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Next-Generation Vaccines: Nanoparticle-Mediated DNA and mRNA Delivery

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Nucleic acid vaccines are a method of immunization aiming to elicit immune responses akin to live attenuated vaccines. In this method, DNA or messenger RNA (mRNA) sequences are delivered to the body to generate proteins, which mimic disease antigens to stimulate the immune response. Advantages of nucleic acid vaccines include stimulation of both cell-mediated and humoral immunity, ease of design, rapid adaptability to changing pathogen strains, and customizable multiantigen vaccines. To combat the SARS-CoV-2 pandemic, and many other diseases, nucleic acid vaccines appear to be a promising method. However, aid is needed in delivering the fragile DNA/mRNA payload. Many delivery strategies have been developed to elicit effective immune stimulation, yet no nucleic acid vaccine has been FDA-approved for human use. Nanoparticles (NPs) are one of the top candidates to mediate successful DNA/mRNA vaccine delivery due to their unique properties, including unlimited possibilities for formulations, protective capacity, simultaneous loading, and delivery potential of multiple DNA/mRNA vaccines. This review will summarize the many varieties of novel NP formulations for DNA and mRNA vaccine delivery as well as give the reader a brief synopsis of NP vaccine clinical trials. Finally, the future perspectives and challenges for NP-mediated nucleic acid vaccines will be explored.

1. Introduction

Since the conceptualization of vaccines and their implementation on a governmental scale, live attenuated or killed whole organism-based vaccines have wiped out or nearly eradicated many once great killers of humanity, including smallpox, polio, measles, mumps, rubella, diphtheria, pertussis, and tetanus.[1–4] However, the quick emergence of diseases such as SARS-CoV-2, H1N1 as well as quickly evolving deadly diseases like Ebola create a challenge for conventional vaccines such as live attenuated viral vaccines (LAV), and inactivated/killed viral vaccines,[5,6] which with the traditional vaccine development pathway may take on average over 10 years to develop, or with Ebola requiring an accelerated 5-year development,[7] and even more time needed to scale up manufacturing and stockpile for a large country. As a result, during the vaccine development, scale-up, and implementation many get sick and lose their lives. The most recent and relevant case for discussion is the ongoing SARS-CoV-2 pandemic, which is considered one of the most crucial global health calamities of the century. According to the report from the World Health Organization (WHO as of December 6, 2020), the current outbreak of SARS-CoV-2 has caused over 1.54 million deaths in more than 200 countries throughout the world, as well as a worldwide economic shutdown of many countries leading to widespread disorder, continental lockdowns and fiscal uncertainty.

With pandemics certain to re-emerge in the future, a modernized vaccination system must be developed to ensure that future pandemics are controlled rapidly with minimal loss of life and minimal disruption of the economy. Conventional seasonal annual vaccine formulations often fail to match strains in circulation due to antigenic drift of viral strains, especially when they occur late in the flu season.[8–10] Vaccines for new viral strains like SARS-CoV-2 are time-consuming to create and distribute; identifying the virus, developing, testing, obtaining approval from regulatory agencies and mass production take at least several months to years, as evidenced in the 2009 H1N1 pandemic as well as the 2014–2016 outbreaks of Ebola. It is clear that a new model of vaccine production should emerge to tackle this urgent problem. In the past decades, there have been attempts to replace inactivated or live attenuated vaccines, through development of modern vaccine technologies such as virus-like particles, peptide-based vaccines, as well as nucleic acid-based vaccines.[11] These newer developments aimed to improve vaccine stability, safety, and cost.[12] Nucleic acid vaccines in particular are a recent and

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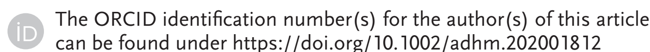

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cost-effective development in the biomedical field which have attracted great amounts of attention. DNA and mRNA vaccines aim to use host cell machinery to produce coded protein antigens, stimulating humoral and cell-mediated immunity through production of neutralizing antibodies and cytotoxic T lymphocytes (CTLs). Therefore nucleic acid vaccines may be the key to an effective vaccine, as they offer quick turnaround utilizing generic DNA/mRNA manufacturing processes, ease of sequence modification to adapt to changing pathogen strains, and the ability to elicit both antibody and cytotoxic T-lymphocyte responses.[13] Further advantages of DNA/mRNA vaccines include the absence of living or killed organisms in the vaccine and the ability to specifically direct immune responses toward only the coded antigens in the vaccine.

Additionally, administration of nucleic acid vaccines generates endogenous proteins displaying the native conformation with posttranslational modifications like those found in natural pathogen infection.[14] Although plasmid DNA and mRNA vaccines display these myriad advantages, and have been widely evaluated in clinical trials, so far none have been licensed for human use and it seems as of now current therapies fail to harness the full potential of this promising therapeutic strategy.[5,15] This is largely because efficient delivery to cells remains elusive. In the human body, nucleic acids are fragile and are degraded rapidly due to endogenous nucleases. DNA/mRNA must also cross many cellular barriers to reach the cytoplasm (for mRNA vaccines) and nucleus (in the case of DNA vaccines). As a result, the immunogenicity of the nucleic acid vaccines which have been developed is low. Finally, due to these reasons, the delivery of DNA and mRNA vaccines has yet to be clinically validated in humans through Phase III.[1,2]

As viral vector delivery has the risk of increasing public health concerns which may hinder widespread adoption, nonviral methods of gene delivery are being increasingly explored. Nanoparticle-mediated delivery is one such nonviral delivery method which holds great promise for efficient delivery of nucleic acids and may represent the future of next generation vaccines. NPs possess many advantages to facilitate vaccine delivery; they protect the nucleic acid payload from degradation and offer versatile formulation strategies with a variety of biomaterial options to overcome the barriers of cell internalization, improve specific immune cell targeting through surface modifications and may utilize pH-sensitive materials to enhance endosomal escape. NPs improve the stability along with the efficacy of the vaccine and act as a robust adjuvant strategy. Another major advantage of NPs is the ability to create cocktail vaccines within one particle; NPs enable codelivery of multiple nucleic acid vaccines to the same target cell which may enable synergistic effects to further enhance immunity. This is a distinct advantage over traditional vaccine cocktails which cannot ensure that each cell receives the combinatorial dose. This review will give the reader an overview of the different promising and cutting-edge NP strategies used to efficiently deliver DNA and mRNA vaccines in vivo, including liposomal, polymeric, inorganic and peptide types. Moreover, an overview of the current clinical trials will be laid out and summarized. Future prospects for development of NP-based DNA and mRNA vaccines will also be discussed.

2. DNA and mRNA Present Many Advantages for Vaccine Development

DNA is the genetic material located within the nucleus, while mRNA is the intermediate which ferries the information from the nuclear DNA to be translated into functional proteins in the cytoplasm. They both participate in the cellular protein translation pathway. The double stranded DNA represents the gene itself and is located in the nucleus, whereas single stranded mRNA is the transcribed version of the gene which associates with the ribosomes to translate codons for protein production. Nucleic acid vaccines have many advantages: they avoid issues associated with recombinant protein vaccines such as improper protein folding or high protein purification cost, they do not display the infectious risks associated with attenuated or inactivated vaccines produced from live infectious organisms, and they can activate both the humoral and cellular immune response, leading to vastly improved protective immune responses.[15] Furthermore, improved transfection efficiency and stability of mRNA can be achieved through various chemical modifications, leading to enhanced immunity. Plasmid DNA (pDNA) sequences contain a promoter region, an intron, antigen sequence, and a polyA signal. mRNAs are usually produced by in vitro transcription of a cDNA template such as pDNA with RNA polymerase.[16] Synthetic mRNA comprises a protein-encoding open reading frame (ORF) with a 5' cap," and a 3' poly(A) tail. 5′ and 3′ untranslated regions (UTRs) flanking the ORF will increase the translation and stability.[17] Chemical modifications may be made to the DNA and mRNA to improve transfection and stability, including codon optimization, prefusion stabilization mutations and additions to support trimerization[18,19] Additionally, for DNA, promoter selection and modifying the plasmid backbone (i.e., removing bacterial elements) can play a large role in increasing the gene expression.[19–23] Finally, for mRNA the optimization of the 5'- and 3'- UTRs have been utilized to improve translational efficacy[17,24] and the incorporation of modified nucleosides such as pseudouridine (Ψ) and 5-methylcytidine (5 mC) have been shown to reduce immune recognition of the mRNA.[1,25–27] Self-amplifying mRNA has been recently developed as another nucleic acid-based vaccine technology. The self-amplifying mRNAs are termed replicons, and are derived from RNA viruses where the structural viral proteins are replaced with mRNA encoding antigens and RNA polymerases. The net effect is that the mRNAs prolong protein expression and increase immunogenicity, which increases the efficiency of the dosage.[28,29] Self-amplifying mRNAs code for the antigens of interest as well as the RNA-dependent polymerase for replicon amplification.[30]

2.1. Mechanism of Nucleic Acid Vaccines

DNA vaccines are simply plasmid DNAs which encode the antigen of interest under the control of a mammalian promoter. For DNA vaccines, after in vivo administration (via intramuscular, intradermal or subcutaneous injection[31]), the DNA must be internalized to the nucleus and translated to protein antigen product (**Figure 1**). mRNA vaccines are similar to DNA vaccines, however mRNA vaccines do not have to enter the nucleus to produce

