

1

A Historical Overview on mRNA Vaccine Development

Rein Verbeke^{1,2}, Miffy H.Y. Cheng², and Pieter R. Cullis²

¹Ghent University, Ghent Research Group on Nanomedicines, Faculty of Pharmaceutical Sciences, Ottergemsesteenweg 460, 9000 Ghent, Belgium

²University of British Columbia, Department of Biochemistry and Molecular Biology, Health Sciences Mall 2350, BC V6T 1Z4 Vancouver, Canada

1.1 Introduction

In less than one year after the COVID-19 pandemic outbreak, two mRNA vaccines received the first emergency use authorization from the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), i.e. BNT162b2 (Comirnaty) from Pfizer/BioNTech and mRNA-1273 (Spikevax) from Moderna. In Phase 3 clinical trials, these mRNA vaccines were found to be generally safe and up to 95% efficacious after the second dose of vaccination in preventing symptomatic SARS-CoV-2 infection [1, 2]. The outstanding efficacy and unprecedented speed with which these mRNA vaccines were produced and distributed, strongly helped to curtail the burden of the pandemic and prevented millions of deaths [3, 4].

Now proven against COVID-19, there is explosive growth in research and investments in mRNA technology. Most notably, the platform of nucleoside-modified mRNA encapsulated in lipid nanoparticles (LNPs) that is utilized in today's COVID-19 mRNA vaccines is poised to have a rapid transformative effect on the future of medicine. Vaccines based on this platform are now being tested in Phase 3 clinical trials for several viral diseases other than COVID-19, such as against influenza (BNT161 and mRNA-1010), cytomegalovirus (mRNA-1647), and respiratory syncytial virus (mRNA-1345), while many other mRNA vaccines are being (pre)clinically studied to target diseases, such as bacterial and parasitic infections, cancer, and autoimmune diseases. In addition, the ability of the mRNA-LNP platform to deliver genetic information for the temporal production of proteins inside cells makes it a potential key technology to enable gene editing, protein replacement, and other immunotherapeutic approaches [5].

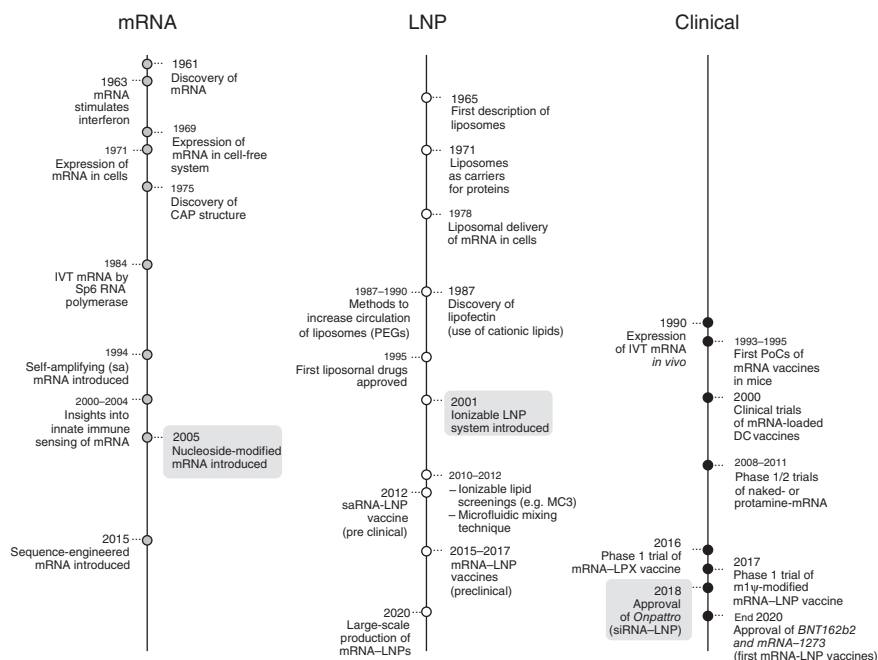


Figure 1.1 Discoveries and milestones in the development of mRNA-based vaccines are subdivided into three parallel timelines of mRNA-, LNP design, and clinical development.

It may seem that the COVID-19 mRNA vaccines came out of the blue, but in fact, decades of research were needed to develop this novel vaccine technology. To understand why it took so long for mRNA vaccines to breakthrough, we need to appreciate the collective efforts made by many scientists and the various problems they have tackled and solved that ultimately led to the development of this first generation of mRNA vaccines. As elucidated by Dowdy, the fundamental problem is that a billion years of evolutionary defenses need to be tackled to successfully deliver RNA. This includes both cellular barriers that have kept foreign RNAs on the outside of cells from invading the inside of cells, as well as the many innate immune defense mechanisms evolved to recognize and destroy foreign RNA [6]. Nonetheless, mRNA represents a most excellent vaccine modality to mimic viral infections, this is to trick the immune system to develop memory against the encoded, pathogen antigen. Indeed, when successfully delivered, mRNA has the potential to process and present encoded antigens through the same cellular machinery as occurring during viral infections, while it may also benefit from the specifically designed immune mechanisms against viruses to prime and promote durable adaptive immune responses, i.e. the immune adjuvant potential of mRNA.

In this first chapter section, a short historical overview is given of the development of mRNA vaccines (Figure 1.1).

1.2 The Path of mRNA as an Unstable and Toxic Product to a New Class of Medicine

1.2.1 The Discovery and *In Vitro* Production of mRNA

In 2015, Cobb wrote an essay that addressed the question of who discovered mRNA. By reconstructing the collective insights and different kinds of evidence gathered during the 1950s through the 1960s, Cobb concluded that mRNA was the product of many years of work by a community of researchers [7]. Ultimately, this research process gained momentum in the summer of 1961, when the nature and properties of mRNA were for the first time described in a theoretical model by Jacob and Monod [8]. In this review article on genetic regulation of protein synthesis, they proposed the existence of an intermediate molecule, or “messenger ribonucleotide” that is produced from DNA and that brings the genetic information to the ribosomes for protein synthesis. At about the same time, experimental support for the existence of mRNA was provided by two different research teams [9, 10]. Both research teams had succeeded in isolating mRNA and demonstrating its association with “pre-existing” ribosomes. This replaced the prevailing theory of the time that new specialized ribosomes are synthesized from the gene, and that these ribosomes are specific for the production of the corresponding protein, i.e. the “one gene – one ribosome – one protein” hypothesis. For detailed information about the remarkable series of events and some of the outstanding experiments that led to the discovery of mRNA, see reference [11].

In 1969, protein synthesis from mRNA inside ribosomes was first demonstrated in cell-free systems. In these experiments, RNA fractions purified from reticulocytes dictated the synthesis of globin when incubated with ribosomes obtained from *Escherichia coli* [12] or a different mammalian species [13]. Later on, the translation of mRNA into hemoglobin was also proven in living cells after the microinjection of 9 s RNA from rabbit reticulocytes into frog oocytes [14, 15]. While these studies might have sparked one’s imagination to use mRNA for therapeutic applications, at the time, the focus was solely on understanding its biological function.

In 1984, a simple and efficient method was established for *in vitro* mRNA synthesis using template DNA and a bacteriophage SP6 polymerase that initiates transcription at an SP6 promoter located upstream of the gene of interest [16]. In the following years, T7 and T3 RNA polymerases were also reported for successful *in vitro* transcribed (IVT) RNA synthesis [17–20]. These methods still represent the foundation of how mRNA is manufactured in today’s COVID-19 mRNA vaccines. However, a limitation of IVT mRNA production using phage polymerases is that it can give rise to multiple contaminants in the form of short and long double-stranded RNA (dsRNA) strands [21, 22]. These dsRNA byproducts have been shown to be largely responsible for the innate immune response to IVT mRNA, and when not controlled, have the potential to jeopardize the safety and functionality of mRNA vaccines (discussed in more detail below). There is, therefore, continuing interest in optimizing

the IVT process of mRNA in order to reduce the formation of dsRNA byproducts, as well as in finding (more) cost-effective purification methods [23–25].

Decades of basic research into the structural characteristics of mRNA and the biological interactions of mRNA with numerous proteins inside the cell not only brought new insights into mRNA metabolism and mRNA translation process but also leveraged IVT mRNA to reach a more optimal design [26]. The genetic information in mRNA is encoded in a codon sequence, where a triplet of adjacent nucleotides specifies an amino acid to be incorporated in a protein, also referred to as the open reading frame (ORF). The ORF is flanked at the 5' and 3' positions with start and stop codons. Because most amino acids are encoded by more than one codon, the codon usage in the ORF can be varied, also referred to as synonymous codon usage. Over the years, several strategies have been proposed to optimize codons so as to improve the translation and half-life of the mRNA, including methods of adjusting codons to match host transfer RNA abundances [27] for the enrichment of GC content [28, 29], and to optimize mRNA folds in the construct [30, 31]. It is important, however, to consider that these methods may have unintended effects on the performance of mRNA vaccines, such as altering protein folding and changing the sites of posttranslational modifications that may affect the immunogenicity and function of the encoded antigen, reviewed in [32].

Different nontranslated structural elements are present in eukaryotic mRNA, which were found to have essential roles in different stages of the mRNA life cycle. In 1975, the cap structure was discovered, which is an N7-methylated guanosine linked to the first nucleotide of the RNA via a reverse 5' to 5' triphosphate linkage [33–36]. By binding to the eukaryotic initiation factor (eIF) 4E, the cap structure enables the recruitment of translating ribosomes to mRNA. The cap structure and its methylation state are also significant determinants of how the host cell distinguishes itself from nonself RNA, as well as they are important for mRNA stability. At the 3' end of mRNA, a poly(A) tail made up of a long stretch of repeating adenosine nucleotides was found [37, 38], which functions synergistically with the cap structure to promote translation and regulate mRNA stability [39]. Furthermore, directly upstream and downstream of the ORF, two untranslated regions (UTRs) are positioned, which contain multiple regulatory elements [40]. Since their discovery, continuous efforts and progress have been made in optimizing these structural elements for improved stability and expression of IVT mRNA through empirical screening approaches and computational models [30, 31, 41–43].

