Chapter

Perspective Chapter: Next-Generation Vaccines Based on Self-Amplifying RNA

Fatemeh Nafian, Simin Nafian, Ghazal Soleymani, Zahra Pourmanouchehri, Mahnaz Kiyanjam, Sharareh Berenji Jalaei, Hanie Jeyroudi and Sayed Mohammad Mohammdi

Abstract

Recently, nucleic acid-based RNA and DNA vaccines have represented a better solution to avoid infectious diseases than "traditional" live and non-live vaccines. Synthetic RNA and DNA molecules allow scalable, rapid, and cell-free production of vaccines in response to an emerging disease such as the current COVID-19 pandemic. The development process begins with laboratory transcription of sequences encoding antigens, which are then formulated for delivery. The various potent of RNA over live and inactivated viruses are proven by advances in delivery approaches. These vaccines contain no infectious elements nor the risk of stable integration with the host cell genome compared to conventional vaccines. Conventional mRNA-based vaccines transfer genes of interest (GOI) of attenuated mRNA viruses to individual host cells. Synthetic mRNA in liposomes forms a modern, refined sample, resulting in a safer version of live attenuated RNA viruses. Self-amplifying RNA (saRNA) is a replicating version of mRNA-based vaccines that encode both (GOI) and viral replication machinery. saRNA is required at lower doses than conventional mRNA, which may improve immunization. Here we provide an overview of current mRNA vaccine approaches, summarize highlight challenges and recent successes, and offer perspectives on the future of mRNA vaccines.

Keywords: vaccine (s), self-amplifying RNA (saRNA), in vitro transcription (IVT), design of experiments, nucleic acid, messenger RNA (mRNA), innate immune stimulation

1. Introduction

Vaccines have been the most successful biomedical invention to prevent the morbidity and mortality caused by infectious diseases [1]. A vaccine stimulates the immune system to produce antibodies against target antigens, preventing infection, reducing disease severity, and decreasing the rate of hospitalization. Early vaccines were based on live, non-live (inactivated), or attenuated replicating strains of the relevant pathogenic organism from those that had only segments of a pathogen or killed whole organisms. In the second half of the last century, the development of the industrial production of a new series of vaccines was known as the advancing years of vaccinology.

It started by generating vaccines against Rubella, Mumps, and Measles in the 1960s, which was extended with the production of the Chickenpox vaccine, and deactivating Japanese encephalitis. The induction of a protective immune response could be a target for new advanced vaccines. Cultivation techniques under dominated conditions have been used to process mass vaccine production. In the 1980s, "conjugate vaccines" were used to stimulate immune responses against capsular polysaccharides and proteins of pathogens. Polysaccharide antigens can also cause protective immune responses and are the basis of vaccines that have been evolved to prevent several bacterial infections, such as pneumonia and meningitis caused by Streptococcus pneumonia, since the late 1980s. Conjugate vaccines were presented either in the form of whole or inactivated pathogens or as structural parts. To gain complete prevent, the vaccine must contain antigens that are either led by the pathogen or produced synthetically to represent the segment of the pathogen. The main component of most vaccines is one or more protein antigens that breed immune responses that create protection. Therefore, "recombinant vaccines" were advanced using genetic engineering to produce multivalent vaccines and balance the efficiency of the immune response and the safety of antigens for immunogenicity.

2. Nucleic acid-based vaccines

In 1990, vaccine development came into its golden age with the introduction of nucleic acid-based vaccines, including viral vectors, plasmid DNA (pDNA), and

