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Review Article

The Emergence of the Next-Generation Vaccines

Léa Monet[1], Nicolas Grandchamp[2]*

[1]Biology department, Ecole Polytechnique, Institut Polytechnique de Paris, France

[2]Biosource / GEG Tech, Paris, France

***Corresponding author:** Nicolas Grandchamp, Biosource / GEG Tech, Paris, France; E-mail: nicolas.grandchamp@geg-tech.com

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Introduction

Vaccination is a medical technique that was first scientifically experimented by the physician Edward Jenner in the 18th century [1]. At that time, smallpox, a disease of viral origin, killed those infected and disfigured the survivors. Edward Jenner observed that farmers did not contract smallpox. However, they were often infected with vaccinia, a virus that induces a disease in cattle similar to smallpox but is beguiling to humans. E. Jenner hypothesizes that inoculating the contents of a vaccinia pustule taken from a female farmer would induce an immune response in humans that would protect them from subsequent infection by viruses similar to vaccinia such as the smallpox virus. He therefore carried out this experiment on a child and then voluntarily infected him with smallpox. As hoped, the child did not contract smallpox [2]. This discovery has been a breakthrough in science and became known as "vaccination".

Today, vaccination is a type of therapy that involves stimulating the immune system by injecting an antigen to produce a specific response [3]. The antigen injected is often based on viral or bacterial elements and should induce protective immunity and immune memory. Vaccination can either be preventive, in which case its aim is to prepare the individual's immune system for an encounter with a pathogen, or it can be therapeutic, in which case its aim is to redirect the patient's immune response to help defend against a pathogen already present in the body [4,5].

The injection of a preventive vaccine induces the activation of the innate and adaptive immune response [6]. During the adaptive immune response, a proliferation of T and B cells specific to the injected antigen takes place and leads to the formation of memory lymphocytes. The B cells and their differentiation into plasma cells enable the production of antigen-specific antibodies. When the vaccine injection is successful, subsequent exposure to the pathogen induces a strong immune response that either eliminates the pathogen or deactivates the toxin responsible for the disease, including a large and rapid production of antibodies due to the presence of memory lymphocytes [4]. Today, there are a variety of vaccine types. One type of vaccine is based on live attenuated microorganisms. It is based on the use of microorganisms that have lost their pathogenicity. This type of vaccination makes it possible to expose several specific epitopes of the pathogen to the immune system and reproduces the characteristics of an infection like the active pathogen, without developing the disease. These vaccines often require only one immunization because they induce a very strong immune response and the production of memory lymphocytes [7,8]. Moreover, one of its limitations is the possibility of reversion to a form that is dangerous to the body through a mechanism called reversion [9]. This type of vaccine is, however, gradually being replaced by vaccines based on killed or inactivated microorganisms. The purpose of inactivating the pathogen using heat or chemicals is to induce in the virus the inability to replicate within its host. Vaccines based on killed or inactivated microorganisms are frequently used because they have low toxicity and are stable. However, upon first injection, a weaker immune response is (removed weakly) induced because the microorganism is unable to replicate [10]. Several booster shots of the vaccine are therefore necessary. A second injection of the same pathogen leads to a greater immune response than that induced after the first injection.

With the advent of genetic engineering, new types of vaccines are being developed to avoid some of the risks of vaccines being based on whole microorganisms. These new vaccines contain only one or more molecules specific to the pathogen. They are called subunit vaccines [11]. They can be used against toxins or specific protein elements of microorganisms which are isolated and cloned to generate subunit vaccines [12,13]. However, some antigens do not elicit a sufficient immune response when injected alone. It is then necessary to fuse the weakly immunogenic antigen with a highly immunogenic protein. In this case, the vaccines are called conjugates [10].

The advent of genetic engineering has also made it possible to design another type of vaccine, the recombinant vaccine. They are based on the use of genetically modified living microorganisms to eliminate the genes responsible for their pathogenicity and to render them harmless. The interest of this type of vaccine is that it is based on genetically attenuate microorganisms without losing its immunogenicity effect and without reversion mechanism [10].

Furthermore, the recent development of sequencing techniques opens the door to new developments in vaccination processes. Pathogen genome sequencing enables access to all the specific epitopes of each microorganism, even those that cannot be grown in the laboratory and therefore cannot be attenuated or modified. The development of

vaccines based on the use of new sequencing techniques and the identification of new antigens is called reverse vaccinology [14].

The various preventive vaccination approaches described are based on the same principle which consists of injecting a whole microorganism or a specific antigen to stimulate the immune system and prepare it for subsequent infection.

Another approach of vaccination is explored since several years and has recently done a technology leap thanks to the advances in genetic engineering. This type of vaccine is based on the use of nucleic acids coding the antigens [15].

I. Nucleic acid vaccines:

Principle and mode of operation

Nucleic acid-based vaccines used cDNA (coding DNA) or messenger RNA (mRNA) coding an antigen [16–18]. The DNA form is a plasmid, containing the cDNA of an antigen under the control of a suitable promoter. For mRNA, there are two possibilities, either vaccines are based on a standard mRNA or on a self-amplifying mRNA system. Non-replicating mRNAs have a simple open reading frame with untranslated regions (UTR). These are transcribed in vitro from a linear DNA template using a phage RNA polymerase [19]. A 5' cap that protects it and allows ribosome recruitment and a 3' polyadenosine tail, which increases stability, are added to form a mature mRNA. The self-amplifying mRNAs are based on a modified viral genome containing the genes coding for the RNA replication mechanism and the structural protein sequences are replaced by the gene of interest. Once the DNA or RNA is internalized in the cell, the cellular transcription or translation mechanism produces an endogenous protein that undergoes post-translational modifications, resulting in a correctly folded and functional protein.

