Expert Review of Vaccines

ISSN: 1476-0584 (Print) 1744-8395 (Online) Journal homepage: https://www.tandfonline.com/loi/ierv20

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To cite this article: Carlo Iavarone, Derek T. O'hagan, Dong Yu, Nicolas F. Delahaye & Jeffrey B. Ulmer (2017) Mechanism of action of mRNA-based vaccines, Expert Review of Vaccines, 16:9, 871-881, DOI: 10.1080/14760584.2017.1355245

To link to this article: https://doi.org/10.1080/14760584.2017.1355245

Accepted author version posted online: 13 Jul 2017.
Published online: 28 Jul 2017.

EXPERT REVIEW OF VACCINES, 2017
VOL. 16, NO. 9, 871–881
https://doi.org/10.1080/14760584.2017.1355245

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REVIEW

Mechanism of action of mRNA-based vaccines

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ABSTRACT

Introduction: The present review summarizes the growing body of work defining the mechanisms of action of this exciting new vaccine technology that should allow rational approaches in the design of next generation mRNA vaccines.

Areas covered: Bio-distribution of mRNA, localization of antigen production, role of the innate immunity, priming of the adaptive immune response, route of administration and effects of mRNA delivery systems.

Expert commentary: In the last few years, the development of RNA vaccines had a fast growth, the rising number of proof will enable rational approaches to improving the effectiveness and safety of this modern class of medicine.

ARTICLE HISTORY
Received 10 March 2017
Accepted 11 July 2017

KEYWORDS
Intrinsic adjuvanticity; innate immunity; antiviral response; type I interferon; delivery system; optimization; self-amplifying mRNA (SAM)

1. Introduction

Live attenuated vaccines have had a substantial impact on human health. However, these vaccines have limitations, such as risk of reversion to virulence and complicated cell-based production processes, which hamper their use against certain pathogens. A more recent approach has been to use individual subunit antigens derived from the pathogen to focus immune responses only against the relevant targets. In general, though, subunit vaccines are less potent and require adjuvants to enhance immune responses. In addition, these vaccines are not effective at eliciting CD8T cell responses in humans, which are important for clearing infections and eradication of tumors. These limitations have provided the impetus to investigate other vaccine strategies [1]. Nucleic acid-based vaccines represent an attractive alternative to live attenuated and subunit-based vaccines, due to their capacity to triggering both antibody-mediated and cell-mediated immunity, as well as offering the potential for low-cost and simplified production processes [1].

During the 1990s, the genomic era triggered the expansion of the nucleic acid vaccination strategies; pioneers in this area were Wolff and colleagues [2] who demonstrated that injection of plasmid DNA (pDNA) or mRNA encoding for reporter genes resulted in local production of protein in myocytes. Subsequently, several reports demonstrated that the immunization with nucleic acids was able to elicit immune responses against the encoded antigens [3–6]. Most ensuing publications focused on the use of pDNA vaccines, but in 1996 Boczowski and coworkers [7] showed that murine dendritic cells (DCs) pulsed with *in vitro* transcribed ovalbumin (OVA) mRNA, delivered with N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP), were capable of presenting antigen *in vitro* as well as *in vivo*, thereby ushering a new cell-based approach to immunotherapy.

More recently, mRNA-based vaccines have been investigated extensively in animal models of infectious and noninfectious disease, and several are in clinical development. Like viral vectors and pDNA vaccines, mRNA-based vaccines can induce both humoral and cellular immunity, but may avoid some of their limitations such as anti-vector immunity and potential integration into the host cell genome. In addition, antigen expression after mRNA vaccination is transient, thereby avoiding T cell exhaustion that may occur with persistent antigen exposure [8]. Finally, RNA functions in the cytoplasm and does not need to enter the nucleus of target cells; hence the efficiency of functional cellular delivery of mRNA is likely to be higher [1].

Recent publications have provided insight on mRNA vaccines innovations [9,10], with particular attention to self-amplifying alphavirus RNA vaccines [11,12] and a broad overview on clinical development [13].

2. Main characteristics of mRNA vaccines

RNA vaccines can be divided into two types: conventional mRNA-based vaccines and self-amplifying mRNA vaccines (SAM) (Figure 1), both of which utilize the host cell translational machinery to produce the antigen target and launch an adaptive immune response [9,13]. Conventional mRNA vaccines are conceptually similar to host cell mRNA molecules and encode only the antigen of interest. In contrast, SAM vaccines encode an engineered RNA virus genome, such as those derived from an alphavirus single-stranded, positive-sense (+)RNA molecule, containing nonstructural proteins (nsPs) and an antigen cassette substituting the genes encoding for the structural proteins [14]. The resultant RNAs, termed replicons, express high levels of the antigen gene due to RNA template amplification in host cells. Since these replicons lack the viral structural protein genes, they are incapable of

