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In these last years, we are witnessing the emergence of new class of biopharmaceuticals based on messenger RNA (mRNA). One of the most promising applications of mRNA is their use as vaccines. Many reports, including ours, demonstrated the preclinical efficacy of mRNA vaccines. mRNA vaccines have several advantages over traditional vaccines or even DNA vaccines. Unlike attenuated or inactivated vaccines, mRNA encodes for a specific antigen that will be expressed *in situ* and stimulates both the innate immune system and an adaptive immunity to promote both humoral and cellular immune responses.

The COVID-19 pandemic and search for rapid and adaptable vaccination strategies revealed the potential of messenger RNA (mRNA)-based vaccines providing a solution to the global crisis of our age. Amongst more than 180 vaccine platforms that have been proposed to tackle the SARS-Cov2 infection, those based on mRNA have been developed at unprecedented speed. This brought to light the required interplay between mRNA production and its intracellular delivery. Any antigenic protein can be encoded by mRNA allowing the development of preventive and therapeutic vaccines to fight against infections, cancer and allergies. Here, we present current knowledge regarding crucial aspects on mRNA structure, stability, formulations, cellular delivery and translation that should be considered when developing mRNA-based vaccines.

Design of mRNA vaccine for potent translation

In 1961, Brenner, Jacob and Meselson validated the hypothesis proposed by Jacob and Monod that ribosomes receive the genetic information from the gene in a form of an unstable intermediate or messenger that is translated into a protein by the ribosomes. Since then, much knowledge has been acquired concerning the structure and physico-chemical features as well as the *in vitro* synthesis of mRNA. Today,

mRNA is considered as the most promising biomedicine of the future. When manipulated correctly in absence of RNases or at pH lower than 8, mRNA is highly stable. mRNA can be precipitated and lyophilized without affecting its translation efficacy upon resuspension in aqueous buffer after long term storage at room temperature.

Compared to DNA therapy, mRNA therapy is considered as safe as contrary to DNA, mRNA does not integrate into the genome and is transiently expressed. The translation machinery being located in the cytosol, mRNA expression does not require nuclear import. Lack of required nuclear import is particularly beneficial for differentiated cells and non-dividing cells. Unlike antigenic peptides, mRNA leads to production of antigens by the host, avoiding the need to develop vaccines for each ethical haplotype. Upon cell uptake, the duration and level of exogenous mRNA expression will be dependent on mRNA sequences and the delivery vehicle used. Once available for the translation machinery, its stability and translation will be governed by the same mechanisms as that of *in-situ* produced mRNA. The resulting produced proteins will be in their native folding with all post-translational modifications.

Currently, the only existing method to produce mRNA therapeutics is *in vitro* transcription (IVT), a cell-free method. It allows mRNA production from a DNA template by bacteriophage (T7, SP6) RNA polymerase in the presence of nucleotide building blocks. The DNA template encodes all the structural elements of a functional mRNA. Once transcribed, this template is degraded with DNases and different purification steps including precipitation and chromatography to eliminate the reagents,any truncated mRNA fragments that have formed due to abortive enzymatic processing and any double stranded RNA that has been created.

Functional mRNA contains 4 structural parts: cap, 5'UTR (untranslated region), ORF (Open Reading Frame), 3'UTR and polyA tail (Figure 1).
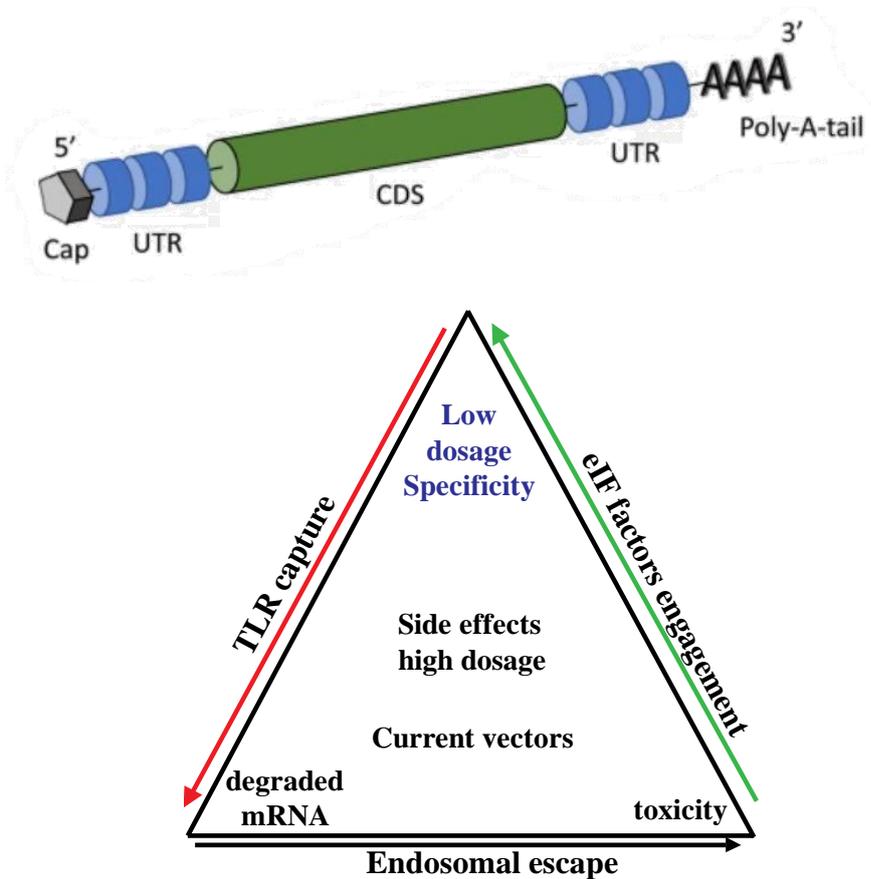
Figure 1: Schematic IVT mRNA structure and intracellular recognition of mRNA

