Acta Pharmaceutica Sinica B 2024;14(1):170–189

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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REVIEW

Advanced nano-based strategies for mRNA tumor vaccine

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Received 17 April 2023; received in revised form 2 July 2023; accepted 18 July 2023

KEY WORDS

mRNA vaccine;
Nanoparticle;
Tumor vaccine;
Delivery system;
Stability;
Targeting;
Transfection;
Immunogenicity

Abstract Tumor vaccine is a promising strategy for cancer immunotherapy by introducing tumor antigens into the body to activate specific anti-tumor immune responses. Along with the technological breakthroughs in genetic engineering and delivery systems, messenger ribonucleic acid (mRNA) technology has achieved unprecedented development and application over the last few years, especially the emergency use authorizations of two mRNA vaccines during the COVID-19 pandemic, which has saved countless lives and makes the world witness the powerful efficacy of mRNA technology in vaccines. However, unlike infectious disease vaccines, which mainly induce humoral immunity, tumor vaccines also need to activate potent cellular immunity to control tumor growth, which creates a higher demand for mRNA delivery to the lymphatic organs and antigen-presenting cells (APCs). Here we review the existing bottlenecks of mRNA tumor vaccines and advanced nano-based strategies to overcome those challenges, as well as future considerations of mRNA tumor vaccines and their delivery systems.

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Peer review under the responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2023.07.025

1. Introduction

In recent years, tumor vaccines have gained prominent momentum in the field of cancer immunotherapy[1]. Through immunizing the body with tumor antigens (inactivated tumor cells, tumor-related proteins or peptides, genes expressing tumor antigens, etc.), tumor vaccines can trigger the patient's adaptive immune responses that specifically recognize and destroy antigen-overexpressing malignant cells, achieve long-term therapeutic response and eventual clearance because of immune memory[2]. However, in the past two decades, the only therapeutic tumor vaccine approved by the U.S. Food and Drug Administration (FDA) is Dendreon's Provenge (Sipuleucel-T), which just prolongs the average survival time of patients by a few months. One of the accepted reasons is that, unlike infectious diseases, tumor antigens are usually diverse and weakly immunogenic, resulting in the inability of traditional recombinant protein or peptide-based vaccines to match the demand for potent tumor-specific immune responses[3]. Therefore, more effective and versatile vaccine platforms are urgently needed.

During the past coronavirus disease 2019 (COVID-19) epidemic, the emergency use authorizations of messenger ribonucleic acid (mRNA) vaccines have saved countless lives and let the world witness the powerful efficacy of mRNA technology in vaccines because of its rapid production, high safety, and effectiveness[4]. To date, three types of COVID-19 mRNA vaccine (Spikevax for Moderna, BNT162b2 for Pfizer, and SYS6006 for CSPC Pharma) have been approved and fully tested in the clinic. The excellent performance of the COVID-19 mRNA vaccine arouses people's interest and confidence in tumor vaccine, especially in mRNA tumor vaccine.

Compared with traditional vaccine platforms, including peptides, dendritic cells (DCs), viral vectors and DNA, the mRNA tumor vaccines have several incomparable advantages[5]: (1) short preparation cycle, for any target protein with known sequence, its mRNA can be synthesized in a short time by *in vitro* transcription, to strive for more treatment time[6]. (2) In general, antigen peptides with high HLA affinity usually are hydrophobic, and the use of mRNA to express these peptides *in vivo* can perfectly solve their poor druggability[7]. (3) mRNA can easily deliver over 20 tumor antigen fragments at the same time, increasing the antigenic diversity and overcoming vaccine resistance[8]. (4) Unlike DNA-based or viral vectors-based vaccines, mRNA is expressed in the cytoplasm, and the dose not enter the nucleus, without risk of insertional mutagenesis[9]. (5) Adjuvant effect, mRNA itself can activate immune cells to release cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-α (IFN-α), to enhance the immune effect of the expressed antigen[10].

Although mRNA vaccines have many of the advantages described above, it is not easy to deliver mRNA intact into cells and translate it effectively. As a large polyanion, mRNA cannot cross non-polar cellular and tissue barriers and also is easily degraded by nucleases[11]. Thus, protecting mRNA with an appropriate delivery vector is essential for its efficient expression *in vivo*. Currently, lipid nanoparticles (LNPs), polymeric nanoparticles, inorganic nanoparticles, and biomimetic nanoparticles are the commonly used nano-based platforms for mRNA delivery[12], among which LNPs are relatively mature platforms validated for mRNA delivery during the COVID-19 pandemic[13]. Nevertheless, some common limitations still restrict the application of mRNA-based drugs for tumor vaccines: (1) against the complex *in vivo* environment, the most commonly used LNP is still weak in improving the in-vivo stability and metabolized quickly[14]. (2) Lack of specific targeting to the immune system, most of the current mRNA vaccines are remained at the injection site or trapped in liver tissue[15]. (3) More than 90% of mRNA internalized by the delivery system is degraded by lysosomes, it's still necessary to increase the lysosomal escape of mRNA to amplify antigen expression and induce a more powerful immune response[16]. (4) In addition, the therapeutic ideas of tumor vaccine and infectious disease vaccine are not the same, for which the protective effect of mRNA vaccines against infectious disease is mainly conferred by strong humoral immunity, and therapeutic mRNA vaccines against cancer, furthermore, also requires potent cytotoxic $CD8^+$ thymus dependent lymphocyte (T cell)-mediated cellular immunity to eradicate malignant cells[3].

In this review, we summarize the targets of mRNA tumor vaccine design, and detail the latest progress in the nano-based strategies to overcome the bottleneck encountered in the delivery of mRNA tumor vaccine from administration to final immune response, eventually enhancing the therapeutic effect. As shown in Fig. 1, it mainly includes some engineering methods based on traditional nano-carriers (*e.g.*, LNPs, polymeric nanoparticles, inorganic nanoparticles) that can optimize performance in terms of stability, targeting specificity, transfection efficiency, and immunogenicity. Finally, we make a brief talk about the future development of mRNA tumor vaccines and their delivery systems.

2. The classification of targets of mRNA tumor vaccines

Traditionally, the targets of mRNA tumor vaccines can be divided into two major categories based on their expression and localization: tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs)[17].

TAAs refer to the non-mutated protein that is highly expressed in tumors and poorly or not expressed in normal tissues, including differentiation antigens, overexpression antigens, and cancer testicular antigens[18]. Differentiation antigens are expressed in tumor cells and cognate healthy cells: For example, prostate-specific antigen (PSA) and prostate acid phosphatase (PAP) are present in normal prostate and prostate cancer. Sipuleucel-T, an autologous cell-derived immunotherapy drug targeting PAP, was approved by the US FDA in 2010 for castration-resistant prostate cancer[19]. Another widely presented category of TAAs is overexpression antigens, which are up-regulated in tumor cells while also expressed in normal cells, such as EGFR and HER2. Overexpression antigens can be directly involved in carcinogenesis by promoting tumor cell growth and survival and are potential targets since T-cell presentation and recognition require a certain threshold level of antigens to activate a specific T-cell response. Immunotherapy targeting the overexpressed antigen HER2/NEU has demonstrated clinical efficacy, and anti-HER2/NEU monoclonal antibodies (trastuzumab, pertuzumab) are approved for the treatment of HER2/NEU overexpression in breast and gastric cancers[20]. Cancer testicular antigens are commonly expressed in germ cells (testis, fetal ovary) and abnormally expressed in lung or bladder cancer and melanoma, and the identified testicular antigens include MAGE family antigens and NY-ESO1. Because germ cells do not express MHC molecules on their surface and cannot present antigens to T cells, the restricted expression of

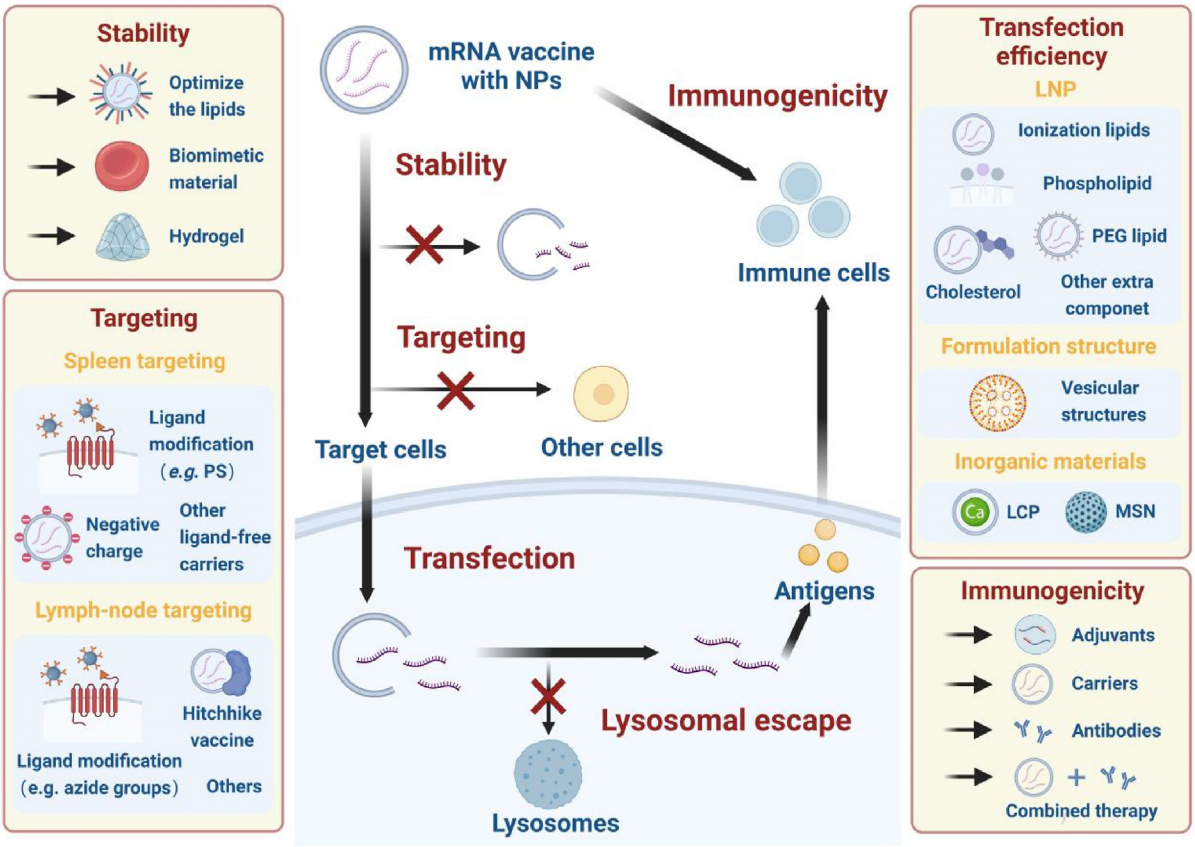

Figure 1 Mechanism of mRNA vaccine *in vivo* and improved engineering strategies to enhance the properties of vaccine based on NPs. Created with Biorender.com.

cancer testicular antigens in tumor cells is a very attractive target for immunotherapy[21]. However, since most of the TAAs are also expressed in non-tumor tissues, which may lead to a paradoxical immune response: (1) failure to elicit anti-tumor immune responses due to autoimmune tolerance mechanisms or (2) induction of autoimmune toxicity due to off-target effects in non-tumor regions, the choice of TAAs as a vaccine target is not optimal to some extent[22].

In contrast to TAAs, TSAs are only expressed in tumor cells, usually derived from oncogenic viral antigens in virus-induced cancers, antigens mutated in somatic cells, or neoantigens generated by abnormal translation and splicing, and considered as "foreignness" by the immune system[23]. The use of TSAs as targets will reduce the damage to normal tissues and can generate T cells with higher specificity[24]. Currently, the antigens identified in HPV-related cancers (head and neck cancer, cervical cancer, anal cancer, etc.) or hepatitis B virus-associated hepatocellular carcinoma are virus-induced oncogenic viral antigens. In the treatment of HPV-related cancers, the first approved prophylactic vaccines, Gardasil® and Cervarix, have been used successfully reducing endocervical incidence in young women. In addition, many studies have shown that TSAs are highly immunogenic, and spontaneous neoantigen-specific T cells have been detected in cancer patients. Therefore, TSAs can be used as preferred targets in the design of mRNA tumor vaccines, which have higher immunogenicity and affinity than TAAs, reducing the risk of autoimmunity[24].