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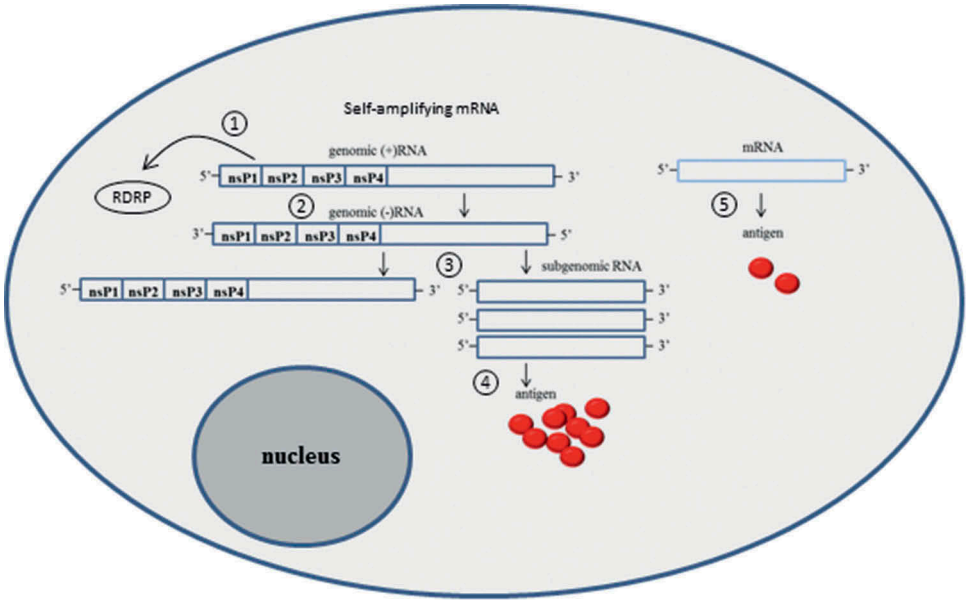

Figure 1. Schematic illustration of difference between self-amplifying RNA and 'conventional' mRNA translation.

After the cell delivery, self-amplifying RNA produce the antigen in four phases. 1) The ORF of the genomic (+) RNA encodes for the nonstructural proteins (nsP1, nsP2, nsP3, nsP4) that produce a RNA-dependent RNA-polymerase (RDRP) complex; 2) RDRP generates a genomic (-) strand; 3) RDRP generrates from the RNA (-) strand a genomic (+) strand and the subgenomic RNA; 4) the translation of the subgenomic RNA produces the antigen. 5) The conventional mRNA can directly express the antigen.

producing infectious virions and spreading to neighboring cells. The alphavirus-based replicon contains the sequences of two open reading frames (ORFs) flanked by untranslated regions (UTRs) at 5′- and 3′-ends. The ORF at 5′-end encodes for a viral polyprotein, which is proteolytically processed into four nsPs, and is translated from the genomic (+)RNA. The function of these individual nsPs of alphaviruses has recently been reviewed [15]. nsP1 is an enzyme which catalyzes reaction required for the viral RNA capping and represents the crucial element to settle the replication complex (RC) at the host membrane. nsP2 has both protease activity that cleaves the polyprotein into individual nsPs and helicase activity that unwinds RNA duplex during replication. The role of nsP3 is less defined but it is an essential component of the RC. Finally, nsP4, the RNA-dependent RNA polymerase, structures the RC and synthesizes complementary negative-sense (–)RNA intermediates and later positive-sense genomic and subgenomic (+)RNAs [14,15]. The second ORF is translated from the subgenomic RNA and expresses the antigen that replaces the viral structural proteins. The packaging of virus-like replicon particles (VRPs) can be made by cell cultures that co-express the viral structural proteins. Such replicons have demonstrated to be efficacious in animal models and immunogenic in clinical trials [12,16]. Alternatively, replicons can now also be delivered with synthetic delivery vehicles [9], which will be discussed in detail later.

In 1993, Martinon et al. [6] reported that mRNA expressing the influenza nucleoprotein, delivered with liposomes, elicited an antigen-specific cytotoxic T lymphocytes (CTL) response in mice. Both conventional and self-amplifying mRNA vaccines can prime adaptive immunity, including antibody-producing B cells, CD4+ and CD8+ T– cells, in homologous [9,11,17] as well as in heterologous modality (prime-boost) in combination with subunit vaccines [18–21]. Protective immunity based on functional antibodies [20,22–24] and polyfunctional CD4+ and CD8+ T cells that home to the lung after respiratory virus infection [25] have been demonstrated.

The effectiveness of RNA vaccines has been established in various animal models (Table 1) and in clinical trials (Table 2).

Table 1. Preclinical proof of principle with conventional non-amplifying mRNA (mRNA) or with self-amplifying mRNA (SAM) vaccines against human pathologies.

	Target	RNA	Vehicle	Antigen	Route	Host	Correlates	Ref.
Cancer	Colon carcinoma	mRNA	LPX	CT26-M90	iv	Mouse	APCs activation	[26]
	Melanoma	SAM	VRP	TRP2	sc	Mouse	T cells, survival	[27]
	prostate cancer	mRNA	protamine	PSMA	id	Mouse	IgG, T cells	[28,29]
Infectious disease	CMV	SAM	VRP	Pentamer	im	Mouse	Nab	[30]
	Dengue	SAM	VRP	E	fp	Mouse, macaque	Nab	[27,31]
	Ebola	SAM	VRP	G	sc	NHP	IgG, Nab	[32]
	HIV	SAM	CNE	gp140	im	NHP	IgG, Nab, T cells	[33]
	Influenza	SAM	CNE	HA	im	Mouse, ferret	IgG, Nab, T cells, protection	[34]
	Rabies	mRNA	Protamine	G	id	Mouse, pig	IgG, T cells, protection	[35]
	RSV	SAM	CNE	F	im	Mouse	IgG, Nab	[17]
	Zika	mRNA	LNP	prM-E	im	Mouse	IgG, Nab, protection	[23,24]

CNE, cationic nanoemulsion; LNP, lipid nanoparticle; VRP, virus-like replicon particle; LPX, lipoplex; fp, footpad; im, intramuscular; id, intradermal; iv, intravenous; sc, subcutaneous; NHP, non-human primates; APC, antigen presenting cell; CMV, cytomegalovirus; RSV, respiratory syncytial virus.