To be stable and functional, mRNA must bear a cap at its 5' end, a cap that is incorporated either co-transcriptionally or added enzymatically after transcription. The cap is crucial for the translation as it binds to eukaryotic translation initiation factor (eIF4E). The simplest structure is cap0 consisting of m7GpppG dinucleotide at the 5'end. The cap 1 structure is found in mammalian mRNAs and it is created by the subsequent methylation of the first nucleotide at the ribose 2'O position. Co-transcriptional capping is highly improved by chemically modified cap analogs (cap 0) or trinucleotide cap analog (cap1, Cleancap®, TriLink) that are incorporated only in the correct orientation.

The ORF corresponds to the coding sequence; it can be codon-optimized to get the highest translation rate. At the 3' end, mRNA must have a polyA tail inserted directly as poly(d[A/T]) sequence in the template or enzymatically added in the transcribed mRNA. This polyA tail is also essential for mRNA stability and translation. The optimization of its length (>60-120) is an important factor. Nevertheless, there is no clear dependency between the length and the translation efficiency as it depends on the cell type. Untranslated regions 5'UTR and 3'UTR are located after the 5'cap and the coding sequence, respectively. Those regions are of importance since they are involved in the regulation of translation and mRNA stability. UTRs from very stable

genes as those coding for α and β globins are used and can be put as tandem repeats in the backbone of IVT mRNA.

Once delivered inside the cells, IVT mRNA are sensed by innate pattern recognition receptors leading to an immunostimulation, resulting in mRNA degradation, translation inhibition and cytotoxicity (Figure 1). The addition of chemically modified nucleosides as pseudouridine during IVT highly reduced this immunostimulation. This is not mandatory for IVT mRNA vaccines as the sequence can be as well optimized in a way to highly reduce the recognition by innate immune sensors. The presence of short RNA fragments due to abortive transcription enhances the inflammatory state of IVT. Removal of short RNA fragments by HPLC (High Pressure Liquid Chromatography) purification helps to reduce those issues.

For vaccination, the mRNA coding for the antigen must be tailored in a way to improve its stability and translation albeit keeping its self-adjuvating effect (immunostimulation) to enhance the immune response. The mRNA are designed to produce antigens targeted into cellular compartments where they can be processed to produce epitopes that will be presented either by class I and/or class II major histocompatibility molecules (MHC) at the cell surface of antigen presenting cells.

Compared to other vaccine candidates the mRNA platform is advantageous for manufacturing because of its flexibility. Any modification of the coding sequence of the antigen does not affect the mRNA backbone allowing a standardized production.

There are two possible forms of mRNA: the conventional mRNA or non-replicating and the self-amplifying mRNA. The conventional mRNA has a transient nature and requires higher doses to achieve high protein expression. The self-amplifying mRNA (saRNA) has in addition a viral replicase gene enabling the mRNA to be self-amplified. The viral replicase is derived from sequences of single-stranded RNA viruses, such as alphaviruses, flaviviruses, and picornaviruses. In addition to the self-amplification, the saRNA-based vaccines are more immunogenic than conventional mRNA vaccines. Therefore, a much lower dose is required to get a potent vaccination. But, one of the disadvantages of saRNA is their long size (> 10 kB).