3. The process of delivery *in vivo*

Antigen-presenting cells (APCs) play an indispensable role in the immune response of the body. As the sites for antigen synthesis, processing, and presentation, APCs express antigen-major histocompatibility complex (MHC) complexes on the cell surface and present them to naïve T cells, thereby activating T cells and initiating cellular immunity. The downregulation or loss of tumor-associated antigen presentation by APCs represents a major immune evasion mechanism of cancer cells, which is exactly the issue that mRNA-based tumor vaccines aim to address. upon administration through various delivery methods, mRNA tumor vaccines enter tissues or circulation, where a portion of the vaccine is taken up by APCs and releases mRNA within the cells. The tumor-associated antigens translated from mRNA are degraded by endogenous pathways and form antigen-MHC Class I complexes, which are recognized by $CD8^+$ T cells, leading to cell-mediated immune responses and specific tumor-killing effects[25]. Another part of vaccines transfects nucleated cells such as muscle cells and neutrophils upon entering the body. The antigens expressed intracellularly are then released *via* exocytosis and taken up by APCs. Through an exogenous pathway, the antigens are presented as antigen-MHC Class II complexes to $CD4^+$ T cells, further activating $CD8^+$ T cells and B cells and inducing cellular and humoral immune responses against tumors. Studies have shown that the quantity and quality of activated, tumor-specific $CD8^+$ T cells at the tumor site significantly influence the efficacy of

immunotherapy[26]. Therefore, promoting mRNA acquisition and expression in APCs is a key focus to enhance the efficacy of mRNA tumor vaccines, and efficiently delivering the vaccine to APCs is the first step. However, since APCs and lymphocytes are primarily enriched in peripheral immune organs and tissues, with scattered distribution in other locations, only a small fraction of the vaccine can be directly taken up by APCs at the injection site or in the bloodstream. The rest of the vaccine particles remain unacquired by APCs. To improve the efficacy of the vaccine, it is necessary to target these remaining free vaccines to peripheral immune organs and tissues. Nevertheless, the complex biological environment *in vivo* presents various physiological barriers, posing risks such as degradation, neutralization, and off-target effects for the vaccine. This places higher demands on the safe transport and precise targeting of the mRNA delivery system in the body. In the following sections, we summarize some optimization strategies for vaccine delivery vectors to enhance stability and targeting *in vivo*.

3.1. Improving stability in the internal environment

Vaccines administered *via* intravenous injection undergo a period of circulation in the bloodstream before targeting the spleen, while vaccines administered *via* intramuscular, subcutaneous, or intradermal injection, apart from being partially absorbed by capillaries into the bloodstream, require a physiological process involving tissue fluid to lymphatic vessels before targeting the lymph nodes. Whether in the bloodstream or interstitial tissues, the delivery systems with a positive charge are susceptible to elimination by anionic surface structures of large molecules (such as mucopolysaccharides, serum proteins, extracellular matrix) and non-target cells (such as red blood cells), thereby facing the risk of clearance in the bloodstream or entrapment in interstitial tissues. Thus, negatively charged surface materials are more favorable for the stable transport of vaccines *in vivo*[27]. In addition to the influence of charge, the major challenges for mRNA nanocarriers in the bloodstream are the capture by the mononuclear phagocyte system (MPS) and rapid clearance by the kidneys, which are mediated by opsonization. Opsonin in plasma will be adsorbed to the surface of foreign nanoparticles (NPs) according to the size and surface characteristics of NPs, leading to the invalidation of nano-drugs and inappropriate accumulation in healthy organs. In the interstitial tissues, the main challenge for mRNA carriers is the hydrophilic environment of the interstitial matrix. The transport of vaccine delivery systems within the interstitium mainly relies on water channels, which preferentially transport carriers with hydrophilic surface properties. This necessitates surface modification of NPs with hydrophilic materials[28]. In addition to the commonly used hydrophilic PEG, lipid optimization, novel biomimetic materials, and hydrogels can also enhance the stability of the carrier.

3.1.1. Optimizing the lipids

Currently, modifying the surface of NPs with polyethylene glycol (PEG) is a common strategy to improve the stability of vaccines in the body. In the bloodstream, conjugation of NPs and PEG reduces the binding of plasma proteins, prevents clearance of NPs by MPS, and isolates mRNA from the plasma enzyme with the hydrophilic steric hindrance[5]. Moreover, the hydrophilic nature of PEG also facilitates the transport of vaccines in the cellular interstitium. However, an excessive amount of PEG-lipid can have detrimental effects on cellular uptake and transfection of the delivery system[13]. Therefore, the benefits of enhancing the stability of the delivery system by increasing the content of PEG are limited. Based on of PEGylated LNPs, Chander et al.[29] achieved improved carrier stability by adjusting the ratio of helper lipids. The research suggested that the proportion of high levels of helper lipids, such as distearoyl phosphatidylcholine (DSPC) or egg sphingomyelin (ESM), significantly influences the properties of LNPs. Results demonstrated that LNPs containing 40% (mol/mol) DSPC or ESM can form an aqueous compartment bounded by a lipid bilayer, exhibiting enhanced stability in the presence of serum. In comparison to systems with 10% (mol/mol) DSPC or ESM, LNP-mRNA systems with 40% (mol/mol) DSPC or ESM showed significantly improved transfection properties *in vitro*, along with prolonged circulation lifespan and significant improvement in gene expression in the spleen, bone marrow, and liver upon intravenous injection. This adjustment of helper lipid proportions also provides new insights for the design of carriers for tumor vaccines.

Although PEG has shown its ability to increase retention time *in vivo*, studies demonstrated that linear PEG-modified NPs will induce anti-PEG-Immunoglobulin M (IgM) at the first injection, which causes accelerated blood clearance (ABC) phenomenon upon repeated injection[30,31]. Research on mice has shown that after the injection of polymeric nanoparticles, the anti-PEG IgM raised, which had significant neutralizing effects on subsequent doses of PEGylated NPs *in vivo*[32]. Furthermore, anti-PEG antibodies may also trigger severe complement activation-related pseudoallergy (CARPA), greatly reducing the safety of linear PEG-modified NPs[33]. Hence, the search for alternative materials for PEG becomes an urgent issue. 15,826 adverse drug reaction reports (aged 46−64 years, approximately 67% female) from the Italian SRS database showed that the frequency of hypersensitivity reactions reporting was higher among PEGylated *versus* non-PEGylated medicinal products (11.7% *vs.* 9.4%, $P < 0.0001$)[34]. Therefore, the search for alternative materials to substitute for PEG has become an urgent task.

Zwitterionic polymers and some other polymers are also considered alternatives for PEG. Among them, poly (sarcosine) (pSar) is a material that has received much attention in recent years. As the repeating unit of pSar, sarcosine (*N*-methylglycine) is an endogenous amino acid metabolic intermediate, pSar is endowed with excellent biocompatibility, and as the degradation products of pSar are completely harmless, which reduces adverse biological reactions. At the same time, pSar is uncharged and also has excellent hydrophilicity and stealth properties, so equipping it with the ability to prolong circulation time *in vivo* like PEG[35]. Many studies have shown the excellent performance of pSar in replacing PEG. Nogueira et al.[36] used pSar lipids with different polymeric chain lengths and molar fractions to modify LNPs. The result showed that assembled LNPs have high mRNA transfection efficiency and safety after intravenous injection. Moreover, PSarylated LNPs showed lower proinflammatory cytokine secretion and decreased complement activation than PEGylated LNPs, which testified that PSarylated LNPs are competitive carriers to deliver mRNA. Taking advantage of pSar's excellent performance, Zhu et al.[37] used the disulfide functionalized pSar prepared by ring-opening polymerization to modify the surface of gold nanorods (AuNRs) to produce more efficient and safer nanocarriers. It turned out that pSar-coated AuNRs have negligible cytotoxicity and enhanced colloidal stability. In comparison to PEG with similar molecular weight, pSar endows AuNRs with longer circulation time and higher accumulation in solid tumors.

In addition, many newly synthesized materials are employed to construct PEG-free nano-carriers. For example, a lately developed

material, tB-UC18 (including a benzene-ring scaffold and three unsaturated lipid tails), has been used to self-assemble with the help lipid, 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine, to form lipid-like nanoassemblies (LLNs)[38]. The LLNs have been found to be thermostable and resistant to nuclease degradation, so when mRNA was encapsulated in LLNs, the delivery system could maintain thermal stability for at least two weeks without PEG lipids. In addition, the research data showed that the PEG-lipid-free two-component mRNA vaccine (PFTCmvac) triggered strong humoral and cellular immune responses in mice, but did not cause significant treatment-related adverse reactions and activation of the complement system in human serum. This PEG-free delivery system provides a new nano-based strategy for rapid construction of mRNA vaccines.

3.1.2. *Using biomimetic materials*

After years of research, encapsulating of NPs with a single layer of the biomimetic cell membrane has become a relatively mature method to replace PEG. This is because the biomimetic cell membrane has high biocompatibility and immune escape capabilities, effectively reducing the occurrence of the ABC phenomenon and CARPA effect, and also achieving the effect of prolonging the circulation time of NPs. Taking the red blood cell membrane as an example, CD47, an integrin-associated protein that is expressed on the red blood cell membrane, acts as a self-protective protein that recognizes the signal-regulating protein α (SIRP-α) on macrophage membranes, sending inhibitory signals to prevent macrophage phagocytosis. This allows NPs coated with red blood cell membranes to possess both long-term circulation ability and low adverse reactions in the blood circulation system[31,39]. However, as the cell membrane is a biological material, the selection, preparation, and storage of the cell membrane must be particularly stringent to prevent unwanted biological effects, which increases the cost and risk and limits the application of biomimetic cell membrane nanocarriers[40].

Segel et al.[41] utilized PEG10, a naturally occurring RNA transport protein in humans, to develop a novel RNA delivery platform called SEND. PEG10 is a retrovirus-like protein, which can directly bind to and secrete its own mRNA in extracellular viral-like capsids. These viral-like particles were then pseudotyped with fusogens to deliver functional mRNA cargo into mammalian cells. By modifying the PEG10 protein, SEND enables the delivery of different RNAs to specific cells or organs, and due to PEG10's natural presence in the human body, SEND could effectively circumvent immune attacks compared to other RNA delivery methods. This platform provides a new endogenous carrier for RNA-based gene therapy and offers valuable insights for the design of tumor mRNA vaccines.

3.1.3. *Protection by hydrogel*

Hydrogels are a class of polymers with hydrophilic groups, which can swell in water but are insoluble in water and have a three-dimensional network structure. Hydrogels exhibit excellent biocompatibility and tunable physicochemical properties, enabling them to be engineered to encapsulate RNA and enhance its stability *in vivo*, while precisely controlling the release of RNA for sustained effects[42]. Yin et al.[43] developed an injectable hydrogel by combining graphene oxide (GO), polyethyleneimine (PEI), adjuvants, and mRNA vaccines (as shown in Fig. 2). Typically, mRNA vaccines have a retention time in the body of 1−2 days, while this hydrogel could generate mRNA (ovalbumin, a model antigen) and adjuvants (R848)-laden nano vaccines even after 30 days in melanoma-bearing mice after upon injection, which presented a significantly prolonged retention time *in vivo*. The data also indicated that the transformable hydrogel could significantly increase the number of antigen-specific $CD8^+$ T cells and suppress tumor growth, effectively preventing tumor growth and metastasis. Subsequently, they further investigated the method of embedding LNPs in transformable hyaluronan dynamic hydrogel[44]. They assembled highly encapsulating mOVA mRNA and adjuvant R848-loaded LNP nanoparticles (mRLNPs) by using a microfluidic mixing device. The prepared mRLNPs were embedded in a hyaluronan dynamic hydrogel (HA-mRLNPs) and maintained in a mildly acidic environment (pH∼6.5) to inhibit RNA degradation and LNP aggregation by interstrand interaction, thereby stabilizing the LNPs at room temperature. Under physiological conditions, the gelatinous hyaluronan undergoes a state transition to control the release of mRLNPs, efficiently delivering tumor antigen-encoding mRNA to DCs, and inducing of antigen-specific $CD8^+$ T cell-mediated tumor cell killing. Overall, this research demonstrates that LNP-hydrogel systems can be effectively utilized for cancer immunotherapy.

