RNA Combined with Nanoformulation to Advance Therapeutic Technologies

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Nucleic acid-based therapies have the potential to address numerous diseases that pose significant challenges to more traditional methods. RNA-based therapies have emerged as a promising avenue, utilizing nanoformulation treatments to target a range of pathologies. Nanoformulation offers several advantages compared to other treatment modalities, including targeted delivery, low toxicity, and bioactivity suitable for drug loading. Various types of nanoformulations are available, such as liposomes, polymeric nanoparticles (NPs), magnetic NPs, nanoshells, and solid lipid nanoparticles (SLNs). RNA-based therapy utilizes intracellular gene nanoparticles with messenger RNA (mRNA) emerging prominently in cancer therapy and immunotechnology against infectious diseases. The approval of mRNA-based technology opens doors for future technological advancements, particularly self-amplifying replicon RNA (repRNA). RepRNA is a novel platform in gene therapy, comprising viral RNA with a unique molecular property that enables the amplification of all encoded genetic information countless times. As a result, repRNA-based therapies have achieved significant levels of gene expression.

RNA replicon therapy RNA replicon vaccines self-amplifying RNA

1. Introduction

While drug development has made significant progress over the years, there remains a pressing need for further advancements in this field [1]. Technological innovations and genomic research have opened numerous treatment possibilities [2]. Moreover, nucleic acid-based therapies hold the potential to address or even cure many diseases that have proven resistant to traditional treatment methods [2]. The discovery of small interfering RNA (siRNA) and antisense RNA, which effectively suppress the expression of specific genes in cells affected by viruses or cancer, has sparked increased interest in RNA treatments [3]. These advances encompass a range of mechanisms, including approaches such as inhibiting messenger RNA (mRNA) translation, gene expression interference via siRNA, self-amplifying replicon RNA (repRNA), catalytically active ribozymes, and protein-binding RNA molecules. Consequently, RNA-based therapies find applications in diagnostics and certain treatment modalities [4].

Synthetic mRNA serves as a versatile tool for producing proteins and peptides, finding extensive use in pharmaceutical applications, including cancer immunotherapy [5]. It is generated in a cell-free system through in vitro transcription (IVT) from a DNA template [6]. Acting as a natural ligand for various receptors, exogenous mRNA

stimulates the release of type I interferon and pro-inflammatory cytokines, providing it with inherent adjuvanticity [5] On the other hand, repRNA, with sizes ranging from 12 to 15 kilobases, stem from viral genomes lacking at least one structural protein gene, enabling sustained antigen production without the risk of producing infectious progeny [7]. However, their susceptibility to RNase and inefficient uptake by dendritic cells represent key limitations. Compared to conventional mRNA, repRNA triggers prolonged gene expression lasting weeks to months after a single injection, making it a promising candidate for sustained antigen expression in vaccine design [8]. repRNA vaccines have emerged as promising candidates, showcasing exemplary safety and immunogenicity, and have already demonstrated encouraging results in various clinical trials [9][10].

Regarding siRNA, its mechanism relies on post-transcriptional gene silencing, known for its specificity in targeting disease-related genes [11]. However, siRNAs face challenges such as low cellular uptake and susceptibility to degradation, necessitating protective carriers for efficient delivery into target cells [12][13]. Various delivery systems, including nanocarriers, aptamers, peptides, sugars, proteins, and antibodies, have been developed to address these limitations [12][13]. In mammalian cells, siRNAs play a crucial role in RNA interference (RNAi), with minimal concentrations as low as 2000 siRNAs per cell being sufficient for effective gene knockdown [14]. Compared to DNA, RNA has several distinctive qualities that make it a potent biomaterial. Notably, RNA is capable of folding into several tertiary structures with unique activities [3]. The effectiveness of RNA therapy is significantly enhanced by its synergistic association with encapsulated nanoparticles, which facilitate targeted and controlled delivery, thereby increasing its therapeutic potential.