After a patient has been injected, plasmid DNA or mRNA enters the cells and the synthesis of the coding antigens and the production of antibodies playing a key role in protecting against further infection [20,21]. To do this, DNA or RNA vaccines must generate the antigen in sufficient quantities but must also produce it either within professional antigen presenting cells (APCs), such as dendritic cells (CDs), or in a cell from which the antigen can be presented to APCs. APCs strongly activate CD4+ and CD8+ T cells by presenting peptide antigens on class I and II major histocompatibility complex (MHC) molecules and induce the release of co-stimulating molecules [4,6]. Activation of APCs results in the production of antigen-specific antibodies by B cells and plasma cells. However, it is difficult to target these types of cells in vivo with RNA or DNA vectors. The most efficient way to transmit an antigen to professional APCs is to transduce or transfect them in vitro before administration to a patient, conferring a high level of complexity of the therapeutic protocol [22].

DNA and mRNA vaccines induce transient expression of the antigen in a manner that mimics the in vivo antigen production of viral pathogens [23–25]. These types of vaccines trigger humoral and cellular immune responses [26–31]. However, self-amplifying RNA vaccines systems occur the RNA replication through the synthesis of the RNA-

dependent RNA polymerase complex. This type of vaccine generates multiple copies of the mRNA by encoding the antigen and expressing high levels of the antigen. Compared to the rapid expression of classical RNA vaccines, published results have shown that vaccination with self-amplifying RNA vaccines results in higher, albeit delayed, levels of antigen expression that persist for several days in vivo. Equivalent protection of a non-replicating RNA vaccine is conferred but at a much lower dose of mRNA [32].

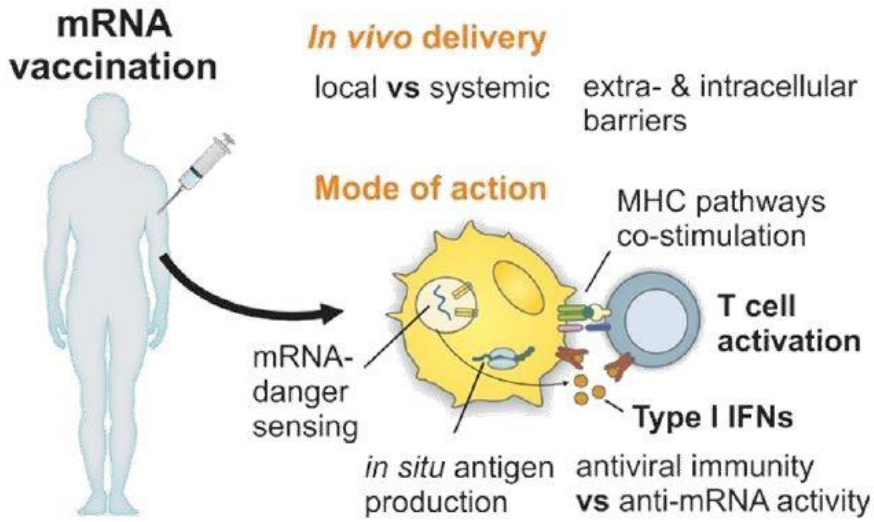

Figure 1: Principle of RNA vaccination

*Verbeke R, Lentacker I, De Smedt SC, Dewitte H. Three decades of messenger RNA vaccine development. Nano Today. 1 oct 2019 ; 28:100766.

Advantages of DNA and RNA vaccines

The major advantages of DNA and RNA vaccines are their speed and low cost production, ease of manufacture and lower handling risks compared to current vaccines [33]. Synthesizing the sequence of a given antigen is much simpler and faster than trying to inactivate or attenuate the pathogenicity of a microorganism or making recombinant proteins. The manufacturing technique for DNA or RNA vaccines also avoids the potential risks associated with working with live pathogens [34,35]. Moreover, these vaccines induce a temporary *in vivo* presence of the synthesized antigen. In addition, injected DNA and mRNA molecules with a good efficiency of transfection or transduction produce more antigen molecules with a better quality compared to traditional approaches [35–38].

Furthermore, DNA and mRNA molecules have immunostimulatory properties that enable nucleic acids vaccines to induce the innate immune response [37–41] and also adaptive immune response [37,42,43]. DNA plasmids do this through their CpG motifs, which stimulate the Toll-like receptor 9, because plasmid DNA also acts on the TBK1-STING pathway through cytosolic receptors [16,38,44,45]. This results in the production of type 1 interferons (IFN

type I), which act as adjuvants and generate the immune response against the antigen encoded by the plasmid DNA vaccine. IFN type I is a family of proteins, including interferon-β (IFNβ) and the multiple isoforms of IFNα, which are released by cells in response to viral infections and pathogens. Detection of IFN type I results in upregulation of genes stimulated by interferon and an antiviral cellular state [39,46]. The double-stranded structure of DNA is also considered an immune stimulant [47].