Alternative forms of RNA have also been investigated as a therapeutic modality, such as those based on self-amplifying RNA (saRNA) and circular RNA (circRNA). saRNA includes the genetic information for a viral replicase in addition to the vaccine antigen or gene of interest in the ORF sequence and was already introduced in the mid-1990s for vaccine development [44, 45]. This replicase complex typically derived from alphaviruses allows the intracellular amplification of RNA and, in theory, can significantly increase and/or prolong the antigen expression capacity. The replication kinetics of saRNA may provide potential dose-sparing effects, while more prolonged antigen exposure may benefit the quality and duration of vaccine-induced immunity. Another more recent research avenue is the exploration of circRNA. This form of RNA characterized by its closed-ring structure was first reported in 1976

as an independent plant pathogens known as viroids, and later on also found to be prevalent in eukaryotic cells [46, 47]. Due to its unique structure, circRNA is protected from exonuclease-mediated degradation, which may confer improved stability compared to linear mRNA counterparts. Since circRNA lacks a cap and poly(A) tail structure, translation from circRNA into proteins is typically enabled through the insertion of an internal ribosome entry site [48].

1.2.2 The Inflammatory Nature of mRNA

A key bottleneck impeding the therapeutic use of RNA has been its inflammatory capacity. In the early years, it was already demonstrated that mRNA can induce antiviral innate immunity in mammalian cells. In 1957, it was found that adding heat-inactivated influenza virus to cells makes a protein substance that interferes with the replication of viruses, which was called “interferon” [49]. A few years later, it became clear that IFN must act by interfering with the viral RNA metabolism in virus-infected cells [50] and that the viral RNA itself was the component responsible for this antiviral immune reaction [51]. More than 30 years later, the discovery of pathogen recognition receptors by Hoffman and Beutler laid the groundwork for further studies into how mRNA triggers an innate immune response. Several intracellular sensors that are activated by structural features of mRNA were identified. As an example, dsRNA species were shown to be recognized by Toll-like receptor (TLR) 3 in the endosomes [52], and upon arrival in the cytosol by receptors such as Retinoic acid-inducible gene I (RIG-I) [53] and Melanoma differentiation-associated gene 5 (MDA5) [54]. TLR7 and TLR8 were identified as receptors for viral and synthetic single-stranded RNAs and their degradative products [55–58], while the cap1 structure (m7GpppNm) on mRNA was found to be critical to avoid recognition by RIG-I [59, 60]. These discoveries brought explanations of how mammalian cells are capable of distinguishing nonself from self RNA. The reader should refer to the following reviews [61, 62] on this topic for a comprehensive list of RNA sensors, the involved signal transduction pathways, and more details on the molecular basis of mRNA recognition.

The immune recognition of mRNA activates downstream signaling pathways, which leads to the production of inflammatory cytokines, including type I IFNs, inducing in the cells an antiviral state. This has some very important implications for the design of mRNA vaccines and therapeutics. First of all, the immune recognition of mRNA is considered as one of the mechanisms underlying the cause of reactogenicity symptoms, and thus, to a large extent, will determine the safety of mRNA therapeutics. Second, the innate immune response to mRNA can drastically impair the translation of IVT mRNA, thereby reducing the antigen availability for T-cell and B-cell recognition. For example, dsRNA elements in mRNA products are sensed by intracellular enzymes, such as protein kinase R (PKR) and 2′–5′-oligoadenylate synthetase (OAS), which in turn activate other proteins that cause translational shutdown and cell death [63, 64]. Yet, on the upside, the innate immune response to mRNA has the potential to provide an important adjuvant effect on vaccine potency, and it can be argued, maybe even play a crucial role in the success of mRNA vaccines. Indeed, downstream signaling from the RNA sensing receptors also activates the expression of several genes involved in the mobilization

and activation of antigen-presenting cells (APCs), while RNA-driven cytokines are capable of exerting direct immune-stimulatory effects on T cells and B cells. Therefore, the most challenging task with mRNA vaccines was to find an optimal mRNA design that could minimize the problems posed by its immune recognition on mRNA translation and vaccine safety while still providing enough adjuvant properties to drive strong vaccine responses against the encoded antigen.

At least part of this problem was overcome by the work of Karikó, Weissman, and colleagues at the University of Pennsylvania (UPenn), who were studying the immune-stimulatory effects of IVT mRNA on dendritic cells (DCs) *in vitro*, i.e. the innate immune cell type specialized in antigen presentation and T-cell activation [65]. By comparing the immune-stimulatory activity of IVT mRNA with RNA types derived from different sources, they found that both bacterial (total) RNA, mammalian mitochondrial RNA, and IVT mRNA primed the DCs to produce high levels of tumor necrosis factor- α (TNF- α). In contrast, they found that total-, nuclear-, and cytoplasmic RNAs from mammalian sources were less potent inducers of DC activation, while mammalian transfer RNA was noninflammatory. This finding led the researchers to hypothesize that posttranscriptional modifications commonly present in mammalian RNA, such as pseudouridine and methylated nucleosides, might serve as another molecular signature by which cellular RNA sensors discriminate self-RNA from foreign RNA. When applied to IVT mRNA, modified nucleotides, in particular pseudouridines and their derivatives, indeed protected IVT mRNA from activating the aforementioned RNA sensors; TLR3, TLR7, TLR8, PKR, OAS, RIG-I, and MDA5 [22, 63–66]. Consequently, the use of modified uridines strongly improved the translation and safety profile of IVT mRNA products [67, 68]. It is noteworthy that the following studies also evidenced that the use of modified uridines in the IVT reaction reduces the formation of dsRNA byproducts, which might at least in part explain the reduced immunogenicity of uridine-modified mRNA [22]. In addition, it was shown that pseudouridine not only acts as a major controller of the innate immune activity of IVT mRNA but can also contribute to the intrinsic stability and the dynamics of mRNA molecules with ribosomes, reviewed in [69]. Taken together, the use of modified uridines was a great leap forward for producing more translatable, less immunogenic, and safer IVT mRNA products. Note that for the COVID-19 mRNA vaccines BNT162b2 and mRNA-1273, every uridine residue in the mRNA is replaced with N1-methylpseudouridine (m1 ψ).

1.3 How Studying Lipid Bilayer Structures in Cell Membranes Gave Rise to the Eventual Development of Lipid Nanoparticles for RNA Delivery

1.3.1 From Biological Cell Membranes to Liposomal Drugs

RNAs are inherently unstable as they are easily degraded by ribonucleases (RNases), which act as the first line of defense against foreign RNAs as well as allow our cells to regulate RNA metabolism. In addition, the lipid bilayer structure that forms

the foundation of cell membranes is uniquely designed to prevent the permeation of such large, negatively charged RNA molecules, protecting the cells from exogenous RNA entering them. Ironically, long-standing efforts to understand the physical properties and functional roles of lipids in cell membranes led scientists to model lipid membrane systems, which have been instrumental in the development of the delivery technology enabling intact nucleic acids to cross these biological barriers; LNPs containing ionizable cationic lipids.

The history of LNPs began in 1964 when Bangham and Horne first described electron microscopy observations of dispersed lecithin (phosphatidylcholine) in water, showing the formation of lamellar “spherulites” structures [70]. In follow-on papers, Bangham and his colleagues described the bimolecular leaflet structure of lecithin vesicles and proved the dispersed phospholipids were spontaneously forming a closed membrane system, i.e. a lipid bilayer completely enclosing an aqueous space [71]. It was Weissmann who named these lipid vesicles “liposomes.” Subsequently, the development and characterization of the physical properties of lipids and liposomes quickly followed [72–76]. These studies on the structures that lipids adopt [72] bilayer permeability [77], and membrane fusion [78] helped lay down the cornerstone for the understanding of the biophysical properties of liposomes [79, 80].

In parallel to understanding the membrane biophysics of liposomes, liposome research has extended into areas of therapeutic application. In 1970, Sessa and Weissman first demonstrated that these liposomes have the ability to entrap lysozyme [81], followed by others that started to explore the therapeutic potential of liposomes as drug carriers for enzymes and proteins in rodent models [82–84]. With the experimental methods available at the time, these studies demonstrated that liposomes were removed from the blood within minutes and accumulated in the liver and spleen of the rats, in which liposome-entrapped protein eventually localized in the lysosomes of cells. However, the major initial advances of liposomes as drug delivery systems came with applications for the delivery of anticancer drugs. Two important advances made in the 1980s were the development of the extrusion process for the rapid production of unilamellar vesicles with diameters in the 100 nm size range [85] and the “remote loading” pH gradient technique for efficiently loading liposomal systems with cancer drugs [86, 87].

The third advance involved the development of techniques to increase the circulation lifetime of liposomes following i.v. administration. The incorporation of cholesterol in phosphatidylcholine liposomes reduced bilayer permeability and increased the stability of these systems *in vitro* and *in vivo* [88, 89]. The presence of gangliosides and sphingomyelin reduced liposome clearance rates, which led to the concept of “stealth” liposomes [90]. Incorporation of polyethylene glycol (PEG) lipids further improved the blood circulation half-life of liposomes [91]. PEGylation quickly became popular to improve pharmacokinetics and biodistribution of liposomes, which along with the improvement of liposomal generation (by extrusion) and drug-loading procedures (using pH gradients) have led to the first liposomal drugs for systemic delivery of small molecule drugs to treat fungal infections and a variety of cancers.