RNA nanotechnology offers several advantages for therapeutic applications. These advantages encompass passive targeting due to its nano-scale size and branched structure, precise synthesis control, limited nonspecific cell membrane crossing, high water solubility, and reduced immunogenicity. Additionally, RNA nanotechnology provides multivalency for module conjugation, favorable in vivo profiles, and specific delivery potential [15]. Furthermore, RNA's classification as a chemical reagent implies more favorable regulatory processes compared to protein-based clinical reagents.

It is important to note that lipid-encapsulated nanoparticles containing small interfering double-stranded RNA (siRNA) have been employed to target transthyretin (TTR) mRNA, leading to the degradation of TTR deposits in patients with TTR-mediated hereditary amyloidosis [9]. This RNA interference drug, Patisiran (ONPATTRO™), has received approval in both the US and Europe for single intravenous infusion [16]. In recent years, mRNAs generated through IVT have gained prominence as promising candidates for the development of new drugs and vaccines [1][16].

Despite considerable progress in RNA nanotechnology for medical applications, several challenges persist. These include RNA's chemical instability, the need for thermodynamic stability in RNA nanoparticles, construction costs, the requirement for favorable pharmacological profiles, specific cell targeting, and the prevention of degradation in the endocytic pathway [3]. Overcoming these challenges is crucial for realizing the full potential of RNA nanotechnology in therapeutics.

2. Nanotechnology's Impact on Healthcare: Advancements, Applications, and RNA Nanotechnology

The prefix "nano", derived from Greek, signifies something minuscule, measuring as small as less than one-millionth of a meter [17]. This minuscule scale is fundamental to nanotechnology's core concept. Conversely, technology denotes the systematic study of processes within a specific domain [18]. However, nanotechnology takes this a step further. Following this initial definition, nanotechnology is regarded as a scientific discipline dedicated to comprehending matter at nanometric scales, typically ranging from 1 to 100 nanometers, with the aim of precise control and manipulation to usher in technological advancements across diverse domains [19].

Our civilization has greatly benefited from nanotechnology, which has made significant contributions to numerous prominent industries [20]. Among these industries, over the past two decades, nanotechnology's advancement in the medical and healthcare sectors have played a crucial role in preventing, diagnosing, and treating various illnesses [20].

Within the realm of healthcare, the application of nanotechnology is termed "nanomedicine," a field that has yielded a profound understanding of cellular mechanisms in living organisms [21]. This profound understanding has paved the way for various applications encompassing pharmaceutical therapy and the prevention, diagnosis, monitoring, and treatment of many diseases [22].

The implications of nanotechnology in scientific research are highly promising, transcending the limitations of conventional therapies by enhancing the mechanisms employed, particularly in safeguarding the desired drug [21]. To achieve these advancements, nanotechnology employs top-down and bottom-up assembly methods to create and utilize materials at the nanometer scale. Notably, DNA, RNA, and protein macromolecules possess inherent properties at the nanoscale, making them promising building blocks for the bottom-up construction of nanostructures and nanodevices [15].

Research into the folding and structure of RNA has a long history. However, it is important to note that RNA nanotechnology distinguishes itself from conventional RNA structure and folding studies [23]. RNA nanotechnology is the study of RNA-based structures with a primary framework at the nanoscale [24]. This unique focus necessitates a comprehensive understanding of not only intramolecular interactions and folding but also intermolecular interactions. A crucial aspect of RNA nanotechnology involves characterizing the physical, chemical, biological, and pharmacological properties of nanoparticles that researchers can homogenize through purification [23]. An important milestone in the field was the assembly of RNA dimer, trimer, and hexamer nanoparticles using reengineered RNA fragments derived from pRNA (packaging RNA), a vital component responsible for driving the DNA packaging motor in bacteriophage phi29 [25].

