An update on self-amplifying mRNA vaccine development

Anna K. Blakney,¹,* Shell Ip,² Andrew J. Geall²

Vaccines Special Issue: “The Past, Present and Future of mRNA Vaccines”

¹. Department of Infectious Disease, Imperial College London, Norfolk Place, London, W2 1PG, United Kingdom
². Precision NanoSystems Inc., Vancouver, British Columbia, V6P 6T7, Canada

*Corresponding author. Email: anna.blakney@msl.ubc.ca

Abstract: This review will explore the four major pillars required for design and development of an saRNA vaccine: antigen design, vector design, non-viral delivery systems, and manufacturing (both saRNA and lipid nanoparticles (LNP)). In will report on the major innovations, preclinical and clinical data reported in the last five years and will discuss future prospects.

Keywords: RNA; self-amplifying RNA; replicon; vaccine; drug delivery
1. Introduction: The Four Pillars of saRNA Vaccines

In December 2019, the SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) virus emerged, causing a respiratory illness, coronavirus disease 2019 (COVID-19), in Hubei province, China.[1, 2] The virus has spread globally, with the World Health Organization (WHO) declaring it a Public Health Emergency of International concern on January 30, 2020 and a pandemic officially on March 7, 2020.[3] There is a strong consensus globally that a COVID-19 vaccine is likely the most effective approach to sustainably controlling the COVID-19 pandemic.[4] There has been an unprecedented research effort and global coordination which has resulted in the rapid development of vaccine candidates and initiation of human clinical trials. This has included conventional vaccine technologies such as viral vectors and adjuvanted subunits, but we have witnessed a renaissance in the field of RNA vaccines and a shift towards synthetic RNA platforms (Figure 1).[5, 6] In fact, one of the first vaccine to start clinical trials was a non-replicating mRNA vaccine from Moderna, mRNA-1273;[7-9] the first patient was vaccinated on March 16th at the same time as a Chinese clinical trial was initiated with an adenovirus type-5 (Ad5) vector.[10] Furthermore, the BioNTech/Pfizer vaccine, BNT162b2, was the first COVID-19 vaccine to receive approval, first in the United Kingdom and then Canada, with an impressive 95% efficacy.[11] Since this time there have been several mRNA vaccine trials initiated, and publication of corresponding preclinical and clinical data, see Table 1.

Figure 1. A comparison of vaccine platforms including vaccines derived from the virus itself and are formulated as a part or whole modified version of the virus (left) and nucleic acid vaccines, such as self-amplifying RNA vaccines (right). Nucleic acid vaccines are derived from knowledge of the viral genome, where glycoproteins are encoded into nucleic acids and delivered with either a synthetic carrier such as a lipid nanoparticle or an inert viral delivery system such as adenoviruses. The encoded antigen sequences are then expressed by the host cells.
Table 1: Published preclinical and clinical trial data with mRNA COVID-19 vaccines.

| Sponsor            | Type of mRNA | Delivery system | Preclinical data | Clinical data |
|--------------------|--------------|-----------------|------------------|---------------|
| Moderna            | bmRNA        | LNP             | [9, 25]          | [7, 8]        |
| BioNTech/Pfizer    | bmRNA        | LNP             | [148]            | [11, 149-152] |
| ICL                | saRNA        | LNP             | [78]             |               |
| Arcturus           | saRNA        | LNP             | [153]            |               |
| CureVac            | mRNA         | LNP             | [154]            |               |

Imperial College London (ICL), conventional non-amplifying messenger ribonucleic acid (mRNA), conventional base-modified non-amplifying mRNA (bmRNA) and self-amplifying messenger RNA (saRNA)

The use of mRNA vaccines for pandemic response has been well described previously in preclinical[12-25] and clinical settings,[16] but this is the first time we have seen the platforms deployed in a real pandemic setting. The core principle behind mRNA vaccines is to encode the antigen in the mRNA and then to deliver the transcript to the host cell cytoplasm using a non-viral delivery system, allowing antigen expression and induction of an antigen-specific immune response. mRNA is made using a cell-free enzymatic transcription reaction, which allows rapid and scalable manufacturing, as is evident from the swift pursuit of RNA vaccines in the current pandemic. Currently, there are three major types of RNA vaccines: conventional, non-amplifying mRNA molecules (mRNA), base-modified, non-amplifying mRNA molecules (bmRNA), which incorporate chemically modified nucleotides, and self-amplifying mRNA (saRNA or replicons) that maintain auto-replicative activity derived from an RNA virus vector. Self-amplifying RNA is beneficial compared to non-amplifying RNA as it maintains the advantages of mRNA vaccines, such as rapid development, modular design, cell-free synthesis, but requires a lower dose of RNA due to the self-replication. This reduces the burden of manufacturing for both the drug substance and product and is potentially advantageous in the context of pandemic response as it would enable a greater number of the population to be vaccinated.

This review will explore the four major pillars required for design and development of an saRNA vaccine (Figure 2): antigen design, vector design, non-viral delivery systems, and manufacturing (both saRNA and lipid nanoparticles (LNP)). In will report on the major innovations, preclinical and clinical data reported in the last five years and will discuss future prospects (Figure 3).

Figure 2. The Four Pillars of successful saRNA vaccine development. The antigens, vectors, delivery and manufacturing each represent modular components that need to be combined to make a successful drug product. Each pillar has its set of design and development considerations and associated technologies that are explored in this review.
2. Antigen Design

saRNA vaccines have been primarily investigated for active vaccination strategies for prevention of infectious diseases, wherein the host’s cells produce a pathogenic antigen encoded in saRNA to induce a humoral and cellular immune response. saRNA encoding viral glycoproteins are the most predominant application, although this has recently been expanded to include bacterial infections (Chlamydia trachomatis,[26] Group A and B Streptococci[27]), parasites (Toxoplasma gondii[28, 29]) and cancer (colon carcinoma,[30, 31] melanoma[31]). A more novel approach to saRNA antigen design includes encoding monoclonal antibodies for passive vaccination.[32] While it is possible to incorporate relatively large (>4,000 nt) or multiple antigens into an saRNA construct, the pDNA construct does have size limitations, so it may be advantageous to use separate saRNA constructs to encode multiple antigens if necessary.[33]

2.1 Infectious Diseases

2.1.1 Viral glycoproteins

Recent advances in saRNA vaccines against infectious diseases include development of vaccines against a variety of viral pathogens. The breadth of these vaccines includes respiratory-transmitted viruses (SARS-CoV-2, respiratory syncytial virus, influenza), insect-transmitted viruses (VEEV, Zika, Ebola), animal-transmitted viruses (rabies) and sexually transmitted viruses (HIV-1) (Table 2). Samsa et al. observed that a codon-modified VEEV backbone, with the positively charged amino acid residues at the N-terminal region of the CP mutated to non-charged residues, induced lower IgG and neutralization titers compared to the wild type, although these mutations had been previously observed to increase VEEV replication.[34] Importantly, Magini et al. showed that it’s possible to co-deliver saRNA encoding multiple antigens, in this case the influenza nuclear and M1 proteins, to induce heterospecific neutralizing antibodies that protect against heterologous challenge.[35] All the clinical trials currently underway for saRNA vaccines have viral glycoproteins as the target (Table 3).
| Disease Target | Disease | Replicon backbone | Antigen | Delivery platform | Preclinical animal model | Ref. |
|---------------|---------|-------------------|---------|-------------------|--------------------------|-----|
| Infectious Disease | Chlamydia trachomatis | VEEV | MOMP | CAF, PEI | Mice | [26] |
| | Ebola | VEEV | Glycoprotein (EBOV) | Dendrimer | Mice | [28] |
| | Group A Streptococci | VEE-SINV | GAS SLOdm | CNE | Mice | [27] |
| | Group B Streptococci | VEE-SINV | GBS BP-2a | CNE | Mice | [27] |
| | HCV | VEEV | E1-E2 | CNE | Mice | [94] |
| | HCMV | VEEV | gH/gL | LNP | Mice | [94] |
| | HIV-1 | VEE-SINV | TV1 Env gp140 | CNE | NHP | [87] |
| | SFV | VEEV | eOD-GT8 gp120 | LNP | Mice | [81] |
| | HIV-1 | VEEV | Env gp140 | Lipoplex | Mice | [82] |
| | SFV | VEEV | HIV-1C Env, Gag, PolRT | Naked | Mice | [66] |
| | Malaria | VEE-SINV | PMIF | CNE | Mice | [36] |
| | Influenza | VEEV | HA (H1N1, A/WSN/33) | Dendrimer | Mice | [28] |
| | | VEE-SINV | HA (H1N1, A/Cal/7/09) | CNE | Mice, Ferrets | [88] |
| | | VEE-SINV | NP (H1N1, A/PR/34/07) | LNP | Mice | [52] |
| | | VEE-SINV | NP, M1 or NP+M1 (H1N1, A/PR/8/34) | LNP | Mice | [35] |
| | CSFV | VEEV | HA, NP (H5N1/Yamaguchi/2004) | PEI with CPP | Mice, Pigs | [70] |
| | | CSFV | NP (H3N2, Brisbane 2007) | Cationic lipid | Mice | [83] |
| | | n.s. | HA (H1N1, A/PR/8, A/Cal/7/09) | PEI | Mice | [50] |
| | CSFV | VEEV | HA, NP (H5N1/Yamaguchi/2004) | PEI | Mice | [69] |
| | | VEEV | HA (A/PR/8/34) | LPPs | Mice | [156] |
| | | n.s. | HA (H1N1, A/Cal/7/09) | MLNPs | Mice | [157] |
| Pathogen | Virus | Antigen | Adjuvant | Delivery | Species |文献 |
|----------|-------|---------|----------|----------|---------|------|
| SFV      | taRNA| HA (H1N1, A/Cal/7/09) | Naked | Mice | [57] |
| VEEV     |     | HA (H1N1, A/Cal/7/09) | pABOL | Mice | [74] |
| VEEV     |     | NP, GMCSF | CNE | Mice | [89] |
| Rabies   | VEEV | Glycoprotein G | CNE | Rats | [158] |
| VEEV     |     | Glycoprotein G | PNPs, Liposomes, CNE | Mice | [86] |
| VEEV     |     | Glycoprotein G | LNP, CNE | Mice | [80] |
| VEEV     |     | Glycoprotein G | LNP, CNE | Mice | [94] |
| Respiratory syncytial virus | VEEV | Glycoprotein F | LNP | Mice | [51] |
| SARS-CoV-2 | VEEV | Pre-fusion stabilized spike protein | LNP | Mice | [78] |
| VEEV     |     | Spike protein | LION emulsion | Mice, NHP | [159] |
| VEEV     |     | Pre-fusion spike protein | LNP | Mice | [31] |
| VEEV     |     | Spike protein | LNP | Mice | [153] |
| Toxoplasma gondii | VEEV | GRA6, ROP2a, ROP18, SAG1, SAG2A, AMA1 | Dendrimer | Mice | [28] |
| SFV      |      | NTPase-II | LNP | Mice | [29] |
| VEEV     |      | E3-E2-6K-E1 | CNE | Mice | [34] |
| Zika     | VEEV | prM-E | Dendrimer | Mice | [71] |
| VEEV     |      | prM-E | NLC | Mice, Guinea pigs | [123] |
| VEEV     |      | prM-E | Naked | Mice | [67] |
| VEEV     |      | ZIKV-117 Ab | NLC | Mice | [32] |
| n.s.     |      | prM-E | CNE | Mice, NHPs | [160] |
| VEEV     |      | NS3, prM-E | LNP | Mice | [161] |
| Cancer   | Melanoma | VEEV | IL-12 | LNP | Mice | [30] |
| VEEV     |      | IL-2 | LNP | Mice | [31] |
| Colon carcinoma | VEEV | IL-12 | LNP | Mice | [30] |
Table 3: Clinical trials of saRNA vaccines since 2015

| Disease Target | Institution | Vaccine components (Route of administration) | Target | Clinical trial number (Phase) | Status          |
|----------------|-------------|-----------------------------------------------|--------|------------------------------|-----------------|
| Rabies         | GlaxoSmithKline | VEE-SINV saRNA with CNE (IM) | Rabies glycoprotein G | NCT04062669 (I) | Ongoing, recruiting |
| SARS-CoV-2     | Arcturus Therapeutics | STARR™ (VEEV) saRNA with LUNAR® LNP (IM) | Pre-fusion stabilized spike protein of SARS-CoV-2 | NCT04480957 (I) | Ongoing, recruiting |
|                | HDT Bio Corp. | VEEV saRNA with LION emulsion (IM) | Spike protein of SARS-CoV-2 | - | Pre-recruiting |
|                | Imperial College London | VEEV saRNA with LNPs (IM) | Pre-fusion stabilized spike protein of SARS-CoV-2 | ISRCTN17072692 (II) | Ongoing, recruiting |
|                | Imperial College London, University of Oxford | VEEV saRNA with LNPs OR ChAdOx (IN) | Pre-fusion stabilized spike protein of SARS-CoV-2 | - | Pre-recruiting |
| Non-Small Cell Lung Cancer, Colorectal Cancer, Gastroesophageal Adenocarcinoma, Urothelial Carcinoma | Gritstone Oncology, Inc. | GRT-C901, GRT-R902 | Personalized neoantigens | NCT03639714 (I/II) | Recruiting |
| Non-Small Cell Lung Cancer, Colorectal Cancer, Pancreatic Cancer, Solid Tumor, Shared Neoantigen-Positive Solid Tumors | Gritstone Oncology, Inc. | GRT-C903 GRT-R904 | Personalized neoantigens | NCT03953235 (I/II) | Recruiting |

Cationic nanoemulsion (CNE), Chimpanzee adenovirus-vectored vaccine (ChAdOx), Intranasal (IN), Intramuscular (IM), Lipid nanoparticles (LNPs), Lipid-enabled and Unlocked Nucleomonomer Agent (LUNAR®), Self-amplifying RNA (saRNA), Self-amplifying and replicating RNA (STARR™), Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Venezuelan equine encephalitis virus (VEEV), Venezuelan equine encephalitis and Sindbis virus replicon chimera (VEE-SINV)
Antibody (Ab), Apical membrane antigen 1 (AMA1), Cationic adjuvant formulation (CAF), Cationic nanoemulsion (CNE), Cell penetrating peptides (CPP), Classical swine fever virus (CSFV), Dense granule protein 6 (GRA6), E1-E2 glycoproteins of hepatitis C virus (E1-E2), GAS Streptolysin-O (SLOdm), GBS pilus 2a backbone protein (BP-2a), gh and gL glycoproteins of human cytomegalovirus (gH/gL), Granular-macrophage colony-stimulating factor (GM-CSF), Group specific antigen (Gag), Envelope protein (Env), Hemagglutinin (HA), human cytomegalovirus (HCMV), hepatitis C virus (HCV), Interleukin-2 (IL-2), Interleukin-12 (IL-12), Lipid inorganic nanoparticle (LION) emulsion, Lipid nanoparticles (LNPs), Major Outer Membrane Protein (MOMP), Mannosylated lipid nanoparticles (MLNPs), Membrane protein 1 (M1), Nanostructured lipid carrier (NLC), Nonhuman primate (NHP), Nucleoprotein (NP), Nucleoside Triphosphate Hydrolase-II (NTPase-II), poly(CBA-co-4-amino-1-butanol (pABOL), Poly(ethylene imine) (PEI), Plasmodium macrophage migration inhibitory factor (PMIF), Polymerase protein (Pol), Polymeric nanoparticles (PNPs), Pre-membrane and envelope protein (prM-E), Reverse transcriptase (RT), Rhopty protein 2A (ROP2A), Rhopty protein 18 (ROP18), Self-amplifying RNA (saRNA), Self-amplifying and replicating RNA (STARR™), Semliki Forest Virus (SFV), Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Surface antigen 1 (SAG1), Surface antigen 2A (SAG2A), Venezuelan equine encephalitis virus (VEEV), trans- amplifying RNA (taRNA), Venezuelan equine encephalitis and Sindbis virus replicon chimera (VEE-SINV)

2.1.2 Bacterial antigens

saRNA vaccines against bacterial antigens have also been investigated, although are limited to protein targets as opposed to polysaccharides and non-protein surface markers. Maruggi et al. investigated the immunogenicity and efficacy of saRNA against Group A and Group B Streptococci, as model bacterial pathogens.[27] They used saRNA encoding Streptolysin-O (SLOdm) and pilus 2a backbone protein (BP-2a), and achieved partial protection against intraperitoneal infection in a maternal immunization/pup challenge model, although protection was higher in both cases with the recombinant protein vaccine. Blakney et al. investigated the use of saRNA encoding the major outer membrane protein (MOMP) of Chlamydia trachomatis as a model antigen, complexed with cationic adjuvant formulations (CAFs).[26] The three saRNA formulations all exhibited antigen-specific humoral and cellular immunity against MOMP, although a challenge study was not included as part of this study. These studies show that it is possible to use saRNA vaccines against bacterial pathogens as a means of disease prevention.