With regard to nucleic acid-based drugs, the idea of using liposomes to deliver DNA and RNA into host cells was introduced simultaneously by Dimitriadis and Ostro [92, 93]. Both studies demonstrated the encapsulation and delivery of rabbit globin mRNA and rabbit reticulocyte 9S mRNA into eukaryotic cells by using large unilamellar liposomes. However, these studies were not pursued due to limited transfection potency, lack of scalable methods of manufacture, and poor-encapsulation efficiencies. The introduction of cationic lipids such as 1,2-di-*O*-octadecenyl-3-trimethylammonium propane (DOTMA) rekindled interest in the late 1980s [94, 95]. The electrostatic interaction between the positively charged quaternary ammonium head group of the lipid and the negatively charged phosphate backbone of nucleic acids leads to the formation of “complexes” with high-encapsulation efficiencies. In addition, the cationic lipid has membrane destabilization properties enabling the delivery of nucleic acid payloads across cellular barriers [96, 97]. In 2001, Hafez et al. proposed that the membrane destabilization properties of cationic lipids were related to their ability to form disruptive nonbilayer structures in combination with the negatively charged lipids found in biological membranes ([96]). This research resulted in products such as Lipofectin™, a liposomal composition of the cationic lipid DOTMA and 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl-ethanolamine (DOPE), followed by other polyvalent cationic lipid containing liposomal formulations such as Lipofectamine™ and Transfectam™. These lipofection reagents are commonly used in biochemistry and molecular biology research for the *in vitro* delivery of DNA and RNA in eukaryotic cells.

1.3.2 Ionizable Lipid Nanoparticles for Systemic Delivery of Nucleic Acids

Delivery of nucleic acid-based drugs presents significantly more problems than the delivery of small molecular drugs. In addition to reaching target tissue, the delivery system has to protect the cargo from degradation, facilitate uptake into target cells, and subsequently deliver the cargo into the cytoplasm of target cells. Complexes of DNA or RNA with cationic liposomes result in a heterogeneous mixture of complexes that are relatively unstable and can change properties such as size over time [98]. In addition, cationic lipids can be toxic and cause membrane disruption, hemolysis and induce inflammatory responses limiting therapeutic applications [99, 100].

The major breakthrough for delivering nucleic acid was the introduction of ionizable cationic lipids. In 1994, Bailey and Cullis synthesized 1,2-dioleoyl-3-dimethylammonium propane (DODAP), an ionizable version of a cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) to study the influence of lipid asymmetry on the fusion of liposomal systems [101]. Subsequently, Cullis and colleagues used lipid-based systems containing ionizable lipids to encapsulate nucleic acids [102]. They showed that DODAP had an apparent pK (pK_a) of approximately 6.5 and that these systems could be used to encapsulate nucleic acids at pH values where the lipids are protonated and positively charged (e.g. pH 4) and that the nucleic acid polymers were retained when the pH was raised to physiological values (pH 7.4). Further investigations showed that these lipid systems had a “solid

core” hydrophobic interior surrounded by a lipid monolayer [103]. These systems were called “LNPs” to distinguish them from liposomes, which exhibit bilayer structures. An important benefit of encapsulation using ionizable lipids such as DODAP is that the LNP are effectively neutral at physiological pH, rendering them less toxic than lipid-based systems containing permanently positively charged lipids, allowing systemic administration. In 2001, the first report was published on the therapeutic potential of a lipid formulation composed of DODAP, cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, and PEG ceramides for the systemic delivery of antisense oligodeoxynucleotides in mice [104].

Subsequent efforts focused on the delivery of small interfering RNA (siRNA) to the liver following i.v. administration to silence genes in hepatocytes. LNPs containing DODAP were able to induce gene silencing in the liver but only at very high doses (see supplemental information for [105]). This led to the synthesis of 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA), a more stable version of DODAP, which, when formulated in LNP siRNA systems, showed early preclinical promise in nonhuman primates for silencing of apolipoprotein B expression in the liver [106]. Further studies guided by a “rational design” approach to adjust the ionizable lipid pK_a and shape properties to drive the formation of membrane disruptive nonbilayer structures and encourage endosomal escape of siRNA contents resulted in DLin-KC2-DMA. LNP siRNA systems containing DLinKC2DMA exhibited improved gene-silencing properties [105], leading to an intense screening program involving the synthesis of hundreds of ionizable lipids and testing in preclinical animal models to find ionizable lipids with optimal silencing properties when incorporated into LNP siRNA systems. This led to DLin-MC3-DMA [107], which was incorporated into the first RNAi drug to be approved by the FDA [108]. Further iterations focused on the degradability of ionizable lipids led to the inclusion of bio-cleavable ester functions within the lipid acyl chains [109]. Later studies aimed at optimizing the liver transfection properties of LNP systems containing mRNA led to new generations of ionizable lipids incorporating branched structures as illustrated in the SM-102 and ALC-0315 lipid utilized in the mRNA-1273 and BNT162b2 vaccine, respectively [109–111].

A different screening approach was taken by Langer and Anderson from the Massachusetts Institute of Technology, who synthesized a library of ionizable lipid-like polyvalent material called lipidoids; these screens identified potent lipidoids such as C12-200 and generated lipidoid-based LNPs that were used to successfully deliver siRNAs in mice and nonhuman primates [112–114].

The PEG-lipid components also required improvement. A PEG-conjugated lipid is required during formulation to generate small, stable LNP systems [115], however a disadvantage is that the steric barrier created by the PEG coating inhibits the cellular uptake, and thus the transfection potency, of LNP containing nucleic acid-based drugs. Thus, the PEG-lipids employed were designed to diffuse away from the LNP following systemic administration [116]. Previous work has shown that PEG-lipids with short (C_{14}) acyl chains readily exchange out of lipid-based systems if a suitable “sink” is available [117]. In addition to enhanced cellular uptake, another benefit was that the “diffusible” PEG-conjugated

lipids mitigate the risk of inducing anti-PEG antibody responses [118, 119]. This unwanted immunogenicity has been associated with accelerated blood clearance and pseudo-allergic reactions upon repeated injections of PEGylated LNPs in preclinical models [120]. Further optimization to balance out the effects of the PEG-conjugated lipid on the efficacy, safety and stability of nucleic-acid loaded LNPs together with the synthesis of the more easily produced PEG-diacylglycerol lipids [121], eventually resulted in the identification of what was to become the gold standard PEG-lipid; 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2000) [122].

A final challenge in the establishment of the LNP technology was to find a reproducible and scalable manufacturing method that could obtain LNPs with high encapsulation efficiencies. Initial work on LNP systems made use of a method based on the ethanol dilution principle first described by Batzri and Korn [123] and followed a three-step procedure: (i) lipid components dissolved in ethanol are diluted in an acidic citrate solution containing nucleic acid to form vesicular structures; (ii) the intermediate mixture is downsized using extrusion; (iii) the mixture is dialyzed to remove ethanol and to raise the pH to 7.4 [104]. This procedure was further optimized by using rapid-mixing devices such as by means of a T-tube junction [124] and microfluidics [125], which allowed for the scalable production of LNP systems with higher encapsulation efficiencies and smaller particle sizes under well defined, reproducible conditions. For more in-depth information on the production of LNPs and how these methods were improved and simplified, the reader is referred to the following reviews [126–128].

Starting from the 2010s, the LNP system became the preferred nonviral delivery system for nucleic acids, which eventually culminated in the clinical approval of the first siRNA-LNP-based product to treat neuropathies associated with hereditary transthyretin amyloidosis, Onpattro® (patisiran) [129]. The clinical approval of Onpattro® was a milestone not only for gene therapies in general but also as a precedent that enabled the clinical progress of mRNA vaccines, as will be discussed in Section 1.4.

1.4 The Journey of Developing Clinical mRNA Vaccines

The exploration of the therapeutic use of IVT mRNA started in the early 1990s with Felgner and coworkers first providing proof-of-concept that naked IVT mRNA or DNA can be translated into protein in living animals [130]. The therapeutic potential of IVT mRNA was first demonstrated in a study where vasopressin-encoding mRNA was injected into the hypothalamus, achieving a temporary reversal of diabetes insipidus in rats [131]. Next, a few pioneering studies reported the usefulness of RNA for immunization in mice; Martinon et al. demonstrated that liposomes containing mRNA encoding the influenza virus nucleoprotein were capable of inducing virus-specific cytotoxic T-cell responses [132]; Conry et al. reported the activation of humoral immune responses by using a naked mRNA vaccine encoding

a carcinoembryonic antigen [133]; while Zhou et al. were the first to show the feasibility of a saRNA vaccine [45].

However, before the development of the COVID-19 mRNA vaccines, there was plenty of trial and error, testing many different mRNA designs and delivery approaches. IVT mRNA was first tested in humans using an *ex vivo* approach, where monocyte-derived DCs are transfected with tumor antigen mRNA and reinfused into patients with cancer as a cellular vaccine [134, 135]. Later on, CureVac, a German biotech company founded in 2000, organized the first-in-human clinical trials testing two RNA cancer vaccine approaches for the treatment of melanoma; one based on the direct dermal administration of naked mRNA in combination with granulocyte macrophage colony-stimulating factor as an adjuvant, and the other using mRNA complexed with protamine [136, 137]. Applying the lessons learned from these early clinical trials helped CureVac to systematically develop and test the next generations of mRNA technologies.

These pioneering clinical trials also inspired other researchers/entrepreneurs to come up with novel concepts. Before COVID-19, BioNTech's activities were mainly focused on the field of immuno-oncology, where they have clinically tested various RNA-based technologies for active and passive immunization strategies. In early work, BioNTech evidenced that lymph node-resident DC could be transfected and activated by the direct "intranodal" administration of naked mRNA (using calcium-rich buffers), which resulted in an efficient expansion of T cells against the encoded antigen [138]. The feasibility of this naked mRNA vaccine approach was further demonstrated in patients with melanoma, showing clinical promise in developing T-cell responses against patient-specific neo-epitopes [139]. In 2016, BioNTech introduced another delivery approach for the systemic delivery of unmodified mRNA cancer vaccines using DOTMA/DOPE liposomes [140]. They found that the charge ratio between cationic lipids and mRNA could be used to change the *in vivo* organ transfection of these systems; with lipoplexes exhibiting an excess negative charge specifically targeting DCs in the spleen and lymph nodes. This mRNA vaccine platform is currently being investigated by the company in Phase 1 and Phase 2 clinical trials for various cancer indications, such as monotherapy or in combination with immune checkpoint inhibition [141].