RNA nanotechnology, as opposed to conventional RNA biology research, focuses on using the characteristics of RNA to create structures for use in nanomedicine [24]. This approach is particularly promising for therapeutic applications, where nanoformulations offer synergistic actions and efficient delivery systems, notably in the

treatment of diverse cancer types [26]. Beyond medicinal technology, a range of industrial sectors, including electronics, environmental science, food production, and textiles, have expressed keen interest in nanotechnology due to its potential to enhance product quality, safety, and durability [22].

2.1. Nanoformulation

Researchers engineer nanoformulations to optimize drug delivery, increase drug solubility, improve bioavailability, and enable targeted drug release, thereby enhancing the overall efficacy and safety of pharmaceuticals [26]. In terms of therapeutic effectiveness, researchers have observed that nanoparticles exhibit optimal internalization by cancer cells, especially in the treatment of diseases like breast cancer [27]. Their enhanced permeation into the target cells not only improves drug delivery, but also renders the nanoformulation more cytotoxic when compared to free drugs [28].

These nanodelivery technologies can also apply to phytocompounds. Various phytocompounds, including cannabidiol (CBD), have received attention for delivery using nanocarriers as a viable platform, with the aim of restricting the variety of negative consequences [29]. Nanodelivery technologies have improved the stability of phytocompounds, enhanced their absorption, shielded them from early enzymatic depletion or metabolism within the body, and prolonged their circulation duration [29].

Therefore, the significance of this technology lies in advancing the treatment of various diseases and offering expanded therapeutic possibilities [30]. Furthermore, its economic advantages are compelling, as it allows for easy industrial scale-up, making it a practical choice in this sector. These innovative nanoformulations hold the potential to revolutionize drug delivery and substantially improve patient outcomes across a wide range of medical conditions. With this in mind, **Table 1** shows some nanoformulations and their main components that have already been approved for therapeutic use by regulatory agencies such as the FDA.

Table 1. Nanoformulations and their main components approved for therapeutic use by FDA and regulatory agencies.

Classification	Name (Trade Tame)	Main Component	Delivery Route	Indication	Approval (Year)
Liposome	AmBisome	Liposomal amphotericin B	Intravenous	Fungal/protozoal infections	FDA (1997) EMA (2006) ANVISA (1997)

Classification	Name (Trade Tame)	Main Component	Delivery Route	Indication	Approval (Year)
	Doxil/ Caelyx	Liposomal doxorubicin	Intravenous	Antineoplastic agents (ovarian and breast cancer; multiple myeloma; Karposi's Sarcoma)	FDA (1995, 2005, 2008) EMA (1996) ANVISA (2011)
	Myocet (Myoce liposomal)	Liposomal doxorubicin	Intravenous	Antineoplastic agents (breast neoplasms)	FDA (2000) EMA (2000)
	Visudyne	Liposomal verteporfin	Intravenous	Ophthalmic agents (myopia; ocular histoplasmosis; macular degeneration, wet age-related)	FDA (2000) ANVISA (2004) EMA (2007)
	Marqibo	Liposomal vincristine	Intravenous	Antineoplastic agents (hematologic malignancies and solid tumors)	FDA (2012)
	Onivyde	Liposomal irinotecan	Intravenous	Antineoplastic agents (pancreatic	FDA (2015)

Classification	Name (Trade Tame)	Main Component	Delivery Route	Indication	Approval (Year)
	(Onivyde pegylated liposomal)			cancer)	EMA (2016)
Polymer-based nanoparticles	Eligard	Leuprolide acetate and polymer (PLGH (poly (DL-Lactide-co-glycolide)))	Subcutaneous	Antineoplastic agents (prostate cancer)	FDA (2002) ANVISA (2006)
	Mircera	Methoxy polyethylene glycol-epoetin beta	Subcutaneous/ Intravenous	Anemia associated with chronic kidney disease	FDA (2007) EMA (2007) ANVISA (2008)
	Cimzia	PEGylated antibody fragment (Certolizumab)	Subcutaneous	Anti-inflammatory action (Crohn's disease; rheumatoid arthritis; psoriatic arthritis; ankylosing spondylitis)	FDA (2008, 2009, 2013) EMA (2009) ANVISA (2017)
	PegIntron	PEGylated IFN alpha-2b protein	Subcutaneous	Immunomodulator (hepatitis C)	FDA (2001)