2.1.3 Parasitic antigens

saRNA vaccines have been applied to two parasitic indications, Toxoplasma gondii and Plasmodium. Chahal et al. demonstrated that a hexaplex saRNA vaccine with six T. gondii-specific antigens, including dense 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), protected mice against lethal T. gondii challenge with a dose of 6.67 µg per replicon (40 µg total) after a single IM injection.[28] Luo et al. utilized saRNA encoding nucleoside triphosphate hydrolase-II (NTPase-II) with a prime-boost regimen of 10 µg doses and observed partial protection, prolonged survival time and reduction in brain parasitic load.[29] Baeza Garcia et al. vaccinated mice with a replicon encoding Plasmodium macrophage migration inhibitory factor (PMIF) and showed that the vaccine delayed blood-stage patency after sporozoite infection, increased anti-Plasmodium antibody titers, and completely protected from reinfection.[36] These studies demonstrate that the saRNA platform can also prevent parasitic infection.
2.1.4 Monoclonal antibodies for passive vaccination

As opposed to active vaccination, passive vaccination with monoclonal antibodies (mAb) provides more immediate protection against a pathogen. Because monoclonal antibodies are expensive to produce and difficult to administer, mRNA is a highly useful alternative platform. Previous studies have utilized mRNA[37] or pDNA[37] encoding a neutralizing antibody against chikungunya virus or influenza and Ebola viruses, respectively. Though the mRNA LNP formulation is protective against chikungunya virus challenge, the required doses of 40-200 µg in mice preclude application of this technology in a human trial. Erasmus et al. encoded ZIKV-117, a potent neutralizing mAb, and observed that a 40 µg dose of saRNA induced higher levels of systemic antibody than an equivalent dose of mRNA. While the circulating antibody levels were protective against Zika virus challenge, the titers reached a maximum concentration of 2 µg/mL, which could likely be improved with molecular and delivery platform optimization. While this strategy is advantageous for infectious diseases with a known neutralizing antibody, it may also be applied to mAb treatments against cancers or rare and inherited diseases.

2.2 Cancer

While mRNA vaccines have been widely applied to oncology,[38, 39] including for the generation of neoantigen cancer vaccines, recent advances in saRNA cancer vaccines are more limited. Li et al. used a clever in vitro evolution approach to introduce mutations into the VEEV replicon backbone that enhanced the magnitude and duration of protein expression in vivo.[31] Compared to the wild-type replicon, the evolved saRNA showed a 5.5-fold increase in the intra-tumoral interleukin-2 (IL-2) levels and increased infiltrating CD8^+ T-cells, which resulted in significantly slowed melanoma tumor growth. Li et al. also developed a LNP-formulated saRNA encoding IL-12 to stimulate immunogenic cancer cell death (ICD) by utilizing an LNP composition that itself stimulates ICD, saRNA that triggers cellular activation, and interleukin-12 (IL-12) for immunomodulation. The observed that the saRNA LNPs induced a highly inflamed tumor microenvironment, eradicated large established tumors and regression of distal un-injected tumors. Gritstone Oncology, Inc. has two ongoing clinical trials using saRNA personalized neoantigen vaccines against non-small cell lung cancer, colorectal cancer, gastroesophageal adenocarcinoma, urothelial carcinoma, solid tumors, and pancreatic ductal adenocarcinoma (Table 3), although preclinical studies and trials results have not yet been published. Virus replicon particles (VRP) have been utilized more extensively clinically in the cancer vaccine space, with encouraging data presented in these reviews;[21, 40] these strategies will likely transition to non-viral delivery approaches in the future. Together these studies set a precedent for future use of saRNA vaccines for cancer applications.

3. Vector Design

As discussed in the introduction, there are three major forms of RNA vaccines based on the auto-replicative capacity of the mRNA and the inclusion of mammalian base-modifications. This section will focus on saRNA, or replicons, that maintain replicative activity derived from an RNA virus vector. Historically, positive-sense single-stranded RNA viruses, such as alphaviruses, flaviviruses, and picornaviruses have been used, but the best-studied self-amplified mRNA molecules are derived from alphavirus genomes, such as those of the Sindbis virus (SINV), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEEV), for reviews see references.[5, 15, 21, 41, 42] This section explores how saRNA self-amplifies and any new published insights that might help in the rational design of improved vectors. In addition, it highlights any innovations reported in the last 5 years on the design of saRNA vectors.
3.1 Mechanisms of self-amplification of RNA

saRNAs are considerably larger (~9-12kb) than non-amplifying mRNAs (Figure 4). They contain the basic elements of mRNA (a cap, 5´ UTR, 3´ UTR, and poly(A) tail of variable length) but have a large open reading frame (ORF) at the 5´ end that encodes four non-structural proteins (nsP1-4) and a subgenomic promoter. Genes in the viral genome that are normally downstream of the subgenomic promoter and encode the viral structural proteins are replaced by gene(s) encoding the vaccine antigen(s). Deletion of the viral structural proteins renders the mRNA incapable of producing an infectious virus. After delivery into the cytosol of a cell, the released mRNA is translationally competent, and engagement with the host cell ribosome produces the four functional components of RNA-dependent RNA polymerase (RDRP) or viral genome replication apparatus: nsP1, nsP2, nsP3 and nsP4 (Figure 5). Studies on the regulation of alphavirus RNA synthesis, the roles of the viral non-structural proteins in this process and the functions of cis-acting RNA elements in replication have led to a greater understanding, but there are still knowledge gaps that restrict rational design of new vectors.[43] Formation of the RDRP is a complex, multistage process, with each of the nsPs having several functions.[44-46] These proteins are expressed as a polyprotein and processed in a highly regulated manner into individual proteins by the viral protease (nsP2). nsP1 is required for plasma membrane association of the replicase complex and 5´ capping of viral RNA species, nsP2 serves as RNA helicase and protease for polyprotein processing, nsP3 exerts a crucial function in mediating multiple virus-host protein-protein interactions, and nsP4 is the RNA-dependent RNA polymerase. Viral RNA synthesis requires the appropriate recognition of sequence and structural elements in the template RNAs by the viral RNA synthetic complex.[43] For alphaviruses cis-acting elements predominantly correspond to UTRs, of which there are three: one at the 5´ end, one at the 3´ end, and one at the junction region between the non-structural and structural ORFs. These UTRs have functions and new research is starting to provide greater insights.[47] In addition, elements exist in coding regions of the genome and subgenome that function in the synthesis of viral RNA, viral protein expression and viral genome packaging. These elements are conserved to varying degrees across the genus, and their role(s) in alphavirus replication continues to be clarified and refined.[47-49]

A. Conventional non-amplifying mRNA

B. Self-amplifying mRNA (replicon)

Figure 4. A comparison of mRNA vectors. Both conventional (A) and self-amplifying (B) mRNAs share basic elements including a cap, 5´ UTR, 3´ UTR, and poly(A) tail of variable length. Self-amplifying RNA (saRNA) also encode four non-structural proteins (nsP1-4) and a subgenomic promoter derived from the genome of the alphavirus. nsP1-4 encode a replicase responsible for amplification of the saRNA that enable lower doses than non-replicating mRNA.
Figure 5. Mechanism of self-amplifying mRNA. (1) Following delivery to the cytoplasm, translation of the saRNA produces the non-structural proteins 1-4 (nsP 1-4) that form the (RDRP). (2) RDRP is responsible for replication of the saRNA producing copies of the saRNA. Multiple copies of the subgenomic RNA (3) are hence produced from each saRNA originally delivered. This leads to translation of many more copies of the antigen (4) when compared to a non-amplifying RNA (5).

The RDRP complex is tethered to the plasma membrane (PM) in a bulb-shaped membrane invagination, where it can hide from host cell immune surveillance.[45] The viral replicase first uses the positive sense genome as template to synthesize complementary negative sense RNA which subsequently serves as template for the synthesis of genomic and subgenomic plus-strand RNA, with the subgenomic RNA being produced in excess of the viral genome.[15] This process leads to high and sustained levels of antigen expression relative to conventional mRNA and is certainly one of the reasons saRNA vaccines work at much lower doses.[50] RNA self-amplification in transfected cells also leads to cellular exhaustion, immune stimulation through dsRNA intermediates and a host cell antiviral response leading to apoptosis. This process in many ways mimics a viral infection and leads to enhance antigen-specific B and T cell responses.[51, 52] In parallel to the self-amplification process primarily in myocytes at the site of intramuscular vaccination,[52] the input saRNA leads to stimulation of the innate immune system, which is mediated by pattern-recognition receptors (PRRs), which detect conserved pathogen-associated molecular patterns (PAMPs) on the nucleic acid.[53] Detection of PAMPs by PRRs leads to the induction of inflammatory responses and innate host defenses. In addition, the sensing of saRNA by PRRs expressed by antigen-presenting cells, particularly dendritic cells (DCs), leads to the activation of adaptive immune responses.[53] Over the last 5 years, saRNA vaccine mechanism of action studies and a better understanding of the RNA amplification process have led to new areas of vector innovation.[54, 55]
3.2 Innovative self-amplifying RNA vector designs

In the last five years, there have been progressive designs of RNA replicons to introduce superior mutations and pioneer the use of trans-amplifying RNA systems. Li et al. developed an in vitro evolution strategy and identified six mutations in nonstructural proteins (nsPs) of Venezuelan equine encephalitis (VEE) replicon that promoted subgenome expression in cells.[31] Furthermore, a research team at Imperial College London developed a split replicon (splitzicon) system wherein the non-structural proteins (NSPs) and the gene of interest are encoded on separate RNA molecules, but still exhibit the self-amplification properties of replicon RNA.[56] They designed both positive and negative strand splitzicons encoding firefly luciferase as a reporter protein to determine which structural components affect amplification. In vitro proof of concept was demonstrated, highlighting a system for screening the components required for amplification from the positive and negative strand intermediates of RNA replicons that might lead to future vector improvements. Subsequent to this work, Beissert et al. have developed a novel bipartite vector system using trans-amplifying RNA (taRNA).[57] The vector cassette encoding the vaccine antigen originates from an alphaviral self-amplifying RNA (saRNA), from which the replicase was deleted to form a trans-replicon. Replicase activity is provided in trans by a second molecule, an optimized non-replicating mRNA (nrRNA). Expression driven by the nrRNA-encoded replicase in the taRNA system was as efficient as a conventional monopartite saRNA system in a mouse influenza challenge model.[57]

3.3 Improving immunogenicity with molecular interferon modulators

It is well known that saRNA activates the type I interferon (IFN) response through both endosomal sensing, via toll-like receptor (TLR) 3, 7 and 8, and cytosolic sensing via melanoma differentiation-associated protein 5 (MDA5), retinoic acid-inducible gene I (RIG-I), protein kinase R (PKR), 2’-5’oligoadenylate synthetase (OAS) as well as other possibly unknown pathways.[58, 59] While this is advantageous for enhancing the immunogenicity of saRNA vaccines, IFN activation is also known to lead to inhibition of translation[60] and degradation of cellular mRNA,[61] which may hinder the potency of the vaccine. To counter IFN activation, the co-delivery of viral immune evasion proteins (E3, K3, and B18/B18R from vaccinia virus and NS1 from flu) are being explored to reduce immune signaling and have shown potential.[5, 62-64] Beissert et al. co-delivered non-replicating mRNA encoding vaccinia virus immune evasion proteins E3, K3 and B18 with saRNA.[62] They observed that co-delivery of the E3 protein, which counteracts translation arrest by ensuring eIF2α functionality, enhanced saRNA expression both in vitro and in vivo. The downfall of this approach is that the 2 µg dose of saRNA required co-delivery of either 6 or 12 µg of E3 mRNA, which significantly increases the amount of administered RNA. Furthermore, trans-encoding these proteins may limit the number of cells that take up and express both types of RNA. Blakney et al. improved upon this approach by encoding an interferon inhibiting protein (IIP), screened from a library of known viral immune evasion proteins, directly in the saRNA.[33] They observed that the parainfluenza virus 5 (PIV-5) V protein and the Middle East Respiratory Syndrome (MERS) ORF4a protein enhanced expression both in vitro and in vivo in mice, and immunogenicity of saRNA encoding the rabies G glycoprotein in rabbits. Interestingly, they also observed that ruxolitinib, a JAK/STAT inhibitor,[65] increased protein expression in vivo, but did not test the effects on immunogenicity. These approaches provide proof-of-concept that saRNA expression and immunogenicity can be favorably impacted by expression of interferon inhibiting proteins.

4. Delivery Systems

The main challenge for saRNA vaccines is achieving sufficient delivery of saRNA to the target cells or tissue. saRNA constructs are relatively large (9,000 to 15,000 nt), anionic molecules, which precludes efficient cellular uptake of unformulated saRNA. Despite the use of
‘naked’ saRNA in some studies, three predominant delivery platforms have emerged: polymeric nanoparticles, lipid nanoparticles and nanoemulsions. These delivery strategies share a central dogma wherein the anionic saRNA is condensed by a cationic (or ionizable cationic) carrier to a nanoparticle of ~100 nm in size, that protects the saRNA from degradation and encourages uptake into target cells (Figure 6). Relevant studies with recent advances (since 2015) using saRNA vaccines can be found in Table 2.

![Diagram of saRNA delivery systems](image)

**Figure 6. Non-viral saRNA delivery systems.** Lipid-, polymer-, and emulsion-based delivery systems all use cationic groups to mediate condensation of the anionic RNA as well as delivery across the cell membrane. LNP systems, which have been found to be the most potent vaccine formulations, utilize a pH-sensitive ionizable cationic lipids and are taken up in cells through receptor-mediated endocytosis. In the endosome, the lower pH environment ionizes the cationic lipids, which then interacts electrostatically with anionic lipids in the endosomal membrane. These ion pairs cause a phase transition into a porous hexagonal phase (HII) that disrupts the endosome and facilitates release of the RNA into the cytoplasm.

#### 4.1 Naked saRNA
Naked saRNA has been successfully used for in vivo immunizations against HIV-1 subtype C,[66] influenza[57] and Zika viruses.[67] While these studies observed that the naked saRNA induced humoral and/or cellular responses, the required dose was significantly higher than other saRNA vaccine studies, and similar to doses used for mRNA. Abjani et al. observed Env-specific antibodies and induction of gag-specific IFN-γ secreting splenocytes after three intramuscular immunizations of 20 µg of saRNA.[66] Similarly, Beissert et al. immunized mice intradermally against H1N1 influenza using a trans-amplifying replicon system comprised of 20 µg of the replicase and varying doses (0.05 to 31.25 µg) of the HA antigen, and observed complete protection of mice against influenza challenge.[57] Zhong et al. utilized electroporation to deliver a dose of 1 or 10 µg of saRNA intramuscularly and observed moderate antibody and cellular responses against the PrM and E proteins of Zika virus.[67] These studies demonstrate that while it is possible to induce immune responses using naked saRNA, the dose required eliminates any advantage of using saRNA over non-replicating mRNA. Interestingly, Huysmans et al. observed that electroporating saRNA significantly enhanced the expression kinetics compared to naked or LNP-formulated saRNA, which they postulate was due to a limited innate immune response after intradermal injection.[68] This important finding highlights that the innate response to the saRNA delivery platform can profoundly impact immunogenicity.

