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Delivering the Messenger: engineering nanoparticles for advanced mRNA therapies

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DELIVERING THE MESSENGER: ENGINEERING NANOPARTICLES FOR ADVANCED mRNA THERAPIES

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CONFLICT OF INTEREST

I, Laura Taina González, author of this thesis, declare the inventorship of a patent requested to the European Patent Office during the present Doctoral Thesis.

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Details of the Patent submission can be found in the Annex Section (Annex I), at the end of this thesis.

PUBLICATIONS INCLUDED IN THIS THESIS

The following publication has been adapted and included in the Introduction of this PhD Thesis.

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I, Laura Taina González, has been involved in Conceptualization, Methodology, Investigation, and Writing the original draft and reviews.

The permission to reproduce this paper as well as the full publication can be found in the Annexes of this thesis.

A todas las mujeres que lucharon para que nosotras pudiéramos llegar aquí,
por todas las que aún están silenciadas

A mis padres, por darme alas,

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'Lock up your libraries if you want, but there is no gate, no lock, no bold that you can set upon the freedom of my mind.'

Virginia Woolf

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**'Cierra con llave las bibliotecas, si quieres, pero no hay barrera, cerradura, ni cerrojo que puedas imponer a la libertad de mi mente.'*

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ABSTRACT

Messenger RNA (mRNA) has advanced to the forefront of innovative therapies in the past years. With the approval of COVID-19 vaccines, mRNA has demonstrated its potential in vaccinology. Nevertheless, multiple biomedical applications can benefit from its advantages over other genetic therapies. Unlike DNA therapies, mRNA exerts its action in the cytoplasm, and, therefore, does not integrate into the nucleus of the host, a fact that is translated to higher safety profiles. For the same reason, the delivery of exogenous mRNA is a more efficient process, facilitating its use as a therapeutic. Furthermore, mRNA can be synthesized in the laboratory following straight forward techniques, which, at the same time are adaptable to industrial scale, including for GMP manufacturing. Importantly, mRNA sequence can be modified and adjusted to specific needs, such as emergency situations or personalized medicine.

Nevertheless, important drawbacks have delayed mRNA therapies development since its discovery in 1961. Due to its physicochemical properties, this large and anionic biopolymer is impeded from functionality when naked administered, as it possesses a short half-life, is hampered from crossing cellular membranes and prompts immunological reactions. As such, researchers have engineered ways to tackle these disadvantages. First, the modification of mRNA chemistry has permitted its translational application, by increasing *in vivo* potency without causing immunological responses. Second, the engineering of nanoparticles that protect, transport, and deliver mRNA to target cells has allowed its use as a therapeutic agent.

The term 'nanoparticle' is comprised in the term 'nanomedicine', which is described as formulations of various materials (organic or inorganic) in the range of the nanometer scale. With several clinical products such as Doxil® or Abraxane®, nanomedicine has played a vital role in the success of mRNA therapies. In RNA space, the approval of Onpattro®, a siRNA-based therapy for the treatment of hereditary transthyretin-mediated amyloidosis (hATTA) in 2018 has pivoted the way forward to today's most advanced delivery vehicle for mRNA, Lipid Nanoparticles (LNPs). As of 2024, three mRNA therapies using LNPs have reached the market, and many others are in clinical development. The success of these particles relies on the discovery of ionizable lipids, lipids which are protonated only at acidic pH, as opposed to the traditional approach of using permanently charged lipids. As such, upon LNPs formulation at acidic pH, ionizable lipids permit the encapsulation of mRNA, however, after final formulation buffer adjustments, LNPs become neutral; overcoming the disadvantages of cationic-charged particles. So far, the most advanced LNPs are composed of an ionizable lipid, cholesterol, DSPC and DMG-PEG. Thus, the research of new lipids that can help push forward the development of more potent and specific or tailored particles is vital. As well, the use of new and more tolerable lipids and

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alternatives to PEG-lipids are essential to grant higher safety profiles, especially important for several biomedical applications.

In this context, the main objective of this thesis was to design and develop a new type of nanoparticles that can effectively protect and deliver mRNA, to fully characterize them and understand their biological behavior, and eventually to explore their application in biomedicine, more specifically in oncology, through the development of nanoimmunotherapeutics, and in tissue regeneration, for cell reprogramming.

To this aim, the design, development and fully characterization of these nanoparticles is presented in Chapters I and II. Upon composition-based screenings and detailed study of the physicochemical properties, *in vitro* and *in vivo* performance, results showed that size, composition and particle concentration dramatically impact formulation performance. Additionally, our results indicate that the developed nanoparticles can be modulated in terms of composition and physicochemical properties to modify organ tropism. On top of that, the design of totally biodegradable nanoparticles is also presented, overcoming the main drawbacks of current LNPs, and allowing for safer and more translational approaches.

In Chapter III, the study of the nanoparticles' efficacy in a relevant therapeutic model is proposed, with a focus on an innovative application within immuno-oncology, which uses the liver as a factory of immunomodulatory proteins. *In vitro* results confirm the potency of the developed nanoparticles, while *in vivo* data show remarkable tumor size decrease as well as tumor regression in 50% of the individuals of the treated group, confirming the high potential of the developed nanomedicines in oncology.

In Chapter IV, the use of biodegradable nanoparticles for tissue reprogramming is studied. This novel application aims at tissue reprogramming using mRNA upon delivering of transcription factors. Results show how the developed nanoparticles are capable of encapsulating all mRNA sequences and the first insights that support the potential of these nanoparticles in regenerative medicine.

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SUMMARY

Genetic medicines can be described as therapeutics which aim to deliver genetic material to prevent, treat or cure a disease. According to the Central Dogma of Molecular Biology, any protein in the body is synthesized following the information encoded in the DNA, such processes only occurring in the described direction. Following the conversion of DNA to messenger RNA (mRNA) in the cell nucleus, *i.e.* transcription, the mRNA travels to the cytoplasm where the ribosomes use the encoded information for protein production, in a process called translation.

As such, genetic medicines can be delivered using either DNA or mRNA. Nevertheless, mRNA therapies bring forward several important advantages when considering their use as therapeutics. First, mRNA does not need to reach the nucleus of the cell to be therapeutically active, therefore, making the delivery more efficient. Second, for the same reason, mRNA does not possess the risk of integrating into the genome of the host cell, as opposed to viral vectors or DNA therapies. Third, mRNA is easily synthesized in the laboratory as well as easy to produce following industrial processes in agreement with Good Manufacturing Practices (GMP) regulations. Finally, mRNA sequence can be easily modified and adapted, accelerating the development of such therapeutics, opening doors to personalized medicine or fast responses in emergency situations.

Messenger RNA is a single strand biopolymer composed of nucleotide subunits linked by phosphodiester bonds. Each nucleotide is composed of a ribose sugar, a phosphate group, and a nitrogenous base (cytosine, C, guanine, G, adenine, A, and uracil, U). Such physicochemical properties impede its use as a therapeutic, as naked mRNA is highly unstable and precluded from crossing cell lipid bilayers. As well, it prompts important immunological responses. Such drawbacks have been overcome by two main approaches: 1) the modification of mRNA sequence to decrease immunogenicity and increase stability, proven important for clinical translation; and 2) the use of delivery vehicles that protect mRNA from degradation and allow its intracellular delivery. This strategy, in essence, incorporated the know-how researchers had been applying to small molecules and biomolecules over the years, where nanomedicine played a vital role. The term nanomedicine is described as formulations in the nanometer scale (or nanoformulations) composed of organic materials such as lipids or polymers, or inorganic materials such as metals, that can be used to deliver small molecules or biomolecules namely proteins, peptides or nucleic acids. Such nanomedicines can be used for therapeutic purposes or diagnostics.

The mechanism of action of mRNA nanomedicines within the cell is described as a challenging process, with several steps that can difficult the efficient delivery of mRNA to its site of action, the

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cytoplasm. As a first contact, nanoparticles interact with receptors, in a process known as endocytosis. In fact, several types of endocytosis have been described and, moreover, different nanomedicines can undergo different endocytic pathways depending on their physicochemical properties or materials, dictating their performance. The target cell type also influences endocytosis mechanisms, and actually, this fact has been linked to the mechanism of action of liver targeting mRNA nanoparticles. Once the interaction has taken place, nanoparticles are now located in the endosome, a highly acidic environment which has been described to be the bottleneck to efficient cargo delivery. Once here, the nanoparticles capable of disrupting the endosomal membrane would efficiently release the mRNA to the cytosol, *i.e.* endosomal escape, where it would be ready to be translated to proteins by the ribosomes. Endosomal escape, nevertheless, has been described as a low efficient process, and in fact, an important amount of research aiming to find more efficient nanomedicines is centered around the investigation of this process. In fact, it is believed that only a small percentage of the cargo is released to the cytoplasm, as many endosomes would be transported to late endosomes and later, to lysosomes, where they will be either degraded or exocytosed.

Specifically for gene delivery, all the research performed in the last 30 years resulted in safe and effective delivery platforms for nucleic acids, such as antisense oligonucleotides (ASOs), small interfering RNAs (siRNA) or DNA, by using different types of delivery systems, both viral-based or synthetic nanoparticles. For example, the development and later commercialization of lipofectamine in 1989 prompted the development of non-viral vectors, as well as the commercialization of Doxil® in 1995 and Abraxane® in 2005 were a huge push to the field of clinical nanomedicine. As of mRNA field, the discovery of the decreased immunogenicity of modified mRNA by Dr. Katalin Karikó funded the basis of the research that would later allow mRNA therapeutics, providing a solution to the initial studies reporting immunogenicity of mRNA in 1963. In fact, Dr. Karikó and Dr. Weismann were awarded with the Nobel Prize of Physiology and Medicine in 2023.

Nonetheless, mRNA therapeutics did not reach the market until the approval of COVID-19 vaccines in 2020. Various delivery vehicles such as lipoplexes, liposomes or cationic nanoemulsions had been subject of studies since the discovery of mRNA, nevertheless, the clinical translation of such remained poor. Indeed, the commercialization of COVID-19 vaccines was possible thanks to the use of Lipid Nanoparticles (LNPs), previously marketed with Onpattro® in 2018, and remains a milestone in both mRNA and nanomedicine field, opening new possibilities to future envisioned therapeutics. As of 2024, a third mRNA-based vaccine for Respiratory Syncytial Virus (RSV) has been approved by the FDA and EMA, highlighting the fast advancement of these therapeutics (Figure 1).

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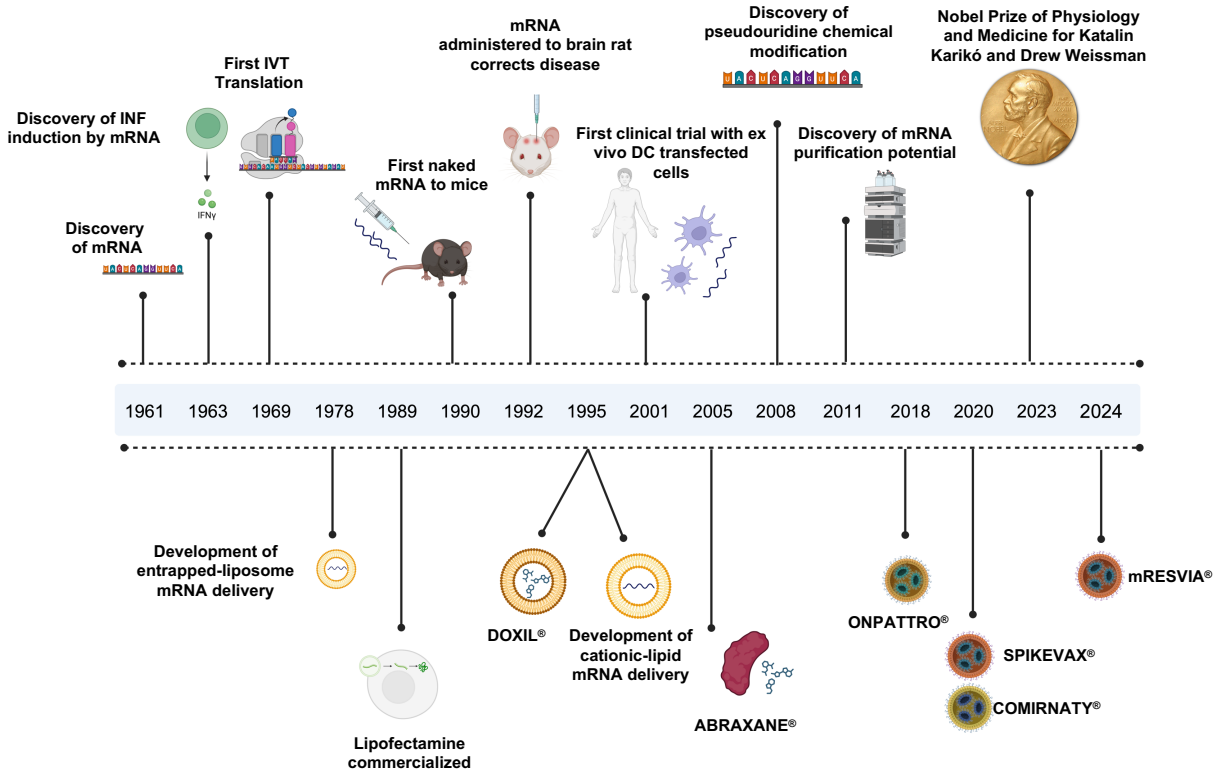


Figure 1. mRNA field meets Nanomedicine field. Timeline of important breakthroughs in both mRNA (upper) and nanomedicine field (lower), which allowed the development of current mRNA therapeutics. This figure is the author's own design. Created with Biorender.

Remarkably, all these marketed mRNA medicines, as well as the majority in the clinical development space, are formulated using LNPs. Originated from liposomes, these particles prevail over traditional approaches which entail the use of cationic lipids (*i.e.* positively charged lipids at any pH) to complex nucleic acids following electrostatic interactions, but which encompassed stability and efficacy problems. LNPs are based on the use of ionizable lipids only positively charged at acidic pH allowing, thus, the formation of neutral particles which encapsulate the mRNA inside their core, permitting longer circulation times, higher endosomal escape and increased efficacy. In fact, since their discovery, a lot of research has been performed, with new structures and materials being investigated.

Nevertheless, important challenges still lie ahead. Most used LNPs today are composed of an ionizable lipid, helper lipids and a PEG-derivative. The ionizable lipid has proven to be the main potency driving lipid, being the key element in LNPs. Helper lipids, *i.e.*, phospholipids and cholesterol, provide structural functionalities. Indeed, cholesterol or sterol derivatives are used to provide rigidity, similarly to what cholesterol does in cell membranes. Commonly used phospholipids such as DSPC or DOPE contribute to structural arrangements, and their structure is described to contribute to endosomal escape

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as well. Similarly, a PEG-lipid derivative is included to provide colloidal stability and increase half-life in circulation. In fact, the total removal of this polymer has proven to decrease stability of LNPs.

Thus, the research of new components could provide insights for the development of more specific, tailored or targeted particles. As well, the understanding of how the modulation of such components can further impact physicochemical properties, *in vitro* and *in vivo* performance, biodistribution and functional delivery would enormously allow for new therapeutic avenues. Finally, the substitution of PEG-derivatives for different alternatives together with the discovery of new biodegradable ionizable materials would further grant higher safety profiles, especially important for repeated administrations.

In this direction, the aim of this thesis is to advance in the development of novel formulations specifically designed for mRNA delivery for different biomedical applications.

Sphingomyelin Nanoemulsions (SNs, WO 2019/138139A1[224]), composed of sphingomyelin and vitamin E have been previously investigated in the laboratory. These two components have demonstrated their ability to self-assembly into nanostructures upon a single-step formulation process. As well, preliminary scale-up data demonstrated the industrial possibilities of these nanoparticles. Indeed, sphingolipids represent one of the main types of lipids in eukaryotic cells, and they are known to provide structural features to membranes. As well, their common backbone, sphingosine, has been associated with bioactive activities such as cell growth and modulation of cell-to-cell interactions. Sphingomyelin has been previously marketed in Marqibo®, a vincristine liposomal formulation approved by the FDA in 2012. Similarly, Vitamin E has been explored as a drug delivery component for years now. Of note, SNs demonstrated positive results in gene therapy. When modified with DOTAP, stearylamine or putrescine, SNs efficiently associate and deliver small interfering RNA (siRNA), micro RNA (miRNA) and plasmid DNA (pDNA) in vitro. More recently, *in vivo* results confirmed that SNs loaded with therapeutic pDNA can contribute to the development of novel anticancer gene therapies. These results are the foundations of this thesis work.

The restrained delivery to DCs and natural accumulation of nanoparticles in the liver, which will be also studied in this thesis, might be one of the most challenging and important issues to tackle, as it averts the potential of this application to other organs. This issue will be also covered in this thesis, with the study of composition adjustments on nanoparticle biodistribution.

Lastly, the therapeutic potential of the designed mRNA nanoformulations will be explored in two different therapeutic areas of interest, which are currently being subject of numerous investigations, oncology, through the development of immunotherapeutics, and regenerative medicine, to provide reprogramming tools for treating cardiovascular acute diseases.

To this aim, the design, development and fully characterization of these nanoparticles is presented in Chapters I and II. Upon composition-based screenings and detailed study of the physicochemical properties, *in vitro* and *in vivo* performance, results showed that size, composition and particle concentration dramatically impact formulation performance. Additionally, our results indicate that the developed nanoparticles can be modulated in terms of composition and physicochemical properties to modify organ tropism. On top of that, the design of totally biodegradable nanoparticles is also presented, overcoming the main drawbacks of current LNPs, and allowing for safer and more translational approaches.