The injected mRNA stimulates the innate and adaptative response by being recognized by a variety of innate cell surfaces, endosomal and cytosolic immune receptors, notably thanks to two types of sensors: Toll-like receptors (TLR) 3, 7 and 8 which are located in the endosomal compartment of professional immune monitoring cells such as CDs, macrophages and monocytes and pattern recognition receptors (PRR) such as RIG-I and MDA5 [37,40]. Thus, activation of TLR7 leads to positive regulation of chemokines, which in turn recruit innate immune cells such as CDs and macrophages at the injection site. Plasmid DNA has fewer immunostimulatory properties than mRNA, but these are better defined [35].

At present, most of the vaccines used, except for some animal vaccines, have to be transported and stored at low temperatures. In poor rural areas of tropical countries, the cold chain may be broken, and the injected vaccine will present a potential hazard. This is why there is growing interest in the development of heat-stable vaccines. The optimization of RNA vaccines has shown that it is possible to produce heat-stable vaccines [48]. A freeze-dried RNA vaccine has been shown to be stable for 36 months at 5-25◦C and 6 months at 40◦C [49]. Moreover, a conventional rabies vaccine based on mRNA encapsulated in protamine was subjected to temperatures ranging from 4 to 56◦C for 20 cycles and exposure to 70◦C. The immunogenicity of this vaccine and its protective effects were not compromised [50].

DNA and RNA vaccines have many advantages over conventional vaccines, such as speed and ease of manufacture, low production costs, and residence at high heat. These vaccines induce an innate and adaptive immune response, thanks to the immunostimulant properties of plasmid DNA or mRNA. Although these two vaccines share some common advantages and characteristics, they differ in some respects.

Differences between DNA and RNA vaccines:

The main difference between DNA and mRNA vaccines is the cell compartment to which the different molecules are delivered.

The injected DNA is in the nucleus of the cell while the injected mRNA is in the cytoplasm. To be correctly located, the plasmid DNA must cross the plasma and nuclear membrane and the mRNA must cross the plasma and endosome membranes. These vaccines must deliver the antigen sequence through the injection of plasmid DNA or mRNA and they must also activate the danger signals of the innate immune response in order for the immune response to occur correctly [34,51,52]. The DNA and mRNA danger sensors are not the same and are located in

different parts of the cell. Sensors of a DNA fragment, such as cyclic GMP-AMP synthase-stimulator of interferon genes, DEAH box nucleic acid helicase and AIM2 absent in melanoma 2, are located in the cytoplasm. RNA sensors, such as Toll-like receptors 7 and 8, are located in the endosomes to avoid chronic inflammatory reactions [53,54].

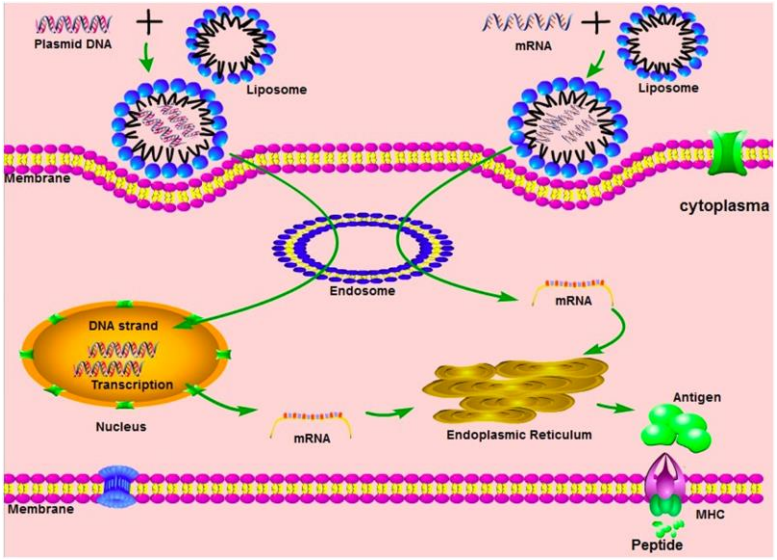

Figure 2: Antigen admittance mechanisms of DNA or RNA vaccines*

MH: Major histocompatibility complex

*Zhang C, Maruggi G, Shan H, Li J. Advances in mRNA Vaccines for Infectious Diseases. Front Immunol [Internet]. March 27th 2019

Furthermore, mRNA is synthesized *in vitro* from plasmid DNA. The production of RNA vaccines therefore has an additional step compared to that of DNA vaccines. In addition, DNA vaccines have been shown to produce the given antigen for a longer period of time than RNA vaccines due to the greater stability of plasmid DNA compared to mRNA [23,55]. However, unlike plasmid DNA, both classical and self-amplifying RNAs cannot integrate into the host genome and will be naturally degraded during the antigen expression process [52]. During the use of DNA vaccines, plasmid DNA integrate events have been observed but at a very low rate and is not considered as a biosafety problem [55], namely all measures to ensure the safe use of biological resources are respected. However, these characteristics indicate that RNA vaccines therefore have a higher level of biosecurity.