#### 4.2 Polymeric Nanoparticles
Polymeric nanoparticle delivery platforms for saRNA can segregated into non-degradable and degradable polymers. Polyethyleneimine (PEI) is a non-degradable, cationic
polymer that has been used by a number of groups for delivery of saRNA. Vogel et al. demonstrated that PEI-formulated saRNA protected against three strains of influenza (H1N1, H3N2 and B), and required a 64-fold lower dose compared to mRNA.[50] Démoulins et al. observed that linear PEI induced humoral and cellular immune responses against influenza HA and NP through efficient internalization in dendritic cells (DC).[69] Following on this work, Démoulins et al. demonstrated that increasing the molecular weight (MW) of PEI inhibits internalization of polyplexes, but that adding Arg9, a cell penetrating peptide (CPP), modestly enhanced cellular responses to PEI-formulated saRNA in pigs.[70] Chahal et al. aimed to improve saRNA polyamine delivery by utilizing monodisperse, molecular defined dendrimers, and showed induction of protective immunity against influenza, Ebola and Toxoplasma gondii challenges using modified dendrimer nanoparticles (MDNP).[71] Because PEI is known to be cytotoxic, especially at higher molecular weights,[72] but higher MW PEI-based polymers enhanced the transfection efficiency of saRNA,[73] Blakney et al. developed pABOL, a bioreducible, cationic polymer which was shown to enhance transfection efficiency, but not cytotoxicity, at higher MW and to protect mice from influenza challenge at a dose as low as 1 µg.[74]

While the ideal target cells for saRNA vaccines are not yet defined, recent polymeric nanoparticles have been developed to target saRNA polyplexes to different cell populations. Gurnani et al. observed that increasing the hydrophobicity of poly(dimethylaminoethyl) acrylate (pDMAEA) copolymers enhances saRNA expression in epithelial cells in human skin explants after intradermal injection.[75] Blakney et al. observed that mannosylated-PEI polyplexes similarly enhanced saRNA expression in epithelial cells in human skin explants in a mannose-dependent manner.[76] Saviano et al. showed that increasing the branching of orthenine-derived dendrimers enriched saRNA uptake and expression specifically in epithelial, NK and Langerhans cells.[77] Ultimately, these targeting strategies may enable targeted delivery of saRNA vaccines to enhance efficiency.

4.3 Lipid Nanoparticles

Lipid nanoparticle formulations of saRNA are currently the most potent, requiring as little as 10 ng of saRNA to induce a robust immune response.[78] saRNA LNPs are predominantly based on formulations optimized for siRNA and mRNA delivery, which include an ionizable lipid, phospholipid, cholesterol and PEGylated lipid.[79] These LNPs have been used for a variety of saRNA vaccine infectious disease indications, including SARS-CoV-2,[78] influenza,[35] rabies,[80] Toxoplasma gondii,[29] respiratory syncytial virus,[51] as well as recent advances in saRNA cancer vaccines, including melanoma[31] and colon carcinoma.[30] Melo et al. used LNPs based on the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and showed high titers of gp120-specific antibodies after a single intramuscular injection, as well as increased levels of antigen-specific germinal center B cells compared to protein immunization.[81]

While saRNA has historically been encapsulated on the interior of lipid nanoparticles, there have also been recent advances of LNP formulations wherein the lipid particle is formed and then the saRNA is complexed on the surface of the particle. Blakney et al. showed induction of HIV-1 gp140 antibody responses was higher with cationic-based lipoplexes, although protein expression was highest when saRNA was encapsulated within an ionizable LNP.[82] Furthermore, Blakney et al. observed that lipoplexes prepared with dimethylidioctadecylammonium (DDA) induced humoral and cellular immune responses against Chlamydia trachomatis.[26] Interestingly, Englezou et al. demonstrated that it was possible to deliver saRNA and induce immune responses against influenza by simply complexing the saRNA with DOCTOR, a cationic lipid.[83] These studies demonstrate the versatility and potency of the lipid-based delivery platforms.
4.4 Nanoemulsions

Cationic nanoemulsions (CNE) are also a leading strategy for delivery of saRNA vaccines. The emulsions are typically a water-in-oil emulsion, similar to the license MF59 adjuvant, that consists of squalene, sorbitan trioleate, polysorbate 80 and DOTAP.[84] The main advantage of this platform is that MF59 has a well-defined safety profile in humans.[85] Anderluzzi et al. observed that CNE had the highest induction of antibodies against rabies in a direct comparison with DOTAP polymeric nanoparticles, DOTAP liposomes and DDA liposomes.[86] Bogers et al. showed in the first in nonhuman primate study that CNE enabled immunogenicity equivalent to an adjuvanted protein vaccine against a clade C glycoprotein of HIV-1.[87] CNE is also a versatile delivery platform, and has been shown to generate immune responses against a variety of pathogens including Group A and B Streptococci,[27] HIV-1,[87] influenza,[88] rabies,[86] and VEEV.[34] These studies show that nanoemulsions are a potent and versatile delivery platform for saRNA vaccines.

4.5 Adjuvanted delivery systems

saRNA is considered to be self-adjuvanting due to the dsRNA structures, replicon intermediates and other motifs that are sensed intracellularly.[59] However, recent studies have investigated the role of both the delivery vehicle and molecular components in adjuvanting saRNA vaccines. Blakney et al. observed that the adjuvancy of incorporating 3M-052, a TLR 7/8 agonist, into lipoplexes was eclipsed by the self-adjuvanting effects of saRNA. Démoulins et al. found that incorporating Pam3Cys-SK4 (P3C), a bacterial lipoprotein, promoted saRNA internalization by DCs in vitro but did not enhance humoral or cellular immunogenicity in vivo.[69] Manara et al. found that encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), a chemoattractant, directly in saRNA increased the recruitment of antigen presenting cells (APCs) to the site of injection and increased antigen-specific CD8+ T-cell responses, but did not affect humoral immunity.[89] These studies give insight into strategies regarding enhancing the immunogenicity saRNA vaccines using either molecular or biomaterial adjuvants.

4.5 Delivery platforms in the clinic

The momentum of the field of RNA gene delivery has accelerated in recent years given the 2018 FDA approval of the LNP-formulated siRNA drug, Onpattro,[90] and the recent shot of adrenaline to RNA vaccines in general provided by the COVID-19 global pandemic. There are currently three ongoing saRNA vaccine clinical trials and two in the pre-recruiting phase (Table 3), all of which use either LNP or CNE as a delivery platform. GSK is currently evaluating a VEE-SINV chimeric replicon encoding the rabies glycoprotein formulated with CNE at three different doses; this study is in Phase I and is estimated to complete in April 2021. The Shattock laboratory at Imperial College London is evaluating a VEEV replicon encoding the rabies glycoprotein formulated with CNE at three different doses; this study is in Phase I and is estimated to complete in April 2021. The Shattock laboratory at Imperial College London is evaluating a VEEV replicon encoding the rabies glycoprotein formulated with CNE at three different doses; this study is in Phase I and is estimated to complete in April 2021. Finally, Arcturus Therapeutics is also testing a saRNA vaccine encoding the prefusion spike protein of SARS-CoV-2 formulated in LNP at four doses and is in Phase II of combined Phase I/II trial slated to complete in December 2020. Two upcoming clinical trials will test a VEEV saRNA vaccine given IN directly against ChAdOx[91] and a saRNA VEEV vaccine formulated with CNE against SARS-CoV-2. The major considerations for these clinical trials, other than the humoral and cellular immunity, are the required dose, the vaccine schedule and storage parameters for the formulations.
5. Manufacturing
5.1 Production of self-amplifying mRNA

saRNA is produced \textit{in vitro} using an enzymatic transcription in a similar process to the production of conventional shorter mRNA, although the reaction conditions need to be optimized to increase yields for this longer mRNA. The process for the synthesis of \textit{in vitro} transcribed RNAs was established in the 1990s,[92] predominantly using phage RNA polymerases, and is now a robust and well-established for the large-scale production of synthetic RNA.[93] The production method avoids complex manufacturing and safety issues associated with cell culture production of live viral vaccines, recombinant subunit proteins, and viral vectors (Figure 7). The enzymatic reaction is catalyzed by a phage RNA polymerase, and commercial \textit{in vitro} transcription kits that produce milligram quantities of RNA for research purposes have been available for several years.[94] Pharmaceutical grade mRNA is currently offered as a CDMO service by several companies: TriLink (www.trilinkbiotech.com), Aldevron (www.aldevron.com), Eurogentec (www.eurogentec.com), Biomay (www.biomay.com), Creative Biolabs (www.creative-biolabs.com) and several more will enable capacity in the near future. There are no publications describing the large-scale manufacture of saRNA, but Figure 8 describes the unit operations that would be found in a typical cell-free RNA production process.[94] Capped mRNA is produced enzymatically in a bioreactor and the DNA template is digested. DNA fragments, transcription enzymes, reagents, and byproducts are removed using chromatographic purification followed by tangential flow filtration (TFF). During TFF, due to the large size of the saRNA, lower molecular weight species are removed if the appropriate molecular weight cut-off membrane is selected, and the RNA can diafiltered into the appropriate buffer and adjusted to the required concentration. RNA is then sterile filtered and stored in bulk ready for further downstream processing and formulation.
Figure 7. A comparison of vaccine drug product manufacturing processes for egg- and cell-based manufacturing of conventional vaccines, as well as vaccines produced from viral genome sequence information such as the RNA, protein subunit, and viral vectored DNA vaccines against SARS-CoV-2 from Moderna, Novavax, and Johnson & Johnson respectively. RNA vaccines offer a cell-free manufacturing process that is responsible for many advantages of the platform, allowing facile and rapid vaccine manufacturing. Moderna’s mRNA vaccine against SARS-CoV-2 (mRNA-1273) began clinical trials just 63 days following the publication of the SARS-CoV-2 genome.
Figure 8. Schematic diagram of the manufacturing process for the RNA drug substance. The process involves a cell-free enzymatic in-vitro transcription reaction followed by purification to remove the DNA template, followed by tangential flow filtration (TFF) for buffer exchange and concentration, followed by sterile filtration through a 0.2 µm filter.

In addition to the polymerase enzyme, in vitro transcription reactions typically includes: a linearized DNA template with a promoter sequence (~23 bases) that has a high binding affinity for its respective polymerase; ribonucleotide triphosphates (rNTPs) for the four required bases (adenine, cytosine, guanine and uracil); a ribonuclease inhibitor to inactivate any contaminating RNase; a pyrophosphatase to degrade pyrophosphate, which will inhibit transcription; MgCl\textsubscript{2}, which supplies Mg\textsuperscript{2+} as a co-factor for the polymerase; and a pH buffer, which also contains an antioxidant and a polyamine at the optimal concentrations.[95] If co-transcriptional capping is utilized, the addition of a cap analogue as an initiator of transcription is required.

The recombinant plasmid is propagated in Escherichia coli, linearized using a unique restriction site downstream of the transcription cassette’s 3’ end, and isolated and purified using standard molecular biology techniques. During the in vitro transcription reaction, the bacteriophage polymerase binds the promoter sequence to initiate transcription, and the enzyme then moves along the template towards its 5’ end, elongating the RNA transcript as it travels. Termination of transcription occurs when the enzyme runs off the end of the template (run-off transcription). The poly(A) tail can be encoded into the DNA template, or, alternatively, it can be added enzymatically post-transcription.[96] When the in vitro transcription reaction is complete, the DNA template is fragmented with a DNase, and RNA is recovered using several methods, including precipitation or chromatography. The quality and quantity of RNA produced in an in vitro transcription reaction depends upon a number of factors, including RNA transcript size, template concentration, reaction time and temperature, Mg\textsuperscript{2+} concentration, and NTP concentration.[97] Typically, the conditions require some optimization for each type of construct being produced.

While there is no published data on a large-scale production process for saRNA, the following sections on capping, purification, immunostimulatory by-products, and stability highlight areas that should be consider during process development.

5.1.1 Capping strategies for saRNA

The IVT mRNA can be capped either by post-transcriptional modification using capping enzymes [98, 99] based on the recombinant vaccinia virus, or by the addition of a cap analog during in vitro transcription.[94, 98] Enzymatic capping is more complex but provides much higher yields, capping efficiency is nearly 100% efficient and all capped structures are added in the proper orientation.[98] Enzymatic capping is being used for large-scale and laboratory production, and cap 0 and cap 1 structures can be produced.[94] Co-transcriptional capping with a cap analog is another approach to prepare the IVT mRNA, where a cap analog is
provided in excess in the transcription reaction. This process is much simpler compared to the enzymatic capping reaction, but the overall yields tend to be lower and various cap structures can be incorporated with more diverse designs. [100-102] The historical issue with the pseudo-symmetrical cap have now been circumvented with anti-reverse cap analogues (ARCAs),[102] which results in a cap 0 structure on approximately 70% of the transcripts and 30% with a 5’ triphosphate. To increase capping efficiency, trimer analogues such as CleanCap[93] have been introduced, which incorporate a cap 1 structure. For saRNA applications, there have not been any published studies comparing the potency of the different capping strategies.

5.1.2 Purification strategies for saRNA

mRNA has a negatively charged phosphodiester backbone, and many of the purification techniques used for pDNA could potentially be adapted to the purification of this molecule. DNA purification techniques include: Size-exclusion chromatography (SEC), reversed-phase chromatography (RPC), anion-exchange chromatography (AIEX), hydrophobic interaction (HIC) and thiophilic adsorption chromatography (TOC).[103] For routine pre-clinical work and in vivo immunization studies, RNA can be precipitated. The polar nature of the negatively charged backbone makes RNA highly soluble in water and several cations (lithium chloride is the most widely used) in combination with ice-cold ethanol as a co-solvent can neutralize the backbone charges and decrease solubility to precipitate the RNA out of solution.[104] However, implementing such a process for GMP production would be extremely challenging. Self-amplifying mRNA with sizes in the order of 10,000 bases (MW ~3MDa), have additional challenges over smaller conventional mRNAs and no commercially viable scalable process has been disclosed to date. Review articles on RNA purification[94, 105, 106] indicate that several techniques could be potentially be utilized and these include: Ion exchange (IE), affinity (AC) and SEC. Thus, there remains a need for improved RNA purification methods for saRNA, that will enable cost and time efficient purification at an industrial scale with high yield and pharmaceutical grade purity, while retaining the stability, biological potency and functionality of the RNA. Large-scale chromatographic purification of saRNA is complex and is an active area of research for many companies and academic institutions.

5.1.3 Immunostimulatory IVT reaction by-products

Theoretically the capping strategy could have a positive or negative influence of the potency of the vaccine, since uncapped RNA and different cap structures are known to trigger an antiviral responses.[107] Mechanism of action studies with saRNA vaccines have shown this could potentially lead to reduced potency,[54] but there is no published data exploring how the capping strategy could influence the potency of a saRNA vaccine.

The IVT reaction is known to produce by-products that are immune-stimulatory in the form of double stranded RNA (dsRNA). Recent studies have identified two main types of byproducts in the IVT reaction that result in the formation of dsRNA molecules.[108, 109] The first is formed by 3’-extension of the run-off products annealing to complementary sequences in the body of the run-off transcript either in cis (by folding back on the same RNA molecule) or trans (annealing to a second RNA molecule) to form extended duplexes.[110] The second type of dsRNA molecules is formed by the hybridization of an antisense RNA molecule to the run-off transcript,[93] produced by promoter-independent transcriptional initiation. dsRNA has been removed from conventional base-modified mRNA using ion pair reversed-phase HPLC,[111] but this method is not scalable, the acetonitrile eluent is very toxic, and there is no evidence it would work for saRNA. A better approach, although not tested with saRNA, would be to utilize the selective binding of dsRNA to cellulose in an ethanol-containing buffer.[111] Alternatively, as described recently, mRNA can be produced by combining high-temperature IVT with template-encoded poly(A) tailing.[112] This process reduced the formation of both kinds of dsRNA by-products, generating functional mRNAs with reduced immunogenicity. It should be noted that
neither of these techniques were used with larger saRNA. Theoretically, the presence of dsRNA could have a positive or negative influence of the potency of the vaccine since dsRNA is known to trigger antiviral responses.[103] Mechanism of action studies with saRNA vaccines have shown this could potentially lead to reduced potency,[104] but there are no published data exploring how the presence of dsRNA could influence the potency of a saRNA vaccine.

5.1.4 Stability of mRNA

There are considerable differences in stability between DNA and RNA. With over 20 years of extensive research and development of pDNA vaccines, a rationally designed liquid formulation that is stable for 1 year at 30 °C has been developed.[113] This degree of stability is unlikely in mRNA vaccines, because RNA contains a 2’hydroxyl group on the ribose, which is hydrolytically much less stable than the deoxyribose. Theoretical calculations for the 5 °C stability for a “naked” 4000 nucleotide mRNA in bulk solution (PBS, pH 7.4, no Mg$^{2+}$) estimate a half-life of 900 days.[114] However, a rise in temperature to 37 °C is predicted to lead to a reduced half-life of 5.4 days. Longer self-amplifying mRNA (12 kB) was calculated to have exacerbated hydrolysis (3-fold higher), with an expected half-life of 314 days at 5 °C and 2 days at 37 °C.