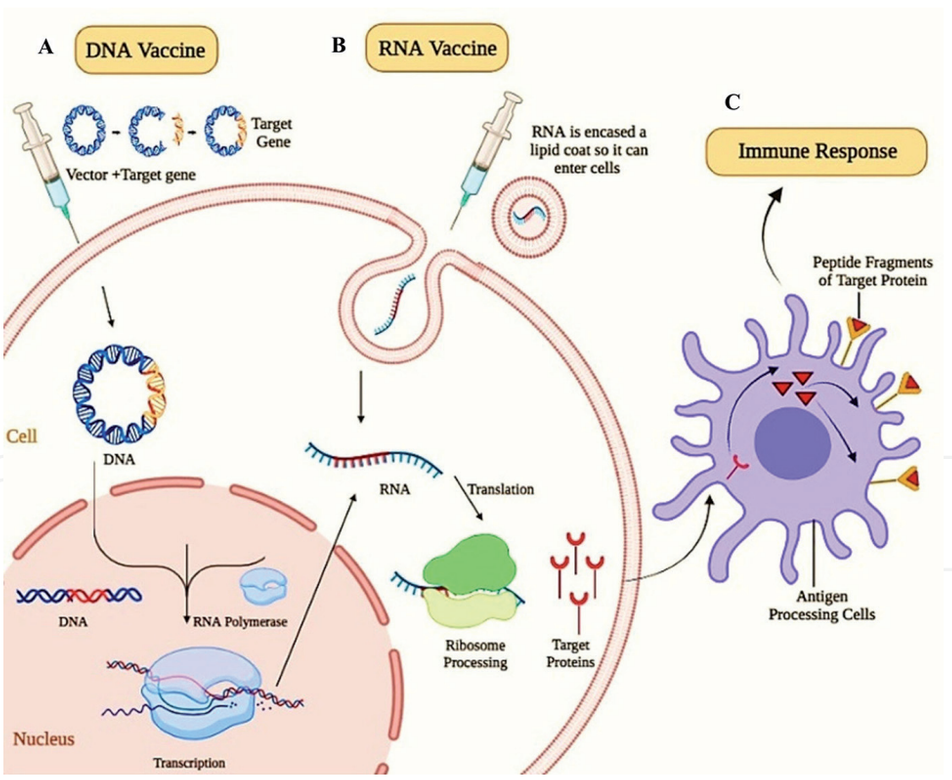

Figure 1.
Molecular basis for the immunostimulatory activity of next-generation nucleic acid vaccines. A) In the DNA vaccine, the target antigen is inserted into a vector. After DNA vaccine injection, the inserted antigen must cross the cell and nuclear membranes to use cellular enzymes which allows the antigen transcripts into mRNAs in the nuclear and then translates into immunogen proteins in the cytoplasm. B) In RNA vaccines the RNA encoding the immunogen protein is formulated into nanoparticles to be delivered into the cell, then its endocytosis and mRNA are releases into the cytoplasm. By entering mRNA into the cytoplasm, the RNA translates to immunogenic proteins. C) the produced protein will be presented upon the surface of the cell to trigger an immune response through antigen-presenting cells (APCs), such as macrophages and dendritic cells. APCs process proteins, break them into peptides and present them in conjunction with MHC molecules on the cell surface where they may interact with appropriate T cell receptors. This figure was created using BioRender (http://www.biorender.com).

mRNA as safer alternatives. They consist of one or more genes of interest (GOI) encoding practically active antigens from an addressed pathogen. After intramuscular injection of RNA or DNA vectors, they are up-taken by immune effector cells and host-arbitrated expressed, resulting in induction of both cellular and humoral immunity (**Figure 1**) [2]. It has been demonstrated that they can induce broadly protective immune responses with a safe approach against infectious and non-infectious diseases. However, nucleic acid vaccines are basically therapeutic agents for cancer. The main challenge is developing ways to prevent or treat infectious diseases such as COVID-19 and human immunodeficiency virus (HIV). With the advent of nucleic acid vaccines, the time and cost of vaccine design and production have been considerably cut, since once the platform has been established for GOI synthesis and insertion into an appropriate expression vector [3]. Accordingly, it might be useful for the development of vaccines against emerging pandemic infectious diseases [4]. Upon vaccination, they mimic a viral infection to express antigens in situ and lower doses are required to stimulate both humoral and cellular responses [5]. RNA vaccines have some advantages over DNA vaccines, such as being able to enter non-dividing cells to the cytosolic expression of proteins. In contrast, using DNA shows the perceived risk of integration into the host genome, since it uptakes into cells and enters into the nucleus due to the breakdown of the nuclear membrane during cell division. However, there are difficulties in producing the quantities of mRNA required to be produced in vivo [4]. A "vaccine on-demand" approach can provide a rapid research and development process, large-scale production, and distribution for nucleic acid based-vaccines [6].