Classification	Name (Trade Tame)	Main Component	Delivery Route	Indication	Approval (Year)
					EMA (2000) ANVISA (2011)
Magnetic nanoparticles	NanoTherm	Iron oxide coated with amino silane	Intratumoral injection	Antineoplastic agents (glioblastoma)	FDA (2010) EMA (2013)
	Feraheme	Iron oxide and a polyglucose sorbitol carboxymethyether	Intravenous	Treatment of anemia	FDA (2009) EMA (2012)
Lipid nanoparticle	Patisiran (Onpattro)	Phospholipids, cholesterol, ionizable cationic lipid (DLin-MC3-DMA), and polyethylene glycol-modified lipid	Intravenous	Polyneuropathy	FDA (2018)

2.2. Nanotoxicology Challenges and Opportunities

Nanotoxicology, while an expanding field, still has significant gaps, with existing research primarily focused on acute toxicity, necessitating an in-depth exploration of long-term toxicity and chronic exposure effects [31]. The comprehensive evaluation of the safety and toxicity of nanodrug delivery systems remains challenging due to various potential factors contributing to nanotoxicity and the potential for unpredictable interactions with biological systems [31][32].

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The integration of validated analytic methodologies and well-designed experiments can elucidate toxicity mechanisms, ensuring the safe utilization of nanoformulations in therapeutic and diagnostic applications [33][34]. Advancements in nanobiotechnology, material synthesis, and computer simulation studies offer promising prospects for engineering nanoformulations that prioritize both safety and efficacy, thereby mitigating potential toxicities [33]. Notably, regulatory bodies such as EMA and FDA have established guidelines to oversee the application of nanoformulations in medicine [31].

Consequently, further toxicological investigations are imperative to enhance an in-depth understanding of nanoparticle behavior, allowing for the enhancement of their properties to minimize toxicity and maximize therapeutic efficacy [32][35]. Studies have documented various toxic effects associated with different nanoparticles, including increased oxidative stress and potential intracellular infiltration by metal-based nanoparticles, as well as observed hepatotoxicity and nephrotoxicity from protein-based nanoparticles [32][36][37].

3. Virus-Based Delivery System

Viral vectors, which make use of viruses' ability to transfer genetic information into host cells, have shown promise in gene therapy systems [38]. Numerous altered viral vector genomes have been created, such as lentiviruses, herpes viruses, adenoviruses, adeno-associated viruses, retroviruses, human foamy viruses (HFVs), and herpes viruses [39][40] These modified genomes have certain limitations, including immunogenicity, toxin production, insertional mutagenesis, and constraints in transgenic capacity size [41][42]. Recent advancements have led to the design of viral vectors with specific receptors for the transfer of transgenes to non-natural target cells, a concept known as retargeting [41].

Retroviral vectors are commonly used for gene delivery in both somatic and germline gene therapies due to their ability to transfect dividing cells by passing through the nuclear pores of mitotic cells [43]. This feature renders retroviruses suitable candidates for in situ treatments, distinguishing them from adenoviral and lentiviral vectors [41]. These viruses integrate with the host genome, allowing the production of viral proteins, such as gag, pol, and env, that are extracted during gene delivery [44]. The coordinated design of packaging cell lines and retroviral expression vectors is the foundation of retroviral gene transfer technology, which makes it easier to package recombinant retroviral RNAs into infectious, replication-incompetent particles [45].

