccines containing MF59 adjuvant. The same cationic nanoemulsions have been described previously for delivery of plasmid DNA [105].

7 Preclinical and clinical applications

7.1 Cancer immunotherapy

Their ability to induce antigen-specific effector T cells makes mRNA vaccines of tremendous interest for cancer immunotherapy. They can encode either tumor-associated antigens or neo-antigens [75, 106]. To be effective, they are selectively internalized by DCs. Once inside, the mRNA is translated in the cytoplasm, and the resulting polypeptide is processed into epitope peptides that bind to MHC class I molecules, which

are presented to naïve T cells, initiating a tumor-specific T cell response.

Anti-cancer mRNA vaccines can be administered directly by injection, in which case the administration route and delivery vehicle can greatly influence immunization outcomes [21, 107]. Lipoplexes, LNPs, and lipopolyplexes can deliver anti-cancer mRNA vaccines and induce potent anti-tumor immune responses [62, 75, 96]. Anti-cancer mRNA vaccines can also be pre-loaded into DCs *in vitro* [54, 108]. These DC-based vaccines sidestep the issue of targeting DCs *in vivo* [35]. Clinical trials have been performed for mRNA vaccines either injected directly or loaded into DCs [62, 109], and these vaccines have targeted a range of tumors including melanoma, renal cell carcinoma, pancreatic cancer, breast cancer, prostate cancer, glioblastoma, mesothelioma, brain metastases, and ovarian cancer [107, 110].

The efficacy of mRNA vaccines can be improved by designing greater self-adjuvanticity, or by co-delivering various adjuvants [7]. For example, combining naked mRNA with granulocyte-macrophage colony-stimulating factor (GM-CSF) induces a primarily Th1 immune response, while naked mRNA alone induces a Th2 response [111]. Combining naked mRNA and an adjuvant comprising protamine-mRNA complexes induces a balanced TLR 7-dependent adaptive response [112, 113]. DC responses can be strengthened using a so-called "TriMix" of mRNAs encoding CD40L, CD70, and truncated, constitutively active TLR 4 [114].

In fact, mRNA vaccines can be used in combination with other agents or therapies. For example, mRNA vaccines can synergize with radiation therapy to eliminate tumors in preclinical animal models [115]. Data from an ongoing clinical trial suggests that the combination of an mRNA vaccine and tyrosine kinase inhibitor called Sutent (sunitinib) can extend survival time for patients with renal cell carcinoma [116]. Preclinical animal studies [117, 118] and clinical trials [119] have tested the effects of combining mRNA vaccines with immune checkpoint inhibitors.

In addition, personalized anti-cancer therapy becomes easily accessible with mRNA vaccines, since mRNAs can be rapidly and affordably synthesized to encode tumor-associated mutations unique to each patient [120]. These mutations can be identified using next-generation sequencing and their immunogenicity can be predicted using immunoinformatics, paving the way for clinical trials [121].

7.2 Vaccines against infectious diseases

For targeting infectious disease, mRNA vaccines present several advantages over traditional ones. Vaccines based on mRNA can be optimized and produced rapidly on-demand, making them well-suited as rapid responses to emerging pathogens or sudden outbreaks [122]. A new modified dendrimer-RNA nanoparticle vaccine system can be developed in approximately 1 week [122], while traditional vaccines based on cell cultures or fertilized eggs can require at least 6 months [123, 124]. In addition, mRNA vaccines can induce a CTL response to clear virus-infected cells and eradicate the cellular reservoir of pathogens [5]. To date, about 10 mRNA vaccines against HIV-1, rabies virus, Zika virus, and influenza virus have entered clinical trials [107, 110].

Both non-replicating and self-amplifying mRNAs have been used as vaccines against infectious disease. LNPs, polyplex, and micelleplex nanoparticles have been shown to efficiently deliver non-replicating mRNA-encoding viral antigens *in vivo*, where they elicited potent immune responses. DC mRNA vaccines have been exploited primarily to treat HIV-infected individuals [107]. In one study, a series of 4 vaccinations with autologous DCs previously electroporated with mRNAs encoding HIV antigens elicited specific responses in HIV-1 infected patients who were stable on combination antiretroviral therapy, and it did not cause severe adverse events [125].

The best-studied self-amplifying mRNAs are derived from alphavirus genomic sequences [126] in which structural genes have been replaced with antigen(s)-encoding sequences while genes encoding replication machinery remain intact [107] (Fig. 2). The advantages of self-amplifying mRNA include a relatively small dose needed to induce immune responses [56] and intrinsic adjuvant property produced by replication intermediates [107]. Self-amplifying mRNAs can be delivered by viral vectors, but the use of packaging cell lines or large-scale electroporation remains a concern [127]. However, the non-viral delivery is a challenge, for self-amplifying mRNA is usually ca.

9 kb long, which is much bigger than that of non-replicating mRNA. Multiple self-amplifying mRNAs have been delivered in dendrimer-RNA nanoparticles and cationic nanoemulsions as well as PEI-based polyplexes [103, 122, 128].

8 Conclusion and future perspective

The promising safety and efficacy of mRNA vaccines against cancer and infectious diseases in preclinical studies make them quite attractive and has led to several clinical trials. Appropriate delivery systems can overcome the limited stability, translation efficiency, and cell targeting of naked mRNA. However, most mRNA vaccines in clinical trials are administered without such delivery systems, suggesting that their further development is needed in order to exploit mRNA vaccines to their full potential.