Optimizing the delivery systems

In addition to their sensitivity to nuclease degradation, naked mRNAs are hardly taken up by cells due their negative charge. Different mRNA delivery systems have been proposed to protect them from degradation and to promote their cellular uptake into the targeted cells. In most of the case, those systems are made with cationic carriers that compacting the mRNA *via* electrostatic interactions

complexation or encapsulation leading to the formation of nanoparticles that can be taken up by the cell by endocytosis. Once inside the cells, those nanoparticles must be released in the cytosol and the mRNA must dissociate from its carrier to be available for the translation machinery. The most challenging issue for the design of mRNA delivery system is to find the right balance between the compaction and the dissociation of the mRNA from its carrier. If this interaction is too strong, mRNA will be hardly released in the cytosol for its translation.

The delivery system needs to ensure complexation and protection of the mRNA, endocytosis and intracellular release (Fig. 2). Ligands can be grafted onto the surface of the delivery system for cellular targeting. Dendritic cells are the major cells for antigen presentation to naïve lymphocytes and are frequently targeted. After endocytosis, mRNA resides in acidic endosomes where it is destined for degradation in the lysosomes. Delivery systems possess protonable units to allow endosomal escape. Once in the cytosol, the presence of optimized sequence on mRNA favoring the engagement of translation initiation factors will result in ribosome recruitment. To foster antigen presentation by dendritic cells, the protein sequence must contain signal peptides to improve antigen routing and presentation to lymphocytes.
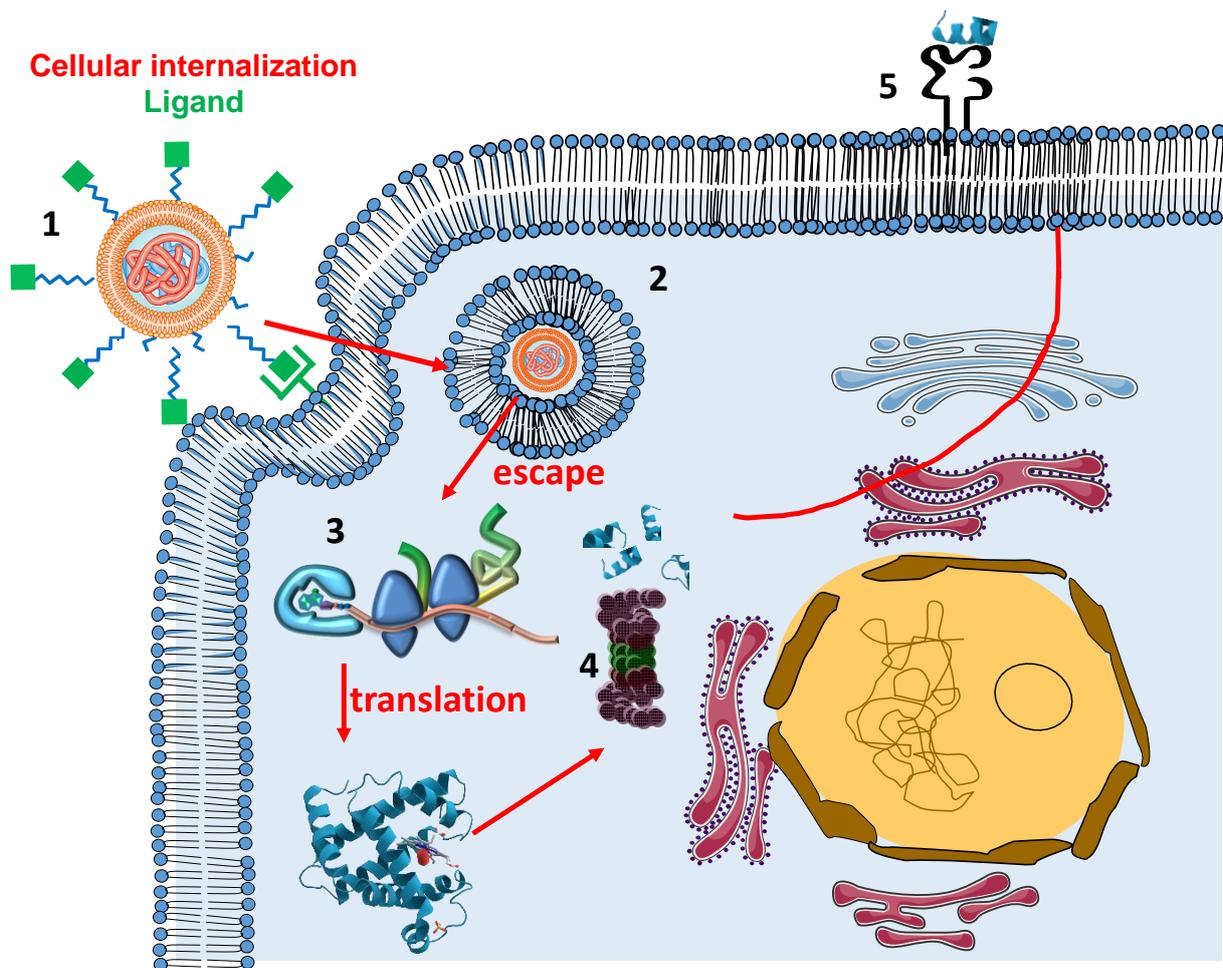
Figure 2: Critical steps in mRNA vaccine delivery: 1-recognition of targeted formulation by the cognate receptor in DC; 2- confinement of mRNA formulation inside endosome; 3-mRNA recruitment to the ribosome; 4-proteasome degradation of antigen; 5-antigenic presentation *via* major histocompatibility class I (MHC I) as an example).