Table 2. Overview of clinical trials using RNA vaccine.

	Sponsor	Target	Status	Trial ID	Outcome	Ref.
Cancer	BioNTech	Melanoma	Complete	NCT01684241	nr	[36]
	BioNTech, Sanofi	Melanoma	Phase I	NCT02410733	Assessing safety and tolerability	[26]
	Curevac	Prostate cancer	Complete	NCT00831467	Failed to meet primary end point of improving overall survival[a]	[37]
	Curevac	NSCLC	Phase I	NCT01915524	Improved survival	[19]
	TübingenUniversity	Melanoma	Phase II	NCT00204607	MAAs immune responses	[38,39]
	TübingenUniversity	RCC	Phase I/II	–	Improved survivalNo side effects	[40,41]
Infectious disease	Curevac	Rabies	Phase I	NCT02238756	Assessing safety and tolerability	[35]
	Moderna	Flu	Phase I	NCT03076385	Acceptable tolerability, protective immunity	[42]
	Moderna	Zika	Phase II	NCT03014089	Assessing safety, tolerability, and immunogenicity	[24]

MAA, melanoma-associated antigen; NSCLC, non-small cell lung carcinoma; RCC, renal cell carcinoma; nr, non-reported; NCT, Clinical trial ID registered in ClinicalTrials.gov.
[a]Curevac clinical update at 35th Annual J.P. Morgan Healthcare Conference.

3. Yin and yang of innate immunity

3.1. The intrinsic adjuvant properties of RNA vaccines

The innate immune system consists of pattern-recognition receptors (PRRs), which function to detect pathogens (pathogen-associated molecular patterns, PAMPs). Interaction among PRRs and PAMPs triggers the inflammatory response, the link between the innate immunity and the adaptive immunity [43].

Most traditional vaccines consisting of subunit antigens fail to activate PRRs, consequently requiring the addition of adjuvants to provide innate immune stimulation and induce effector responses. RNA, on the other hand, can directly engage some PRRs and stimulate innate immune responses [3].

Internalization of mRNA vaccines occurs primarily by nonimmune cells at injection site. Probst and colleagues have shown that after injection with naked β-galactosidase-coding mRNA in mice, the molecule is internalized and expressed mainly by muscle cells, fibroblast, and keratinocytes [44]. Expression and cellular localization of PRRs detecting the internalized RNA depends on the cell type. For example, the cytoplasmic RIG-I-like receptors (RLRs) RIG-I and MDA5 sense the exogenous RNA in nonimmune cells, resulting in upregulation of cytokines and chemokines at the immunization site [45]. Antigen expression in these nonimmune cells can result in priming of antigen-specific antibodies and the induction of CD8+ T cell responses by means of cross-presentation [46]. For example, after intramuscular vaccination, antigen expression occurs mainly in myocytes, yet bone marrow-derived APCs are required for priming major histocompatibility complex (MHC) class-I-restricted CD8+ T cells. Therefore, direct transfection of APCs with mRNA vaccines is not a prerequisite for effectiveness.

mRNA vaccines induce a robust innate immune response resulting in chemokine and cytokine production at the inoculation site [47,48], which may play an important role in successful immunization. Intradermal (id) immunization with mRNA vaccines upregulates chemokines CXCLs and CCLs, which recruit innate immune cells such as DCs and macrophages, to the site of injection [45]. mRNA vaccines also trigger proinflammatory cytokines, such as TNF-α that in turn induces not only immunostimulatory effects but also increases the expression of matrix metalloproteinases on the cellular membrane of migrating DCs. This facilitates the degradation of the neighboring tissues and trafficking of DCs toward the draining lymph nodes (dLNs) [49]. Consistently, both mRNA and encoded antigen are detectable in the inoculated tissues and in the dLNs, shortly after immunization [45]. In addition, there is an increase in the number of activated immune cells within the dLNs, mainly determined by the recruitment and the proliferation of B cells and granulocytes [45], which have a crucial role in promoting DC maturation by glycosylation-dependent interaction and a boosted antigen presentation [45,50]. Finally, the upregulation of CD69 on immune cells within the dLNs together with enhanced cytokine production implies effective immune priming [51].

Delivery of mRNA to the cytoplasm is necessary for antigen expression but the route of entry has not been elucidated and could include endosomal uptake and/or direct entry through the plasma membrane. In immune cells, such as macrophages and DCs, endosomal toll like receptors (TLRs), for instance TLR7, can sense the internalized mRNA vaccines resulting in the activation of these cells [47,52]. Furthermore, murine and human B cells [53], macrophages, DCs, and pDCs [54–56] express TLR7 and can be activated by synthetic single-stranded RNA (ssRNA). The early activation of B cells by RNA via MYD88/TLR7-dependent signaling pathway may provide a stimulus to regulate adaptive immunity induced by mRNA vaccines. Besides stimulating B-cell activity, TLR7 signaling can increase antigen presentation, promote cytokine secretion, regulate immunoglobulin isotype switching, and improve memory B cell survival. The role of TLR7-mediated RNA sensing illustrates the interplay between innate signaling and stimulation of B-cell response [57].

