AIMS Biophysics

AIMS Biophysics, 7(4): 323–338.
DOI: 10.3934/biophy.2020023
Received: 21 June 2020
Accepted: 16 August 2020
Published: 20 August 2020

http://www.aimspress.com/journal/biophysics

Review

Nanoparticle-based delivery platforms for mRNA vaccine development

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Abstract: Conventional vaccines have saved millions of lives, and new vaccines have also been developed; however, an urgent need for an efficient vaccine against SARS-CoV-2 showed us that vaccine development technologies should be improved more to obtain prophylactic agents rapidly during pandemic diseases. One of the next-generation vaccine technologies is utilization of mRNA molecules encoding antigens. The mRNA vaccines offer many advantages compared to conventional and other subunit vaccines. For instance, mRNA vaccines are relatively safe since they do not cause disease and mRNA does not integrate into the genome. mRNA vaccines also provide diverse types of immune responses resulting in the activation of CD4+ and CD8+ T cells. However, utilization of mRNA molecules also has some drawbacks such as degradation by ubiquitous nucleases in vivo. Nanoparticles (NPs) are delivery platforms that carry the desired molecule, a drug or a vaccine agent, to the target cell such as antigen presenting cells in the case of vaccine development. NP platforms also protect mRNA molecules from the degradation by nucleases. Therefore, efficient mRNA vaccines can be obtained via utilization of NPs in the formulation. Although lipid-based NPs are widely preferred in vaccine development due to the nature of cell membrane, there are various types of other NPs used in vaccine formulations, such as virus-like particles (VLPs), polymers, polypeptides, dendrimers or gold NPs. Improvements in the NP delivery technologies will contribute to the development of mRNA vaccines with higher efficiency.

Keywords: adjuvant; delivery system; mRNA vaccine; nanoparticle; vaccine development; immune response

1. Introduction

A messenger RNA (mRNA) is a single-stranded RNA molecule that functions in the biosynthesis of proteins through ribosomes in the cytoplasm [1]. Changes in the production levels of proteins due to the mutations affecting mRNA maturation, ribosome biogenesis or translation may cause many ailments such as cancer while infections may play a major role in the sudden change of protein levels [2]. Therefore, mRNA level in the cytoplasm is important for many diseases. For instance, in case of choroideremia, an X-linked disease of retinal degeneration, the male patients carry the variants of *CHM* gene causing deficiency in REP1 protein. However, some patients have c.940 + 3delA variation affecting the splice site of *CHM* intron 7. Thus, the level of correctly spliced mRNA for *CHM* variant is decreased, and the disease progression is decelerated [3].

In eukaryotes, mature mRNA covers 5' methylguanosine (m7G or 5' cap), 5'–untranslated region (UTR), coding region, 3'–UTR, and polyadenylated [poly(A)] tail [4]. Biosynthesis of proteins begins with the recognition of mRNA sequence by ribosome at 5'–UTR. The 3'–UTR is involved in mRNA stabilization, containing various microRNA (miRNA) binding sites. When the poly(A) tail is less than 12 adenosine nucleotides, the mRNA is separated from the 5' cap structure; therefore, this tail is critical for mRNA to continue or stop translation [5].

In addition to its role in cellular biological activities, mRNA has also been shown as a promising molecule for various therapeutic and vaccine applications over the past few years [6–8]. Especially in 2020, a liposome nanoparticle (NP)-based mRNA vaccine encoding S protein (mRNA-1273) became a candidate vaccine (in phase 3 trial) against the new type of coronavirus (SARS-CoV-2) which spread globally from Wuhan, China [9].