Over the years, the feasibility of the LNP technology has been demonstrated in academic literature for various applications and different nucleic acid cargo. It also became clear that exploiting the interaction of LNPs with immune cells, rather than escaping it, provides opportunities for vaccination and other immunotherapeutic approaches [142, 143]. The first example of combining LNP and RNA as a new vaccine platform was reported by Geall and his colleagues at Novartis in 2012 [144]. An LNP formulation with the ionizable lipid DLinDMA was used to encapsulate a saRNA encoding the fusion glycoprotein of the respiratory syncytial virus. After intramuscular immunization, the saRNA-LNP vaccine demonstrated noninferior immune responses compared to a viral delivery technology in mice with the major advantage of removing the inherent limitations of viral vectors. In the search for a suitable delivery system, the Weissman lab (UPenn) partnered with the LNP-specialized company Acuitas Therapeutics founded by Cullis, Madden, and

Hope in 2009. They first reported on a study that showed the potential of delivering LNPs loaded with m1 ψ -modified mRNA via different administration routes in mice [145], followed by several reports on preclinical studies demonstrating the outstanding capacity of this vaccine platform to elicit immune responses against viral pathogens, such as zika, influenza, herpes simplex virus, and human immunodeficiency virus [146–149]. In 2018, this success led to collaborations between BioNTech and Pfizer to co-develop an mRNA influenza vaccine based on these technologies, where it was recently announced by Pfizer that first volunteers have entered a Phase 3 clinical trial [150]. Likewise, CureVac entered an agreement with Acuitas Therapeutics, and before COVID-19, already worked on a prophylactic mRNA–LNP vaccine against rabies by applying their sequence-optimized mRNA platform and Acuitas’ LNP technology [151, 152]. In 2017, Moderna was the first company to report interim results from a Phase 1 clinical trial on the immunogenicity and reactogenicity of a m1 ψ -modified mRNA–LNP vaccine against H10N8 and H7N9 influenza viruses [153, 154].

So, when the SARS-CoV-2 pandemic hit, several mRNA vaccine platforms had already reached a substantial degree of maturity. This allowed companies such as CureVac, BioNTech/Pfizer, and Moderna to produce clinical-grade mRNA vaccine candidates against SARS-CoV-2 in record times. However, none of these mRNA vaccine platforms had made it to Phase 3 clinical testing before, which made the COVID-19 pandemic the first great opportunity to investigate and compare the potential and safety of these different mRNA vaccine platforms in large-scale clinical trials [155]. The m1 ψ -modified mRNA vaccines, BNT162b2 and mRNA-1273, have been the first to get results from Phase 3 clinical trials, and although no direct comparisons were made between the different vaccine candidates, these vaccines have demonstrated a significantly greater efficacy for preventing symptomatic COVID-19 disease (~95%) compared to other mRNA vaccine candidates, e.g. CVnCoV, CureVac’s sequence-optimized mRNA vaccine candidate: 47% [156] and ARCT-154, Arcturus’s saRNA vaccine candidate: 55% [157]. Note that, all of these mRNA vaccine candidates were using LNPs to enable their delivery following the intramuscular administration route.

1.5 Concluding Remarks

In this chapter section, we focused on some of the fundamental discoveries and technological improvements that contributed to the development of the COVID-19 mRNA vaccines. Here, we highlighted nucleoside modification and ionizable LNPs as two key enabling technologies of this novel vaccine platform, which have drastically increased the therapeutic index and potency of mRNA vaccines.

However, there are many aspects to the success of mRNA vaccines, and we believe that we might soon learn that alternative mRNA vaccine platforms will be clinically useful as well. For SARS-CoV-2 as an example, Providence Therapeutics recently announced positive results from an unmodified uridine mRNA–LNP vaccine (PTX-COVID19-B) that showed noninferior immunogenicity and equal

safety compared to BNT162b2 in Phase 2 clinical trial [158]. Also, CureVac has further optimized its COVID-19 mRNA vaccine candidate by adjusting the UTRs and poly(A) tail structure, which achieved greatly improved immunogenicity and protective efficacy compared to their first-generation COVID-19 vaccine in nonhuman primates [41]. For saRNA-based vaccines, it was suggested that modifications or additional measures that could minimize the innate immune response early during saRNA vaccination may prove to be critical to increasing primary expression and the resulting clinical performance [159, 160]. Moreover, there might also be a need to select and/or optimize mRNA vaccine design depending on the disease indication and the types of immune response that correlate with protection. For instance, it was recently demonstrated that LNPs loaded with purified unmodified mRNA elicited a higher CD8⁺ T-cell response and achieved more effective anti-tumor immunity in B16-melanoma bearing mice compared to a m1 ψ -modified mRNA–LNP counterpart, which highlights the positive effect of unmodified mRNA and the induction of type I IFN responses for cancer vaccination [161]. Interestingly, several studies have also recently demonstrated that the LNP formulation not only enables the delivery of mRNA vaccines but also contributes to their reactogenicity and immunogenicity by providing innate immune stimulation [162–164]. These findings could open up new avenues for the design of more effective and potentially safer mRNA vaccine generations. However, the underlying mechanisms of these LNP adjuvant properties and how these properties can be more fine-tuned remain largely unknown and will require further exploration [165].

Finally, it needs to be mentioned that other factors also helped the rapid development and production of the COVID-19 mRNA vaccines, which were not discussed in this chapter section, such as the prior knowledge that was available on SARS-CoV-1 and other coronaviruses and the huge financial investments and scientific collaborations which allowed some of the steps in the research and development process to be conducted in parallel. Future development of mRNA medicines is poised to benefit from the enormous steps and hurdles taken with respect to various industrial aspects, such as the establishment of supply chain systems for starting materials, the infrastructure that was built for large-scale production of LNP–mRNA products, and the better regulation guidelines that are now set out to bring these products to the market.

Taken together, the development of the COVID-19 mRNA vaccines was a great achievement, which was built on decades of fundamental and translational science. Based on the continuous effort being made by a growing field of multidisciplinary researchers, we believe that multiple therapeutic modalities will become available using (m)RNA technologies for a variety of diseases.

References

- 1 Baden, L.R., El Sahly, H.M., Essink, B. et al. (2021). Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. *N. Engl. J. Med.* 384 (5): 403–416. <https://doi.org/10.1056/NEJMoa2035389>.

- 2 Polack, F.P., Thomas, S.J., Kitchin, N. et al. (2020). Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N. Engl. J. Med.* 383 (27): 2603–2615. <https://doi.org/10.1056/NEJMoa2034577>.
- 3 Di Fusco, M., Marczell, K., Deger, K.A. et al. (2022). Public health impact of the Pfizer-BioNTech COVID-19 vaccine (BNT162b2) in the first year of rollout in the United States. *J. Med. Econ.* 25 (1): 605–617. <https://doi.org/10.1080/13696998.2022.2071427>.
- 4 Watson, O.J., Barnsley, G., Toor, J. et al. (2022). Global impact of the first year of COVID-19 vaccination: a mathematical modelling study. *Lancet Infect. Dis.* 22 (9): 1293–1302. [https://doi.org/10.1016/S1473-3099\(22\)00320-6](https://doi.org/10.1016/S1473-3099(22)00320-6).
- 5 Rohner, E., Yang, R., Foo, K.S. et al. (2022). Unlocking the promise of mRNA therapeutics. *Nat. Biotechnol.* 40 (11): 1586–1600. <https://doi.org/10.1038/s41587-022-01491-z>.
- 6 Dowdy, S.F. (2017). Overcoming cellular barriers for RNA therapeutics. *Nat. Biotechnol.* 35 (3): 222–229. <https://doi.org/10.1038/nbt.3802>.
- 7 Cobb, M. (2015). Who discovered messenger RNA? *Curr. Biol.* 25 (13): R526–R532. <https://doi.org/10.1016/j.cub.2015.05.032>.
- 8 Jacob, F. and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* 3: 318–356. [https://doi.org/10.1016/s0022-2836\(61\)80072-7](https://doi.org/10.1016/s0022-2836(61)80072-7).
- 9 Brenner, S., Jacob, F., and Meselson, M. (1961). An unstable intermediate carrying information from genes to ribosomes for protein synthesis. *Nature* 190: 576–581. <https://doi.org/10.1038/190576a0>.
- 10 Gros, F., Hiatt, H., Gilbert, W. et al. (1961). Unstable ribonucleic acid revealed by pulse labelling of *Escherichia coli*. *Nature* 190: 581–585. <https://doi.org/10.1038/190581a0>.
- 11 Judson, H.F. (1979). *The Eighth Day of Creation*. New York: CSH Press.
- 12 Laycock, D.G. and Hunt, J.A. (1969). Synthesis of rabbit globin by a bacterial cell free system. *Nature* 221 (5186): 1118–1122. <https://doi.org/10.1038/2211118a0>.
- 13 Lockard, R.E. and Lingrel, J.B. (1969). The synthesis of mouse hemoglobin beta-chains in a rabbit reticulocyte cell-free system programmed with mouse reticulocyte 9S RNA. *Biochem. Biophys. Res. Commun.* 37 (2): 204–212. [https://doi.org/10.1016/0006-291x\(69\)90720-7](https://doi.org/10.1016/0006-291x(69)90720-7).
- 14 Gurdon, J.B., Lane, C.D., Woodland, H.R. et al. (1971). Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. *Nature* 233 (5316): 177–182. <https://doi.org/10.1038/233177a0>.
- 15 Lane, C.D., Marbaix, G., and Gurdon, J.B. (1971). Rabbit haemoglobin synthesis in frog cells: the translation of reticulocyte 9 s RNA in frog oocytes. *J. Mol. Biol.* 61 (1): 73–91. [https://doi.org/10.1016/0022-2836\(71\)90207-5](https://doi.org/10.1016/0022-2836(71)90207-5).
- 16 Melton, D.A., Krieg, P.A., Rebagliati, M.R. et al. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12 (18): 7035–7056. <https://doi.org/10.1093/nar/12.18.7035>.