During production (IVT reaction and downstream processing) the mRNA is going to be subject to high concentrations of Mg$^{2+}$ and elevated temperatures,[115] which need to be mitigated as much as possible to limit hydrolysis. A largely unexplored strategy and theoretical basis to reduce mRNA hydrolysis is to redesign RNAs to form double-stranded regions, which are protected from in-line cleavage and enzymatic degradation, while coding for the same proteins.[114]

RNA is more sensitive than DNA to oxidation, alkylation or electrophilic additions which result in hydrolysis of glycosidic bonds.[113] In addition, RNA is prone to enzymatic degradation with three major classes of RNA-degrading enzymes (ribonucleases or RNases): endonucleases (which cut RNA internally), 5’ exonucleases (which hydrolyze RNA from the 5’ end), and 3’ exonucleases (which degrade RNA from the 3’ end).[113] Therefore, after production of the saRNA it is generally stored at -80 °C and great care is taken to avoid the introduction of RNases. The optimal pH to store RNA is in the range of pH=4-5 [4], since RNA is susceptible to alkaline hydrolysis at pH>6, and acid hydrolysis only occurs at pH<2.

During delivery after intramuscular vaccination, saRNA is susceptible to hydrolysis due to the presence of high levels of Mg$^{2+}$ ions and a body temperature of 37 °C, and RNase degradation. Encapsulation in an LNP has been shown to limit enzymatic degradation,[79] but it should be noted that the saRNA encapsulated in lipid formulations may be subject to increased hydrolysis if the lipid’s cationic headgroups lower the pKa of the ribose 2’ hydroxyl group.[114]

5.2 Manufacturing considerations for formulated mRNA drug product

While the manufacturing and production process for the formulated mRNA drug product can differ considerably depending on the type of formulation, a clinically relevant manufacturing process can be generalized into four steps: 1) formulation, which involves one or more mixing steps, 2) downstream processing and purification, 3) sterile filtration through a 0.2 µm filter, 4) fill and finish. Presently, little information has been published regarding the specific manufacturing processes utilized for saRNA vaccine candidates currently in clinical trials. Hence, the preclinical processes for each formulation type will be generalized from methods published in the literature for lipid nanoparticles and nanoemulsions. To focus on potential clinical production, scalable continuous flow process steps are favored over fixed-volume processes.
5.2.1 Production of lipid nanoparticles

Anderluzzi et al. demonstrated the versatility of the NanoAssemblr microfluidic mixers to produce a variety of saRNA formulations such as liposomes, solid lipid nanoparticles and polymer nanoparticles by continuous flow solvent/antisolvent precipitation.[86] Lou et al. also demonstrated the use of the same platform for saRNA formulations with ionizable LNPs.[80] The technology has been established for producing other RNA-LNP formulations [116-120] including base-modified mRNA vaccines.[18, 121, 122] The process involved rapid advective mixing of a water-miscible organic solvent containing dissolved lipids or polymers with an aqueous phase containing dissolved saRNA at optimized flow rates and organic/aqueous flow rate ratios to control the precipitation conditions. When using ionizable cationic lipids, the aqueous phase containing dissolved saRNA is buffered at pH 4 - below the pKa of the ionizable lipid. Other in-line mixing methods have also been employed for saRNA-LNP formulations including alternative microfluidic architectures[82] and in-line macro-mixing in a T-tube.[35, 51, 52, 78, 79] To remove solvent and adjust the final concentration, tangential flow filtration has been employed as a high-throughput method.[51, 78]

5.2.2 Product of nanoemulsions

Cationic nanoemulsions employ two immiscible phases and thus required a different method for production. The processes described in the literature generally involved dissolving the cationic lipid and a hydrophobic surfactant in squalene. The resulting oil phase is mixed with a mildly acidic buffer containing a hydrophilic surfactant to create a primary emulsion. The primary emulsion is then repeatedly passed through a high-pressure homogenizer to obtain a more homogeneous nanoemulsion. The resulting emulsion is complexed with saRNA by mixing and incubating at 4°C for 30 to 120 min. [86-88] The formulation is then sterilized by passage through a 0.2 µm filter.[123] High-pressure homogenization has been established for large scale production of lipid-based colloids for drug delivery.[124-128]

6. Future Outlook

While historical (pre-2015) preclinical studies of saRNA vaccines were predominantly focused on viral replicon particles and cancer applications, the field has more recently shifted to applications in viral infectious diseases, although a few studies have also explored prevention of parasitic and bacterial infections. The investigation of saRNA to passive immunization by encoding a monoclonal antibody is also a highly promising application that warrants further development. The clinical trials for rabies and SARS-CoV-2 are an exciting opportunity for the field of saRNA vaccines, and will no doubt be informative as to the characteristics of the immune response, required dose, duration of immunity, and required regimen. The field is also starting to consider methods to modulate the innate response to saRNA, which will no doubt be imperative to the clinical success of these vaccines, so that the lesson of DNA vaccine clinical trials are not forgotten.[129] One strategy that may facilitate efficacious saRNA vaccines is utilizing evaluation models, such as skin explants that have human immune cells and innate sensing, in order to optimize molecular and delivery components. While the SARS-CoV-2 global pandemic has been detrimental to economies and health, it’s provided a valuable opportunity to test saRNA vaccines in the clinical that otherwise might have taken decades. Given the short timespan required to design and test new saRNA vaccines (reportedly as little as 14 days in the case of Imperial College London),[130] it is clear that this platform is particularly well-suited to outbreaks, and also possibly seasonal vaccines, such as influenza. The rapid and easy manufacture of saRNA vaccines may also pave the way for a distributed manufacturing model where vaccines are produced locally in order to minimize logistical and cold-chain issues that could hinder widespread distribution of a vaccine. Overall, saRNA vaccines have made
monumental strides in the past five years, and the next five years will be telling as to the clinical utility and success of this promising vaccine platform.

REFERENCES

1. Oberfeld, Blake, Aditya Achanta, Kendall Carpenter, Pamela Chen, Nicole M. Gillette, Pinky Langat, Jordan Taylor Said, Abigail E. Schiff, Allen S. Zhou, Amy K. Barczak, and Shiv Pillai. "Snapshot: Covid-19." Cell 181, no. 4 (2020): 954-54.e1.

2. Zhou, Peng, Xing-Lou Yang, Xian-Guang Wang, Ben Hu, Lei Zhang, Wei Zhang, Hao-Rui Si, Yan Zhu, Bei Li, Chao-Lin Huang, Hui-Dong Chen, Jing Chen, Yun Luo, Hua Guo, Ren-Di Jiang, Mei-Qin Liu, Ying Chen, Xu-Rui Shen, Xi Wang, Xiao-Shuang Zheng, Kai Zhao, Quan-Jiao Chen, Fei Deng, Lin-Lin Liu, Bing Yan, Fa-Xian Zhan, Yan-Yi Wang, Geng-Fu Xiao, and Zheng-Li Shi. "A Pneumonia Outbreak Associated with a New Coronavirus of Probable Bat Origin." Nature 579, no. 7798 (2020): 270-73.

3. Sohrabi, Catrin, Zaid Alsafi, Niamh O’Neill, Mehdi Khan, Ahmed Kerwan, Ahmed Al-Jabir, Christos Iosifidis, and Riaz Agha. "World Health Organization Declares Global Emergency: A Review of the 2019 Novel Coronavirus (Covid-19)." International Journal of Surgery 76 (2020): 71-76.

4. Koirala, Archana, Ye Jin Joo, Ameneh Khatami, Clayton Chiu, and Philip N. Britton. "Vaccines for Covid-19: The Current State of Play." Paediatric Respiratory Reviews 35 (2020): 43-49.

5. Bloom, Kristie, Fiona van den Berg, and Patrick Arbuthnot. "Self-Amplifying Rna Vaccines for Infectious Diseases." Gene Therapy (2020).

6. Funk, Colin D., Craig Laferrière, and Ali Ardakani. "A Snapshot of the Global Race for Vaccines Targeting Sars-Cov-2 and the Covid-19 Pandemic." Frontiers in Pharmacology 11, no. 937 (2020).

7. Anderson, Evan J., Nadine G. Rouphael, Alicia T. Widge, Lisa A. Jackson, Paul C. Roberts, Mamodikoe Makhene, James D. Chappell, Mark R. Denison, Laura J. Stevens, Andrea J. Prijssers, Adrian B. McDermott, Britta Flach, Bob C. Lin, Nicole A. Doria-Rose, Sijy O’Dell, Stephen D. Schmidt, Kizzmekia S. Corbett, Phillip A. Swanson, Marcelino Padilla, Kathy M. Neuzil, Hamilton Bennett, Brett Leav, Mat Makowski, Jim Albert, Kaitlyn Cross, Venkata Viswanadh Edara, Katharine Floyd, Mehul S. Suthar, David R. Martinez, Ralph Baric, Wendy Buchanan, Catherine J. Luke, Varun K. Phadke, Christina A. Rostad, Julie E. Ledgerwood, Barney S. Graham, and John H. Beigel. "Safety and Immunogenicity of Sars-Cov-2 Mrna-1273 Vaccine in Older Adults." New England Journal of Medicine (2020).

8. Jackson, Lisa A., Evan J. Anderson, Nadine G. Rouphael, Paul C. Roberts, Mamodikoe Makhene, Rhea N. Coler, Michele P. McCullough, James D. Chappell, Mark R. Denison, Laura J. Stevens, Andrea J. Prijssers, Adrian McDermott, Britta Flach, Nicole A. Doria-Rose, Kizzmekia S. Corbett, Kaitlyn M. Morabito, Sijy O’Dell, Stephen D. Schmidt, Phillip A. Swanson, Marcelino Padilla, John R. Mascola, Kathleen M. Neuzil, Hamilton Bennett, Wellington Sun, Etza Peters, Mat Makowski, Jim Albert, Kaitlyn Cross, Wendy Buchanan, Rhonda Pikaart-Taute, Julie E. Ledgerwood, Barney S. Graham, and John H. Beigel. "An Mrna Vaccine against Sars-Cov-2 — Preliminary Report." New England Journal of Medicine (2020).

9. Corbett, Kizzmekia S., Darin Edwards, Sarah R. Leist, Olubukola M. Abiona, Seyhan Boyoglu-Barnum, Rebecca A. Gillespie, Sunny Himansu, Alexandra Schäfer, Cynthia T. Ziwa wo, Anthony T. DiPiazza, Kenneth H. Dinnon, Sayda M. Elbashir, Christine A. Shaw, Angela Woods, Ethan J. Fritch, David R. Martinez, Kevin W. Bock, Mahnaz Minai, Bianca M. Nagata, Geoffrey B. Hutchinson, Kapil Bahl, Dario Garcia-Dominguez, LingZhi Ma,
Isabella Renzi, Wing-Pui Kong, Stephen D. Schmidt, Lingshu Wang, Yi Zhang, Laura J. Stevens, Emily Phung, Lauren A. Chang, Rebecca J. Loomis, Nedim Emil Altaras, Elisabeth Narayanan, Mihr Metkar, Vlad Presnyak, Catherine Liu, Mark K. Louder, Wei Shi, Kwanyee Leung, Eun Sung Yang, Ande West, Kendra L. Gully, Nianshuang Wang, Daniel Wrapp, Nicole A. Doria-Rose, Guillaume Stewart-Jones, Hamilton Bennett, Martha C. Nason, Tracy J. Ruckwardt, Jason S. McLellan, Mark R. Denison, James D. Chappell, Ian N. Moore, Kaitlyn M. Morabito, John R. Mascola, Ralph S. Baric, Andrea Carfi, and Barney S. Graham. "Sars-Cov-2 Mrna Vaccine Development Enabled by Prototype Pathogen Preparedness." bioRxiv (2020): 2020.06.11.145920.

10. Cohen, Jon. "Vaccine Designers Take First Shots at Covid-19." Science 368, no. 6486 (2020): 14.

11. Polack, Fernando P., Stephen J. Thomas, Nicholas Kitchin, Judith Absalon, Alejandra Gurtman, Stephen Lockhart, John L. Perez, Gonzalo Pérez Marc, Edson D. Moreira, Cristiano Zerbini, Ruth Bailey, Kena A. Swanson, Satrajit Roychoudhury, Kenneth Koury, Ping Li, Warren V. Kalina, David Cooper, Robert W. Frenck, Laura L. Hammitt, Özlem Türeci, Haylene Nell, Axel Schaefer, Serhat Ünal, Dina B. Tresnan, Susan Mather, Philip R. Dormitzer, Ügur Şahin, Kathrin U. Jansen, and William C. Gruber. "Safety and Efficacy of the Bnt162b2 Mrna Covid-19 Vaccine." New England Journal of Medicine (2020).

12. Corbett, Kizzmekia S., Barbara Flynn, Kathryn E. Foulds, Joseph R. Francica, Seyhan Boyoglu-Barnum, Anne P. Werner, Britta Flach, Sarah O’Connell, Kevin W. Bock, Mahnaz Minai, Bianca M. Nagata, Hanne Andersen, David R. Martinez, Amy T. Noe, Naomi Douek, Mitzi M. Donaldson, Nadesh N. Nji, Gabriela S. Alvarado, Darin K. Edwards, Dillon R. Flebbe, Evan Lamb, Nicole A. Doria-Rose, Bob C. Lin, Mark K. Louder, Siy O’Dell, Stephen D. Schmidt, Emily Phung, Lauren A. Chang, Christina Yap, John-Paul M. Todd, Laurent Pessaint, Alex Van Ry, Shanai Browne, Jack Greenhouse, Tammy Putman-Taylor, Amanda Strasbaugh, Tracey-Ann Campbell, Anthony Cook, Alan Dodson, Katelyn Steinigrebe, Wei Shi, Yi Zhang, Olubukola M. Abiona, Lingshu Wang, Amarendra Pegu, Eun Sung Yang, Kwanyee Leung, Tongqing Zhou, I. Ting Teng, Alicia Widge, Ingelise Gordon, Laura Novik, Rebecca A. Gillespie, Rebecca J. Loomis, Juan I. Moliva, Guillaume Stewart-Jones, Sunny Himansu, Wing-Pui Kong, Martha C. Nason, Kaitlyn M. Morabito, Tracy J. Ruckwardt, Julie E. Ledgerwood, Martin R. Gaudinski, Peter D. Kwong, John R. Mascola, Andrea Carfi, Mark G. Lewis, Ralph S. Baric, Adrian McDermott, Ian N. Moore, Nancy J. Sullivan, Mario Roederer, Robert A. Seder, and Barney S. Graham. "Evaluation of the Mrna-1273 Vaccine against Sars-Cov-2 in Nonhuman Primates." New England Journal of Medicine 383, no. 16 (2020): 1544-55.

13. Hekele, Armin, Sylvie Bertholet, Jacob Archer, Daniel G. Gibson, Giuseppe Palladino, Luis A. Brito, Gillis R. Otten, Michela Brazzoli, Scilla Buffato, Alessandra Bonci, Daniele Casini, Domenico Maione, Zhi-Qing Qi, John E. Gill, Nicky C. Caiazzu, Jun Urano, Bolyn Hubby, George F. Gao, Yuelong Shu, Ennio De Gregorio, Christian W. Mandl, Peter W. Mason, Ethan C. Settembre, Jeffrey B. Ulmer, J. Craig Venter, Philip R. Dormitzer, Rino Rappuoli, and Andrew J. Geall. "Rapidly Produced Sam® Vaccine against H7n9 Influenza Is Immunogenic in Mice." Emerging Microbes & Infections 2, no. 1 (2013): 1-7.

14. Kis, Zoltán, Robin Shattock, Nilay Shah, and Cleo Kontoravdi. "Emerging Technologies for Low-Cost, Rapid Vaccine Manufacture." Biotechnology Journal 14, no. 1 (2019): 1800376.

15. Maruggi, G., C. Zhang, J. Li, J. B. Ulmer, and D. Yu. "Mrna as a Transformative Technology for Vaccine Development to Control Infectious Diseases." Mol Ther 27, no. 4 (2019): 757-72.