3. RNA vaccines

Over the past years, mRNA has provided a promising technology in the field of vaccine development with several beneficial features overkilled, live attenuated viruses, and subunit as well as DNA vaccines [7]. First, mRNA is a safe platform with no potential risk of infection or genomic integration. mRNA half-life can be regulated in vivo using various modifications and delivery methods [8]. The mRNA immunogenicity can be down-modulated to further increase the safety profile [9]. Second, various modifications can increase efficacy, stability, and expression levels of mRNA [10]. In vivo delivery can be well-organized by formulating mRNA into carrier molecules, allowing rapid uptake and expression in the cytoplasm [11]. Third, mRNA vaccines can be manufactured rapidly and inexpensively on a large scale with a high yield of IVT reactions.

The mRNA vaccine falls into two basic types: conventional non-replicating mRNA and self-amplifying RNA (saRNA). Both approaches show essential elements of a eukaryotic mRNA: a cap structure [m7Gp3N], a 5′ UTR, an open reading frame (ORF) encoding the gene of interest (GOI), a 3′ UTR, and a tail of 40–120 adenosine residues [poly (A) tail] (**Figure 2**) [12]. Self-amplifying mRNA vaccines are derived from the engineered RNA genomes of plus-strand RNA viruses such as alphaviruses or flaviviruses, and picornaviruses [13, 14]. Therefore, it encodes not only the antigen of interest flanked by 5′ and 3′ UTRs but also contains an amplicon required for intracellular RNA amplification enhancing antigen expression levels [13, 15, 16].

Both groups can be produced in vitro transcription of mRNA (IVT mRNA), as a cell-free system, using an enzymatic transcription reaction from a linearized pDNA template [17]. The RNA manufacturing begins with the construction of the pDNA molecule that is used as a template for an IVT mRNA using a promoter with a high binding affinity for a DNA-dependent RNA polymerase, and a restriction site for