Although DNA and mRNA vaccines show differences and use different ways and mechanisms of action, these two types of nucleic acid vaccines have several advantages compared to traditional vaccines and seems very promising. However, advancements in nucleic acid vaccine development are still hindered by several technical points which could be addressed by the recent advancements in genetic engineering.

Limitations of DNA and RNA vaccines

The major difficulty to DNA and RNA vaccines is that nucleic acids must cross biological barriers to enter the desired cells. Furthermore, although sequencing techniques have made it possible to synthesize plasmid DNA or mRNA bearing the coding sequence of an antigen, for many diseases, scientists do not know which is the best immune response/combination of immune responses, and which are the most immunogenic antigenic targets [16,39,56].

The optimal protocol for administering the DNA or RNA vaccine in humans can be difficult to find because we cannot predict whether the vaccine administered will respond in the same way as in the animal model [49,57]. Despite preclinical studies that have shown the efficacy of DNA vaccines for various diseases in animal models, the human doses of several milligrams of DNA can produce an insufficient antigen expression and the efficacy of the vaccine has been disappointing [58]. In humans, DNA vaccines have not yet shown sufficient protection in Phase III [59]. The major limitation of these technologies is related to their *in vivo* administration. Indeed, DNA and RNA can be rapidly degraded after *in vivo* administrations, the genic vectors used so far showed a poor efficiency of transfection or transduction and they are not able to target the specific cell populations inducing the immune response [52,60,60–63]. Consequently, the quantity of DNA or RNA coding an antigen which penetrates in the APCs is not enough to induce a strong immune response conferring an immune protection.

In addition, during the manufacture of RNA vaccines, other molecules such as contaminating nucleoside triphosphate remnants, DNA matrices and dsRNA, which are introduced and then remain, are also highly immunostimulatory [64]. Recognition by dsRNA also induces activation of IRF3 and NF-kB, which then leads to an increase in the production of type I IFN [65–67] dsRNA contamination due to aberrant RNA polymerase activities, which leads to decreased translation and degradation of cellular mRNA and ribosomal RNA and thus decreases protein expression by interrupting the translation machine [68]. Suppression of dsRNA can dramatically increase translation. Vaccine mRNA should therefore be purified as much as possible from excess components and DNA matrices or dsRNA during manufacturing. Purification by Fast Protein Liquid Chromatography (FPLC) or High-Performance Liquid Chromatography (HPLC) can be used to remove any remaining product and produce purified mRNA on a large scale [68–70]. However, the mechanisms of mRNA inflammation are relevant to the efficacy of the vaccine, particularly as an adjuvant. An effective adjuvant strategy, TriMix, a combination of mRNAs encoding three immune activating proteins: CD70, CD40 ligand and the constitutively active TLR4 protein, has increased the immunogenicity of naked, unmodified and unpurified mRNA in multiple cancer vaccine studies and has been particularly associated with increased CDs maturation and cytotoxic T cell responses [57,71–73].

These new vaccines have limitations, particularly in terms of the choice of antigens to be synthesized or the constraints to cross the various cell barriers in order to deliver the DNA or mRNA molecule into the right cell

compartment. However, some limitations may prove useful, for example the inflammation mechanisms of the mRNA can be used as an adjuvant.

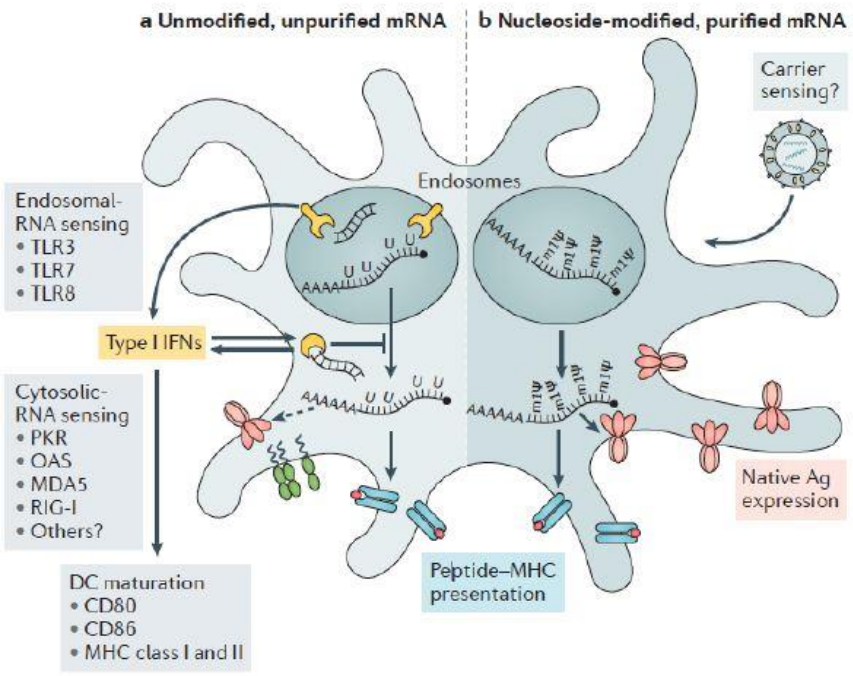

Figure 3: Innate immune reaction induced by purified mRNAs with modified or unmodified nucleosides in a dendritic cell.