16. Ulmer, J. B., M. K. Mansoura, and A. J. Geall. "Vaccines 'on Demand': Science Fiction or a Future Reality." Expert Opin Drug Discov 10, no. 2 (2015): 101-6.
17. Feldman, R. A., R. Fuhr, I. Smoloven, A. Mick Ribeiro, L. Panther, M. Watson, J. J. Senn, M. Smith, Ö Almarsson, H. S. Pajar, M. E. Laska, J. Thompson, T. Zaks, and G. Ciaramella. "Mrna Vaccines against H10n8 and H7n9 Influenza Viruses of Pandemic Potential Are Immunogenic and Well Tolerated in Healthy Adults in Phase 1 Randomized Clinical Trials." *Vaccine* 37, no. 25 (2019): 3326-34.

18. Bahl, Kapil, Joe J. Senn, Olga Yuzhakov, Alex Bulychev, Luis A. Brito, Kimberly J. Hassett, Michael E. Laska, Mike Smith, Örn Almarsson, James Thompson, Amilcar Ribeiro, Mike Watson, Tal Zaks, and Giuseppe Ciaramella. "Preclinical and Clinical Demonstration of Immunogenicity by Mrna Vaccines against H10n8 and H7n9 Influenza Viruses." *Molecular Therapy* 25, no. 6 (2017): 1316-27.

19. Petsch, Benjamin, Margit Schnee, Annette B. Vogel, Elke Lange, Bernd Hoffmann, Daniel Voss, Thomas Schlake, Andreas Thess, Karl-Josef Kallen, Lothar Stitz, and Thomas Kramps. "Protective Efficacy of in Vitro Synthesized, Specific Mrna Vaccines against Influenza a Virus Infection." *Nature biotechnology* 30, no. 12 (2012): 1210-16.

20. Scorza, F. B., and N. Pardi. "New Kids on the Block: Rna-Based Influenza Virus Vaccines." *Vaccines (Basel)* 6, no. 2 (2018).

21. Lundstrom, Kenneth. "Self-Amplifying Rna Viruses as Rna Vaccines." *International journal of molecular sciences* 21, no. 14 (2020): 5130.

22. DeFrancesco, L. "The 'Anti-Hype' Vaccine." *Nat Biotechnol* 35, no. 3 (2017): 193-97.

23. Jackson, Nicholas A. C., Kent E. Kester, Danilo Casimiro, Sanjay Gurunathan, and Frank DeRosa. "The Promise of Mrna Vaccines: A Biotech and Industrial Perspective." *npj Vaccines* 5, no. 1 (2020): 11.

24. Lutz, Johannes, Sandra Lazzaro, Mohamed Habbeddine, Kim Ellen Schmidt, Patrick Baumhof, Barbara L. Mui, Ying K. Tam, Thomas D. Madden, Michael J. Hope, Regina Heidenreich, and Mariola Fotin-Mleczek. "Unmodified Mrna in Lnps Constitutes a Competitive Technology for Prophylactic Vaccines." *npj Vaccines* 2, no. 1 (2017): 29.

25. Pardi, Norbert, Kaela Parkhouse, Ericka Kirkpatrick, Meagan McMahon, Seth J. Zost, Barbara L. Mui, Ying K. Tam, Katalin Karikó, Christopher J. Barbosa, Thomas D. Madden, Michael J. Hope, Florian Krammer, Scott E. Hensley, and Drew Weissman. "Nucleoside-Modified Mrna Immunization Elicits Influenza Virus Hemagglutinin Stalk-Specific Antibodies." *Nature Communications* 9, no. 1 (2018): 3361.

26. Blakney, Anna K., Paul F. McKay, Dennis Christensen, Bárbara Ibarzo Yus, Yoann Aldon, Frank Follmann, and Robin J. Shattock. "Effects of Cationic Adjuvant Formulation Particle Type, Fluidity and Immunomodulators on Delivery and Immunogenicity of Sarna." *Journal of Controlled Release* 304 (2019): 65-74.

27. Maruggi, Giulietta, Emiliano Chiarot, Cinzia Giovani, Scilla Buccato, Stefano Bonacci, Elisabetta Frigimelica, Immaculada Margarit, Andrew Geall, Giuliano Bensi, and Domenico Maione. "Immunogenicity and Protective Efficacy Induced by Self-Amplifying Mrna Vaccines Encoding Bacterial Antigens." *Vaccine* 35, no. 2 (2017): 361-68.

28. Chahal, J. S., O. F. Khan, C. L. Cooper, J. S. McPartlan, J. K. Tsoie, L. D. Tilley, S. M. Sidik, S. Lourido, R. Langer, S. Bavari, H. L. Ploegh, and D. G. Anderson. "Dendrimer-Rna Nanoparticles Generate Protective Immunity against Lethal Ebola, H1n1 Influenza, and Toxoplasma Gondii Challenges with a Single Dose." *Proc Natl Acad Sci U S A* 113, no. 29 (2016): E4133-42.

29. Luo, Fangjun, Lina Zheng, Yue Hu, Shuxian Liu, Yan Wang, Zhongkui Xiong, Xin Hu, and Feng Tan. "Induction of Protective Immunity against Toxoplasma Gondii in Mice by Nucleoside Triphosphate Hydrolase-Ii (Ntase-Ii) Self-Amplifying Rna Vaccine Encapsulated in Lipid Nanoparticle (Lnp)." *Frontiers in Microbiology* 8, no. 605 (2017).

30. Li, Yingzhong, Zhijun Su, Weiuyu Zhao, Xinfu Zhang, Noor Momin, Chengxiang Zhang, K. Dane Wittrup, Yizhou Dong, Darrell J. Irvine, and Ron Weiss. "Multifunctional Oncolytic
Nanoparticles Deliver Self-Replicating IL-12 Rna to Eliminate Established Tumors and Prime Systemic Immunity." Nature Cancer 1, no. 9 (2020): 882-93.

31. Li, Yingzhong, Brian Teague, Yuan Zhang, Zhijun Su, Ely Porter, Brian Dobosh, Tyler Wagner, Darrell J. Irvine, and Ron Weiss. "In Vitro Evolution of Enhanced Rna Replicons for Immunotherapy." Scientific Reports 9, no. 1 (2019): 6932.

32. Erasmus, Jesse H., Jacob Archer, Jasmine Fuerte-Stone, Amit P. Khandhar, Emily Voigt, Brian Granger, Robin G. Bombardi, Jennifer Govero, Qing Tan, Lorellin A. Durnell, Rhea N. Coler, Michael S. Diamond, James E. Crowe, Steven G. Reed, Larissa B. Thackray, Robert H. Carnahan, and Neal Van Hoeven. "Intramuscular Delivery of Replicon Rna Encoding Zikv-117 Human Monoclonal Antibody Protects against Zika Virus Infection." Molecular Therapy - Methods & Clinical Development 18 (2020): 402-14.

33. Blakney, A. K., P. F. McKay, C. R. Bouton, K. Hu, K. Samnuan, and R. J. Shattock. "Innate Inhibiting Proteins Enhance Expression and Immunogenicity of Self-Amplifying Rna." Molecular Therapy In Press (2020).

34. Samsa, Marcelo M., Lesley C. Dupuy, Clayton W. Beard, Carolyn M. Six, Connie S. Schmaljohn, Peter W. Mason, Andrew J. Geall, Jeffrey B. Ulmer, and Dong Yu. "Self-Amplifying Rna Vaccines for Venezuelan Equine Encephalitis Virus Induce Robust Protective Immunogenicity in Mice." Molecular Therapy 27, no. 4 (2019): 850-65.

35. Magini, Diletta, Cinzia Giovani, Simona Mangiavacchi, Silvia Maccari, Raffaella Cecchi, Jeffrey B. Ulmer, Ennio De Gregorio, Andrew J. Geall, Michela Brazzoli, and Sylvie Bertholet. "Self-Amplifying Mrna Vaccines Expressing Multiple Conserved Influenza Antigens Confer Protection against Homologous and Heterosubtypic Viral Challenge." PloS one 11, no. 8 (2016): e0161193.

36. Baeza Garcia, Alvaro, Edwin Siu, Tiffany Sun, Valerie Exler, Luis Brito, Armin Hekele, Gib Otten, Kevin Augustijn, Chris J. Janse, Jeffrey B. Ulmer, Jürgen Bernhagen, Erol Fikrig, Andrew Geall, and Richard Bucala. "Neutralization of the Plasmodium-Encoded Mif Ortholog Confers Protective Immunity against Malaria Infection." Nature Communications 9, no. 1 (2018): 2714.

37. Kose, Nurgun, Julie M. Fox, Gopal Sapparapu, Robin Bombardi, Rashika N. Tennekoon, A. Dharshan de Silva, Sayda M. Elbashir, Matthew A. Theisen, Elisabeth Humphris-Narayanan, Giuseppe Ciaramella, Sunny Himansu, Michael S. Diamond, and James E. Crowe. "A Lipid-Encapsulated Mrna Encoding a Potently Neutralizing Human Monoclonal Antibody Protects against Chikungunya Infection." Science Immunology 4, no. 35 (2019): eaaw6647.

38. Diken, M., L. M. Kranz, S. Kreiter, and U. Sahin. "Mrna: A Versatile Molecule for Cancer Vaccines." Curr Issues Mol Biol 22 (2017): 113-28.

39. Fiedler, K., S. Lazzaro, J. Lutz, S. Rauch, and R. Heidenreich. "Mrna Cancer Vaccines." Recent Results Cancer Res 209 (2016): 61-85.

40. Lundstrom, Kenneth. "Alphavirus-Based Antigen Preparation." In Vaccine Delivery Technology: Methods and Protocols, edited by Blaine A. Pfeifer and Andrew Hill, 63-81. New York, NY: Springer US, 2021.

41. Tews, B. A., and G. Meyers. "Self-Replicating Rna." Methods Mol Biol 1499 (2017): 15-35.

42. Ljungberg, K., and P. Liljeström. "Self-Replicating Alphavirus Rna Vaccines." Expert Rev Vaccines 14, no. 2 (2015): 177-94.

43. Rupp, J. C., K. J. Sokoloski, N. N. Gebhart, and R. W. Hardy. "Alphavirus Rna Synthesis and Non-Structural Protein Functions." J Gen Virol 96, no. 9 (2015): 2483-500.

44. Götte, B., L. Liu, and G. M. McInerney. "The Enigmatic Alphavirus Non-Structural Protein 3 (Nsp3) Revealing Its Secrets at Last." Viruses 10, no. 3 (2018).

45. Pietilä, M. K., K. Hellström, and T. Ahola. "Alphavirus Polymerase and Rna Replication." Virus Res 234 (2017): 44-57.
46. Fros, Jelke J., and Gorben P. Pijlman. "Alphavirus Infection: Host Cell Shut-Off and Inhibition of Antiviral Responses." *Viruses* 8, no. 6 (2016): 166.

47. Hyde, J. L., R. Chen, D. W. Trobaugh, M. S. Diamond, S. C. Weaver, W. B. Klimstra, and J. Wilusz. "The 5' and 3' Ends of Alphavirus Rnas--Non-Coding Is Not Non-Functional." *Virus Res* 206 (2015): 99-107.

48. Carrasco, L., M. A. Sanz, and E. González-Almela. "The Regulation of Translation in Alphavirus-Infected Cells." *Viruses* 10, no. 2 (2018).

49. Lello, Laura Sandra, Age Utt, Koen Bartholomeeusen, Sainan Wang, Kai Rausalu, Catherine Kendall, Sandra Coppens, Rennos Fragkoudis, Andrew Tuplin, Luke Alphey, Kevin K. Ariën, and Andres Merits. "Cross-Utilisation of Template Rnas by Alphavirus Replicases." *PLoS Pathogens* 16, no. 9 (2020): e1008825.

50. Vogel, Annette B., Laura Lambert, Ekaterina Kinnear, David Busse, Stephanie Erbar, Kerstin C. Reuter, Lena Wicke, Mario Perkovic, Tim Beissert, Heinrich Haas, Stephen T. Reece, Ugur Sahin, and John S. Tregoning. "Self-Amplifying Rna Vaccines Give Equivalent Protection against Influenza to Mrna Vaccines but at Much Lower Doses." *Molecular Therapy* 26, no. 2 (2018): 446-55.

51. Pepini, Timothy, Anne-Marie Pulichino, Thomas Carsillo, Alicia L. Carlson, Farid Sari-Sarraf, Katrin Ramsauer, Jason C. Debasitis, Giulietta Maruggi, Gillis R. Otten, Andrew J. Geall, Dong Yu, Jeffrey B. Ulmer, and Carlo Iavarone. "Induction of an Ifn-Mediated Antiviral Response by a Self-Amplifying Rna Vaccine: Implications for Vaccine Design." *The journal of immunology* 198, no. 10 (2017): 4012.

52. Lazzaro, Sandra, Cinzia Giovani, Simona Mangiavacchi, Diletta Magini, Domenico Maione, Barbara Baudner, Andrew J. Geall, Ennio De Gregorio, Ugo D’Oro, and Cecilia Buonsanti. "Cd8 T-Cell Priming Upon Mrna Vaccination Is Restricted to Bone-Marrow-Derived Antigen-Presenting Cells and May Involve Antigen Transfer from Myocytes." *Immunology* 146, no. 2 (2015): 312-26.

53. Iwasaki, Akiko, and Ruslan Medzhitov. "Control of Adaptive Immunity by the Innate Immune System." *Nature Immunology* 16, no. 4 (2015): 343-53.

54. Pepini, T., A. M. Pulichino, T. Carsillo, A. L. Carlson, F. Sari-Sarraf, K. Ramsauer, J. C. Debasitis, G. Maruggi, G. R. Otten, A. J. Geall, D. Yu, J. B. Ulmer, and C. Iavarone. "Induction of an Ifn-Mediated Antiviral Response by a Self-Amplifying Rna Vaccine: Implications for Vaccine Design." *J Immunol* 198, no. 10 (2017): 4012-24.

55. Iavarone, C., T. O’Hagan D, D. Yu, N. F. Delahaye, and J. B. Ulmer. "Mechanism of Action of Mrna-Based Vaccines." *Expert Rev Vaccines* 16, no. 9 (2017): 871-81.

56. Blakney, A. K., P. F. McKay, and R. J. Shattock. "Structural Components for Amplification of Positive and Negative Strand Veev Splitzicons." *Front Mol Biosci* 5 (2018): 71.

57. Beissert, Tim, Mario Perkovic, Annette Vogel, Stephanie Erbar, Kerstin C. Walzer, Tina Hempel, Silke Brill, Erik Haefner, René Becker, Özlem Türeci, and Ugur Sahin. "A Trans-Amplifying Rna Vaccine Strategy for Induction of Potent Protective Immunity." *Molecular Therapy* 28, no. 1 (2020): 119-28.

58. Kallen, K. J., R. Heidenreich, M. Schnee, B. Petsch, T. Schlake, A. Thess, P. Baumhof, B. Scheel, S. D. Koch, and M. Fotin-Mleczek. "A Novel, Disruptive Vaccination Technology: Self-Adjuvanted Rnactive(®) Vaccines." *Hum Vaccin Immunother* 9, no. 10 (2013): 2263-76.

59. Pardi, Norbert, Michael J. Hogan, Frederick W. Porter, and Drew Weissman. "Mrna Vaccines — a New Era in Vaccinology." *Nature Reviews Drug Discovery* 17, no. 4 (2018): 261-79.

60. De Haro, César, Raúl Méndez, and Javier Santoyo. "The Eif-2α Kinases and the Control of Protein Synthesis1." *The FASEB Journal* 10, no. 12 (1996): 1378-87.

61. Liang, S. L., D. Quirk, and A. Zhou. "Rnase L: Its Biological Roles and Regulation." *IUBMB life* 58, no. 9 (2006): 508-14.
62. Beissert, T., L. Koste, M. Perkovic, K. C. Walzer, S. Erbar, A. Selmi, M. Diken, S. Kreiter, Ö Türeci, and U. Sahin. "Improvement of in Vivo Expression of Genes Delivered by Self-Amplifying Rna Using Vaccinia Virus Immune Evasion Proteins." *Hum Gene Ther* 28, no. 12 (2017): 1138-46.

63. Liu, Yi, Jas Min Chin, En Lin Choo, and Kyle K. L. Phua. "Messenger Rna Translation Enhancement by Immune Evasion Proteins: A Comparative Study between Ekb (Vaccinia Virus) and Ns1 (Influenza a Virus)." *Scientific Reports* 9, no. 1 (2019): 11972.