A further indication of the intrinsic adjuvant properties of RNA vaccines delivered with synthetic delivery vehicles, such as cationic lipid DOTAP, has come from evaluating the differentiation of bone marrow-derived DCs (BMDCs) transfected *in vitro* with DOTAP complexed with mRNA. Transfected DCs showed an increase of the activation markers CD40, CD80, and CD86, a moderate rise in pro-inflammatory cytokines, and a robust increase of the type I interferons (IFNs) [58]. Importantly, mRNA formulated with cationic lipid also induced a rapid and transient IFNα response in the blood of immunized mice, and a rapid but transient recruitment of inflammatory monocytes to the dLNs, such as occurs after an infection [58]. These results suggest that cationic lipids can potentiate the adjuvant effect of RNA molecules. Mechanistically, delivery materials may facilitate the transport of RNA molecules across cell membranes in a manner that

facilitates engagement of PRRs. Cationic lipids are believed to enhance RNA uptake and facilitate endosomal escape [59]. Hence, delivered mRNA can interact with endosomal TLRs in the immune cells or with the cytosolic RLRs of nonimmune cells. Cationic lipids and cationic polymers have been utilized as nonviral carriers to deliver effectively DNA vaccines; this procedure has shown the ability to boost the transfection effectiveness and improve the *in vivo* vaccine potency. Treatment with cationic liposomes is able to induce the release of cytokines and chemokines from antigen-presenting cells, and the expression on DCs of maturation and activation markers such as CD11c, CD80, and CD86 [60]. Delivery of self-amplifying mRNA by a lipid nanoparticle was recently shown to have strong local effects on gene expression in mice within 24 h after injection, mapping to innate immune, anti-viral, and inflammatory signaling pathways [47]. Thus, the potentiating effect of delivery vehicles on exogenous RNA-mediated immune activity may be mediated through a variety of cellular pathways.

Finally, while RNA molecules encoding a gene of interest can trigger an adjuvant effect in conjunction with its delivery system, noncoding RNA molecules can also enhance innate immunity and function as a vaccine adjuvant. Heidenreich et al. [61] have recently demonstrated that a synthetic RNA combined with a polymeric carrier can efficiently activate the innate immune system by acting at the injection site, generating a local immunostimulatory environment, and increasing the immunogenicity of subunit vaccines. This approach has also been utilized to improve the efficacy of conventional mRNA vaccines [13].

3.2. Innate antiviral responses against RNA vaccines – two sides of the coin

RNA vaccines seem to have 'self-adjuvanting' properties by activating host sensing machinery. However, the innate immune system may also establish an antiviral response and thereby creating a potentially unfavorable environment for translation of mRNA vaccines that could reduce vaccine effectiveness. Data supports both sides of the coin.

mRNA vaccines elicit a robust type I IFN response enhancing the capacity to expand cytolytic CD8+ T cells, thereby enabling eradication of tumor or infected cells [62]. For example, Kranz et al. [26] immunized intravenously with mRNA formulated with cationic lipid, resulting in the induction of type-I-IFN-mediated innate immune response as well as CD8+ T cell responses in murine models as B16-OVA melanoma and CT26-gp70 colon carcinoma. The absence of IFN type I response impaired vaccine efficacy [26]. This work was extended to a clinical setting where three patients with malignant melanoma (NCT02410733) were immunized intravenously with mRNAs expressing the tumor-associated antigens (TAAs) NY-ESO-1, MAGE-A3, tyrosinase and TPTE. The patients showed an immediate induction of antigen-specific CD8+ T cells, 2 weeks after the first immunization; interestingly, the patients' blood level of IFNα peaked at 6 h similar to the results in mice [26].

Similar observations were made by Broos and colleagues [63]. Intravenous administration of OVA mRNA in IFNAR$^{-/-}$ mice increased the magnitude of CTL responses, despite a negatively affected gene expression with a luciferase mRNA.

In addition to the positive effects of IFN on mRNA vaccine effectiveness, data also shows that a type I INF response can be detrimental for RNA vaccines.

As reported by Cruz et al. [64], specific single mutations in nsP1 sequence of alphaviruses increase type I IFN levels and play a role in virus virulence attenuation. Based on this finding, Maruggi et al. [65] have shown that specific single mutations in nsP1 of SAM increased IFN type I levels and reduced vaccine potency. In particular, the mutation A533I induced elevated type I IFN and decreased antigen expression of SAM in infected cells. Importantly, this mutation also reduced *in vivo* antigen expression and vaccine immunogenicity.

Consistent with these observations, transfection of murine DCs IFNAR$^{-/-}$ with mRNA vaccines increased the efficiency of cell transfection and immunization in IFNAR$^{-/-}$ mice raised the number of T cells antigen-specific compared to the control mice [58]. Similar observations were made with self-amplifying mRNA vaccines where increased gene expression and adaptive immune responses were seen in IFNAR$^{-/-}$ mice compared to control mice [47]. Furthermore, De Beuckelaer et al. [66] have shown that vaccination with mRNA lipoplexes in IFNAR$^{-/-}$ mice resulted in enhanced priming of antigen-specific T cells. The priming determined effector functions in T cells and, consequently, was able to eliminate the target B16 cells. When challenged with B16 melanoma cells, vaccinated IFNAR$^{-/-}$ mice have longer survival time compared to vaccinated wild-type mice. The effect of IFN was also investigated by co-administration of MAR1-5A3 antibody, an IFNAR antagonist, at the time of immunization in wild-type mice [67]. This block of IFNAR by MAR1-5A3 enhanced survival in response to both prophylactic and therapeutic RNA vaccination, confirming that interferon type I can substantially impair the efficacy of mRNA vaccines. These results suggest that mRNA vaccines be engineered to achieve the correct balance of type I IFN induction to capture an adjuvant effect but not interfere with launch of the mRNA vaccines.