Safety is the primary issue that hinders the development of delivery systems, which is mainly determined by the cationic nature of the vectors. Positively charged particles are likely to bind with negatively charged proteins *in vivo*, precipitate in huge clusters, and adhere to cell surfaces, which could destabilize the plasma-membrane and induce the immediate toxicity [129]. Although near-neutral or slightly negatively charged particles can be obtained by adjusting the ratio of cationic lipids or polymers to anionic mRNAs, free cationic vectors are positively charged and are toxic [130]. As alternatives, ionizable lipids are introduced to formulate LNPs. Modifications to lipid or polymer structures are also made to reduce toxicity.

In addition to safety issues, ideal delivery systems should be as simple as possible and synthesized with relative ease in a scalable manner. Currently, lipoplexes and LNPs are the most promising delivery systems. The lipoplex formulation for melanoma immunotherapy and LNP formulation against Zika virus have entered clinical trials, although further research is needed to determine their effectiveness in humans. Researchers should be more dedicated to the development of polymer-based vectors in the future, moving beyond the current focus on lipid-based carriers. Polymers possess high transfection activity, and they are more stable and much cheaper than lipids. More polymers should be investigated for their potential to deliver mRNA vaccines.

Acknowledgements

We acknowledge financial support from the National Natural Science Foundation of China (Nos. 81673362 and 81690261).

References

[1] Iavarone, C.; O'Hagan D, T.; Yu, D.; Delahaye, N. F.; Ulmer, J. B. Mechanism of action of mRNA-based vaccines. *Expert Rev. Vaccines* **2017**, *16*, 871–881.

[2] Martinon, F.; Krishnan, S.; Lenzen, G.; Magné, R.; Gomard, E.; Guillet, J. G.; Lévy, J. P.; Meulien, P. Induction of virus-specific cytotoxic T lymphocytes *in vivo* by liposome-entrapped mRNA. *Eur. J. Immunol.* **1993**, *23*, 1719–7122.

[3] Geall, A. J.; Ulmer, J. B. Introduction to RNA-based vaccines and therapeutics. *Expert Rev. Vaccines* **2015**, *14*, 151–152.

[4] Rodríguez-Gascón, A.; del Pozo-Rodríguez, A.; Solinís, M. Á. Development of nucleic acid vaccines: Use of self-amplifying RNA in lipid nanoparticles. *Int. J. Nanomedicine* **2014**, *9*, 1833–1843.

[5] Pollard, C.; De Koker, S.; Saelens, X.; Vanham, G.; Grooten, J. Challenges and advances towards the rational design of mRNA vaccines. *Trends Mol. Med.* **2013**, *19*, 705–713.

[6] Hekele, A.; Bertholet, S.; Archer, J.; Gibson, D. G.; Palladino, G.; Brito, L. A.; Otten, G. R.; Brazzoli, M.; Buccato, S.; Bonci, A. et al. Rapidly produced SAM® vaccine against H7N9 influenza is immunogenic in mice. *Emerg. Microbes Infect.* **2013**, *2*, e52.

[7] Schlake, T.; Thess, A.; Fotin-Mleczek, M.; Kallen, K. J. Developing mRNA-vaccine technologies. *RNA Biol.* **2012**, *9*, 1319–1330.

[8] Bettinger, T.; Carlisle, R. C.; Read, M. L.; Ogris, M.; Seymour, L. W. Peptide-mediated RNA delivery: A novel approach for enhanced transfection of primary and post-mitotic cells. *Nucleic Acids Res.* **2001**, *29*, 3882–3891.

[9] Sharova, L. V.; Sharov, A. A.; Nedorezov, T.; Piao, Y.; Shaik, N.; Ko, M. S. H. Database for mRNA half-life of 19 977 genes obtained by DNA microarray analysis of pluripotent and differentiating mouse embryonic stem cells. *DNA Res.* **2009**, *16*, 45–58.

[10] Pollard, C.; Rejman, J.; De Haes, W.; Verrier, B.; Van Gulck, E.; Naessens, T.; De Smedt, S.; Bogaert, P.; Grooten, J.; Vanham, G. et al. Type I IFN counteracts the induction of antigen-specific immune responses by lipid-based delivery of mRNA vaccines. *Mol. Ther.* **2013**, *21*, 251–259.

[11] Weide, B.; Garbe, C.; Rammensee, H. G.; Pascolo, S. Plasmid DNA- and messenger RNA-based anti-cancer vaccination. *Immunol. Lett.* **2008**, *115*, 33–42.

[12] Sahin, U.; Karikó, K.; Türeci, Ö. mRNA-based therapeutics—Developing a new class of drugs. *Nat. Rev. Drug Discov.* **2014**, *13*, 759–780.

[13] Granstein, R. D.; Ding, W. H.; Ozawa, H. Induction of anti-tumor immunity with epidermal cells pulsed with tumor-derived RNA or intradermal administration of RNA. *J. Invest. Dermatol.* **2000**, *114*, 632–636.

[14] Kreiter, S.; Selmi, A.; Diken, M.; Koslowski, M.; Britten, C. M.; Huber, C.; Türeci, Ö.; Sahin, U. Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. *Cancer Res.* **2010**, *70*, 9031–9040.

[15] Probst, J.; Weide, B.; Scheel, B.; Pichler, B. J.; Hoerr, I.; Rammensee, H. G.; Pascolo, S. Spontaneous cellular uptake of exogenous messenger RNA *in vivo* is nucleic acid-specific, saturable and ion dependent. *Gene Ther.* **2007**, *14*, 1175–1180.