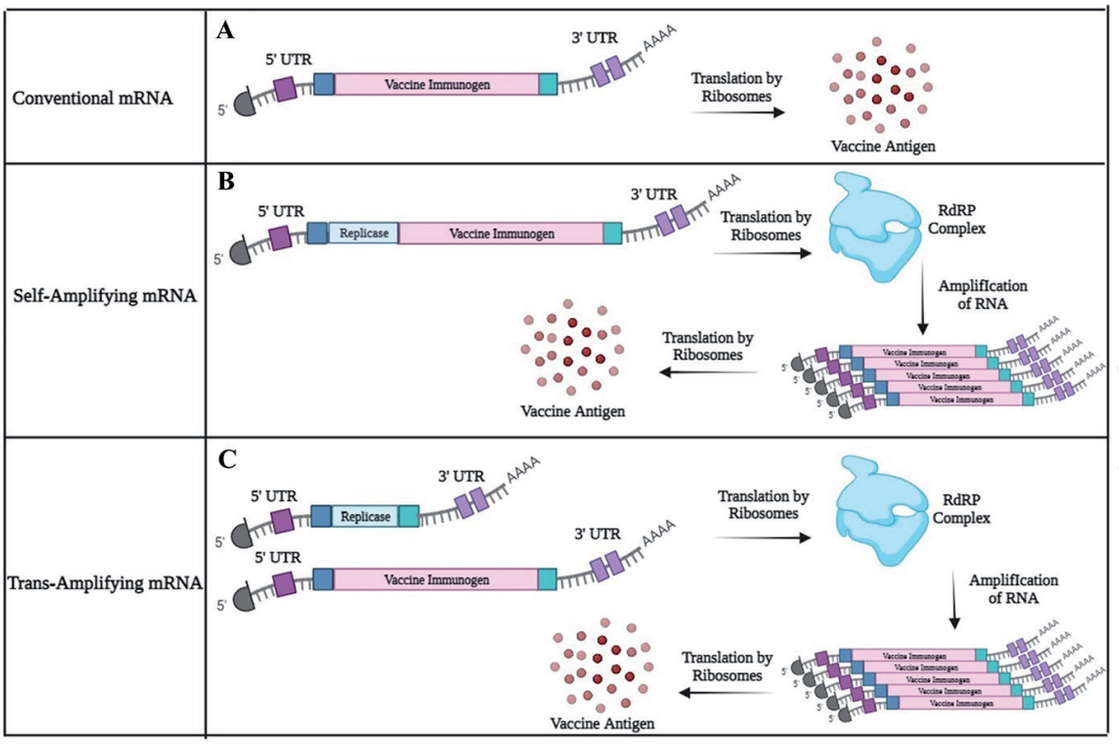

Figure 2.
Schematic of different parts of conventional mRNA, self-amplifying mRNA, and trans-amplifying mRNA vaccines. A) In conventional mRNA, the essential elements are presented, included: a cap structure, a 5′ UTR, an ORF encoding immunogenic protein, a 3′ UTR, and a 3'poly (A) tail, and also the mRNA translates by ribosomes. B) self-amplifying mRNA contains a cap structure, a 5′ UTR, an ORF encoding immunogenic protein, a 3′ UTR, and a 3'poly (A) tail and extra region called replicase. Replicase is a replicating polyprotein complex which this constructs from capping enzyme, helicase, poly (A) polymerase, and an RNA-dependent RNA-polymerase (RdRP) which causes high expression of downstream antigens and increasing immunogenicity. First, the mRNA translates by ribosomes to assemble the RdRp complex to amplify the mRNA then in the next step these amplified mRNAs translate to the vaccine target protein. C) In trans-amplifying mRNA, the replicase and ORF encoding immunogenic protein is separated into two mRNA, but co-delivered to the target cells, which both have a cap structure, a 5′ UTR, a 3′ UTR, and a 3'poly (A) tail. First of the replicase translates to the RdRp complex then utilizes it to amplify. At the end mRNAs, containing ORF encoding immunogenic protein will be translated by ribosomes. This figure was created using BioRender (http://www.biorender.com).

insertion of the specific sequence encoding the target antigen. The ORF must be inserted without affecting the overall physicochemical characteristics of the mRNA molecule. IVT mRNA occurs in three steps of transcription; initiation, elongation, and termination with the help of a linearized plasmid DNA that has a promoter, NTPs, RNA polymerase, and mg2+. The enzyme elongates the RNA transcript until it runs off the end of the template. Then, pDNA is degraded by incubation with DNase, and a cap or a synthetic cap analog is enzymatically added to the 5′ end of the mRNA [18–20]. A co-transcriptional capping strategy was also developed to add a natural 5′ cap structure to a specific start sequence during IVT [21]. This strategy results in innate immune activation when the IVT mRNA is prepared [22, 23]. The presence of a 5'cap structure protects mRNA from intracellular nuclease digestion and is also crucial for efficient translation in vivo [24, 25]. In the end, the mRNA is purified to remove reaction agents, including residual pDNA, enzymes, truncated or double-stranded transcripts [26, 27]. There are several factors that must be addressed before using mRNA, including the ability to express sufficient levels of antigen, immunogenicity, stability, and toxicity of formulated RNA, as well as the possibility of having negative consequences on unexpected and undesired tissues [28]. The establishment of valuable guidance for rapid, simple, and inexpensive mass production of mRNA is a critical requirement for the future implementation of mRNA vaccines [29].