The means by which type I IFNs exert their negative impact remains unclear, but it is conceivable that they might impair mRNA vaccine-elicited adaptive immunity at multiple levels [62]. Type I IFNs can activate an intracellular antiviral response and consequently prevent exogenous RNA replication and expression, as shown *in vitro* in BMDCs incubated with mRNA lipoplexes [58,66] and *in vivo* in IFNAR$^{-/-}$ mice [47]. Alternatively, type I IFNs may directly impact at the level of T cells. While type I IFNs can determine the differentiation of antigen-primed CD8+ T cells into cytolytic effectors, they may also promote T cell exhaustion [66]. The net impact of these two opposing effects depends on the kinetics of T cell exposure to type I IFNs. T cell inhibition could prevail if triggering of type I IFN receptors precedes that of T cell receptors. The mRNA lipoplex vaccine induces type I IFNs rapidly and RNA-sensing TLRs are triggered in endosomal compartments of the DCs that take up the RNA lipoplexes. This activation of type I IFNs likely occurs prior to release of mRNA into the cytoplasm for translation and translocation of the DCs to the lymph nodes (LNs) to present the antigen [66]. Further studies using mice selectively deficient in IFNAR on different cell

types will shed light on the mechanism of type I IFN interference on T cell immunity in mRNA vaccination.

Alphaviruses, like many viruses, encode functions to antagonize the interferon response: for example, type I IFNs via Jak/STAT pathway can generate the expression of numerous IFN-stimulated genes (ISGs) involved in antiviral activities [68]. Simmons et al. [69] reported that nsP2 of VRPs from Venezuelan equine virus (VEEV) bound the nuclear importin karyopherin (KPNA1), preventing the interaction with the transcription factor STAT1 and the resulting nuclear translocation [69]. Consequently, with STAT1 sequestered in the cytoplasm of infected cells, STAT1-dependent transcription of ISGs was inhibited.

4. Optimization of mRNA vaccines

An mRNA vaccine that can elicit robust immune response requires the engagement of innate immunity as well as the adaptive immunity. Exogenous RNA interacts with intracellular pathways network at multiple levels. RNA viruses, such as alphaviruses, have also evolved evasion mechanisms to overcome host antiviral responses. The insight of these interactions can guide the efforts of optimization of RNA vaccine backbones and delivery systems to enable the rational designs and improve vaccine effectiveness and safety.

4.1. Backbone optimization

In the last decade, significant progress has been achieved in improving the efficacy of the RNA vaccines. Modifications of the 5′ cap, poly (A) tail, coding and UTRs, and nucleoside bases are some approaches employed to enhance RNA stability and gene expression, and resulting vaccine potency [3,70,71].

For self-amplifying mRNA vaccines, insight into alphavirus biology has elucidated the mechanism of viral genomic replication, RNA transcription, gene translation, and significant features of the cross talk between virus and cell. This knowledge has provided the opportunity to improve alphavirus replicon vector design and, consequently, improve antigen expression [72].

4.1.1. Cap

Eukaryotic mRNAs, including viral RNAs such as those from alphaviruses, at the 5′ present a methylguanosine cap containing two types of methylation. The 7-methylguanosine (m7G) cap (cap 0), which is added during transcription via a triphosphate bridge, prevents RNA premature degradation and is essential for mRNA maturation, export, and translation initiation [73]. The 2′-O methylation (cap 1), which is added to the 7-methylguanosine (m7G) cap, prevents the induction of the innate immunity against exogenous RNA. The effect of the 5′ cap on the mRNA vaccines was demonstrated by Kuhn and coworkers [74], where they showed that an encoding-luciferase mRNA vaccine, capped with phosphorothioate anti-reverse cap analogs enhanced RNA stability and expression in immature DCs, as well as antigen expression and immunogenicity in immunized mice.

For mRNA vaccines, the 5′ cap can be added by using a capping enzyme or using nucleotide cap analog after the *in vitro* transcription reaction [3].

RNA viruses initiate translation in a cap-independent manner via internal ribosomal entry, such as the pestivirus CSFV (classical swine fever virus). These viruses use an internal ribosomal entry site in the 5′-UTR to initiate translation [75]; replicons derived from them have a cap-independent translation initiation and provide an alternative system [76].

4.1.2. Untranslated regions

The mRNA gene replication and translation can be influenced by mRNA UTRs. Multiple sequence elements have been identified within 5′ and 3′ UTRs of both cellular and viral mRNAs that have the ability to affect mRNAs stability and expression [77]. For example, 5′ UTRs of many orthopoxvirus mRNAs can inhibit cap removal and exonuclease degradation of these RNAs [77]. RNA UTRs regulate in alphaviruses genomic replication, expression, and interactions with the host [78]. Besides, alphaviruses adopt secondary structural sequences within the 5′ UTR to alter the binding and the function of interferon-induced antiviral-binding RNA protein, IFIT1, a sensor for the viral single-stranded RNA (ssRNA) [79].

Bell et al. [80] reported an interesting UTR modification for RNA vaccine development, whereby engineered riboswitches were introduced into the 3′ UTR of an SAM vaccine to regulate RNA amplification and gene expression. These riboswitches consisted of a hammerhead ribozyme from the satellite RNA of Tobacco Ringspot Virus actuated by an aptamer sensor specific for theophylline. These riboswitches were capable of modulating gene expression of the RNA vaccine, when the compound was added to the cell host. Consequently, this approach has the potential to provide tunable expression of vaccine antigens.