[16] Lorenz, C.; Fotin-Mleczek, M.; Roth, G.; Becker, C.; Dam, T. C.; Verdurmen, W. P. R.; Brock, R.; Probst, J.; Schlake, T. Protein expression from exogenous mRNA: Uptake by receptor-mediated endocytosis and trafficking via the lysosomal pathway. *RNA Biol.* **2011**, *8*, 627–636.

[17] Peiser, L.; Mukhopadhyay, S.; Gordon, S. Scavenger receptors in innate immunity. *Curr. Opin. Immunol.* **2002**, *14*, 123–128.

[18] Diken, M.; Kreiter, S.; Selmi, A.; Britten, C. M.; Huber, C.; Türeci, Ö.; Sahin, U. Selective uptake of naked vaccine RNA by dendritic cells is driven by macropinocytosis and abrogated upon DC maturation. *Gene Ther.* **2011**, *18*, 702–708.

[19] Selmi, A.; Vascotto, F.; Kautz-Neu, K.; Türeci, Ö.; Sahin, U.; von Stebut, E.; Diken, M.; Kreiter, S. Uptake of synthetic naked RNA by skin-resident dendritic cells via macropinocytosis allows antigen expression and induction of T-cell responses in mice. *Cancer Immunol. Immunother.* **2016**, *65*, 1075–1083.

[20] Wolff, J. A.; Malone, R. W.; Williams, P.; Chong, W.; Acsadi, G.; Jani, A.; Felgner, P. L. Direct gene-transfer into mouse muscle *in vivo*. *Science* **1990**, *247*, 1465–1468.

[21] Midoux, P.; Pichon, C. Lipid-based mRNA vaccine delivery systems. *Expert Rev. Vaccines* **2015**, *14*, 221–234.

[22] Lazzaro, S.; Giovani, C.; Mangiavacchi, S.; Magini, D.; Maione, D.; Baudner, B.; Geall, A. J.; De Gregorio, E.; D'Oro, U.; Buonsanti, C. Cd8 T-cell priming upon mRNA vaccination is restricted to bone-marrow-derived antigen-presenting cells and may involve antigen transfer from myocytes. *Immunology* **2015**, *146*, 312–326.

[23] Kowalczyk, A.; Doener, F.; Zanzinger, K.; Noth, J.; Baumhof, P.; Fotin-Mleczek, M.; Heidenreich, R. Self-adjuvanted mRNA vaccines induce local innate immune responses that lead to a potent and boostable adaptive immunity. *Vaccine* **2016**, *34*, 3882–3893.

[24] Alexopoulou, L.; Holt, A. C.; Medzhitov, R.; Flavell, R. A. Recognition of double-stranded RNA and activation of NF-κB by toll-like receptor 3. *Nature* **2001**, *413*, 732–738.

[25] Botos, I.; Liu, L.; Wang, Y.; Segal, D. M.; Davies, D. R. The toll-like receptor 3:dsRNA signaling complex. *Biochim. Biophys. Acta* **2009**, *1789*, 667–674.

[26] Ceppi, M.; Ruggli, N.; Tache, V.; Gerber, H.; McCullough, K. C.; Summerfield, A. Double-stranded secondary structures on mRNA induce type I interferon (IFN α/β) production and maturation of mRNA-transfected monocyte-derived dendritic cells. *J. Gene Med.* **2005**, *7*, 452–465.

[27] Diebold, S. S.; Kaisho, T.; Hemmi, H.; Akira, S.; Reis e Sousa, C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **2004**, *303*, 1529–1531.

[28] Diebold, S. S.; Massacrier, C.; Akira, S.; Paturel, C.; Morel, Y.; Reis e Sousa, C. Nucleic acid agonists for toll-like receptor 7 are defined by the presence of uridine ribonucleotides. *Eur. J. Immunol.* **2006**, *36*, 3256–3267.

[29] Forsbach, A.; Nemorin, J. G.; Montino, C.; Müller, C.; Samulowitz, U.; Vicari, A. P.; Jurk, M.; Mutwiri, G. K.; Krieg, A. M.; Lipford, G. B. et al. Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. *J. Immunol.* **2008**, *180*, 3729–3738.

[30] Heil, F.; Hemmi, H.; Hochrein, H.; Ampenberger, F.; Kirschning, C.; Akira, S.; Lipford, G.; Wagner, H.; Bauer, S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **2004**, *303*, 1526–1529.

[31] Sabbah, A.; Chang, T. H.; Harnack, R.; Frohlich, V.; Tominaga, K.; Dube, P. H.; Xiang, Y.; Bose, S. Activation of innate immune antiviral responses by Nod2. *Nat. Immunol.* **2009**, *10*, 1073–1080.

[32] Schlee, M.; Roth, A.; Hornung, V.; Hagmann, C. A.; Wimmenauer, V.; Barchet, W.; Coch, C.; Janke, M.; Mihailovic, A.; Wardle, G. et al. Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* **2009**, *31*, 25–34.

[33] Yoneyama, M.; Kikuchi, M.; Matsumoto, K.; Imaizumi, T.; Miyagishi, M.; Taira, K.; Foy, E.; Loo, Y. M.; Gale, M., Jr.; Akira, S. et al. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* **2005**, *175*, 2851–2858.