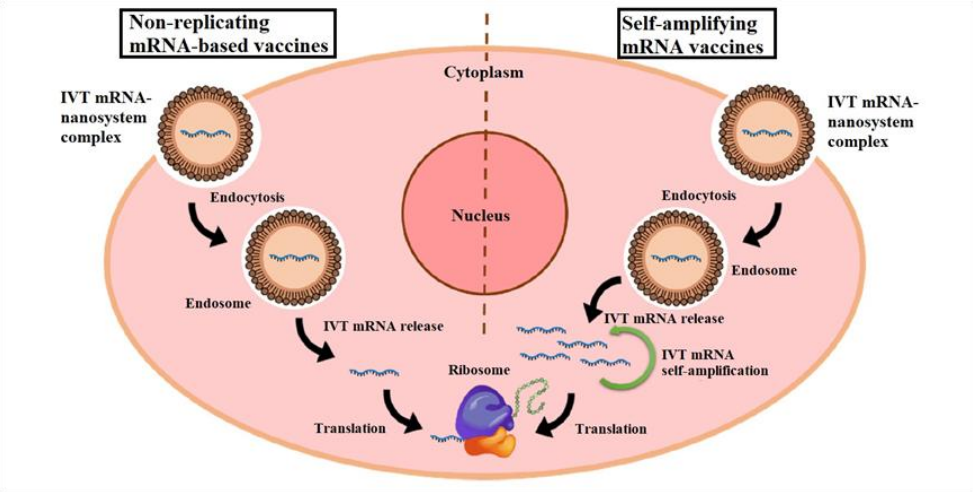

Figure 1. Schematic representation of two types of mRNA vaccines. In vitro transcribed (IVT) mRNA molecules are loaded into nanoparticles (NPs), and this complex enters into the cell via endocytosis forming an endosome. Later, the IVT mRNA releases from the complex, and directly joins to ribosome for translation of antigen in non-replicating mRNA vaccines. Self-amplifying mRNA (SAM) vaccines, also called replicon, contain two different open reading frames (ORFs). Following the release from endosome, one ORF encodes the antigen of interest, and the other encodes proteins for RNA capping and replication [18].

mRNA vaccines provide relatively reliable, simple, and inexpensive vaccine solutions which are suitable for mass production [10–12]. In these vaccine systems, mRNAs enable the expression of encoded antigens in the transfected cells providing a strong T cell response. mRNA vaccines offer advantages such as producing a strong immune response without a separate adjuvant compared to subunit vaccines. Also, mRNA vaccines do not possess the drawbacks such as reversibility of live attenuated vaccines, and weak cellular immunity in DNA vaccines [13,14]. Single-stranded RNAs may integrate into the genome. However, mRNAs used for vaccine purposes do not enter into the nucleus, and their integration into genomic DNA is prevented. [15]. One of the most important advantages of mRNA vaccines is their capacity to transfect dendritic cells (DCs) higher than other types of vaccines [16]. mRNA vaccines can be produced using non-replicating or self-amplifying mRNA molecules (Figure 1) [17,18].