64. Yoshioka, N., E. Gros, H. R. Li, S. Kumar, D. C. Deacon, C. Maron, A. R. Muotri, N. C. Chi, X. D. Fu, B. D. Yu, and S. F. Dowdy. "Efficient Generation of Human Ipscs by a Synthetic Self-Replicative Rna." *Cell stem cell* 13, no. 2 (2013): 246-54.

65. Elli, Elena Maria, Claudia Baratè, Francesco Mendicino, Francesca Palandri, and Giuseppe Alberto Palumbo. "Mechanisms Underlying the Anti-Inflammatory and Immunosuppressive Activity of Ruxolitinib." *Frontiers in oncology* 9 (2019): 1186-86.

66. Ajbani, Seema P., Shilpa M. Velhal, Ravindra B. Kadam, Vainvra Patel, and Atmaram H. Bandivdekar. "Immunogenicity of Semliki Forest Virus Based Self-Amplifying Rna Expressing Indian Hiv-1c Genes in Mice." *International Journal of Biological Macromolecules* 81 (2015): 794-802.

67. Zhong, Zifu, João Paulo Portela Catani, Séan Mc Cafferty, Liesbeth Couck, Wim Van Den Broeck, Nina Gorlë, Roosmarijn E. Vandenbroucke, Bert Devriendt, Sebastian Ulbert, Lieselotte CNops, Johan Michels, Kevin K. Arien, and Niek N. Sanders. "Immunogenicity and Protection Efficacy of a Naked Self-Replicating Mrna-Based Zika Virus Vaccine." *Vaccines* 7, no. 3 (2019): 96.

68. Huysmans, Hanne, Zifu Zhong, Joyca De Temmerman, Barbara L. Mui, Ying K. Tam, Séan Mc Cafferty, Arlieke Gitsels, Daisy Vanrompay, and Niek N. Sanders. "Expression Kinetics and Innate Immune Response after Electroporation and Lnp-Mediated Delivery of a Self-Amplifying Mrna in the Skin." *Molecular Therapy - Nucleic Acids* 17 (2019): 867-78.

69. Démoulins, Thomas, Panagiota Milona, Pavlos C. Englezou, Thomas Ebensen, Kai Schulze, Rolf Suter, Chantal Pichon, Patrick Midoux, Carlos A. Guzmán, Nicolas Ruggli, and Kenneth C. McCullough. "Polyethylenimine-Based Polyplex Delivery of Self-Replicating Rna Vaccines." *Nanomedicine: Nanotechnology, Biology and Medicine* 12, no. 3 (2016): 711-22.

70. Démoulins, Thomas, Thomas Ebensen, Kai Schulze, Pavlos C. Englezou, Maria Pelliccia, Carlos A. Guzmán, Nicolas Ruggli, and Kenneth C. McCullough. "Self-Replicating Rna Vaccine Functionality Modulated by Fine-Tuning of Polypelex Delivery Vehicle Structure." *Journal of Controlled Release* 266 (2017): 256-71.

71. Chahal, Jasdave S., Tao Fang, Andrew W. Woodham, Omar F. Khan, Jingjing Ling, Daniel G. Anderson, and Hadde L. Ploegh. "An Rna Nanoparticle Vaccine against Zika Virus Elicits Antibody and Cd8+ T Cell Responses in a Mouse Model." *Scientific Reports* 7, no. 1 (2017): 252.

72. Kunath, K., A. von Harpe, D. Fischer, H. Petersen, U. Bickel, K. Voigt, and T. Kissel. "Low-Molecular-Weight Polyethylenimine as a Non-Viral Vector for DNA Delivery: Comparison of Physicochemical Properties, Transfection Efficiency and in Vivo Distribution with High-Molecular-Weight Polyethylenimine." *J Control Release* 89, no. 1 (2003): 113-25.

73. Blakney, Anna K., Gokhan Yilmaz, Paul F. McKay, C. Remzi Becer, and Robin J. Shattock. "One Size Does Not Fit All: The Effect of Chain Length and Charge Density of Poly(Ethylene Imine) Based Copolymers on Delivery of Pdna, Mrna, and Reprma Polyplexes." *Biomacromolecules* 19, no. 7 (2018): 2870-79.

74. Blakney, Anna K., Yunqing Zhu, Paul F. McKay, Clément R. Bouton, Jonathan Yeow, Jiaqing Tang, Kai Hu, Kanyart Samnuan, Christopher L. Grigsby, Robin J. Shattock, and Molly M. Stevens. "Big Is Beautiful: Enhanced Sarna Delivery and Immunogenicity by a
Higher Molecular Weight, Bioreducible, Cationic Polymer." ACS nano 14, no. 5 (2020): 5711-27.

75. Gurnani, Pratik, Anna K. Blakney, Roberto Terracciano, Joshua E. Petch, Andrew J. Blok, Clément R. Bouton, Paul F. McKay, Robin J. Shattock, and Cameron Alexander. "The in Vitro, Ex Vivo, and in Vivo Effect of Polymer Hydrophobicity on Charge-Reversible Vectors for Self-Amplifying Rna." Biomacromolecules 21, no. 8 (2020): 3242-53.

76. Blakney, Anna K., Yamin Abdouni, Gokhan Yilmaz, Renjie Liu, Paul F. McKay, Clément R. Bouton, Robin J. Shattock, and C. Remzi Becer. "Mannosylated Poly(Ethylene Imine) Copolymers Enhance Sarna Uptake and Expression in Human Skin Explants." Biomacromolecules 21, no. 6 (2020): 2482-92.

77. Saviano, Francesca, Tatiana Lovato, Annapina Russo, Giulia Russo, Clément R. Bouton, Robin J. Shattock, Fabiana Quaglia, Anna K. Blakney, Pratik Gurnani, and Claudia Conte. "Ornithine-Derived Oligomers and Dendrimers for in Vitro Delivery of DNA and Ex Vivo Transfection of Skin Cells Via Sarna." Journal of Materials Chemistry B 8, no. 22 (2020): 4940-49.

78. McKay, Paul F., Kai Hu, Anna K. Blakney, Karnyart Samnuan, Jonathan C. Brown, Rebecca Penn, Jie Zhou, Clément R. Bouton, Paul Rogers, Krunal Polra, Paulo J. C. Lin, Christopher Barbosa, Ying K. Tam, Wendy S. Barclay, and Robin J. Shattock. "Self-Amplifying Rna Sars-Cov-2 Lipid Nanoparticle Vaccine Candidate Induces High Neutralizing Antibody Titers in Mice." Nature Communications 11, no. 1 (2020): 3523.

79. Geall, A. J., A. Verma, G. R. Otten, C. A. Shaw, A. Hekele, K. Banerjee, Y. Cu, C. W. Beard, L. A. Brito, T. Krucker, D. T. O'Hagan, M. Singh, P. W. Mason, N. M. Valiante, P. R. Dormitzer, S. W. Barnett, R. Rappuoli, and C. W. Mandl. "Nonviral Delivery of Self-Amplifying Rna Vaccines." Proc Natl Acad Sci U S A 109, no. 36 (2012): 14604-9.

80. Lou, Gustavo, Giulia Anderluzzi, Signe Tandrup Schmidt, Stuart Woods, Simona Gallorini, Michela Brazzoli, Fabiola Giusti, Ilaria Ferlenghi, Russell N. Johnson, Craig W. Roberts, Derek T. O'Hagan, Barbara C. Baudner, and Yvonne Perrie. "Delivery of Self-Amplifying Mrna Vaccines by Cationic Lipid Nanoparticles: The Impact of Cationic Lipid Selection." Journal of Controlled Release 325 (2020): 370-79.

81. Englezou, Pavlos C., Cedric Sapet, Thomas Démoulins, Panagiota Milona, Thomas Ebensen, Kai Schulze, Carlos-Alberto Guzman, Florent Poulhes, Olivier Zelphati, Nicolas Ruggli, and Kenneth C. McCullough. "Self-Amplifying Replicon Rna Delivery to Dendritic Cells by Cationic Lipids." Molecular Therapy - Nucleic Acids 12 (2018): 118-34.

82. Blakney, Anna K., Paul F. McKay, Bárbara Ibarzo Yus, Yoann Aldon, and Robin J. Shattock. "Inside Out: Optimization of Lipid Nanoparticle Formulations for Exterior Complexation and in Vivo Delivery of Sarna." Gene Therapy 26, no. 9 (2019): 363-72.

83. Brito, Luis A., Michelle Chan, Christine A. Shaw, Armin Hekele, Thomas Carsillo, Mary Schaefer, Jacob Archer, Anja Seubert, Gillis R. Otten, Clayton W. Beard, Antu K. Dey, Anders Lilja, Nicholas M. Valiante, Peter W. Mason, Christian W. Mandl, Susan W. Barnett, Philip R. Dormitzer, Jeffrey B. Ulmer, Mannmohan Singh, Derek T. O'Hagan, and Andrew J. Geall. "A Cationic Nanoemulsion for the Delivery of Next-Generation Rna Vaccines." Molecular Therapy 22, no. 12 (2014): 2118-29.

84. Ansaldi, Filippo, Paola Canepa, Valentina Parodi, Sabrina Bacilieri, Andrea Orsi, Federica Compagnino, Giancarlo Icardi, and Paolo Durando. "Adjuvanted Seasonal Influenza Vaccines and Perpetual Viral Metamorphosis: The Importance of Cross-Protection." Vaccine 27, no. 25 (2009): 3345-48.
86. Anderluzzi, Giulia, Gustavo Lou, Simona Gallorini, Michela Brazzoli, Russell Johnson, Derek T. O'Hagan, Barbara C. Baudner, and Yvonne Perrie. "Investigating the Impact of Delivery System Design on the Efficacy of Self-Amplifying Rna Vaccines." *Vaccines* 8, no. 2 (2020): 212.

87. Bogers, Willy M., Herman Oostermeijer, Petra Mooij, Gerrit Koopman, Ernst J. Verschoor, David Davis, Jeffrey B. Ulmer, Luis A. Brito, Yen Cu, Kaustuv Banerjee, Gillis R. Otten, Brian Burke, Antu Dey, Jonathan L. Heeney, Xiaoying Shen, Georgia D. Tomaras, Celia Labranche, David C. Montefiori, Hua-Xin Liao, Barton Haynes, Andrew J. Geall, and Susan W. Barnett. "Potent Immune Responses in Rhesus Macaques Induced by Nonviral Delivery of a Self-Amplifying Rna Vaccine Expressing Hiv Type 1 Envelope with a Cationic Nanoemulsion." *The Journal of infectious diseases* 211, no. 6 (2015): 947-55.

88. Brazzoli, Michela, Diletta Magini, Alessandra Bonci, Scilla Buccato, Cinzia Giovani, Roland Kratzer, Vanessa Zurli, Simona Mangiavacchi, Daniele Casini, Luis M. Brito, Ennio De Gregorio, Peter W. Mason, Jeffrey B. Ulmer, Andrew J. Geall, and Sylvie Bertholet. "Induction of Broad-Based Immunity and Protective Efficacy by Self-Amplifying Mrna Vaccines Encoding Influenza Virus Hemagglutinin." *Journal of virology* 90, no. 1 (2015): 332-44.

89. Manara, Cristina, Michela Brazzoli, Diego Piccioli, Marianna Taccone, Ugo D'Oro, Domenico Maione, and Elisabetta Frigimelica. "Co-Administration of Gm-Csf Expressing Rna Is a Powerful Tool to Enhance Potency of Sam-Based Vaccines." *Vaccine* 37, no. 30 (2019): 4204-13.

90. Akinc, Akin, Martin A. Maier, Muthiah Manoharan, Kevin Fitzgerald, Muthusamy Jayaraman, Scott Barros, Steven Ansell, Xinyao Du, Michael J. Hope, Thomas D. Madden, Barbara L. Mui, Sean C. Semple, Ying K. Tam, Marco Ciufolini, Dominik Witzigmann, Jayesh A. Kulkarni, Roy van der Meel, and Pieter R. Cullis. "The Onpattro Story and the Clinical Translation of Nanomedicines Containing Nucleic Acid-Based Drugs." *Nature Nanotechnology* 14, no. 12 (2019): 1084-87.

91. Folegatti, Pedro M., Katie J. Ewer, Parvinder K. Aley, Brian Angus, Stephan Becker, Sandra Bellj-Rammerstorfer, Duncan Bellamy, Sagida Bibi, Mustapha Bittaye, Elizabeth A. Clutterbuck, Christina Dold, Saul N. Faust, Adam Finn, Amy L. Flaxman, Bassam Hallis, Paul Heath, Daniel Jenkin, Rajeka Lazarus, Rebecca Makinson, Angela M. Minassian, Katrina M. Pollock, Maheshi Ramasamy, Hannah Robinson, Matthew Snape, Richard Tarrant, Merryn Voysey, Catherine Green, Alexander D. Douglas, Adrian V. S. Hill, Teresa Lambe, Sarah C. Gilbert, Andrew J. Pollard, Jeremy Aboagye, Kelly Adams, Aabidah Ali, Elizabeth Allen, Jennifer L. Ellison, Rachel Anslow, Edward H. Arbo-Barnes, Gavin Babbage, Kenneth Baillie, Megan Baker, Natalie Baker, Philip Baker, Ioana Baleanu, Juliana Ballaminut, Eleanor Barnes, Jordan Barrett, Louise Bates, Alexander Batten, Kirsten Beadon, Rebecca Beckley, Eleanor Berrie, Lisa Berry, Amy Beveridge, Kevin R. Bewley, Else Margreet Bijker, Tracey Bingham, Luke Blackwell, Caitlin L. Blundell, Emma Bolam, Elena Boland, Nicola Borthwick, Thomas Bower, Amy Boyd, Tanja Brenner, Philip D. Bright, Charlie Brown-O'Sullivan, Emily Brutn, Jamie Burbage, Sharon Burge, Karen R. Buttigieg, Nicholas Byard, Ingrid Cabera Puig, Anna Calvert, Susana Camara, Michelangelo Cao, Federica Cappuccini, Melanie Carr, Miles W. Carroll, Victoria Carter, Katrina Cathie, Ruth J. Challis, Sue Charlotte, Irina Chelysheva, Jee-Sun Cho, Paola Cicconi, Liliana CIFuentes, Helen Clark, Elizabeth Clark, Tom Cole, Rachel Colim-Jones, Christopher P. Conlon, Aislinn Cook, Naomi S. Coombes, Rachel Cooper, Catherine A. Cosgrove, Karen Coy, Wendy E. M. Crocker, Christina J. Cunningham, Brad E. Damratski, Lynne Dando, Mehreen S. Datoo, Hannah Davies, Hans De Graaf, Tesfaye Demisse, Claudio Di Maso, Isabelle Dietrich, Tao Dong, Francesca R. Donnellan, Naomi Douglas, Charlotte Downing, Jonathan Drake, Rachael Drake-Brockman, Ruth Elizabeth Drury, Susanna Jane Dunachie, Nick J. Edwards, Frances D. L. Edwards, Chris J.
Edwards, Sean C. Elias, Michael J. Elmore, Katherine R. W. Emary, Marcus Rex English, Susanne Fagerbrink, Sally Felle, Shuo Feng, Samantha Field, Carine Fixmer, Clare Fletcher, Karen J. Ford, Jamie Fowler, Polly Fox, Emma Francis, John Frater, Julie Furze, Michelle Fusko, Eva Galiza, Diane Gbesemete, Ciaran Gilbride, Kerry Godwin, Giacomo Gorini, Lyndsey Goulston, Caroline Grabau, Lara Gracie, Zoe Gray, Lucy Belle Guthrie, Mark Hackett, Sandro Halwe, Elizabeth Hamilton, Joseph Hamlyn, Brama Hanumunthadu, Iraisha Harding, Stephanie A. Harris, Andrew Harris, Daisy Harrison, Clare Harrison, Thomas C. Hart, Louise Haskell, Sophia Hawkins, Ian Head, John Aaron Henry, Jennifer Hill, Susanne H. C. Hodgson, Mimi M. Hou, Elizabeth Howe, Nicola Howell, Cecilia Hutlin, Sabina Ikram, Catherine Isitt, Poppy Iveson, Susan Jackson, Frederic Jackson, Sir William James, Megan Jenkins, Elizabeth Jones, Kathryn Jones, Christine E. Jones, Bryony Jones, Reshma Kailath, Konstantinos Karampatsas, Jade Keen, Sarah Kelly, Dearbhla Kelly, David Kerr, Simon Kerridge, Liaquat Khan, Uzma Khan, Annabel Killen, Jasmin Kinch, Thomas B. King, Lloyd King, Jade King, Lucy Kingham-Page, Paul Kleuerman, Francesca Knapper, Julian C. Knight, Daniel Knott, Stanislava Koleva, Alexandra Kupke, Colin W. Larkworthy, Jessica P. J. Larwood, Anna Laskey, Alison M. Lawrie, Arlene Lee, Kim Yee Ngan Lee, Emily A. Lees, Helen Legge, Alice Lelliott, Nana-Marie Lemm, Amelia M. Lias, Aline Linder, Samuel Lipworth, Xin Xue Liu, Shuchang Liu, Raquel Lopez Ramon, May Lwin, Francesca Mabesa, Meera Madhavan, Garry Mallett, Kushal Mansatta, Ines Marcal, Spyridoula Marinou, Emma Marlow, Julia L. Marshall, Jane Martin, Joanne McEwan, Lorna McInroy, Gretchen Meddaugh, Alexander J. Mentzer, Neginsadat Mirtorabi, Maria Moore, Edward Moran, Ella Morey, Victoria Morgan, Susan Jane Morris, Hazel Morrison, Gertraud Morshed, Richard Morter, Yama F. Mujadidi, Jilly Muller, Tatiana Munera-Huertas, Claire Munro, Alasdair Munro, Sarah Murphy, Vincent J. Munster, Philomena Mweu, André Noé, Fay L. Nugent, Elizabeth Nuthall, Katie O’Brien, Daniel O’Connor, Blanché Oguti, Jennifer L. Oliver, Catarina Oliveira, Peter John O’Reilly, Mairead Osborn, Piper Osborne, Cathy Owen, Daniel Owens, Nelly Owino, Mihaela Pacurar, Kaye Parker, Helena Parracho, Maia Patrick-Smith, Victoria Payne, Jennifer Pearce, Yanchun Peng, Marco Polo Peralta Alvarez, James Perring, Katja Pfafferott, Dimitra Pipini, Emma Phested, Helen Pluess-Hall, Katrina Pollock, Ian Poulton, Laura Presland, Samuel Provstgaard-Morys, David Pulido, Kajal Radia, Fernando Ramos Lopez, Jade Rand, Helen Ratcliffe, Thomas Rawlinson, Sarah Rhead, Amy Ridell, Adam John Ritchie, Hannah Roberts, Joanna Robson, Sophie Roche, Cornelius Rohde, Christine S. Rollier, Rossana Romani, Indra Rudiansyah, Stephen Saich, Sara Sajjad, Stephannie Salvador, Lida Sanchez Riera, Helen Sanders, Katherine Sanders, Shari Sapaun, Chloe Sayce, Ella Schofield, Gavin Screaton, Beatrice Selby, Calum Semple, Hannah R. Sharpe, Imam Shaik, Adam Shea, Holly Shelton, Sarah Silk, Laura Silva-Reyes, Donal T. Skelly, Heather Sme, Catherine C. Smith, David J. Smith, Rinn Song, Alexandra J. Spencer, Elizabeth Stafford, Amy Steele, Elena Stefanova, Lisa Stockdale, Anna Szegiti, Abdessamad Tahiri-Alaoui, Moira Tait, Helen Talbot, Rachel Tanner, Iona Jennifer Taylor, Victoria Taylor, Rebecca Te Water Naude, Nazis Thakur, Yrene Themistocleous, Andreas Themistocleous, Merin Thomas, Tonia M. Thomas, Amber Thompson, Samantha Thomson-Hill, Jennifer Tomlins, Susan Tonks, James Towner, Nguyen Tran, Julia A. Tree, Adam Truby, Kate Turkentine, Cheryl Turner, Nicola Turner, Sally Turner, Toby Tuthill, Marta Ulaszewska, Rachel Varughese, Neelitje Van Doremalen, Kristin Veighel, Marije K. Verheul, Iason Vichos, Elia Vitale, Laura Walker, Marion E. E. Watson, Benjamin Welham, Julie Wheat, Caroline White, Rachel White, Andrew T. Worth, Danny Wright, Suzie Wright, Xin Li Yao, and Yasmine Yau.