[34] Probst, J.; Brechtel, S.; Scheel, B.; Hoerr, I.; Jung, G.; Rammensee, H. G.; Pascolo, S. Characterization of the ribonuclease activity on the skin surface. *Genet. Vaccines Ther.* **2006**, *4*, 4.

[35] Boudreau, J. E.; Bonehill, A.; Thielemans, K.; Wan, Y. H. Engineering dendritic cells to enhance cancer immunotherapy. *Mol. Ther.* **2011**, *19*, 841–853.

[36] Jiang, H.; Wang, Q.; Sun, X. Lymph node targeting strategies to improve vaccination efficacy. *J. Control. Release* **2017**, *267*, 47–56.

[37] Li, M.; Zhao, M. N.; Fu, Y.; Li, Y.; Gong, T.; Zhang, Z. R.; Sun, X. Enhanced intranasal delivery of mRNA vaccine by overcoming the nasal epithelial barrier via intra- and paracellular pathways. *J. Control. Release* **2016**, *228*, 9–19.

[38] Kranz, L. M.; Diken, M.; Haas, H.; Kreiter, S.; Loquai, C.; Reuter, K. C.; Meng, M.; Fritz, D.; Vascotto, F.; Hefesha, H. et al. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* **2016**, *534*, 396–401.

[39] Pascolo, S. Vaccination with messenger RNA (mRNA). In *Toll-Like Receptors (TLRs) and Innate Immunity. Handbook of Experimental Pharmacology*; Bauer, S.; Hartmann, G., Eds.; Springer: Berlin, Heidelberg, 2008; Vol. 183, pp 221–235.

[40] Sonenberg, N.; Hinnebusch, A. G. Regulation of translation initiation in eukaryotes: Mechanisms and biological targets. *Cell* **2009**, *136*, 731–745.

[41] Pasquinelli, A. E.; Dahlberg, J. E.; Lund, E. Reverse 5' caps in RNAs made *in vitro* by phage RNA polymerases. *RNA* **1995**, *1*, 957–967.

[42] Stepinski, J.; Waddell, C.; Stolarski, R.; Darzynkiewicz, E.; Rhoads, R. Synthesis and properties of mRNA containing the novel "anti-reverse" cap analogs 7-methyl (3'-O-methyl)gpppg and 7-methyl (3'-deoxy)GpppG. *RNA* **2001**, *7*, 1486–1495.

[43] Kowalska, J.; Lewdorowicz, M.; Zuberek, J.; Grudzien-Nogalska, E.; Bojarska, E.; Stepinski, J.; Rhoads, R. E.; Darzynkiewicz, E.; Davis, R. E.; Jemielity, J. Synthesis and characterization of mRNA cap analogs containing phosphorothioate substitutions that bind tightly to eIF4E and are resistant to the decapping pyrophosphatase DcpS. *RNA* **2008**, *14*, 1119–1131.

[44] Martin, S. A.; Paoletti, E.; Moss, B. Purification of mRNA guanylyltransferase and mRNA (guanine-7-) methyltransferase from vaccinia virions. *J. Biol. Chem.* **1975**, *250*, 9322–9329.

[45] Holtkamp, S.; Kreiter, S.; Selmi, A.; Simon, P.; Koslowski, M.; Huber, C.; Türeci, Ö.; Sahin, U. Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. *Blood* **2006**, *108*, 4009–4017.

[46] Körner, C. G.; Wahle, E. Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. *J. Biol. Chem.* **1997**, *272*, 10448–1056.

[47] Krieg, P. A.; Melton, D. A. Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cdnas. *Nucleic Acids Res.* **1984**, *12*, 7057–7070.

[48] Bossi, L.; Roth, J. R. The influence of codon context on genetic code translation. *Nature* **1980**, *286*, 123–127.

[49] Zhong, F.; Cao, W. P.; Chan, E.; Tay, P. N.; Cahya, F. F.; Zhang, H. F.; Lu, J. H. Deviation from major codons in the toll-like receptor genes is associated with low toll-like receptor expression. *Immunology* **2005**, *114*, 83–93.

[50] Gustafsson, C.; Govindarajan, S.; Minshull, J. Codon bias and heterologous protein expression. *Trends Biotechnol.* **2004**, *22*, 346–353.

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

[52] Karikó, K.; Buckstein, M.; Ni, H. P.; Weissman, D. Suppression of RNA recognition by toll-like receptors: The impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* **2005**, *23*, 165–175.

[53] Pardi, N.; Weissman, D. Nucleoside modified mRNA vaccines for infectious diseases. In *RNA Vaccines. Methods in Molecular Biology*; Kramps, T.; Elbers, K., Eds.; Humana Press: New York, NY, 2017; Vol. 1499, pp 109–121.

[54] DeFrancesco, L. The "anti-hype" vaccine. *Nat. Biotechnol.* **2017**, *35*, 193–197.

[55] Yin, H.; Kanasty, R. L.; Eltoukhy, A. A.; Vegas, A. J.; Dorkin, J. R.; Anderson, D. G. Non-viral vectors for gene-based therapy. *Nat. Rev. Genet.* **2014**, *15*, 541–555.

[56] Vogel, A. B.; Lambert, L.; Kinnear, E.; Busse, D.; Erbar, S.; Reuter, K. C.; Wicke, L.; Perkovic, M.; Beissert, T.; Haas, H. et al. Self-amplifying RNA vaccines give equivalent protection against influenza to mRNA vaccines but at much lower doses. *Mol. Ther.* **2018**, *26*, 446–455.