In the first chapter, the design and development of a new type of nanoparticles, SNPs, is shown. To this aim, new nanoparticles were developed by using different combinations of lipids and loaded with Firefly Luciferase mRNA (FLuc mRNA). Characterization experiments refer to analysis of the colloidal properties, association efficiencies, and *in vitro* and *in vivo* transfection efficiencies. For reference, SNs previously developed by the research group and efficient for the association of nucleic acids, were also formulated and tested. Results confirmed the superiority of the new nanoparticle formulations.

Next, the proportion of the lipids was adjusted, to understand the influence of the lipid composition on the physicochemical properties and transfection efficiencies. Four different SNPs compositions were formulated and fully characterized (1-SNPs, 2-SNPs, 3-SNPs, 4-SNPs). Physicochemical properties, encapsulation efficiency, colloidal stability and morphology, were determined by Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM) and indirect mRNA fluorescent-based quantification, and are shown in Figure 2. The cell internalization was tested on HEK293 and HEPG2 cell-lines, with fluorescently-labelled nanoparticles, labelled mRNA and double-labelling (nanoparticles and mRNA), via confocal microscopy and flow cytometry. No differences were observed among the four prototypes. Next, *in vitro* transfection efficiency and cytotoxicity were determined. In terms of expression of the encapsulated FLuc mRNA (bioluminescence was measured 24h post-transfection), two of the formulations shown the most promising results (3-SNPs, 4-SNPs), which moreover were as potent as to the positive control, LNPs formulated with Onpattro® composition (ONP). When eGFP-mRNA was encapsulated, the efficient translation of the protein was confirmed via confocal microscopy. In terms of cytotoxicity (studies were carried out by direct quantification of the live-cell protease activity), cells remain ed fully viable upon 24h and 48h incubation with the nanoparticles, as opposed to what it was observed with MessengerMax (Invitrogen), underlaying the cell compatibility of SNPs. Next, SNPs were evaluated *in vivo*. Their potency as well as the functional delivery to main organs (liver, spleen, lungs, kidneys and omenta) was assessed by bioluminescence quantification (ph/s/cm^2/sr) in C57/BL6 female mice. Potency achieved after 6h post IV-SNPs administration was quantified. In agreement with *in vitro* potency results, 3-SNPs and 4-SNPs

exhibited higher protein production, and were comparable to the positive control ONP, underlaying the positive prospective of the newly developed mRNA formulations. In a second experimental analysis, 24h post-administration, mice were administered luciferin, sacrificed, and the bioluminescence from each organ was quantified. The percentage of luminescence in each organ was calculated by considering the total organ bioluminescence. As compared to ONP, some SNPs exhibited preferred spleen delivery. In addition, the most potent prototype, 3-SNPs, was compared against Spikevax® LNPs composition (Moderna, MOD) and JetPEI®, a commonly used transfection reagent for *in vivo* dosing. Results showed a remarkable overperformance of SNPs as compared to JetPEI®, although they did not reach MOD potency.

To sum up, the first chapter of this thesis dives into the description of a new delivery vehicle for mRNA. By carefully studying from colloidal properties to *in vivo* fate, this chapter provides insights on delivery design, opening new doors for future formulation development works.

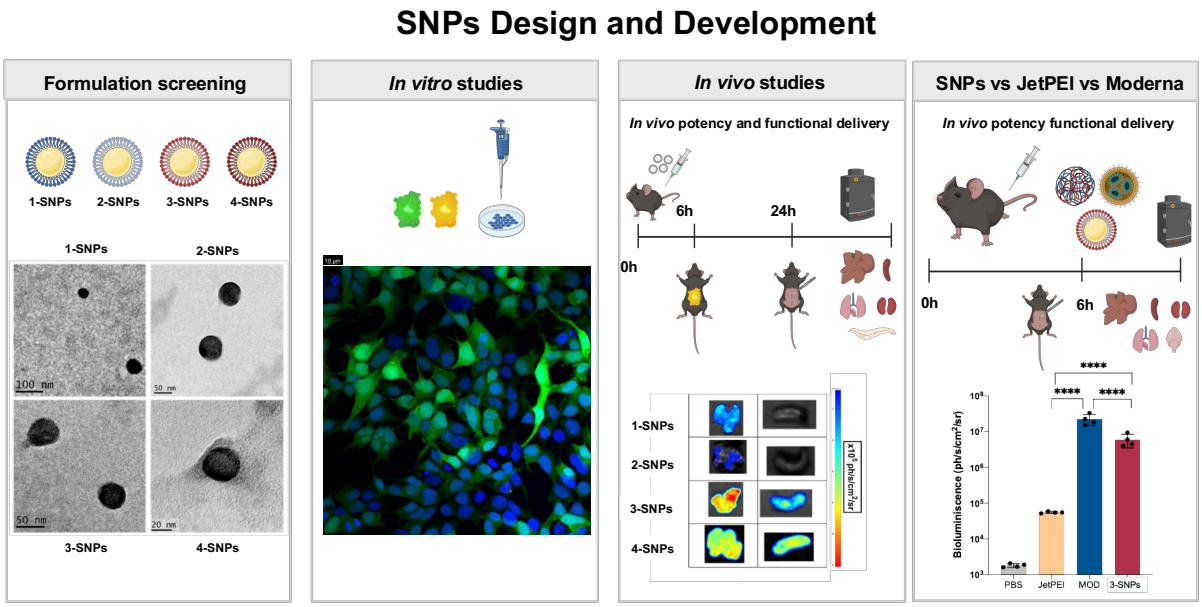


Figure 2. Design and development of the new nanoparticle formulations described in Chapter I. Formulation screening was developed to select the most promising prototypes, and *in vitro* and *in vivo* studies were conducted to assess performance. TEM images and eGFP translated protein upon SNPs administration are shown. Nanoparticles were lastly compared to the commercial reagent JetPEI® and LNPs prepared with the composition of Moderna (MOD). This figure is the author's own design. Created with BioRender.com

In the second chapter, we aimed at providing a response to the current limitations of today's technologies. The need of designing biodegradable and more biocompatible delivery systems as well as the study and understanding of targeted delivery to specific organs were subject of this chapter.

For the design of totally biodegradable SNPs, the removal and substitution of PEG for a biodegradable hydrophilic polymer was performed. In addition, all lipids included in the nanoparticle

composition, as disclosed in Chapter II, were biodegradable. This is of great advantage as PEG derivatives have been previously described as immunogenic upon IV administration, and even though they have extensively been used for many years thanks to their capacity to prolong circulation time as well as decrease opsonization, the field is currently evolving towards limiting their use. However, their complete removal has been associated with decrease stability, and so, our results demonstrated the possibilities to find alternatives that maintain stability and potency. Formulated nanoparticles resulted stable homogeneous and show activity *in vivo*. Unsurprisingly, these replacements led to a decrease in potency, as observed in transfection experiments in vitro and in vivo, possibly due to rapid degradation upon administration, according to previous reports in the field. Thus, maintaining the potency while providing with biodegradable features remained an important challenge. To overcome this limitation, a helper lipid, was included in the formulation to enhance potency. Indeed, its addition provided a more potent formulation. The study of functional delivery of these biodegradable nanoparticles, BD-SNPs, was also performed. In relation to previous formulations, BD-SNPs exhibited an increase in spleen delivery.

For studying and modulating organ distribution, different formulations were prepared, fully characterized and tested. Comparative studies refer to the 4-SNPs formulation, as described in Chapter I, and upon inclusion of a positively charged lipid (P-SNPs) and a negatively charged lipid, (N-SNPs). An increase in lung and spleen delivery was respectively observed (Figure 3), revealing the possibility of modulating biodistribution and defining improved formulations for specific organ targeting. The mechanisms of action should be further explored and can be related to specific functionality of the lipids leading by structure and charge, as well as to specific arrangement of components in SNPs modulating the surface properties and therefore specific features as can be the formation of the protein corona. More studies would be necessary to deepen the understanding of these results. Overall, this chapter provides data on the development of biodegradable SNPs, as well as pivots the study of specific organ delivery for SNPs.

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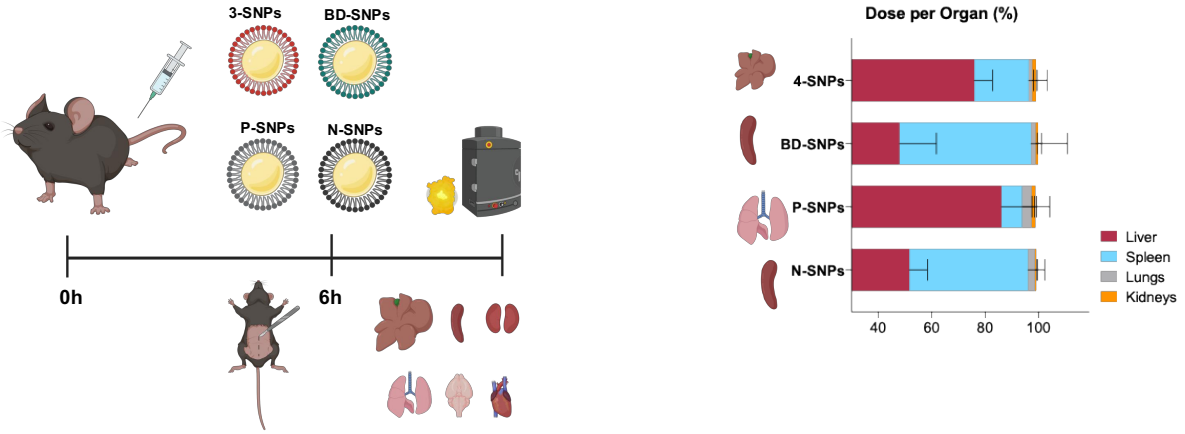


Figure 3. Schematic representation of the work presented in Chapter II. Functional delivery studies were performed with several prototypes. This figure is the author's own design. Created with BioRender.com

In Chapter III, the study of the nanoparticles' efficacy in a relevant therapeutic model is proposed, with a focus on an innovative application within immuno-oncology, which uses the liver as a factory of immunomodulatory proteins.

The use of the developed SNPs for the delivery of immunomodulatory therapies of interest in oncology is proposed. In this specific case, the new nanoparticle formulations, in particular 1-SNPs and 3-SNPs, were loaded with a therapeutic mRNA encoding for a immunomodulatory protein, well known by its capacity to arrest tumor cell growth but with severe toxic side effects, as a first proof-of-concept. Additionally, by delivering immunomodulatory proteins in the form of mRNA, important drawbacks of protein therapeutics can be overcome, such as short half-life and difficult and expensive engineering manufacturing processes. In this context, the use of the liver as a factory of proteins that can be secreted to the bloodstream and exert their therapeutic action is a novel approach currently being investigated in preclinical and clinical settings. As such, mRNA loaded SNPs administered IV would be a viable therapeutic strategy to treat difficult-to-access or distal tumors (Figure 4).

First, a kinetic experiment to provide evidence of the secretion of the encoded protein to the bloodstream after SNPs administration was performed. Upon analysis of the serum protein levels, 3-SNPs were selected as the most promising formulation, leading to protein serum levels still detectable at 96h post-administration. Once the successful production of the encoded protein was confirmed, the efficacy of the proposed therapy was analysed in a relevant model of disease, B16OVA tumor-bearing mice. Upon two administrations of 10µg of mRNA per mice at day 10 and 14 post tumor inoculation and monitorization of tumor size, mice weight and overall survival, results were remarkable. mRNA-

loaded 3-SNPs were able to significantly reduce tumor size with respect to the controls (mice treated with PBS and nanoparticles loaded with a mock mRNA sequence). Furthermore, 50% of individuals experienced tumor remission, which was translated to an overall increased survival in the treatment group. These results highlight the potential of SNPs for therapeutic delivery of mRNA, with a special focus on immuno-oncology.

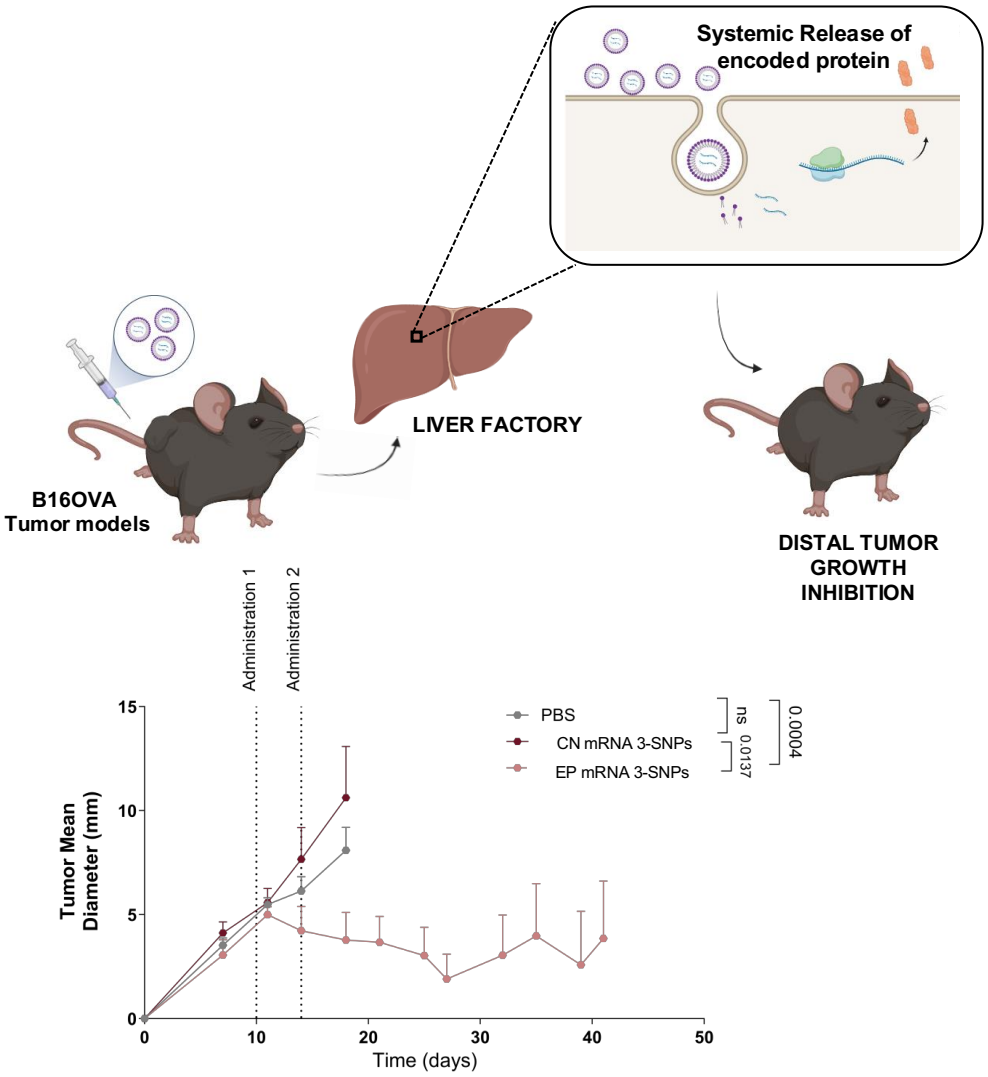


Figure 4. Graphical representation of the work performed in Chapter III. Encoded Protein (EP) mRNA loaded 3-SNPs were administered Intravenously (IV) to B16 OVA bearing tumor mice models for the treatment of distal tumors, achieving decreased tumor size and increased survival. CN stands for Control, which was a mock-mRNA sequence. This figure is the author's own design. Created with Biorender.com.

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In Chapter IV, the use of biodegradable nanoparticles for tissue reprogramming is studied. This novel application aims at tissue reprogramming using mRNA encoding different transcription factors. We proposed the use of the reported biodegradable SNPs in Chapter II. Cell reprogramming has become an important strategy for the treatment of degenerative diseases. By overexpressing transcription factors, multiple laboratories have shown that it is possible to revert somatic cells to a pluripotency state. Based on this and by using mRNA and carefully studying factors ratios and doses, rejuvenation of cells could be potentially achieved. Nevertheless, little work has been performed with mRNA and no work has been published using both mRNA and nanoparticle delivery. First, the delivery of mCherry mRNA loaded into BD-SNPs was successfully achieved in human fibroblasts and cardiomyocytes. Then, evidence of the effective synthesis of four different transcription factors related to reprogramming (TF1, TF2, TF3, TF4) mRNA sequences and encapsulation capacity of SNPs, as well as transfection efficacy of (TF1 and TF2) in human fibroblasts is provided. A first pilot study for reprogramming using several treatments and doses was also performed. Although reprogramming results were not positive, and need more optimization, an important readout is that SNPs showed a great biocompatibility profile, with cells remaining healthy after 8 administrations over the course of 16 days, in contrast to what observed for control cells treated with the commercial transfection reagent Messenger Max®. Next experiments aim to further dive into treatment conditions.

