Self-amplifying RNA vaccines for infectious diseases

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Abstract

Vaccinology is shifting toward synthetic RNA platforms which allow for rapid, scalable, and cell-free manufacturing of prophylactic and therapeutic vaccines. The simple development pipeline is based on in vitro transcription of antigen-encoding sequences or immunotherapies as synthetic RNA transcripts, which are then formulated for delivery. This approach may enable a quicker response to emerging disease outbreaks, as is evident from the swift pursuit of RNA vaccine candidates for the global SARS-CoV-2 pandemic. Both conventional and self-amplifying RNAs have shown protective immunization in preclinical studies against multiple infectious diseases including influenza, RSV, Rabies, Ebola, and HIV-1. Self-amplifying RNAs have shown enhanced antigen expression at lower doses compared to conventional mRNA, suggesting this technology may improve immunization. This review will explore how self-amplifying RNAs are emerging as important vaccine candidates for infectious diseases, the advantages of synthetic manufacturing approaches, and their potential for preventing and treating chronic infections.

Introduction

Since the 18th century vaccines have been developed to prevent and eliminate the spread of infectious diseases [1]. They form the foundation of global public health programs and have major socioeconomic benefits [2]. Prophylactic immunization offers pre-exposure protection and supports development of herd immunity. Therapeutic vaccination, a form of immunomodulation, assists in the treatment of infectious diseases and cancers. The longstanding clinical value of currently licensed vaccines continues to encourage further research on novel vaccination approaches. These are aimed at improving prophylactic and therapeutic efficacy, developing new technologies, streamlining manufacturing processes, and enabling a rapid response to emerging infectious diseases. Nucleic acid vaccines represent one such approach where synthetic sequences are used to express antigenic peptides or proteins in situ [3]. Genetic immunization may promote superior adaptive immunity by activating both humoral and cell-mediated responses, and

has manufacturing advantages over traditional vaccines. Initial studies focused heavily on developing DNA rather than RNA candidates [4], because there were concerns about stability and large-scale production of RNA-based therapeutics. However DNA vaccines have generally performed poorly in human clinical trials [5], which has led to a renewed interest in RNA vaccinology for infectious diseases. To some extent this shift has been attributed to successes of cancer immunotherapy research. The transient nature and cytosolic location of RNA improves the safety profile of these nucleic acid vaccines. This is in contrast to their more stable DNA counterparts, which require nuclear delivery, promoter-driven expression, and risk integration within the host genome. These properties have led to major investments in RNA-based therapeutics over the past few years [6].

There are currently two different types of synthetic RNA vaccines: conventional mRNA and self-amplifying RNA (saRNA) (Fig. 1). Use of conventional mRNA strategies (also referred to as nonreplicating or non-amplifying mRNA) against infectious diseases and cancers has been investigated in several preclinical and clinical trials [7]. In vitro transcribed mRNAs encoding viral antigens have been explored as vaccines, while those encoding therapeutic proteins, such as antibodies or immune modulators, have been considered for immunotherapy. The incorporation of chemically modified nucleotides, sequence optimization, and different purification strategies improve efficiency of

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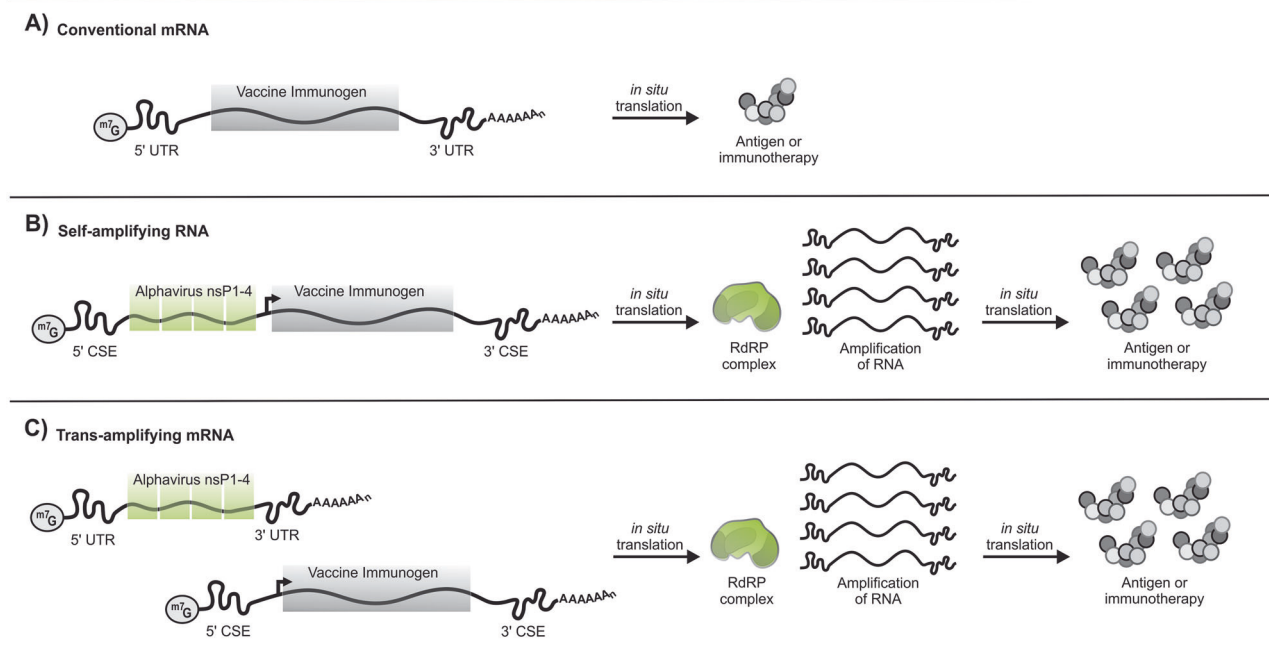
Fig. 1 Conventional, self-amplifying, and trans-amplifying RNA vaccine designs. A 5′ cap (m7G) and poly A tail are common to all RNA transcripts. **A** Conventional mRNAs encode the vaccine immunogen and flanking 5′ and 3′ UTRs. An antigen or immunotherapy is translated from the nonreplicating transcript. **B** Self-amplifying RNA encodes 5′ and 3′ CSE sequences, the nsP1-4 genes, a subgenomic promoter, and the vaccine immunogen. Following in situ translation, the nsP1-4 proteins form an RdRP complex which recognizes flanking CSE sequences and amplifies vaccine-encoding transcripts. This results in an accumulation of the antigen or immunotherapy within the cell. **C** Trans-amplifying mRNAs use two different transcripts to achieve a similar effect to self-amplifying RNAs. A conventional mRNA encoding the nsP1-4 genes flanked by 5′ and 3′ UTRs is co-delivered with a separate transcript that encodes the viral CSE sequences, the subgenomic promoter, and the vaccine immunogen. In situ translation of the conventional mRNA results in the formation of the RdRP complex, which subsequently amplifies the vaccine-encoding transcript to result in the accumulation of the antigen or immunotherapy. UTR untranslated region, CSE conserved sequence elements, nsP1–4 nonstructural proteins 1–4, RdRP RNA-dependent RNA polymerase.