Lentiviruses, a subclass of retroviruses, have emerged as significant gene delivery vectors, distinguished by their unique capacity to naturally integrate with nondividing cells [46]. This characteristic sets them apart from other retroviruses, which can solely infect dividing cells [47]. The strong tropism of lentiviruses for neural stem cells has rendered them invaluable for ex vivo gene transfer in the central nervous system, exhibiting no significant immune responses or unwanted side effects [48][49]. These vectors offer several advantages, including the high-efficiency infection of both dividing and nondividing cells, long-term stable expression of a transgene, low immunogenicity, and the ability to accommodate larger transgenes [41][50]. Due to their ability to integrate the transgene into the host genome and enable long-term expression, lentiviral vectors have remained a compelling option for clinical gene delivery [51].

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4. Advantages and Challenges of Replicon RNA Therapy

The application of nucleic acid-based therapy offers promise in addressing diseases that traditional treatments struggle to manage [58][59]. Self-replicating RNA, known as replicon RNA (RepRNA), represents a novel frontier in gene therapy [58][60]. These RepRNA molecules, derived from RNA viruses with specific gene deletions, can amplify all encoded genetic information, resulting in high antigen expression levels [58]. This sets RepRNA apart from plasmid DNA and conventional mRNA therapies [60].

mRNA therapy, another breakthrough, leverages nanoparticles as intracellular delivery systems for normal genes [59][61]. These synthetically manipulated mRNAs can replace missing or defective genes and express transient proteins to correct genetic disorders and promote beneficial mechanisms or pathways, ultimately aiming to cure or treat pathologies [16][62].

repRNA's favorable characteristics are evident in its therapeutic potential [60]. RepRNA-based vaccines have proven safe and potent in clinical trials against various infectious diseases and cancers [63]. RepRNA serves as a template for increased RNA molecule translation, leading to more rounds of antigen production [64]. Unlike DNA, which requires entry into the cell nucleus, both mRNA and RepRNA perform protein translation in the cytoplasm, reducing the risk of gene interactions [60].

In the comprehensive exploration of nanoformulations integrated with repRNA technology, a multifaceted approach unfolds. The synergy between various nanoformulations and repRNA is visually depicted in **Figure 1**a, illustrating the diverse forms of association that empower this innovative therapeutic approach. From liposomes to polymeric nanoparticles, this mix enhances the pharmacological potential of repRNA. These nanoformulations also delve into the realm of immunomodulation, as illustrated in **Figure 1**b. They possess the remarkable capacity to either

stimulate or suppress the immune response, opening the door to a range of therapeutic applications. Whether due to their potential for immunostimulation to combat infections or employing them for immunodepression in autoimmune disorders, the versatility of nanoformulations associated with repRNA holds immense promise in shaping the future of medical treatments [65][66].