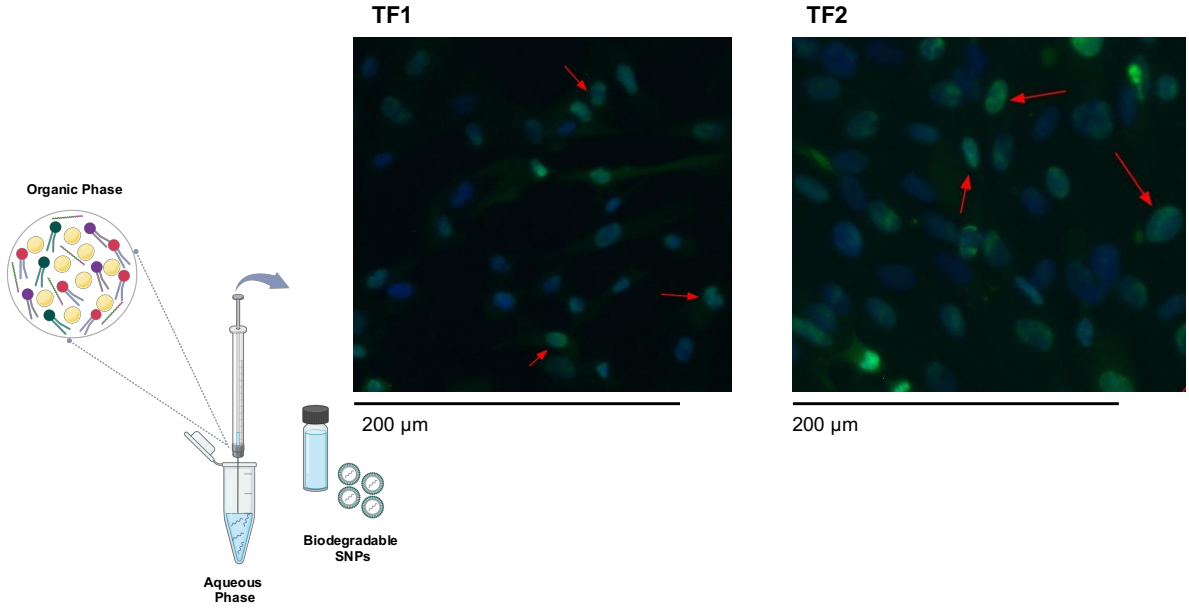


Figure 5. Schematical representation of the work from Chapter IV. Fluorescence microscopy images of cells transfected with SNPs loaded with mRNA encoding for TF1 and TF2 after immunofluorescent staining with specific antibodies. mCherry loaded SNPs were used as negative controls (not shown). Nuclei was stained with Hoechst (Blue, 1:1000, 10 min). Green signal (secondary antibody labelled with AlexaFluor 488) successfully confirms the presence of the transcription factors upon efficient translation. This figure is the author's own design. Created with Biorender.com

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In summary, this thesis shows the rational design of a new type of nanoparticles specifically designed for mRNA delivery, SNPs, and explores their potential for applications in the biomedical field. As such, we accentuate their versatility, by providing insights on composition-based screenings, and how such can drive performance. In addition, we provide efficacy data on a relevant tumor model and investigate their potential for the development of the regenerative medicine field. In summary, the data presented in this thesis allows us to conclude that 1) SNPs are a valid and versatile delivery platform that work with a broad range of mRNA sequences; 2) SNPs potency, physicochemical properties, functional delivery and biocompatibility can be modulated by adjusting the composition; 3) the use of SNPs for immuno-oncology applications has been proven, providing remarkable efficacy data, and; 4) the ability of SNPs to transfect fibroblast and cardiomyocytes as well as to deliver reprogramming factors opens up new doors for tissue regeneration applications.

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RESUMEN

Los medicamentos genéticos pueden describirse como productos terapéuticos cuyo objetivo es entregar material genético para prevenir, tratar o curar una enfermedad. Según el Dogma Central de la Biología Molecular, cualquier proteína del organismo es sintetizada a partir de la información contenida en el ADN. Después de la conversión del ADN en ARN mensajero (ARNm) en el núcleo celular, es decir, la transcripción, el ARNm viaja al citoplasma donde los ribosomas utilizan la información codificada para la producción de proteínas, en un proceso llamado traducción.

Como tal, los medicamentos genéticos pueden administrarse utilizando ADN o ARNm. Sin embargo, las terapias con ARNm aportan varias ventajas importantes al considerar su uso como terapéutico. En primer lugar, no es necesario que el ARNm llegue al núcleo de la célula para ser terapéuticamente activo, lo que hace que la entrega sea más eficiente. En segundo lugar, por la misma razón, el ARNm no presenta el riesgo de integrarse en el genoma de la célula huésped, a diferencia de los vectores virales o las terapias de ADN. En tercer lugar, el ARNm se sintetiza fácilmente en el laboratorio y es fácil de producir siguiendo procesos industriales de acuerdo con las regulaciones de Buenas Prácticas de Fabricación (GMP). Finalmente, la secuencia de ARNm se puede modificar y adaptar fácilmente, acelerando el desarrollo de dichas terapias, y abriendo puertas a la medicina personalizada o a respuestas rápidas en situaciones de emergencia.

El ARN mensajero es un biopolímero monocatenario compuesto por subunidades de nucleótidos unidas por enlaces fosfodiéster. Cada nucleótido está compuesto por un azúcar ribosa, un grupo fosfato y una base nitrogenada (citosina, C, guanina, G, adenina, A y uracilo, U). Tales propiedades fisicoquímicas impiden su uso como terapéutico, ya que el ARNm desnudo es muy inestable y no puede cruzar las bicapas lipídicas de las células. Además, provoca importantes respuestas inmunológicas. Estos inconvenientes se han superado mediante dos enfoques principales: 1) la modificación de la secuencia del ARNm para disminuir la inmunogenicidad y aumentar la estabilidad, lo que ha demostrado ser importante para la traducción clínica; y 2) el uso de vehículos de administración que protejan el ARNm de la degradación y permitan su administración intracelular. Esta estrategia, en esencia, incorporó los conocimientos que los investigadores habían estado aplicando a fármacos tradicionales y biomoléculas a lo largo de los años, donde la nanomedicina desempeñó un papel vital. El término nanomedicina se describe como el conjunto de formulaciones a escala nanométrica (o nanoformulaciones) compuestas de materiales orgánicos como lípidos o polímeros, o materiales inorgánicos como metales, que pueden usarse para administrar moléculas o biomoléculas, por ejemplo, proteínas, péptidos o ácidos nucleicos. Estos nanomedicamentos pueden utilizarse con fines terapéuticos o de diagnóstico

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El mecanismo de acción de los nanomedicamentos de ARNm dentro de la célula está descrito como un proceso arduo, con varios pasos que pueden dificultar la entrega eficiente de ARNm a su sitio de acción, el citoplasma. Como primer contacto, las nanopartículas interactúan con los receptores, en un proceso conocido como endocitosis. De hecho, se han descrito varios tipos de endocitosis y, además, diferentes nanomedicamentos pueden seguir diferentes vías endocíticas en función de sus propiedades fisicoquímicas o materiales, lo que dicta su rendimiento. El tipo de célula diana también influye en los mecanismos de endocitosis y, de hecho, este hecho se ha relacionado con el mecanismo de acción de las nanopartículas de ARNm que se dirigen al hígado. Una vez que se ha producido la interacción, las nanopartículas ahora se ubican en el endosoma, un ambiente altamente ácido que se ha descrito como el cuello de botella para la liberación eficiente de ácidos nucleicos. Una vez aquí, las nanopartículas capaces de alterar la membrana endosomal liberarían eficientemente el ARNm al citosol, en un proceso conocido como escape endosomal, donde estaría listo para ser traducido a proteínas por los ribosomas. El escape endosomal, sin embargo, ha sido descrito como un proceso poco eficiente y, de hecho, una importante cantidad de investigaciones encaminadas a encontrar nanomedicinas más eficientes se centran en la investigación de este proceso. De hecho, se cree que sólo un pequeño porcentaje de la carga se libera al citoplasma, ya que muchos endosomas serían transportados a endosomas tardíos y posteriormente a lisosomas, donde serán degradados o exocitados.

Específicamente para la entrega de genes, todas las investigaciones realizadas en los últimos 30 años dieron como resultado plataformas de entrega seguras y efectivas para ácidos nucleicos, como oligonucleótidos antisentido (ASO), pequeños ARN de interferencia (siRNA) o ADN, mediante el uso de diferentes tipos de sistemas de entrega, nanopartículas tanto de base viral como sintéticas. Por ejemplo, el desarrollo y posterior comercialización de la lipofectamina en 1989 impulsó el desarrollo de vectores no virales, así como la comercialización de Doxil® en 1995 y Abraxane® en 2005 supusieron un gran impulso en el campo de la nanomedicina clínica. En el campo del ARNm, el descubrimiento de la inmunogenicidad disminuida del ARNm modificado por la Dra. Katalin Karikó fundó la base de la investigación que luego permitiría la terapéutica del ARNm, dando solución a los estudios iniciales que reportaban la inmunogenicidad del ARNm en 1963. De hecho, la Dra. Karikó y el Dr. Weismann recibieron el Premio Nobel de Fisiología y Medicina en 2023 por este descubrimiento.

Sin embargo, las terapias de ARNm no llegaron al mercado hasta la aprobación de las vacunas COVID-19 en 2020. Varios vehículos de administración, como lipoplexos, liposomas o nanoemulsiones catiónicas, habían sido objeto de estudios desde el descubrimiento del ARNm, sin embargo, la traducción clínica de los mismos seguía siendo difícil. De hecho, la comercialización de las vacunas contra la COVID-19 fue posible gracias al uso de nanopartículas lipídicas (LNP), comercializadas anteriormente con Onpattro® en 2018, y sigue siendo un hito tanto en el campo del ARNm como en la

nanomedicina, al abrir nuevas posibilidades para futuras terapias. A partir de 2024, la FDA y la EMA aprobaron una tercera vacuna basada en ARNm para el virus sincitial respiratorio (VRS), lo que destaca el rápido avance de estas terapias.

Sorprendentemente, todos estos medicamentos de ARNm comercializados, así como la mayoría en el ámbito del desarrollo clínico, se formulan utilizando nanopartículas lipídicas. Originadas a partir de liposomas, estas partículas prevalecen sobre los enfoques tradicionales que implican el uso de lípidos catiónicos (es decir, lípidos cargados positivamente a cualquier pH) para acomplejar ácidos nucleicos por medio de interacciones electrostáticas, pero que abarcaban problemas de estabilidad y eficacia. Las LNP se basan en el uso de lípidos ionizables cargados positivamente sólo a pH ácido permitiendo, así, la formación de partículas neutras que encapsulan el ARNm dentro de su núcleo, lo que permite tiempos de circulación en sangre más prolongados, mayor escape endosomal y, en definitiva, mayor eficacia. De hecho, desde su descubrimiento se han realizado muchas investigaciones, buscando nuevas estructuras y materiales.

Sin embargo, aún quedan importantes desafíos por delante. Las LNP más utilizadas en la actualidad están compuestas por un lípido ionizable, lípidos auxiliares y un derivado de PEG. El lípido ionizable ha demostrado ser el principal lípido impulsor de potencia, siendo el elemento clave en las LNP. Los lípidos auxiliares, es decir, los fosfolípidos y el colesterol, proporcionan funcionalidades estructurales. De hecho, el colesterol o los derivados de esteroles se utilizan para proporcionar rigidez, de forma similar a lo que hace el colesterol en las membranas celulares. Los fosfolípidos de uso común, como DSPC o DOPE, contribuyen a las disposiciones estructurales y se describe que su estructura también contribuye al escape endosomal. De manera similar, se incluye un lípido derivado de PEG para proporcionar estabilidad coloidal y aumentar la vida media en circulación. De hecho, se ha demostrado que la eliminación total de este polímero disminuye la estabilidad de las LNP.

Por tanto, la investigación de nuevos componentes podría proporcionar información para el desarrollo de partículas más específicas, adaptadas o dirigidas. Además, comprender cómo la modulación de dichos componentes puede afectar aún más a las propiedades fisicoquímicas, el rendimiento *in vitro* e *in vivo*, la biodistribución y la liberación funcional permitiría el desarrollo de nuevas vías terapéuticas. Finalmente, la sustitución de los derivados de PEG por alternativas más biocompatibles junto con el descubrimiento de nuevos materiales ionizables biodegradables otorgaría perfiles de seguridad más altos, especialmente importantes para administraciones repetidas.

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Las nanoemulsiones de esfingomielina (SNs, WO 2019/138139A1224), compuestas de esfingomielina y vitamina E, se han investigado previamente en el laboratorio. Estos dos componentes han demostrado su capacidad para autoensamblarse en nanoestructuras mediante un proceso de formulación de un solo paso. Además, los datos preliminares de escalado demostraron las posibilidades industriales de estas nanopartículas. De hecho, los esfingolípidos representan uno de los principales tipos de lípidos en las células eucariotas y se sabe que proporcionan características estructurales a las membranas. Además, su estructura común, la esfingosina, se ha asociado con actividades bioactivas como el crecimiento celular y la modulación de las interacciones entre células. La esfingomielina se comercializó anteriormente en Marqibo®, una formulación liposomal de vincristina aprobada por la FDA en 2012. De manera similar, la vitamina E se ha explorado como componente de administración de fármacos durante años. Es de destacar que las SN demostraron resultados positivos en la terapia génica. Cuando se modifican con DOTAP, estearilamina o putrescina, los SN se asocian y liberan de manera eficiente pequeños ARN de interferencia (ARNi), microARN (miARN) y ADN plásmido in vitro. Más recientemente, los resultados in vivo confirmaron que las SNs cargadas con pDNA terapéutico pueden contribuir al desarrollo de nuevas terapias genéticas contra el cáncer. Estos resultados son las bases de este trabajo de tesis.

La entrega restringida a las Células Dendríticas y la acumulación natural de nanopartículas en el hígado, que también se estudiarán en esta tesis, podría ser una de las cuestiones más desafiantes e importantes de abordar, ya que evita el potencial de esta aplicación a otros órganos. Este tema también será cubierto en esta tesis, con el estudio de los ajustes de composición en la biodistribución de nanopartículas.

Por último, se explorará el potencial terapéutico de las nanoformulaciones de ARNm diseñadas en dos áreas terapéuticas diferentes de interés, que actualmente están siendo objeto de numerosas investigaciones, la oncología, a través del desarrollo de inmunoterapéuticas, y la medicina regenerativa, para proporcionar herramientas de reprogramación en el tratamiento de enfermedades cardiovasculares agudas. enfermedades.

En resumen, el diseño, desarrollo y caracterización completa de estas nanopartículas se presenta en los Capítulos I y II. Tras evaluaciones basadas en la composición y un estudio detallado de las propiedades fisicoquímicas y el rendimiento *in vitro* e *in vivo*, los resultados mostraron que el tamaño, la composición y la concentración de las partículas impactan dramáticamente el rendimiento de la formulación. Además, nuestros resultados indican que las nanopartículas desarrolladas pueden modularse en términos de composición y propiedades fisicoquímicas para modificar el tropismo a los órganos. Asimismo, también se presenta el diseño de nanopartículas totalmente biodegradables,

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superando los principales inconvenientes de las LNP actuales y permitiendo enfoques más seguros y traslacionales.

En el primer capítulo se muestra el diseño y desarrollo de un nuevo tipo de nanopartículas, los SNP. Para ello, se desarrollaron nuevas nanopartículas utilizando diferentes combinaciones de lípidos y cargadas con ARNm de Firefly Luciferase (FLuc mRNA). Los experimentos de caracterización se refieren al análisis de las propiedades coloidales, eficiencias de asociación y eficiencias de transfección in vitro e in vivo. Como referencia, también se formularon y probaron SN previamente desarrollados por el grupo de investigación y eficientes para la asociación de pDNA y mRNA. Los resultados confirmaron la superioridad de las nuevas formulaciones de nanopartículas.

A continuación, se ajustó la proporción de lípidos para comprender la influencia de la composición de los lípidos en las propiedades fisicoquímicas y las eficiencias de transfección. Se formularon y caracterizaron completamente cuatro composiciones diferentes de SNP (1-SNP, 2-SNP, 3-SNP, 4-SNP). Las propiedades fisicoquímicas, la eficiencia de encapsulación, la estabilidad coloidal y la morfología se determinaron mediante dispersión dinámica de luz (DLS), análisis de seguimiento de nanopartículas (NTA), microscopía electrónica de transmisión (TEM) y cuantificación indirecta basada en fluorescencia de ARNm, y se muestran en la Figura 2. La internalización celular se probó en líneas celulares HEK293 y HEPG2, con nanopartículas marcadas con fluorescencia, ARNm marcado y doble marcaje (nanopartículas y ARNm), mediante microscopía confocal y citometría de flujo. No se observaron diferencias entre los cuatro prototipos. A continuación, se determinaron la eficacia de la transfección in vitro y la citotoxicidad. En términos de expresión del ARNm de Fluc encapsulado (la bioluminiscencia se midió 24 h después de la transfección), dos de las formulaciones mostraron los resultados más prometedores (3-SNP, 4-SNP), que además eran tan potentes como el control positivo, LNP. formulado con la composición Onpattro® (ONP). Cuando se encapsuló el ARNm de eGFP, la traducción eficiente de la proteína se confirmó mediante microscopía confocal. En términos de citotoxicidad (los estudios se llevaron a cabo mediante cuantificación directa de la actividad de la proteasa de células vivas), las células permanecen completamente viables tras 24 y 48 horas de incubación con las nanopartículas, a diferencia de lo que se observó con MessengerMax (Invitrogen), lo que subyace a la Compatibilidad celular de los SNP. A continuación, se evaluaron los SNP in vivo. Su potencia, así como el suministro funcional a los órganos principales (hígado, bazo, pulmones, riñones y epiplón) se evaluaron mediante cuantificación de bioluminiscencia (ph/s/cm2/sr) en ratones hembra C57/BL6. Se cuantificó la potencia alcanzada después de 6 h después de la administración de IV-SNP. De acuerdo con los resultados de potencia in vitro, los 3-SNP y 4-SNP exhibieron una mayor producción de proteínas y fueron comparables al ONP de control positivo, lo que sustenta las perspectivas positivas de las formulaciones de ARNm recientemente desarrolladas. En un segundo análisis experimental, 24 h

después de la administración, a los ratones se les administró luciferina, se sacrificaron y se cuantificó la bioluminiscencia de cada órgano. El porcentaje de luminiscencia en cada órgano se calculó considerando la bioluminiscencia total del órgano. En comparación con la ONP, algunos SNP mostraron una entrega preferente en el bazo. Además, el prototipo más potente, los 3-SNP, se comparó con la composición de LNP de Spikevax® (Moderna, MOD) y JetPEI®, un reactivo de transfección de uso común para dosificación in vivo. Los resultados mostraron un notable rendimiento superior de los SNP en comparación con JetPEI®, aunque no alcanzaron la potencia MOD.