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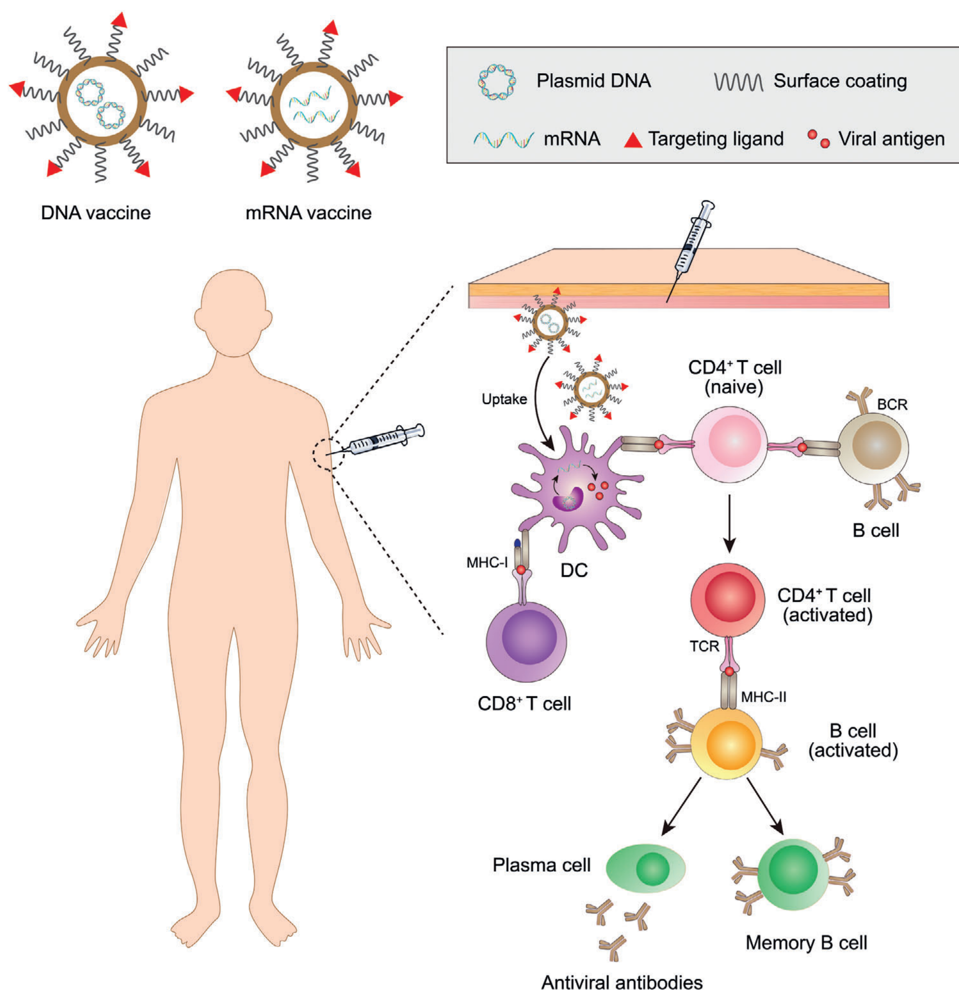

Figure 1. Scheme detailing the general steps of nanoparticle vaccine immunization strategy. After encapsulation of the pDNA/mRNA, the nucleic acid-nanoparticle vaccine is administered and either taken up by local cells or APCs, where the nucleic acid payload is released and processed to create antigens which are further processed for MHCI and MHCII presentation. This leads to CD8+ cytotoxic T cells or CD4+ T helper cell activation and cell mediated immunity. Further activation of B cells mediates the humoral immunity. DC: dendritic cell, TCR: T cell receptor.

antigen protein, which removes a barrier of the nuclear envelope that exists for DNA vaccines. Therefore, the introduction of mRNA into the cell cytosol is enough to subject it to ribosomal translation and antigen production. Since mRNA does not involve entering the nucleus to produce protein, mRNA vaccines function to produce immunity without crossing the extra nuclear barrier, which increases the efficacy per dose. For mRNA, the production of the antigen protein is transient, and then the mRNA is degraded naturally in the cell. Modified nucleosides can also make the mRNA less immunogenic and increase translational efficacy.[32] These are some of the major benefits of mRNA, however, DNA is a more stable molecule, which may yield a more robust vaccine and longer shelf life. Regardless, DNA/mRNA gene constructs which code for antigens are simpler and faster to produce than inactivating viruses or making recombinant proteins and avoids risks of working with live virus/pathogen. Furthermore, in either case the designed vaccine construct can code for merely the key antigens and exclude other deleterious proteins that may present in the live/attenuated virus.[33]

In terms of mechanism of DNA/mRNA vaccines, it has been proposed that i) the DNA plasmid/mRNA is expressed by somatic cells at site of injection and presented through MHC class I complexes to CD8+ cells, ii) antigen presenting cells (APCs) such as dendritic cells (DCs) at the site of injection are directly transfected by the plasmid DNA and T cell antigen presentation proceeds through MHC class I and II complexes, or iii) APCs phagocytose DNA/mRNA-transfected somatic cells to trigger cross-priming and presentation of antigen to CD4+ and CD8+ T cells.[19] DCs are crucial in the immune response because they proceed from the site of administration (for example muscle tissue injection) to lymph nodes where they acquire expression of costimulatory factors, and present antigen peptide fragments loaded onto MHCI and MHCII molecules to naïve CD4+ and CD8+ T cells.[34] CD4+ T cells will also aid in activation of cytotoxic CD8+ T cells and B cells.[35] Activated B cells will produce a humoral immune response. Direct DC transfection by DNA/mRNA vaccines will activate the CD8+ T cells and CD4+ helper T cells in parallel via MHCI and MHCII as well as through the costimulating

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receptors.[36] Therefore, nucleic acid vaccines can elicit both cell-mediated and humoral immunity.

2.2. Challenges and Limitations of Nucleic Acid Delivery

Though DNA and mRNA show promising potential in gene therapy and vaccine development, clinical translation is limited by several drug delivery hurdles including phagocytosis, enzymatic degradation, protein absorption, nonspecific immunogenesis as well as cellular internalization barriers.[37] DNA vaccines have generated much excitement since the 1990s, but poor performance in larger animal models have stalled their progress. However, over the previous few decades the DNA vaccine platform has been improved upon, particularly newly designed antigens and expression vectors, the development of novel vaccine adjuvants, the development of mRNA vaccines, and new gene delivery methods.[38] Even with these new developments, nucleic acid vaccines still face strong challenges of poor immunogenicity. The cellular uptake of naked DNA is very inefficient and it has been shown that the vast majority of the injected DNA remains extracellular,[39] with 95–98% of intramuscularly injected plasmid DNA remaining in the interfibrillar space.[40] Therefore, adjuvants must be used to increase the immune response of these nucleic acid vaccines. The two broad categories of adjuvants can be classified into immunostimulant molecules like Toll-like receptor ligands, bacterial toxins, saponins, and cytokines[41–45] as well as delivery systems such as particle bombardment, high pressure delivery, dermal patches, electroporation and nanoparticles.[5,42] Here we will focus on the latter, with nanoparticles as a viable and quickly developing delivery system for increasing the efficacy of nucleic acid vaccines.

To achieve successful DNA and mRNA vaccine development, a safe and efficient gene delivery system is one of the most important factors and arguably the rate-limiting step for a viable nucleic acid vaccine product. There were early attempts at utilizing viral vectors as a delivery method for gene delivery due to high in vivo delivery and transfection efficiency,[46] however major issues with viral vectors such as the immune response of the host, possible activation of oncogenes which cause malignancies[47,48] as well as complications from inflammatory response have hindered their development significantly.[49] Other methods of gene delivery such as electroporation and microinjection have been utilized to some effect, but they also present with cell damage and drawbacks such as painful administration.[50] Nonviral vectors on the other hand, have many advantages compared with viral vectors such as increased safety, reduced pathogenicity, reduced capacity for insertional mutagenesis and convenient large scale preparation.[46] Sustained gene payload release from the vector is important for successful vaccination as it increases the window of antigen expression while protecting functionality of the encapsulated nucleic acids and reducing the number of administrations.[51,52] An exemplary model and class of non-viral vectors which will be the focus of this review are nanoparticles.