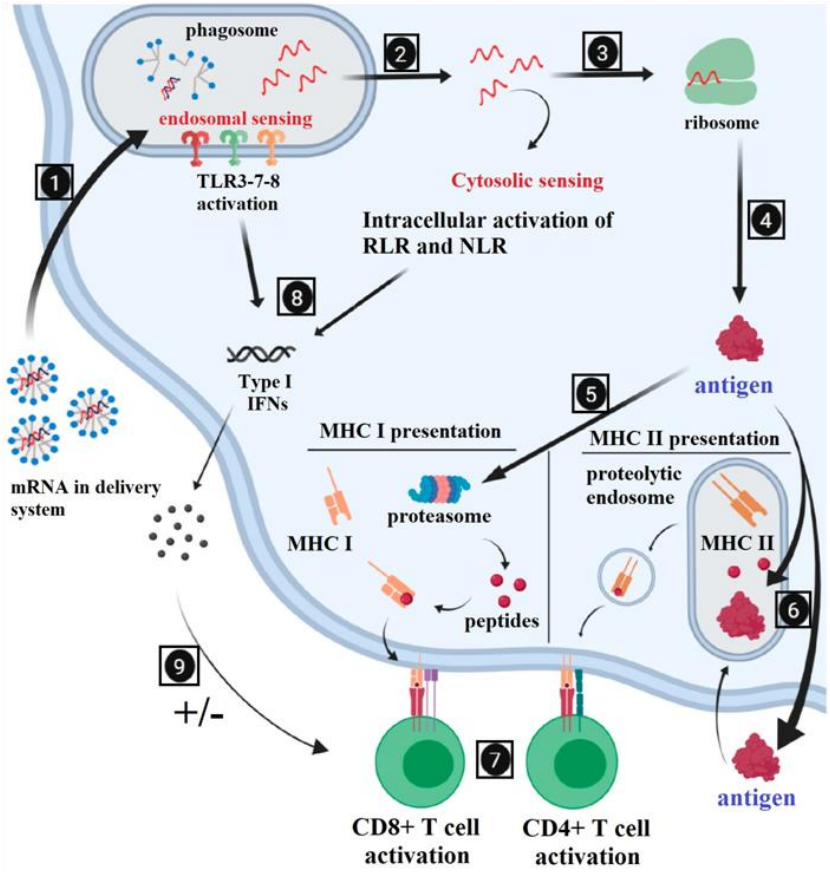

Figure 2. Stimulation of the different arms of immune responses by mRNA vaccines. 1) The mRNA delivery system is processed in phagosome, and Toll-like receptors (TLRs) are activated. 2) Intracellular activation of retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) occurs via cytosolic sensing of mRNA. 3) mRNA binds to ribosome. 4) Antigen is produced in ribosome. 5-6) Antigens stimulate immune response via MHC I or MHC II presentation. 7) In MHC I presentation, peptides are produced via proteasomal activity, and CD8+ T cells are activated. In MHC II presentation, peptides are produced via endosomal activity, and CD4+ T cells are activated. 8) Activation of TLRs, RLRs and NLRs stimulates production of type I interferons (IFNs) without antigen presentation. 9) Type I IFNs may have positive or negative effective on T cell activation [19].

mRNA vaccines highly target DCs to provide an increased T cell response. As shown in Figure 2, mRNA molecules enter into the antigen presenting cells (APCs), are transported to appropriate vesicles, and enable coding of antigens with ribosomal activity. The peptide antigens activate CD8+ T cells by binding to major histocompatibility complex class I (MHC I) through proteosomal activities, and activate CD4+ T cells by binding to MHC II molecules through endosomal activity [19]. Humoral immunity is usually provided by mRNA vaccines via antigen-specific antibodies produced by B cells [20,21]. Follicular T cells, one of the T cell subtypes induced by mRNA-NP immunization in mice, increase the responses of B cells that can produce long-lived antibodies with strong affinity to bacterial and viral pathogens [22,23]. The CD8+ cytotoxic T cells (CTLs), providing cellular immunity, were shown to attack viruses or destroy cancer cells. mRNA vaccines exhibit strong CTL and Th1 immune responses, especially with an increase in cytokine levels. Cellular immune responses are promising for the treatment of serious illnesses such as AIDS and cancer [24,25].

There are some drawbacks in the development of mRNA vaccines [26]. In particular, mRNA molecules possess low stability, and cannot easily cross the cell membrane [27]. Difficulties in intracellular delivery also pose a problem since mRNA molecules are sensitive to catalytic hydrolysis by the omnipresent ribonucleases [28]. Therefore, when administered to the body on their own, mRNA molecules may not reach to the desired target. Many strategies have been developed to overcome this problem encountered during in vivo studies of mRNA vaccines. RNA conjugations and modifications, viral vector transfections, micro and nanoparticles have been used for RNA delivery [29–31]. Structure of mRNA can also be improved for enhanced expression of encoded antigen. Codon modifications in protein coding regions of mRNA sequences can significantly increase protein expression levels [32,33]. The 5' cap modification of mRNA can induce translation by increasing the resistance of RNA to hydrolytic catalysis [34]. Eventually, efficiency of mRNA vaccines can be advanced via optimization of mRNA structure and utilization of proper delivery systems [35–37].