[57] Bonehill, A.; Heirman, C.; Tuyaerts, S.; Michiels, A.; Breckpot, K.; Brasseur, F.; Zhang, Y.; Van Der Bruggen, P.; Thielemans, K. Messenger RNA-electroporated dendritic cells presenting MAGE-A3 simultaneously in HLA class I and class II molecules. *J. Immunol.* **2004**, *172*, 6649–6657.

[58] Torchilin, V. P. Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discov.* **2005**, *4*, 145–160.

[59] Perri, V.; Pellegrino, M.; Ceccacci, F.; Scipioni, A.; Petrini,

S.; Gianchecchi, E.; Lo Russo, A.; De Santis, S.; Mancini, G.; Fierabracci, A. Use of short interfering RNA delivered by cationic liposomes to enable efficient down-regulation of *PTPN22* gene in human T lymphocytes. *PLoS One* **2017**, *12*, e0175784.

[60] Zhi, D. F.; Zhang, S. B.; Cui, S. H.; Zhao, Y.; Wang, Y. H.; Zhao, D. F. The headgroup evolution of cationic lipids for gene delivery. *Bioconjugate Chem.* **2013**, *24*, 487–519.

[61] Mintzer, M. A.; Simanek, E. E. Nonviral vectors for gene delivery. *Chem. Rev.* **2009**, *109*, 259–302.

[62] Grabbe, S.; Haas, H.; Diken, M.; Kranz, L. M.; Langguth, P.; Sahin, U. Translating nanoparticulate-personalized cancer vaccines into clinical applications: Case study with RNA-lipoplexes for the treatment of melanoma. *Nanomedicine* **2016**, *11*, 2723–2734.

[63] Hess, P. R.; Boczkowski, D.; Nair, S. K.; Snyder, D.; Gilboa, E. Vaccination with mRNAs encoding tumor-associated antigens and granulocyte-macrophage colony-stimulating factor efficiently primes CTL responses, but is insufficient to overcome tolerance to a model tumor/self antigen. *Cancer Immunol. Immun.* **2006**, *55*, 672–683.

[64] Lv, H. T.; Zhang, S. B.; Wang, B.; Cui, S. H.; Yan, J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release* **2006**, *114*, 100–109.

[65] Hajj, K. A.; Whitehead, K. A. Tools for translation: Non-viral materials for therapeutic mRNA delivery. *Nat. Rev. Mater.* **2017**, *2*, 17056.

[66] Whitehead, K. A.; Langer, R.; Anderson, D. G. Knocking down barriers: Advances in siRNA delivery. *Nat. Rev. Drug Discov.* **2009**, *8*, 129–138.

[67] Reichmuth, A. M.; Oberli, M. A.; Jeklenec, A.; Langer, R.; Blankschtein, D. mRNA vaccine delivery using lipid nanoparticles. *Ther. Deliv.* **2016**, *7*, 319–334.

[68] Walsh, C. L.; Nguyen, J.; Tiffany, M. R.; Szoka, F. C. Synthesis, characterization, and evaluation of ionizable lysine-based lipids for siRNA delivery. *Bioconjugate Chem.* **2013**, *24*, 36–43.

[69] Granot, Y.; Peer, D. Delivering the right message: Challenges and opportunities in lipid nanoparticles-mediated modified mRNA therapeutics—An innate immune system standpoint. *Semin. Immunol.* **2017**, *34*, 68–77.

[70] Chen, S.; Tam, Y. Y. C.; Lin, P. J. C.; Sung, M. M. H.; Tam, Y. K.; Cullis, P. R. Influence of particle size on the *in vivo* potency of lipid nanoparticle formulations of siRNA. *J. Control Release* **2016**, *235*, 236–244.

[71] Jayaraman, M.; Ansell, S. M.; Mui, B. L.; Tam, Y. K.; Chen, J. X.; Du, X. Y.; Butler, D.; Eltepu, L.; Matsuda, S.; Narayanannair, J. K. et al. Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing *in vivo*. *Angew. Chem., Int. Ed.* **2012**, *51*, 8529–8533.

[72] Maier, M. A.; Jayaraman, M.; Matsuda, S.; Liu, J.; Barros, S.; Querbes, W.; Tam, Y. K.; Ansell, S. M.; Kumar, V.; Qin, J. et al. Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Mol. Ther.* **2013**, *21*, 1570–1578.

[73] Pardi, N.; Hogan, M. J.; Pelc, R. S.; Muramatsu, H.; Andersen, H.; DeMaso, C. R.; Dowd, K. A.; Sutherland, L. L.; Scearce, R. M.; Parks, R. et al. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. *Nature* **2017**, *543*, 248–251.

[74] Richner, J. M.; Himansu, S.; Dowd, K. A.; Butler, S. L.; Salazar, V.; Fox, J. M.; Julander, J. G.; Tang, W. W.; Shresta, S.; Pierson, T. C. et al. Modified mRNA vaccines protect against Zika virus infection. *Cell* **2017**, *168*, 1114–1125.e10.

[75] Oberli, M. A.; Reichmuth, A. M.; Dorkin, J. R.; Mitchell, M. J.; Fenton, O. S.; Jaklenec, A.; Anderson, D. G.; Langer, R.; Blankschtein, D. Lipid nanoparticle assisted mRNA delivery for potent cancer immunotherapy. *Nano Lett.* **2017**, *17*, 1326–1335.