3.2. *Improving targeting specificity*

Cancer immunotherapy necessitates a robust immune response, particularly a strong $CD8^+$ T cell response[27]. Targeting the free vaccine to peripheral immune organs and tissues enriched with APCs and T lymphocytes can induce a potent cellular immune response, reduce vaccine retention and clearance in the blood and interstitial tissues, and enhance the efficacy of the vaccine. Therefore, improving the targeting ability of tumor vaccines is of paramount importance. Peripheral immune organs and tissues can be categorized into lymph nodes, spleen, and mucosa-associated tissues. Typically, intravenous administration targets the spleen, while intramuscular, subcutaneous, and intradermal injections target lymph nodes. The delivery methods targeting mucosa-associated tissues are relatively less utilized in mRNA tumor vaccine research. Hence, this section primarily focuses on the strategies for modifying delivery systems targeting the spleen and lymph nodes.

3.2.1. *Spleen targeting*

The spleen is the largest lymphoid organ in the human body, which contains a substantial number of APCs. Once successful

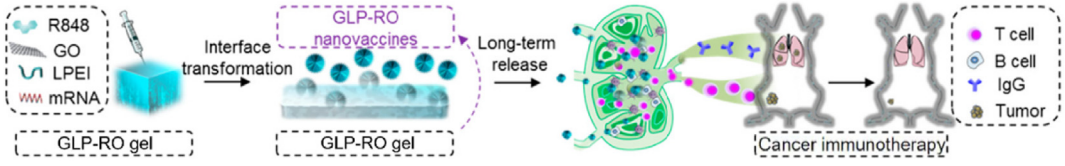

Figure 2 A schematic illustration and characterizations of transformable hydrogel. Reprinted with permission from Ref. 43. Copyright © 2021 American Chemical Society.

transfection of APCs in the spleen can be achieved, it would be expected to generate a stronger immune response. Thus, designing mRNA vaccine delivery systems that target the spleen is a promising strategy. However, Delivery systems for intravenous or intramuscular administration have been found to exhibit significant mRNA expression in the liver. As reported in pharmacokinetic data provided by Pfizer to the European Medicines Agency (EMA), Pfizer-BioNTech's COVID-19 mRNA vaccine (BNT162b2), administered through intramuscular injection, primarily distributed in the liver and the injection site, which led to reversible liver damage and $CD8^+$ T cell-dominant hepatitis[45,46]. Additionally, vaccines that are retained or cleared at the injection site are unable to exert their effects, also resulting in reduced vaccine efficacy. Therefore, enhancing the targeting specificity of mRNA-loaded NPs is requisite to minimize vaccine side effects and maximize therapeutic efficacy[47]. There have been numerous studies on spleen-targeted mRNA vaccines, while they also face some challenges, such as hepatic uptake of mRNA and degradation of the injection system[48]. In response to these challenges, some strategies to improve spleen targeting are presented below.

The most common design for spleen-targeted mRNA vaccines is to modify the vaccine with the ligand. Several membrane lectins belonging to the pattern recognition receptors (PRRs) binding mannose residues are expressed by DCs like the Mannose-Receptor/CD206, DC-specific ICAM3-grabbing-nonintegrin/CD209 (also known as DC-SIGN) or Langerin/CD207[49], and induce cargo internalization. Thus, mannose-modified NPs facilitate the targeting of DCs in the spleen and lymphoid Le Moignic et al.[49] found the delivery to the splenic DCs was improved after intravenous injection of mannosylated lipid-polymer-RNA lipopolyplexes (LPR) after functionalization of liposomal mannose residues. Compared to non-mannose-modified (bare) lipopolyplexes, the mannose-modified lipopolyplexes carrying mRNA encoding the mar-1 antigen exhibited superior preventive effects against B16F10 melanoma in mice. Perche et al.[50] also prepared mannose-modified NPs loaded with mRNA. They incorporated mannose-modified and histidylated liposomes into mRNA-PEGylated histidylated polylysine complexes to form mannose-modified and histidylated lipopolyplexes (Man11-LPR100). After intravenous injection, the expression of enhanced green fluorescent protein (EGFP) mRNA in spleen DCs was fourfold higher in the mice treated with EGFP mRNA-loaded Man11-LPR100 compared to mice injected with non-mannose-modified LPR100, demonstrating that mannose-modified and histidylated LPRs can enhance transfection of DCs *in vivo* and serve as an efficient system for delivering tumor antigen mRNA to spleen DCs to induce anticancer immune responses. Phosphatidylserine (PS) is a signaling molecule that promotes phagocytic uptake and viral envelope entry into cells. It has been utilized by Luozhong et al.[51] to develop an effective carrier for targeting secondary lymphoid organs (SLOs). They employed biomimetic strategies and incorporated PS into a standard tetra-component MC3-based LNP formulation (PS-LNP) to facilitate cellular uptake of RNA by immune cells, demonstrating its superior efficiency compared to commonly used charge-driven targeting principles. Results also showed efficient expression of PS-LNPs in lymph nodes and spleen after intravenous injection. Moreover, *in vitro* and *in vivo* characterization of PS-LNPs revealed a monocyte/macrophage-mediated SLO-targeting delivery mechanism, providing a novel idea for spleen-targeted mRNA tumor vaccine carriers.

Kranz et al.[52] found that optimizing the net charge of intravenously administered RNA-lipid complexes (RNA-LPX) can achieve precise and effective targeting of DCs *in vivo* without the need for functionalizing particles with molecular ligands. In their research, they unexpectedly discovered that negatively charged RNA-LPX exhibited spleen-targeting specificity. By applying RNA NPs encoding the reporter gene firefly luciferase (Luc-RNA), they observed that a gradual reduction in cationic lipid content in LPX resulted in a shift of Luc expression from the lungs to the spleen. Neutral to slightly negatively charged particles, such as those with a charge ratio of 1.7:2, provided a specific spleen signal. They also tested various other well-characterized lipid components, such as DOTAP and cholesterol, and the result showed that all particles with excess negative charge displayed desirable pharmacological and physicochemical properties and induced selective antigen expression in spleen cell populations. However, transfection efficiency decreased gradually with an increased negative charge, possibly due to an increase in the amount of uncomplexed free RNA. Therefore, adjusting the negative charge content of lipids can effectively target the spleen and achieve transfection of RNA, the underlying mechanisms of which is currently no conclusive evidence, but may be related to the formation and entrapment of protein corona[53]. Based on the conclusion that adjusting charge could modulate tissue tropism, Cheng et al.[54] developed a novel organ-selective targeting nanoparticle platform called SORT (as shown in Fig. 3). On the basis of a four-component LNP, the SORT introduced a fifth component (called SORT molecules), either a cationic lipid (*e.g.*, DOTAP), an ionizable lipid (*e.g.*, DODAP), or an anionic lipid [*e.g.*, 1,2-dioleoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) (18 PA)], into a widely used four-component LNP system. By adjusting the proportions of SORT molecules, intravenous administration of mRNA drugs selectively targeted the lungs, spleen, or liver in mice. These additional components could modify the delivery characteristics of mRNA *in vivo*, and by modulating the incorporation levels of SORT components, the charge and biophysical properties of LNPs could be altered, resulting in different tissue targeting. Among them, the content of DOTAP played a key role, as an increase in the mole percentage of DOTAP led to a gradual shift of the produced luciferase protein expression from the liver to the spleen and then to the lungs, demonstrating a clear and precise organ-specific delivery trend. An optimal content of 10%–15% DOTAP aids spleen delivery. This approach is applicable to various nanoparticle systems and provides a novel method for predictable LNP design for targeting relevant cells. However, the anionic lipid 18 PA used in SORT is an artificial anionic phospholipid, so the worry is that the complex synthesis process of these artificial anionic lipids with unknown biocompatibility may limit the clinical application of spleen-selective LNPs modified with synthetic anionic lipids, thus Pan et al.[48] utilized endogenous anionic dietary stearic acid as a substitute for 18 PA and developed stearic acid-doped LNPs. The LNPs for spleen-selective mRNA tumor vaccine co-loaded ovalbumin (OVA)-coding mRNA and Toll-like receptor 4 agonist (MPLA). The data showed that sLNPs-OVA/MPLA selectively translated the protein antigen encoded by the mRNA in the spleen after intravenous injection, and enhanced the immunogenicity of the mRNA tumor vaccine by activating Toll-like receptors 4 (TLR4) on the cell membrane of APCs and TLR7/8 inside the cells, triggering a T helper cell 1 (Th1)-type immune response and inducing durable immune memory, thus achieving potent tumor immunotherapy effects.

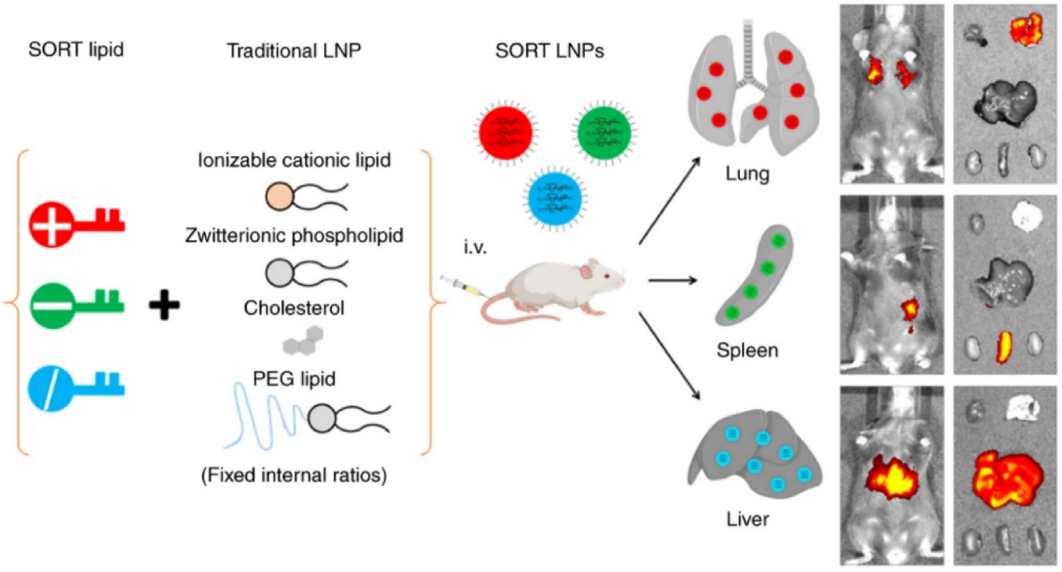

Figure 3 Schematic illustration of SORT. Reprinted with permission from Ref. 54. Copyright © 2020 Nature Publishing Group.

In addition, many novel ligand-free spleen-targeting carriers are under research. Zhao et al.[55] established a structure-based screening method. In the first step, a library of lipidoids with significantly different chemical structures was screened, and the result demonstrated that lipidoids containing imidazole groups had a certain promoting effect on mRNA delivery into T lymphocytes, suggesting that imidazole groups are one of the key structures for primary T lymphocyte delivery. In the second step of screening, a lipid library containing imidazole and its analogs was tested in FLuc mRNA-loaded LNP complexes, which turned out that amine head 93 with O17O and O17S tails exhibited strong and specific luminescent expression and transfection efficiency in the spleen of BALB/c mice after intravenous injection. Amine head 9322 with O17O and O17S tails showed high transfection efficiency in spleen $CD4^+$ and $CD8^+$ T lymphocytes and luminescence in both the liver and spleen but with higher intensity in the spleen. Additionally, some piperazine-based lipids also possess spleen-targeting functionality. Ni et al.[56] first synthesized ionizable lipids consisting of a piperazine core and two tertiary amines as ionizable headgroups linked to hydrophobic carbon chains, called "Pi-lipids", and then successfully synthesized eight novel piperazine-based ionizable lipids (PPZ). Through average normalized delivery of LNPs with different Pi-lipids, they found that Pi-LNP containing PPZ-A10, which has a linkage consisting of two carbons and a short C10 carbon chain, exhibited the highest delivery efficiency. LNP-A10 delivered Cre mRNA showed high signal expression in Kupffer cells, splenic macrophages, and splenic DCs, indicating that LNP-A10 preferentially delivers nucleic acids to immune cells in the liver and spleen.