3. Nanoparticles Are an Advantageous Method for Delivering Nucleic Acid Vaccines

As injecting naked DNA/mRNA into the body will lead to quick degradation via endonucleases, there is a need for special methods to enhance the nucleic acid vaccine delivery. Vectors are systems which enable gene delivery into the cell, provide protection from degradation, and enhance gene transcription in the cell.[53] The two types of vectors in use are viral vectors such as retroviral, adenoviral, adeno-associated viral and lentiviral vectors; and nonviral vectors such as nanoparticles which are synthesized from lipids, polymers, and inorganic molecules. Viruses present with high efficiency transfection and the viral vectors used are engineered through viral replication, assembly or infection gene deletion.[54] Nonviral vectors, on the other hand present advantages such as increased safety, almost limitless transgene size and ability for repeated administration.[54]

Due to these advantages, there has been a steady shift in focus from viral-based nucleic acid delivery to synthetic vectors, as the inherent perceived risks of viral delivery to many are outweighed by advantages of nonviral vectors.[55] NPs are a prominent and promising nonviral vector used in a wide variety of applications, most notably diagnostic imaging and drug delivery. NPs are typically less than 200 nm in diameter, and their nanoscopic size facilitates intracellular uptake. Since nanoparticles and viruses exist at the same size scale, nanotechnology can have drastic and novel impacts on vaccine development. NPs can encapsulate therapeutics and are capable of controlled delivery to target diseased cells and encapsulation of therapeutics with NPs also enhances the solubility of the therapeutic.[56] NPs have a large ratio of volume to surface area, modifiable external shell, biodegradability, and low cytotoxicity, advantageous characteristics of a payload delivery system.[57] The ability to functionalize the NP surface with targeting moieties not only improves drug effectiveness but concurrently reduces dosage to optimize therapeutic pharmacokinetics.[58] Moreover, toward the goal of endosomal escape to release the DNA/mRNA payload, nanomaterials sensitive to the endolysosome environment have been designed and studied.[59] The delivery of NPs to the lymph nodes (LNs) has also been explored for NP design as there are large populations of B cells, T cells, follicular dendritic cells, and subcapsular sinus macrophages residing in the LNs.[59] This can be achieved through the NP delivery to APCs which migrate to LNs, and also through drainage of small NPs 10–100 nm in size to LNs.[60] Finally, NP cocktail vaccines loaded with combinations of DNAs/mRNAs can be designed to co-deliver vaccine in pre-defined ratios to each single target cell for synergistic immune effect.

As mentioned previously, delivering nucleic acids into cells is difficult as nucleic acids are susceptible to endogenous nucleases, dense negative charges of the nucleic acids impede cell internalization, and the nonspecific interferon response triggered by the presence of foreign nucleic acids in the cytoplasm is also a major impediment to clinical translation.[61] Therefore a NP nucleic acid delivery system should efficiently encapsulate the negatively charged nucleic acids, protect against endogenous enzymes, and facilitate cellular uptake and intracellular release (**Figure 2**). It is an additional benefit if the NPs preferably target APCs or the LNs. Here, we outline the fact that nanoparticles provide a robust nucleic acid delivery platform capable of endless customizability and rapid clinical translation of nucleic acid vaccines. For example, incorporation of cationic polymers/lipids to complex with negatively charged nucleic acids may protect the DNA/mRNA from endonuclease degradation as well as immunorecognition,

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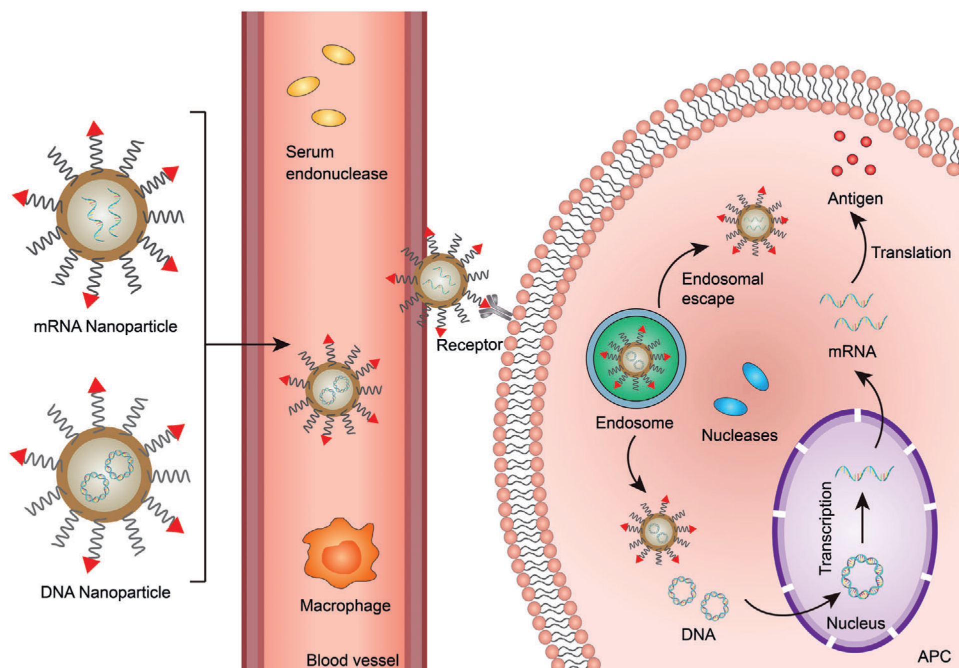

Figure 2. Challenges with nucleic acid vaccines and solution through NP-based delivery. If injected intravenously, DNA/mRNA vaccines must be protected from many barriers to successful translation of encoded antigen/epitope. First, NPs can protect nucleic acids from degradation via endonucleases and general phagocytic elimination via the reticuloendothelial system. Second, the naked nucleic acids will face barriers entering the negatively charged cell membrane. NPs may target cells via surface ligand presentation matching a specific cell receptor and enter the cell through receptor-mediated endocytosis. Within the cell, the NP vaccine must escape the endosome to deliver the payload. NPs have been designed to respond to the acidic pH of the endosome, triggering endosomal escape and intracellular payload release. Once in the cytosol, DNA vaccines must further translocate to the nucleus to be transcribed.

inorganic NPs which have the nucleic acid functionalized, and other design features such as multivalent targeting ligand modification of the nanoparticle components may maintain the therapeutic dose for longer periods of time and target specific immune cells as well as LNs, increasing vaccine effectiveness. Cell penetrating peptides are able to complex with nucleic acids for increased delivery efficacy. Additionally, direct modifications to the DNA and mRNA molecules can be employed to increase effectiveness of the formulation.

The following sections will concisely summarize the recent progress in nanoparticles for nucleic acid vaccine delivery with an emphasis on newly developed nanoparticle platforms, clinical and preclinical trials. The promising characteristics of nanocarrier platforms needed to surmount the nucleic acid vaccine delivery will also be highlighted.

3.1. Liposomal Nanoparticles

Liposomes are minute artificial vesicles with at least one lipid bilayer. In liposomal formulations of nucleic acids, self-assembly into spherical or amorphous structures is most common, with lipids and nucleic acids interspersed throughout the bilayer. The majority of liposomal gene delivery methods have employed cationic lipids to facilitate encapsulation of negatively charged nucleic acids; neutral lipids on the other hand may be used to enable stability and transfection efficiency.[62] Cationic lipids employed for gene delivery share similar characteristics: a hydrophilic head which bears a positive charge associates with negatively charged nucleic acids and the hydrophobic lipid tail becomes a linker between them.[50,63] The transfection efficiency is dependent on geometric shape, number of charged groups per molecule, nature of lipid anchor and linker bond.[50] There are some concerns with the use of cationic lipids, as cellular toxicity stemming from the positive charge has been shown.[46,64] Recent developments with LNPs include the targeting of lymph nodes, antigen presenting cells and cargo release from a response to a changing cellular microenvironment (tumors, endosomes).

Oberli et al. formulated a lipid nanoparticle to deliver mRNA vaccine for cancer immunotherapy. Cancer immunotherapy, in the broadest sense, involves utilizing the body's immune system to combat cancer. NPs can be engineered to be responsive to their environment, for example the acidic environment within solid tumors or within cellular endosomes to release their cargo. The aim is to diminish toxicity in off target areas while increasing delivery to target cancer cells.[65] The authors' formulation combines an ionizable lipid, a phospholipid, cholesterol, a polyethylene glycol (PEG) containing lipid, and an additive for the delivery of mRNA vaccines (**Figure 3**).[66] Experimenters replaced 1% of the molar composition of PEG in the optimized LNP formulation with lipopolysaccharide (LPS), a very potent TLR4 agonist. Furthermore, the NP was modified with an ionizable lipid as it is positively charged at low pH which aids in complexing with the negatively charged mRNA and will also aid in cellular uptake and endosomal escape. LNP formulation B-11 showed transfection in different immune cell populations, including dendritic

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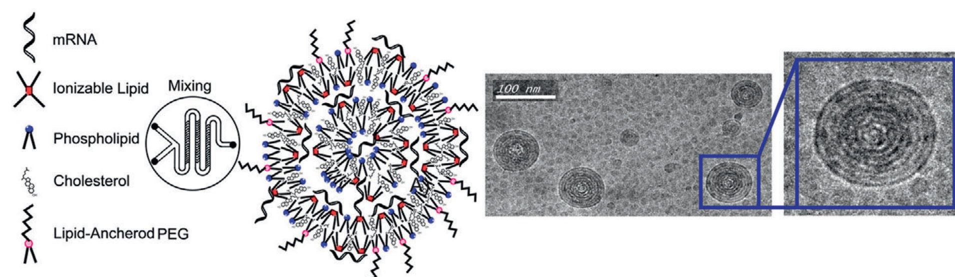

Figure 3. Lipid nanoparticle design for cellular uptake and endosomal escape. Left: Ionizable lipid complexes with the negatively charged mRNA at low pH. This facilitates endocytosis and endosomal escape. Phospholipid provides structural integrity to the bilayers while supporting endosomal escape of the mRNA to the cytosol. Cholesterol aids to stabilize the LNPs, promoting membrane fusion. Lipid-anchored PEG prevents LNP aggregation and reduces nonspecific interactions. Right: Cryogenic transmission electron microscopy image of spherical LNPs with multilamellar structure. Reproduced with permission.[66] Copyright 2017, American Chemical Society.

cells, macrophages, neutrophils, and B cells in Ai14D reporter mice. Additionally, mRNA coding for the tumor associated self-antigens, TRP2 and gp100, showed efficacy in an a B16F10 tumor model and extended the overall mice survival. Adding LPS to the LNP formulation increases the effectiveness of mRNA vaccines delivered by LNPs and will encourage future modifications by other groups in their own liposomal-nucleic acid formulations.