[76] Gary, D. J.; Lee, H.; Sharma, R.; Lee, J. S.; Kim, Y.; Cui, Z. Y.; Jia, D.; Bowman, V. D.; Chipman, P. R.; Wan, L. et al. Influence of nano-carrier architecture on *in vitro* siRNA delivery performance and *in vivo* biodistribution: Polyplexes vs micelleplexes. *ACS Nano* **2011**, *5*, 3493–3505.

[77] Wang, W.; Li, W.; Ma, N.; Steinhoff, G. Non-viral gene delivery methods. *Curr. Pharm. Biotechnol.* **2013**, *14*, 46–60.

[78] Elouahabi, A.; Ruysschaert, J. M. Formation and intracellular trafficking of lipoplexes and polyplexes. *Mol. Ther.* **2005**, *11*, 336–347.

[79] Lungwitz, U.; Breunig, M.; Blunk, T.; Göpferich, A. Polyethylenimine-based non-viral gene delivery systems. *Eur. J. Pharm. Biopharm.* **2005**, *60*, 247–266.

[80] Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: Polyethylenimine. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297–7301.

[81] Démoulins, T.; Milona, P.; Englezou, P. C.; Ebensen, T.; Schulze, K.; Suter, R.; Pichon, C.; Midoux, P.; Guzmán, C. A.; Ruggli, N. et al. Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines. *Nanomedicine* **2016**, *12*, 711–722.

[82] Li, M.; Li, Y.; Peng, K.; Wang, Y.; Gong, T.; Zhang, Z. R.; He, Q.; Sun, X. Engineering intranasal mRNA vaccines to enhance lymph node trafficking and immune responses. *Acta Biomater.* **2017**, *64*, 237–248.

[83] Üzgün, S.; Nica, G.; Pfeifer, C.; Bosinco, M.; Michaelis, K.; Lutz, J. F.; Schneider, M.; Rosenecker, J.; Rudolph, C. Pegylation improves nanoparticle formation and transfection efficiency of messenger RNA. *Pharm. Res.* **2011**, *28*, 2223–2232.

[84] McKinlay, C. J.; Vargas, J. R.; Blake, T. R.; Hardy, J. W.; Kanada, M.; Contag, C. H.; Wender, P. A.; Waymouth, R. M. Charge-altering releasable transporters (CARTs) for the delivery and release of mRNA in living animals. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E448–E456.

[85] Jarzębińska, A.; Pasewald, T.; Lambrecht, J.; Mykhaylyk, O.; Kümmerling, L.; Beck, P.; Hasenpusch, G.; Rudolph, C.; Plank, C.; Dohmen, C. et al. A single methylene group in oligoalkylamine-based cationic polymers and lipids promotes enhanced mRNA delivery. *Angew. Chem., Int. Ed.* **2016**, *55*, 9591–9595.

[86] Almeida, M.; Magãlhes, M.; Veiga, F.; Figueiras, A. Poloxamers, poloxamines and polymeric micelles: Definition, structure and therapeutic applications in cancer. *J. Polym. Res.* **2018**, *25*, 31.

[87] Jhaveri, A. M.; Torchilin, V. P. Multifunctional polymeric micelles for delivery of drugs and siRNA. *Front. Pharmacol.* **2014**, *5*, 77.

[88] Zhao, M. N.; Li, M.; Zhang, Z. R.; Gong, T.; Sun, X. Induction of HIV-1 gag specific immune responses by cationic micelles mediated delivery of gag mRNA. *Drug Deliv.* **2016**, *23*, 2596–2607.

[89] Matsui, A.; Uchida, S.; Ishii, T.; Itaka, K.; Kataoka, K. Messenger RNA-based therapeutics for the treatment of apoptosis-associated diseases. *Sci. Rep.* **2015**, *5*, 15810.

[90] Aini, H.; Itaka, K.; Fujisawa, A.; Uchida, H.; Uchida, S.; Fukushima, S.; Kataoka, K.; Saito, T.; Chung, U. I.; Ohba, S. Messenger RNA delivery of a cartilage-anabolic transcription factor as a disease-modifying strategy for osteoarthritis treatment. *Sci. Rep.* **2016**, *6*, 18743.

[91] Uchida, S.; Kinoh, H.; Ishii, T.; Matsui, A.; Tockary, T. A.; Takeda, K. M.; Uchida, H.; Osada, K.; Itaka, K.; Kataoka, K. Systemic delivery of messenger RNA for the treatment of pancreatic cancer using polyplex nanomicelles with a cholesterol moiety. *Biomaterials* **2016**, *82*, 221–228.

[92] Baba, M.; Itaka, K.; Kondo, K.; Yamasoba, T.; Kataoka, K. Treatment of neurological disorders by introducing mRNA *in vivo* using polyplex nanomicelles. *J. Control. Release* **2015**, *201*, 41–48.

[93] Guan, S.; Rosenecker, J. Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems. *Gene Ther.* **2017**, *24*, 133–143.

[94] Mockey, M.; Bourseau, E.; Chandrashekhar, V.; Chaudhuri, A.; Lafosse, S.; Le Cam, E.; Quesniaux, V. F. J.; Ryffel, B.; Pichon, C.; Midoux, P. mRNA-based cancer vaccine: Prevention of B16 melanoma progression and metastasis by systemic injection of MART1 mRNA histidylated lipopolyplexes. *Cancer Gene Ther.* **2007**, *14*, 802–814.