En resumen, el primer capítulo de esta tesis profundiza en la descripción de un nuevo vehículo de entrega de ARNm. Al estudiar cuidadosamente desde las propiedades coloidales hasta el destino *in vivo*, este capítulo proporciona información sobre el diseño de estas formulaciones, abriendo nuevas puertas para futuros trabajos de desarrollo de formulaciones.

En el segundo capítulo, nuestro objetivo era dar una respuesta a las limitaciones de las tecnologías actuales. La necesidad de diseñar sistemas de administración biodegradables y más biocompatibles, el uso de alternativas de PEG, así como el estudio y la comprensión de la administración dirigida a órganos específicos fueron objeto de estudio en este capítulo.

Para el diseño de SNPs totalmente biodegradables se realizó la eliminación y sustitución de PEG por un polímero hidrófilo biodegradable. Además, todos los lípidos incluidos en la composición de nanopartículas, como se describe en el Capítulo II, eran biodegradables. Esto supone una gran ventaja ya que los derivados de PEG han sido descritos previamente como inmunogénicos tras su administración intravenosa, y aunque se han utilizado ampliamente durante muchos años gracias a su capacidad para prolongar el tiempo de circulación y disminuir la opsonización, actualmente el campo está evolucionando hacia la limitación. su uso. Sin embargo, su eliminación completa se ha asociado con una disminución de la estabilidad, por lo que nuestros resultados demostraron las posibilidades de encontrar alternativas que mantengan la estabilidad y la potencia. Las nanopartículas formuladas resultaron estables, homogéneas y muestran actividad in vivo. Como era de esperar, estos reemplazos condujeron a una disminución de la potencia, como se observó en experimentos de transfección in vitro e in vivo, posiblemente debido a la rápida degradación tras la administración, según informes anteriores en el campo. Por lo tanto, mantener la potencia y al mismo tiempo proporcionar características biodegradables siguió siendo un desafío importante. Para superar esta limitación, se incluyó un lípido auxiliar en la formulación para mejorar la potencia. De hecho, su adición proporcionó una formulación más potente. También se realizó el estudio de la liberación funcional de estas nanopartículas biodegradables, BD-SNP. En relación con formulaciones anteriores, los BD-SNP mostraron un aumento en la administración al bazo.

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Para estudiar y modular la distribución de órganos, se prepararon, caracterizaron y probaron diferentes formulaciones. Los estudios comparativos se refieren a la formulación de 4-SNP, como se describe en el Capítulo I, y a la inclusión de un lípido cargado positivamente (P-SNP) y un lípido cargado negativamente (N-SNP). Se observó un aumento en la administración al pulmón y al bazo, respectivamente (Figura 3), lo que revela la posibilidad de modular la biodistribución y definir formulaciones mejoradas para dirigirse a órganos específicos. Los mecanismos de acción deben explorarse más a fondo y pueden relacionarse con la funcionalidad específica de los lípidos liderados por estructura y carga, así como con la disposición específica de los componentes en los SNP que modulan las propiedades de la superficie y, por lo tanto, características específicas como puede ser la formación de la proteína. corona. Serían necesarios más estudios para profundizar la comprensión de estos resultados. En general, este capítulo proporciona datos sobre el desarrollo de SNP biodegradables, además de orientar el estudio de la administración de órganos específicos para SNP.

En el Capítulo III se propone el estudio de la eficacia de las nanopartículas en un modelo terapéutico relevante, centrándose en una aplicación innovadora dentro de la inmunooncología, que utiliza el hígado como fábrica de proteínas inmunomoduladoras.

Se propone el uso de los SNP desarrollados para la administración de terapias inmunomoduladoras de interés en oncología. En este caso específico, las nuevas formulaciones de nanopartículas, en particular 1-SNP y 3-SNP, se cargaron con un ARNm terapéutico que codifica una proteína inmunomoduladora, bien conocida por su capacidad para detener el crecimiento de células tumorales pero con graves efectos secundarios tóxicos, como una primera prueba de concepto. Además, al administrar proteínas inmunomoduladoras en forma de ARNm, se pueden superar importantes inconvenientes de la terapia proteica, como una vida media corta y procesos de fabricación de ingeniería difíciles y costosos. En este contexto, el uso del hígado como fábrica de proteínas que pueden secretarse al torrente sanguíneo y ejercer su acción terapéutica es un enfoque novedoso que actualmente se investiga en entornos preclínicos y clínicos. Como tal, los SNP cargados de ARNm administrados por vía intravenosa serían una estrategia terapéutica viable para tratar tumores distales o de difícil acceso (Figura 4).

Primero, se realizó un experimento cinético para proporcionar evidencia de la secreción de la proteína codificada al torrente sanguíneo después de la administración de SNP. Tras el análisis de los niveles de proteína sérica, se seleccionaron 3-SNP como la formulación más prometedora, lo que llevó a niveles séricos de proteína aún detectables 96 h después de la administración. Una vez confirmada la producción exitosa de la proteína codificada, se analizó la eficacia de la terapia propuesta en un modelo relevante de enfermedad: ratones portadores de tumor B16OVA. Tras dos administraciones de 10 μg de

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ARNm por ratón los días 10 y 14 después de la inoculación del tumor y la monitorización del tamaño del tumor, el peso de los ratones y la supervivencia general, los resultados fueron notables. Los 3-SNP cargados con ARNm fueron capaces de reducir significativamente el tamaño del tumor con respecto a los controles (ratones tratados con PBS y nanopartículas cargadas con una secuencia de ARNm simulada). Además, el 50 % de los individuos experimentaron la remisión del tumor, lo que se tradujo en una mayor supervivencia general en el grupo de tratamiento. Estos resultados resaltan el potencial de los SNP para la administración terapéutica de ARNm, con especial atención a la inmunooncología.

En el Capítulo IV se estudia el uso de nanopartículas biodegradables para la reprogramación de tejidos. Esta novedosa aplicación tiene como objetivo la reprogramación de tejidos utilizando ARNm que codifica diferentes factores de transcripción. Propusimos el uso de los SNP biodegradables informados en el Capítulo II. La reprogramación celular se ha convertido en una estrategia importante para el tratamiento de enfermedades degenerativas. Al sobreexpresar factores de transcripción, múltiples laboratorios han demostrado que es posible revertir las células somáticas a un estado de pluripotencia. En base a esto y mediante el uso de ARNm y el estudio cuidadoso de las proporciones y dosis de los factores, se podría lograr potencialmente el rejuvenecimiento de las células. Sin embargo, se han realizado pocos trabajos con ARNm y no se ha publicado ningún trabajo que utilice tanto ARNm como administración de nanopartículas. En primer lugar, la entrega de ARNm de mCherry cargado en BD-SNP se logró con éxito en fibroblastos y cardiomiocitos humanos. Luego, se proporciona evidencia de la síntesis efectiva de cuatro factores de transcripción diferentes relacionados con la reprogramación de secuencias de ARNm (TF1, TF2, TF3, TF4) y la capacidad de encapsulación de SNP, así como la eficacia de transfección de (TF1 y TF2) en fibroblastos humanos. También se realizó un primer estudio piloto de reprogramación utilizando varios tratamientos y dosis. Aunque los resultados de la reprogramación no fueron positivos y necesitan más optimización, una lectura importante es que los SNP mostraron un gran perfil de biocompatibilidad, con células que permanecieron sanas después de 8 administraciones en el transcurso de 16 días, en contraste con lo observado para las células de control tratadas con el producto comercial. reactivo de transfección Messenger Max®. Los próximos experimentos tienen como objetivo profundizar en las condiciones de tratamiento.

En resumen, esta tesis muestra el diseño racional de un nuevo tipo de nanopartículas diseñadas específicamente para la entrega de ARNm, SNP, y explora su potencial para aplicaciones en el campo biomédico. Como tal, acentuamos su versatilidad al brindar información sobre las proyecciones basadas en composición y cómo éstas pueden impulsar el rendimiento. Además, proporcionamos datos de eficacia sobre un modelo tumoral relevante e investigamos su potencial para el desarrollo del campo de la medicina regenerativa. En resumen, los datos presentados en esta tesis nos permiten concluir que 1) los SNP son una plataforma de entrega válida y versátil que funciona con una amplia gama de secuencias

de ARNm; 2) La potencia de los SNP, las propiedades fisicoquímicas, la entrega funcional y la biocompatibilidad se pueden modular ajustando la composición; 3) se ha demostrado el uso de SNPs para aplicaciones de inmunooncología, aportando datos de eficacia destacables, y 4) la capacidad de los SNP para transfectar fibroblastos y cardiomiocitos, así como para administrar factores de reprogramación, abre nuevas puertas para aplicaciones de regeneración de tejidos.

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RESUMO

Os medicamentos xenéticos poden describirse como produtos terapéuticos cuxo obxectivo é entregar material xenético para previr, tratar ou curar unha enfermidade. Segundo o Dogma Central da Bioloxía Molecular, calquera proteína do organismo sintetízase seguindo a información codificada no ADN, estes procesos só se realizan na dirección descrita. Despois da conversión do ADN en ARN mensaxeiro (ARNm) no núcleo celular, é dicir, a transcrición, o ARNm viaxa ao citoplasma onde os ribosomas utilizan a información codificada para a produción de proteínas, nun proceso chamado tradución.

Como tal, os medicamentos xenéticos poden administrarse utilizando ADN ou ARNm. Con todo, as terapias con ARNm achegan varias vantaxes importantes ao considerar o seu uso como terapéutico. En primeiro lugar, non é necesario que o ARNm chegue ao núcleo da célula para ser terapéuticamente activo, o que fai que a entrega sexa máis eficiente. En segundo lugar, pola mesma razón, o ARNm non presenta o risco de integrarse no xenoma da célula hóspede, a diferenza dos vectores virais ou as terapias de ADN. En terceiro lugar, o ARNm sintetízase facilmente no laboratorio e é fácil de producir seguindo procesos industriais dacordo coas regulacións de Boas Prácticas de Fabricación (GMP). Finalmente, a secuencia de ARNm pódese modificar e adaptar fácilmente, acelerando o desenvolvemento das devanditas terapias, e abrindo portas á medicina personalizada ou a respostas rápidas en situacións de emerxencia.

O ARN mensaxeiro é un biopolímero monocatenario composto por subunidades de nucleótidos unidas por ligazóns fosfodiéster. Cada nucleótido está composto por un azucre ribosa, un grupo fosfato e unha base nitroxenada (citosina, C, guanina, G, adenina, A e uracilo, Ou). Tais propiedades fisicoquímicas impiden o seu uso como terapéutico, xa que o ARNm espido é moi inestable e non pode cruzar as bicapas lipídicas das células. Ademáis, provoca importantes respostas inmunolóxicas. Estes inconvintes superáronse mediante dous enfoques principais: 1) a modificación da secuencia do ARNm para diminuír a inmunoxenicidade e aumentar a estabilidade, o que demostrou ser importante para a tradución clínica; e 2) o uso de vehículos de administración que protexan o ARNm da degradación e permitan a súa administración intracelular. Esta estratexia, en esencia, incorporou os coñecementos que os investigadores estiveron aplicando a fármacos tradicionais e biomoléculas ao longo dos anos, onde a nanomedicina desempeñou un papel vital. O termo nanomedicina descríbese como o conxunto de formulacións a escala nanométrica (ou nanoformulaciones) compostas de materiais orgánicos como lípidos ou polímeros, ou materiais inorgánicos como metais, que poden usarse para administrar moléculas ou biomoléculas, por exemplo, proteínas, péptidos ou ácidos nucleicos. Estes nanomedicamentos poden utilizarse con fins terapéuticos ou de diagnóstico.

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O mecanismo de acción dos nanomedicamentos de ARNm dentro da célula está descrito como un proceso arduo, con varios pasos que poden dificultar a entrega eficiente de ARNm ao seu sitio de acción, o citoplasma. Como primeiro contacto, as nanopartículas interactúan cos receptores, nun proceso coñecido como endocitose. De feito, describíronse varios tipos de endocitose e, ademais, diferentes nanomedicamentos poden seguir diferentes vías endocíticas en función das súas propiedades fisicoquímicas ou materiais, o que determina o seu rendemento. O tipo de célula diana tamén inflúe nos mecanismos de endocitose e, en efecto, este feito relacionouse co mecanismo de acción das nanopartículas de ARNm que se dirixen ao fígado. Unha vez que se produciu a interacción, as nanopartículas agora sitúanse no endosoma, un ambiente altamente ácido que se describiu como o principal factor limitante para a liberación eficiente de ácidos nucleicos. Unha vez aquí, as nanopartículas capaces de alterar a membrana endosomal liberarían eficientemente o ARNm ao citosol, nun proceso coñecido como escape endosomal, onde estaría listo para ser traducido a proteínas polos ribosomas. O escape endosomal, con todo, foi descrito como un proceso pouco eficiente e, de feito, unha importante cantidade de investigacións encamiñadas a atopar nanomedicinas máis eficientes céntranse na investigación deste proceso. De feito, crese que só unha pequena porcentaxe da carga se libera ao citoplasma, xa que moitos endosomas serían transportados a endosomas tardíos e posteriormente a lisosomas, onde serán degradados ou exocitados.

Específicamente para a entrega de xenes, todas as investigacións realizadas nos últimos 30 anos deron como resultado plataformas de entrega seguras e efectivas para ácidos nucleicos, como oligonucleótidos antisentido (ASO), pequenos ARN de interferencia (siRNA) ou ADN, mediante o uso de diferentes tipos de sistemas de entrega, nanopartículas tanto de base viral como sintéticas. Por exemplo, o desenvolvemento e posterior comercialización da lipofectamina en 1989 impulsou o desenvolvemento de vectores non virais, así como a comercialización de Doxil® en 1995 e Abraxane® en 2005 supuxeron un gran impulso no campo da nanomedicina clínica. No campo do ARNm, o descubrimento da inmunoxenicidade diminuída do ARNm modificado pola Dra. Katalin Karikó fundou a base da investigación que logo permitiría a terapéutica do ARNm, dando solución aos estudos iniciais que reportaban a inmunoxenicidade do ARNm en 1963. De feito, a Dra. Karikó e o Dr. Weismann recibiron o Premio Nobel de Fisioloxía e Medicina en 2023 por este descubrimento.

Con todo, as terapias de ARNm non chegaron ao mercado ata a aprobación das vacinas COVID-19 en 2020. Varios vehículos de administración, como lipoplexos, liposomas ou nanoemulsións catiónicas, foron obxecto de estudos desde o descubrimento do ARNm, con todo, a tradución clínica dos mesmos seguía sendo difícil. De feito, a comercialización das vacinas contra a COVID-19 foi posible grazas ao uso de nanopartículas lipídicas (LNP), comercializadas anteriormente con Onpattro® en 2018, e segue sendo un fito tanto no campo do ARNm como na nanomedicina, ao abrir novas

posibilidades para futuras terapias. A partir de 2024, a FDA e a EMA aprobaron unha terceira vacina baseada en ARNm para o virus sincitial respiratorio (VRS), o que destaca o rápido avance destas terapias.

Sorprendentemente, todos estes medicamentos de ARNm comercializados, así como a maioría no ámbito do desenvolvemento clínico, formúlanse empregando nanopartículas lipídicas (LNP). Orixinadas a partir de liposomas, estas partículas prevalecen sobre os enfoques tradicionais que implican o uso de lípidos catiónicos (é dicir, lípidos cargados positivamente a calquera pH) para acomplexar ácidos nucleicos por medio de interaccións electrostáticas, pero que abarcaban problemas de estabilidade e eficacia. As LNP baséanse no uso de lípidos ionizables cargados positivamente só a pH ácido permitindo, así, a formación de partículas neutras que encapsulan o ARNm dentro do seu núcleo, o que permite tempos de circulación en sangue máis prolongados, maior escape endosomal e, en definitiva, maior eficacia. De feito, desde o seu descubrimento realizáronse moitas investigacións, buscando novas estruturas e materiais.

Con todo, aínda quedan importantes desafíos por diante. As LNP máis empregadas na actualidade están compostas por un lípido ionizable, lípidos auxiliares e un derivado de PEG. O lípido ionizable demostrou ser o principal lípido impulsor de potencia, sendo o elemento clave nas LNP. Os lípidos auxiliares, é dicir, os fosfolípidos e o colesterol, proporcionan funcionalidades estruturais. De feito, o colesterol ou os derivados de esterois utilízanse para proporcionar rixidez, de forma similar ao que fai o colesterol nas membranas celulares. Os fosfolípidos de uso común, como DSPC ou DOPE, contribúen ás disposicións estruturais e descríbese que a súa estrutura tamén contribúe ao escape endosomal. De maneira similar, inclúese un lípido derivado de PEG para proporcionar estabilidade coloidal e aumentar a vida media en circulación. De feito, demostrouse que a eliminación total deste polímero diminúe a estabilidade das LNP.

Por tanto, a investigación de novos compoñentes podería proporcionar información para o desenvolvemento de partículas máis específicas, adaptadas ou dirixidas. Ademáis, comprender como a modulación dos devanditos compoñentes pode afectar aínda máis ás propiedades fisicoquímicas, a o rendemento *in vitro* e *in vivo*, á biodistribución e á liberación funcional permitiría o desenvolvemento de novas vías terapéuticas. Finalmente, a substitución dos derivados de PEG por alternativas máis biocompatibles xunto co descubrimento de novos materiais ionizables biodegradábeis outorgaría perfís de seguridade máis altos, especialmente importantes para administracións repetidas.