2. Nanoparticles used in mRNA vaccine formulations

Many of the next-generation vaccines were observed to provide weak immune responses [38]. Therefore, new applications have been introduced, and more technological materials are needed to increase the immunogenicity of technological vaccines. NPs produced from various biocompatible materials with sizes ranging from 1-100 nanometers (nm) have many advantageous properties [39]. Since the NPs can be obtained smaller than the size of a cell, molecules capable of cellular entry through endocytosis or pinocytosis can be synthesized [40].

NP systems have been used for the delivery of diverse pharmaceuticals, and offer many advantages for mRNA vaccines, such as increased pharmacokinetic efficacy. Thus, the potential of mRNAs to be used in gene therapy, immunotherapy, and cancer therapies as well as in therapeutic and prophylactic vaccine applications was increased [41,42]. The nano-carrier systems used in formulations can increase the stability of the mRNAs by protecting them from enzymatic degradation in bloodstream, and can also provide adjuvant properties, easily delivering vaccines to APCs [43]. Encapsulation of mRNA molecules with NPs facilitates receptor interactions of APCs by expanding surface adsorption, and provides controlled release [44]. Due to their small size, NPs can quickly pass through the epithelial barriers, and enter into the bloodstream in invasive applications. However, our innate immune system might constitute a risk to damage the NP system after

introduction into the body. In addition to vaccine agents (mRNA, DNA, peptide etc.), NPs can also have immunotoxicological effects [45].

Various biocompatible polymeric, lipid-based, and inorganic NPs such as gold, carbon, liposome, dendrimer, poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), and silica have been applied in the vaccine delivery studies [46,47]. PLGA, PEG and polylactic acid (PLA) are also approved by The U.S. Food and Drug Administration (FDA). NPs are produced with desired size, shape and surface modifications, and used effectively in the successful and stable delivery of antigens. For instance, gold NPs (AuNPs) delivering antigens of pathogens such as influenza virus or human immunodeficiency virus (HIV) showed a robust immune response in vivo [48,49].