Once the purified mRNA enters the cytosol, the cellular translation and post-translation machinery produce a properly folded, fully functional protein. IVT mRNA is finally degraded by normal physiological processes, thus reducing the risk of metabolite toxicity. In some cases, it has validated the immunogenicity of various mRNA vaccine platforms in recent years [30–33]. Engineering of the RNA sequence has increased translation and prolonged antigen expression in vivo. The first successful vaccination by the IVT mRNA was reported around three decades ago when an intramuscular injection in mice resulted in the local production of an encoded reporter protein and generalization of immune responses against the antigen [34]. However, the early results led to no substantial investment in developing mRNA vaccines, due to concerns about mRNA instability, inefficient in vivo delivery, and high innate immunogenicity. Instead, the field pursued DNA-based and protein-based therapeutic approaches [35, 36].

3.1 Conventional mRNA vaccines

A conventional mRNA vaccine only encodes the sequence of the specific antigen flanked by transcription regulatory regions. The major advantages of the conventional mRNA vaccine are the simplicity and small size of the RNA molecule. However, the stability and efficiency of conventional mRNA in vivo is limited and needs to be optimized in RNA structural elements and formulation approach [37]. The cap or its analogs, UTRs, and the poly (A) tail are crucial elements for stability, accessibility, and interaction with the translation machinery of the mRNA vaccine [38–41]. Codon usage is required to enhance protein expression from DNA, RNA, and viral vector vaccines [42, 43]. The nucleoside base of mRNA can be chemically modified coupled with chromatographic purification to remove dsRNA contaminants, which are improper immune-stimulatory [44, 45]. Although activation of innate immunity is required for vaccination, its excessive activation interferes with antigen production and adaptive immunity [46, 47]. When modified mRNA is highly purified, the highest levels of protein expression and immunogenicity are observed [48].

3.2 Self-amplifying RNA (saRNA) vaccines

Self-amplifying RNA (also called replicon RNA) is one of the most immune-responsive types of mRNA since it activates several Toll-like receptors (TLRs) to generate very strong immune responses. saRNA not only has the basic parts of eukaryotic mRNA (a cap, an ORF, a sub-genomic promoter, a poly (A) tail, 3′ and 5′ UTR flanks) but also encodes a replicating polyprotein complex including an RNA-dependent RNA-Polymerase, capping, helicase, and poly (A) polymerase (**Figure 2**). The replicative features of positive-stranded RNA viruses are mimicked to highly express the antigen and increase immunogenicity. The gene of interest is placed downstream of the replicon construct, which is under the control of the promoter. Entering saRNA into the cell cytoplasm immediately couples with the translation of a replicase complex that recognizes a subgenomic promoter and amplifies a smaller mRNA (subgenomic RNA). The most predominant antigens in saRNA are viral glycoproteins, although this has recently been expanded to include the proteins of bacterial infections, parasites, and cancer. A more novel saRNA encodes monoclonal antibodies for passive vaccination. If necessary to encode multiple antigens, it can be advantageous to use separate saRNA constructs since the pDNA construct does have size limitations [49]. saRNA vaccines against bacterial antigens are limited to protein targets, as opposed to polysaccharides and non-protein surface markers.

The innate immune system has advanced to recognize pathogen-associated molecular patterns (PAMPs) via binding pattern recognition receptors (PRRs) with them. PRRs can be represented as cytosolic receptors including nucleotide-like receptors (NLRs) and RIG-I like receptors (RLRs) or as endosomal toll-like receptors (TLRs) [50, 51]. Innate immune activation can recognize both conventional mRNA and saRNA and arrange signaling pathways to strongly battle the pathogen [52]. saRNA is more immunogenic than conventional mRNA. The optimization of both translation and purity of mRNA vaccines (conventional mRNA or saRNA) can be used to overcome the problems of immune-stimulating activities. Some discoveries emphasize the capacity to synthesize high quality and quantity of mRNA through IVT [53]. The full-length mRNA molecule can be produced by IVT from a pDNA template and delivered as either synthetically unformulated RNA, or as formulated into nanoparticles if structural genes are provided in trans. Alternatively, saRNA can also be produced directly in vivo, by delivering a pDNA containing the replicon complex and GOI into the target cells [54]. The efficacy of nanoparticles as a vaccine carrier must be engineered to correctly condense processed heterologous proteins and deliver antigen to specific cell types, such as APCs [54, 55]. Activation of dendritic cells by mRNA nanoparticles results in a wave of cytokine cascade and subsequently a vaccine-elicited adaptive immune response [56]. One of the challenges with mRNA vaccines is the determination of sufficient quantities of RNA sequence, integrity, and purity for use in the target population (**Figure 3**) [57–59].