Yellow: the RNA sensors, **Red:** the antigen, **Green:** the CD maturation factors and **Light Blue and Red:** the major histocompatibility complexes (MHC). An example of a lipid nanoparticle carrier is shown in the upper right. A list of the main known RNA sensors that contribute to the recognition of unmodified double-stranded and single-stranded RNA is given. Unmodified, unpurified mRNAs (part a) and nucleoside-modified, purified by rapid protein liquid chromatography (FPLC) (part b) illustrates the two formats of mRNA vaccines where known forms of mRNA detection are present and absent, respectively. The dotted arrow represents reduced expression of the antigen. Ag, antigen; PKR, interferon-induced, RNA-activated, double-stranded protein kinase; MDA5, protein 1 containing the interferon-induced helicase C domain (also known as IFIH1); IFN, interferon; m1Ψ, 1 methylpseudouridine; OAS, 2′ 5′ oligoadenylate synthetase; TLR, Toll-like receptor

Vaccines	Advantages	Disadvantages
	• Based on non-infectious pathogen. • Stable, rapid and scalable	• Potential integration into the human genome. • Low *in vivo* delivery efficiency.

DNA Vaccines	production. • Potential to trigger a strong immune response. • Stimulation of innate immune response. • Induction of T and B cell immune response.	• Need to target cells of interest. • Need for adjuvants to improve efficacy of *in vivo* administration.
ARN Vaccines	• Based on non-infectious pathogen • Rapid and scalable production. • Potential to trigger a strong immune response. • Stimulation of innate immune response. • Induction of T and B cell immune response. • Non-infectious, non-integrating, natural degradation. • Degraded by normal cellular processes. • Modulation of antigen half-life.	• RNA Instability. • Low *in vivo* delivery efficiency. • Need to target cells of interest. • Need for adjuvants to improve efficacy of *in vivo* administration.

Table 1: The main advantages and disadvantages of DNA vaccines and RNA vaccines

*Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines — a new era in vaccinology. Nat Rev Drug Discov. avr 2018;17(4):261-7

Applications, pre-clinical and clinical trials of DNA and RNA vaccines

Despite some limitations observed in these new vaccines, several laboratories initiated clinical trials [45,74,75]. Four DNA vaccines have received marketing authorization for animals. They are used to vaccinate farmed salmon against infectious hematopoietic necrosis and pancreatic disease caused by a subtype of alphavirus, chickens against avian influenza, and to treat dogs with oral melanoma [76–79]. However, for now, no DNA vaccine is currently available for humans even clinical trials on humans are underway [80–82]. In clinical trials for DNA vaccines against the Ebola and Marburg viruses or West Nile virus, individuals have produced antibodies that are considered protective [83,84]. These observations show that DNA vaccines are therefore capable of inducing enough antibodies in humans. The production of effective antibodies would therefore not be a limitation of this technology. On the other hand, the choice of the right antigenic target remains a major one. The first two Zika vaccines in human clinical trials were also DNA vaccines [85], further clinical trials of DNA vaccines are underway against cancer

[80,86–90]. Plasmid DNA codes for fusion proteins, including cytotoxic tumor T cell epitopes and helper T cell stimulators. A clinical trial against cellular hepatocarcinoma using DNA vaccine is ongoing [53]. The DNA codes for the embryonic antigen α-fetoprotein, which is over-expressed in 70% of individuals with this cancer. The DNA is vectorized using a nanovector called Nanotaxi, a tetra-functional amphiphilic copolymer consisting of four hydrophobic-hybrophilic polymer blocks centered on an ionizable core. The results of the regulatory safety study demonstrated that the amphiphilic tetra-functional copolymer alone or in combination with DNA is very well tolerated, inducing neither mortality nor toxicity.

For a decade, mRNA was of much less interest to the scientific community because of its instability relative to plasmid DNA. mRNA has a number of immunostimulatory mechanisms that can be useful or harmful to vaccines [27,40]. However, the use of modified nucleosides has solved key problems concerning the stability of mRNA, increased production of the encoded protein and some decrease in innate immunogenicity [68,91–93]. The mRNA is also stable in liquid or lyophilized form [49,50]. As a result, mRNA has begun to generate a lot of interest in vaccine manufacturing and clinical trials have been conducted [49,57,94]. The delivery of mRNA to antigen-presenting cells and lymph nodes at the injection site was made possible by the lipid nanovector CHOLK (cholesterol-kanamycin), derived from a natural sugar [53,95]. It was also shown that activation of specific mRNA hazard signals and immunogen synthesis took place when this vector was used [53,95]. However, the largest number of ongoing clinical trials of mRNA vaccines, particularly for cancer immunotherapy, involve the *ex vivo* transfection of cells with mRNA encoding tumor antigens, followed by re-infusion of the transfected cells to the patient [35,37,39]. Cancer RNA vaccines have been designed to express tumor-associated antigens that stimulate cell-mediated immune responses to eliminate or inhibit cancer cells. Another approach that is currently under development is to move towards personalized immunotherapeutic cancer vaccine products using a library of mRNAs encoding antigens that can be combined to be personalized for an individual. A significant number of cancer trials use mRNA by *ex vivo* transfection of CDs that are then re-infused into the patient. In addition, a company recently announced in a press release that a clinical trial has been launched for a Chikungunya RNA vaccine where the mRNA codes for a monoclonal antibody [96]. Similarly, it has been shown that an mRNA encoding the light and heavy chains of an HIV antibody and encapsulated in lipid nanoparticles protects mice against intravenous HIV infection [97]. The rabies vaccine using a vaccine with non-adjuvanted RNA has been replaced by a vaccine using mRNA encoding the glycoprotein of the rabies virus. Following an intradermal or intramuscular injection of this rabies mRNA vaccine, effective antibodies have been obtained. However, 78% of those tested had systemic adverse events and 10% had serious but non-life-threatening (grade III) adverse events [35,49]. Nevertheless, the vaccine has been described as generally safe with a reasonable tolerance profile which shows that sometimes there is some margin of acceptance. Self-amplifying RNA vaccines have also been produced, the polyethyleneimine (PEI) formulation of self-amplifying mRNA encoding H1N1/PR8-HA resulted in significantly higher antibody titer and longer lasting antigen expression than the use of unformulated self-amplifying mRNA [32,98]. Chitosan and PEI were also used to provide the self-amplifying mRNA in nanoparticle form 98,99. Other new modified nanoparticles are currently being investigated, such as polyplexes, nanoplex and scaffold release porous polymers [100,101].