As DCs are the main antigen presenting cells for CD4+ and CD8+ T-cell activation, they are a natural target for transfection.[67] Unfortunately, DCs are notoriously hard to transfect, therefore nanoparticle approaches which exploit targeting to their intrinsic receptors are being developed. Since mannose receptors (MRs) are expressed on the surfaces of DCs, Voshavar and co-workers designed liposomal DNA vaccine carriers using mannose-mimicking shikimoylated cationic amphiphiles containing a 6-amino hexanoic acid spacer group in the head-group region in complexation with DNA vaccine which encodes for melanoma antigen (MART1), termed (pCMV-MART1). The group showed that this formulation induces long lasting antimelanoma immune responses with ex vivo immunization of mice.[68] Structure−activity investigations have also been carried out which demonstrated that mannose-receptor selective cationic amphiphiles containing five methylene units in the spacer arm between the hydrophobic tail and mannose-mimicking shikimoyl and quinoyl headgroups (lipids 5 and 10) are most useful for ex vivo DC-DNA vaccination. Furthermore, lipoplexes of pCMV-MART1 and the designed lipid 5 in ex vivo DC transfection were found most efficient in for effective antimelanoma immune response in mice challenged with B16F10 cells.

Fan et al. explored the use of cationic lipid-assisted nanoparticles (CLAN) to carry and deliver mRNA vaccine. CLAN were constructed from a copolymer of poly(ethylene glycol)-block-poly(lactic-*co*-glycolic acid) (PEG-*b*-PLGA) and cationic lipid, for the delivery of nucleic acids, and the formulation was previously shown to be successful in vectoring CRISPR/Cas9 plasmids.[69–72] It was found that CLAN encapsulating mRNA encoding antigen successfully caused the maturation of DCs as well as the activation and proliferation of antigen-specific T cells, enhancing the maturation of CD11c+ cells and proliferation of CD8+ T cells in lymphoid tissue. Mice injected i.v. with CLAN/mRNA encoding ovalbumin (OVA) showed marked OVA-specific T-cell response and reduced tumor development in an aggressive E.G7-OVA lymphoma model.[73]

As mentioned previously, the optimization of 5' and 3' UTRs can improve the performance of nucleic acid vaccines by improving protein production. Dong and co-workers recently developed an optimal combination of 5′ and 3′ UTR, termed NASAR mRNA which are 5- to 10-fold more efficient than the tested endogenous UTRs. Moreover, Dong et al. previously created and optimized N1, N3, N5-tris(2-aminoethyl)benzene-1,3,5- tricarboxamide lipid-derived (TT3) NPs through an orthogonal array design, which demonstrated improved delivery efficiency of mRNA encoding luciferase in vitro by over 350-fold.[74] In a recent work, they utilized a combination of rationally engineered NASAR mRNA with TT3 NPs to deliver mRNA vaccine for SARS-CoV-2.[75] NASAR mRNAs were produced through a rigorous process of global gene expression bioinformatics scanning including analysis of copies produced/mRNA molecule, amino acids produced/mRNA molecule, along with many other factors such as endogenous/de novo UTR selection, nucleotide length/composition optimization, removal of miRNA target sites and integration of beneficial RNA motifs. The TT3-formulated NASAR mRNA vaccine for SARS-CoV-2 was shown to induce 300-fold more anti-S1 antibodies than MC3 (an FDA-approved lipid-based delivery vehicle), and additionally intramuscular injection induced fivefold more antigen-specific antibodies than subcutaneous injection. Researchers uncovered several crucial details towards the development of UTRs for nucleic acid delivery. They found that the optimal length for the 5′ UTR found was 70 nt, and should not contain certain regulatory elements such as TOP motifs, secondary structures, upstream open-reading frames, and microRNA binding sites. Results also showed that in the 3′UTR, secondary structures such as R3U may enhance mRNA expression. The NASAR mRNA is an example of the engineering potential of mRNA therapeutics.

Perhaps most excitingly, lipid nanoparticles from Moderna therapeutics are in human clinical trials for the SARS-CoV-2 virus. The spike (S) protein is the primary target for neutralizing antibodies as it is the major surface protein on the SARS-CoV-2 virus and modification of the protein code can confer stability and increase effectiveness of the mRNA vaccine. The group previously showed that prefusion-stabilized protein immunogens which preserve neutralization-sensitive epitopes can be effective as a vaccine strategy against respiratory syncytial virus (RSV).[76–79] The group also identified 2 proline substitutions (2P) at the apex of the central helix and heptad repeat

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1 which stabilized Middle East Respiratory Syndrome (MERS-CoV), SARS-CoV, and human CoV-HKU1 S proteins in the prefusion conformation and this 2P protein was transferable to other beta-CoV spike proteins and was incorporated into future designs such as mRNA-1273.[80–82] The mRNA-1273 vaccine encodes the S-2P antigen, made up of the SARS-CoV-2 glycoprotein with a transmembrane anchor and an intact S1–S2 cleavage site. mRNA-1273 is an LNP dispersion formulated from 4 lipids (1 proprietary and 3 commercially available): the proprietary ionizable lipid SM-102; cholesterol; 1,2-distearoyl-snglycero-3 phosphocholine (DSPC); and 1 monomethoxypolyethyleneglycol-2,3-dimyristylglycerol with polyethylene glycol of average molecular weight 2000 (PEG2000-DMG). Corbett et al. showed that with this LNP formulation, 1 µg of mRNA-1273 was enough to induce robust pseudovirus neutralizing activity and CD8 T-cell responses, balanced Th1/Th2 antibody isotype responses, and protection from viral replication for more than 3 months in mice. Additionally, the induction of protective immunity was achieved after a single dose. This study, along with immunogenicity data from nonhuman primates and subjects in early Phase 1 clinical trials, were used to apprise the dosing and regimen of mRNA-1273 in human clinical efficacy trials. Earlier this year, Moderna conducted a phase I, dose-escalation, open-label trial including 45 healthy adults, 18 to 55 years of age, who received two vaccinations 28 d apart.[83] After the first vaccination, antibody responses were higher with higher dose (day 29 enzyme-linked immunosorbent assay anti-S-2P antibody geometric mean titer [GMT]. After the second vaccination, the titers increased (day 57 GMT, 299751, 782719, and 1192154, respectively). After the second vaccination, serum-neutralizing activity was detected by two methods in all participants evaluated. mRNA-1273 is currently undergoing simultaneous phase II and III clinical trials. Recently, Moderna released data from their preliminary phase III clinical trial which has enrolled over 30 000 participants, and shows that their SARS-CoV-2 vaccine is 95% effective in preventing Covid-19. Pfizer/BioNTech has also released data from their phase III trial showing that their vaccine is also 95% effective, and caused no safety concerns. If successful, this would be a very quick (<1 year) and unprecedented triumph for mRNA vaccine development worldwide.

Self-amplifying RNA (saRNA) has also been recently used by McKay and co-workers, who encapsulated saRNA encoding the SARS-CoV-2 spike protein encapsulated within a LNP vaccine. The LNPs used in this study were composed of an ionizable cationic lipid (proprietary to Acuitas)/phosphatidylcholine/cholesterol/PEG-lipid. The ionizable amino lipid through electrostatic interaction with polyanionic nucleic acids promotes self-assembly into nanoparticles encapsulating saRNA. Following endocytosis of LNPs by target cells, this formulation enables saRNA to escape the endosome for cytoplasmic delivery.[84,85] Authors observed high and dose-dependent SARS-CoV-2 specific antibody titers in mouse sera leading to neutralization of pseudotyped as well as wild type virus. The immunogenicity of the SARS-CoV-2 saRNA LNP vaccine in comparison to natural infection in COVID-19 recovered patients revealed that neutralization is proportional to the quantity of specific IgG and higher in magnitude than recovered COVID-19 patients. Authors concluded that the potent LNP formulation played a role in inducing such a robust cellular response for their vaccine, and indeed the LNP-formulated saRNA showed higher antibody titers, viral neutralization (IC50) and cellular response over electroporated pDNA.[85] This method shows great translation potential since the potent LNP-formulated saRNA vaccine is amenable to needle injection, which may facilitate widespread use without electroporation.