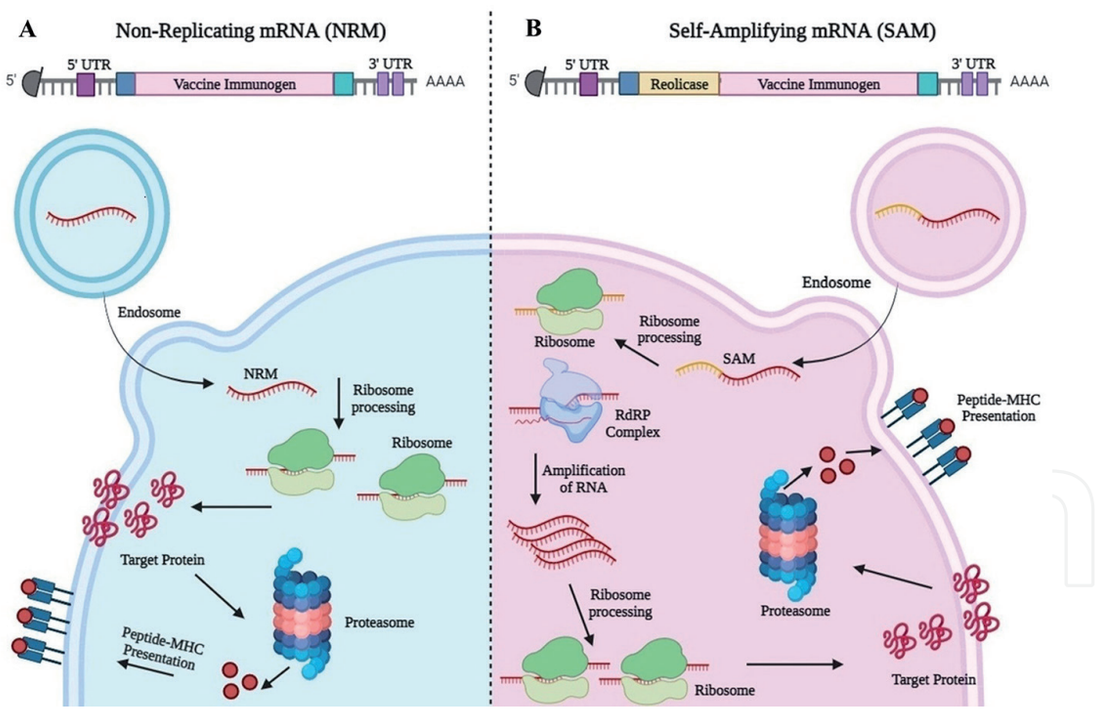

Figure 3.
Schematic of different protein production of conventional mRNA and self-amplifying mRNA in APCs. Both mRNAs (A and B) can be encapsulated in nanoparticles (NPs) to preserve them from degradation and facilitate cellular uptake. Membrane-derived endocytic pathways are commonly used for cellular uptake of mRNA with its delivery system. A) after entry of SAM into cytoplasm the translation begins to build up the RdRp complex and in the next step the RdRp complex amplifies the mRNAs. mRNAs translate by ribosomes and then some of them will be delivered directly on the surface and others will be degraded by proteasome which leads to MHC presentation. B) In NRM, the mRNA is just translated and does not have replication. Like to SAM the numbers of the target proteins are expressed on the surface directly on the rest of them through MHC presentation. Once the formulated mRNA enters the cytosol, it directly translates and post-translates into a fully functional antigen to be ready for MHC presentation on the cell surface. The presented protein can induce both innate and adaptive immune responses. Furthermore, self-amplifying mRNA encodes the replicating complex that is required for intracellular RNA amplification. This figure was created using BioRender. (http://www.biorender.com).