- 17 Milligan, J.F., Groebe, D.R., Witherell, G.W. et al. (1987). Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* 15 (21): 8783–8798. <https://doi.org/10.1093/nar/15.21.8783>.
- 18 Studier, F.W. and Moffatt, B.A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189 (1): 113–130. [https://doi.org/10.1016/0022-2836\(86\)90385-2](https://doi.org/10.1016/0022-2836(86)90385-2).
- 19 Tabor, S. and Richardson, C.C. (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. U. S. A.* 82 (4): 1074–1078. <https://doi.org/10.1073/pnas.82.4.1074>.
- 20 van der Werf, S., Bradley, J., Wimmer, E. et al. (1986). Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* 83 (8): 2330–2334. <https://doi.org/10.1073/pnas.83.8.2330>.
- 21 Baiersdorfer, M., Boros, G., Muramatsu, H. et al. (2019). A facile method for the removal of dsRNA contaminant from in vitro-transcribed mRNA. *Mol. Ther. Nucleic Acids* 15: 26–35. <https://doi.org/10.1016/j.omtn.2019.02.018>.
- 22 Mu, X., Greenwald, E., Ahmad, S. et al. (2018). An origin of the immunogenicity of in vitro transcribed RNA. *Nucleic Acids Res.* 46 (10): 5239–5249. <https://doi.org/10.1093/nar/gky177>.
- 23 Dousis, A., Ravichandran, K., Hobert, E.M. et al. (2022). An engineered T7 RNA polymerase that produces mRNA free of immunostimulatory byproducts. *Nat. Biotechnol.* 41 (4): 560–568. <https://doi.org/10.1038/s41587-022-01525-6>.
- 24 Nelson, J., Sorensen, E.W., Mintri, S. et al. (2020). Impact of mRNA chemistry and manufacturing process on innate immune activation. *Sci. Adv.* 6 (26): eaaz6893. <https://doi.org/10.1126/sciadv.aaz6893>.
- 25 Piao, X.J., Yadav, V., Wang, E.D. et al. (2022). Double-stranded RNA reduction by chaotropic agents during in vitro transcription of messenger RNA. *Mol. Ther. Nucleic Acids* 29: 618–624. <https://doi.org/10.1016/j.omtn.2022.08.001>.
- 26 Sahin, U., Kariko, K., and Tureci, O. (2014). mRNA-based therapeutics - developing a new class of drugs. *Nat. Rev. Drug Discov.* 13 (10): 759–780. <https://doi.org/10.1038/nrd4278>.
- 27 Kane, J.F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* 6 (5): 494–500. [https://doi.org/10.1016/0958-1669\(95\)80082-4](https://doi.org/10.1016/0958-1669(95)80082-4).
- 28 Kudla, G., Lipinski, L., Caffin, F. et al. (2006). High guanine and cytosine content increases mRNA levels in mammalian cells. *PLoS Biol.* 4 (6): e180. <https://doi.org/10.1371/journal.pbio.0040180>.
- 29 Thess, A., Grund, S., Mui, B.L. et al. (2015). Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals. *Mol. Ther.* 23 (9): 1456–1464. <https://doi.org/10.1038/mt.2015.103>.
- 30 Leppek, K., Byeon, G.W., Kladwang, W. et al. (2022). Combinatorial optimization of mRNA structure, stability, and translation for RNA-based therapeutics. *Nat. Commun.* 13 (1): 1536. <https://doi.org/10.1038/s41467-022-28776-w>.

- 31 Mauger, D.M., Cabral, B.J., Presnyak, V. et al. (2019). mRNA structure regulates protein expression through changes in functional half-life. *Proc. Natl. Acad. Sci. U. S. A.* 116 (48): 24075–24083. <https://doi.org/10.1073/pnas.1908052116>.
- 32 Mauro, V.P. and Chappell, S.A. (2014). A critical analysis of codon optimization in human therapeutics. *Trends Mol. Med.* 20 (11): 604–613. <https://doi.org/10.1016/j.molmed.2014.09.003>.
- 33 Adams, J.M. and Cory, S. (1975). Modified nucleosides and bizarre 5'-termini in mouse myeloma mRNA. *Nature* 255 (5503): 28–33. <https://doi.org/10.1038/255028a0>.
- 34 Furuichi, Y. and Miura, K. (1975). A blocked structure at the 5' terminus of mRNA from cytoplasmic polyhedrosis virus. *Nature* 253 (5490): 374–375. <https://doi.org/10.1038/253374a0>.
- 35 Martin, S.A., Paoletti, E., and Moss, B. (1975). Purification of mRNA guanylyl-transferase and mRNA (guanine-7-) methyltransferase from vaccinia virions. *J. Biol. Chem.* 250 (24): 9322–9329. [10.1016/S0021-9258\(19\)40646-7](https://doi.org/10.1016/S0021-9258(19)40646-7).
- 36 Muthukrishnan, S., Both, G.W., Furuichi, Y. et al. (1975). 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. *Nature* 255 (5503): 33–37. <https://doi.org/10.1038/255033a0>.
- 37 Burr, H. and Lingrel, J.B. (1971). Poly A sequences at the 3' termini of rabbit globin mRNAs. *Nature* 233 (36): 41–43. <https://doi.org/10.1038/newbio233041a0>.
- 38 Lim, L. and Canellakis, E.S. (1970). Adenine-rich polymer associated with rabbit reticulocyte messenger RNA. *Nature* 227 (5259): 710–712. <https://doi.org/10.1038/227710a0>.
- 39 Passmore, L.A. and Collier, J. (2022). Roles of mRNA poly(A) tails in regulation of eukaryotic gene expression. *Nat. Rev. Mol. Cell Biol.* 23 (2): 93–106. <https://doi.org/10.1038/s41580-021-00417-y>.
- 40 Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* 12 (2): 857–872. <https://doi.org/10.1093/nar/12.2.857>.
- 41 Gebre, M.S., Rauch, S., Roth, N. et al. (2022). Optimization of non-coding regions for a non-modified mRNA COVID-19 vaccine. *Nature* 601 (7893): 410–414. <https://doi.org/10.1038/s41586-021-04231-6>.
- 42 Holtkamp, S., Kreiter, S., Selmi, A. et al. (2006). Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. *Blood* 108 (13): 4009–4017. <https://doi.org/10.1182/blood-2006-04-015024>.
- 43 Orlandini von Niessen, A.G., Poleganov, M.A., Rechner, C. et al. (2019). Improving mRNA-based therapeutic gene delivery by expression-augmenting 3' UTRs identified by cellular library screening. *Mol. Ther.* 27 (4): 824–836. <https://doi.org/10.1016/j.ymthe.2018.12.011>.
- 44 Johanning, F.W., Conry, R.M., LoBuglio, A.F. et al. (1995). A Sindbis virus mRNA polynucleotide vector achieves prolonged and high level heterologous gene expression in vivo. *Nucleic Acids Res.* 23 (9): 1495–1501. <https://doi.org/10.1093/nar/23.9.1495>.

- 45 Zhou, X., Berglund, P., Rhodes, G. et al. (1994). Self-replicating semliki forest virus RNA as recombinant vaccine. *Vaccine* 12 (16): 1510–1514. [https://doi.org/10.1016/0264-410x\(94\)90074-4](https://doi.org/10.1016/0264-410x(94)90074-4).
- 46 Hsu, M.T. and Coca-Prados, M. (1979). Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. *Nature* 280 (5720): 339–340. <https://doi.org/10.1038/280339a0>.
- 47 Sanger, H.L., Klotz, G., Riesner, D. et al. (1976). Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. *Proc. Natl. Acad. Sci. U. S. A.* 73 (11): 3852–3856. <https://doi.org/10.1073/pnas.73.11.3852>.
- 48 Wesselhoeft, R.A., Kowalski, P.S., and Anderson, D.G. (2018). Engineering circular RNA for potent and stable translation in eukaryotic cells. *Nat. Commun.* 9 (1): 2629. <https://doi.org/10.1038/s41467-018-05096-6>.
- 49 Isaacs, A. and Lindenmann, J. (1957). Virus interference. I. The interferon. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 147 (927): 258–267. <https://doi.org/10.1098/rspb.1957.0048>.
- 50 De Somer, P., Prinzie, A., Denys, P. Jr., et al. (1962). Mechanism of action of interferon. I. Relationship with viral ribonucleic acid. *Virology* 16: 63–70. [https://doi.org/10.1016/0042-6822\(62\)90202-7](https://doi.org/10.1016/0042-6822(62)90202-7).
- 51 Isaacs, A., Cox, R.A., and Rotem, Z. (1963). Foreign nucleic acids as the stimulus to make interferon. *Lancet* 2 (7299): 113–116. [https://doi.org/10.1016/s0140-6736\(63\)92585-6](https://doi.org/10.1016/s0140-6736(63)92585-6).
- 52 Alexopoulou, L., Holt, A.C., Medzhitov, R. et al. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413 (6857): 732–738. <https://doi.org/10.1038/35099560>.
- 53 Yoneyama, M., Kikuchi, M., Natsukawa, T. et al. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5 (7): 730–737. <https://doi.org/10.1038/ni1087>.
- 54 Pichlmair, A., Schulz, O., Tan, C.P. et al. (2009). Activation of MDA5 requires higher-order RNA structures generated during virus infection. *J. Virol.* 83 (20): 10761–10769. <https://doi.org/10.1128/JVI.00770-09>.
- 55 Diebold, S.S., Kaisho, T., Hemmi, H. et al. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science (New York, N.Y.)* 303 (5663): 1529–1531. <https://doi.org/10.1126/science.1093616>.
- 56 Heil, F., Hemmi, H., Hochrein, H. et al. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science (New York, N.Y.)* 303 (5663): 1526–1529. <https://doi.org/10.1126/science.1093620>.
- 57 Tanji, H., Ohto, U., Shibata, T. et al. (2015). Toll-like receptor 8 senses degradation products of single-stranded RNA. *Nat. Struct. Mol. Biol.* 22 (2): 109–115. <https://doi.org/10.1038/nsmb.2943>.
- 58 Zhang, Z., Ohto, U., Shibata, T. et al. (2016). Structural analysis reveals that toll-like receptor 7 is a dual receptor for guanosine and single-stranded RNA. *Immunity* 45 (4): 737–748. <https://doi.org/10.1016/j.immuni.2016.09.011>.
- 59 Devarkar, S.C., Wang, C., Miller, M.T. et al. (2016). Structural basis for m7G recognition and 2'-O-methyl discrimination in capped RNAs by the innate