Cationic nanoemulsion (CNE) utilizes nanoemulsion in conjunction with cationic lipids.[86] Through hydrophobic and hydrophilic surfactants which stabilize oil core in the aqueous phase, nanoemulsion can generate particles via vigorous agitation, ultrasound, and microfluidics.[86,87] Brito and co-coworkers used CNE composed of the cationic lipid (1,2-dioleoyl-sn-glycero-3-phosphocholine) (DOTAP) and MF59, a well-established emulsion adjuvant to deliver a self-amplifying mRNA which produced potent immune responses against the respiratory syncytial virus (RSV), human cytomegalovirus(hCMV), and human immunodeficiency virus (HIV) in mice, rats, rabbits, and nonhuman primates.[88] These results were at comparable levels to adjuvanted subunit vaccine or viral replicon particle delivery, with lower doses than required for pDNA vaccines. Additionally, Samsa et al. generated two novel Venezuelan equine encephalitis virus (VEEV) vaccine candidates using saRNA and CNE. This engineered replication-defective VEEV-based vaccine demonstrated 100% protection against aerosol VEEV challenge in mice.[89] These studies show that saRNA platforms may modernize the development of preclinical studies and lay the groundwork for more clinical studies in humans.

The incorporation of LNPs with DC targeting, lipopolymer design, UTR optimization, ionizable lipids and self-amplifying RNAs toward nucleic acid vaccination over the last few years has moved the field forward perhaps more than any other type of NP. However there are concerns about potential toxicity of cationic LNP components and decreased interaction of LNPs with endosomal membranes which may hinder endosomal escape without the proper modifications.[30] This presents challenges for future LNP formulations as toxicity stems from disruption of membrane structures, which may cause cytoplasm vacuolization, cell lysis and necrosis.[90,91] Cationic lipids can also influence the expression of multiple genes in undesirable manner.[91] However, attempts at mitigation of toxicity via the reduction of cationic charges in LNPs reduces the nucleic acid encapsulation efficiency and transfection efficiency. Therefore a careful balance between toxicity and therapeutic effectiveness must be struck in their continued development. Nevertheless, LNPs represent the largest portion of NP-mediated nucleic acid vaccine delivery and have found many preclinical uses in treating a very wide variety of diseases. LNPs have also progressed the farthest and have the most candidates in human clinical trials.

3.2. Polymeric Nanoparticle Systems

Although LNPs are by far the most popular vehicle for NP vaccination, polymeric nanoparticles represent an excellent choice for formulation. Recently, polymers have been extensively investigated for nucleic acid delivery. Polymeric nanoparticles (PNPs) are usually prepared from biocompatible and biodegradable polymers where the drug is dissolved, entrapped, encapsulated or attached to a nanoparticle matrix. The use of biodegradable

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polymeric nanoparticles for controlled drug delivery has shown significant therapeutic potential.[92,93] They have widely varying chemistries and physical characteristics, can protect the payload from degradation, enable controlled release of the gene material, and are amenable to structural modification to adjust their physiochemical properties as well as show biocompatibility and biodegradability.[94]

The most abundant type of polymers for nucleic acid delivery are the cationic polymers used to bind and condense nucleic acids via electrostatic interactions between the positively charged polymer and negatively charged nucleic acids' phosphate groups, forming polymer-nucleic acid polyplexes which can protect the nucleic acids against nuclease degradation.[94,95] Widely used examples of such polymers include polyamidoamine dendrimers (PAMAM) and polyethylenimine (PEI). The PEI polymer easily forms complexes with nucleic acids due to electrostatic interactions between negatively charged nucleic acid phosphate groups and positively charged PEI amine groups.[96] This creates nanoparticles which protect the nucleic acids and facilitates their cellular entry. PEI has other notable characteristics which aid in nucleic acid complexation, including a high buffer capacity over a broad range of pH values and increased protonation ratio of amine groups at low pH than at high pH.[97,98] The PAMAM dendrimers are hydrophilic, biocompatible, highly branched cationic polymers with unique 3D structure that allows for functionalization and also for conjugation/entrapment with therapeutics and nucleic acids.[99] PAMAM branches are based on methyl acrylate and ethylenediamine, and end in amine and carboxyl terminal groups.[100] Also worthy of mention are polyanhydrides, which are biocompatible FDA-approved polymers that degrade through surface erosion,[101] and polysulfenamides with sulfenamide bonds (R_2N-SR) that are synthesized easily at room temperature which have also been used to deliver nucleic acid vaccines in microparticle and nanoparticle form.[102,103] Poly lactic-*co*-glycolic acid (PLGA) has also been used as an FDA-approved biomaterial to deliver CpG as well as doxorubicin for cancer co-immunotherapy.[104] Moreover, it has been demonstrated that combinations of PLGA–PEG coblock polymer along with cationic polymer PBAE can show unique particle-in-particle morphology and deliver nucleic acids with high transfection efficiency and sustained release for up to 8 d.[105]

As mentioned above, highly branched cationic polymers such as PAMAM dendrimers have been used to deliver nucleic acids. Chahal et al. modified PAMAM dendrimer and used microfluidics to form modified dendrimer NP (MDNP) saRNA vaccine which protected mice against lethal viral infection of H1N1 influenza virus or the Ebola virus.[106] Furthermore, multiplexed vaccine carrying multiple replicons protects mice against lethal Toxoplasma gondii challenges. The authors were able to show that the MDNP produced multiple antigens and induced protective immune response in mice over a range of disease models. Additionally, it is of note that from DNA sequence access to milligram-scale, injection-ready MDNP vaccine, the production timeline was only 7 d.

Intradermal administration of polypeptide viruses, inactivated viruses and DNA vaccines have shown that microneedle (MN) delivery systems display greater immunogenicity than intramuscular injections.[107–109] Seok and co-workers developed an intradermal pH1N1 DNA vaccine delivery platform using stainless steel MNs coated with a cationic polyplex containing poly lactic-*co*-glycolic acid/polyethyleneimine (PLGA/PEI) nanoparticles. The coated polyplex dissolved in porcine skin within 5 min and generated a greater humoral immune response than that of intramuscular polyplex delivery or naked pH1N1 DNA vaccine delivery by a dry-coated MN.[110] This research potentially provides a platform for other intradermal DNA vaccines. However, the authors note that expression level of the exogenous genes with this formulation was low and resulting immunogenicity was weak, and therefore improvements can be made with the NP formulation and MN composition.[110]

Dhakal and co-workers developed a polyanhydride nanoparticle-based inactivated intranasal swine influenza vaccine[111] (termed KAg + CpG-nanovaccine) encapsulating inactivated/killed soluble antigen (KAg) and Toll-like receptor (TLR)-9 agonist (CpG-ODN). CpG oligodeoxynucleotides bind to and activate Toll-like receptor 9 (TLR9), which triggers an innate immune response that supports the subsequent development of adaptive immunity and improve antigen presentation as well as cellular and humoral immune response.[112] The NP vaccine 20:80 CPTEG:CPH copolymer was created via melt polycondensation reaction[113] and in pigs, the prime-boosted KAg + CpG-nanovaccine induced remarkably improved levels of antigen-specific IgA antibody responses in the nasal cavity, higher lymphoproliferative response in peripheral blood mononuclear cells (PBMCs), as well as greater IFN-γ secretion during antigen-induced recall responses of PBMCs and tracheobronchial lymph nodes cells over KAg alone. Viral fever, viral shedding and lung virus titers were also reduced.[111] Intranasal administration presents antigens in a manner similar to natural infection with large surface area, high vascularization and lower enzymatic and chemical degradation than oral route. This, in combination with the successful demonstration of NP vaccine in a larger animal model show that the field of NP vaccine therapeutics is greatly improving. Many vaccines require storage in very low temperature freezers, which is a significant drawback for diseases such as Ebola which are present in developing countries without wide availability of low temperature storage. Furthermore, biodegradability of MNs is preferable because of less material waste and sharps disposal. In pursuit of these two goals, the Yang group presented a novel method of Ebola vaccination using a DNA vaccine coated on polylactic-*co*-glycolic acid–polyl-lysine/poly-γ-glutamic acid (PLGA-PLL/γPGA) nanoparticles administered using a PVA microneedle (MN) patch which dissolves in the skin (**Figure 4**). This formulation relies on the cationic nature of PLGA-PLL nanoparticles to bind the Ebola DNA vaccine (EboDNA) vaccine. The formulation was shown to induce immune response in mice and MN were shown to be stable at 37 °C for at least 2 weeks. The MN patch delivery system enables vaccination by personnel with minimal training, vaccine stability without refrigeration, all at low cost.[114] These studies show that focus in the MN-NP field should shift to transfection efficiency of formulations in microneedles in vivo and optimization of the MN-NP systems.