3.2.2. *Lymph-node targeting*

Lymph nodes (LNs) are also important immune organs in the human body, which serve as crucial sites for adaptive immune-related lymphocytes. Therefore, exploring lymphoid organ-specific mRNA vaccines may be a potential strategy for developing next-generation mRNA vaccines. Studies have shown that the efficacy of T cell-mediated immune responses activated by vaccination is related to the efficiency of APCs activated by the vaccine being transported to the LNs[57]. For interstitial administration, the size of the delivery carrier initially affects the efficiency of the vaccine entering the interstitial fluid and subsequently entering the LNs. Research has demonstrated that NPs with a diameter smaller than 5 nm could easily enter capillaries and induce rapid clearance, while NPs larger than 100 nm remain at the injection site without entering lymphatic vessels. Therefore, particles with diameters ranging from 15 to 70 nm are considered more suitable for vaccines to effectively transfer through the interstitium, enter lymphatic capillaries, and ultimately reach LNs[58]. Except for size tuning, increasing interstitial osmotic pressure and lymph flow velocity can also enhance absorption at LNs, and compared to adjusting the physical properties of the delivery system, utilizing the inherent biological characteristics of the human body enables more specific transport to the lymph nodes, such as modifying ligands that bind to the receptors on the surface of cells surrounding the LNs, binding to albumin in the bloodstream to facilitate homing and other approaches that do not require active targeting ligands.

By modifying the delivery system with the corresponding ligands based on the receptors present on the surface of LN surrounding cells (such as endothelial cells and lymphocytes), the vaccine can actively be home to the LNs under the driving force of ligand−receptor interactions. Receptors can naturally exist like the highly expressed 33D1 and CD205 receptors on the surface of LN DCs[15], or they can be artificially modified. For example, Qin et al.[59] achieved effective delivery of vaccines to the LNs by using azide groups to modify the surface of lymphatic endothelial cells, which provided targets for DBCO-modified liposomes. Under the ligand−receptor interaction, the vaccine-induced enhanced $CD8^+$ T cell responses and significantly prolonged the median survival of mice. Compared to DBCO-modified liposomes treatment without azide-targeting, the survival rate of mice treated with azide-targeted vaccines increased by 100% within 60 days, demonstrating significantly enhanced anti-tumor immune responses. Mannose, as a commonly used ligand for DC targeting, also plays an important role in lymphatic targeting. Liu et al.[60] used mannose-modified lipid calcium phosphate (LCP) to deliver mRNA encoding MUC1 to DCs in LNs. They synthesized

an HA-tagged MUC1 gene to distinguish the exogenous from endogenous expression and inject LCP loaded with HA-tagged mRNA encoding MUC1 fusion protein into mice. On Day 7 after vaccination, the Western blot detected HA-tagged MUC1 in the draining lymph nodes from immunized mice, indicating that mannose-modified LCP could deliver mRNA to the LNs and mRNA was translated into the target protein correctly.

Furthermore, the "hitchhike vaccine" is another biological approach for LN targeting, which binds to albumin in the blood and travels to the LNs along with albumin movement. Liu et al.[61] constructed a hitchhike vaccine that comprises an antigen or adjuvant linked to a lipophilic albumin-binding tail *via* a solubilizing PEG chain. By exploiting albumin as a fatty acid transporter, the vaccine accumulated in the LNs after subcutaneous injection, resulting in a 30-fold increase in T cell priming and strong inhibition of tumor growth.

Delivery vehicles that do not require modification with any active targeting ligands are also being investigated for the development of mRNA tumor vaccines. Chen et al.[62] screened and optimized a series of LNPs with targeting specificity, and explored an endogenous LN-targeting LNP called 113-O12B for therapeutic mRNA tumor vaccines. Next, the research team used OVA as a model antigen to analyze the immune activation effect of the mRNA vaccine encoding OVA *in vivo*. The result showed that compared to LNP formulated with synthetic lipid ALC-0315, which was used in COVID-19 vaccines, the 113-O12B exhibited significantly reduced expression levels in the liver, while its expression in the LNs was enhanced and specific. 113-O12B also demonstrated antibody activation levels comparable to ALC-0315 and enhanced activation of $CD8^+$ T cells, which proved that 113-O12B could increase the response of $CD8^+$ T cells to the encoded full-length OVA model antigen. Subsequently, the team evaluated the inhibitory capacity of this mRNA vaccine as a therapeutic vaccine against established tumors by using the full-length OVA and tumor-specific peptide TRP2180-188 as antigens, respectively, and B16F10-OVA or B16F10 cells as tumor models. In both systems, 113-O12B significantly suppressed tumor growth. Two mice (40%) even achieved complete tumor regression after combined treatment with PD-1 antibodies. Furthermore, the mice that achieved complete regression were able to prevent the formation of any new tumors after subsequent injection of metastatic tumor cells, indicating the long-lasting immune memory effect of the mRNA tumor vaccine with 113-O12B as a carrier.

4. Cell expression level of mRNA tumor vaccine

In addition to the smooth delivery of the vaccine to target cells, the promotion of transfection and expression of relevant tumor antigens in APCs is also very important for the subsequent antigen presentation and immune process. In this process, achieving successful transfection and escaping lysosomal degradation are key points, which decide the amount of antigen that can be ultimately expressed and the intensity of cellular immune response[63]. In the following sections, we review the improvement of the RNA tumor vaccine delivery system from the perspective of formulating efficient transfection reagents with low cytotoxicity of mRNA vaccine and achieving lysosomal escape at the cellular level.

4.1. Optimization strategies based on LNP

LNP delivery systems have advantages such as easy preparation, self-assembly, excellent biocompatibility, high bioavailability, and high payload capacity. However, there are still limitations in practical applications. LNPs typically consist of four components: ionizable lipids, phospholipids, cholesterol, and PEG-lipid conjugates. Among them, ionizable lipids can assemble with mRNA to form virus-sized particles and facilitate mRNA release in the cytoplasm. Natural phospholipids forge a lipid monolayer structure to encapsulate mRNA. PEG could extend the half-life of the formulation, helping to avoid LNP being taken up by phagocytic cells and prolonging circulation time in the blood. Cholesterol primarily acts as a stabilizer, enhancing the stability of LNP. Currently, there is ongoing research to improve these four components of LNPs, aiming to further enhance the functionality of LNP delivery carriers and achieve higher transfection efficiency.

4.1.1. Ionizable lipids

Ionizable lipids with positive charges can interact with negatively charged nucleic acids, allowing the mRNA vaccine to be encapsulated in the LNP delivery carrier, which makes the process of mRNA transfection and intracellular escape possible[64]. Ionizable lipids usually have three parts: headgroups, hydrophobic tails, and internal linkers. Based on the number of amino groups in the headgroups, ionizable lipids can be classified as single-charge or multi-charge lipids. Well-known ionizable lipids, DLin-MC3-DMA (MC3), SM-102, and ALC-0315 are the only three FDA-approved ionizable cationic lipids for RNA delivery, and all of them belong to single-charge lipids. However, these three single-charge ionizable lipids are non-biodegradable and can probably accumulate *in vivo*, leading to cytotoxicity. Because of this fault, recent research has focused more on multi-charge lipids, such as C12-200, G0-C14, cKK-E12, OF-2, TT3, and 306OI10. When ionizable lipids have equal mass ratios to mRNA, multi-charge lipids typically have a higher nitrogen-to-phosphorus (N/P) ratio than single-charge lipids, which provides advantages in mRNA encapsulation, uptake, and endosomal escape processes. Chen et al.[65] constructed a new series of multi-charge ionizable lipids. These lipids consist of four amino nitrogen atoms (4N4T), having a hydrophilic center with a tertiary amine and four hydrophobic tails. The mRNA gene expression levels presented by 4N4T liposomes with different core structures, such as piperazine and amide, acylpiperazine, and piperazine were different. Among these lipids, the ones with a piperazine and amide or acylated piperazine core structure were demonstrated to have the highest gene expression level. The 4N4T lipids were proved to have the ability to significantly improve gene expression efficiency compared to the use of single-charge lipid SM-102. Therefore, multi-charge lipids, with their higher N/P ratio, are likely to facilitate endosomal escape and achieve higher gene expression efficiency, leading to higher transfection efficiency in experiments.

Another safe and effective mRNA delivery system provided by cationic liposomes was developed by the team of Arya[66]. They reported a liposome-based transfection reagent, InstantFECT, which could help mRNA modified with pseuoguanosine at the tail residue to be concentrated into a nano complex. This improvement can make mRNA vaccines transfect multiple organs more efficiently by being delivered locally. Among all those tries of it, the mRNA complex prepared with 4ul of InstantFECT has the highest level of transfection activity. Compared with the commercial transfection reagent liposome Mirus, the bioluminescence signal intensity of the luciferase mRNA-InstantFECT complex is several orders of magnitude greater, meaning that it significantly improves the gene transfection level. The bioluminescence also shows that

the injection site has a bright center and the fluorescence area in the periphery is larger but the overall intensity is lower. That is to say, the method that uses InstantFECT as a transfection vector breaks the restriction of transfection range, expands its transfection area, and greatly controls the obvious expression in the non-injection site during transfection *in vivo*. In terms of safety tests, there was nearly no obvious toxicity sign in 50 mice vaccinated with multiple doses of the vaccine by different injection methods in the experiment. And even when injecting a full dose of luciferase mRNA-InstantFECT nanocomplex intravenously through the tail vein, the signal could only be identified in the tail after 24 h of tracking. If a portion of the nanocomposite does leak into the circulation at the site of injection, the transfection in other vital organs could be substantially prevented, preventing a safety hazard.

Based on the traditional type of LNP, Haabeth et al.[67] have proposed to use of a dynamic drug delivery system of mRNA-CRAT for delivering therapeutic tumor vaccines. This method makes use of the charge-releasable transport protein (CART) to aid the delivery process of mRNA, whose properties could change with the controllable change of the physical properties. Using the specific degradation mechanism that could change its charge, CART can convert the initial polycationic skeleton into neutral small molecule by-products. Thus, the transformed mRNA delivery complex can release from the lysosome electrostatically, avoid degradation, and enhance the efficiency of the following translation and expression process, realizing high transfection efficiency.

4.1.2. *Natural phospholipid*

Phospholipids, as auxiliary lipids, could contribute to the formation of lipid nanoparticles and the escape from lysosomes. However, traditional phospholipids such as DSPC and DOPE often have limitations in terms of structural flexibility and accessibility to reactions. Liu et al.[68] developed hundreds of ionizable phospholipids called iPhos. Among them, 9A1P9, which has a zwitterionic headgroup and three alkyl tails, exhibited 40 times higher efficacy than DSPC and 965 times higher efficacy than DOPE. The unique structure of iPhos lipids makes it easier for them to adopt a conical shape in intracellular environments, promoting membrane fusion and enhancing the endosomal escape process of the delivery system, thereby improving the transfection efficiency of mRNA.

Furthermore, through *in vitro* screening of phospholipid structures, Alvarez-Benedicto et al.[69] discovered that incorporating phosphatidylethanolamine (PE) headgroup phospholipids in liposomes also can facilitate membrane fusion and endosomal escape of LNPs, reducing the degradation of the LNP delivery system by lysosomes *in vivo*, and enhancing mRNA expression, which could ultimately maximize the functionality of mRNA vaccines, thereby improving the transfection efficiency of the vaccine.