2.1. Polymeric nanoparticles

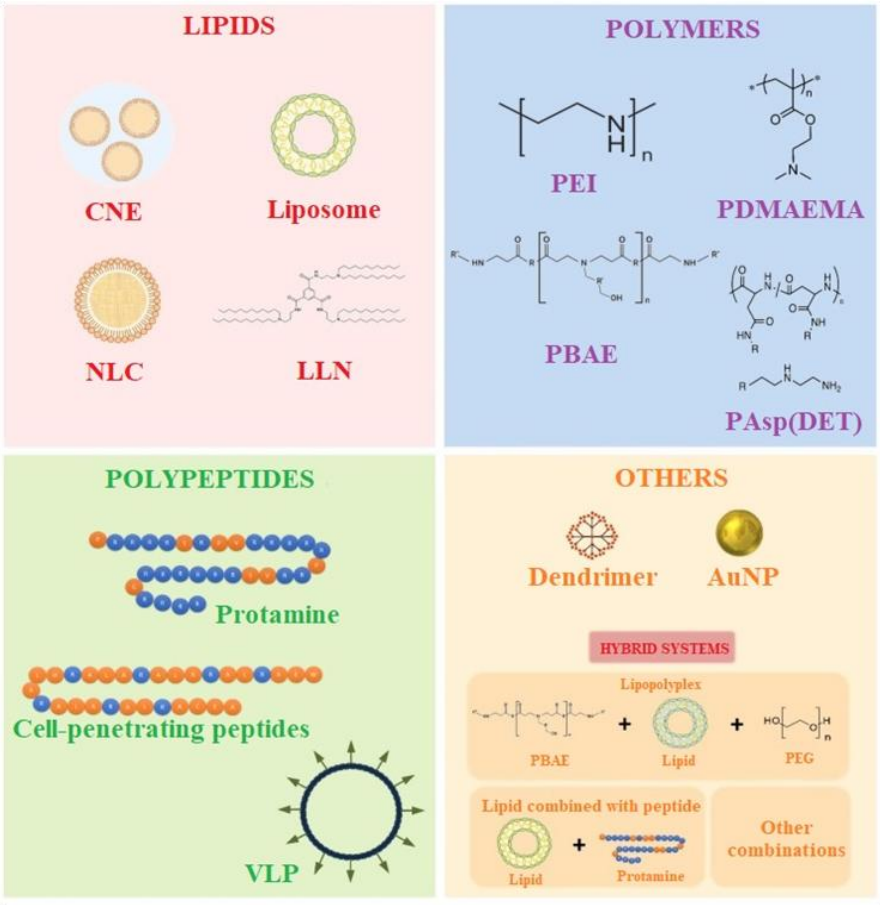

Figure 3. Nanoparticle systems frequently used for the delivery of mRNA molecules into cells for vaccine purposes. AuNP: gold nanoparticle, CNE: cationic nanoemulsion, LLN: lipid-like nanoparticle, NLC: nanostructured lipid carrier, PAsp(DET): poly(aspartamide) bearing 1,2-diaminoethane side chains, PBAE: poly(β-amino ester), PDMAEMA: poly[2-(dimethylamino)ethyl methacrylate], PEG: polyethylene glycol, PEI: poly(ethyleneimine), VLP: virus-like particle **[**18].

Polymeric NPs are frequently preferred in the delivery of vaccines due to their biodegradability and biocompatibility, lack of toxicity, easy surface modifications, and low cost of synthesis [50].

Various types of polymeric materials are used for NP production, such as polyamines and polypeptides as well as bipolar and triblock polymers (Figure 3). Especially, poly(ethyleneimine) (PEI) is a cationic polymer commonly used for the delivery of nucleic acids [51,52]. The combination of PEI and PLGA can also be used for the efficient delivery of IVT mRNA to DCs [53]. Uchida et al. [54] added a cholesterol moiety to PEG-polycation block copolymers in the formulation with IVT mRNA encoding anti-angiogenic protein (sFlt-1), and it was shown to inhibit growth of pancreatic tumor tissues significantly.

Biocompatible and pH-sensitive poly(β-amino ester)s (PBAEs) are synthesized by the addition of amines and acrylates, and interact with IVT mRNAs electrostatically from their tertiary amine groups. Antigen production can be obtained after 24 hours in the lung using properly dispersed PBAE-mRNA formulations in mice via inhalation. They showed very high stability in the blood serum when administered intravenously [55,56]. PBAE nanosystems are also used to form copolymers with polymers such as PEG, PLA and poly(ε-caprolactone) (PCL) [57]. Capasso Palmiero et al. [58] used PBAE-co-PCL terpolymers for the delivery of mRNA molecules, and showed that the terpolymer had higher transfection efficiency than PEI. Palam à et al. [59] produced highly stable PCL NPs loaded with mRNA-protamine complex for the delivery of mRNA molecules into the cell. Recently, mRNA transfection with PLA micelles capable of targeting DCs was also reported [60]. Structure-activity relationship analysis of poly(glycoamidoamine) (PGAA) showed that increased number of amino groups elevated the transfection efficiency of mRNA [61].

2.2. Dendrimers

Dendrimers are highly branched, globular, polymeric macromolecules. The architecture of dendrimers is uniform and well-defined with three distinct components: a core domain at the center, repetitive hyperbranched units, and corona with modifiable functional groups. Desired properties can be given to dendrimers via controlling their architecture, and functional nanocarriers can be obtained. It is possible to encapsulate pharmaceuticals in the internal cavity or bound them to the surface of dendrimers via electrostatic or hydrophobic interactions. Attachment between pharmaceutical and dendrimer can also be obtained through covalent bonds at the terminal functional groups [62,63].