mRNA translation and reduce intrinsic immunogenic properties [7]. However, antigen expression is proportional to the number of conventional mRNA transcripts successfully delivered during vaccination. Achieving adequate expression for protection or immunomodulation may thus require large doses or repeat administrations. saRNA vaccines, which are genetically engineered replicons derived from self-replicating single-stranded RNA viruses [8, 9], address this limitation. They can be delivered as viral replicon particles (VRPs) with the saRNA packaged into the viral particle, or as a completely synthetic saRNA produced after in vitro transcription. To generate replication-defective VRPs, envelope proteins are provided in trans as defective helper constructs during production. Resulting VRPs therefore lack the ability to form infectious viral particles following a first infection, and only the RNA is capable of further amplification. VRPs may be derived from both positive-sense and negative-sense RNA viruses, however the latter are more complex and require reverse genetics to rescue the VRPs [10]. As with gene therapy, there are several issues associated with the use of viral vectors for vaccine development. These include immunogenicity of the vector itself, which can elicit an undesirable immune response and prevent subsequent booster administrations using the same vector [11]. Preexisting immunity to the viral vector can also render a vaccine ineffective. As with live-attenuated vaccines, replication-competent alphavirus vectors also pose the threat of viral reactivation [9]. To circumvent this, saRNA vaccines can be produced and delivered in a similar manner to conventional mRNA vaccines. Positive-sense alphavirus genomes that have been commonly used for saRNA vaccine design include the Venezuelan equine encephalitis virus (VEE), Sindbis virus (SINV), and Semliki forest virus (SFV) (Table 1). The alphavirus replicase genes encode an RNA-dependent RNA polymerase (RdRP) complex which amplifies synthetic transcripts in situ. The antigenic or therapeutic sequence is expressed at high levels as a separate entity and further proteolytic processing of the immunogen is not required. As a result of their self-replicative activity, saRNAs can be delivered at lower concentrations than conventional mRNA vaccines to achieve comparable antigen expression [12]. This review will explore the recent preclinical progress of prophylactic and therapeutic saRNA vaccine candidates,

Table 1 Clinical and preclinical synthetic saRNA vaccine studies for infectious diseases.