4.1.3. *Cholesterol*

Cholesterol in LNPs contributes to increasing stability and facilitating cell membrane fusion. Optimizing the structure of cholesterol also is a good way to improve the delivery efficiency of LNPs. In line with this research direction, Herrera et al.[70] reported the use of a cell reporting system, Galectin 8-GFP, to visualize the endosomal escape capability of LNP-mediated mRNA delivery systems. Using this system, they found that LNPs with β-sitosterol, a plant sterol substitute for cholesterol, exhibited a detectable tenfold increase in endosomal perturbation time compared to standard cholesterol LNPs, indicating that β-sitosterol-replaced LNPs could enhance endosomal escape capability. Building on these screening results, the group of Kim[71] replaced cholesterol with β-sitosterol to construct a novel delivery system. Experimental results showed a significant increase in transfection efficiency in the group using β-sitosterol-replaced LNPs, and that group had a 12-fold higher expression level of the observed luciferase enzyme compared to the pre-replacement group. The underlying principle may be that introducing β-sitosterol into lipid nanoparticles with a high-density PEG layer creates a lighter multilayered, polyhedral PEG structure that facilitates subcellular delivery of mRNA. This structure promoted the diffusion of the delivery system in the pulmonary mucus environment, prevented its uptake and degradation by endosomes, and increased the amount of mRNA entering the cells, thereby achieving high transfection efficiency.

4.1.4. *PEG lipid conjugates*

Incorporating PEG lipid conjugates into LNPs can reduce nanoparticle aggregation and evade phagocytosis by mononuclear cells, thereby extending the half-life of the liposomes in the body and prolonging their circulation time. However, PEGylation of liposomes also hinders the interaction between LNPs and target cells and the process of endosomal escape, leading to a decrease in transfection efficiency. Studies have found that by adjusting the carbon chain length of PEGylated lipids, the rate of interaction can be enhanced, thereby improving their effectiveness. It was identified that 1.5% (mol/mol) PEG2000 can effectively form stable LNP structures, prolong the half-life of LNPs *in vivo*, and ensure the transfection delivery efficiency of mRNA, thus addressing the impact of PEGylation on the efficacy of mRNA vaccines to some extent.

In previous studies, it was found that the PolyU complex has a good anti-tumor effect on targeting DCs and triggering $CD8^+$T cell response. On the basis of the excellent anti-tumor auxiliary function of this complex, Lou et al.[72] developed single-stranded polyuridine (PolyU) complexes and post-modified them with PEG, forming pegylated RNA complexes (as shown in Fig. 4). Post-functionalization of the PEG chain ends exposed at the surface with different membrane-active peptides, such as cationic, hemolytic, melittin, a pH-sensitive fusion peptide GALA, an antimicrobial peptide LEDE, allows the mRNA-presenting complex to acquire an effective endosomal escape capability. In the case of using GALA to modify it, the complex modified in this way can selectively bind to sialic acid and terminate glycans on DC cell, which promotes the destruction of phagosome membranes and forbids it from being degraded. Then the transfection efficiency of PEG lipids could realize an improvement. At the same time, using poly U complex, the complex has a better anti-tumor function.

Yan et al.[73] formulated a novel mRNA composite nanoparticle preparation, and during that process, optimizing PEG modification plays an important role in its improvement. A polymeric library used through the process contains 480 different modifications with different functional groups. It was screened by *in vitro* delivery of luciferase-encoded mRNA to IGROV1 cells to identify the delivery capacity of lead polyester substances. It shows that the polyester modified by cysteamine has a better delivery efficiency. Aimed at the issue that it is easy to be absorbed and aggregated by protein, Pluronic F127 is used for the treatment of the prepared nanoparticles. After the treatment, the PPO segment of Pluronic

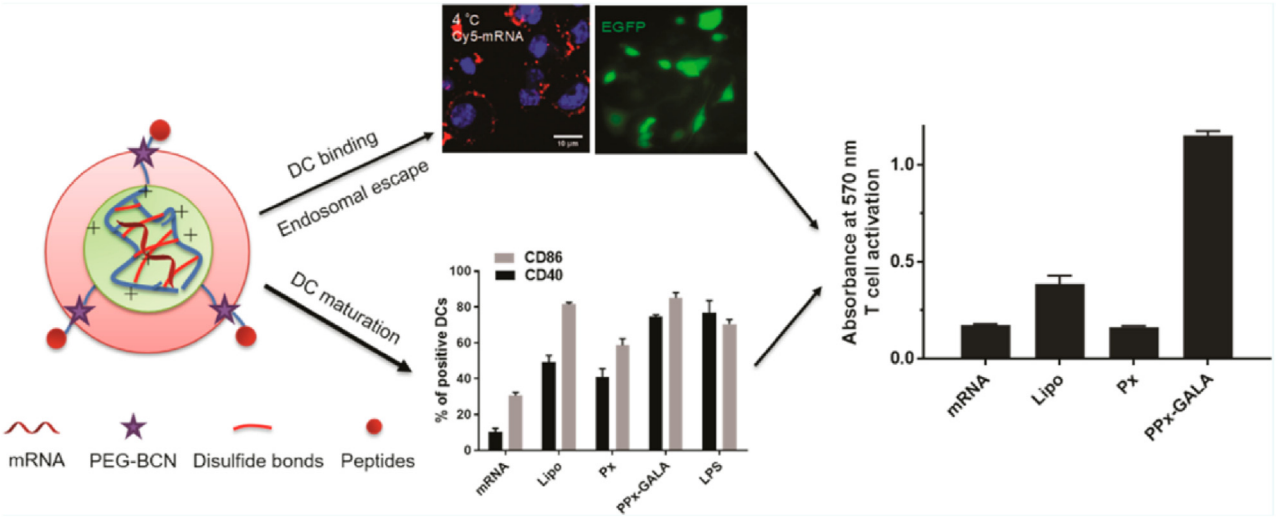

Figure 4 The mechanism of GALA modified mRNA polyplexes (PPx-GALA). Reprinted with permission from Ref. 72. Copyright © 2018 American Chemical Society.

F127 can be inserted into the hydrophobic domain of the prepared mRNA poly complex NP, leaving a hydrophilic PEG end on the surface, which can inhibit the adsorption and aggregation of the nucleic acids. However, excessive exposure of PEG ends on the surface can also neutralize the charges of the nanoparticle, which could significantly improve the stability of mRNA nanoparticle complex *in vivo*, but at the same time, it would influence the interaction process between nanoparticle and cells, as well as the uptake process of the nanoparticle by cells. Thus, the group of Yunfeng Yan further studied the appropriate action function of Pluronic F127. Among the three test conditions (5% F127, 10% F127 and 5% PEG2000DMG), they found that using the mRNA NPs treated by 5% F127 showed the best effects. Furthermore, all of the functional polyesters in the screening library are degradable, so to some extent, this method also conquers the difficulty of polyester degradation.

4.1.5. Introducing the fifth component

Although cationic lipid carriers play an important role in fully encapsulating negatively charged mRNA drugs, they also have some non-negligible defects in the process of mRNA drugs. For example, the high positive charge carried by cationic liposomes can cause cell damage and produce cytotoxicity. Moreover, a high positive charge will also hinder the transport of delivered mRNA to immune organs, reduce the uptake of mRNA by immune cells, and affect the function of the mRNA tumor vaccine. Thus, the surface charge of mRNA cationic lipids is a challenge for formulating a successful mRNA delivery. Duan et al.[74] improved the cationic lipid delivery system from a new perspective of combining commercial natural anionic drug excipients with existing cationic lipid carriers through the coating. Negative-charged SA@DOTAP-mRNA nanovaccines were prepared by introducing the natural anionic polymer sodium alginate (SA) to coat DOTAP-mRNA. Using classical DOTAP/CHOL-GFP as a positive control, it was found that the transfection efficiency of SA@DOTAP-mRNA was significantly higher than that of the DOTAP-GFP group and positive control group and the optimal mRNA: SA to mass ratio was 1:1. In addition, while having high transfection efficiency, the transfection system modified by SA can also significantly reduce hemolysis and have lower cytotoxicity than the unmodified iso-cationic lipid carrier in the hemolysis experiment. At the organ level, SA modification can also reduce the damage to the liver, kidney, and other important organs of mice. Overall, SA modification greatly reduced the cytotoxicity and tissue toxicity of the cationic lipid delivery system and established a safer and higher transfection efficiency of the cationic liposome/mRNA complex formulation.

Zhang et al.[75] introduced protamine into cationic liposome DOTAP to prepare liposome-protamine lipid complex (CLPP) to deliver IVT mRNA encoding survivin-T34A gene. Protamine is a positively charged polycationic protein (as shown in Fig. 5). Its introduction can help the negatively charged mRNA to be concentrated into a solid core, and at the same time, it could help liposomes to be prepared into lipid bilayer shells, forming a novel core−shell nanoparticle preparation. Through lipid raft-mediated endocytosis, the CLP liposome complex could stimulate the complex to enter cells and complete the transfection process, realizing a higher gene expression efficiency. According to the experiment result, the average fluorescence intensity of the developed CLPP vector was 1.8 times higher than that of the commercial vector lipofectamine™. Under the protection of this special nuclear structure, the degradation of mRNA can be reduced, and in terms of safety, by microscopic examination, nearly no significant pathological changes were found in the main organs of the CLPP/mSur-T34A-treated group, indicating that this vector has high security.

Ayad et al.[76] developed an innovative carrier called LipoParticles (LP) by incorporating poly (lactic acid) (PLA) into the core of LNP liposomes. This delivery system utilized a particle layer-by-layer formulation strategy, where the nucleic acid is adsorbed onto the surface of LP by electrostatic interactions induced by the addition of the LAH 4-L1 peptide. Compared to standalone LNPs, the introduction of a PLA core in LP significantly enhanced its transfection efficiency in HeLa and DC 2.4 cells. That kind of delivery system belongs to lipid/polymer hybrid nanoparticles (LPHNs), which are nanostructures composed of a lipid shell and a polymer core. LPHNs could enable cellular penetration, efficient endosomal release, and high protein translation, and realizing enhanced transfection efficiency of mRNA by up to 80%.

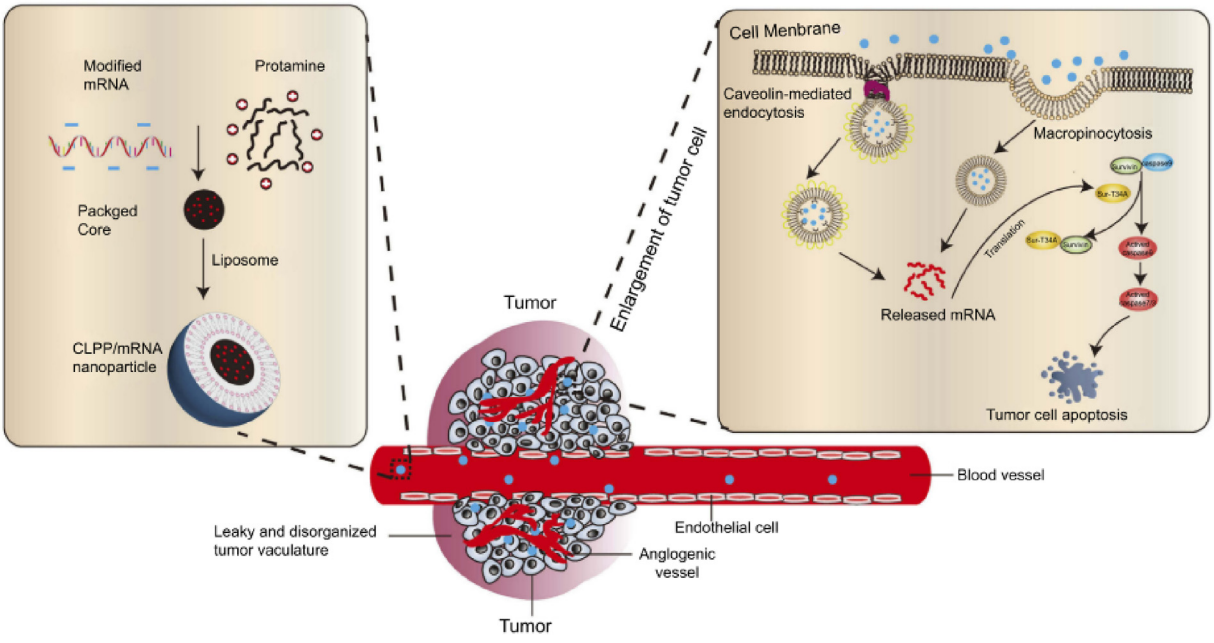

Figure 5 The mechanism of introducing protamine into cationic liposome DOTAP to prepare liposome-protamine lipid complex (CLPP). Reprinted with permission from Ref. 75. Copyright © 2019 Dove Medical Press Ltd.