4.1.3. Codon usage

The nucleotide content of the coding regions of nucleic acid vaccines can have a substantial effect on the magnitude of gene expression in cells. For instance, in contrast to viral genes, the codons of mammalian genes frequently present a guanine (G) or a cytosine (C) at third codon position and are expressed with a better efficiency than those presenting A or T at third position [81]. In addition, antigen expression from an alphavirus-derived SAM vaccine can also be enhanced by altering the replication mode of the viral RNA. Kim and coworkers modified the VEEV replicon so that not only genomic RNA but also subgenomic RNA could replicate [72]. As a result, the levels of intracellular subgenomic RNAs and consequently antigen expression were significantly increased leading to protective immunogenicity in mice [72].

Nucleotide replacement can also be introduced for generating modifications that can hamper the RNA cytosolic exposure and expression, such as secondary structures [82,83].

4.1.4. Nucleoside base modification

Nucleoside base modification can reduce the potential for innate antiviral immunity directed toward RNA-based vaccines. While natural mRNA can stimulate PRRs, mRNA with

base modifications, such as 2-thiouridine, 5-methylcytidine, or pseudouridine, can limit the effects of the innate immunity [84–86]. In addition, such changes can protect the mRNA from degradation by ribonucleases, thereby enhancing the antigen expression [87]. However, Probst et al. [88] have shown that 2′ modification of mRNA has only a modest effect on mRNA stability and may inhibit translation.

Therefore, certain nucleoside base modifications of the RNA have the potential to enhance the efficacy of RNA vaccines by modulating the interaction and/or the activity of PRRs, and consequently type I IFN induction.

4.2. Delivery systems and route of administration

To produce an antigen-specific immune response, an mRNA vaccine must reach the cytosol of recipient cells and express the antigen. Uptake and expression *in vivo* is in some cases can be better than spontaneous uptake observed *in vitro* and, often, comparable to cells transfected *in vitro* under optimal conditions [70]. Wolff [2] demonstrated that uptake and expression of exogenous RNA can be achieved without a delivery system. In addition, Hoerr et al. [89] reported that both naked and liposome-encapsulated mRNA expressing beta-galactosidase elicited antigen-specific cytotoxic T lymphocytes and antibodies responses following the id vaccination in mice. The biodistribution and cellular uptake of mRNA after administration are influenced by several parameters, including the vascular system, endothelial barriers, molecule size, and interactions between the molecule and host cell receptors. RNA molecules are large, hydrophilic, and negatively charged; consequently, diffusion across membranes is thermodynamically unfavorable and efficient delivery of RNA into the cytoplasm of target cells requires a delivery system. The ideal vehicle should protect RNA from ribonucleases present in the tissues, avoid entry into off-target cells, and facilitate release into the target cell cytoplasm.

Several strategies have been evaluated for RNA vaccine delivery, such as nanoparticles carriers. Particulate formulations have been shown to protect mRNA from degradation leading to enhanced cellular uptake, increase antigen expression and vaccine potency [17,90]. In addition, formulations can influence the quantity and quality of local gene expression patterns [47], innate immune stimulation, [59] and can provide a synergistic adjuvant effect [91]. Approaches to nonviral delivery of mRNA have included injection of naked mRNA, formulation with liposomes, lipoplexes, polyplexes, particulate carrier-mediated, electroporation, and gene gun [13]. Cationic formulations effectively condense RNA and can facilitate uptake by cells and delivery across cellular membranes of cellular compartments. In this way, facilitated RNA delivery has the potential to interact with endosomal TLRs (e.g. TLR7, TLR8) of immune cells and with cytosolic RLRs (e.g. RIG-I, MDA-5) in nonimmune cells. Recent human clinical trials for cancer immunotherapy have been performed by id immunization with mRNA encoding TAAs, either as naked RNA or formulated with protamine. Encouraging data has pointed out the feasibility and the efficacy of this method [19,26,38–41,92].

One of the barriers of cytosolic delivery is release of RNA from the endosomal compartment. Several polymers and lipid-based formulations have the potential to disrupt the endosomal membrane. For example, successful intranasal and systemic delivery *in vivo* have been reported by Su et al. using luciferase mRNA complexed to a hydrophobic poly β-amino ester surrounded by a phospholipid bilayer [93]. This pH-responsive delivery system appears well-suited for RNA vaccines. These particles are able to efficiently deliver oligonucleotides to the cytoplasm facilitated by the core–shell system that produces a proton sponge effect and promotes endosomal disruption. In addition, the polymeric shell isolates the hydrophobic core within the cationic hydrophilic shell and reduces cytotoxicity [90,93–95].

However, while several studies show efficient uptake and endosomal escape of condensed RNA *in vitro*, these results are sometimes not recapitulated *in vivo*, where the RNA remains trapped in endosomal vesicles [70]. Extensive data *in vitro* with cell lines and primary cells has demonstrated that uptake of naked mRNA can depend on factors such as temperature and composition of the injection buffer [44], and endosome permeation for cytosolic RNA delivery. Mockey et al. [96] used replicating mRNA expressing the melanoma-associated antigen MART1 delivered by histidine-rich cationic polymers or histidylated cationic lipids in B16F10 model by systemic immunization to increase the cytosolic RNA release by endosomal permeation.