Currently, the SARS-CoV-2 coronavirus pandemic, known as Covid-19, which was declared on 16 March 2020 by the World Health Organization, is enabling start-ups that have long been working on DNA or RNA vaccines and companies to use their technology to work for health. On March 27, Sanofi Pasteur announced a collaboration with Translate Bio on an mRNA vaccine against covid-19 [102]. According to the World Health Organization, 80% of cases of Covid-19 contamination are harmless, 14% are serious and 5% are critical, causing severe respiratory problems where the lungs fill with fluids preventing oxygen from reaching the organs. Patients with mild cases recover in one to two weeks, while severe cases take an average of six weeks to recover and 1% of those infected die. The search for a vaccine against Covid-19 is ongoing in many countries. On March 16, 2020, the first phase 1 clinical trials were launched in the United States to develop a vaccine against Covid-19 by the start-up Moderna Therapeutics [103]. Its Covid-19 mRNA vaccine, *mRNA-1273*, was injected into 45 people at the Kaiser Permanente Washington Health Research Institute in Seattle. Modern Therapeutics developed its first vaccine in 63 days illustrating the speed of the technology. They encapsulated the mRNA encoding the Covid-19 surface antigen in lipids to enter the cytoplasm of cells by endocytosis. On April 6, 2020, Inovio Pharmaceuticals announced that the Food and Drug Administration had approved its application to test its DNA vaccine named *INO-4800* against the SARS-CoV-2 coronavirus [104]. To inject DNA into the cells, Inovio Therapeutics uses an electroporation method called Cellectra. At the injection site, needles inserted into the muscle or dermis induce electrical impulses that disrupt the cell membranes and enable the DNA to enter them. However, even with the launches of these first clinical trials, the American authorities have estimated that it will still be a year and a half before a vaccine is available if all goes according to plan.

The numerous applications, preclinical and clinical trials of DNA and RNA vaccines on going show that more and more nucleic acid vaccines have tended to be developed in recent years in contrast to conventional vaccines. This illustrates that, the recent progresses in our understanding in immunology and the new technologies emerging in genetic engineering enable, step by step, to undo the technological locks of nucleic acid vaccines use.

II. Approaches to improve DNA and RNA vaccines

The number of studies and clinical trials developing and using nucleic acids vaccination demonstrates the interest in this technology. However, the low number of studies leads to a marketing authorization showing that improvements of the technology are necessary. The principle issue is to achieve enough antigen level expression in the immune cells to generate a protective immune response. Improvement of this parameter is crucial to make this technology available and several approaches are developed in this purpose.

Nucleic acid sequence design

The immunogenicity level induced by nucleic acid vaccination needs to be improved by increasing protein production, abundance and stability. In this purpose, strategies to optimize the plasmid DNA backbone or by modifying rare, low-use codons and promoters must be undertaken.

Efforts are also being made to increase the potency of mRNA including sequence optimization and the use of modified nucleosides, such as pseudo uridine, 5-methylcytidine, cap-1 structure and optimized codons, which improve translation efficiency. These modified nucleosides avoid recognition by immune sensors and thus prevent degradation of the mRNA before it reaches its site of arrival [19,91,105–108]. However, the observed toxicity of RNA drugs using non-naturally occurring modified nucleotide analogues raises questions about the possible toxic effects of RNA vaccines [109–111]. Different ways to improve the stability of mRNA are underway, such as the use of circular RNA molecules that are more stable and result in a more potent protein production than linear mRNA [112–114]. In addition, mRNA can also be modified at the codon and its guanine and cytosine content, as well as at the poly-A tails [18].