Lipid-based systems comprising liposomes or lipid nanoparticles (LNPs) are the most used mRNA delivery carriers. LNP development proved efficacious for the delivery of siRNA drugs. Onpattro® (Alnylam Pharmaceuticals, Inc., Cambridge, MA, USA) that consists of siRNA directed against the transtyrethin gene defect formulated as LNP, was the first drug based on siRNA licensed in the USA in 2018.

For COVID-19 mRNA vaccines, the mRNA coding for spike protein is encapsulated in a LNP. The mRNA is encapsulated in a solid lipid structure that is composed of ionizable lipids which are neutral or slightly charged at physiological pH and become positive at acidic pH. The structure contains cholesterol, helper lipids and PEGylated lipids for complexation, stabilization of nanoparticle cytosolic release and reduction of non-specific interactions, respectively. Once mRNAs are encapsulated into LNPs at low pH, the particles have a neutral surface charge in the extracellular space to reduce non-specific interactions.

Other types of mRNA carriers are based on polymer and polymer/lipid hybrid particles. Those carriers are not as widely used compared to the lipid-based systems but they have interesting features that support a wider diversity and increased flexibility. We have designed an mRNA formulation based in a polymer/lipid hybrid particle.

Lipopolyplexes (LPR) are ternary Lipid-Polycation-RNA complexes in which mRNA is first complexed by a cationic polymer before engulfment in liposomes. In our studies, the liposomes contain a mannosylated lipid to engage the mannose receptor expressed by antigen presenting cells such as dentrictic cells. Using murine models of melanoma, we demonstrated that presence of the mannosylated lipid enhanced *in vivo* splenic dendritic cell transfection and protection against tumor growth. One of the interesting features of LPR is the fact that this delivery system does not rely on type I IFN for effective T-cell immunity in contrast to most mRNA delivery systems. This property led to a similar protection against melanoma growth after vaccination with unmodified or m1Ψ-modified mRNA encoding a tumor antigen. Intramuscular injection of LPR prepared with saRNA encoding Influenza antigens in mice induced CD4 and CD8 antigen-specific lymphocytes, establishing the potential of LPR RNA vaccines.

Conclusions

mRNA-based vaccination has shown promising results in the past few years. mRNA vaccines and mRNA therapeutics have been shown to be safe and well-tolerated in clinical trials. The flexibility of the mRNA to encode for various antigens and the technological advances in genomics to determine neoantigens allow the development of personalized vaccination. The approval of SARS-CoV2 mRNA vaccines has fueled the maturation of large-scale mRNA vaccines production facilities ensuring application of mRNA vaccines for other therapeutic fields. This will surely broaden the scope of mRNA to other therapeutic fields of applications such as protein replacement therapy and tissue regeneration. The next challenge will be to implement different actions that will provide access to mRNA-based medicines all over the world without social disparities.

Future reading

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Acknowledgments

Authorship

Chantal Pichon is Full Professor at the University of Orléans and leads the Innovative therapies and Nanomedicine research lab at the Center for Molecular Biophysics (CBM, CNRS, Orléans, France). She defended her PhD in Marseille university (France) and performed her Post-doc training at AFRC in Cambridge (UK). Her research activity is related to the development of innovative therapies based on nucleic acids (DNA, siRNA/miRNA and mRNA) therapeutics and non-viral delivery systems (chemical and ultrasound-based deliveries). These last years, she is mainly interested in mRNA delivery and production.

Federico Perche is a researcher at the CBM (CNRS Orléans, France). He received his PhD from the University of Orléans in 2010 under the supervision of Dr P. Midoux. He then did a postdoctoral fellowship at Northeastern University (Boston, 2011 – 2013) under the supervision of Prof V. Torchilin and, a second postdoctoral fellowship at the University of Tokyo (2013-2015) under the supervision of Prof K. Miyata. In 2016, he joined the CBM as a researcher in the lab of Chantal PICHON. His major research interests are related to biomaterials for mRNA delivery and, optimization of the intracellular processing of mRNA.