4. Delivery systems for mRNA vaccines

Naked mRNA is quickly degraded by extracellular RNases and is not internalized efficiently. Anionic saRNA constructs are relatively large (9000 to 15,000 n) and need to be condensed by a cationic carrier into a nanoparticle of ~100 nm in size that encourages uptake into target cells and protects the saRNA from degradation [60]. A great variety of in vitro and in vivo transfection reagents can facilitate cellular uptake of mRNA and protect it from degradation. Unmodified RNA has limited stability in the bloodstream and passes through the cell membrane and immunogenicity. RNA is a single hydrophilic molecule and is likely to form a secondary or territory structure, leading to an unfavorable problem with delivery approaches. The vectors used for effective cell delivery of mRNA vaccines include viral vectors such as adenoviral, adeno-associated viral, retroviral, and lentiviral vectors, as well as nanoparticles made of lipids, polymers, and inorganic compounds. Viral vectors present high-efficiency transfection by design through infection gene deletion, viral replication, and assembly. On the other hand, nanoparticle (NPs) vectors offer advantages such as greater safety, flexible administration, wider adoption, and unlimited transgene size and may represent the future of next-generation vaccines.

The early delivery system was a combination of minor arginine-rich cationic proteins, protamine, and mRNA. Protamine-complexed mRNA reduced protein expression. That's why a mixture of free and protamine-complexed mRNA is used [61]. NPs offer a variety of biomaterial alternatives to formulate mRNA, facilitate cell internalization, increase specific immune cell targeting through surface modifications, and boost endosomal escape using pH-sensitive materials [62]. NPs, as a strong adjuvant, enhance protection through synergistic effects since they allow for the generation of cocktail vaccines in a single particle and for the delivery of numerous nucleic acids to the same target cell [62]. There has been new research into liposomal, polymeric, inorganic, lipidoid, and peptide-based nanoparticles, with a wide range of different 3D structures, sizes, and modifications, all aimed at increasing the effectiveness of nucleic acid delivery. The recent systems for mRNA vaccine delivery are liposome nanoparticles (LNP), which are lipid bilayer-coated artificial vesicles. Cationic lipids bind to negatively charged nucleic acids through their positive-charged hydrophilic heads, and the hydrophobic lipid tails encapsulate them. On the other hand, neutral lipids can be used to improve transfection efficiency and stability. The quantity of charged groups per molecule, geometric shape, and type of the lipid anchor are all important factors in transfection efficiency [62]. The LNP-encapsulated saRNAs induce higher antibody titers, pathogen neutralization (IC50), and antigen-specific CD4+ and CD8+ T cell responses than electroporated pDNA [62]. Recently, the combination of LNPs with dendritic cell (DC) targeting, UTR optimization, lipopolymer design, and ionizable lipids has advanced the field of saRNA vaccination. However, there are concerns about the toxicity of cationic LNPs, caused by membrane disruption, as well as endosomal escapes that interact with LNPs.

Polymeric nanoparticles (PNPs) that are also used for mRNA vaccine delivery are typically made from biocompatible and biodegradable polymers. They have a wide range of physicochemical properties, which may be structurally modified for regulated release of the gene contents [62]. Cationic polymers encapsulate mRNA via electrostatic interactions to generate polymer-mRNA polyplexes. Polyamidoamine dendrimers (PAMAM) and polyethylenimine (PEI) are two commonly used polymers. PNPs preserve mRNAs and also facilitate their entrance into cells. PEI offers additional benefits, including a higher protonation ratio of amine groups and a high buffer capacity over a wide pH range [62]. PAMAM is a biocompatible and highly branched cationic polymer that enables functionalization

to deliver multiple antigen-expressing replicons at once. The low transfection effectiveness and cytotoxicity of PNPs-mRNA delivery are remaining challenges.