- immune receptor RIG-I. *Proc. Natl. Acad. Sci. U. S. A.* 113 (3): 596–601. <https://doi.org/10.1073/pnas.1515152113>.
- 60 Schubert-Wagner, C., Ludwig, J., Bruder, A.K. et al. (2015). A conserved histidine in the RNA sensor RIG-I controls immune tolerance to N1-2'O-methylated self RNA. *Immunity* 43 (1): 41–51. <https://doi.org/10.1016/j.immuni.2015.06.015>.
 - 61 Alameh, M.-G. and Weissman, D. (2022). Chapter 7 - Nucleoside modifications of in vitro transcribed mRNA to reduce immunogenicity and improve translation of prophylactic and therapeutic antigens. In: *RNA Therapeutics* (ed. P.H. Giangrande, V. de Franciscis, and J.J. Rossi), 141–169. Academic Press.
 - 62 Mu, X. and Hur, S. (2021). Immunogenicity of in vitro-transcribed RNA. *Acc. Chem. Res.* 54 (21): 4012–4023. <https://doi.org/10.1021/acs.accounts.1c00521>.
 - 63 Anderson, B.R., Muramatsu, H., Jha, B.K. et al. (2011). Nucleoside modifications in RNA limit activation of 2'-5'-oligoadenylate synthetase and increase resistance to cleavage by RNase L. *Nucleic Acids Res.* 39 (21): 9329–9338. <https://doi.org/10.1093/nar/gkr586>.
 - 64 Anderson, B.R., Muramatsu, H., Nallagatla, S.R. et al. (2010). Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. *Nucleic Acids Res.* 38 (17): 5884–5892. <https://doi.org/10.1093/nar/gkq347>.
 - 65 Karikó, K., Buckstein, M., Ni, H. et al. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 23 (2): 165–175. <https://doi.org/10.1016/j.immuni.2005.06.008>.
 - 66 Durbin, A.F., Wang, C., Marcotrigiano, J. et al. (2016). RNAs containing modified nucleotides fail to trigger RIG-I conformational changes for innate immune signaling. *mBio* 7 (5): e00833-16. <https://doi.org/10.1128/mBio.00833-16>.
 - 67 Andries, O., Mc Cafferty, S., De Smedt, S.C. et al. (2015). N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J. Control. Release* 217: 337–344. <https://doi.org/10.1016/j.jconrel.2015.08.051>.
 - 68 Karikó, K., Muramatsu, H., Welsh, F.A. et al. (2008). Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol. Ther.* 16 (11): 1833–1840. <https://doi.org/10.1038/mt.2008.200>.
 - 69 Morais, P., Adachi, H., and Yu, Y.T. (2021). The critical contribution of pseudouridine to mRNA COVID-19 vaccines. *Front. Cell Dev. Biol.* 9: 789427. <https://doi.org/10.3389/fcell.2021.789427>.
 - 70 Bangham, A.D. and Horne, R.W. (1964). Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol.* 8 (5): 660–668. [https://doi.org/10.1016/S0022-2836\(64\)80115-7](https://doi.org/10.1016/S0022-2836(64)80115-7).
 - 71 Bangham, A.D., Standish, M.M., Watkins, J.C. et al. (1967). The diffusion of ions from a phospholipid model membrane system. *Protoplasma* 63 (1): 183–187. [10.1007/BF01248030](https://doi.org/10.1007/BF01248030).

- 72 Cullis, P.R. and de Kruijff, B. (1979). Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* 559 (4): 399–420. [https://doi.org/10.1016/0304-4157\(79\)90012-1](https://doi.org/10.1016/0304-4157(79)90012-1).
- 73 Papahadjopoulos, D., Mayhew, E., Poste, G. et al. (1974). Incorporation of lipid vesicles by mammalian cells provides a potential method for modifying cell behaviour. *Nature* 252 (5479): 163–166. <https://doi.org/10.1038/252163a0>.
- 74 Papahadjopoulos, D., Poste, G., and Schaeffer, B.E. (1973). Fusion of mammalian cells by unilamellar lipid vesicles: influence of lipid surface charge, fluidity and cholesterol. *Biochim. Biophys. Acta Biomembr.* 323 (1): 23–42. [https://doi.org/10.1016/0005-2736\(73\)90429-X](https://doi.org/10.1016/0005-2736(73)90429-X).
- 75 Papahadjopoulos, D., Poste, G., Schaeffer, B.E. et al. (1974). Membrane fusion and molecular segregation in phospholipid vesicles. *Biochim. Biophys. Acta Biomembr.* 352 (1): 10–28. [https://doi.org/10.1016/0005-2736\(74\)90175-8](https://doi.org/10.1016/0005-2736(74)90175-8).
- 76 Papahadjopoulos, D., Vail, W.J., Jacobson, K. et al. (1975). Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. *Biochim. Biophys. Acta* 394 (3): 483–491. [https://doi.org/10.1016/0005-2736\(75\)90299-0](https://doi.org/10.1016/0005-2736(75)90299-0).
- 77 Deamer, D.W. and Bramhall, J. (1986). Permeability of lipid bilayers to water and ionic solutes. *Chem. Phys. Lipids* 40 (2–4): 167–188. [https://doi.org/10.1016/0009-3084\(86\)90069-1](https://doi.org/10.1016/0009-3084(86)90069-1).
- 78 Cullis, P.R. and Hope, M.J. (1978). Effects of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion. *Nature* 271 (5646): 672–674. <https://doi.org/10.1038/271672a0>.
- 79 Cullis, P.R., Hope, M.J., Bally, M.B. et al. (1997). Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. *Biochim. Biophys. Acta* 1331 (2): 187–211.
- 80 Nicolau, C. and Gregoriadis, G. (1998). Demetrios Papahadjopoulos (1934–98). *Nature* 396 (6707): 118. <https://doi.org/10.1038/24052>.
- 81 Sessa, G. and Weissmann, G. (1970). Incorporation of lysozyme into liposomes: a model for structure-linked latency. *J. Biol. Chem.* 245 (13): 3295–3301. [https://doi.org/10.1016/S0021-9258\(18\)62994-1](https://doi.org/10.1016/S0021-9258(18)62994-1).
- 82 Gregoriadis, G., Leathwood, P.D., and Ryman, B.E. (1971). Enzyme entrapment in liposomes. *FEBS Lett.* 14 (2): 95–99. [https://doi.org/10.1016/0014-5793\(71\)80109-6](https://doi.org/10.1016/0014-5793(71)80109-6).
- 83 Gregoriadis, G. and Ryman, B.E. (1972). Fate of protein-containing liposomes injected into rats. *Eur. J. Biochem.* 24 (3): 485–491. <https://doi.org/10.1111/j.1432-1033.1972.tb19710.x>.
- 84 Gregoriadis, G. and Ryman, B.E. (1972). Lysosomal localization of β -fructofuranosidase-containing liposomes injected into rats. Some implications in the treatment of genetic disorders. *Biochem. J.* 129 (1): 123–133. <https://doi.org/10.1042/bj1290123>.
- 85 Hope, M.J., Bally, M.B., Webb, G. et al. (1985). Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* 812 (1): 55–65. [https://doi.org/10.1016/0005-2736\(85\)90521-8](https://doi.org/10.1016/0005-2736(85)90521-8).