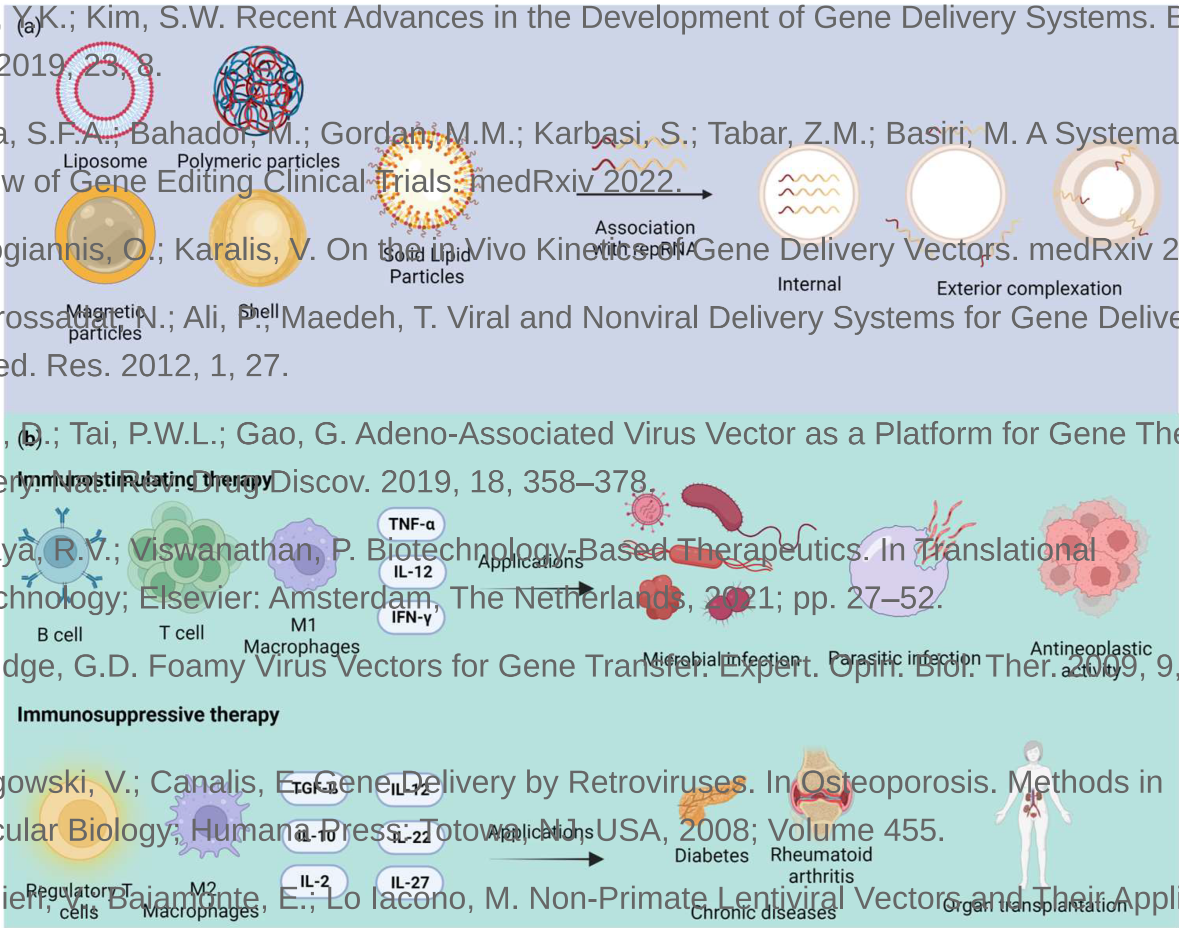

Figure 1. Overview of the use of nanoformulations associated with repRNA: (a) examples of nanoformulations and forms of association with repRNA; (b) immunomodulatory potential of nanoparticles associated with repRNA and therapeutic application, either by immunostimulation or immunodepression. The information contained in this illustration was based on the work of Blakney [65] and Feng et al. [66]. Created with BioRender.com (accessed on 2 October 2023).

The RepRNA production system is based on the loading of nanoparticles with RepRNA, followed by the efficient uptake of this formulation by target cells. These nanoparticles have a protective role against RNAses and degradation processes, ensuring the integrity of the encapsulated RNA [67]. Once inside the cellular cytoplasm, the nanoparticles underwent controlled disintegration, facilitating the amplification of RNA and a subsequent increase in the number of copies and protein expression [67]. This orchestrated process ultimately led to the presentation of RNA-derived antigens to the major histocompatibility complex (MHC), eliciting a robust immune response and highlighting the potential of nanoparticle-based RNA delivery systems.

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Furthermore, RepRNA production is considered simpler and more cost-effective than mRNA production, as it generates superior immune responses with significantly lower RNA doses due to the presence of RNA replicons [60]. However, despite these advantages, RepRNA therapy still faces major challenges [1][16].

The large molecular weight of RepRNA presents a significant obstacle to efficient cytosolic delivery, particularly when compared to smaller mRNA molecules [8]. Additionally, its susceptibility to ribonucleases (RNases) and the ineffective translation process within dendritic cells (DCs) [7] Consequently, one of the primary hurdles to expanding the extensive potential of RepRNA vaccines lies in the imperative requirement for safe and effective in vivo delivery strategies [8].

These limitations underscore the critical need for innovative strategies to mitigate the impact of these obstacles and enhance the overall efficacy of RepRNA vaccines.