Stimulants use

To increase the protective power of current nucleic acid vaccines, admiration of enhancers is used such as adjuvants, immunostimulants like cytokines and co-stimulant molecules either as recombinant proteins or encoded by plasmid DNA or mRNA. Different strategies such as prime-boost combinations, usually using plasmid DNA as a primer, followed by a heterologous booster with a viral vector or protein [35]. However, this could also increase the potential for toxicity due to inflammation mechanisms. This booster could have an impact in particular on the potency due to the inflammatory effects of the nucleic acid that would lead to a decrease in its translation [115]. The search for a better balance between inflammation and possible deleterious toxicities is therefore underway by exploiting the adjuvant activity of nucleic acids while limiting or eliminating its toxicity. This would require optimization of nucleoside substitutions, nucleic acid building blocks, immunostimulants in vaccines, delivery devices and specific ways of administration [35].

Administration mode

The route of administration of nucleic acid vaccines is also an important parameter affecting the level of antigen expression and the strength of the immune response. Nucleic acid vaccines are administered by a systemic or local method depending on the desired location of antigen expression. For example, intravenous administration results in rapid digestion of naked mRNA not modified by ribonucleases and stimulation of the innate immune response [116]. However, these detrimental effects can be overcome with appropriate delivery systems and mRNA modifications. For infectious diseases, direct intramuscular (i.m.), intradermal (i.d.) or subcutaneous injection is the main route of nucleic acids vaccines administration, while intraperitoneal (i.p.) and intravenous (i.v.) administration is more used for therapeutic applications. Several studies have shown that i.m. and i.d. injections offer the best levels and duration of effect, with protein production peaking at 4 h and remaining locally 8 to 10 days after injection, depending on the dose [117,118]. However, this may vary between vaccines. In an influenza vaccine assay, intranodal (i.n.) administration of naked mRNA triggered potent CD4 and CD8 T cell immune responses in mice, and repeated i.n. injection of modified mRNA resulted in priming of antigen-specific CD4 and CD8 T cells, whereas subcutaneous,

i.d. administration did not [119]. Combination with two or more methods of administration has been studied and used in the development of mRNA cancer vaccines [120].

Targeting the cells of interest

Once DNA or RNA is administered in the body, it must enter into specific immune cells to trigger an immune response. The development of strategies that directly target immune cells such as APCs could enable to improve nucleic acid vaccines. An effective approach for DC targeting of mRNA vaccines after systemic administration has recently been described. A complex delivery platform was generated using neutral cationic lipids and auxiliary lipids and mRNA [121]. It was found that the lipid to mRNA ratio, and thus the net particle load, has a profound impact on the biodistribution of the vaccine. While a positively charged lipid particle primarily targeted the lung, a negatively charged particle targeted CDs in secondary lymphoid tissues and bone marrow. The negatively charged particle induced potent immune responses against specific tumors that were associated with impressive tumor reduction in various mouse models. As no toxic effects were observed in mice or non-human primates, trials using this approach are underway to treat patients with melanoma or breast cancer [121]. In addition, certain formulations, such as the administration of a lipoplex mRNA vaccine, have been shown to be specifically absorbed by CDs through micropinocytosis [122]. Finally, for RNA vaccines, cells absorb mRNA by endocytosis, so efforts are being made to design delivery systems that increase the endosomal release of mRNA into the cytoplasm to improve the correct delivery of mRNA [64].

Vector design

The type of the vector and techniques used to deliver nucleic acids are also critical parameters. Indeed, the vector employed needs to have a good stability *in vivo* and a high efficiency to deliver DNA or RNA molecules into the cells. Until now, most of the vectors used were largely degraded *in vivo*, limiting the quantity of DNA or RNA delivered to the cells and finally, the quantity of antigens expressed. New delivery techniques such as electrolocation are designed to improve the quantity of nucleic acids delivered in the cells *in vivo* [61,62,121,123–126]. Cell penetration peptides, a type of cationic peptide, represent promising tools for the mRNA delivery to intracellular target sites. Encapsulation of mRNA with a cationic liposome or cell penetrating peptide protected the mRNA from degradation by RNase. Protamine, a cationic peptide rich in arginine, can bind to the mRNA and transport it into the cytoplasm [20,73,127]. This molecule has been widely used as a delivery system for cancer vaccines and viral RNA vaccines. Another approach to improve the potency of the vaccine could be to increase the amount of protein produced through a new vector design, protecting the nucleic acids from digestion by the ribonuclease, enabling efficient absorption by the target cells and easy release of the RNA into the cytoplasm without toxic effects. Several approaches have been explored and progress has been made in the design of mRNA delivery vectors [60,61,63,91,98,99,117,121]. However, several of these delivery vectors have demonstrated *in vivo* toxicity, which may limit their use in humans [128]. Self-amplifying RNA vaccines with lipid nanoparticles as the delivery vector induce a potent cellular and humoral immune response through different routes of administration [97]. Although

progress has been made in the development of delivery tools, further efforts in understanding the mechanism of action may be required.

The use of viral vectors could be a relevant option to design stable vectors *in vivo* [129,130]. Indeed, this class of vector is naturally protected from the degradation as a wild type virus thanks to its capsid or envelope. Furthermore, this class of vectors shows a high efficiency to deliver nucleic acids in cells without adding adjuvants which could induce toxicity. In addition, some of viral vectors have a high flexibility of design enabling to add specific envelopes targeting immune cells such as DCs [131]. Despite all these advantages, until now, they have been scarcely used in vaccination because their level of biosafety was low and hardly compatible with this field of application. The principal issue about the use of vectors derived from lentivirus is to avoid the potential genotoxic effects due to the integration of their genomes into cell [132–138]. To answer this challenge, new versions of lentiviral vectors were designed to be non-integrating [139–141].