Infectious disease	Replicon	Immunogen	Delivery	Animal	Year (reference)
Clinical studies					
Rabies	–	Glycoprotein G	CNE	Human	2019 (NCT04062669)
COVID-19	VEE	Spike protein	LNP	Human	2020 (ISRCTN17072692)
Preclinical studies					
RSV	SFV	F glycoprotein	Naked	Mice[b]	2001 [80]
	VEE–SINV	F glycoprotein	LNP	Mice, rats[b]	2012 [81]
	VEE–SINV	F glycoprotein	CNE	Mice	2014 [68]
Influenza	SFV	NP	Naked	Mice	1994 [79]
	SFV	HA	Naked	Mice[b]	2001 [80]
	VEE–SINV	HA	LNP	Mice	2013 [14]
	CSFV	HA/NP	Chitosan NGA	Mice, rabbit	2014 [71]
	VEE–SINV	HA	CNE	Mice[b], ferret[b]	2015 [125]
	VEE–SINV	NP	LNP	Mice	2015 [126]
	VEE–SINV	M1/NP	LNP	Mice[b]	2016 [85]
	VEE	HA	MDNP	Mice[b]	2016 [127]
	CSFV	HA/NP	CPP PEI	Pigs	2017 [128]
	CSFV	NP	Cationic lipid	Mice	2018 [129]
	–	HA	PEI	Mice[b]	2018 [12]
	VEE	HA	Neutral LPP	Mice	2019 [55]
	–	HA	MLNP	Mice	2019 [54]
	Trans-amplifying	HA	Naked	Mice[b]	2020 [62]
	VEE	HA	pABOL	Mice[b]	2020 [50]
Coronavirus	VEE	Spike protein	LNP	Mice	2020 [86]
LIV	SFV	prM-E	Naked	Mice[b]	2001 [80]
TBEV	TBEV	Δ TBEV capsid	Gene gun	Mice[b]	2004 [130]
	TBEV	Δ TBEV capsid	Gene gun	Mice[b]	2005 [131]
HIV	VEE–SINV	Env	LNP	Mice	2012 [81]
	VEE–SINV	Env	Electroporation	Mice	2013 [132]
	VEE–SINV	Env	CNE	Rabbit	2014 [68]
	VEE–SINV	Env	CNE	NHP	2015 [121]
	SFV	Gag/Pol mosaic	PEI	Mice	2019 [123]
	VEE	eOD-GT8	LNP	Mice	2019 [120]
	VEE	Env	Exterior LNP	Mice	2019 [58]
CMV	VEE–SINV	gB/pp65-IE1	CNE	NHP	2014 [68]
Ebola	VEE	Glycoprotein	MDNP	Mice[b]	2016 [127]
Toxoplasma gondii	VEE	Multimer[a]	MDNP	Mice[b]	2016 [127]
	SFV	NTPase-II	LNP	Mice[b]	2017 [133]
GAS	VEE–SINV	SLOdm	CNE	Mice[b]	2017 [134]
GBS	VEE–SINV	BP-2a	CNE	Mice[b]	2017 [134]
Zika	VEE	prM-E	MDNP	Mice	2017 [91]
	VEE	prM-E	NLC	Mice[b], guinea pigs	2018 [90]
	VEE	prM-E	Naked	Mice[b]	2019 [89]
VEE	VEE	Attenuated VEE	CNE	Mice[b]	2019 [88]
Rabies	VEE–SINV	Glycoprotein G	CNE	Rats	2020 [92]
	VEE–SINV	Glycoprotein G	Liposome, nanoparticle, CNE	Mice	2020 [59]

BP-2a GBS pilus 2a backbone protein, *CMV* cytomegalovirus, *CSFV* classical swine fever virus, *CNE* cationic nanoemulsion, *Env* envelope, *GAS* group A streptococci, *GBS* group B streptococci, *gB* glycoprotein B, *HA* haemagglutinin, *HIV* human immunodeficiency virus, *LIV* louping ill virus, *LNP* lipid nanoparticle, *LPP* lipopolyplexes, *M1* matrix protein 1, *MLNP* manosylated LNP, *MDNP* modified dendrimer nanoparticle, *NGA* nanogel alginate, *NHP* nonhuman primate, *NLC* nanostructured lipid carrier, *NP* nucleoprotein, *pABOL* poly(CBA-co-4-amino-1-butanol (ABOL)), *PEI* polyethylenimine, *Pol* polymerase, *prM-E* premembrane and envelope glycoproteins, *RSV* respiratory syncytial virus, *SFV* Semliki forest virus, *SINV* Sindbis virus, *SLOdm* double-mutated GAS Streptolysin-O, *TBEV* tick-borne encephalitis virus, *VEE* Venezuelan equine encephalitis virus, *VEE–SINV* alphavirus chimera based on the VEE and SINV replicons.

[a]Multimer comprised of granule protein 6 (GRA6), rhoptry protein 2A (ROP2A), rhoptry protein 18 (ROP18), surface antigen 1 (SAG1), surface antigen 2A (SAG2A), and apical membrane antigen 1 (AMA1).

[b]Vaccination conferred protection.

particularly for chronic infectious diseases such as the human immunodeficiency virus 1 (HIV-1), and emphasize their potential for innovative vaccine development.

Producing RNA vaccines

The need for rapid vaccine development in response to emerging pathogens has become devastatingly clear during the SARS-CoV-2 pandemic. A major caveat of live-attenuated, inactivated, toxin, or subunit vaccine manufacturing is the requirement for intricate cell culture technologies. These need dedicated facilities to produce individual vaccines as well as lengthy safety assessments to exclude risks posed by biological contaminants. In comparison RNA vaccine production is simple, can be easily adapted to accommodate new candidates within an established manufacturing pipeline, and is cost effective [13]. The in vitro transcription reaction used to produce both conventional mRNA and saRNA vaccines is cell-free and Good Manufacturing Practice-compliant reagents are available, facilitating quick turnaround times. This has been illustrated by Hekele et al. who produced a lipid nanoparticle (LNP) formulated saRNA vaccine for H7N9 influenza in 8 days [14]. Prompt RNA therapeutic manufacturing capabilities have more recently been revealed amidst the COVID-19 pandemic. The first SARS-CoV-2 vaccine to enter phase 1 clinical trials is the LNP-encapsulated mRNA-1273 developed by Moderna and the Vaccine Research Center at the National Institute of Health (ClinicalTrials.gov—NCT04283461) [15, 16]. Impressively it took only 25 days to manufacture the first clinical batch which commenced testing on the 16th of March 2020. With LNP mRNA-1273 receiving fast-track designation to phase 3 (NCT04470427), the efficiency of the vaccine as well as the capacity of the manufacturing pipeline will be tested.