Chahal et al. [52] produced a dendrimer-based mRNA vaccine composed of an ionizable modified dendrimer NP, a lipid-anchored PEG, and mRNA molecules encoding H1N1 hemagglutinin (HA), Ebola virus (EBOV) glycoprotein (GP) or multiplexed antigens of *Toxoplasma gondii*. This vaccine was shown to protect mice against lethal viral and *T. gondii* challenges. Moreover, Islam et al. [64] obtained a polymer-lipid hybrid NP using a modified polyamidoamine (PAMAM) dendrimer, ceramide-PEG, and PLGA. The hybrid NP successfully delivered mRNA encoding phosphatase and tensin homolog (PTEN) to prostate cancer cells, and tumor growth was inhibited in mice.

2.3. Polysaccharide-based nanoparticles

Polysaccharide-based NPs have been used efficiently for the targeted delivery of pharmaceuticals. Chitosan is composed of N-acetyl-D-glucosamine monomers, and chitosan NPs have been used for the delivery of mRNA molecules. The mRNA vaccine encoding influenza proteins H9N2 HA2 and M2e formulated with chitosan NPs provided increased immune responses and protection in chickens against challenge with avian influenza viruses H7N9 or H9N2 [65].

McCullough et al. [66] also reported that chitosan NPs successfully delivered mRNA molecules, encoding HA and nucleoprotein of influenza virus, to DCs. Additionally, chitosan-coated selenium NPs were used for the delivery of F*luc* mRNA, and induced apoptosis was observed in the targeted colorectal and colon carcinoma cells in vitro [67]. Another polysaccharide used for NP production is mannan. Son et al. [68] reported that the mannan capsules were efficient in the delivery of mRNA and activation of DCs. Moreover, Siewert et al. [69] showed that cationic polysaccharide diethylaminoethylen (DEAE)-dextran system can be used for mRNA delivery.

2.4. Peptide-based nanoparticles

Peptides used for the mRNA delivery should be cationic, containing positively charged amino acids like lysine and arginine to have electrostatic interactions with the negatively charged phosphate groups of nucleic acids. Encapsulation efficiency can be enhanced via increasing the amount of charged amino groups compared to phosphate groups [70].

Protamine is a small, cationic, arginine-rich nuclear protein playing role in DNA stability during spermatogenesis in testis. Due to its association with nucleic acids, protamine was shown to stabilize and deliver mRNA molecules [71,72]. Protamine-mRNA complex is protected from nucleases, and has adjuvant effect via TLR7 activation. However, the mRNA in this complex might be translated poorly [70]. Fotin-Mleczek et al. [73] showed that protamin-complexed mRNA vaccine stimulated TLR7-mediated immune responses and displayed antitumor activity against ovalbumin (OVA)-expressing lymphoma cells in mice. Moreover, Schnee et al. [74] reported that the mRNA encoding glycoprotein of rabies virus (RABV-G) formulated with protamine induced immune responses and provided protection against viral challenge in mice and pigs.

Cell-penetrating peptides (CPPs) are also cationic molecules promising in mRNA delivery. Arginine-rich RALA peptide (WEARLARALARALARHLARALARALRACEA) was used to obtain a condensed nanocomplex with OVA-mRNA, providing specific CTL response in mice [75]. Additionally, Coolen et al. [76] used RALA, LAH4 (KKALLALALHHLAHLALHLALALKKA), and LAH4-L1 (KKALLAHALHLLALLALHLAHALKKA) amphipathic CPPs to vector mRNA molecules onto PLA NPs, and showed that LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA formulations were promising platforms for the development of mRNA vaccines.

Virus-like particles (VLPs) are other successful peptide-based mRNA delivery systems. VLPs can efficiently package mRNAs, and carry them to target cells protecting from degradation by RNases [77]. The recombinant bacteriophage MS2 VLP-based mRNA vaccine was shown to provide high humoral and cellular responses in mice delaying the tumor growth [78]. Sun et al. [79] also used MS2 VLPs for the delivery of mRNA encoding Gag protein of HIV-1, and showed increased antibody response in mice specific to Gag antigen. Recently, a chimeric VLP system was obtained by the fusion of a ribosomal protein (L7Ae) from *Archaeoglobus fulgidus* and the protein G of Vesicular Stomatitis Virus, and effective delivery of mRNA was achieved into the cell lines difficult to transfect [80]. Moreover, artificial VLPs can be produced using synthetic peptides. Jekhmane et al. [77] composed an artificial VLP composed of an oligolysine, a midblock similar to silk protein, and a hydrophilic C-terminal random coil. Self-assembly of these peptides resulted in the rod-shaped VLPs each containing one to five mRNAs.