[95] Perche, F.; Benvegnu, T.; Berchel, M.; Lebegue, L.; Pichon, C.; Jaffres, P. A.; Midoux, P. Enhancement of dendritic cells transfection *in vivo* and of vaccination against B16F10 melanoma with mannosylated histidylated lipopolyplexes loaded with tumor antigen messenger RNA. *Nanomedicine* **2011**, *7*, 445–453.

[96] Persano, S.; Guevara, M. L.; Li, Z. Q.; Mai, J. H.; Ferrari, M.; Pompa, P. P.; Shen, H. F. Lipopolyplex potentiates anti-tumor immunity of mRNA-based vaccination. *Biomaterials* **2017**, *125*, 81–89.

[97] Rezaee, M.; Oskuee, R. K.; Nassirli, H.; Malaekeh-Nikouei, B. Progress in the development of lipopolyplexes as efficient non-viral gene delivery systems. *J. Control. Release* **2016**, *236*, 1–14.

[98] Singh, R. S.; Gonçalves, C.; Sandrin, P.; Pichon, C.; Midoux, P.; Chaudhuri, A. On the gene delivery efficacies of pH-sensitive cationic lipids via endosomal protonation: A chemical biology investigation. *Chem. Biol.* **2004**, *11*, 713–723.

[99] Kumar, V. V.; Pichon, C.; Refregiers, M.; Guerin, B.; Midoux, P.; Chaudhuri, A. Single histidine residue in head-group region is sufficient to impart remarkable gene transfection properties to cationic lipids: Evidence for histidine-mediated membrane fusion at acidic pH. *Gene Ther.* **2003**, *10*, 1206–1215.

[100] Pichon, C.; Gonçalves, C.; Midoux, P. Histidine-rich peptides and polymers for nucleic acids delivery. *Adv. Drug Deliv. Rev.* **2001**, *53*, 75–94.

[101] Anderson, D. G.; Lynn, D. M.; Langer, R. Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery. *Angew. Chem., Int. Ed.* **2003**, *42*, 3153–3158.

[102] Zugates, G. T.; Peng, W. D.; Zumbuehl, A.; Jhunjhunwala, S.; Huang, Y. H.; Langer, R.; Sawicki, J. A.; Anderson, D. G. Rapid optimization of gene delivery by parallel end-modification of poly(β-amino ester)s. *Mol. Ther.* **2007**, *15*, 1306–1312.

[103] Brito, L. A.; Chan, M.; Shaw, C. A.; Hekele, A.; Carsillo, T.; Schaefer, M.; Archer, J.; Seubert, A.; Otten, G. R.; Beard, C. W. et al. A cationic nanoemulsion for the delivery of next-generation RNA vaccines. *Mol. Ther.* **2014**, *22*, 2118–2129.

[104] Ott, G.; Barchfeld, G. L.; Chernoff, D.; Radhakrishnan, R.;

van Hoogevest, P.; Van Nest, G. MF59 design and evaluation of a safe and potent adjuvant for human vaccines. In *Vaccine Design. Pharmaceutical Biotechnology*; Powell, M. F.; Newman, M. J., Eds.; Springer: Boston, MA, 1995; Vol. 6, pp 277–296.

[105] Choi, W. J.; Kim, J. K.; Choi, S. H.; Park, J. S.; Ahn, W. S.; Kim, C. K. Low toxicity of cationic lipid-based emulsion for gene transfer. *Biomaterials* **2004**, *25*, 5893–5903.

[106] Forghanifard, M. M.; Gholamin, M.; Moaven, O.; Farshchian, M.; Ghahraman, M.; Aledavood, A.; Abbaszadegan, M. R. Neoantigen in esophageal squamous cell carcinoma for dendritic cell-based cancer vaccine development. *Med. Oncol.* **2014**, *31*, 191.

[107] Pardi, N.; Hogan, M. J.; Porter, F. W.; Weissman, D. mRNA vaccines—A new era in vaccinology. *Nat. Rev. Drug Discov.* **2018**, *17*, 261–279.

[108] Kreiter, S.; Diken, M.; Selmi, A.; Türeci, Ö.; Sahin, U. Tumor vaccination using messenger RNA: Prospects of a future therapy. *Curr. Opin. Immunol.* **2011**, *23*, 399–406.

[109] Kyte, J. A.; Aamdal, S.; Dueland, S.; Sæbøe-Larsen, S.; Inderberg, E. M.; Madsbu, U. E.; Skovlund, E.; Gaudernack, G.; Kvalheim, G. Immune response and long-term clinical outcome in advanced melanoma patients vaccinated with tumor-mRNA-transfected dendritic cells. *Oncoimmunology* **2016**, *5*, e1232237.

[110] Kaczmarek, J. C.; Kowalski, P. S.; Anderson, D. G. Advances in the delivery of RNA therapeutics: From concept to clinical reality. *Genome Med.* **2017**, *9*, 60.

[111] Carralot, J. P.; Probst, J.; Hoerr, I.; Scheel, B.; Teufel, R.; Jung, G.; Rammensee, H. G.; Pascolo, S. Polarization of immunity induced by direct injection of naked sequence-stabilized mRNA vaccines. *Cell. Mol. Life Sci.* **2004**, *61*, 2418–2424.

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

[113] Fotin-Mleczek, M.; Zanzinger, K.; Heidenreich, R.; Lorenz, C.; Thess, A.; Duchardt, K. M.; Kallen, K. J. Highly potent mRNA based cancer vaccines represent an attractive platform for combination therapies supporting an improved therapeutic effect. *J. Gene Med.* **2012**, *14*, 428–439.