Conventional and synthetic saRNA vaccines are essentially produced in the same manner [13, 17, 18]. Briefly, an mRNA expression plasmid (pDNA) encoding a DNA-dependent RNA polymerase promoter (typically derived from the T7, T3, or SP6 bacteriophages) and the RNA vaccine candidate is designed as a template for in vitro transcription. The flexibility of gene synthesis platforms is a key advantage. For conventional mRNA vaccines the antigenic or immunomodulatory sequence is flanked by 5′ and 3′ untranslated regions (UTRs). A poly(A) tail can either be incorporated from the 3′ end of the pDNA template, or added enzymatically after in vitro transcription [19]. saRNA vaccine pDNA templates contain additional alphavirus replicon genes and conserved sequence elements (Fig. 1). The nonstructural proteins 1, 2, 3, and 4 (nsP1-4) are essential for replicon activity as they form the RdRP complex [20]. In vitro transcription is performed on the linear pDNA template, typically with a T7 DNA-dependent RNA

polymerase, resulting in multiple copies of the RNA transcript. After the RNA is capped at the 5′ end and purified, it is ready for formulation and delivery.

Refining saRNA pharmacokinetics

Substantial effort has gone into understanding and improving RNA production, stability, translation, and pharmacokinetics. Revising the 5′ cap structure, controlling the length of the poly(A) tail, including modified nucleotides, codon or sequence optimization, as well as altering the 5′ and 3′ UTRs are just some of the factors under consideration (recently reviewed in [21]). Balancing the intrinsic and extrinsic immunogenic properties of the synthetic RNA, the vaccine antigen, and delivery formulation are equally important for longer saRNA transcripts. As the field of synthetic RNA vaccinology is still relatively new it is difficult to decipher which technologies are indispensable. Some studies show that incorporating various pseudouridine-modified nucleotides during transcription enhanced translation and reduced RNA-associated immunogenicity [22, 23], whilst others show no discernible advantage of such modifications [24, 25]. As saRNAs use host-cell factors for mRNA replication, the addition of modified nucleotides may prove less valuable as they would be lost during amplification [26]. One practical approach to improving translation of saRNA vaccines is through optimization of 5′ and 3′ UTRs which is based on the evolution of naturally occurring alphaviruses [27]. The single-stranded RNA genome forms a variety of secondary structures to allow alphaviruses to bypass requirements of normal host-cell translation processes [28, 29] and evade immune responses [30–32]. Revising the sequence encoding the nsP1-4 replicon genes may also prove beneficial. An in vitro evolution strategy using interferon- (IFN-) competent cells was adopted by Li et al. to identify mutations within the VEE nonstructural proteins that improve in situ expression of the subgenomic RNA [33].

Five-prime Caps

A well-researched approach to protecting in vitro transcribed RNAs from nuclease digestion and augmenting translation is modification of the synthetic 5′ cap structure. The anti-reverse cap analog [34] and recent phosphorothioate derivatives [35] have been shown to improve in situ translation of RNAs by only capping transcripts in the forward orientation. Post-transcription capping enzymes derived from the vaccinia virus [36, 37] have high capping efficiencies, and when combined with 2′-O-methyltransferases, generate Cap 1 structures that mimic natural eukaryotic mRNAs [38]. To further improve co-transcriptional capping, TriLink BioTechnologies has

recently developed the CleanCap™ system [39]. This technology can achieve Cap 1, Cap 2, and Cap 1 epitranscriptomic derivatives such as m^6A_m, which may further enhance RNA stability by evading de-capping metalloenzymes encoded by *DCP2* [40, 41]. Interestingly the CleanCap Reagent AU has specifically been designed to generate Cap 1 structures on saRNA transcripts derived from positive-sense alphaviruses. Cap analogs play an important role in evading cells' innate immune response as uncapped and Cap 0 RNAs may stimulate IFN responses. However, certain alphaviruses bypass the necessity for 2′-O-methylated caps [30], suggesting that careful design of the 5′ UTRs of may negate Cap 0 immunity.

Synthetic RNA immunogenicity

Stimulation of the innate immune system by in vitro transcribed saRNA vaccines is a complex topic which still requires empirical study. Immunization should ideally elicit an antigen-specific immune response, however it is currently unclear whether peripheral innate immune activation from in vitro transcribed RNAs enhances or compromises this response. Improving the in vitro transcription reaction [42, 43] and liquid chromatography purification [44] may reduce unintended immune stimulation by spurious by-products, including double-stranded RNAs (dsRNAs). For saRNA vaccines, the immunogenic benefits of purification may be less significant than for conventional mRNAs. Although purification may remove nonspecific dsRNAs, the formation of new dsRNA intermediates is inevitable during self-amplification. The co-delivery of immune modulating transcripts, particularly pattern recognition receptors (PRRs) antagonists, may help mitigate these effects. Conventional mRNAs encoding the E3, K3, and B18/B18R vaccinia virus immune evasion proteins, or the influenza A virus nonstructural protein 1 (NS1) have shown potential in this regard [45–47]. Beissert et al. augmented saRNA reporter gene expression in vitro and in vivo by combining conventional mRNAs encoding E3, K3, and B18, to reduce protein kinase R and IFN signaling in a synergistic manner [45].