3.2.1. Natural Polymers

Natural polymers, such as chitosan and alginate, are polymers produced by the cells of living organisms.[115] Natural polymer

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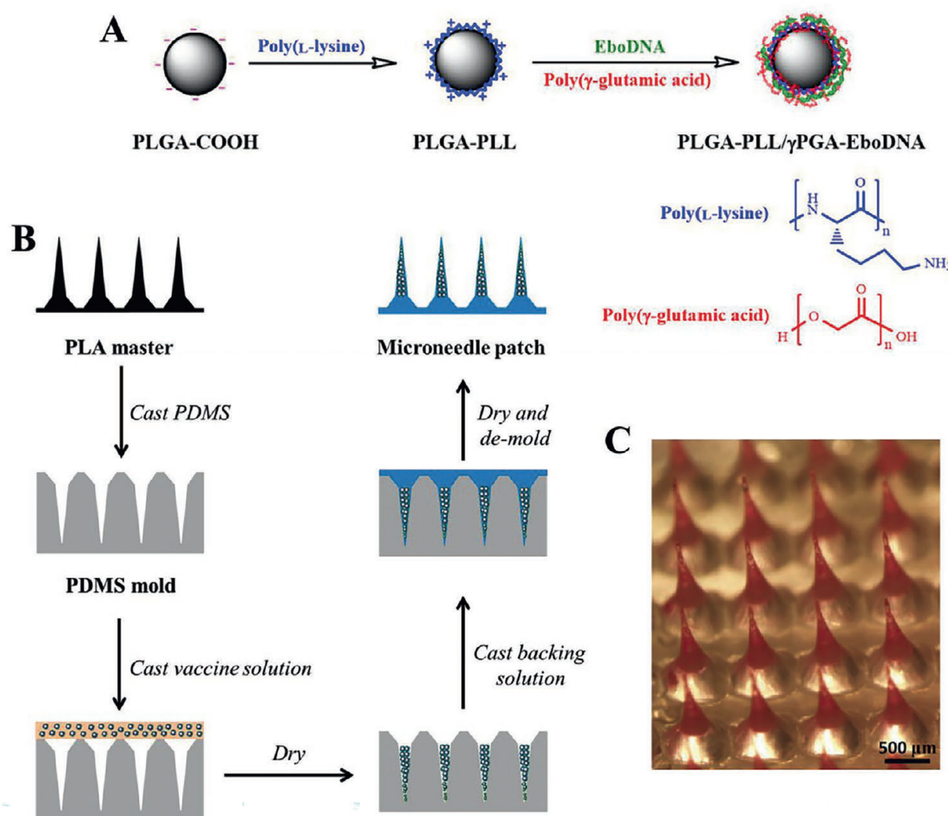

Figure 4. A) Schematic of the preparation procedure of PLGA-PLL/γPGA-EboDNA. B) Schematic of dissolving MN patch fabrication. C) Bright-field image of MN patch with PLGA-PLL-SRB (red) encapsulated in the MNs. Reproduced with permission.[114] Copyright 2017, Wiley-VCH GmbH.

based nanoparticles are suitable for clinical application due to their versatile traits, including biocompatibility, biodegradability, and low immunogenicity.[116] As mentioned previously, conventional DNA vaccines are intrinsically unstable in the body, may require multiple booster doses, and are low in immunogenicity. Chitosan, a cationic polysaccharide and natural biopolymer, has been used as an adjuvant and vaccine delivery system. It is nontoxic, biocompatible, can penetrate mucosal surfaces of epithelial cells and tight intercellular connections.[117,118] Chitosan has mucoadhesive properties which may enhance the absorption of vaccines and drugs at mucosal surfaces, which are one of the main entryways of pathogens to the body.[119,120] Zhao et al. formulated NDV F gene plasmid DNA with C3d6 molecular adjuvant (pVAX I-F(o)-C3d6) encapsulated in the of N-2-hydroxypropyl trimethylammonium chloride chitosan (N-2-HACC) N,O-carboxymethyl chitosan (CMC) nanoparticles (N-2-HACC-CMC/pFDNA-C3d6 NPs). The intranasal immunization of chickens with N-2-HACC-CMC/pFDNA-C3d6 NPs produced greater anti-NDV IgG and sIgA antibody than chickens in other groups did and considerably stimulated lymphocyte proliferation and triggered higher levels of IL-2, IL-4, and IFN-γ. This work shows that quaternized chitosan nanoparticles may be efficient mucosal immunity delivery carriers for DNA vaccines.[121]

Hyaluronic acid (HA) is an immunoneutral polysaccharide that occurs naturally in all living organisms.[122,123] Self-assembled HA nanoparticles (HA-NPs) have been extensively investigated for biomedical and pharmaceutical applications due to their biocompatibility and receptor-binding properties.[124] Naturally anionic polysaccharide hyaluronic acid (HA)-based NPs have been studied as delivery systems to target tumor cells which overexpress CD44, which is a receptor of HA.[125,126] Regarding delivery to macrophages, CD44 receptor is also overexpressed in peritoneal macrophages, making HA NPs a natural choice for targeted delivery of vaccine. As DNA/mRNA is also negatively charged, modifications to HA such as with cationic polymers like poly(ethyleneimine) (PEI) have been utilized to help form complexes with the nucleic acids. PEI can also facilitate endosomal escape via the "proton sponge effect" to aid the payload escape once internalized to the cell.[127]

Tran and co-workers synthesized HA-PEI conjugate NPs for encapsulation and targeted delivery of plasmid DNA expressing IL4 and IL10 genes (termed HA-PEI/pDNA) to macrophages and modulated their functional polarity toward anti-inflammatory M2a and M2c phenotypes in both J774A.1 macrophages and in peritoneal macrophages of C57BL/6 mice.[128] The HA-PEI/pDNA NPs were demonstrated to self-assemble and internalized by J774A.1 macrophages overexpressing CD44 receptor. HA-PEI/pDNA-IL4 and HA-PEI/pDNA-IL10 transfected macrophages showed a high level of IL4 and IL10 genes in the macrophages. C57BL/6 mice were subject to IP administration and transfection of HA-PEI/pDNA-IL4 and HA-PEI/pDNA-IL10 in stimulated peritoneal macrophages showed the upregulation of IL4 and IL10 genes and amplified peritoneal and serum IL10 levels, converting LPS and IFN-γ stimulated

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peritoneal macrophages towards the M2 phenotype. HA-PEI/pDNA-IL10 NPs IP administration also reduced local inflammation induced by LPS. In general, it has been shown that the targeting of CD-44 overexpressing macrophages with HA-NPs is another promising method of gene delivery.

Polymeric NPs display biocompatibility, stability, and ease of modification of the chemical structure. Polymers such as PAMAM dendrimers are able to deliver multiple antigen-expressing replicons at the same time to confer multipronged immunity, microneedle loading of polymeric NPs further increase stability, reduction of sharps waste and are able to be administered by unskilled medical personnel, and natural polymers such as chitosan and HA enhance mucosal absorption for intranasal delivery as well as display tumor and macrophage targeting ability, respectively. Further modifications with HA-PEI show proton sponge effect which changes the osmolarity of endosomes, leading to their rupture and payload escape. Because of these great materials benefits, polymeric NPs have long been a staple along with liposomes for delivery of nucleic acids due to customizability and advantageous natural properties.

Challenges of polymeric nanoparticle nucleic acid delivery include the relatively low transfection efficiency and potential cytotoxicity as well as the current limited understanding of the protein corona's interactions with polymeric vectors.[129] Furthermore, side-chain groups for advantageous charge density and hydrophilic/hydrophobic customization must be optimized as these factors influence the strength of polyplex–cell membrane interactions, NP stability, and intracellular release of nucleic acids.[130] Finally, the preparation of newer generations of polymeric NPs involve more intricate synthesis processes such as the protection–deprotection of functional groups, and in situ polymerization, which present potential issues for scale-up.[131]

3.3. Inorganic Nanoparticles

Inorganic nanoparticles have been broadly researched for nucleic acid delivery. Inorganic NPs in general display smaller size than polymeric/liposomal NPs, narrow size distribution and surface chemistry amenable to ligand conjugation.[132] Gold nanoparticles (AuNPs) are very stable inorganic nanoparticles, and they display wide-ranging electromagnetic properties which have aroused attention to their biomedical applications in nucleic acid delivery.[133,134] AuNPs are extensively used in research and development for their intrinsic optical properties and facile surface chemical modification with many types of ligands.[135] The Meka group further develop the concept of shikimoyl ligands to transfect DCs by using gold nanoparticles conjugated to mannose-mimicking shikimoyl ligand (SL) via a 6-amino hexane thiol spacer (AuNPs-SL) for use in ex vivo DC transfection based genetic immunization via electrostatic complexation with DNA vaccine.[136] Subcutaneous administration of C57BL/6J mice with DCs ex vivo transfected with electrostatic complex of AuNPs-SL & melanoma antigen (MART1) encoded DNA vaccine (p-CMV-MART1) induced long lasting (200 d) anti-tumor immune response in immunized mice upon subsequent challenge with lethal dose of melanoma.