- 86 Madden, T.D., Harrigan, P.R., Tai, L.C. et al. (1990). The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. *Chem. Phys. Lipids* 53 (1): 37–46. [https://doi.org/10.1016/0009-3084\(90\)90131-a](https://doi.org/10.1016/0009-3084(90)90131-a).
- 87 Mayer, L., Bally, M., and Cullis, P. (1986). Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient. *Biochim. Biophys. Acta* 857 (1): 123–126.
- 88 Gregoriadis, G. and Davis, C. (1979). Stability of liposomes in vivo and in vitro is promoted by their cholesterol content and the presence of blood cells. *Biochem. Biophys. Res. Commun.* 89 (4): 1287–1293. [https://doi.org/10.1016/0006-291x\(79\)92148-x](https://doi.org/10.1016/0006-291x(79)92148-x).
- 89 Papahadjopoulos, D., Jacobson, K., Nir, S. et al. (1973). Phase transitions in phospholipid vesicles. Fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. *Biochim. Biophys. Acta* 311 (3): 330–348. [https://doi.org/10.1016/0005-2736\(73\)90314-3](https://doi.org/10.1016/0005-2736(73)90314-3).
- 90 Allen, T.M. and Chonn, A. (1987). Large unilamellar liposomes with low uptake into the reticuloendothelial system. *FEBS Lett.* 223 (1): 42–46. [https://doi.org/10.1016/0014-5793\(87\)80506-9](https://doi.org/10.1016/0014-5793(87)80506-9).
- 91 Klibanov, A.L., Maruyama, K., Torchilin, V.P. et al. (1990). Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.* 268 (1): 235–237. [https://doi.org/10.1016/0014-5793\(90\)81016-H](https://doi.org/10.1016/0014-5793(90)81016-H).
- 92 Dimitriadis, G.J. (1978). Translation of rabbit globin mRNA introduced by liposomes into mouse lymphocytes. *Nature* 274 (5674): 923–924. <https://doi.org/10.1038/274923a0>.
- 93 Ostro, M.J., Giacomoni, D., Lavelle, D.O.N. et al. (1978). Evidence for translation of rabbit globin mRNA after liposomemediated insertion into a human cell line. *Nature* 274 (5674): 921–923. <https://doi.org/10.1038/274921a0>.
- 94 Felgner, P.L., Gadek, T.R., Holm, M. et al. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U. S. A.* 84 (21): 7413–7417. <https://doi.org/10.1073/pnas.84.21.7413>.
- 95 Malone, R.W., Felgner, P.L., and Verma, I.M. (1989). Cationic liposome-mediated RNA transfection. *Proc. Natl. Acad. Sci. U. S. A.* 86 (16): 6077–6081. <https://doi.org/10.1073/pnas.86.16.6077>.
- 96 Hafez, I.M., Maurer, N., and Cullis, P.R. (2001). On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther.* 8 (15): 1188–1196. <https://doi.org/10.1038/sj.gt.3301506>.
- 97 Zelphati, O. and Szoka, F.C. Jr., (1996). Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. U. S. A.* 93 (21): 11493–11498. <https://doi.org/10.1073/pnas.93.21.11493>.
- 98 Chonn, A. and Cullis, P.R. (1998). Recent advances in liposome technologies and their applications for systemic gene delivery. *Adv. Drug Deliv. Rev.* 30 (1): 73–83. [https://doi.org/10.1016/S0169-409X\(97\)00108-7](https://doi.org/10.1016/S0169-409X(97)00108-7).
- 99 Campbell, P.I. (1983). Toxicity of some charged lipids used in liposome preparations. *Cytobios* 37 (145): 21–26.
- 100 Filion, M.C. and Phillips, N.C. (1997). Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector

- cells. *Biochim. Biophys. Acta* 1329 (2): 345–356. [https://doi.org/10.1016/S0005-2736\(97\)00126-0](https://doi.org/10.1016/S0005-2736(97)00126-0).
- 101 Bailey, A.L. and Cullis, P.R. (1994). Modulation of membrane fusion by asymmetric transbilayer distributions of amino lipids. *Biochemistry* 33 (42): 12573–12580. <https://doi.org/10.1021/bi00208a007>.
 - 102 Semple, S.C., Klimuk, S.K., Harasym, T. et al. (1998). High efficiency encapsulation of charged therapeutic agents in lipid vesicles. WO/1998/051278, filed 14 May 1998.
 - 103 Leung, A.K.K., Hafez, I.M., Baoukina, S. et al. (2012). Lipid nanoparticles containing siRNA synthesized by microfluidic mixing exhibit an electron-dense nanostructured core. *J. Phys. Chem. C* 116 (34): 18440–18450. <https://doi.org/10.1021/jp303267y>.
 - 104 Semple, S.C., Klimuk, S.K., Harasym, T.O. et al. (2001). Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures. *Biochim. Biophys. Acta* 1510 (1–2): 152–166. [https://doi.org/10.1016/s0005-2736\(00\)00343-6](https://doi.org/10.1016/s0005-2736(00)00343-6).
 - 105 Semple, S.C., Akinc, A., Chen, J. et al. (2010). Rational design of cationic lipids for siRNA delivery. *Nat. Biotechnol.* 28 (2): 172–176. <https://doi.org/10.1038/nbt.1602>.
 - 106 Zimmermann, T.S., Lee, A.C., Akinc, A. et al. (2006). RNAi-mediated gene silencing in non-human primates. *Nature* 441 (7089): 111–114. <https://doi.org/10.1038/nature04688>.
 - 107 Jayaraman, M., Ansell, S.M., Mui, B.L. et al. (2012). Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. *Angew. Chem. Int. Ed. Eng.* 51 (34): 8529–8533. <https://doi.org/10.1002/anie.201203263>.
 - 108 Adams, D., Gonzalez-Duarte, A., O’Riordan, W.D. et al. (2018). Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N. Engl. J. Med.* 379 (1): 11–21. <https://doi.org/10.1056/NEJMoa1716153>.
 - 109 Maier, M.A., Jayaraman, M., Matsuda, S. et al. (2013). Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Mol. Ther.* 21 (8): 1570–1578.
 - 110 Ansell, S.M. and Du, X. (2016). Lipid and lipid nanoparticle formulation for delivery of nucleic acids. US Patent 10,166,298 B2, filed 28 October 2016.
 - 111 Hassett, K.J., Benenato, K.E., Jacquinet, E. et al. (2019). Optimization of lipid nanoparticles for intramuscular administration of mRNA vaccines. *Mol. Ther. Nucleic Acids* 15: 1–11. <https://doi.org/10.1016/j.omtn.2019.01.013>.
 - 112 Akinc, A., Goldberg, M., Qin, J. et al. (2009). Development of lipidoid-siRNA formulations for systemic delivery to the liver. *Mol. Ther.* 17 (5): 872–879. <https://doi.org/10.1038/mt.2009.36>.
 - 113 Akinc, A., Zumbuehl, A., Goldberg, M. et al. (2008). A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat. Biotechnol.* 26 (5): 561–569. <https://doi.org/10.1038/nbt1402>.
 - 114 Love, K.T., Mahon, K.P., Levins, C.G. et al. (2010). Lipid-like materials for low-dose, in vivo gene silencing. *Proc. Natl. Acad. Sci. U. S. A.* 107 (5): 1864–1869. <https://doi.org/10.1073/pnas.0910603106>.

- 115 Maurer, N., Wong, K.F., Stark, H. et al. (2001). Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-destabilized cationic liposomes. *Biophys. J.* 80 (5): 2310–2326. [https://doi.org/10.1016/S0006-3495\(01\)76202-9](https://doi.org/10.1016/S0006-3495(01)76202-9).
- 116 Mui, B.L., Tam, Y.K., Jayaraman, M. et al. (2013). Influence of polyethylene glycol lipid desorption rates on pharmacokinetics and pharmacodynamics of siRNA lipid nanoparticles. *Mol. Ther. Nucleic Acids* 2 (12): e139. <https://doi.org/10.1038/mtna.2013.66>.
- 117 Holland, J.W., Hui, C., Cullis, P.R. et al. (1996). Poly(ethylene glycol)–lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry* 35 (8): 2618–2624. <https://doi.org/10.1021/bi952000v>.
- 118 Judge, A., McClintock, K., Phelps, J.R. et al. (2006). Hypersensitivity and loss of disease site targeting caused by antibody responses to PEGylated liposomes. *Mol. Ther.* 13 (2): 328–337. <https://doi.org/10.1016/j.ymthe.2005.09.014>.
- 119 Semple, S.C., Harasym, T.O., Clow, K.A. et al. (2005). Immunogenicity and rapid blood clearance of liposomes containing polyethylene glycol-lipid conjugates and nucleic acid. *J. Pharmacol. Exp. Ther.* 312 (3): 1020–1026. <https://doi.org/10.1124/jpet.104.078113>.
- 120 Dams, E.T., Laverman, P., Oyen, W.J. et al. (2000). Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J. Pharmacol. Exp. Ther.* 292 (3): 1071–1079. <https://www.ncbi.nlm.nih.gov/pubmed/10688625>.
- 121 Ambegia, E., Ansell, S., Cullis, P. et al. (2005). Stabilized plasmid–lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression. *Biochim. Biophys. Acta Biomembr.* 1669 (2): 155–163. <https://doi.org/10.1016/j.bbamem.2005.02.001>.
- 122 Heyes, J., Hall, K., Tailor, V. et al. (2006). Synthesis and characterization of novel poly (ethylene glycol)-lipid conjugates suitable for use in drug delivery. *J. Control. Release* 112 (2): 280–290. <https://doi.org/10.1016/j.jconrel.2006.02.012>.
- 123 Batzri, S. and Korn, E.D. (1973). Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta* 298 (4): 1015–1019. [https://doi.org/10.1016/0005-2736\(73\)90408-2](https://doi.org/10.1016/0005-2736(73)90408-2).
- 124 Jeffs, L.B., Palmer, L.R., Ambegia, E.G. et al. (2005). A scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA. *Pharm. Res.* 22 (3): 362–372. <https://doi.org/10.1007/s11095-004-1873-z>.
- 125 Belliveau, N.M., Huft, J., Lin, P.J. et al. (2012). Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA. *Mol. Ther. Nucleic Acids* 1: e37. <https://doi.org/10.1038/mtna.2012.28>.
- 126 Evers, M.J.W., Kulkarni, J.A., van der Meel, R. et al. (2018). State-of-the-art design and rapid-mixing production techniques of lipid nanoparticles for nucleic acid delivery. *Small Methods* 2 (9): 1700375. <https://doi.org/10.1002/smtd.201700375>.
- 127 Kulkarni, J.A., Cullis, P.R., and van der Meel, R. (2018). Lipid nanoparticles enabling gene therapies: from concepts to clinical utility. *Nucleic Acid Ther.* 28 (3): 146–157. <https://doi.org/10.1089/nat.2018.0721>.