4.2. Altering the structure of formulation

The functionality of LNP mRNA delivery systems heavily relies on the composition of ionizable lipids, and based on the existing surveys, LNP mRNA delivery systems composed of optimized ionizable lipids can all exhibit unique vesicular structures enriched with mRNA. Cheng et al.[77] discovered that similar vesicular structures can also be formed in the presence of high concentrations of pH 4 buffer solutions (such as sodium citrate). Therefore, they explored the relationship between the bubble-like structures and the transfection capability of the delivery system in order to try to reveal an innovative method to improve the LNP delivery system. The results showed that when the LNP mRNA system encoding firefly luciferase with KC2 was treated with the optimized pH 4 buffers, it exhibited comparable or even stronger transfection efficiency compared to LNP formulations containing SM-102 (an approved LNP nucleic acid drug). This improvement may be attributed to the formation of vesicular structures in LNPs, which encapsulate mRNA and enhance its stability, thus improving the transfection efficiency of delivery systems composed of less active ionizable lipids. This study revealed that besides the composition of ionizable lipids, the structural arrangement of the lipid formulation also plays a role in the transfection efficiency of LNP mRNA delivery systems, suggesting that improving the formulation structure is also a pathway to enhance the transfection efficiency of LNP mRNA delivery systems.

4.3. Using inorganic materials

4.3.1. Achieve intracellular protection

Wang et al.[78] formulated a lipid-coated calcium phosphate NPs. When using the complex as a carrier, the CaP core of it would solute in an acid lysosomal chamber. The high ion concentration caused by the solution raises the osmotic pressure, which ruptures the endosomal membrane due to excessive absorption of water and makes the contents release, thus significantly reducing the amount of degraded complex, increasing the release efficiency of mRNA vaccine, and promoting its transfection efficiency. As the results demonstrated by flow cytometry, up to 68% of the DCs express the corresponding substance after using the vector modified by LCP to deliver mRNA for 24 h. As a comparison, the mRNA vaccine delivered by vector without LCP modification only transfected about 41% of the DCs *in vitro*. Hence, it shows that using LCP NPs could efficiently make endosomal escape and cargo release come true, enhancing the efficiency of transfection.

4.3.2. Achieve sustained intracellular release

Achieving intracellular sustained release is another approach to improving the transfection efficiency of mRNA vaccines. In light of this, Zhang et al.[79] developed a subcutaneous delivery system using mesoporous silica nanoparticles loaded with mRNA (MSN-mRNA). The mesoporous silica nanoparticle system possesses high surface area and well-ordered pore structures, which enable efficient adsorption and separation of the protein kinase R (PKR) inhibitor C19, an imidazole and hydroxy indole RNA activator. After loading C19, the system allows for sustained release of it, which inhibits PKR and maintains/enhances mRNA translation processes *in vivo*, thereby prolonging the kinetic expression of mRNA and improving the transfection efficiency of vaccines in the body.

5. Enhancing immunogenicity

Moreover, ample preclinical and clinical trials suggest that the tumor-killing effect induced by immunogenicity based solely on the expression of antigenic peptides is inadequate. The development of therapeutic cancer vaccines still faces many challenges. Unlike preventive vaccines for infectious diseases, therapeutic cancer vaccines must ensure the induction of robust cytotoxic

$CD8^+$ T cell responses to eradicate cancer cells. Although preventive cancer vaccines are possible, there are currently only two FDA-approved cancer-related vaccines, both targeting known oncogenic viruses [Human Papillomavirus (HPV) and Hepatitis B virus]. Another challenge is the high antigenic variability among individuals, necessitating the selection of appropriate antigens to successfully induce highly tumor-specific immune responses. The increasing trend of patient-specific neoantigens aims to address this challenge. Finally, even if the antigens can induce a cellular immune response, the inhibitory tumor microenvironment can prevent T cell infiltration into the tumor, leading to T cell exhaustion.

Therefore, to better exploit mRNA cancer vaccines, strategies to enhance their immunogenicity and the resultant immune responses must be employed. According to the central dogma, mRNA translates into encoded proteins in cells, eliciting an immune response. mRNA vaccines can be presented through both MHC-I and MHC-II antigen presentation pathways. They first trigger the body's innate immune response, and then initiate the adaptive immune response. Upon injection, mRNA expresses antigenic proteins in antigen-presenting cells. When these proteins are processed into antigenic peptides in lysosomes or proteasomes, these peptides are presented to $CD4^+$ and $CD8^+$ T cells or released to be recognized by B cells, thereby activating the body's comprehensive humoral and cellular immunity. In this process, strategies such as the use of adjuvants or monoclonal antibodies, optimizing delivery carriers, and combination drug use can enhance the immunogenicity and antitumor effects of mRNA vaccines.

5.1. Adjuvants

Designing optimized adjuvants with immune-activating activity can enhance the immune response triggered by mRNA cancer vaccines, thereby enhancing antigen expression efficiency. For adjuvant design, three strategies are commonly used: (1) independent synthesis of adjuvants and antigens: encoding the antigen mRNA sequence and adjuvant molecules are encapsulated together into the same polymer to ensure both essential components are delivered into the same antigen-presenting cell. This is the most conventional method. (2) Packaging material as adjuvant: the adjuvant is integrated into the mRNA packaging material to simplify production. For example, ionizable lipids used as LNP packaging components have natural immune-stimulating activity. However, this strategy requires a balance between delivery efficiency and adjuvant activity, which must be achieved through extensive screening and optimization to find suitable packaging materials. (3) The antigen itself fuses with adjuvants: The adjuvant is integrated into the mRNA sequence itself, maximizing the delivery efficiency of the packaging material and the immune-stimulating effect of the adjuvant. This is the most innovative and simple method, which can be achieved using existing, optimized delivery materials. The immune activation of adjuvants can target different points, including the transmembrane innate immune receptor TLR that can recognize endosomal nucleic acids, another set of innate immune receptors RLR that can recognize cytoplasmic RNA, and the stimulator of interferon genes (STING) that can recognize exogenous DNA in the cytoplasm.

TLRs are critical innate immune receptors, encoding different proteins to recognize different pathogen-associated molecular patterns (PAMPs), activating downstream signaling pathways, and initiating immune responses. TLR3/7/8/9 are located inside endosomes formed during phagocytosis, and they identify intracellular nucleic acid-related products. Tri-palmitoyl-*S*-glyceryl-cysteine peptide (Pam3), which binds with TLR 2 and 1, is a well-known lipid adjuvant that can synergistically enhance the immune response with LNP due to the interaction between Pam3's lipid tail and the lipid component of LNP. Moreover, since TLR 2 and 1 are present on the cell membrane surface, Pam3-LNP can be recognized by receptors when LNP interacts with cells. Lee et al.[80] successfully incorporated Pam3 as an adjuvant into LNP containing OVA mRNA, expressing tumor antigen and enhancing the immune response, demonstrating that the synergistic effect of Pam3-LNP can effectively improve the cancer prevention effect of mRNA vaccines. In this process, Pam3-modified LNP (Pam-LNP) can be recognized by TLR7 and 8, synergistically enhancing immune activation. Pan et al.[48] focused on the combination of TLR4 agonists and mRNA, delivering LNPs that carry unmodified antigen mRNA and TLR4 agonist MPLA, performing potent tumor immunotherapy through synergistic different immune stimulations and Th1-type immune responses.

Retinoic-acid-inducible gene I (RIG-I) acts as the body's primary immune recognition receptor for virus detection, belonging to the family of RIG-I-like receptors (RLRs) and playing a crucial role in the body's antiviral immune response, especially in the induction of Type I interferons. Recent studies have found that short double-stranded RNA (dsRNA) of 20 base pairs (bp) can activate RIG-I. Tockary et al.[81] developed a tumor vaccine that directly integrates dsRNA targeting RIG-I as an adjuvant into the antigen-encoding mRNA chain. By adjusting the structure and microenvironment of dsRNA by changing its length and sequence, RIG-I can be effectively stimulated, thus determining the structure of nucleic acid vaccines. The optimized structure of the dsRNA-mRNA tumor vaccine obtained from experiments can effectively activate mouse and human dendritic cells and drive them to secrete a wide range of pro-inflammatory cytokines without increasing the secretion of anti-inflammatory cytokines. At the same time, the intensity of immune stimulation can be regulated by adjusting the number of dsRNA on the mRNA chain, thereby preventing excessive immune stimulation.

Interferon gene stimulant signal agonists are currently one of the most promising immune adjuvants. They can promote the activation of STING signaling in antigen-presenting cells, and induce the secretion of Type I interferons (IFN−I), thus promoting the cross-presentation of tumor antigens and the subsequent proliferation and activation of T lymphocytes. However, the free form of STING agonists, when used as immune adjuvants in the design of cancer vaccines, still has problems like poor lymphatic organ delivery efficiency and inability to improve the release of antigen cytoplasm, which are key reasons for the poor clinical therapeutic effect of current water-soluble vaccines. Therefore, developing a new generation of vaccine formulations to synergistically promote the cytoplasmic delivery of tumor antigens in lymphoid organs and efficient activation of STING signaling is of great significance for improving cancer vaccine therapy. In 2022, Chen et al.[82] found that clinically used magnetic contrast agent iron oxide nanoparticles (IONPs) can significantly increase the spectrum of Type I interferon production of STING signal agonist MSA-2, and achieve a 16-fold dose-sparing effect in human STING haplotypes. Acid ions can ionize copolymers to assemble with IONPs and MSA-2 into iron nanoparticle adjuvants, to concentrate the STING activation in the draining lymph nodes. The preferred iron nanoparticle adjuvant (PEIM) effectively delivers the model antigen ovalbumin (OVA) to $CD169^+$ APCs, promotes antigen

cross-presentation, and triggers an antigen-specific $CD8^+$ cytotoxic T lymphocyte response 55 times higher than soluble antigens. This nanoparticle vaccine can induce effective and durable anti-tumor immunity to prevent tumor lung metastasis and eliminate established tumors. In addition, in B16-OVA melanoma and MC38 colorectal tumor models, PEIM nanoparticle adjuvants are also suitable for delivering autologous tumor antigens, and they synergize with immune checkpoint blockade therapy to prevent postoperative tumor recurrence and distant metastasis. Also, Chen et al.[83] showed that a therapeutic cancer vaccine composed of a protamine/mRNA core and a lipid shell is highly potent in promoting cytotoxic $CD8^+$ T cell responses and mediating anti-tumor immunity.

5.2. *Carrier*

Effective intracellular delivery is a key factor for the success of mRNA vaccines. Exogenous mRNA must cross the barrier of the lipid membrane to enter the cytoplasm and then successfully translate into functional proteins. There are two obstacles to delivering mRNA to cells: enzyme degradation during delivery and membrane barriers caused by charge repulsion. The ideal delivery system must meet several conditions: effectively package and protect mRNA, maintain its stability before reaching the target site; effectively help mRNA enter cells efficiently; and release it into the cytoplasm before reaching the lysosomes. The stability and efficient delivery of mRNA can be achieved by modifying the carrier, thereby enhancing the immunogenicity and anti-tumor performance of the vaccine. At present, various carriers have been developed for mRNA vaccine delivery to protect mRNA from degradation and promote its function. The main non-viral carriers include arginine, liposomes, and many new carriers, such as glucan vesicles.

As a positively charged polyionic protein, arginine can protect mRNA from degradation by nucleases[84,75]. Adding arginine to the carrier can completely wrap up mRNA, making the formulation more stable, and it has a significant effect on antigen accumulation in the spleen and lymph nodes. Mai et al.[85] have developed an arginine-based nasal delivery of mRNA (as shown in Fig. 6). This complex is a multi-cation-mRNA complex wrapped by DOTAP/Chol/DSPE-PEG cationic liposomes, with high stability. The results show that this cationic liposome arginine complex (LPC) has a higher uptake efficiency of vaccine particles *in vitro* and a stronger ability to stimulate the maturation of dendritic cells, further inducing a strong anti-tumor immune response.