Another type of inorganic NP, mesoporous silica nanoparticles (MSNs) are biodegradable and chemically stable nanostructured materials with uniquely large porosity. This porosity allows expansive surface area available for NP surface chemistry modification and drug encapsulation, and allows for many sites to efficiently carry nucleic acids.[134,137,138] An and co-workers describe a cationic silica nanoparticle (SiNP) delivery system to target lymph nodes which efficiently coloaded negatively charged oligonucleotide adjuvant and Ovalbumin (OVA) antigen through electrostatic interactions.[139] Antigen-specific CD4+ and CD8+ T cell mediated immunity are vital for chronic infectious diseases and cancer.[140] As the T cell mediated immunity is induced in secondary lymphoid organs, such as the LNs,[141] recent studies such as this have focused on developing strategies to target vaccines to the LNs, and target the antigen presenting cells (APCs) residing in the lymphoid tissue, such as DCs. As viruses can induce robust T-helper and CTL immune responses through their in vivo LN drainage, authors designed SiNPs to mimic viral particles to accumulate in APCs in LNs. Mouse immunization with SiNPs generated antigen-specific cytotoxic T cells and humoral response which enhanced antitumor efficacy, minimizing the systemic dissemination and reducing vaccine-induced toxicity. The formulation outperformed soluble vaccine in an animal tumor model.[139]

Recent research has shown that MSNs show intrinsic immunological adjuvant activity[142] and spurred the development of MSN-nucleic acid vaccines. Song et al. report the development of a MSN-based DNA vaccine using rambutan-like MSNs as both gene vector and adjuvant.[143] The rambutan-like MSNs have been developed through co-polymerization of resorcinol-formaldehyde (RF) resin and silica to show unique spiky nanotopography, further modified with cationic PEI (**Figure 5**) which have previously been shown to show superior pDNA delivery and effective protection of gene from nuclease degradation.[144] Authors show that rambutan-like MSN containing ovalbumin (OVA)-encoding pDNA (pDNA-OVA) vaccine enhances antigen-specific IgG production and dendritic cell maturation with enhanced CD80 and CD86 expression, and the MSNs improved antigen-specific IgG antibody, cytokine production of IFN-γ, and increased CD8+ T cell activation in mice.[143] Furthermore, the immune response of their MSN-based DNA vaccine outperformed a commercially available transfection agent, in vivo-jetPEI.

It has been shown that AuNPs are able to be conjugated to targeting ligands and complex with DNA vaccine for successful tumor immunization, MSNs were shown to show LN accumulation-based T cell and humoral response, and another study with MSN-modified with PEI and RF showed enhanced pDNA delivery over a commercially available transfection agent.

Although AuNPs show stability, tunable surface and low toxicity owing to their unique size, shape, structure, and optical properties, challenges remain in their design for nucleic acid delivery.[145] For example, researchers must fully elucidate how the conjugated ligands may influence the pharmacokinetics, biodistribution, and side effects that may occur, as cationic ligands are shown to increase cytotoxicity.[146] In terms of MSNs, one major issue which is common is endosomal entrapment of the particles, leading to low cytoplasmic delivery efficiency and lowered performance of nucleic acid payload.[147] Furthermore, many inorganic NPs used for nucleic acid delivery are proof-of-concept studies which necessarily imply that further

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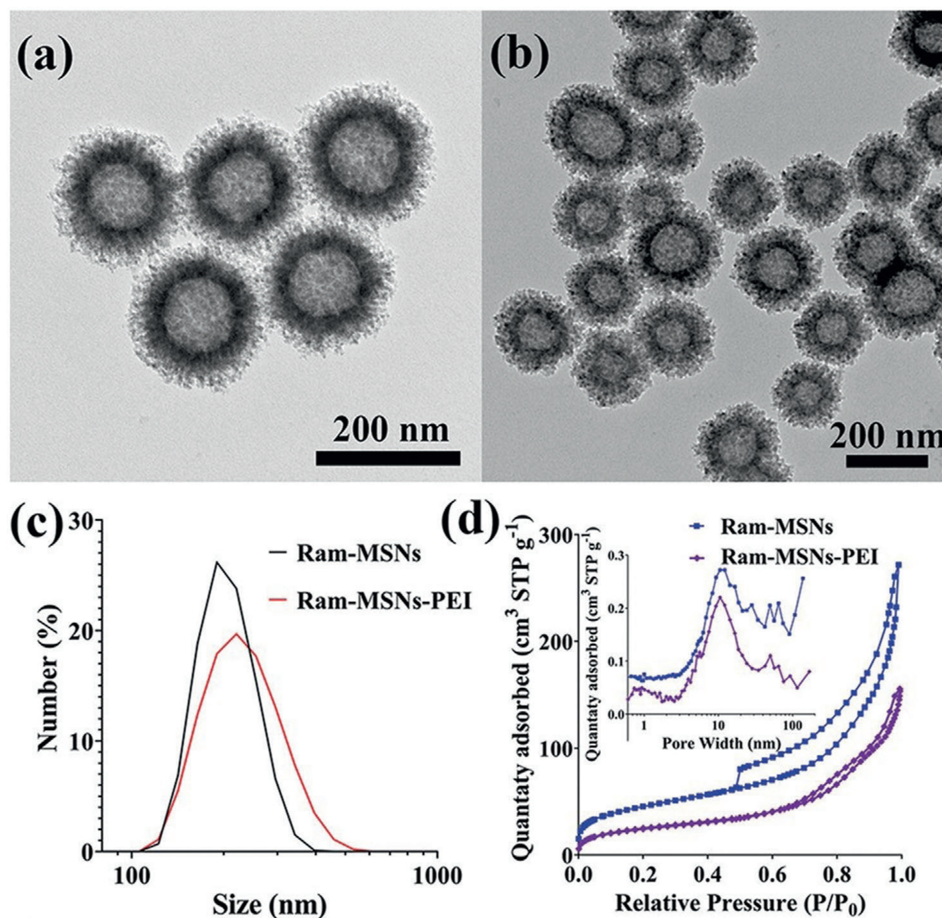

Figure 5. Rambutan-like mesoporous silica nanoparticles which show superior pDNA adsorption. A) TEM images of B) Ram–MSNs and C) Ram-MSNs-PEI corresponding particle size distribution determined by DLS D) nitrogen sorption isotherm Reproduced with permission.[143] Copyright 2019, Wiley-VCH GmbH.

optimization is needed for nucleic acid/ligand ratios, as well as testing in larger animals, clinical trials and improving design for future scalability.[148] In general, inorganic NPs are highly stable NPs which are gradually finding more usages in DNA/mRNA vaccine delivery.

3.4. Peptide-Based Nanoparticle Systems

Peptides, aside from being used as vaccine agents themselves,[149] have been used to facilitate nucleic acid vaccine delivery. Peptides used for nucleic acid delivery are positively charged through inclusion of lysine and arginine residues to electrostatically bind the negatively charged nucleic acids to form nanocomplexes which may be considered nanoparticles, and the positive-negative ratio affects complex formation.[150] Furthermore, virus-like particles (VLPs) have been utilized to deliver nucleic acid vaccines.

For example, Udhayakumar et al. demonstrate the use of cell-penetrating peptides (CPPs) with the amphipathic RALA motif for delivery of mRNA vaccine.[151] RALA is an amphipathic peptide (N-WEARLARALARALARHLARALARALRACEA-C) CPP displaying positively charged arginine residues on one side and neutral leucine residues on the other. RALA was able to condense the mRNA into nanocomplexes with acidic pH-dependence membrane escape ability from endosomes. To show this, RALA-based mRNAs were taken up by DCs with mRNA released from the endosomes resulting in efficient antigen expression. Modification of the mRNA with pseudouridine and 5-methylcytidine showed potent cytolytic T cell responses and superior efficacy as compared to unmodified mRNA nanocomplexes. RALA-mediated mRNA vaccination was also shown to outclass liposomal mRNA formulation with cationic lipid DOTAP and the fusogenic lipid DOPE. This study shows that RALA and other CPPs are highly favorable vehicles for mRNA delivery and further studies.

Poly(lactic acid) (PLA) NPs have been shown to encapsulate and/or adsorb various antigens and immunostimulant molecules and are efficiently taken up by DCs.[152,153] Since both the PLA-NP surface and mRNA biomolecules are negatively charged, cationic CPPs have been used as cationic intermediates for loading mRNA onto PLA-NPs (**Figure 6**). Coolen and co-workers have developed mRNA-PLA NP platforms using three different CPPs (termed RALA, LAH4 and LAH4-L1) as cationic intermediates for vectoring mRNA onto

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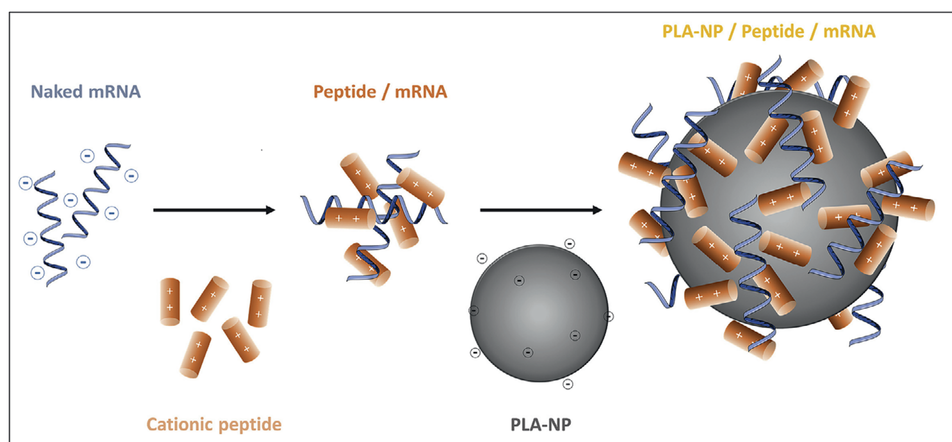

Figure 6. mRNA vectorization by PLA-NPs with cationic peptide intermediates. A schematic representation of the vectorization strategy of mRNAs onto PLA-NPs. The negatively charged mRNA associates with cationic peptides (RALA, LAH4 or LAH4-L1) to form Peptide/mRNA polyplexes. Complex are adsorbed onto PLA-NPs to form PLA-NP/Peptide/mRNA nanocomplexes. Reproduced with permission.[154] Copyright 2019, Elsevier.