- 128 Semple, S.C., Leone, R., Barbosa, C.J. et al. (2022). Lipid nanoparticle delivery systems to enable mRNA-based therapeutics. *Pharmaceutics* 14 (2): 398. <https://doi.org/10.3390/pharmaceutics14020398>.
- 129 Akinc, A., Maier, M.A., Manoharan, M. et al. (2019). The Onpattro story and the clinical translation of nanomedicines containing nucleic acid-based drugs. *Nat. Nanotechnol.* 14 (12): 1084–1087. <https://doi.org/10.1038/s41565-019-0591-y>.
- 130 Wolff, J.A., Malone, R.W., Williams, P. et al. (1990). Direct gene transfer into mouse muscle in vivo. *Science (New York, N.Y.)* 247 (4949 Pt 1): 1465–1468. <https://doi.org/10.1126/science.1690918>.
- 131 Jirikowski, G.F., Sanna, P.P., Maciejewski-Lenoir, D. et al. (1992). Reversal of diabetes insipidus in Brattleboro rats: intrahypothalamic injection of vasopressin mRNA. *Science (New York, N.Y.)* 255 (5047): 996–998. <https://doi.org/10.1126/science.1546298>.
- 132 Martinon, F., Krishnan, S., Lenzen, G. et al. (1993). Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. *Eur. J. Immunol.* 23 (7): 1719–1722. <https://doi.org/10.1002/eji.1830230749>.
- 133 Conry, R.M., LoBuglio, A.F., Wright, M. et al. (1995). Characterization of a messenger RNA polynucleotide vaccine vector. *Cancer Res.* 55 (7): 1397–1400. <https://www.ncbi.nlm.nih.gov/pubmed/7882341>.
- 134 Heiser, A., Coleman, D., Dannull, J. et al. (2002). Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. *J. Clin. Invest.* 109 (3): 409–417. <https://doi.org/10.1172/JCI14364>.
- 135 Su, Z., Dannull, J., Heiser, A. et al. (2003). Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. *Cancer Res.* 63 (9): 2127–2133. <https://www.ncbi.nlm.nih.gov/pubmed/12727829>.
- 136 Weide, B., Carralot, J.P., Reese, A. et al. (2008). Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. *J. Immunother.* 31 (2): 180–188. <https://doi.org/10.1097/CJI.0b013e31815ce501>.
- 137 Weide, B., Pascolo, S., Scheel, B. et al. (2009). Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients. *J. Immunother.* 32 (5): 498–507. <https://doi.org/10.1097/CJI.0b013e3181a00068>.
- 138 Kreiter, S., Selmi, A., Diken, M. et al. (2010). Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. *Cancer Res.* 70 (22): 9031–9040. <https://doi.org/10.1158/0008-5472.Can-10-0699>.
- 139 Sahin, U., Derhovanessian, E., Miller, M. et al. (2017). Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* 547 (7662): 222. <https://doi.org/10.1038/nature23003>.
- 140 Kranz, L.M., Diken, M., Haas, H. et al. (2016). Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* 534 (7607): 396–401. <https://doi.org/10.1038/nature18300>.

- 141 Sahin, U., Oehm, P., Derhovanessian, E. et al. (2020). An RNA vaccine drives immunity in checkpoint-inhibitor-treated melanoma. *Nature* 585 (7823): 107–112. <https://doi.org/10.1038/s41586-020-2537-9>.
- 142 Basha, G., Novobrantseva, T.I., Rosin, N. et al. (2011). Influence of cationic lipid composition on gene silencing properties of lipid nanoparticle formulations of siRNA in antigen-presenting cells. *Mol. Ther.* 19 (12): 2186–2200. <https://doi.org/10.1038/mt.2011.190>.
- 143 Leuschner, F., Dutta, P., Gorbatov, R. et al. (2011). Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat. Biotechnol.* 29 (11): 1005–1010. <https://doi.org/10.1038/nbt.1989>.
- 144 Geall, A.J., Verma, A., Otten, G.R. et al. (2012). Nonviral delivery of self-amplifying RNA vaccines. *Proc. Natl. Acad. Sci. U. S. A.* 109 (36): 14604–14609. <https://doi.org/10.1073/pnas.1209367109>.
- 145 Pardi, N., Tuyishime, S., Muramatsu, H. et al. (2015). Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. *J. Control. Release* 217: 345–351. <https://doi.org/10.1016/j.jconrel.2015.08.007>.
- 146 Awasthi, S., Hook, L.M., Pardi, N. et al. (2019). Nucleoside-modified mRNA encoding HSV-2 glycoproteins C, D, and E prevents clinical and subclinical genital herpes. *Sci. Immunol.* 4 (39): <https://doi.org/10.1126/sciimmunol.aaw7083>.
- 147 Pardi, N., Hogan, M.J., Naradikian, M.S. et al. (2018). Nucleoside-modified mRNA vaccines induce potent T follicular helper and germinal center B cell responses. *J. Exp. Med.* 215 (6): 1571–1588. <https://doi.org/10.1084/jem.20171450>.
- 148 Pardi, N., Hogan, M.J., Pelc, R.S. et al. (2017). Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. *Nature* 543 (7644): 248–251. <https://doi.org/10.1038/nature21428>.
- 149 Pardi, N., LaBranche, C.C., Ferrari, G. et al. (2019). Characterization of HIV-1 nucleoside-modified mRNA vaccines in rabbits and rhesus macaques. *Mol. Ther. Nucleic Acids* 15: 36–47. <https://doi.org/10.1016/j.omtn.2019.03.003>.
- 150 Pfizer. (2022). Pfizer initiates phase 3 study of mRNA-based influenza vaccine. <https://www.pfizer.com/news/press-release/press-release-detail/pfizer-initiates-phase-3-study-mrna-based-influenza-vaccine> (accessed 9 November 2022).
- 151 Aldrich, C., Leroux-Roels, I., Huang, K.B. et al. (2021). Proof-of-concept of a low-dose unmodified mRNA-based rabies vaccine formulated with lipid nanoparticles in human volunteers: a phase 1 trial. *Vaccine* 39 (8): 1310–1318. <https://doi.org/10.1016/j.vaccine.2020.12.070>.
- 152 Lutz, J., Lazzaro, S., Habbeddine, M. et al. (2017). Unmodified mRNA in LNPs constitutes a competitive technology for prophylactic vaccines. *NPJ Vaccines* 2: 29. <https://doi.org/10.1038/s41541-017-0032-6>.
- 153 Bahl, K., Senn, J.J., Yuzhakov, O. et al. (2017). Preclinical and clinical demonstration of immunogenicity by mRNA vaccines against H10N8 and H7N9 influenza viruses. *Mol. Ther.* 25 (6): 1316–1327. <https://doi.org/10.1016/j.ymthe.2017.03.035>.

- 154 Feldman, R.A., Fuhr, R., Smolenov, I. et al. (2019). 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 (25): 3326–3334. <https://doi.org/10.1016/j.vaccine.2019.04.074>.
- 155 Verbeke, R., Lentacker, I., De Smedt, S.C. et al. (2021). The dawn of mRNA vaccines: the COVID-19 case. *J. Control. Release* 333: 511–520. <https://doi.org/10.1016/j.jconrel.2021.03.043>.
- 156 CureVac. (2021). CureVac provides update on phase 2b/3 trial of first-generation COVID-19 vaccine candidate, CVnCoV. <https://www.curevac.com/en/curevac-provides-update-on-phase-2b-3-trial-of-first-generation-covid-19-vaccine-candidate-cvncov/> (accessed 9 November 2022).
- 157 Arcturus. (2022). Arcturus announces self-amplifying COVID-19 mRNA vaccine candidate ARCT-154 meets primary efficacy endpoint in phase 3 study. <https://ir.arcturusrx.com/news-releases/news-release-details/arcturus-announces-self-amplifying-covid-19-mrna-vaccine> (accessed 9 November 2022).
- 158 Providence. (2022). Providence announces positive top-line data from phase 2 primary immunization trial of its mRNA vaccine candidate PTX-COVID19-B in adults. <https://providencetherapeutics.com/press-details/providence-announces-positive-top-line-data-from-phase-2-primary-immunization-trial-of-its-mrna-vaccine-candidate-ptx-covid19-b-in-adults.html> (accessed 9 November 2022).
- 159 Low, J. G., de Alwis, R., Chen, S., et al. (2021). A phase 1/2 randomized, double-blinded, placebo controlled ascending dose trial to assess the safety, tolerability and immunogenicity of ARCT-021 in healthy adults. *medRxiv*: 2021.2007.2001.21259831. <https://doi.org/10.1101/2021.07.01.21259831>
- 160 Pollock, K.M., Cheeseman, H.M., Szubert, A.J. et al. (2022). Safety and immunogenicity of a self-amplifying RNA vaccine against COVID-19: COVAC1, a phase I, dose-ranging trial. *EClinicalMedicine* 44: 101262. <https://doi.org/10.1016/j.eclinm.2021.101262>.
- 161 Sittplangkoon, C., Alameh, M.G., Weissman, D. et al. (2022). mRNA vaccine with unmodified uridine induces robust type I interferon-dependent anti-tumor immunity in a melanoma model. *Front. Immunol.* 13: 983000. <https://doi.org/10.3389/fimmu.2022.983000>.
- 162 Alameh, M.G., Tombacz, I., Bettini, E. et al. (2021). Lipid nanoparticles enhance the efficacy of mRNA and protein subunit vaccines by inducing robust T follicular helper cell and humoral responses. *Immunity* 54 (12): 2877–2892 e2877. <https://doi.org/10.1016/j.immuni.2021.11.001>.
- 163 Ndeupen, S., Qin, Z., Jacobsen, S. et al. (2021). The mRNA-LNP platform's lipid nanoparticle component used in preclinical vaccine studies is highly inflammatory. *iScience* 24 (12): 103479. <https://doi.org/10.1016/j.isci.2021.103479>.
- 164 Tahtinen, S., Tong, A.J., Himmels, P. et al. (2022). IL-1 and IL-1ra are key regulators of the inflammatory response to RNA vaccines. *Nat. Immunol.* 23 (4): 532–542. <https://doi.org/10.1038/s41590-022-01160-y>.
- 165 Verbeke, R., Hogan, M.J., Loré, K. et al. (2022). Innate immune mechanisms of mRNA vaccines. *Immunity* 55 (11): 1993–2005. <https://doi.org/10.1016/j.immuni.2022.10.014>.