"Safety and Immunogenicity of the Chadox1 Ncov-19 Vaccine against Sars-Cov-2: A Preliminary Report of a Phase 1/2, Single-Blind, Randomised Controlled Trial." The Lancet 396, no. 10249 (2020): 467-78.
92. Sahin, Uğur, Katalin Karikó, and Özlem Türeci. "Mrna-Based Therapeutics — Developing a New Class of Drugs." *Nature Reviews Drug Discovery* 13, no. 10 (2014): 759-80.

93. Wu, Monica Z., Haruichi Asahara, George Tzertzinis, and Bijoyita Roy. "Synthesis of Low Immunogenicity Rna with High-Temperature in Vitro Transcription." *RNA (New York, N.Y.)* 26, no. 3 (2020): 345-60.

94. Brito, Luis A., Sushma Kommareddy, Domenico Maione, Yasushi Uematsu, Cinzia Giovani, Francesco Berlanda Scorza, Gillis R. Otten, Dong Yu, Christian W. Mandl, Peter W. Mason, Philip R. Dormitzer, Jeffrey B. Ulmer, and Andrew J. Geall. "Chapter Seven - Self-Amplifying Mrna Vaccines." In *Advances in Genetics*, edited by Leaf Huang, Dexi Liu and Ernst Wagner, 179-233: Academic Press, 2015.

95. Geall, A. J., C. W. Mandl, and J. B. Ulmer. "Rna: The New Revolution in Nucleic Acid Vaccines." *Semin Immunol* 25, no. 2 (2013): 152-9.

96. Davis, Robert H. "Large-Scale Oligoribonucleotide Production." *Current Opinion in Biotechnology* 6, no. 2 (1995): 213-17.

97. Marcotrigiano, Joseph, Anne-Claude Gingras, Nahum Sonenberg, and Stephen K. Burley. "Cocrystal Structure of the Messenger Rna 5' Cap-Binding Protein (Eif4e) Bound to 7-Methyl-Gdp." *Cell* 89, no. 6 (1997): 951-61.

98. Kwon, H., M. Kim, Y. Seo, Y. S. Moon, H. J. Lee, K. Lee, and H. Lee. "Emergence of Synthetic Mrna: In vitro Synthesis of Mrna and Its Applications in Regenerative Medicine." *Biomaterials* 156 (2018): 172-93.

99. Yisraeli, Joel K., and Doug A. Melton. "[4] Synthesis of Long, Capped Transcripts in Vitro by Sp6 and T7 Rna Polymerases." In *Methods in Enzymology*, 42-50: Academic Press, 1989.

100. Jemielity, J., T. Fowler, J. Zuberek, J. Stepinski, M. Lewdorowicz, A. Niedzwiecka, R. Stolarski, E. Darzynkiewicz, and R. E. Rhoads. "Novel "Anti-Reverse" Cap Analogs with Superior Translational Properties." *RNA* 9, no. 9 (2003): 1108-22.

101. Peng, Zheng-Hong, Vivek Sharma, Scott F. Singleton, and Paul D. Gershon. "Synthesis and Application of a Chain-Terminating Dinucleotide Mrna Cap Analog." *Organic Letters* 4, no. 2 (2002): 161-64.

102. Vaidyanathan, S., K. T. Azizian, Akma Haque, J. M. Henderson, A. Hendel, S. Shore, J. S. Antony, R. I. Hogrefe, M. S. D. Kormann, M. H. Porteus, and A. P. McCaffrey. "Uridine Depletion and Chemical Modification Increase Cas9 Mrna Activity and Reduce Immunogenicity without Hplc Purification." *Mol Ther Nucleic Acids* 12 (2018): 530-42.

103. Walker, Sarah E., and Jon Lorsch. "Chapter Nineteen - Rna Purification – Precipitation Methods." In *Methods in Enzymology*, edited by Jon Lorsch, 337-43: Academic Press, 2013.

104. Baronti, Lorenzo, Hampus Karlsson, Maja Marušić, and Katja Petzold. "A Guide to Large-Scale Rna Sample Preparation." *Analytical and Bioanalytical Chemistry* 410, no. 14 (2018): 3239-52.

105. Batey, Robert T. "Advances in Methods for Native Expression and Purification of Rna for Structural Studies." *Current Opinion in Structural Biology* 26 (2014): 1-8.

106. Martins, R., J. A. Queiroz, and F. Sousa. "Ribonucleic Acid Purification." *Journal of Chromatography A* 1355 (2014): 1-14.

107. Deering, R. P., S. Kommareddy, J. B. Ulmer, L. A. Brito, and A. J. Geall. "Nucleic Acid Vaccines: Prospects for Non-Viral Delivery of Mrna Vaccines." *Expert Opin Drug Deliv* 11, no. 6 (2014): 885-99.

108. GholamaliPour, Yasaman, Aruni Karunanayake MudiyanseLage, and Craig T. Martin. "3' End Additions by T7 Rna Polymerase Are Rna Self-Templated, Distributive and Diverse in Character—Rna-Seq Analyses." *Nucleic Acids Research* 46, no. 18 (2018): 9253-63.
109. Triana-Alonso, F. J., M. Dabrowski, J. Wadzack, and K. H. Nierhaus. "Self-Coded 3'-Extension of Run-Off Transcripts Produces Aberrant Products During in Vitro Transcription with T7 Rna Polymerase." *J Biol Chem* 270, no. 11 (1995): 6298-307.

110. Mu, X., E. Greenwald, S. Ahmad, and S. Hur. "An Origin of the Immunogenicity of in Vitro Transcribed Rna." *Nucleic Acids Res* 46, no. 10 (2018): 5239-49.

111. Baiersdörfer, Markus, Gábor Boros, Hiromi Muramatsu, Azita Mahiny, Irena Vlatkovic, Ugur Sahin, and Katalin Karikó. "A Facile Method for the Removal of Dsrna Contaminant from &lt;Em&gt;in&lt;/Em&gt;-Transcribed Mrna." *Molecular Therapy - Nucleic Acids* 15 (2019): 26-35.

112. Moon, Stephanie L., and Jeffrey Wilusz. "In Vitro Transcription of Modified Rnas." In *Recombinant and in Vitro Rna Synthesis: Methods and Protocols*, edited by Graeme L. Conn, 171-80. Totowa, NJ: Humana Press, 2012.

113. Houseley, Jonathan, and David Tollervey. "The Many Pathways of Rna Degradation." *Cell* 136, no. 4 (2009): 763-76.

114. Wayment-Steele, Hannah K., Do Soon Kim, Christian A. Choe, John J. Nicol, Roger Wellington-Oguri, R. Andres Parra Sperber, Po-Ssu Huang, and Rhiju Das. "Theoretical Basis for Stabilizing Messenger Rna through Secondary Structure Design." *bioRxiv : the preprint server for biology* (2020): 2020.08.22.262931.

115. Brunelle, Julie L., and Rachel Green. "Chapter Five - in Vitro Transcription from Plasmid or Pcr-Amplified DNA." In *Methods in Enzymology*, edited by Jon Lorsch, 101-14: Academic Press, 2013.

116. Belliveau, Nathan M., Jens Huft, Paulo Jc Lin, Sam Chen, Alex Kk Leung, Timothy J. Leaver, Andre W. Wild, Justin B. Lee, Robert J. Taylor, Ying K. Tam, Carl L. Hansen, and Pieter R. Cullis. "Microfluidic Synthesis of Highly Potent Limit-Size Lipid Nanoparticles for in Vivo Delivery of Sirna." *Molecular Nanotechnology, Nucleic Acids* 1, no. 8 (2012): e37-e37.

117. Finn, J. D., A. R. Smith, M. C. Patel, L. Shaw, M. R. Youniss, J. van Heteren, T. Dirstine, C. Ciullo, R. Lescarbeau, J. Seitzer, R. R. Shah, A. Shah, D. Ling, J. Growe, M. Pink, E. Rohde, K. M. Wood, W. E. Salomon, W. F. Harrington, C. Dombrowski, W. R. Strapps, Y. Chang, and D. V. Morrissey. "A Single Administration of Crispr/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In vivo Genome Editing." *Cell Rep* 22, no. 9 (2018): 2227-35.

118. Veiga, Nuphar, Meir Goldsmith, Yasmin Granot, Daniel Rosenblum, Niels Dammes, Ranit Kedmi, Srinivas Ramishetti, and Dan Peer. "Cell Specific Delivery of Modified Mrna Expressing Therapeutic Proteins to Leukocytes." *Nature Communications* 9, no. 1 (2018): 4493.

119. Viger-Gravel, Jasmine, Anna Schantz, Arthur C. Pinon, Aaron J. Rossini, Staffan Schantz, and Lyndon Emsley. "Structure of Lipid Nanoparticles Containing Sirna or Mrna by Dynamic Nuclear Polarization-Enhanced Nmr Spectroscopy." *The Journal of Physical Chemistry B* 122, no. 7 (2018): 2073-81.

120. Yaghi, N. K., J. Wei, Y. Hashimoto, L. Y. Kong, K. Gabrusiewicz, E. K. Nduom, X. Ling, N. Huang, S. Zhou, B. C. Kerrigan, J. M. Levine, V. R. Fajt, G. Levine, B. F. Porter, E. G. Marcusson, K. Tachikawa, P. Chivukula, D. C. Webb, J. E. Payne, and A. B. Heimberger. "Immune Modulatory Nanoparticle Therapeutics for Intracerebral Glioma." *Neuro Oncol* 19, no. 3 (2017): 372-82.

121. Richner, J. M., B. W. Jagger, C. Shan, C. R. Fontes, K. A. Dowd, B. Cao, S. Himansu, E. A. Caine, B. T. D. Nunes, D. B. A. Medeiros, A. E. Muruato, B. M. Foreman, H. Luo, T. Wang, A. D. Barrett, S. C. Weaver, P. F. C. Vasconcelos, S. L. Rossi, G. Ciaramella, I. U. Mysorekar, T. C. Pierson, P. Y. Shi, and M. S. Diamond. "Vaccine Mediated Protection against Zika Virus-Induced Congenital Disease." *Cell* 170, no. 2 (2017): 273-83.e12.
122. Kim, Jeonghwan, Antony Jozic, and Gaurav Sahay. "Naturally Derived Membrane Lipids Impact Nanoparticle-Based Messenger Rna Delivery." *Cellular and molecular bioengineering* 13, no. 5 (2020): 1-12.

123. Erasmus, Jesse H., Amit P. Khandhar, Jeff Guderian, Brian Granger, Jacob Archer, Michelle Archer, Emily Gage, Jasmine Fuerte-Stone, Elise Larson, Susan Lin, Ryan Kramer, Rhea N. Coler, Christopher B. Fox, Dan T. Stinchcomb, Steven G. Reed, and Neal Van Hoeven. "A Nanostructured Lipid Carrier for Delivery of a Replicating Viral Rna Provides Single, Low-Dose Protection against Zika." *Molecular Therapy* 26, no. 10 (2018): 2507-22.

124. Vemuri, Sriram, Cheng-Der Yu, Vuthichai Wangsatorntanakun, and Niek Roosdorp. "Large-Scale Production of Liposomes by a Microfluidizer." *Drug development and industrial pharmacy* 16, no. 15 (1990): 2243-56.

125. Jenning, V., A. Lippacher, and S. H. Gohla. "Medium Scale Production of Solid Lipid Nanoparticles (Sln) by High Pressure Homogenization." *J Microencapsul* 19, no. 1 (2002): 1-10.

126. Muchow, M., P. Maincent, and R. H. Muller. "Lipid Nanoparticles with a Solid Matrix (Sln, Nlc, Ldc) for Oral Drug Delivery." *Drug Dev Ind Pharm* 34, no. 12 (2008): 1394-405.

127. Shegokar, R., K. K. Singh, and R. H. Müller. "Production & Stability of Stavudine Solid Lipid Nanoparticles--from Lab to Industrial Scale." *Int J Pharm* 416, no. 2 (2011): 461-70.

128. Sorgi, Frank L., and Leaf Huang. "Large Scale Production of Dc-Chol Cationic Liposomes by Microfluidization." *International journal of pharmaceutics* 144, no. 2 (1996): 131-39.