This type of vector shows a better level of biosafety than the integrating version and has been successfully tested in several models of DNA vaccination142. Nevertheless, several studies have shown that a small fraction of NILVs can be integrated into the genome of transduced cells, between 0.1% and 1% [143], including within vaccination context with primate models [144]. Although these integration events could generate genotoxic effects, they are not deemed an impediment to using NILVs in vaccination [144]. However, this risk must be taken into consideration when vaccines and therapeutic strategies based on NILVs are designed, limiting the scope of application.

Recently, a new version of lentiviral vectors (LVs) has shown a higher level of biosafety than that of NILVs. This type of vector contains a mutation of the reverse transcriptase which is the protein that converts the lentiviral genome from RNA to DNA. Therefore, this version of LV is unable to conduct this step of conversion. After transduction, the genome of the vector remains in RNA form and is taken in charge by the cells as an mRNA to be translated into protein [145]. Furthermore, it has been demonstrated that the mutation entirely abolishes the activity of the reverse transcriptase and no event of pseudotranfection has been observed, demonstrating that this version of LV is fully unable to deliver DNA. This new version of LVs could be used in vaccination, opening new avenues in this field thanks to its high stability, high level of transduction, flexibility of design and biosafety.

Conclusion

Vaccination is one of the major advances in human health. The principle consists of stimulating the immune system by injecting an antigen in order to obtain a specific response. The antigen injected is often of viral or bacterial origin and should induce protective immunity and immune memory. Vaccination can either be preventive or therapeutic. Since its creation in the 18th century, it has made it possible to eradicate smallpox globally and the World Health Organization has announced the eradication of other pathogens, notably polio in 2003. In addition, in France, since 1 January 2018, 11 vaccines have become compulsory.

Over the last few years, a set of so-called "classic vaccines" have been developed. The different types of vaccines vary according to their composition. They can be composed of an entire bacterium or virus, attenuated or inactivated, or only a protein. Recombinant antigens can also be obtained by genetic engineering. These conventional vaccines have facilitated vast improvements in the sphere of human health. However, they show several limitations in terms of quality, safety, production, or scope of disease. A new class of vaccine named nucleic acid vaccine could overcome these limits. They are based on the use of cDNA (coding DNA) or mRNA (messenger RNA) molecules, carrying the coding part of an antigen. Administration of a nucleic acid vaccine results in an endogenous generation of pathogen proteins with native conformation, glycosylation profiles, and other posttranslational modifications that mimic antigen produced during natural viral infection, offering several advantages over conventional vaccines. The principle of this type of vaccination has long been recognized but is impeded by the low level of nucleic acids entering in the cells, not enabling to generate a high quantity of antigens, consequently the immune response induced is weak. Consequently, during a long time, very few studies have validated a vaccine for animals or humans due to several biological barriers limiting the quantity of DNA or RNA entering the immune cells. Until now, the vectors used for *in vivo*.

administrations were synthetic. This type of vector shows a high level of biosafety; however, they do not confer a high level of protection against *in vivo* degradation after injection, their efficiency of transfection is limited, and it is difficult to combine them with a system targeting specific cell populations. On the contrary, the vectors derived from the virus confer a high level of nucleic acids protection, they can very efficiently transduce cells *in vivo* and can be combined with a system that targets specific cell populations. Nevertheless, these vectors' level of biosafety is low and have so far not been compatible for vaccination applications. The recent advances in genetic engineering enable the design of new generations of DNA or RNA vectors combining the biosafety level of synthetic vectors with the high efficiency and design flexibility of viral vectors. These new generations of vectors promise a potential breakthrough, permitting the extension of the nucleic acid vaccines scope in animal health and making their use in human health possible. More than twenty years after this approach was first described, encouraging results have finally been reported. For example, in human clinical trials, even if the immune system's response was lower than expected, compared to those observed in animal model, the results showed good tolerance and activation of antigen-specific T and B cell immune responses.

In addition, RNA vaccines that have historically been more difficult to design due to molecule stability issues but are more promising than DNA in terms of biosafety, have also shown promising results in recent times. Several pre-clinical and clinical trials have shown that RNA vaccines provide, among other things, a safe and durable immune response in animal and human models.

Recently, a new proof of the nucleic acid vaccines development speeding is about the SARS-CoV-2 coronavirus pandemic. Indeed, the first two vaccines developed and tested in humans are nucleic acid vaccines and not conventional vaccines, illustrating its rapid design and its good level of biosafety. The first vaccine to be licensed for

a clinical trial against Covid-19 is an mRNA-1273 RNA vaccine and was developed in just 63 days. Soon after, another clinical trial started, based on INO-4800 DNA vaccine.

For a long time, vaccinations based on nucleic acids has been imagined and described. However, our limited know-how in immunology and genetic engineering prevented us from uncovering its potential. Recent advancements in these areas are game-changing. The increase in the number of clinical trials in a short period of time clearly shows that nucleic acid vaccines are becoming available for more and more diseases. Furthermore, the fields of application for this type of vaccine may extend beyond prophylactically areas to other therapeutically areas such as cancer therapies.

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