One significant challenge lies in the delivery of RNA, given its negative charge and large molecular size, making it difficult to traverse the anionic lipid bilayer of cells [58]. Additionally, its single-stranded nature renders it fragile and susceptible to degradation by RNase enzymes, hindering its intracellular entry and limiting its applications in gene therapy [68]. Recent scientific and technological advances have made strides in overcoming these RNA-associated obstacles, leading to the development of efficient delivery systems that protect RNA from degradation, break through cell membranes, and deliver RNA into the cytoplasm [69].

Another challenge is the route of administration, primarily intravenous (i.v.) delivery, which targets highly vascularized organs like the liver or spleen to achieve therapeutic protein expression in a sufficient number of transfected cells [70]. However, this approach requires prolonged administration times and results in high treatment costs, often necessitating hospitalization or monitoring in infusion centers. Therefore, researchers have increasingly explored subcutaneous or intramuscular applications to address these limitations [63].

In recent years, RepRNA has gained attention as a potential therapy for COVID-19, and its success has stimulated interest in its application for various other pathologies (**Table 2**).

Table 2. Application of RepRNA associated with nanocarriers for therapy of pathologies.

Carrier	RNA Replicon	Results	Reference
Cationic nanocarrier	RepRNA PMIF (macrophage migration inhibitory factor and cytokine).	It improved host cellular and humoral immunity against Plasmodium infection in the liver and blood and conferred complete protection against malaria reinfection in murine mice.	[71]

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Carrier	RNA Replicon	Results	Reference
Nanostructured lipid transporters (NLCs)	RepRNA ZIKV-117 mAb.	Rapid protection against Zika virus infection in mice.	[72]
Lipid InOrganic Nanoparticles (LION)	LION/repRNA-CoV2S	LION/repRNA-CoV2S vaccine intramuscularly to mice, a significant amount of anti-SARS-CoV-2 S protein IgG antibody isotypes, resembling a Type 1 T helper cell response, were produced.	[63]
Cationic nanocarrier	RepRNA CCHFV (Crimean–Congo hemorrhagic fever virus) encoding NP (nucleoprotein), GPC (glycoprotein precursor) or both	It provided robust protection against Crimean–Congo hemorrhagic fever virus in lethal mice.	[73]
Cationic liposomes	samPfRH5 replicon (*Plasmodium falciparum* reticulocyte binding protein homologue 5)	The liposome–replicon complexes showed high transfection efficiencies. They elicited antibodies capable of inhibiting the growth of the parasite in vitro	[74]
Mannosylation of lipid nanoparticles (LNPs)	Self-amplifying mRNA encoded an influenza (hemagglutinin)	Compared to LNPs, mannnosylated lipid nanoparticles (MLNPs) showed higher levels of IgG1 and IgG2a.	[75]
Polymeric nanoparticle	Nanoparticle (MDNP)-delivered VEEV replicon RNA encoding the hemagglutinin protein (HA) of an H1N1 influenza virus (A/WSN/33) or the Ebola virus (EBOV) glycoprotein (GP)	The vaccine elicits both CD8+ T-cell and antibody responses and can be created with numerous antigen-expressing replicons.	[76]

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through [illegible] RNA replicon-based vaccine. A cationic nanoemulsion was prepared by combining squalene, DOTAP, and sorbitan trioleate, and it was characterized for particle size, RNase protection, and endotoxin, as previously described [71][78]. The complexation process was allowed to continue for at least 30 min before immunization [71]. Intriguingly, the study observed a delay in the onset of blood-stage patency following sporozoite infection [71]. Furthermore, vaccination against PMIF resulted in notable improvements in T follicular helper (Tfh) cell and germinal center responses, a reduction in the expression of Th1-associated inflammatory markers such as TNF-α, IL-12, and IFN-γ during the blood-stage infection, elevated anti-*Plasmodium* antibody levels, and the enhanced differentiation of antigen-experienced memory CD4 T cells and liver-resident CD8 T cells [71].