Delivering large saRNA transcripts

Choice of delivery formulations, the inoculation site, and adjuvants are additional immunological considerations [7]. Nonviral formulations including cationic lipids, LNPs, polymers, and protamine sulfate, as well as physical methods such as electroporation may be used to deliver RNA therapeutics and vaccines [48, 49]. The inclusion of the nsP1-4 replicon sequence in saRNAs makes them much longer than their conventional counterparts which is important for formulation. Over the past year several new approaches aimed at improving the delivery of saRNAs have been described. Novel bioreducible polymer formulations using high molecular weight poly(CBA-co-4-amino-1-butanol) (pABOL) [50], ornithine dendrimers [51], mannosylated polyethyleneimine [52], or multiple linear peptides [53], may help facilitate intracellular trafficking and endosomal release. Blakney et al. demonstrated the preclinical efficacy of their 8 kDa pABOL polymer formulation to deliver haemagglutinin- (HA-) encoding saRNAs in mice [50]. Modifications to LNP formulations have also been described, where incorporating a mannose-cholesterol amine conjugate into LNPs improved intradermal (ID) immunization [54]. Manosylation of polyethyleneimine improved delivery of saRNA reporter constructs to human skin explants [52]. Intramuscular (IM) delivery of saRNAs as neutral lipopolyplexes (LPPs) resulted in an increase in antigen-specific T cells with a concurrent loss of antigen-expressing cells [55]. These LPPs were generated by creating core RNA/polyethylenimine polyplexes before encapsulation in PEG-anionic liposome formulations with mannosylated lipids. Inclusion of mannose is aimed at improving vaccine delivery to antigen presenting cells [56, 57]. Interestingly, saRNAs may not require conventional encapsulation to protect them from RNAse degradation. Complexing saRNAs on the exterior of positively charged LNPs formulated with dimethyldioctadecylammonium (DAA) cationic lipids conferred complete protection from direct RNaseA treatment [58]. Mice that were immunized with an HIV-1 Envelope (Env) saRNA complexed to the surface of DDA LNPs developed peak antibody titers following a single injection. When delivered as an encapsulated LNP, a boost injection was required to achieve equivalent antibody titres [58].

With so many different nonviral formulations available, Anderluzzi et al. sought to compare liposomes, solid LNPs, polymeric nanoparticles, and emulsions for the delivery of an saRNA vaccine-encoding the rabies virus glycoprotein [59]. In their hands, low-dose saRNA nanoparticles complexed with the non-ionizable cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) initially resulted in similar antibody titres to Rabipur (RabAvert), a commercially licensed rabies vaccine. However, the proprietary cationic nanoemulsion (CNE) 56 from GlaxoSmithKline (GSK, Rockville, MD, USA) outperformed all DDA and DOTAP formulations [59]. Comparing delivery approaches in side-by-side studies such as this would assist in determining the usefulness of each formulation. Reducing the size of the saRNA vaccine transcript by employing a trans-amplifying approach may also address concerns about inefficient delivery. Here the saRNA is split into two transcripts, the first encoding the nsP1-4 replicon complex and the second encoding the gene of interest as a "transreplicon"

(Fig. 1) [60–62]. This approach was recently adopted by Beissert et al. [62] who generated an influenza vaccine transreplicon based on a SFV saRNA design. The trans-amplifying strategy worked best when the replicon was delivered as a sequence-modified mRNA transcript and not as an saRNA. Mice that received ID injections were successfully immunized, produced functional virus-neutralizing antibodies, and were protected following influenza challenge [62].

Augmenting vaccine immunity

Vaccines traditionally incorporate adjuvants to boost adaptive immunity and shape T-cell responses [63]. Aluminum salts, emulsions, lipid analogs, and virosomes are a few examples of adjuvants that are currently included in licensed vaccines. The importance of such adjuvants in RNA vaccinology remains unclear and may be contingent on the types of modifications included during design and manufacturing. To date, clinical trials with conventional mRNA vaccines have shown limited humoral immunity. As saRNAs contain native alphavirus motifs and mimic viral translation in situ they have the propensity to enhance immunization through stimulation of PRRs [64]. Further efforts to augment or direct RNA vaccine-associated immunity have also been described. RNActive®, a protamine complexed mRNA [65, 66] and RNAdjuvant®, a proprietary immunostimulatory cationic peptide formulation [67] have been developed by CureVac AG to improve the efficacy of vaccines. saRNA CNEs incorporating the adjuvant MF59 (Novartis) have shown comparable immunological responses to a subunit vaccine comprising the same adjuvant [68]. Lipid-based delivery formulations are well established adjuvants [69] and have been adapted to promote RNA vaccine immuno-potentiation [70]. The inclusion of mannosylated conjugates [52, 54, 55] and chitosan-based nanogel alginate [71] may help shape the immune response by enhancing receptor-mediated endocytosis of saRNA vaccines by dendritic cells. Manara et al. recently established a chemokine-adjuvant strategy using a murine granulocyte-macrophage colony-stimulating factor saRNA in conjunction with their influenza saRNA vaccine [72]. Including cytokine or chemokine adjuvants that augment cytolytic T-cell responses would improve the therapeutic potential of RNA vaccines, particularly for chronic infectious diseases. Another study by Blakney et al. described cationic adjuvant formulations with PPR agonists for saRNA delivery [73]. Surprisingly, the incorporation of toll-like receptor (TLR) 7/8 agonists R848 and 3M-052 had little effect on innate immunity as the saRNA dominated the IFN response. Perche et al. [55] included the stimulator of interferon genes (STING) agonist, cyclic diadenylate monophosphate (c-di-AMP), to modulate their LPP