As nanoemulsións de esfingomielina (SN, WO 2019/138139A1224), compostas por esfingomielina e vitamina E, investigáronse previamente no laboratorio no que se desenrolou parcialmente esta tese doutoral. Estes dous compoñentes demostraron a súa capacidade para autoensamblarse en

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nanoestructuras mediante un proceso de formulación dun só paso. Ademáis, os datos preliminares de escalado demostraron as posibilidades industriais destas nanopartículas. De feito, os esfingolípidos representan un dos principais tipos de lípidos nas células eucariotas e sábese que proporcionan características estruturais ás membranas. Ademáis, a súa estrutura común, a esfingosina, asociouse con actividades bioactivas como o crecemento celular e a modulación das interaccións entre células. A esfingomielina comercializouse anteriormente en Marqibo®, unha formulación liposomal de vincristina aprobada pola FDA en 2012. De maneira similar, a vitamina E explorouse como compoñente de administración de fármacos durante anos. É de destacar que as SNs demostraron resultados positivos na terapia xénica. Cando se modifican con DOTAP, estearilamina ou putrescina, as SN asocian e liberan de xeito eficiente pequenos ARN de interferencia (ARNip), microARN (miARN) e ADN plasmídico (ADNp) in vitro. Máis recentemente, os resultados in vivo confirmaron que as SN cargadas con pDNA terapéutico poden contribuír ao desenvolvemento de novas terapias xenéticas contra o cancro. Estes resultados son as bases deste traballo de tese.

A entrega restrinxida ás Células Dendríticas e a acumulación natural de nanopartículas no fígado, que tamén se estudarán nesta tese, podería ser unha das cuestións máis desafiantes e importantes de abordar, xa que evita o potencial desta aplicación a outros órganos. Este tema tamén será cuberto nesta tese, co estudo dos axustes de composición na biodistribución de nanopartículas.

Por último, explorarase o potencial terapéutico das nanoformulacións de ARNm deseñadas en dúas áreas terapéuticas diferentes de interese, que actualmente están a ser obxecto de numerosas investigacións, a oncoloxía, a través do desenvolvemento de novas inmunoterapias , e a medicina rexenerativa, para proporcionar ferramentas de reprogramación no tratamento de enfermidades cardiovasculares agudas.

En resumo, o deseño, desenvolvemento e caracterización completa destas nanopartículas preséntase nos Capítulos I e II. Tras avaliacións baseadas na composición e un estudo detallado das propiedades fisicoquímicas e o rendemento in vitro e in vivo, os resultados mostraron que o tamaño, a composición e a concentración das partículas impactan dramáticamente no rendemento da formulación. Ademáis, os nosos resultados indican que as nanopartículas desenvolvidas poden modularse en termos de composición e propiedades fisicoquímicas para modificar o tropismo aos órganos. Así mesmo, tamén se presenta o deseño de nanopartículas totalmente biodegradábeis, superando os principais inconvintes das LNP actuais e permitindo enfoques máis seguros e traslacionais.

No primeiro capítulo amósase o deseño e desenvolvemento dun novo tipo de nanopartículas, as SNP. Para iso, desenvolvéronse novas nanopartículas utilizando diferentes combinacións de lípidos e cargadas con ARNm de Firefly Luciferase (FLuc mRNA). Os experimentos de caracterización refírense

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á análise das propiedades coloidais, eficiencias de asociación e eficiencias de transfección in vitro e in vivo. Como referencia, tamén se formularon e probaron SN previamente desenvoltas polo grupo de investigación e eficientes para a asociación de ácidos nucleicos. Os resultados confirmaron a superioridade das novas formulacións de nanopartículas.

A continuación, axustouse a proporción de lípidos para comprender a influencia da composición dos lípidos nas propiedades fisicoquímicas e as eficiencias de transfección. Formuláronse e caracterizáronse completamente catro composicións diferentes de SNP (1-SNP, 2-SNP, 3-SNP, 4-SNP). As propiedades fisicoquímicas, a eficiencia de encapsulación, a estabilidade coloidal e a morfoloxía determináronse mediante dispersión dinámica de luz (DLS), análise de seguimento de nanopartículas (NTA), microscopía electrónica de transmisión (TEM) e cuantificación indirecta baseada en fluorescencia de ARNm, e amósanse na Figura 2. A internalización celular probouse en liñas celulares HEK293 e HEPG2, con nanopartículas marcadas con fluorescencia, ARNm marcado e dobre marcaxe (nanopartículas e ARNm), mediante microscopía confocal e citometría de fluxo. Non se observaron diferenzas entre os catro prototipos. A continuación, determináronse a eficacia da transfección in vitro e a citotoxicidad. En termos de expresión do ARNm de Fluc encapsulado (a bioluminiscencia mediuse 24 h despois da transfección), dúas das formulacións mostraron os resultados máis prometedores (3-SNP, 4-SNP), que ademáis eran tan potentes como o control positivo, LNP. formulado coa composición Onpattro® (ONP). Cando se encapsulou o ARNm codificando para a proteína fluorescente eGFP, a tradución eficiente da proteína confirmouse mediante microscopía confocal. En termos de citotoxicidade (os estudos levaronse a cabo mediante cuantificación directa da actividade da proteasa de células vivas), as células permaneceron completamente viables tras 24 e 48 horas de incubación coas nanopartículas, a diferenza do que se observou con MessengerMax (Invitrogen), o que subxace á compatibilidade celular dos SNP. A continuación, avaliáronse os SNP in vivo. A súa potencia, así como a administración funcional aos órganos principais (fígado, bazo, pulmóns, riles e epiplón) avaliáronse mediante cuantificación de bioluminiscencia (ph/s/cm2/sr) en ratos femia C57/BL6. Cuantificouse a potencia alcanzada despois de 6 h despois da administración de IV-SNP. Dacordo cos resultados de potencia in vitro, os 3-SNP e 4-SNP exhibiron unha maior produción de proteínas e foron comparables ao ONP de control positivo, o que sustenta as perspectivas positivas das formulacións de ARNm recentemente desenvolvidas. Nunha segunda análise experimental, 24 h despois da administración, aos ratos administróuselles luciferina, sacrificáronse e cuantificouse a bioluminiscencia de cada órgano. A porcentaxe de luminiscencia en cada órgano calculouse considerando a bioluminiscencia total do órgano. En comparación coa ONP, algúns SNP mostraron unha entrega preferente no bazo. Ademáis, o prototipo máis potente, os 3-SNP, comparouse coa composición de LNP de Spikevax® (Moderna, MOD) e JetPEI®, un reactivo de transfección de uso común para dosaxe in vivo. Os resultados

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mostraron un notable rendemento superior dos SNP en comparación con JetPEI®, aínda que non alcanzaron a potencia MOD.

En resumo, o primeiro capítulo desta tese profunda na descrición dun novo vehículo de entrega de ARNm. Ao estudar coidadosamente desde as propiedades coloidais ata o destino in vivo, este capítulo proporciona información sobre o deseño destas formulacións, abrindo novas portas para futuros traballos de desenvolvemento de formulacións.

No segundo capítulo, o noso obxectivo era dar unha resposta ás limitacións das tecnoloxías actuais. A necesidade de deseñar sistemas de administración biodegradábeis e máis biocompatibles, o uso de alternativas de PEG, así como o estudo e a comprensión da administración dirixida a órganos específicos foron obxecto de estudo neste capítulo.

Para o deseño de SNPs totalmente biodegradábeis realizouse a eliminación e substitución de PEG por un polímero hidrófilo biodegradable. Ademais, todos os lípidos incluídos na composición de nanopartículas, como se describe no Capítulo II, eran biodegradábeis. Isto supón unha gran vantaxe xa que os derivados de PEG foron descritos previamente como inmunogénicos tras a súa administración intravenosa, e aínda que se utilizaron amplamente durante moitos anos grazas á súa capacidade para prolongar o tempo de circulación e diminuír a opsonización, actualmente o campo está a evolucionar cara á limitación. o seu uso. Con todo, a súa eliminación completa asociouse cunha diminución da estabilidade, polo que os nosos resultados demostraron as posibilidades de atopar alternativas que manteñan a estabilidade e a potencia. As nanopartículas formuladas resultaron estables, homoxéneas e mostran actividade in vivo. Como era de esperar, estas substitucións conduciron a unha diminución da potencia, como se observou en experimentos de transfección in vitro e in vivo, posiblemente debido á rápida degradación tras a administración, segundo informes anteriores no campo. Por tanto, manter a potencia e ao mesmo tempo proporcionar características biodegradábeis seguiu sendo un desafío importante. Para superar esta limitación, incluíuse un lípido auxiliar na formulación para mellorar a potencia. De feito, a súa adición proporcionou unha formulación máis potente. Tamén se realizou o estudo da liberación funcional destas nanopartículas biodegradábeis, BD-SNP. En relación con formulacións anteriores, os BD-SNP mostraron un aumento na administración ao bazo.

Para estudar e modular a distribución de órganos, preparáronse, caracterizáronse e probáronse diferentes formulacións. Os estudos comparativos refírense á formulación de 4-SNP, como se describe no Capítulo I, e á inclusión dun lípido cargado positivamente (P-SNP) e un lípido cargado negativamente (N-SNP). Observouse un aumento na administración ao pulmón e ao bazo, respectivamente (Figura 3), o que revela a posibilidade de modular a biodistribución e definir formulacións melloradas para dirixirse a órganos específicos. Os mecanismos de acción deben explorarse máis a fondo e poden relacionarse

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coa funcionalidade específica dos lípidos liderados por estrutura e carga, así como coa disposición específica dos compoñentes nos SNP que modulan as propiedades da superficie e, polo tanto, características específicas como pode ser a formación da coroa proteica. Serían necesarios máis estudos para profundizar na comprensión destes resultados. En xeral, este capítulo proporciona datos sobre o desenvolvemento de SNP biodegradábeis, ademais de orientar o estudo da administración para dirección das SNP a órganos específicos.

No Capítulo III proponse o estudo da eficacia das nanopartículas nun modelo terapéutico relevante, centrándose nunha aplicación innovadora dentro da inmunooncología, que utiliza o fígado como fábrica de proteínas inmunomoduladoras.

Proponse o uso das SNP desenvolvidas para a administración de terapias inmunomoduladoras de interese en oncoloxía. Neste caso específico, as novas formulacións de nanopartículas, en particular 1-SNP e 3-SNP, cargáronse cun ARNm terapéutico que codifica unha proteína inmunomoduladora, ben coñecida pola súa capacidade para deter o crecemento de células tumorales pero con graves efectos secundarios tóxicos, como unha primeira proba de concepto. Ademáis, ao administrar proteínas inmunomoduladoras en forma de ARNm, pódense superar importantes inconveintes da terapia proteica, como unha vida media curta e procesos de fabricación de enxeñería difíciles e custosos. Neste contexto, o uso do fígado como fábrica de proteínas que poden segregarse ao torrente sanguíneo e exercer a súa acción terapéutica é un enfoque novo que actualmente se investiga en contornas preclínicos e clínicos. Como tal, os SNP cargados de ARNm administrados por vía intravenosa serían unha estratexia terapéutica viable para tratar tumores distales ou de difícil acceso (Figura 4).

Primeiro, realizouse un experimento cinético para proporcionar evidencia da secreción da proteína codificada ao torrente sanguíneo despois da administración de SNP. Tras a análise dos niveis de proteína sérica, seleccionáronse 3-SNP como a formulación máis prometedora, o que levou a niveis séricos de proteína aínda detectables 96 h despois da administración. Unha vez confirmada a produción exitosa da proteína codificada, analizouse a eficacia da terapia proposta nun modelo relevante de enfermidade: ratos portadores de tumor B16OVA. Tras dúas administracións de 10 μg de ARNm por rato os días 10 e 14 despois da inoculación do tumor e a monitorización do tamaño do tumor, o peso dos ratos e a supervivencia xeral, os resultados foron notables. Os 3-SNP cargados con ARNm foron capaces de reducir significativamente o tamaño do tumor con respecto aos controis (ratos tratados con PBS e nanopartículas cargadas cunha secuencia de ARNm simulada). Ademáis, o 50 % dos individuos experimentaron a remisión do tumor, o que se traduciu nunha maior supervivencia xeral no grupo de tratamento. Estes resultados resaltan o potencial dos SNP para a administración terapéutica de ARNm, con especial atención á inmunooncoloxía.

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No Capítulo IV estúdase o uso de nanopartículas biodegradábeis para a reprogramación de tecidos. Esta nova aplicación ten como obxectivo a reprogramación de tecidos utilizando ARNm que codifica diferentes factores de transcrición. Propuxemos o uso dos SNP biodegradábeis informados no Capítulo II. A reprogramación celular converteuse nunha estratexia importante para o tratamento de enfermidades dexenerativas. Ao sobreexpresar factores de transcrición, múltiples laboratorios demostraron que é posible reverter as células somáticas a un estado de pluripotencia. En base a isto e mediante o uso de ARNm e o estudo coidadoso das proporcións e doses dos factores, poderíase lograr potencialmente o rexuvenecemento das células. Con todo, realizáronse poucos traballos con ARNm e non se publicou ningún traballo que utilice tanto ARNm como administración de nanopartículas. En primeiro lugar, a entrega de ARNm de mCherry cargado en BD-SNP logrouse con éxito en fibroblastos e cardiomiocitos humanos. Logo, proporciónase evidencia da síntese efectiva de catro factores de transcrición diferentes relacionados coa reprogramación de secuencias de ARNm (TF1, TF2, TF3, TF4) e a capacidade de encapsulación de SNP, así como a eficacia de transfección de (TF1 e TF2) en fibroblastos humanos. Tamén se realizou un primeiro estudo piloto de reprogramación utilizando varios tratamentos e doses. Aínda que os resultados da reprogramación non foron positivos e necesitan máis optimización, unha lectura importante é que os SNP mostraron un gran perfil de biocompatibilidade, con células que permaneceron sas despois de 8 administracións no transcurso de 16 días, en contraste co observado para as células de control tratadas co produto comercial. reactivo de transfección Messenger Max®. Os próximos experimentos teñen como obxectivo profundar nas condicións de tratamento.

En resumo, esta tese mostra o deseño racional dun novo tipo de nanopartículas deseñadas especificamente para a entrega de ARNm, SNP, e explora o seu potencial para aplicacións no campo biomédico. Como tal, acentuamos a súa versatilidade ao brindar información sobre as proxeccións baseadas en composición e como estas poden impulsar o rendemento. Ademais, proporcionamos datos de eficacia sobre un modelo tumoral relevante e investigamos o seu potencial para o desenvolvemento do campo da medicina rexenerativa. En resumo, os datos presentados nesta tese permítennos concluír que 1) as SNP son unha plataforma de entrega válida e versátil que funciona cunha ampla gama de secuencias de ARNm; 2) A potencia das SNP, as propiedades fisicoquímicas, a entrega funcional e a biocompatibilidade pódense modular axustando a composición; 3) demostrouse o uso de SNPs para aplicacións de inmunooncoloxía, achegando datos de eficacia destacables, e 4) a capacidade dos SNP para transfectar fibroblastos e cardiomiocitos, así como para administrar factores de reprogramación, abre novas portas para aplicacións de rexeneración de tecidos.

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Introduction

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1. Prospective and limitations of mRNA

Messenger RNA (mRNA) is a large and negatively charged biomolecule whose main function is to translate the information encoded in the DNA. By being the direct precursor of proteins, mRNA's potential as a therapeutic has been investigated for years. mRNA first isolation was published in Nature in 1961 [2,3], starting to build up the path towards the full understanding of this molecule [4]. The potential to synthetically produce mRNA from DNA in the eighties, i.e., *In Vitro* Transcribed mRNA (IVT mRNA), opened up a broad spectrum of preclinical applications [5]. Since then, multiple efforts have been conducted to understand its mechanism of action and the path towards developing mRNA-based drugs. Nevertheless, it was not until 2020 that the development of such therapies was remarkably accelerated.

Therapeutic mRNA potential lies in its capacity to induce the expression of proteins [5,6] for preventing or altering a particular disease state. mRNA therapeutics possess many opportunities, which can depend on the targeted cells, organ selective accumulation and encoded protein of interest. However, up to the present day, two main strategies have been more extensively investigated , namely i) targeting of dendritic cells (DC) for immune activation, and ii) endogenous production of therapeutic proteins. This will be discussed more in detail in Section 4.

Importantly, mRNA presents several advantages over other gene therapies such as those using double stranded DNA (ds) or plasmid DNA (pDNA): i) mRNA does not need to reach the nucleus of the cell as opposed to DNA, therefore, the delivery is more efficient; ii) mRNA does not integrate into the genome of the host cell, a fact associated with risk of mutagenesis and, therefore, is considered to be a safer approach; iii) mRNA can be synthesized with fairly easy protocols, following scalable processes in agreement with GMP regulations, and iv) mRNA sequences can be easily modified and updated, an important fact to adapt its use for different diseases conditions, and specifically relevant in vaccinology (*i.e.,* when mutations of the target protein occur) or for personalized medicine (*i.e.,* cancer vaccines). These advantages are also important when comparing mRNA technology to protein delivery technology, which normally comes up with higher productions costs, short half-lives and expensive and tedious industrial processes [5–7].

However, despite all these important advantages, two major drawbacks had been holding up the clinical development of this technology: its high instability and its potential immunogenicity. On one hand, naked mRNA is prone to degradation by Ribonucleases (RNAases) present in extracellular fluids upon administration. Additionally, exogenous RNA molecules are recognized by Toll Like Receptors (TLR) and trigger immunological responses [8]. Besides, naked mRNA is not capable of crossing lipid

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bilayers to reach its target site. To overcome these limitations and to improve the pharmacokinetic and pharmacodynamic profile of naked mRNA, two main strategies have been proposed to date: the introduction of chemical modifications on the sequence and the use of a delivery vehicle.