2.5. Lipid-based nanoparticles

Liposomes are generally circular NPs with hydrophilic nuclei from 20 nm to several microns in size, and with one or more lipid layers. Two-layered liposomes are preferred for the formulations due to ease of cellular endocytosis. Cholesterol and PEG are used to stabilize those two layers and to avoid immune cell attack, respectively [81,82]. The first use of liposomes in mRNA vaccines was demonstrated in 1978 by introducing rabbit globin mRNA sequences to mouse lymphocyte cells [83]. Many liposomes were effective in vaccine studies, especially for the antigens weak in cell internalization. The single- or multi-layered liposomes can be degraded in biological fluids and contain many types of units such as phosphatidylserine, phosphatidylcholine, and cholesterol [84,85].

Among the NP systems used for the delivery of mRNA into cells, lipid-based nanomaterials are highly effective [86]. Monslow et al. [87] reported that mRNA encoding gE antigen of Varicella-zoster virus (VZV) formulated with lipid NP conferred higher immune responses than live attenuated VZV. In addition to antigen encoding mRNAs, delivery of exogenous mRNAs encoding monoclonal antibodies (mAbs) for the treatment of infectious diseases has also been studied. Recently, Erasmus et al. [88] showed that intramuscular (i.m.) administration of mRNA encoding ZIKV-117, a neutralizing human mAb, delivered by lipid NPs provided protection against Zika virus challenge in mice. Also, lipid NP-formulated mRNA vaccine encoding multiple conserved antigens of Influenza virus conferred protection against challenge with a panel of Group 1 Influenza A viruses in mice [89]. Additionally, Lo et al. [90] reported that a lipid-based mRNA vaccine encoding the soluble glycoprotein of Hendra virus provided protection against Nipah virus challenge in Syrian hamsters.

A clinically advanced form of lipid NPs, ionizable lipid NPs (iLNPs) are widely used for mRNA delivery. The iLNPs are neutral under physiological conditions but charges are formed in the acidic environments such as endosomes [91]. Moyo et al. [92] developed a tetravalent iLNP-mRNA vaccine "HIVconsvM" against HIV, which conferred strong T-cell responses in mice.

Additionally, utilization of cationic lipid NPs in mRNA vaccine formulations, such as dimethyldioctadecylammonium (DDA)- or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-based SAM vaccines, were shown to provide strong immune responses in mice as much as iLNPs [93]. The mRNA encoding cytokeratin 19 delivered by cationic liposome/protamine complex increased cellular immune responses and anti-tumor activity in a Lewis lung cancer model]94]. Moreover, lipid NPs including cholesterol analogs have higher capacity of gene transfection [95]. The mRNA vaccine formulation containing DOTAP/cholesterol NPs reduced Influenza A viral titers and morbidity in mice [96].

Cationic nanoemulsions (CNEs) are also used in mRNA vaccines, consisted of a dispersion of an oil phase stabilized with an aqueous phase containing a cationic lipid layer of about 200 nm in size [97]. The mRNA vaccine encoding HIV-1 envelope protein formulated with CNEs exhibited strong immune responses in rhesus macaques [98].

Different approaches can be used to enhance the immunological activity of lipid-based NPs. The mRNA vaccines developed using lipid and PLA NPs boosted Th1 responses, and elevated endosomal and cytosolic receptor activity, inducing innate immune response by DC transfection in vitro [76]. Yang et al. [8] used a hybrid PLGA-core/lipid-shell NP system to co-deliver mRNA and gardiquimod, a TLR7 agonist, and showed an efficient gene expression in spleen as well as induced immune responses in mice.