formulated saRNA influenza vaccine. This approach has also been used to improve the immunogenicity of cancer mRNA vaccines, where STING-activatable cyclic lipids were incorporated into LNPs [74].

Whether these adjuvant formulations improve the clinical potential of saRNA vaccines remains to be assessed. Pre-clinical studies indicate that both conventional and saRNAs elicit strong IFN responses that may be detrimental to vaccine efficacy [75, 76]. In this regard modifications to the RNA sequence, cap structure, or the inclusion of PRR antagonists may prove beneficial [77]. When using the VEE replicon architecture, Huysmans et al. showed that expression kinetics of saRNAs delivered by ID electroporation were superior to those of LNP-formulated saRNAs [78]. A rapid and strong innate immune response to the LNP saRNA had a negative effect on the translation and amplification of the bioluminescence reporter sequence. Strong IFN stimulation from both the saRNA and delivery formulation may impede in situ translation of the vaccine, ultimately preventing successful immunization. Extensive comparative and clinical studies will no doubt influence optimization of saRNA vaccine pharmacokinetics and shed light on which technologies are crucial for translating preclinical approaches into clinically relevant vaccines.

Expanding the scope of vaccines for infectious diseases

Licensed vaccines continue to have a major impact on global health as they reduce mortality associated with illnesses such as measles, mumps, pertussis, smallpox, and polio [2]. But for many longstanding and emerging infectious diseases, there is currently no prophylactic or therapeutic immunization available. The notion of using synthetic saRNAs as a vaccine was first described by Zhou et al. when they modified an SFV replicon to express the influenza nucleoprotein (NP) [79]. A few years later the same group described a similar approach to immunize mice against influenza, respiratory syncytial virus (RSV), or louping ill virus (LIV) [80]. These seminal studies demonstrated the potential of in vitro transcribed saRNA vaccines to elicit protective immune responses in mice following IM injection of unformulated RNA. More than a decade later Geall et al. [81] described the first LNP-formulated saRNA vaccine for RSV and HIV-1, using an alphavirus chimera based on the VEE and SINV replicons [82]. An ionizable cationic lipid was used to encapsulate the saRNA, which conferred protection from RNase degradation, and following IM injection increased immunogenicity when compared to an unformulated counterpart. Prophylactic protective immunity was achieved, which was equivalent to that of a VRP, and comparable to neutralizing titres required to protect

infants from infection [81]. Notably, this study preceded the vast array of NVV formulations now used to deliver saRNA vaccines in preclinical models (Table 1) (reviewed by [7, 26, 83]).

Contagious respiratory viruses

Influenza is a popular candidate for saRNA vaccine development and a diverse range of delivery approaches have been established to improve immunization (Table 1). As discussed earlier, recent formulations under preclinical evaluation include the cationic polymer-based pABOLs [50], Mannosylated LNPs (MLNPs) [54], and neutral LPPs [55]. Strong antibody responses were attained with low-dose (1 µg per primer injection) saRNAs complexed with pABOLs, and the addition of mannose improved IgG response times in LNP formulations [50, 54]. Interestingly the pABOLs conferred protection following IM but not ID injections. MLNPs achieved enhanced ID immunization resulting in superior T-cell responses when compared to a monovalent inactivated influenza vaccine. This may facilitate the delivery of saRNA vaccines using needle-free devices. An important consideration for seasonal influenza is how antigenic drift could render a vaccine ineffective [84]. saRNAs can accommodate multiple antigens in a flexible pDNA template [85] which could be modified to include new epitopes. With conventional mRNA vaccines entering phase 3 clinical trials for the highly contagious SARS-CoV-2, an saRNA vaccine candidate developed by researchers at Imperial College London and Acuitas Therapeutics has recently entered a UK-based phase 1/2 clinical trial (isrctn.com—ISRCTN17072692). Preclinical studies in mice showed that the LNP-nCoVsaRNA vaccine, which encodes the SARS-CoV-2 spike protein, achieved a dose-dependent immune response following IM injection [86]. Vaccination elicited antigen-specific IgG antibodies and T-helper type I cells, with the lowest dose (0.01 µg) achieving higher SARS-2 antibody titres than in recovered COVID-19 patients. Dose-dependent neutralization of pseudo- and wild-type SARS-CoV-2 was shown, again with the lowest dose attaining a higher neutralization efficiency than COVID-19 patient samples. According to a report from Imperial College London the vaccine took only 14 days to develop and will embark on larger clinical trials later this year [87].