2. Structural and chemical modifications of mRNA

From a chemical point of view, mRNA is a single strand biopolymer composed by nucleotide subunits linked by phosphodiester bonds. Each nucleotide is composed of a ribose sugar, a phosphate group, and a nitrogenous base (cytosine, C, guanine, G, adenine, A, and uracil, U). mRNA sequence has a 7'methyl guanosine residue (m7G) at the 5' cap and a poly(A) tail at 3' end. As other oligonucleotides, mRNA forms secondary structures thanks to the hydrogen bonding between complementary nucleotides, which is important for its stability [9]. Similarly, another promising technology, self-amplifying RNA (saRNA), is presented when the mRNA molecule is engineered so as to include an RNA virus genome [5], acquiring the capability to self-replicate and encode for multiple protein copies. This platform allows not only for the maintenance of the just mentioned mRNA advantages, but also shows a further benefit by requiring much lower doses [10]. For instance, its pharmacological activity can be maintained for up to two months, increasing the potency even more and reducing manufacturing costs of both excipients and RNA molecules, potentially allowing for even higher speed development processes. The fact that the saRNA molecule is longer, however, could result in a loss of stability and increased risk of immunogenicity [11]. A recently published review on saRNA vaccines further explains and comments the potential of these platforms [12].

As previously mentioned, one of the main advantages of mRNA is that it can be easily synthesized in the laboratory. This process can be performed following two main strategies. The first one, and very commonly used, is based on the *in vitro* transcription of a linearized pDNA. The second one is by making use of PCR, using a template with a bacteriophage promoter, a 5' UTR, an open reading frame (ORF), a 3' UTR and a poly[d(A/T)] sequence [6].

Furthermore, mRNA sequence can be subjected to chemical modifications to achieve an increased *in vivo* stability. Some reported modifications refer to changes in the m7G residue which would prevent the mRNA from being rapidly degraded by RNAases and its recognition via the immune system [13,14]. In this sense, recent advances in 5'cap analogues for enhancing mRNA expression include ARCAs (anti-reverse cap analogues), Beta-S-ARCAs, as well as the incorporation of a 2S analogue [15]. More recently, CleanCap has emerged as one of the most potent alternatives [16] . Other modifications to enhance stability are related to the incorporation of longer PolyA tails, which would lead to a change in the secondary

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structure of mRNA [17,18] . Recently, branched PolyA tails have been described as an enhancer in protein production [19] .

Importantly, substitutions using synthetic nucleotides have demonstrated reduced immunogenicity and improved *in vivo* potency. These include N1-methyladenosine or N6-methyladenosine instead of natural adenosine, cytidine with 5-methylcytidine or uridine with pseudouridine [20,21]. In fact, the latter work by Karikó and Weissman was awarded with Nobel Prize in 2023, recognizing their impact in COVID-19 vaccine development. Lastly, improvements in the purification processes and removal of secondary products from the *in vitro* transcription process have shown to reduce immunogenicity [22]. To extend information regarding the chemical modification of RNA, specialized reviews have been previously published [23,24].

3. Delivery vehicles for mRNA

3.1. Lipid Nanoparticles (LNPs)

Moderna (SpikeVax®) and BioNTech/Pfizer (Comirnaty®) mRNA vaccines were first marketed under emergency authorization in December 2020. Both vaccines were developed in less than a year, proving the potential of mRNA technology to be quickly adapted to emergency situations. These vaccines are composed of Sars-Cov-2 spike protein (S) [25,26] loaded into LNPs and they have not only meant a breakthrough in terms of design and manufacturing speed, but also in terms of efficacy. BioNTech/Pfizer vaccine exhibited a 95% efficacy in the phase 3 of clinical trials [27], lasting over a period of 6 months, irrespective of previous Sars-CoV-2 infections [28]. On its part, Moderna vaccine phase 3 clinical trial results showed an efficacy of 94,1% [29]. A published paper compared the immune response kinetics between mRNA vaccines with Janssen vaccine (of Adeno-Associated Virus, AAV), concluding that mRNA vaccines elicit a high initial peak antibody response which decreases after 8 months, while Janssen vaccine had stable but lower antibody levels over the same period of time [30]. Clinical data on vaccine safety and efficacy in children from 5 to 11 [31] and in adolescents [32,33] and the vaccination campaigns performed later proved the the application of this vaccine technology to the younger population.

All these achievements would have hardly been possible without nanotechnology, which can be defined as the production and application of structures, devices, and systems by controlling the shape and size of materials at a nanometer scale. In recent years, nanotechnology applied to medicine has been referred to as nanomedicine [34,35]. The term nanomedicine, thus, comprises formulations in the nanometer scale (*i.e.,* nanoformulations) composed of organic materials such as lipids or polymers, or inorganic

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materials such as metals, that can be used to deliver small molecules or biomolecules namely proteins, peptides or nucleic acids. Nanomedicine can be used for therapeutic purposes [36], but it also has an application in diagnostics and regenerative medicine [37]. With respect to the specific application of nanomedicine in gene delivery, all the research performed in the last 30 years has resulted in safe and effective delivery platforms for nucleic acids, such as antisense oligonucleotides (ASOs), small interfering RNAs (siRNA), DNA and, more recently, mRNA, using different types of strategies such as Gal-NAc conjugates, and different delivery vehicles such as AAV and more recently LNPs, as recently reviewed in this article [38]. Besides this great effort, at present, there is only one nanoformulation in the market for siRNA delivery, Onpattro®, although many others are undergoing clinical trials, expecting to see more genetic nanomedicines in the near future [38]. In this sense, the approval of Onpattro® for hereditary transthyretin-mediated amyloidosis (hATTR) in 2018 meant a huge breakthrough in nanoparticle design: LNPs, later used for the development of mRNA vaccines, were marketed for the first time. Like so, these nanoparticles have been key for the development of the two marketed COVID-19 vaccines and Respiratory Syncytial Virus (RSV, mRESVIA®) [39,40] vaccine in 2024, and are today the most advanced technology in mRNA delivery. The origin of LNPs lies in the discovery of liposomes, bilayer lipid structures which were firstly described in 1965 by Banghman, setting up the basis of model membrane systems [41]. A few years later, Gregoriadis *at al*. established the idea that these lipidic vesicles could be used for drug delivery purposes, opening a door towards a full development of the liposome field [42–44] and, later on, LNPs [45]. Other lipidic-based delivery vehicles have also been a matter of research, whose most recent advancements will be discussed in **Section 3.2**. The composition, structure, manufacture, and stability considerations of LNPs will be described in the following subsections.

3.1.1. Unraveling LNPs composition

Clinically used LNPs nowadays have a very well-established composition based on a mixture of lipids whose ratios, types and structures are carefully defined. Major constituents are i) ionizable lipids, ii) structural and helper lipids and iii) PEG-lipids.

For each of the vaccines, the detailed composition is described here. Comirnaty® (BioNTech/Pfizer) LNPs composition (ionizable lipid: phospholipid: cholesterol: PEG-lipid) is ALC-0315: DSPC: Chol: ALC-0159 (molar ratio % 46.3:9.4:42.7:1.6), with 30 μg of mRNA dose. On its part, Spikevax® and mRESVIA® (Moderna) composition is SM-102:DSPC:Chol:DMG-PEG (molar ratio % 50:10:38.5:1.5) with 100 μg [46] and 50 μg mRNA dose, respectively. A schematic representation of the composition of LNPs for both vaccines is shown in **Figure 1 and chemical structures of other lipidic components is shown in Figure 2**.

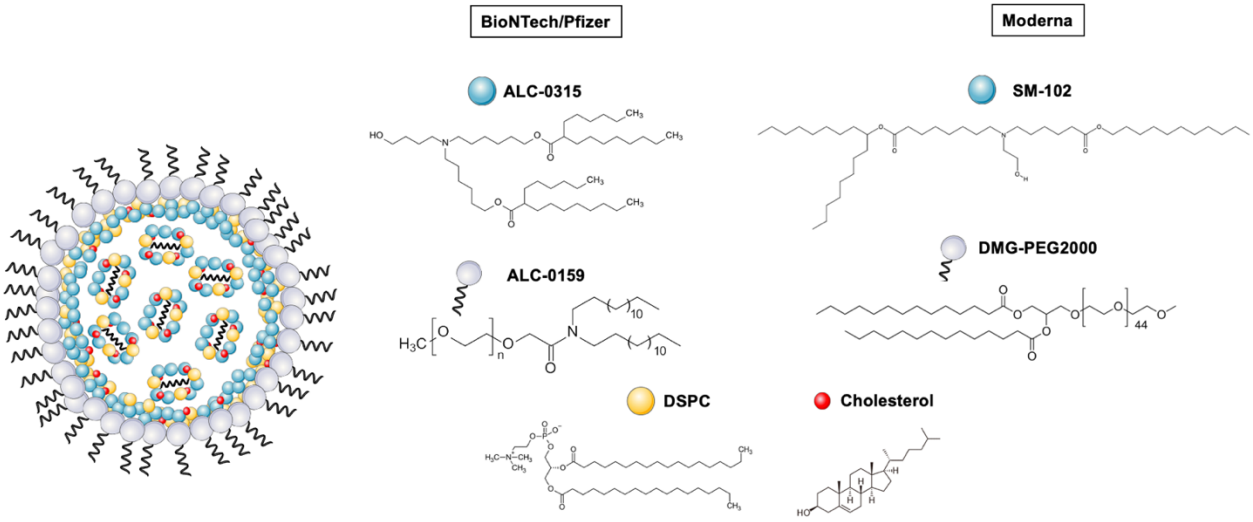


Figure 1. Structure and composition of the marketed mRNA formulations. This figure is the author's own design and was previously published[1]. Created with Medical Art.

Each of these constituents possess a crucial function in the structured nanoparticle, described in detail below.

i) Ionizable lipids are possibly the most innovative component of LNPs, having meant a huge breakthrough in RNA delivery [47]. Their mechanism of action is based on complexing the nucleic acid by electrostatic interactions. Several properties, such as the charge or lipid shape as well as the protein corona formation have been described as important factors to consider when understanding structure-activity relationship studies and, thus, the design of new ionizable lipids.

On behalf of the charge, prior to the use of ionizable lipids, permanently positive charged lipids, such as DOTAP, DOTMA or DDAB, were of preference. However, the positive surface charge contributed to low stability due to aggregation phenomena and had toxicity issues [48]. By contrast, today, the most commonly used lipids are ionizable lipids: pH-titratable lipids which are positively charged only at acidic pH, allowing the formation of complexes with nucleic acids during the formulation process but neutral at pH 7.4, overcoming the inconveniences of cationic lipids. In result, allowing for longer circulation times, increasing accumulation in targeted tissues, and promoting better tolerability [49–51]. Their use, together with the optimization of manufacturing processes, as described in **Section 3.1.2**., allows the mRNA to be encapsulated with high efficiency within the core of the nanoparticle – in contrast to which is reported for conventional cationic lipoplexes or cationic nanoemulsions, described in **Section 3.2.**

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The lipid shape, which comes hand in hand with the degree of the lipid chain's unsaturation and the type of ionizable lipid, also seems to play an important role in promoting endosomal escape. Heyes *et al*. proved that when increasing the number of double bonds, the efficiency in delivery increases dramatically due to the formation of H11 hexagonal phase in the particle that promotes fusogenicity with the cell membrane [52]. Semple *et al*. further discussed this by hypothesizing that, once in the endosome, lipids would get protonated thanks to the acidic environment. This in turn would facilitate the interaction with the anionic phospholipids of the endosomal membrane, disrupting it and liberating the cargo [52,53].

The design of new lipids has been evolving ever since the first proof of concept for ApoB gene silencing using DLinDMA lipid in hepatocytes was achieved [54]. This research led to the discovery of DLin-KC2-DMA, which demonstrated the increased *in vivo* activity at siRNA doses of 0.01 mg/kg in rodents and 0.1 mg/kg in non-human primates in silencing factor VII [53]. On its part, DLin-MC3-DMA (i.e., MC3) lipid, considered even today a gold standard for LNPs formulation development, was first discovered after carrying out a systematic screening to improve gene silencing *in vivo,* which also concluded that optimum pKa for hepatic delivery should stand between 6,2 and 6,5 [55]. This lipid would later be commercialized with Onpattro® in 2018 [56].

As opposed to cationic lipids, a specific uptake mechanism has been postulated for these ionizable lipids closely related to the formation of a protein corona enriched in Apolipoprotein E (ApoE), a well-known phenomenon driving nanoparticle's fate and biodistribution upon administration [57], as firstly disclosed by Akin *et al*. [58]. This work, which compared LNPs formulated with the ionizable lipid DLin-KC2-DMA (iLNPs) versus cationic lipids LNPs (cLNPs) refers to an ApoE dependent mechanism by which iLNPs would get efficiently endocytosed by hepatocytes via LDL receptor, mediating accumulation in this organ. Further studies suggested that the main mechanism driving the endocytosis for ionizable MC3-based LNPs were both clathrin-mediated endocytosis as well as macropinocytosis [59]. On this note, a publication on LNPs structure indicated that upon binding of MC3-based LNPs with ApoE, a rearrangement is induced in the structure of the nanoparticle, which could drive nanoparticle's fate [60]. More recently, other studies with mRNA showed that efficient LNPs-mRNA transfection could also rely on an early narrow endosomal escape window before lysosomal sequestration or exocytosis [61]. Furthermore, another study showed a linkage between exogenously administered mRNA and extracellular vesicles (EVs) after observing mRNA present in these EVs at 1:1 ratio, concluding that mRNA should be neutrally charged by the ionizable lipid to promote endosomal escape [62].

As a step forward in the lipid design, a subtype of ionizable lipids was presented in 2008 - commonly called lipidoids - with a dendrimer-like structure, [63] with the advantage of a straightforward

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synthesis protocol. Later, Dong *et al*. reported the synthesis of cKK-E12, a lipidoid capable of silencing hepatocytes with the highest potency in rats, mice and non-human primates reported until that point [64]. Research on new ionizable lipids has also been focused on obtaining more biocompatible and biodegradable lipids, classified as new-generation lipids. In this term, Maier *et al*. showed that including an ester bond in the alkyl chains improved tolerability and retained potency, as compared to MC3 [65]. Other studies have also reported better cell viability *in vitro* by including disulfide bonds [66], which led to Akita *et al*. to firstly synthesize ssPalmE, a lipid which incorporates a tocopherol-like moiety in its structure [67]. A complete review on ionizable lipid types and structure can be found here [47].

ii) Structural and helper lipids are also constituents of LNPs, playing an important role in terms of stability and fusogenicity [68]. These lipids are mainly phospholipids (such as DOPE, DSPC, DEPC, DSPE) and cholesterol, which forms the main skeleton of LNPs and aid in endosomal escape. Phospholipids play key role possibly due to their nature mimicking biological membranes. However, not all phospholipids seem to be equally efficient. It has been reported that the type of structures formed by phospholipids is an important factor driving fusogenicity. In fact, it seems that lipid species such as unsaturated phosphatidylethanolamines (PEs) adopt non-bilayer structures, which, in turn are less stable, driving the disruption of the LNPs and inducing fusion with cell membrane and the consequent release of the cargo inside the endosome [53,69]. Supporting such, this newer study found the PE-derived phospholipids improved mRNA delivery as compared to other backbones [70] . Therefore, the chemical structure of phospholipids seems crucial to improve endosomal escape too. When *in vivo* biodistribution is considered, helper lipid structure also seems to play a major role. In this regard, this study aimed to systematically screen a library of 96 LNPs compositions, finding that DOPE containing LNPs preferentially accumulated in the liver, as opposed to the DSPC containing LNPs. Furthermore, authors reported that DOPE containing LNPs also exhibited higher ApoE binding affinity, also correlated to higher liver protein expression [71]. Other studies have looked into how phospholipid charge can modify LNPs fate, reporting for the first-time lung and spleen protein production in a strategy called Selective Organ Targeting, 'SORT', studies that will be in detail reviewed in Chapter 2. [72–74]

On the other hand, cholesterol has been shown to give stability to LNPs and improve efficacy by enhancing membrane fusion [51] Patel. *et al*. have also investigated different cholesterol analogues and their function in enhancing intracellular delivery of mRNA, concluding that the type and flexibility of the alkyl tail present in the sterol ring can affect transfection efficiency [75]. More recently, other sterol substitutions such as hydroxycholesterol [76], cationic-cholesterol [77] or bile-acids [78] have been reported to impact mRNA delivery.

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iii) PEG-Lipids. Polyethilenglycol (PEG) is a widely known polymer that has been described and previously used in liposomes and other types of nanoformulations. It seems to play a main role in prolonging circulation time by reducing Macrophage Phagocytic System (MPS) interactions, improving colloidal stability, and preventing the formation of the protein corona[52,79,80]. PEGylated lipids typically included in these formulations consist of two domains, a hydrophilic PEG polymer conjugated to a hydrophobic lipid branch. In LNPs, the hydrophilic domain is located towards the exterior surface of the particle and the lipid branch towards the interior. Additionally, LNPs properties can be tuned by modulating PEG-lipid to total lipid content ratio. For instance, it is known that smaller LNPs are generated at higher PEG-lipid ratios [81] , a matter that is further described in **Section 3.1.3.** PEG-lipids can additionally balance half-life and cellular uptake of LNPs. [53] Like so, the PEG amount is commonly maintained to 1,5 mol% of the total lipid content, after Semple *et al*. reported a 5-fold potency improvement when decreasing PEG content from 10% to 1,5% [53]. Considering that PEG can also affect cell-nanoparticle interactions by preventing the close approach between the two and impede endosomal escape by preventing fusion [82], some studies suggest that getting PEG lipids to diffuse from the LNPs upon administration would allow for intracellular delivery promotion [83]. Currently, the field aims at substituting PEG-lipids for more biocompatible alternatives, as discussed in Chapter 2 of this thesis.