C1 type LNP has efficient antigen expression and adjuvant characteristics and can enhance the anti-tumor effect by stimulating DC and inducing the expression of inflammatory cytokines. Zhang et al.[86] have developed a delivery system containing C1-type LNP. This delivery system can effectively deliver mRNA to APCs through phagocytosis, showing excellent performance in antigen expression and presentation. C1 type LNP can also activate TLR4, induce the activation of T cells by stimulating the TLR4 signaling pathway in DC, and induce the expression of inflammatory cytokines (such as IL-12). The mRNA vaccine using C1 type LNP shows significant anti-tumor effects in the prevention and therapeutic vaccine environment.

Previous research has shown that microbial cell walls contain unique polysaccharides and repeating carbohydrate units, which can be recognized by PRR on DCs to achieve effective immune stimulation[87,88]. Son et al.[89] have developed a new type of nanocapsule called glucan vesicles. Glucan vesicles are composed of polysaccharides in microbial cell walls. Their flexible polysaccharide shell and hollow core can effectively guide mRNA to lymph nodes and activate DCs. Among all glucan vesicles carrying mRNA, those composed of mannose are more effective. They can promote strong activation of DCs, mRNA translation, and antigen presentation on DCs. Mannitol capsules can also induce a strong antigen-specific $CD4^+$ and $CD8^+$ T cell response in the body, demonstrating an anti-tumor effect.

Outer membrane vesicles (OMVs) are extracellular carriers that are universally present around gram-negative bacteria. They are formed by the outward budding of bacterial outer membranes and carry a large number of bacterial-derived substances such as proteins, genetic materials, and toxins. They play a crucial role in bacterial life activities, including protecting bacteria from harmful substances, regulating the formation of bacterial biofilms, mediating communication between bacteria, and promoting the spread of bacterial toxicity. Li et al.[90] have genetically engineered *Escherichia coli* to establish a genetically engineered bacterium-derived oral cancer vaccine based on OMVs, which can generate OMVs carrying tumor antigens *in situ* in the intestine after oral administration. These OMVs can effectively cross the intestinal epithelial barrier together with tumor antigens, be recognized by immune cells in the inherent layer, and then effectively activate tumor antigen-specific immune responses, thereby significantly inhibiting tumor growth, limiting tumor metastasis, and exerting long-term protective effects. Although most genetically engineered bacteria are eliminated 24 h after oral administration, for safety reasons, it is still necessary to control the production of OMVs carrying tumor antigens to avoid immune tolerance caused by long-term antigen stimulation. The research team also introduced arabinose (Ara)-inducible promoter to control the expression of fusion proteins. Only in the presence of arabinose will the expression of fusion proteins be induced; otherwise, the expression remains in the off state. After oral administration of this genetically engineered bacterium and the inducer arabinose, OMVs carrying tumor antigens can be produced in the intestine in a controllable manner *in situ*, which are called OMV-Ag-mFc.

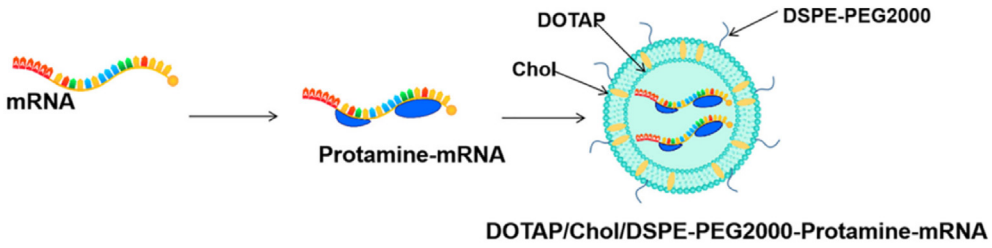

Figure 6 The preparation of the LPC/mRNA composite. mRNA was condensed by protamine and then delivered with cationic liposome. Reprinted with permission from Ref. 85. Copyright © 2020 Elsevier Inc.

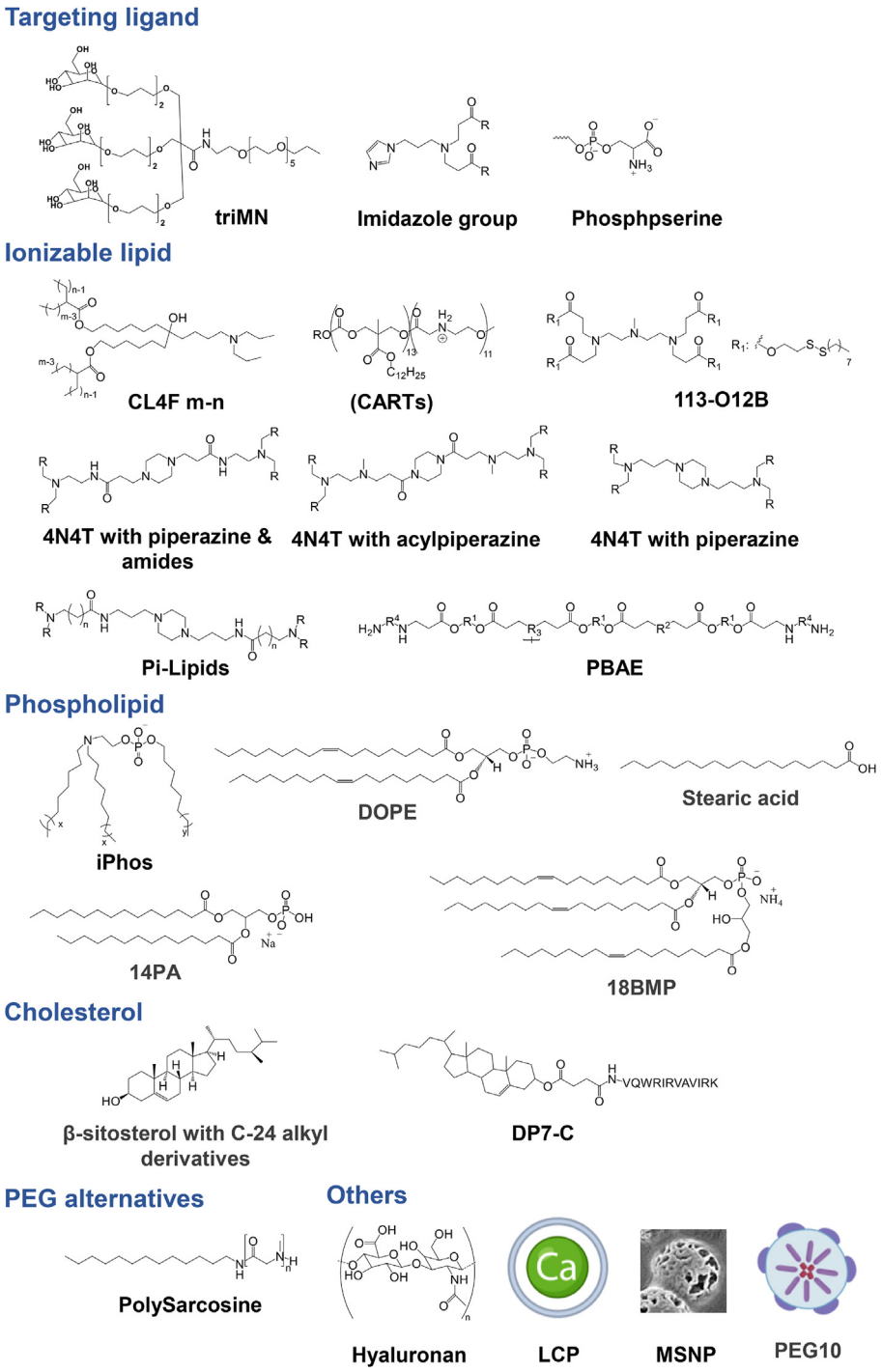

Figure 7 Representative structures of nano-based vectors to enhance the efficacy of mRNA tumor vaccines. Here listed targeting ligand include triMN[49], imidazole group[55], and phosphpserine[51]; phospholipids include iPhos[68], DOPE[68], stearic acid[48], 14 PA[54], 18BMP[54]; ionizable lipids include CL4F m-n[64], charge-altering releasable transporters (CARTs)[67], 113-O12B[62], 4N4T with piperazine & amides[65], 4N4T with acylpiperazine[65], 4N4T with piperazine[65], Pi-lipids[56], and PEAE[48]; cholesterol include β-sitosterol with C-24 alkyl derivatives DP7-C[70]; PEG alternatives include polySarcosine[37]; the others include hyaluronan[27], LCP[78], MSNP[79]. Some structures are created with BioRender.com.

5.3. *Monoclonal antibodies*

Antibodies are a class of immunoglobulins that specifically bind to antigens and play an important role in humoral immunity. When B cells encounter foreign antigens, they are activated to produce high-affinity antibodies, which can specifically recognize pathogens and clear them through various mechanisms, such as activating complement, binding Fc receptors, and antibody-dependent cell-mediated cytotoxicity.

Triple-negative breast cancer (TNBC) is the most common cancer in women worldwide, and its treatment is challenging clinically due to a lack of treatment targets[91,92,93]. Liu et al.[60] have

Table 1 Summary of advanced nano-based strategies for mRNA tumor vaccine.

Targeted function	Strategy	Design	Key advance	Ref.
Improve stability in the internal environment	Optimizing the lipids	Adjusting the ratio of helper lipids, such as DSPC or ESM	40% (mol/mol) DSPC or ESM showed significantly improved transfection properties *in vitro*, along with prolonged circulation lifespan.	29
		Coating the AuNRs with the disulfide functionalized pSar	Has negligible cytotoxicity and enhanced colloidal stability.	37
		Using inorganic material tB-UC18 to form LNNs	Trigger strong humoral and cellular immune responses in mice, but did not cause significant treatment-related adverse reactions and activation of complement system in human serum.	38
	Using biomimetic materials	Utilizing the PEG10 protein to form a new endogenous carrier SEND	Effectively circumvents immune attacks compared to other RNA delivery methods, and enables the delivery of different RNAs to specific cells or organs.	41
	Protection by hydrogel	Developing an injectable hydrogel by combining GO, PEI, adjuvants, and mRNA vaccines	Present a significantly prolonged retention time *in vivo*, and prevent tumor growth and metastasis.	43
		Loading mRLNPs into a hyaluronan dynamic hydrogel	Stabilize the LNPs at room temperature and efficiently delivering tumor antigen-encoding mRNA to DCs.	44
Improving targeting specificity	Spleen targeting	Mannosylated lipid-polymer-RNA lipopolyplexes	Facilitate targeting of DCs in the spleen and lymphoid tissues.	49
		Incorporate PS into a standard tetra-component MC3-based LNP formulation	Promote expression of PS-LNPs in lymph nodes and spleen after intravenous injection.	51
		Optimizing the net charge of intravenously administered RNA-lipid complexes	A gradual reduction in cationic lipid content in LPX results in a shift of Luc expression from the lungs to the spleen.	52
		Adjusting the proportions of SORT molecules to form an organ-selective targeting nanoparticle platform called SORT	An optimal content of 10−15% DOTAP aid spleen delivery.	54
		Lipidoids containing imidazole groups	Amine head 93 with O17O and O17S tails exhibited strong and specific luminescent expression and transfection efficiency in the spleen after intravenous injection.	55
	Lymph-node targeting	Use azide groups to modify surface of lymphatic endothelial cells, providing targets for DBCO-modified liposomes	The survival rate of mice treated with azide-targeted vaccines increased by 100% within 60 days.	59
		Use mannose-modified lipid calcium phosphate (LCP) to deliver mRNA encoding MUC1 to DCs in LNs	Mannose-modified LCP could deliver mRNA to the LNs and mRNA was translated into the target protein correctly.	60
		Construct a hitchhike vaccine that comprises an antigen or adjuvant linked to a lipophilic albumin-binding tail *via* a solubilizing PEG chain	Vaccine accumulated in the LNs after subcutaneous injection, resulting in a 30-fold increase in T cell priming and strong inhibition of tumor growth.	61
		Explore an endogenous LN-targeting LNP called 113-O12B	Significantly reduced expression level in the liver, while its expression in the LNs was enhanced and specific.	62
Improving transfection efficiency	LNP	Introduce InstantFECT modified with pseuoguanosine	Break the limitation of the transfection, enlarge the transfection area, and inhibit wrong transfection in other critical organs.	66
		Use charge changing transporter by RNA (CRAT)	Using the neutral by-products of CART, allowing the polyanionic mRNA cargo to escape from lysosomes.	67
		Developing a unique phospholipid called iPhos, having a zwitterionic headgroup and three alkyl tails.	Promoting membrane fusion, enhancing the endosomal escape process, thereby improving the transfection efficiency of mRNA.	68
		Replacing cholesterol with β-sitosterol	Preventing the uptake and degradation of delivery system by endosomes, then increasing the amount of mRNA entering the cells, thereby achieving high transfection efficiency.	70