Insect-transmitted viruses

Encouraging preclinical results for protection against mosquito-borne diseases have also recently been described. By creating live- and irreversibly attenuated (LAV and IAV) versions of the VEE replicon, Samsa et al. developed a CNE formulated saRNA vaccine for VEE [88]. The safer (reversion-deficient) IAV was less immunogenic than the LAV, but still protected 70% of mice following VEE aerosol challenge. Using the same replicon, Zhong et al. [89] endeavored to immunize mice against Zika virus using an unformulated saRNA, however not all immunocompetent mice developed neutralizing antibodies following ID injection. Protective cellular and humoral immune responses were only detected in type I IFN receptor double knockout mice (Ifnar$^{-/-}$), suggesting the self-adjuvant activity of naked saRNA compromised antigen-specific immunity. Previous Zika virus saRNA vaccine studies achieved protective immunity following IM injection of immunocompetent mice, but only when the RNA was formulated in a nanostructured lipid carrier [90]. Similarly, modified dendrimer nanoparticles [91] elicited antigen-specific IgG response in mice, highlighting the importance of formulation and injection site for vaccine efficacy.

Animal-transmitted virus

Rabies, another infectious disease with a licensed vaccine, has recently been the focus of GSK's vaccinology program (RG-SAM GSK3903133A) which boasts the only clinical trial for a synthetic saRNA to date (NCT04062669). Preclinical evaluations of CNE formulated saRNA encoding the glycoprotein G antigen, showed that the vaccine was well tolerated following multiple IM injections in rats [92]. The saRNA-derived rabies RNA was detected up to 2 months after a single injection, emphasizing the durability of the approach. A second study evaluated immunogenicity in mice using the licensed inactivated rabies virus vaccine Rabipur (RabAvert) as a benchmark for immunization [59]. Positive protective antibody responses similar to Rabipur were observed following a single IM injection of DOTAP nanoparticle-encapsulated or CNE56-absorbed saRNAs (0.5 and 1.5 µg). A second injection boosted antigen-specific IgG titres in all encapsulation formulations (DOTAP nanoparticles, DOTAP liposomes, and DDA liposomes) but only high-dose CNEs elicited responses comparable to the licensed vaccine [59]. The first clinical trial of GSKs CNE formulated RG-SAM vaccine (NCT04062669) will establish the safety of a 3-dose IM regimen and determine the immunogenicity of saRNAs in humans. It will be interesting to compare the results of this trial with those of the CureVac conventional mRNA Rabies vaccine currently under investigation. An initial phase 1 trial of the protamine-formulated conventional mRNA (CV7201) proved disappointing, but following modification and LNP formulation has re-entered clinical testing (CV7202; NCT03713086) [93]. These parallel investigations will help ascertain the clinical relevance of conventional and saRNA vaccines.

The promise of an HIV-1 vaccine

HIV-1 remains an incurable global health challenge and despite intensive studies an effective vaccine is not yet available. Since 1984 scientists have attempted to develop a prophylactic vaccine, however numerous clinical trials have been met with few successes [94]. Sequence diversity of the HIV-1 viral Env glycoprotein required for entry into cells has presented a challenge to vaccine design [95, 96]. It has proven difficult to elicit broadly neutralizing antibodies (bNAbs) that are able to neutralize several HIV-1 strains with conventional vaccines. Protein-based vaccines have been widely investigated, but with some disappointing results in clinical trials [97]. New strategies currently under investigation include novel antibody approaches [98–100], antibody-like inhibitors [101], polyvalent immunogens [102], and adeno-associated viral vectors [103]. VRPs are also popular HIV-1 vaccine candidates which have shown promise in nonhuman primates [104–106] and mice [107]. While these traditional platforms continue to strive toward clinical efficacy, renewed interest in RNA vaccinology has led to development of unique HIV-1 candidates which have the potential to provide both prophylactic and therapeutic efficacy. Conventional mRNAs have been investigated in preclinical and clinical studies [108–110] with saRNA approaches gaining interest over the past few years. Phase I and II clinical trials have demonstrated the safety and feasibility of mRNA vaccines [111–116], but inadequate immune responses have resulted in disappointing clinical efficacy. An RNA (AGS-004) encoding multiple HIV-1 proteins and the CD40 ligand (CD40L) was tested in phase I and IIB clinical trials [113–115], but the vaccine did not reduce viral load or prevent viral rebound. When combined with the latency-reversing agent vorinostat, no substantial impact on the frequency of resting T-cell infection was observed [117]. A naked mRNA (iHIVRNA) encoding HIV-1 immunogens and the dendritic cell activator TriMix (CD40L, CD70, and caTLR4) was safe in a phase I trial, but did not show sufficient immunogenicity in a phase II trial which was recently terminated (NCT02888756) [118, 119].