Apart from the above-described study where DOPE and DSPC LNPs formulations are compared [71], another important study was recently published in Nature about Selective Organ Targeting of LNPs, SORT [72]. In this study, authors conclude that it is possible to include additional lipids to tune LNPs base composition and, thus, modify their surface charge to target specific organs such as the spleen and lungs. Like so, they performed several experiments with different ionizable lipids LNPs (5A2-SC8, MC3 and C12-200) which they tuned with positive or anionic lipids. Results showed that increased amounts of DOTAP achieved an increase in protein expression in the spleen and lungs; and results were reproduced when other cationic lipids were used. On its part, negative lipids drove the protein expression to the spleen. Furthermore, authors described the enhancement in liver delivery when supplementing established LNPs with more ionizable lipid [72]. Recent studies suggest the formation of a different protein corona when different components and ratios are used, and thus, redirecting LNPs to different organs. [72–74,84,85] , as commented in Chapter 2.

Similarly, it has also been described that mannosylation of LNPs may enhance the uptake of nanoparticles by Antigen Presenting Cells (APCs). By incorporating a mannose-cholesterol amine conjugate to LNPs an enhanced *in vitro* uptake in dendritic cells was observed as compared to non-decorated LNPs, a fact that translates into a faster onset of the antibody response upon administration by both intradermal (i.d.) and intramuscular (i.m.) routes [86]. Furthermore, authors further investigated

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how the length of this surface LNPs decoration may influence antibody response, observing that vaccine priming response was improved with a higher chain length [87].

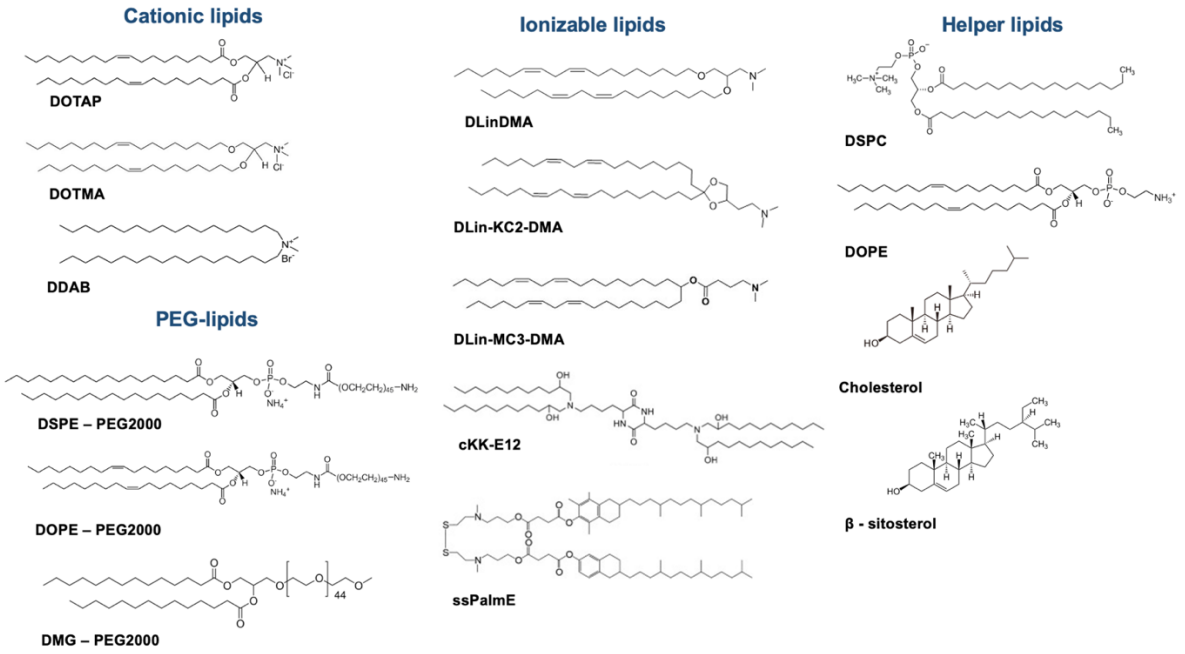


Figure 2. Chemical Structure of some LNP components. This figure is the author's own design and was previously published[1].

3.1.2. Manufacturing processes for LNPs

The production of LNPs is based on the ethanol dilution technique first described in the early 2000's. This method consists of the lipid dissolution in an ethanolic phase followed by rapid mixing, using a T tube mixer, with an aqueous buffer solution containing the nucleic acid at acidic pH [50]. As these two solutions are mixed, the ionizable lipid becomes positively charged and complexes with the negatively charged nucleic acid, at the same time as the overall lipid solubility is reduced by the dilution of ethanol. This forms unstable LNPs, in terms of size, which will then need pH adjustment to raise the value to physiological pH, normally achieved by dialysis processes [49,50]. The use of continuous microfluidics has revolutionized the bench-top formulation and manufacturing process of LNPs, allowing for the accurate formation of LNPs with defined sizes, by altering and optimizing the mentioned parameters as well as reducing manufacturing time [88]. This technique involves the mixture of the lipids with the aqueous phase using a micromixer, being likewise highly reproducible, scalable, and robust [88,89]. Recently, manufacturing considerations for the production of LNPs using both cationic and ionizable lipids have been extensively reviewed in this article [90].

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When considering bench-top formulations, important factors need to be considered to obtain LNPs with desired characteristics. In a recent study, Terada *et al*. investigated the effect of bench-top siRNA - LNPs preparation parameters, *i.e.,* total lipid content, flow rate ratio, and total flow rate, by systematically comparing formulations using the Design of Experiments (DoE) platform, providing a statistically relevant study [91]. Conclusions were driven with respect to the lipid concentration and flow rate ratio, stating that these two factors might be critical for size dispersion when formulating and manufacturing LNPs. Furthermore, this study points to the fact that the presence of the nucleic acids, in this case siRNA, dramatically impacts LNPs behavior within the whole formulation process as compared to empty LNPs [91], also in line with the conclusions driven by Sebastiani *et al*. [60]. This issue will be further described in next **Section 3.1.3.**

3.1.3. Disclosing LNPs structure

LNPs structure has not been fully deconvolved yet, being still a matter of debate. In fact, it seems clear that one structure does not fit all LNPs composition, and that, in fact, the chemistry of the ionizable, helpers and PEG-lipids highly influences the overall structure [46]. Nevertheless, two main general structures have been proposed, explained in this section, and disclosed in **Figure 3 (A and B)**.

First studies on LNPs structure deconvolution have been conducted mainly for siRNA loaded LNPs, and more recently, several studies also report the structural organization of mRNA loaded LNPs. Various studies have been carried out since Leung *et al*. firstly proposed that LNPs have an electron-dense core in which the nucleic acid would be located inside inverted micelles [92]. According to this hypothesis, the initial association of the ionizable lipid with the nucleic acid would form such types of micelles, which in turn would associate with more empty micelles, altogether structuring the core. This core would then be coated with polar lipids, *i.e.,* DSPC and PEG-lipids [92]. Later, other studies suggested that components would be located forming a layered structure, where the phospholipids and the PEG-lipids would be located at the surface, and the cholesterol and ionizable lipids would form the core of the nanoparticle; reporting differences in the structure depending on the cargo [93]. Subsequently, experiments using cryo-TEM and Small Angle X-Ray Scattering (SAXR) approaches suggested that LNPs would be formed upon fusion of smaller particles occurring after the rapid mixing [94]. Later, the same authors performed experiments with larger cargoes such as pDNA, mRNA and gold nanoparticles [81]. Results obtained supported the initial idea that the formation of LNPs would happen through fusion of smaller particles in a pH-dependent manner after the rapid mixing process, and that this fusion would be highly dependent on the amount of PEG-lipid accumulated in the surface. Thus, PEG content would dictate LNPs size, with a higher PEG content leading to smaller LNPs [81] Recently, the same group has

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published a study hypothesizing that the entrapment of siRNA into LNPs occurs through inversion of preformed vesicles [95].

Similarly, studies performed with mRNA formulations firstly suggested that the mRNA would be located inside water cylinders surrounded by ionizable lipids [96]. In a later study using MC3 LNPs, authors concluded that the shell of the particle would be indeed enriched with DSPC, MC3 and cholesterol, the latter being mainly present here. The core would be composed of MC3, where it would be present in double amount as compared to the shell [60]. In the same study, LNPs and ApoE interactions analysis concluded that this protein corona could mediate an irreversible rearrangement of components, affecting LNPs fate and protein expression efficacy [60]. Furthermore, recent evidence showed that cholesterol analogues can also change LNPs structure, by switching spherical particles to polyhedral [97]. Another study using Cryo-TEM techniques described the mRNA to be located in the outer edges of the particles, between layers of lipids, as well as in aqueous compartments surrounded by bilayer structures[98].

Despite these recent advancements and hypothesis, more studies are still needed to better understand the structural organization of LNPs. In this regard, a study performed by Blakney *et al*. [99] aimed to describe how the association strategy of the nucleic acid to the LNPs would dictate LNPs colloidal properties, *in vitro* and *in vivo* behavior. To do so, authors compared two different approaches: encapsulation and complexation (*i.e*., interior vs exterior RNA localization). For this purpose, they used two different types of LNPs: LNPs incorporating a cationic lipid (cLNPs, DDA or DOTAP) or an ionizable lipid (iLNPs, C12-200). In terms of physicochemical characterization, results showed that saRNA localization did not seem to dictate colloidal properties, *i.e*., no difference was seen when comparing LNPs of same composition but different saRNA localization. In regards to the transfection efficiency, authors showed that, for iLNPs, it was higher when the RNA was encapsulated; as opposed to cLNPs, when higher transfection rates were achieved when the RNA was complexed [99]. This study is important as it gives an alternative approach to the paradigm of encapsulation RNA for efficient delivery. The possibility of complexing the saRNA onto the LNPs' surface could have the potential advantage of having a ready-to-use blank formulation for association of RNA when needed, allowing shorter RNA working times, easy updating of the sequence, and so on.

3.1.4. Stability considerations

Undoubtedly, one of the biggest challenges when it comes to mRNA formulation is the stability of the biomolecule. As mentioned before, mRNA is extremely unstable and needs to be stored at -80ºC. The importance of stability lies in the fact that even a small change in the structure can stop mRNA

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translation into proteins. Physical instability comprises denaturation and the loss of secondary structure, which is related to aggregation and precipitation and linked to poor translation efficiency [46]. However, according to Pogocki *et al*., the main stability issue of mRNA is chemical degradation, which occurs via oxidation and hydrolysis via the phosphodiester bonds that form the backbone of the molecule. *In vivo*, there is a major challenge due to the presence of RNAses, converting protection of mRNA in a key issue to guarantee its efficacy[9].

From a pharmaceutical perspective, achieving a formulation that can be stored at higher temperatures while preserving the biomolecule integrity is in fact one of the big challenges. On this behalf, studies evaluating pH, temperature and lyophilization techniques had been conducted in the past with siRNA containing LNPs, showing that when stored in aqueous media, refrigeration kept LNPs stable the longest time, as compared to -20ºC or Room Temperature (RT). Interestingly, pH seemed to not be a factor influencing stability. Remarkably, authors show that stability was protected upon freeze-thaw cycles when used cryoprotectants such as sucrose or trehalose [100], two excipients used in the marketed vaccines. In this regard, more recently, a novel class of mRNA reactivity that leads to loss of activity has been reported: the formation of mRNA-LNP adducts through the covalent union between some reactive lipids and the nucleobase [101] . It is also worth noting that mRNA could potentially have more stability issues than siRNA as it is a longer and more complex molecule. Indeed, Onpattro® siRNA LNPs formulation has a shelf life of three years at 4ºC [102] while the BioNTech/Pfizer vaccine is reported in the technical documents to be stable just for 6 months between -90ºC and -60ºC [26]. However, Pfizer and BioNTech submitted data on increased stability, proposing that the vaccine can be stored at -25ºC to -15ºC, and later on refrigerator storage up to one month once unfrozen. On the other hand, the Moderna vaccine can be stored frozen for up to 7 months and it is stable in the refrigerator for up to 3 months. A review about stability considerations has been published [103].

3.2. Alternative lipid-based delivery vehicles for mRNA

As introduced in **Section 3,** LNPs are today the most researched delivery vehicle for mRNA delivery. Nevertheless, LNPs are not the only vehicle under investigation, and it is worth noting the importance of others. Like so, both lipids and polymer materials hold tremendous potential, especially because it seems apparent that the idea of having one delivery system that fits all kinds of nucleic acids is no longer accepted. As such, polymeric nanoparticles and materials such as polyethyleneimine, polyacrylates, polyesters, and polyamines among others, are currently evolving as an alternative for lipid carriers. Nonetheless, this review will be focusing only on the newest advances in lipidic nanoformulations, leaving aside polymeric materials as it has been extensively covered in recent reviews[104,105].

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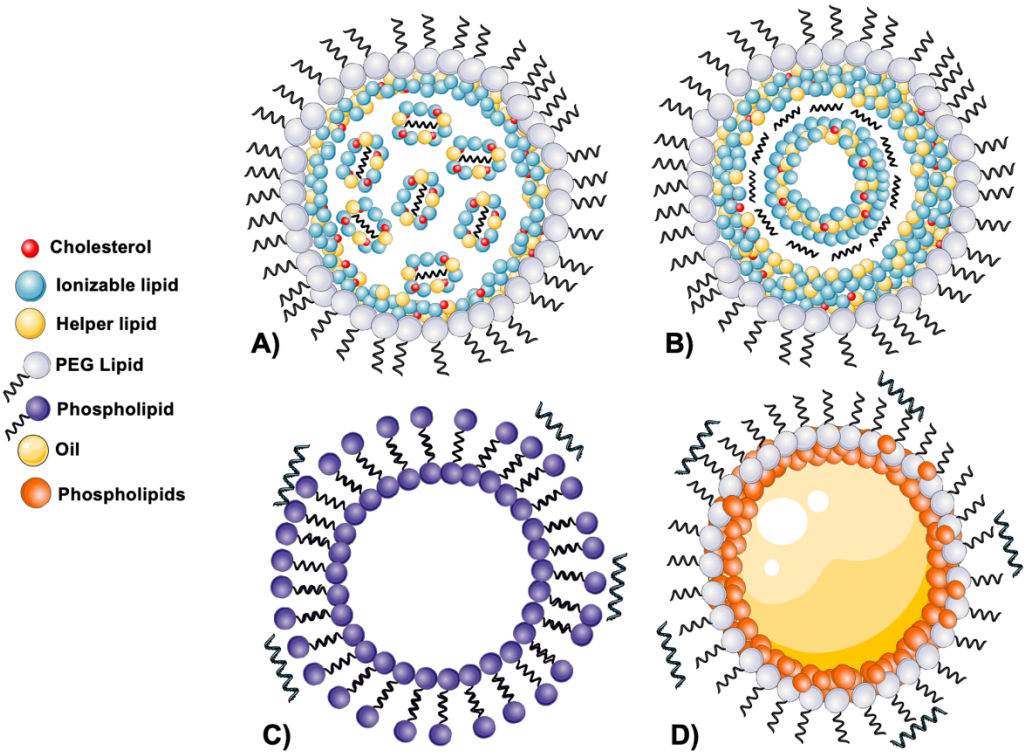


Figure 3. Lipid delivery vehicles for mRNA delivery. A) Lipid Nanoparticles (LNPs), structure proposed [94] B) Lipid Nanoparticles (LNPs), structured proposed [93] C) Lipoplexes (LPX) and D) Nanoemulsions (NEs). This figure is the author's own design and was previously published[1]. Created with Medical Art.

3.2.1 Lipoplexes (LPX)

It was in the 70's when Dimitriadis *et al*. managed to efficiently deliver encoded rabbit globin mRNA to mouse spleen lymphocytes for the first time, using large unilamellar liposomes [106]. At the same time, Dray *et al*. published results on protein translation after treating HEP-2 cells with mRNA encapsulated in liposomes [107]. However, due to the physicochemical characteristics of nucleic acids, traditional approaches used for small molecule encapsulation in response to pH changes had not been successful [50]. As mentioned in **Section 3.1**, LNPs evolved from liposomes; however, also using approaches relying on the use of complexing agents, different delivery vehicles emerged, and are typically disclosed as lipoplexes (LPX, Figure 3.C.).

Regarding the composition of LPX, most commonly described materials include several lipids such as EPC, DOPE, DOPC, DSPC and cholesterol as well as complexing agents such as cationic lipids like DOTAP, DOTMA, DC-Cholesterol; ionizable lipids such as MC3 or C12-200 or other positively charged peptides or polymers. Formulation methods described in the literature include the traditional approach of hydration lipid-film [108], pre-complexation of the cationic component with the nucleic acid

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[109,110] and the simple mixture of lipids dissolved in ethanol with an aqueous phase containing the nucleic acid [111,112].

As mentioned, the use of complexing agents to associate the mRNA to the nanocarrier is a commonly used strategy and it normally involves cationic lipids. For instance, Sahin *et al*. showed in 2018 that intravenously (IV) administered DOTMA/DOPE LPX formulation was capable of triggering INF-γ and T-cell memory upon administration of antigen encoding mRNA [113]. On the same note, DC-Cholesterol/DOPE LPX have been studied for α-1-antitrypsin encoding mRNA protein production, showing minimal animal toxicity [114]. Van Hoecke *et al*. also used cationic LPX to study how the route of administration could impact T-cell activation [115]. More recently, Salomon *et al*. carried out a study with LPX containing DOTMA and DOPE, showing the potential of CD4 neoantigen vaccine in increasing the antitumor activity of local radiotherapy in mice [116]. Regarding Sars-Cov-2, vaccine investigation using LPX has also been carried out. In a very recent study [117], the potential approach of using cholesterol/DOTAP LPX was evaluated preclinically resulting in a 30 μg mRNA dose, achieving immune activation after three doses. Interestingly, some studies have also been conducted for cationic LPX for other routes of administration other than IV and IM. Dhaliwal *et al*. have shown the potential of DPPC/DOTAP/cholesterol LPX for mRNA delivery to the brain via intranasal (IN) administration. Results showed dose-dependent and region-specific distribution of mRNA when LPX are used, as opposed to naked mRNA administration [118].