129. Liu, M. A., and J. B. Ulmer. "Human Clinical Trials of Plasmid DNA Vaccines." *Adv Genet* 55 (2005): 25-40.

130. Scheuber, Andrew. "Imperial Social Enterprise to Accelerate Low-Cost Covid-19 Vaccine." [https://www.imperial.ac.uk/news/198053/imperial-social-enterprise-accelerate-lowcost-covid19/](https://www.imperial.ac.uk/news/198053/imperial-social-enterprise-accelerate-lowcost-covid19/)

131. Release, Alnylam Pharmaceuticals Press. "Alnylam Announces First-Ever Fda Approval of an Rnai Therapeutic, Onpattro (Patisiran) for the Treatment of the Polynuropathy of Hereditary Transthyretin-Mediated Amyloidosis in Adults." 2018.

132. Collaboration, Center for Leading Innovation &. "Safety and Immunogenicity Study of 2019-Ncov Vaccine (Mrna-1273) for Prophylaxis Sars Cov-2 Infection (Covid-19)." 2020.

133. Brenner, S., F. Jacob, and M. Meselson. "An Unstable Intermediate Carrying Information from Genes to Ribosomes for Protein Synthesis." *Nature* 190, no. 4776 (1961): 576-81.

134. Pfizer. "Pfizer and Biontech Achieve First Authorization in the World for a Vaccine to Combat Covid-19." 2020.

135. Pollard, Charlotte, Joanna Rejman, Winni De Haes, Bernard Verrier, Ellen Van Gulck, Thomas Naessens, Stefaan De Smedt, Pieter Bogaert, Johan Grooten, Guido Vanham, and Stefaan De Koker. "Type I Ifn Counteracts the Induction of Antigen-Specific Immune Responses by Lipid-Based Delivery of Mrna Vaccines." *Molecular Therapy* 21, no. 1 (2013): 251-59.

136. Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner. "Direct Gene Transfer into Mouse Muscle in Vivo." *Science* 247, no. 4949 (1990): 1465.

137. Martinon, Frédéric, Sivadasan Krishnan, Gerlinde Lenzen, Rémy Magné, Elisabeth Gomard, Jean-Gérard Guillet, Jean-Paul Lévy, and Pierre Meulien. "Induction of Virus-Specific Cytotoxic T Lymphocytes in Vivo by Liposome-Entrapped Mrna." *European journal of immunology* 23, no. 7 (1993): 1719-22.

138. Bangham, A. D., and R. W. Horne. "Negative Staining of Phospholipids and Their Structural Modification by Surface-Active Agents as Observed in the Electron Microscope." *Journal of Molecular Biology* 8, no. 5 (1964): 660-1N10.
139. Conry, R. M., A. F. LoBuglio, M. Wright, L. Sumerel, M. J. Plke, F. Johanning, R. Benjamin, D. Lu, and D. T. Curiel. "Characterization of a Messenger Rna Polynucleotide Vaccine Vector." *Cancer Res* 55, no. 7 (1995): 1397-400.

140. Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. "Lipofection: A Highly Efficient, Lipid-Mediated DNA-Transfection Procedure." *Proc Natl Acad Sci U S A* 84, no. 21 (1987): 7413-7.

141. Weide, B., S. Pascolo, B. Scheel, E. Derhovanessian, A. Pfugfelder, T. K. Eigentler, G. Pawelec, I. Hoerr, H. G. Rammensee, and C. Garbe. "Direct Injection of Protamine-Protected Mrna: Results of a Phase 1/2 Vaccination Trial in Metastatic Melanoma Patients." *J Immunother* 32, no. 5 (2009): 498-507.

142. Karikó, Katalin, Michael Buckstein, Houping Ni, and Drew Weissman. "Suppression of Rna Recognition by Toll-Like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of Rna." *Immunity* 23, no. 2 (2005): 165-75.

143. Jeffs, Lloyd B., Lorne R. Palmer, Ellen G. Ambegia, Cory Giesbrecht, Shannon Ewanick, and Ian MacLachlan. "A Scalable, Extrusion-Free Method for Efficient Liposomal Encapsulation of Plasmid DNA." *Pharmaceutical research* 22, no. 3 (2005): 362-72.

144. Belliveau, Nathan M., Jens Huft, Paulo J. C. Lin, Sam Chen, Alex K. K. Leung, Timothy J. Leaver, Andre W. Wild, Justin B. Lee, Robert J. Taylor, Ying K. Tam, Carl L. Hansen, and Pieter R. Cullis. "Microfluidic Synthesis of Highly Potent Limit-Size Lipid Nanoparticles for in Vivo Delivery of Sirna." *Molecular Therapy - Nucleic Acids* 1 (2012): e37.

145. Semple, Sean C., Akin Akinc, Jianxin Chen, Ammen P. Sandhu, Barbara L. Mui, Connie K. Cho, Dinah W. Y. Sah, Derrick Stebbing, Erin J. Crosley, Ed Yaworski, Ismail M. Hafez, J. Robert Dorkin, June Qin, Kieu Lam, Kallanthottathil G. Rajeev, Kim F. Wong, Lloyd B. Jeffs, Lubomir Nechev, Merete L. Eisenhardt, Muthusamy Jayaraman, Mikameh Kazem, Martin A. Maier, Masuna Srinivasulu, Michael J. Weinstein, Qingmin Chen, Rene Alvarez, Scott A. Barros, Soma De, Sandra K. Klimuk, Todd Borland, Verbenos Kosovrasti, William L. Cantley, Ying K. Tam, Muthiah Manoharan, Marco A. Ciufolini, Mark A. Tracy, Antonin de Fougerolles, Ian MacLachlan, Pieter R. Cullis, Thomas D. Madden, and Michael J. Hope. "Rational Design of Cationic Lipids for Sirna Delivery." *Nature biotechnology* 28, no. 2 (2010): 172-76.

146. Kreiter, Sebastian, Mustafa Diken, Abderraouf Selmi, Jan Diekmann, Sebastian Attig, Yves Hüsemann, Michael Koslowski, Christoph Huber, Özlem Türeci, and Ugur Sahin. "Flt3 Ligand Enhances the Cancer Therapeutic Potency of Naked Rna Vaccines." *Cancer research* 71, no. 19 (2011): 6132.

147. Bailey, Austin L., and Pieter R. Cullis. "Modulation of Membrane Fusion by Asymmetric Transbilayer Distributions of Amino Lipids." *Biochemistry* 33, no. 42 (1994): 12573-80.

148. Laczkó, Dorottya, Michael J. Hogan, Sushila A. Toulmin, Philip Hicks, Katlyn Lederer, Brian T. Gaudette, Diana Castaño, Fatima Amanat, Hiromi Muramatsu, Thomas H. Oguni, 3rd, Amrita Ojha, Lizhou Zhang, Zekun Mu, Robert Parks, Tomaz B. Manzoni, Brianne Roper, Shirin Stroheimeir, István Tombácz, Leslee Arwood, Raffael Nachbagauer, Katalin Karikó, Jack Greenhouse, Laurent Pessaint, Maciel Porto, Tammy Putman-Taylor, Amanda Strasbaugh, Tracey-Ann Campbell, Paulo J. C. Lin, Ying K. Tam, Gregory D. Sempowski, Michael Farzan, Hyeryun Choe, Kevin O. Saunders, Barton F. Haynes, Hanne Andersen, Laurence C. Eisenlohr, Drew Weissman, Florian Kramer, Paul Bates, David Allman, Michela Locci, and Norbert Pardi. "A Single Immunization with Nucleoside-Modified Mrna Vaccines Elicits Strong Cellular and Humoral Immune Responses against Sars-Cov-2 in Mice." *Immunity* 53, no. 4 (2020): 724-32-e7.

149. Mulligan, Mark J., Kirsten E. Lyke, Nicholas Kitchin, Judith Absalon, Alejandra Gurman, Stephen Lockhart, Kathleen Neuzil, Vanessa Raabe, Ruth Bailey, Kena A. Swanson, Ping Li, Kenneth Koury, Warren Kalina, David Cooper, Camila Fontes-Garfias, Pei-Yong Shi, Özlem Türeci, Kristin R. Tompkins, Edward E. Walsh, Robert French, Ann R. Fals
R. Dormitzer, William C. Gruber, Uğur Şahin, and Kathrin U. Jansen. "Phase i/ii Study of Covid-19 Rna Vaccine Bnt162b1 in Adults." *Nature* 586, no. 7830 (2020): 589-93.

150. Sahin, Ugur, Alexander Muik, Evelyne Derhovanessian, Isabel Vogler, Lena M. Kranz, Mathias Vormehr, Alina Baum, Kristen Pascal, Jasmin Quandt, Daniel Maurus, Sebastian Brachtendorf, Verena L. Loerks, Julian Sikorski, Rolf Hilk, Dirk Becker, Ann-Kathrin Eller, Jan Gruetzner, Carsten Boesler, Corinna Rosenbaum, Marie-Cristine Kuehnle, Ulrich Luxemburger, Alexandra Kemmer-Brueck, David Langer, Martin Bexon, Stefanie Bolte, Katalin Kariko, Tania Palanche, Boris Fischer, Armin Schultz, Pei-Yong Shi, Camila Fontes-Garfias, John L. Perez, Kena A. Swanson, Jakob Loschko, Ingrid L. Scully, Mark Cutler, Warren Kalina, Christos A. Kyratsous, David Cooper, Philip R. Dormitzer, Kathrin U. Jansen, and Oezlem Tuereci. "Concurrent Human Antibody and Th1 Type T-Cell Responses Elicited by a Covid-19 Rna Vaccine." *medRxiv* (2020): 2020.07.17.20140533.

151. Mulligan, Mark J., Kirsten E. Lyke, Nicholas Kitchin, Judith Absalon, Alejandra Gurtman, Stephen P. Lockhart, Kathleen Neuzil, Vanessa Raabe, Ruth Bailey, Kena A. Swanson, Ping Li, Kenneth Koury, Warren Kalina, David Cooper, Camila Fonter-Garfias, Pei-Yong Shi, Ozlem Türeci, Kristin R. Tompkins, Edward E. Walsh, Robert Frenck, Ann R. Falsey, Philip R. Dormitzer, William C. Gruber, Uğur Sahin, and Kathrin U. Jansen. "Phase 1/2 Study to Describe the Safety and Immunogenicity of a Covid-19 Rna Vaccine Candidate (Bnt162b1) in Adults 18 to 55 Years of Age: Interim Report." *medRxiv* (2020): 2020.06.30.20142570.

152. Walsh, Edward E., Robert W. Frenck, Ann R. Falsey, Nicholas Kitchin, Judith Absalon, Alejandra Gurtman, Stephen Lockhart, Kathleen Neuzil, Mark J. Mulligan, Ruth Bailey, Kena A. Swanson, Ping Li, Kenneth Koury, Warren Kalina, David Cooper, Camila Fontes-Garfias, Pei-Yong Shi, Ozlem Türeci, Kristin R. Tompkins, Kirsten E. Lyke, Vanessa Raabe, Philip R. Dormitzer, Kathrin U. Jansen, Uğur Şahin, and William C. Gruber. "Safety and Immunogenicity of Two Rna-Based Covid-19 Vaccine Candidates." *New England Journal of Medicine* (2020).

153. de Alwis, Ruklanthi, Esther S. Gan, Shiwei Chen, Yan Shan Leong, Hwee Cheng Tan, Summer L. Zhang, Clement Yau, Daiki Matsuda, Elizabeth Allen, Paula Hartman, Jenny Park, Maher Alayyoubi, Hari Bhaskaran, Adrian Dukanovic, Belle Bao, Brenda Clemente, Jerel Vega, Scott Roberts, Jose A. Gonzalez, Marciano Sablad, Rodrigo Yelin, Wendy Taylor, Kiyoshi Tachikawa, Sueanne Parker, Priya Karmali, Jared Davis, Sean M. Sullivan, Steve G. Hughes, Pad Chivukula, and Eng Eong Ooi. "A Single Dose of Self-Transcribing and Replicating Rna Based Sars-Cov-2 Vaccine Produces Protective Adaptive Immunity in Mice." *bioRxiv* (2020): 2020.09.03.280446.

154. Rauch, Susanne, Nicole Roth, Kim Schwendt, Mariola Fotin-Mleczek, Stefan O. Mueller, and Benjamin Petsch. "Mrna Based Sars-Cov-2 Vaccine Candidate Cynvov Induces High Levels of Virus Neutralizing Antibodies and Mediates Protection in Rodents." *bioRxiv* (2020): 2020.10.23.351775.

155. Moyo, Nathifa, Annette B. Vogel, Søren Buus, Stephanie Erbar, Edmund G. Wee, Uğur Sahin, and Tomáš Hanke. "Efficient Induction of T Cells against Conserved Hiv-1 Regions by Mosaic Vaccines Delivered as Self-Amplifying Mrna." *Molecular Therapy - Methods & Clinical Development* 12 (2019): 32-46.

156. Perche, Federico, Rudy Clemençon, Kai Schulze, Thomas Ebensen, Carlos A. Guzmán, and Chantal Pichon. "Neutral Lipopolylexes for In vivo Delivery of Conventional and Replicative Rna Vaccine." *Molecular Therapy - Nucleic Acids* 17 (2019): 767-75.

157. Goswami, Roshan, Despo Chatzikelephantous, Gustavo Lou, Fabiola Giusti, Alessandra Bonci, Marianna Taccone, Michelis Brazzoli, Simona Gallorini, Ilaria Ferlenghi, Francesco Berti, Derek T. O'Hagan, Carlo Pergola, Barbara C. Baudner, and Roberto Adamo. "Mannosylation of Lnp Results in Improved Potency for Self-Amplifying Rna (Sam) Vaccines." *ACS Infectious Diseases* 5, no. 9 (2019): 1546-58.
158. Stokes, Alan, Johanne Pion, Ornella Binazon, Benoit LaFontt, Maude Bigras, Guillaume Dubois, Karine Blouin, Jamie K. Young, Michael A. Ringenberg, Nawel Ben Abdeljelil, Julius Haruna, and Luis-Alexander Rodriguez. "Nonclinical Safety Assessment of Repeated Administration and Biodistribution of a Novel Rabies Self-Amplifying Mrna Vaccine in Rats." Regulatory Toxicology and Pharmacology 113 (2020): 104648.

159. Erasmus, Jesse H., Amit P. Khandhar, Megan A. O’Connor, Alexandra C. Walls, Emily A. Hemann, Patience Murapa, Jacob Archer, Shanna Leventhal, James T. Fuller, Thomas B. Lewis, Kevin E. Draves, Samantha Randall, Kathryn A. Guerriero, Malcolm S. Duthie, Darrick Carter, Steven G. Reed, David W. Hawman, Heinz Feldmann, Michael Gale, David Veesler, Peter Berglund, and Deborah Heydenburg Fuller. "An Alphavirus-Derived Replicon Rna Vaccine Induces Sars-Cov-2 Neutralizing Antibody and T Cell Responses in Mice and Nonhuman Primates." Science translational medicine 12, no. 555 (2020): eabc9396.

160. Luisi, Kate, Kaitlyn M. Morabito, Katherine E. Burgomaster, Mayuri Sharma, Wing-Pui Kong, Bryant M. Foreman, Sonal Patel, Brian Fisher, Maya A. Aleshnick, Jason Laliberte, Madison Wallace, Tracy J. Ruckwardt, David N. Gordon, Christine Linton, Nicole Ruggiero, Jessica L. Cohen, Russell Johnson, Kunal Aggarwal, Sung-Youl Ko, Eun Sung Yang, Rebecca S. Pelc, Kimberly A. Dowd, Derek O’Hagan, Jeffrey Ulmer, Sally Mossman, Anna Sambor, Edith Lepine, John R. Mascola, Theodore C. Pierson, Barney S. Graham, and Dong Yu. "Development of a Potent Zika Virus Vaccine Using Self-Amplifying Messenger Rna." Science Advances 6, no. 32 (2020): eaba5068.

161. Elong Ngono, Annie, Thasneem Syed, Anh-Viet Nguyen, Jose Angel Regla-Nava, Mercyla Susantono, Darina Spasova, Allison Aguilar, Melissa West, Jessica Sparks, Andrew Gonzalez, Emilie Branche, Jason L. DeHart, Jerel Boyd Vega, Priya Prakash Karmali, Padmanabh Chivukula, Kurt Kamrud, Parinaz Alihamd, Nathaniel Wang, and Sujan Shresta. "Cd8&lt;sup&gt;&lt;/sup&gt; T Cells Mediate Protection against Zika Virus Induced by an Ns3-Based Vaccine." Science Advances 6, no. 45 (2020): eabb2154.