The disproportion between preclinical and clinical success reinforces the need for strategies that improve RNA vaccine immunogenicity or immunotherapy in humans. The self-adjuvant effect of saRNAs may augment immunity and novel HIV-1 candidates have shown potential in preclinical models of infection (Table 1). In 2012, to highlight the flexibility of their LNP-formulated replicon, Geall et al. [81] described the first HIV-1 Env gp140 saRNA vaccine in mice. Env-specific immune responses were detected following multiple delivery routes (IM, ID, and subcutaneous), with IM injections achieving superior CD8$^+$ T-cell responses. More recently, an saRNA encoding the HIV-1 Env gp140 protein was formulated on the interior or exterior of LNPs and administered to mice by IM injection, resulting in an antigen-specific IgG response [58]. Peak antibody titres were achieved after a single injection of the exterior formulated LNPs or a prime-boost of the encapsulated saRNA. LNPs have also been used to deliver an saRNA encoding a germline-targeting gp120 immunogen (eOD-GT8) designed to self-assemble into a 60-mer protein nanoparticle and prime B-cells capable of producing VRC01-like bNAbs [120]. Administration of this saRNA to transgenic mice expressing human inferred-germline B-cell receptors led to primed B-cell responses and somatic hypermutation characteristic of bNAb development. The efficacy and safety of MF59 CNE formulations was demonstrated in nonhuman primates where an saRNA vaccine encoding the HIV-1 gp140 Env protein was administered intramuscularly to rhesus macaques [121]. Higher levels of T-cell response, neutralizing antibodies and anti-envelope antibodies including V1V2 antibodies, which correlated with reduced risk of infection in the RV144 vaccine trial [122], were observed when compared to delivery using a corresponding VRP. An effective immune response was also achieved using a relatively low-dose (50 μg per primer injection) of the saRNA in the CNE formulation, thus demonstrating clinical utility. A PEI-based polymer formulation was recently used to deliver a mosaic vaccine-encoding six conserved regions of the gag and pol proteins in mice [123]. This vaccine induced plurifunctional CD4+ and CD8+ T cells at relatively high levels that persisted for up to 22 weeks post administration. These results are very promising, but also highlight the need for additional strategies to promote neutralization breadth and antibody durability for long-term prophylaxis or treatment of chronic HIV-1 infection. saRNAs should theoretically reduce the frequency or necessity for booster administrations, however, extensive clinical evaluations are still required.

Conclusions

Successful immunization programs have transformed our heath care systems by providing prophylactic protection against deadly infectious diseases. Pandemics such as those caused by SARS-CoV-2 emphasize not only the health, but also the social and economic impacts that a highly contagious virus can have on our everyday lives. Vaccine development and manufacturing strategies that allow rapid responses to such threats are currently limited. With many advantages, saRNA vaccines have the potential to fill this gap (Table 2). Simple cell-free manufacturing pipelines can easily be adapted to develop new vaccine candidates, ultimately streamlining production. The transient in situ translation of antigenic or immunotherapeutic sequences

Table 2 Advantages and disadvantages of saRNA-based vaccines.

Property	Advantage	Disadvantage
Efficacy	Comparable efficacy to traditional protein-based vaccines	Prime/boost administrations may be required
	High level of RNA amplification in situ	Little information is available on the effects of sustained, high-level amplification and expression of saRNAs
	saRNA activity occurs in the cytoplasm so nuclear import is not required as for DNA vaccines	
	Humoral and cellular responses are elicited against the expressed antigen	
	Protection against infection has been demonstrated in preclinical studies	
Safety	Viral genes for structural proteins are removed from the saRNA replicon to prevent viral assembly	Little information is available regarding immunogenicity of the RdRP complex
	Cytoplasmic mode of action, no danger of integration	Limited clinical data to date
Synthesis	Amenable to large-scale synthesis using GMP in vitro transcription	
	New sequences for different antigens can be synthesized easily	
	Flexibility to incorporate polyvalent or multipathogen sequences	
Delivery by NVVs	Can be delivered using nonviral vectors	Delivery is not typically tissue-specific
	Formulations are amenable to large-scale synthesis	Balancing immunogenicity of NVVs and saRNA
	Expression at delivery site following intramuscular, intradermal, or subcutaneous injection	

elicits humoral and cell-mediated immune responses as proteins can be presented by both major histocompatibility complexes (MHC I and MHC II). Immunopotent saRNAs amplify antigenic sequences as subgenomic transcripts and the accumulation of these immunomodulatory proteins in the cytoplasm may improve genetic immunization strategies, particularly for chronic infectious diseases. Preclinical studies confirm that saRNA vaccines establish antigen-specific immune responses against various infectious diseases and confer protection.

The RNA vaccinology field is constantly evolving as new studies aim to improve in vitro transcription, optimize adjuvant and delivery formulations, and ultimately refine in vivo pharmacokinetics. Yet with such a large variation in vaccination approaches and very few parallel comparisons, it is difficult to decipher which strategies are best. Establishing clinical efficacy is the next important step for synthetic saRNA vaccines as this will guide future design and production endeavors. It will also determine whether the preclinical trends are accurate, and if saRNAs are able to confer immunity at lower doses. Eliciting a strong adaptive immune response could lead to single-dose regimens and reduce the number of nonresponders. Comprehensive studies from a diverse population will help improve vaccine designs as environmental and genetic influences may affect immunization [124]. Balancing the innate immune response to enhance and not avert antigen-specific immunity will be central to clinical development. Establishing exactly how saRNA vaccines should be administered will assist in defining storage, distribution, and handling procedures. With the imminent launch of the first-in-human trials, the future of saRNA vaccinology is certainly exciting.

Compliance with ethical standards

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