Clinical trials of some mRNA vaccines formulated with lipid-based NPs are under assessment. Currently, clinical trials of mRNA-1273 vaccine encoding full-length spike (S) protein of SARS-CoV-2 (NCT04283461) [99], and the mRNA encoding RABV-G (NCT03713086) [100], both formulated with lipid NPs, are ongoing. Also, lipid NP-formulated mRNA vaccines against influenza reached to clinical trials (NCT03076385, NCT03345043) after pre-clinical studies in primates and mice. These vaccines were shown to induce humoral immune response against H10N8 and H7N9 influenza viruses in humans [101,102].

2.6. Gold nanoparticles

Gold NPs (AuNPs) are promising for mRNA delivery because of their small size and scalability as well as nontoxic and immunologically inert properties. Additionally, biodistribution and cytotoxicity of AuNPs can be adjusted according to their surface functionality and particle size [103,104]. Yeom et al. [105] observed that mRNA encoding a pro-apoptotic factor, Bcl-2-associated X (BAX) protein, encapsulated with AuNPs inhibited xenograft tumors.

3. Adjuvant properties of nanoparticles

Adjuvants are immunostimulating agents essential for the success of the vaccine formulation [106]. Adjuvants are compounds that can either stimulate or increase the immune response to the antigens included in the vaccine formulation [107–109]. Many adjuvants such as Freund's adjuvant, lipid A, cholera toxin, aluminum salts, cytokines, saponins and CpG oligodeoxynucleotides have been used in vaccine development studies [110–112]. However, the immune stimulating capacities of many adjuvants are poor or they have toxic properties, making them unsuitable for use in humans. Therefore, safer and more efficient adjuvants are needed for vaccine formulations. NPs are usually taken up effectively by APCs [113], the key elements of the primary innate immune system [114], also responsible for triggering adaptive immune responses. For this reason, NPs generally stimulate immune responses, and increase immunogenic properties of the antigens they carry [115,116].

TLRs are important receptor groups located on APCs that can recognize pathogen-associated molecular patterns (PAMPs). TLRs play an important role in the development of new adjuvants because different DC subsets express distinct TLRs, shaping the type of adaptive immune responses. The main idea behind is to boost immune responses against the infection via vaccine formulations targeting specific type of TLRs [117]. Vasilichin et al. [118] reported that metal oxide NPs increased the expression of TLR4 and TLR6. Moreover, zinc oxide NPs were shown to increase TLR2, TLR4, and TLR6 in mice [119].

A proper adjuvant such as PLGA nucleus/lipid-shell hybrid NP carrier system for mRNA vaccines should have capacity for induction of APCs and CTLs [8]. Dendrimer NPs are also useful as adjuvant. Efficiency of a dendrimer-based mRNA vaccine platform was reported without using any additional adjuvants [52]. Additionally, NPs have been increasingly used to deliver not only antigen of interest but also co-adjuvants such as poly(I:C), CpG and monophosphoryl lipid A [120,121].

4. Conclusion

Vaccines have been saving millions of lives protecting from infectious diseases especially in

childhood. Although there are efficient commercial vaccines against many pathogens, protective vaccines still lack for various infectious agents. Novel technologies and methodologies have been developed to obtain vaccines with better characteristics. RNA-based vaccines, especially mRNA vaccines have many advantages compared to conventional vaccines. However, they also have drawbacks such as degradation by ubiquitous nucleases. NP-based delivery systems are efficient vehicles to target the mRNA molecules safely to the APCs. There are different types of NPs for mRNA delivery, such as widely preferred polymers and liposomes. In addition to utilization of a single material, hybrid NP delivery platforms are also used to increase the efficiency. Studies have been conducted to obtain perfect combination for the NP-based mRNA vaccines. NPs also have the adjuvant capacity inducing TLRs, APCs, and CTLs, so that diverse immune responses are boosted. As the technologies for NP production advance, more efficient vaccines will be obtained for various diseases.

Conflict of interest

The authors declare no conflict of interest.

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