For nucleic acid delivery, inorganic NPs have been extensively studied. Inorganic NPs have a lower size than polymeric/liposomal NPs, a limited size distribution, and ligand conjugation-friendly surface chemistry. Gold nanoparticles (AuNPs) have a wide range of electromagnetic properties required for mRNA delivery and their surfaces are easily modified with a variety of ligands. Mesoporous silica nanoparticles (MSNs) are another form of biodegradable inorganic NPs that have very porous nanostructures. The porosity can provide a large surface area for chemical modification and mRNA encapsulation in multiple targeting carriers. Positively charged peptides can form natural NPs for mRNA vaccine delivery due to the presence of lysine and arginine residues. Cell-penetrating peptides can enhance delivery efficacy by creating complexes with nucleic acids. Furthermore, virus-like particles (VLPs) can be used to deliver nucleic acid vaccines, although they may be cleared by phagocytes. Nowadays, next-generation nucleic acid vaccines have been focused on developing transfection effectiveness, optimizing the safety profiles of NP formulations, inducing protective immune responses, and using mixtures of nucleic acid vaccines to target the same immune cell of interest in vivo.

5. Conclusions

Vaccinology is moving toward artificial polymer platforms that allow for fast, scalable, and non-cellular mass production of vaccines. Using mRNA vaccines, there is no chance for undesirable mutations, including insertion, breakage, frameshift, or rearrangements, caused by genome integration [63, 64]. Both conventional and self-amplifying RNA vaccines can be simply designed without restrictions on the size and sequence of antigens. Additionally, multiple antigens can be applied downstream of a robust promoter to have powerful transcription and translation, thus lower doses will be used. However, this chapter demonstrated how self-amplifying RNAs are more potent than conventional types due to productive amplification of mRNA directly within the cytoplasm, the adaptability of applying delivery vectors, and induction of both humoral and cellular immunity for powerful and long-lasting prevention against chronic infectious diseases. Nucleic acid vaccines are safer than infective agents and certain preclinical safety studies may not be necessary, which would further shorten development time and cost. The mRNA vaccines do not require preservation in a cold chain because they are not a live infective vector. All that is needed for mRNA vaccines is to obtain gene sequence information to manufacture an optimized pDNA template using completely synthetic processes or clone it into appropriate expression vectors. They can be delivered for antigen expression in situ without the need to cross the nuclear membrane barrier for protein expression and can express complex antigens without packaging constraints. Finally, it is important to note that both types of nucleic acid-based vaccines have significant advantages over conventional vaccines and thus could be ideal for rapid responses to newly emerging pathogens.

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Conflict of interest

The authors declare no conflict of interest.

Notes

All authors whose names appear in the submission made substantial contributions to the conception, design, and acquisition of data.

Abbreviations

saRNA	self-amplifying RNA
taRNA	trans-amplifying RNA
IVT	in vitro transcription
GOI	genes of interest
pDNA	plasmid DNA
APC	antigen-presenting cell
DC	dendritic cell
ORF	open reading frame
TLR	Toll-like receptor
NLR	nucleotide like receptor
RLR	RIG-I like receptor
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
RdRp	RNA-dependent RNA polymerase
NPs	nanoparticles
PNP	polymeric nanoparticles
AuNPs	gold nanoparticles
MSNs	mesoporous silica nanoparticles
PAMAM	polyamidoamine dendrimers
PEI	polyethylenimine

Author details

Fatemeh Nafian[1*], Simin Nafian[2], Ghazal Soleymani[3], Zahra Pourmanouchehri[4], Mahnaz Kiyanjam[5], Sharareh Berenji Jalaei[6], Hanie Jeyroudi[4] and Sayed Mohammad Mohammdi[4]

1 Department of Medical Laboratory Science, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

2 Department of Stem Cell and Regenerative Medicine, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

3 Department of Genetics, Islamic Azad University Tehran Medical Sciences, Tehran, Iran

4 Department of Cellular and Molecular Biology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

5 Department of Biochemistry, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

6 Department of Microbiology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

*Address all correspondence to: f.nafian@iautmu.ac.ir

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