To facilitate the delivery of an alphavirus replicon encoding the highly neutralizing human monoclonal antibody (mAb) ZIKV-117, Erasmus et al. (2020) engineered a nanostructured lipid carrier (NLC). These NLC nanoparticles exhibit exceptional colloidal stability attributed to their hybrid core composed of liquid squalene and solid glyceryl trimyristate (Dynasan 114) [79]. Their studies have demonstrated robust protection, both as pre-exposure prophylaxis and post-exposure therapy, following alphavirus-driven expression of ZIKV-117 mRNA delivered via intramuscular injection (IM) [72]. The nanoparticle formulation was manufactured using a specific composition [72][79]. The oil phase was composed of squalene and glyceryl trimyristate, along with a non-ionic sorbitan ester surfactant, and the cationic lipid DOTAP. The aqueous phase consisted of a 10 mM sodium citrate trihydrate buffer containing the non-ionic PEGylated surfactant Tween 80. Additionally, the researchers successfully optimized the expression of human IgG from repRNA, enabling the production of protective levels in mice [72].

Furthermore, the same research group unveiled the LION/repRNA-CoV2S vaccine, exhibiting substantial neutralization of SARS-CoV-2 and inducing potent S-specific T cell responses in vaccinated mice and pigtail macaques [63]. According to Erasmus et al. (2020) LION is a highly stable cationic squalene emulsion containing 15 nm superparamagnetic iron oxide (Fe_3O_4) nanoparticles (SPIO) incorporated in the hydrophobic oil phase. This vaccine’s ability to stimulate memory T cell responses specific to SARS-CoV-2 in macaques may contribute to long-term protection against and recovery from SARS-CoV-2 infection [63].

Recent findings by Leventhal et al. (2022) have significantly improved the understanding of how vaccines against the Crimean–Congo hemorrhagic fever virus (CCHFV) provide protection. Vaccine RNA was synthesized in vitro and subsequently complexed to cationic nanocarrier, following established protocols as described previously by Erasmus et al. (2020). Their research has shed light on the involvement of humoral and cellular immunity in repRNA-mediated protection. Importantly, administering RNA through straightforward intramuscular vaccinations, particularly with the use of cationic emulsions, has emerged as an essential strategy for enhancing RNA stability and transport [73].

In the study conducted by Fotoran et al., tests were managed on a self-amplifying repRNA (repRNA) vaccine formulation, which was based on the SP6 Venezuelan equine encephalitis (VEE) vector incorporated into cationic liposomes. They generated three vaccines that encoded two reporter genes (GFP and nanoLuc) and the *Plasmodium falciparum* reticulocyte binding protein homologue 5 (PfRH5). The liposome–replicon complexes

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exhibited high transfection efficiencies and elicited antibodies capable of inhibiting the growth of the parasite in vitro [74].

To find out how mannans of different lengths (from mono to tetrasaccharide) affected the antibody response of a model repRNA replicon that encodes the respiratory syncytial virus fusion F protein, a new set of LNPs with changed surfaces was created by Goswami et al. (2021). As the mannose chain length increased, the vaccination priming response showed a steady improvement; nevertheless, the response to the booster dose peaked at a length greater than the disaccharide. Mannosylated lipid nanoparticles (MLNPs) were shown to exhibit higher amounts of IgG1 and IgG2a in comparison to LNPs. The potential of mannosylated samRNA LNPs for intramuscular and intradermal distribution is confirmed by this study [75].

Chahala et al. (2016) created a single-dose, completely synthetic, adjuvant-free dendrimer nanoparticle vaccination platform with a fast response, and encapsulated mRNA replicons that encode antigens. Protective immunity against a wide range of deadly pathogen threats, including as the Ebola virus, *Toxoplasma gondii*, and H1N1 influenza, can be produced via this mechanism [76]. The vaccine possesses the ability to induce both CD8+ T-cell and antibody responses and may be designed with various antigen-expressing replicons [76].