		Introduce protamine into DOTAP	Improving the capacity of condensing mRNA, reducing the probability of loss of encapsulated mRNA antigen.	74
	Change formulation structure	Forming vesicular structures in the presence of high concentrations of pH 4 buffer solutions	Encapsulating mRNA and enhancing its stability, thus improving the transfection efficiency of delivery systems composed by less active ionizable lipids.	77
	Inorganic materials	Establish a lipid-coated calcium phosphate formulation called LCP NPs	Utilizing the dissolution of CaP nuclei in the acidic lysosomal compartment, leading to an increase in the osmotic pressure in the lysosome, resulting in the rupture of the endosomal membrane.	78
Enhancing immunogenicity	Adjuvants	Adding Pam3 as an adjuvant to LNPs containing OVA mRNA	The synergistic effect of Pam3 LNPs can effectively improve the efficacy of mRNA vaccine for tumor prevention.	80
		Combination of TLR4 agonists and mRNA, delivering LNPs that carry unmodified antigen mRNA and TLR4 agonist MPLA	Potent tumor immunotherapy through synergistic different immune stimulations and Th1 type immune responses.	48
		Developing a tumor vaccine that directly integrates dsRNA targeting RIG-I as an adjuvant into the antigen-encoding mRNA chain	Effectively activating dendritic cells and drive them to secrete a wide range of pro-inflammatory cytokines without increasing the secretion of anti-inflammatory cytokines.	81
		Acid ions can ionize copolymers to assemble with IONPs and MSA-2 into iron nanoparticle adjuvants	Increasing the spectrum of Type I interferon production of STING signal agonist MSA-2, and achieve a 16-fold dose-sparing effect in human STING haplotypes.	82
	Carrier	Encapsulating the positively charged protamine mRNA vaccine with DOTAP/Chol/DSPE-PEG cationic liposomes	Higher uptake efficiency of vaccine particles and a stronger ability to stimulate DCs maturation *in vitro*.	76
		A nanovaccine containing C1 type LNP with high antigen expression and self-adjuvant properties	Activate TLR4 and induce powerful T cell activation.	86
		Using sugar capsules	Promote strong activation of DC, mRNA translation, and antigen presentation on DC.	89
		Engineering *Escherichia coli* to establish a genetically engineered bacterium-derived oral cancer vaccine based on OMVs	Generating OMVs carrying tumor antigens *in situ* in the intestine after oral administration.	90
	Monoclonal antibodies	Combining monoclonal antibodies against CTLA-4 with the mRNA Vaccine	Enhancing the anti-tumor immune response compared to the vaccine or monoclonal antibody alone.	60
	Combined therapy	BNT122 combined with anti-PD-L1 antibody atezolizumab and mFOLFIRINOX chemotherapy	Significantly delaying the recurrence time of pancreatic cancer patients undergoing surgical resection.	95

AuNRs, gold nanorods; CART, charge releasable transport protein; CTLA-4, cytotoxic T lymphocyte-associated protein 4; DCs, dendritic cells; DSPC, distearoyl phosphatidylcholine; dsRNA, double-stranded RNA; ESM, egg sphingomyelin; GO, graphene oxide; IONPs, iron oxide nanoparticles; LCP, lipid calcium phosphate; LNs, lymph nodes; LNPs, lipid nanoparticles; LPX, lipid complexes; mRNA, messenger ribonucleic acid; OMVs, outer membrane vesicles; OVA, ovalbumin; PEG, polyethylene glycol; PEI, polyethyleneimine; PS, phosphatidylserine; RIG-I, retinoic-acid-inducible gene I; T cells, thymus dependent lymphocyte cells; Th1, T helper cell 1; TLR4, toll-like receptors 4.

Table 2 Summary of Clinical trials with mRNA vaccines against cancer.

ClinicalTrials.gov identifier	Drug administration	Drug combination	Phase
NCT02316457	Breast cancer (triple negative breast cancer, TNBC)	IVAC_W_bre1_uID/IVAC_M_uID	Phase I
NCT02035956	Melanoma	IVAC MUTANOME, RBL001/RBL002	Phase I
NCT04526899	Melanoma stage; melanoma stage IV; Unresectable melanoma	BNT111/cemiplimab	Phase II
NCT05158621	Metastatic colorectal cancer; stage II/III colon cancer	None	Completed
NCT04853017	Minimal residual disease; KRAS G12D; KRAS G12R; NRAS G12D; NRAS G12R; pancreatic ductal; adenocarcinoma; colorectal cancer; non-small cell lung cancer; ovarian cancer; cholangiocarcinoma; bile duct cancer; gallbladder carcinoma	None	Phase I
NCT03313778	Solid tumors	mRNA-4157/pembrolizumab	Phase I
NCT03394937	Resected melanoma (stages II c, III and IV)	None	Phase I
NCT01817738	Metastatic castration-resistant prostate cancer	None	Phase I/II
T00923312	Non-small-cell lung cancer (stages IIIb and IV)	None	Phase I/II
NCT01915524	Non-small-cell lung cancer (stage IV)	With local irradiation (with or without pemetrexed and with or without EGFR tyrosine-kinase inhibitor)	Phase I
NCT02410733	Melanoma	With or without standard PD-1 therapy	Phase I
NCT04503278	CLDN6 (CARVac)	With CLDN6 CAR-T cells	Phase I/II
NCT03480152	Gastrointestinal cancer	None	Phase I/II
NCT03289962	Melanoma; non-small cell lung; cancer bladder; cancer colorectal; cancer triple negative; breast cancer renal; cancer head and neck cancer; other solid cancers	Autogene cevumeran/atezolizumab	Phase I

attempted to treat TNBC with mRNA vaccines by combining monoclonal antibodies against cytotoxic T lymphocyte-associated protein 4 (CTLA-4) with mRNA vaccines to enhance their anti-tumor effect. *In vivo* studies show that the mRNA vaccine targeting mannose receptors on DCs successfully expresses tumor antigens in lymph nodes and induces a strong, antigen-specific cytotoxic T lymphocyte response against TNBC 4T1 cells. They believe that compared with using either treatment method alone, the combined immunotherapy of vaccines and anti-CTLA-4 monoclonal antibodies can significantly enhance the anti-tumor immune response.

5.4. *Combined therapy*

Combining mRNA with some existing clinical therapies, such as PD-1 antibodies, chemotherapy, and CTLA-4 antibody therapy, can effectively enhance the immunogenicity of the nucleic acid vaccine and perform better[94]. In 2022, Moderna and Merck jointly announced that the 2 b clinical trial of the cancer mRNA vaccine mRNA-4157 (V940) combined with Keytruda for the treatment of melanoma reached its primary endpoint, and phase III clinical trials will be launched this year and rapidly extended to other tumors. The study uses the mRNA cancer vaccine and PD-1 antibody as adjuvant treatments after surgery to prevent a recurrence. The results show that this combined treatment method can reduce the risk of recurrence or death by 44%. Similarly, last year, BioNTech announced the launch of the mRNA vaccine BNT116 combined with Libtayo (Cemiplimab) PD-1 antibody therapy for the treatment of late-stage non-small cell lung cancer. Clinical trial results also show that the combined medication group outperforms the single-drug group. In addition, BioNTech recently introduced BNT122 combined with anti-PD-L1 antibody atezolizumab and mFOLFIRINOX chemotherapy, which can significantly delay the recurrence time of pancreatic cancer patients undergoing surgical resection[95]. This therapy induces a large number of T cell activities, which may be related to the delay in PDAC recurrence.

Representative structures of nano-based vectors to enhance the efficacy of mRNA tumor vaccines are in Fig. 7. Examples of advanced nano-based delivery systems for mRNA tumor vaccines are summarized in Table 1.

6. Conclusions and perspectives

Over the past three years, the COVID-19 pandemic has brought the mRNA vaccine into view, and now that the impact of the global epidemic has almost passed, while the mRNA vaccine track has only just been started. mRNA tumor vaccines are an important direction, as shown in Table 2, the clinical trials of mRNA tumor vaccines are in full swing. Although mRNA technology has developed in leaps and bounds during the pandemic, there are still many limitations of the current mRNA delivery system. In this review, we analyze four major aspects of the limitations of mRNA tumor vaccine, including stability, targeting specificity, transfection efficiency, and immunogenicity, and summarize recent advances in nano-based delivery strategies to enhance the therapeutic effect from administration to finally immune response. In terms of stability, although there is indeed no good way to further improve the *in vivo* stability of LNPs, taking advantage of the protection and *in vivo* release capabilities of the hydrogel system, may be a good strategy to load mRNA into hydrogels, as it has been shown to be able to prolong the action time of mRNA after

subcutaneous injection. Nevertheless, whether the final released mRNA is still in LNPs remains to be investigated. In addition, regarding lymph targeting, mannose modification is the commonly used active targeting strategy, which has been proven to be effective in improving peripheral DC recognition; At the same time, reasonable particle size and negative surface charge, from the current point of view, maybe more important for lymph system targeting. However, the mechanism of specific targeting, presumably related to the binding protein corona, still needs further explanation. As for improving transfection, it is mainly achieved by optimizing the four components of LNPs, adding a fifth component, or using the inorganic materials with the function of protection and sustained release. Finally, whether from current preclinical or clinical research results, it can be found that combination therapy is necessary, but the immune system is very delicate, and how the degree of immune up-regulation is optimal needs to be verified by more experiments.

In addition, although the current industry chain of mRNA production and LNP preparation is quite mature at present, it still takes more than 2 months to obtain the final mRNA product from antigen peptide screening, analysis, plasmid construction, and manufacture, as the preliminary calculation and screening is the key step. Therefore, for patients with advanced diseases, it may not be possible to wait for the production of specific vaccines, that's why the research of TAAs is ongoing even with a risk of ineffectiveness. Moreover, the spleen is the largest immune organ in the human body, and theoretically targeting the spleen is an effective method to induce a potent immune response. Indeed, the RNA-LPX delivery technology of BioNTech has achieved several good clinical therapeutic effects by expressing TAAs or TSAs in the spleen after IV administration, combined with popular targeted therapy. At present, many studies have developed mRNA delivery systems with spleen-targeting function, which would be a good breakthrough and interesting attempt to improve the immunotherapy efficacy of vaccines. But meanwhile, the liver remains the organ with the largest accumulation of substances, and hepatic and systemic toxicity after intravenous administration of the spleen-targeting delivery systems must be clarified. Besides, with continuous attempts on optimizing mRNA sequence and research on novel types of mRNA, currently available mRNA varies considerably. There are three types of mRNA vaccine constructs, including non−replicating mRNA vaccine construct, self−amplifying mRNA vaccine construct, and trans−amplifying mRNA vaccine construct[96]. Different types and sizes of mRNA have diverse molecular weight and electrical properties, and thus may have different requirements for delivery systems, which also need further research. In addition to the research on the delivery system of mRNA tumor vaccine, the optimization of mRNA sequence is also a field worthy of deep cultivation. The sequence of mRNA itself is decisive for vaccine target, function, and potency, and is also the foundation of vaccine treatment of tumors.

Acknowledgments

This work was supported by National Natural Science Funds of China (Nos. 92059110 and 81872808), Development Fund for Shanghai Talents (No. 2020090, China), Natural Science Foundation of Shanghai Science and Technology Innovation Plan (No. 22ZR1414100, China), FDU 2025- Excellence Program Fund, Project Supported by Shanghai Municipal Science and Technology Major Project (No. 2018SHZDZX01, China) and ZJLab, Shanghai Post-doctoral Excellence Program (No. 2033016, China), China Postdoctoral Science Foundation Funded Project (No. 2023M730711).

Author contributions

Chen Jiang and Tao Sun designed the review. Yangqi Qu, Jingjing Xu, and Tong Zhang read and analyzed the references and wrote the manuscript. Jiang Chen, Tao Sun and Qinjun Chen revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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