PLA-NPs. Negatively charged mRNA was associated with cationic peptides (RALA, LAH4, or LAH4-L1) to form peptide/mRNA polyplexes. Then, this complex was adsorbed onto PLA-NPs to form PLA-NP/Peptide/mRNA nanocomplexes. LAH4-L1 and PLA-NP/LAH4-L1 formulations showed the highest protein expression in vitro through phagocytosis and clathrin-dependent endocytosis, and further investigation reveals clathrin-mediated endocytosis and phagocytosis pathways are key to PLA-NPs entering DCs.[154]

CPPs with amphipathic RALA motif have been shown to condense mRNA into nanocomplexes, which could escape from endosomes and invoke T cell immunity in vivo, outperforming another liposome-based mRNA formulation. Furthermore, CPP-modification of PLA has been shown to form cationic peptide/mRNA polyplexes for DC delivery. Therefore, CPPs show versatile ability to act alone to condense the nucleic acid payload, or in conjunction with polymer for increased efficacy. Further investigations with CPPs can include further modifications of liposomes, polymeric NPs and inorganic NPs for synergistic effect.

Virus-like particles are viral structural proteins, which are recombinantly produced or produced via cell-free protein synthesis, self-assembling without the viral genetic material present. This renders them as non-infectious antigenic nanoparticles which have generated much interest and development.[155,156] VLPs can load small molecules, proteins as well as nucleic acids. They can further be functionalized with peptides, antibody fragments or PEG for targeting or to extend circulation time.[157] Cheng et al. developed an unmethylated CpG-A motif-rich G10 oligodeoxynucleotide (ODN) encapsulated in virus-like particles, termed CMP-001. The CpG-A ODN stimulates larger amounts of type 1 IFN from pDCs compared with CpG-B and CpG-C,[158] and in this case the VLPs serve as a protecting encapsulation for the ODNs protecting against degradation. Researchers show that in situ vaccination with CMP-001 induced both local and abscopal antitumor immune responses in the presence of anti-Qβ. In fact, CMP-001 dramatically enhanced tumor response to anti-PD-1 therapy and was shown to activate plasmacytoid dendritic cells (pDCs), as well as natural killer (NK) cells in a human papilloma virus-positive (HPV+) tumor mouse model.[159] CMP-001 is currently undergoing clinical trials in combination with PD-1 blockade in multiple tumor types such as melanoma, colon, HNSCC, and lymphoma.

As natural structures present in all biological systems, peptide-based NPs are a natural method to deliver nucleic acid vaccines. Though peptide-based NP systems have recently made an important impact on the field of nucleic acid vaccine delivery, there are only a few small compound libraries that are effective from which to draw from in their development. Therefore, improvements can be made through developing effective new compound libraries and expanding the pool of materials which can create peptide delivery systems. Second, arginine-rich CPPs that are commonly used for their potency also show nephrotoxicity and researchers should be careful in applying these compounds too judiciously in their formulations.[160] VLPs face issues with stability and phagocyte-mediated clearance.[157,161] As these are very recently developed platforms, the challenges regarding optimization, chemical modification, scalability, storage and testing on larger animals/clinical trials also apply to peptide-based NP systems.

3.5. Clinical Trials

Each NP vaccine candidate needs to go through a series of clinical trials to be evaluated for safety, immunogenicity, and protective efficacy in humans. Below is a table describing the NP vaccine therapies currently undergoing different stages of clinical trials (**Table 1**). As is clear, many are liposomal based, and this represents an enormous opportunity to improve and refine the polymeric, inorganic and peptide-based NPs for clinical use. Furthermore, only a minority of the clinical trials involving NPs are nucleic acid-based, indicating that there remains a great opportunity to fill the niche of NP-mediated nucleic acid delivery in clinic with the aforementioned improvements in NP and nucleic acid design.

4. Perspectives, Challenges, and Conclusion

DNA and mRNA vaccines present many benefits towards a modernized vaccination system enabling quick adaptation to

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Table 1. Nanoparticle vaccines which have been/are being evaluated in clinical trials. All data were obtained from clinicaltrials.gov.

Formulation Name	Disease	Clinical Trial Phase	NCT number	Type	Status	Nucleic acid NP vaccine?
2019nCoV-101	SARS-CoV-2	Phase 2	NCT04368988	Recombinant trimeric spike protein	Recruiting	No
W_ova1	Ovarian Cancer	Phase 1	NCT04163094	Liposome	Recruiting	Yes
ConM SOSIP.v7 gp140	HIV-1-infection	Phase 1	NCT03961438	Liposome	Recruiting	No
mRNA-1273	SARS-CoV-2	Phase 3	NCT04470427	Liposome	Recruiting	Yes
L-BLP25	Lung Cancer	Phase 2	NCT00828009	Liposome	Active, not recruiting	No
RSV-F Vaccine	Respiratory Syncytial Virus (RSV)	Phase 2	NCT01704365	Recombinant RSV fusion protein nanoparticle	Completed	No
DNA PEI polyplex Vaccination	Relapsed Neuroblastoma	Phase 1	NCT04049864	DNA-PEI polyplex	Recruiting	Yes
PAN-301-1	Prostate Cancer	Phase 1	NCT03120832	Protein directed nanoparticle vaccine	Completed	No
NanoFlu	Influenza	Phase 1	NCT03293498	Recombinant Trivalent Nanoparticle Influenza Vaccine	Completed	No
CMP-001	Melanoma	Phase 2	NCT04387071	VLP-encapsulated TLR9 Agonist	Not yet recruiting	Yes

changing pathogen strains as well as cheap and swift manufacturing capability. Due to the constraints linked to viral vectors for DNA/mRNA delivery, non-viral vectors, particularly nanoparticle formulations have been developed with various beneficial delivery properties using different materials including lipids, polymers, inorganic molecules, peptides and combinations thereof. NPs offer groundbreaking opportunities to develop highly effective targeted therapies with desired biodistribution, pharmacokinetics, bioavailability, and safety profiles, leading to improved vaccine efficacy.[61] We are currently seeing vast creativity and ingenuity in NP delivery strategies to optimize payload protection, endosomal escape, APC/LN targeting and transfection efficiency. Targeted delivery of nucleic acids to DCs greatly enhances immune response and is just another facet of NP-based delivery.

Since the SARS-Cov-2 global pandemic, there has been an explosion of nucleic acid-based vaccines explored and developed to meet the demand for a suitable and effective vaccine with rapid turnaround. In parallel with advances in design and modification of nucleic acid-based vaccine technologies, a wide variety of nanoparticles have been advanced and employed as delivery vehicles for nucleic acid vaccines in preclinical and clinical research.[162–164] So far some of the developed nanoparticle formulations have demonstrated effectiveness in delivery of nucleic acid-based vaccines in animal studies and early stages of clinical research. However there still remains challenges to be addressed. First, detailed mechanisms of the NP-mediated delivery processes need further investigation to enhance endosomal escape (for mRNA vaccines) and nuclear transport (for DNA vaccines), thereby achieving improved transfection efficiency. It is crucial to note that transfection efficacy is not the only parameter that we need to consider during the vaccine development. Understanding the biological processes in which the transfected cells promote protective immune response effectively is vital to developing and designing next-generation nucleic acid vaccines.

Another challenge is to achieve in vivo targeted delivery of singular or combinations of different nucleic acid vaccines to the same immune cell of interest. A variety of ligands have been decorated to the NP surface to bestow NP vaccines with targeting capacity, which have shown the potential to enhance immunization efficacy.[68,136] Even though effective immunization was observed in animal studies, they may not be applicable to humans. Due to the differences in the immune systems between human and animal models, a comprehensive evaluation of NP formulations, nucleic acid dose and administration routes is required to determine the optimal parameters for the desired immune response in human trials. Novel research has proceeded with liposomal, polymeric, inorganic and peptide-based NPs, with a vast array of different morphologies, sizes, and modifications all aimed to increase the effectiveness of nucleic acid delivery, yet there are a lack of comprehensive studies to compare all of the different delivery platforms, which might provide valuable guidance toward design of optimal NP-mediated nucleic acid vaccines, as well as prediction of in vivo immune response. Finally, it is also crucial to optimize the safety profiles of NP formulations while maintaining their vaccine efficacy.

The governmental fast-tracking of many vaccines due to the SARS-CoV-2 pandemic, including NP-based mRNA vaccines taken to Phase 3 clinical trials, has significantly accelerated the rational design and clinical trials of nucleic acid vaccine NPs. A first-generation NP nucleic acid vaccine may emerge soon if the trend continues. Further refinement on the design of the DNA/mRNA payloads and NP formulations based on the previous successful experience in clinical development process and improved understanding in the immune response induced by NP-mediated nucleic acid vaccines will yield more rapidly implementable vaccines, which may take mere months to develop in response to infectious diseases and cancers. Although the COVID-19 pandemic has caused tremendous damage and loss of life worldwide, it has given the world a wake-up call to design new technologies and concepts for vaccines. By addressing the aforementioned challenges, we believe that the full potential of NP-mediated nucleic acid vaccines will be uncovered in the future.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

DNA, mRNA, nanoparticles, nucleic acid, vaccine delivery, vaccines

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