[114] Van Lint, S.; Goyvaerts, C.; Maenhout, S.; Goethals, L.; Disy, A.; Benteyn, D.; Pen, J.; Bonehill, A.; Heirman, C.; Breckpot, K. et al. Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy. *Cancer Res.* **2012**, *72*, 1661–1671.

[115] Fotin-Mleczek, M.; Zanzinger, K.; Heidenreich, R.; Lorenz, C.; Kowalczyk, A.; Kallen, K. J.; Huber, S. M. mRNA-based vaccines synergize with radiation therapy to eradicate established tumors. *Radiat. Oncol.* **2014**, *9*, 180.

[116] Motzer, R. J.; Escudier, B.; Bukowski, R.; Rini, B. I.; Hutson, T. E.; Barrios, C. H.; Lin, X.; Fly, K.; Matczak, E.; Gore, M. E. Prognostic factors for survival in 1059 patients treated with sunitinib for metastatic renal cell carcinoma. *Br. J. Cancer* **2013**, *108*, 2470–2477.

[117] Wang, Y. H.; Zhang, L.; Xu, Z. H.; Miao, L.; Huang, L. mRNA vaccine with antigen-specific checkpoint blockade induces an enhanced immune response against established melanoma. *Mol. Ther.* **2018**, *26*, 420–434.

[118] Liu, L.; Wang, Y. H.; Miao, L.; Liu, Q.; Musetti, S.; Li, J.; Huang, L. Combination immunotherapy of MUC1 mRNA nano-vaccine and CTLA-4 blockade effectively inhibits growth of triple negative breast cancer. *Mol. Ther.* **2018**, *26*, 45–55.

[119] Wilgenhof, S.; Corthals, J.; Heirman, C.; van Baren, N.; Lucas, S.; Kvistborg, P.; Thielemans, K.; Neyns, B. Phase II study of autologous monocyte-derived mRNA electroporated dendritic cells (TriMixDC-MEL) plus ipilimumab in patients with pretreated advanced melanoma. *J. Clin. Oncol.* **2016**, *34*, 1330–1338.

[120] Diken, M.; Kreiter, S.; Kloke, B.; Sahin, U. Current developments in actively personalized cancer vaccination with a focus on RNA as the drug format. *Prog. Tumor Res.* **2015**, *42*, 44–54.

[121] Boisguérin, V.; Castle, J. C.; Loewer, M.; Diekmann, J.; Mueller, F.; Britten, C. M.; Kreiter, S.; Türeci, Ö.; Sahin, U. Translation of genomics-guided RNA-based personalised cancer vaccines: Towards the bedside. *Br. J. Cancer* **2014**, *111*, 1469–1475.

[122] Chahal, J. S.; Khan, O. F.; Cooper, C. L.; McPartlan, J. S.; Tsosie, J. K.; Tilley, L. D.; Sidik, S. M.; Lourido, S.; Langer, R.; Bavari, S. et al. Dendrimer-RNA nanoparticles generate protective immunity against lethal Ebola, H1N1 influenza, and *Toxoplasma gondii* challenges with a single dose. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E4133–E4142.

[123] Chen, Z. Y.; Wang, W. J.; Zhou, H. L.; Suguitan, A. L., Jr.; Shambaugh, C.; Kim, L.; Zhao, J.; Kemble, G.; Jin, H. Generation of live attenuated novel influenza virus a/california/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs. *J. Virol.* **2010**, *84*, 44–51.

[124] Wurm, F. M. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat. Biotechnol.* **2004**, *22*, 1393–1398.

[125] Allard, S. D.; De Keersmaecker, B.; de Goede, A. L.; Verschuren, E. J.; Koetsveld, J.; Reedijk, M. L.; Wylock, C.; De Bel, A. V.; Vandeloo, J.; Pistoor, F. et al. A phase I/IIa immunotherapy trial of HIV-1-infected patients with Tat, Rev and Nef expressing dendritic cells followed by treatment interruption. *Clin. Immunol.* **2012**, *142*, 252–268.

[126] Deering, R. P.; Kommareddy, S.; Ulmer, J. B.; Brito, L. A.; Geall, A. J. Nucleic acid vaccines: Prospects for non-viral delivery of mRNA vaccines. *Expert Opin. Drug Deliv.* **2014**, *11*, 885–899.

[127] Brito, L. A.; Kommareddy, S.; Maione, D.; Uematsu, Y.; Giovani, C.; Berlanda Scorza, F.; Otten, G. R.; Yu, D.; Mandl, C. W.; Mason, P. W. et al. Self-amplifying mRNA vaccines. *Adv. Genet* **2015**, *89*, 179–233.

[128] Démoulins, T.; Ebensen, T.; Schulze, K.; Englezou, P. C.; Pelliccia, M.; Guzmán, C. A.; Ruggli, N.; McCullough, K. C. Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. *J. Control. Release* **2017**, *266*, 256–271.

[129] Fischer, D.; Bieber, T.; Li, Y. X.; Elsässer, H. P.; Kissel, T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: Effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* **1999**, *16*, 1273–1279.

[130] Xu, Y. H.; Hui, S. W.; Frederik, P.; Szoka, F. C. Physicochemical characterization and purification of cationic lipoplexes. *Biophys. J.* **1999**, *77*, 341–353.