The modality of route administration can also affect RNA uptake and expression. Id delivery of mRNA has been widely employed for mRNA-based vaccination, and antigen-specific immunity is presumed to be mediated by local dermal skin-derived DCs [97]. These DCs can carry the antigen from the site of injection in the skin to the dLNs [98]. The expansion of skin DCs with granulocyte macrophage colony-stimulating factor (GM-CSF) [99] or Flt3 ligand [100] has been reported years ago by Warren and Jefford, respectively, and seems to be beneficial for induction of adaptive immunity with this route of administration [101]. However, other cell types within the skin can internalize mRNA, including nonimmune cells, suggesting that cross-priming may also play a role in immune priming.

Since LNs contain large numbers of DCs and are the site where the antigen is presented, intranodal immunization has appeal [102]. This modality can reduce the antigen and the adjuvant required for immunization [101,103], and the use of ultrasound guidance has facilitated its feasibility [102,104]. This route of administration has been used in mice for mRNA delivery [105] and for evaluating the mechanism involved in the exogenous mRNA uptake. Diken et al. [106] reported that the macropinocytosis is the principal means of mRNA delivery into DCs after intranodal immunization and is affected by state of DC differentiation. Similarly, Selmi et al. [107] reported that id immunization of naked RNA resulted in uptake by infiltrating DCs in mice via macropinocytosis. In addition, as shown by Lorenz et al. [108] mRNA can also enter cells via caveolae/lipid rafts facilitated by scavenger receptors. Interestingly, intranodal delivery of mRNA induces a robust T cell response, whereas id immunization triggers a strong humoral response [101].

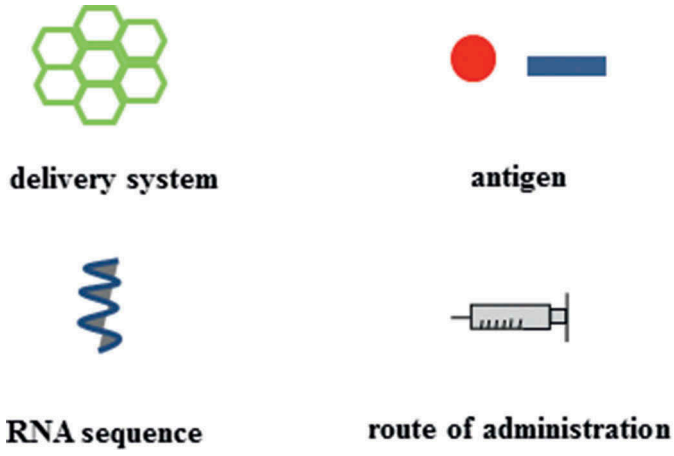

Figure 2. Factors that can influence RNA vaccine potency.

In 2006, Scheel et al. [109] used direct intra-tumoral administration of naked or protamine-formulated RNA in a mouse glioma SMA-560 model and demonstrated tumor regression and long-term immunity. Other work [110–112] has shown that intra-tumoral delivery of mRNA is able to activate resident DCs, induce their migration to dLNs, and provoke the CTL response.

Hence, the route of vaccine administration, nature of the RNA vaccine, and the delivery system play roles in quality and quantity of the ensuing immune response (Figure 2).

4.3. Combination with small immunomodulatory molecules

Modulation of the early effects of the innate immune response is an area for further investigation for increasing the potency of mRNA vaccines. Potential strategies could include RNA sequence modification to generate RNA molecules less susceptible to interference by IFN, formulations to delivery of RNA vaccines in a manner that minimizes the consequences of interacting with innate immune sensors, and small molecule modulators that target various points of the inflammatory signaling cascade. Such an approach was taken by Kim and coworkers [113] using a hepatitis C virus (HCV)-derived replicon to demonstrate an enhancing effect of glucocorticoids by promoting replication independently of the host immunosuppressive activity of these small molecules [113]. Glucocorticoids are also able to improve replicon transfer and expression *in vitro* and *in vivo* [114]. Pretreatment of mice with dexamethasone (a corticosteroid) attenuated the early pro-inflammatory response induced by immunization with an adenoviral vector vaccine [31]. While no improvement in vaccine potency was observed in this study, this approach could be considered for enhancement of replicon-based RNA vaccines.

5. Expert commentary

Synthetic nucleic acids have several potential applications, such as cancer immunotherapies, vaccines for infectious diseases, tolerization to the allergies, genetic editing, and protein supplementation.

Nucleic acid vaccines are effective at inducing broad and potent immune responses, at least in part, because they mimic a live virus (i.e. express antigens *in situ*), but without the complications of a live organism. The main types of such vaccines include viral vectors, pDNA, and mRNA, all of which hold promise for use as human vaccines.

Clinical proof of concept has been achieved for induction of functional immune responses and, in some cases, protective immunity in field efficacy trials. However, practical issues of manufacturing feasibility and anti-vector immunity may limit the broad utility of viral vectors, while lack of potency remains a barrier for pDNA vaccines. Whilst there is less human clinical experience with mRNA-based vaccines, they have the potential to combine the positive attributes of viral vectors and DNA vaccines, without their limitations. Growing insight into mechanism of action is offering the possibility to rationally design the mRNA vaccine components for optimal potency and safety, and thereby take full advantage of this new class of nucleic acid vaccines.

Although several progress in understanding the molecular pathways involved in RNA vaccine action, advancements in the identification and development of new delivery systems, modifications in the RNA sequence to tune the host-innate immune system cross talk, and more appropriate use of suitable routes of administration have been made so far, RNA vaccines have not been approved for use in human, but this topic could be only a matter of time.

In the coming years, beyond the classical cited strategies, other ultimate procedures could represent the deus ex machina to improve the efficacy of RNA vaccines, such as the combination with immunomodulatory molecules able to tune the innate immunity, by impacting the body detrimental response and intensifying the intrinsic adjuvanticity of RNA vaccine. A further approach to pursue is the combination of RNA vaccine with antibodies for immunocheckpoints, which improve the T cells activity or antagonize the T-reg activity. Finally, since therapies efficacy can be affected by human genetic polymorphisms, next-generation sequencing empowers the fast identification of somatic mutations which, in combination with their systematic immunogenicity analysis, allow a personalized vaccine strategy. The vaccinogenetics has the ability to predict the responses to immunization on the basis of host genetic, and the synthetic RNA vaccine offers the possibility for a faster on-demand drug synthesis.

6. Five-year view

More than a half century after the discovery of mRNA (Figure 3), the true therapeutic potential of this dynamic molecule is only beginning to be recognized in fields like gene therapy and vaccination. In the past decade, we have witnessed a rapid increase in the R&D of mRNA therapeutics.

There is now a substantial body of preclinical work in animal models amply demonstrating the broad utility of mRNA vaccines, what remains is validation in human clinical trials. Initial clinical targets have been therapeutic vaccines against cancer, and proof of principle for safety and immunogenicity has been attained. More recently, preventive vaccines against infectious diseases have entered human clinical trials. In addition, there are expanding

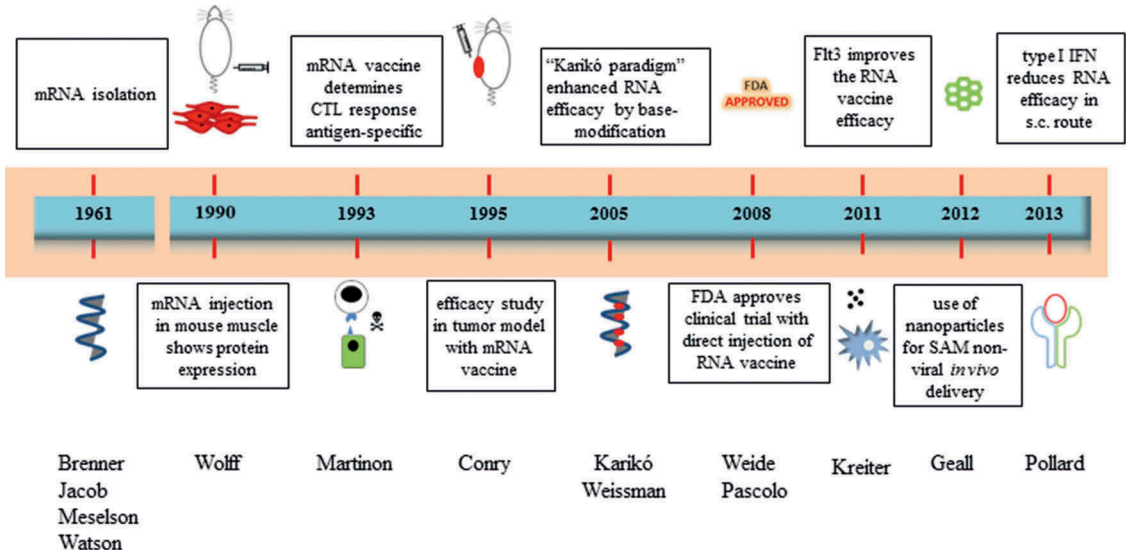

Figure 3. Milestones in RNA Vaccines.

efforts in other areas of mRNA therapeutics, such as treatment of cardiovascular disease, rare diseases, and personalized medicine. The next five years will be a critical period for this new technology, as during this time we will have clear feedback on the safety and potency of conventional mRNA and SAM vaccines from several human clinical trials. We anticipate that at least some of these vaccines will be immunogenic in humans, based on preclinical benchmarks. However, only the results of these clinical trials will tell if the current state of the art is sufficiently potent and safe, or whether further optimization will be needed. In the case that further improvements are needed, mechanistic studies have suggested that modulation of the early innate immune response may be useful. Approaches could include replicon engineering, new delivery systems, and the inclusion of small molecule antagonists to minimize interference by cytokines.

Key issues

Preclinical proof of principle for mRNA vaccines was established in the early 1990's, but there appeared to be no path to commercialization at the time, due to perceived issues of vaccine stability and complexity/cost of manufacture. Today, these two limitations have been largely solved. The remaining key issues relate to potency and tolerability in humans, which will be governed by the nature of the mRNA vaccine and the delivery system utilized. As has been described in some detail in this review, the nature of the delivery system will determine efficiency of cellular delivery, cell type targeted, and intracellular location of delivered mRNA, all of which will have an impact on the magnitude and quality of immune responses. In addition, the components of the delivery system on their own have potential to stimulate innate immune responses and act synergistically with mRNA. Hence, it will be important to comprehensively assess the clinical performance of the mRNA vaccines for potency of antigen-specific immune responses, early innate immune signaling, systemic effects, and local tolerability. In addition, it would be very instructive to, where possible, include licensed vaccines as comparators in the human trials to allow direct and relevant benchmarking. In this way, the true potential of mRNA vaccines can be accurately assessed.

Acknowledgments

Part of the work reported on SAM was supported by DARPA, Defense Advanced Research Project Agency under agreement HR0011-12-3-001. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. The authors thank Ulrike Krause for excellent technical assistance and Giulietta Maruggi for helpful discussions during the critical review of the manuscript.

Funding

The manuscript was funded by GlaxoSmithKline Biologicals SA.

Declaration of interest

All authors are employees of the GSK group of companies. N Delahaye reports owning restricted shares in the GSK group of companies. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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