Despite the fact that cationic lipids usage is still a main approach when formulating LPX, another approach has been described by Zhang *et al*. [109]. This strategy involves the use of a positively charged polymer, *i.e.,* protamine, which complexes the nucleic acid prior to the formation of the nanoparticle. In this study, they managed to efficiently deliver T34A mRNA locally and systemically, improving the results on their DNA counterparts and also with a better safety profile on colon cancer gene therapy [109]. The same team has also proposed the use of this delivery vehicle for immunotherapy when they managed to deliver IL-15 cytokine mRNA *in vitro* and *in vivo* [110]. Later on, they also reported the use of this strategy to efficiently deliver a neoantigen mRNA vaccine using a positively charged peptide as complexing agent, *i.e.,* DP-7 [119]. More recently, LPX using KALA peptide as a complexing agent have also been reported [108] further evidencing the fact that other complexing agents can be used.

An interesting study regarding the complexing lipid type and localization, aimed to characterize how this component affects the cellular uptake and mRNA expression in different cell lines *in vitro* and after intradermal (ID) administration. For this purpose, they used LPX composed of cholesterol, DOPE and different ionizable (C12-200, MC3), cationic (DDA, DOTAP) and zwitterionic (cephalin) lipids.

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The most relevant conclusion was that the resulting overall LPX surface charge was the most important predictive factor for efficient protein expression [111].

Apart from the complexing agent modification, tuning the composition of LPX has also been studied. For instance, Langguth *et al*. published results on how EPC/DOPC/DOTAP LPX could be further modified to achieve controlled release effects [120]. Furthermore, similarly to what Goswami *et al*. [86,87] have studied for LNPs, and previously commented in **Section 3.1.1.**, the development of mannosylation strategies through the incorporation of mannose modified lipids has also been proposed for LPX for specific targeting to macrophages, achieving positive results in the induction of a stimulatory immune response upon mice vaccination [121,122].

3.2.2. Nanoemulsions (NEs)

A different type of lipid nanoformulations that are currently under preclinical and clinical investigation are cationic nanoemulsion (NEs), represented in Figure 3.D. These delivery vehicles have been extensively used in nanomedicine for different kinds of drugs and applications thanks to their versatility and ability to encapsulate different types of therapeutic molecules [123–125]. Emulsions are by definition a mixture of two immiscible phases that are stabilized by an emulsifier or surfactant, normally added to the continuous or external phase to stabilize the internal phase. Depending on the nature of the phases, emulsions can be categorized as oil in water (O/W) or water in oil (W/O) emulsions. Furthermore, emulsions can be classified depending on their droplet size as coarse emulsions or nanoemulsions, and in fact, there has been some debate on the nomenclature when considering nanoemulsions or microemulsions [126]. In this term, nanoemulsions are exclusively formed by spherical nanosized oil droplets, however they are not thermodynamically stable as microemulsions are [127]. Although microemulsions can exist in the nanosized ranges [127], we will refer to nanoemulsions here when considering only kinetically nanosized emulsions.

When considering formulation methods, NEs can be obtained by different methods such as high-pressure homogenization, microfluidization or sonication. The technique employed in the manufacturing process as well as the type of lipids and formulation parameters have a huge impact on physicochemical properties of the final formulations [123,124]. Undoubtedly, the simplest approach to formulate these nanoemulsions is the ethanol injection method [128] . Specifically for gene delivery purposes, NEs usually contain a hydrophobic oily core surrounded by phospholipids and other surfactants; and similarly to what was proposed for LPX, NEs typically contain a cationic component which complexes the nucleic acid. On this behalf, recent modelling of a siRNA loaded nanoemulsion has been performed, finding it had a compact shape, in which the hydrophobic domains of the phospholipids and the oil are placed in

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the core, while the polar groups are forming a hydrophilic shield on the exterior of the particle. Moreover, ethanol molecules would be placed throughout the system; not contributing to the nanoemulsion's size [129]. The siRNA would be attached to the nanoemulsion via non-covalent interactions with the cationic lipid. Interestingly, the physical radius of the nanoemulsion did not increase after the addition of the siRNA [129]. Apart from siRNA [130], NEs have been proposed for pDNA [131–133], miRNA [134], and, of the interest of this review, saRNA delivery [135]. More recently, a new type of nanoemulsion containing ionizable lipids has also been disclosed, as well as its proposed use for delivery of mRNA to the brain [136]. In this work, authors incorporated C12-200 to DOPE and Vitamin E containing nanoemulsions. Interestingly, their results showed improved diffusivity through the brain as compared to LNPs .

The major advancements when considering preclinical and clinical development for nanoemulsion technology has been achieved with saRNA [135,137]. The same formulation, composed of Tween80, Span 85, DOTAP and squalene which initially showed prolonged levels of saRNA protein expression [135], was chosen to be preclinically tested in different animal models and for different applications. Notably, this formulation provided the first evidence in a non-human primate specie that HIV vaccination with saRNA technology is safe and immunogenic [138]. Later on, Brazzoli *et al*. studied the potential application of this formulation against influenza virus, demonstrating good immunogenicity in ferrets and mice, exhibiting both T-cell and antibody responses [139]. Furthermore, it was also investigated for Venezuelan equine encephalitis virus (VEEV)[140] and rabies virus [141]. A recent study described the development of a Zika virus vaccine using this same technology and comparing two different vaccine candidates, both elicited potent neutralizing antibody responses in mice and non-human primates [142].

As it can be acknowledged by the information given so far, several delivery strategies are being proposed for RNA delivery. However, until recently, no study was conducted to make a head-to-head comparison of different formulations. A published study by Blakney *et al*. evaluated how the delivery vehicle might impact saRNA protein expression and vaccine immunogenicity [143]. To do so, authors compared pABOL, a polyplex formulation, which has previously shown its potential in saRNA delivery [144], with several LNPs compositions, varying ionizable lipids and phospholipids (DOPE vs DSPC). Formulation characterization results showed no major differences between colloidal properties and encapsulation efficiency, irrespective of their composition. The main difference was the surface charge, positive for pABOL and neutral for LNPs. However, when *in vivo* studies were performed, results showed an increased protein expression upon IM administration with the pABOL formulation. Within LNPs formulations, DOPE containing nanoparticles showed higher protein expression. Interestingly, when vaccine immunogenicity was investigated, LNPs exhibited higher IgG titers after the second shot, despite pABOL exhibiting higher protein expression levels upon IM administration. Higher antibody

titers were translated to higher protection against influenza virus with LNPs too. When routes of administration were compared (IM vs IN), IM administration of 1 ng of saRNA LNPs exhibited similar antibody titers to 1 μg of saRNA formulated in pABOL nanoparticles. An important factor describing vaccine immunogenicity is the capacity to achieve mucosal antibodies upon IM administration. In this study, mucosal antibodies were found in the majority of the doses given by LNPs, but no with pABOL. When given IN, LNPs formulations exhibited systemic antibody titers, however lower than the same dose IM. Overall, this study shows that the selection of the delivery vehicle can drive the fate and therapeutic application and effect [143].

4. Biomedical applications of mRNA using nanomedicine

The approval of mRNA vaccines using LNPs can indeed be considered the biggest milestone to foster the clinical translation of many other mRNA nanoformulations that can address current clinical needs. Indeed, the technology can be applied to a broad spectrum of applications and therapeutic areas. Vaccines for infectious diseases might be the first application that has reached the market, but nanoformulated mRNA has the full potential to answer clinical demands related to the need of therapeutic proteins. In the next sections, a full overview of the most promising developments to date is provided. Biomedical applications for mRNA therapeutics are pictured in **Figure 4.**

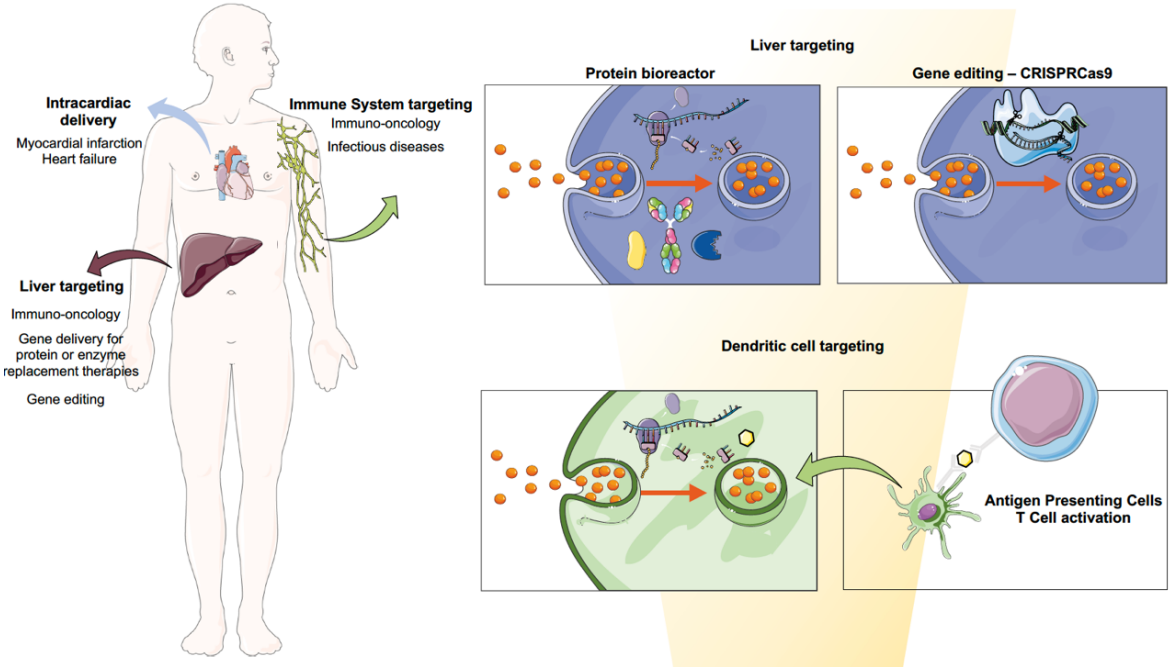


Figure 4. Most advanced biomedical applications for mRNA therapeutics. Immuno-oncology treatments can be envisioned using the liver as a factory of therapeutic proteins such as neoantigens or antibodies, as well as by the use of the APCs physiological function to induce immune activation towards cancerous cells. The latter can also be applied for the development of safe and efficacious mRNA and saRNA vaccines against infectious diseases.

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Gene delivery for therapeutic protein synthesis or restoration of a particular enzymatic function as well as gene editing using CRISPR cas9 technology can be achieved by targeting the liver. Cardiovascular regeneration can be achieved by intracardial delivery of mRNA encoding for growth factors. This figure is the author's own design and was previously published[1]. Created with Medical Art.

4.1. Infectious diseases

Despite the fact that COVID-19 vaccines have been the first marketed mRNA formulations, this technology has been researched for decades using different delivery approaches, from LPX to cationic nanoemulsions to polymeric nanoparticles [10]. Apart from the approved COVID-19 vaccines, an RSV vaccine has just been approved both by the FDA[40] and EMA[145] in 2024, further demonstrating the potential of this technology.

Since mRNA loaded into liposomes demonstrated for the first time that it could trigger a specific T-cell response against influenza virus in mice [146], the field has enormously advanced. In fact, as of June 2024, 70 clinical trials were recruiting patients for mRNA-based vaccines[147] Because this area has been covered in an extensive review [148], in this section the most promising approaches will be showcased. Nonetheless, updated clinical trials for infectious diseases other than COVID-19 are shown in **Table 1**. In this regard, clinically, Respiratory Syncytial Virus (RSV), Influenza virus, Human Metapneurovirus and Parainfluenza Virus (HMPV) are amongst the most advanced clinical candidates. Up until June 2024, a new RSV mRNA vaccine has been approved by the FDA[40] and EMA[145]. The clinical product (mRNA-1345, mRESVIA®) by Moderna exhibited 83,7% efficacy in population above 60 years old for the lower tract infection [39], with a continuous protection over 8 months. As of 2023, as well, the results from a Phase II trial for mRNA vaccine against influenza showed positive safety data, supporting the continuous development of this vaccine (NCT04956575)[149]. Positive results were also reported for H108 and H7N9 influenza viruses (NCT03345043, NCT03076385)[150]. Recent data from HMPV phase 1 clinical trials trials results showed good tolerability and neutralization titers in healthy adults [151]. Recent updates on previous ongoing clinical trials have been recently published for Zika virus (NCT04064905 and NCT03014089)[152], cytomegalovirus (CMV, NCT04232280)[153] and Chikungunya virus (NCT03829384)[154].

Besides, preclinically, one of the most important applications is HIV vaccine. HIV currently affects 38 million people, with these numbers being expected to increase by 2030 [155]. This virus has been extensively researched for over decades, however, its retroviral nature along with the fact that it possesses a very high antigen diversity in its envelope, make it very difficult to achieve good outcomes [156]. In recent years several studies have tried to deliver mRNA vaccines encoding for HIV proteins; however, their success had not been as expected [135,138,157]. Nevertheless, a recently published study has

shown broad neutralization and reduction in the risk of infection in rhesus macaques upon the administration of mRNA encoding for both HIV-1 envelope protein and simian immunodeficiency virus Gag proteins, which would be responsible for forming virus-like particles (VLP). [158]. A different approach based on targeting hepatocytes, and which will be further discussed for immuno-oncology applications in **Section 4.2.2**., has achieved good results. This study involved the delivery of mRNA encoding the light and heavy chains of VRC01, a neutralizing antibody against HIV-1. Using LNPs, they were able to achieve robust protein expression in the liver. Furthermore, prophylactic immunization was also achieved at a dose of 0,7 mg/kg as opposed to the 10 to 20 mg/kg needed if the recombinant protein is administered [159]. As of 2024, a clinical trial started in 2022 is evaluating the efficacy and safety of three mRNA vaccine candidates upon IM administration (NCT05217641).

Another important preclinical advancement was achieved with Ebola vaccine. Ebola virus (EbolV) caused the deaths of more than 11.000 lives across West Africa from 2014 to 2016 [160]. This lethal virus causes a hemorrhagic fever that is fatal in up to 50% of the patients [160]. In 2019, one viral vector-based Ebola vaccine was approved by the FDA, however, clinical trials reported some safety concerns. Like explained in the introduction, from this point of view, mRNA vaccination presents an advantage. In a study in guinea pigs, 20 μg of glycoprotein encoding mRNA were administered using LNPs as delivery vehicle, resulting in good antibody titers and protection from death [161].

Lastly, it is important to note the advancements carried out in other infectious diseases other than viruses. In this regard, *Plasmodium,* the causal agent of Malaria is highlighted next. Very recently, the first malaria vaccine was approved by WHO [162]. The RTS, S malaria vaccine is a protein-recombinant vaccine which has shown to prevent lethal disease, being especially efficacious when given together with the pharmacological treatment [162]. Several studies have also been conducted towards the development of a mRNA vaccine against malaria. The first one, a saRNA based [163] encoding for *Plasmodium* secreted cytokine macrophage migrating inhibitory factor, which had previously shown efficacy [164]. In this study, anti-*Plasmodium* antibodies as well as protective T-cell memory was achieved [163]. On another study, *Plasmodium falciparum* acid-rich protein (PfGARP) mRNA LNPs encoded were dosed to infected aotus monkeys, showing reduced levels of the parasite after three doses [165].

Altogether, all these advancements provide strong evidence on the possibilities ahead regarding mRNA vaccinology, which seems to provide the needed advantages to be the next vaccine technology, especially when considering future pandemics.

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Table *1*. mRNA Nanomedicines for Infectious Diseases in ongoing Clinical Trials. A list of clinical trials for infectious diseases other than COVID-19 is provided in this table. **Abbreviations:** hMPV/PIV3, human metapneumovirus/Parainfluenza virus type 3; RSV, Respiratory Syncytial Virus; CMV, Cytomegalovirus; HPV, human papillomavirus. NIAID, National Institute of Allergy and Infectious Diseases. Clinical trials review searching was based on Sponsor's information (Official website) as well as search in ClinicalTrials.com. Only active trials were included.

Therapeutic	Clinical application	Phase	NCT Number	Sponsor
mRNA-1647	CMV	Phase III	NCT05085366	Moderna
BG505 MD39.3	HIV	Phase I/II	NCT05217641	NIAID
mRNA-1644	HIV	Phase I	NCT05414786	Moderna
mRNA-1644	HIV	Phase I	NCT05001373	Moderna
ARCT-2303	Influenza	Phase III	NCT06279871	Arcturus
MRT5421, MRT5424, and MRT5429	Influenza	Phase I/II	NCT06361875	Sanofi
ARCT-2138	Influenza	Phase I	NCT06125691	Arcturus
GSK4382276A	Influenza	Phase I/II	NCT05823974	GSK
mRNA-1975 mRNA-1982	Lyme Disease	Phase I/II	NCT05975099	Moderna
mRNA-1345	RSV	Phase III	NCT05330975	Moderna
RSV/hMPV mRNA LNP 1/2	RSV	Phase I	NCT06237296	Sanofi
mRNA-1345	RSV	Phase II	NCT06097299	Moderna
mRNA-1345	RSV	Phase III	NCT06067230	Moderna

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4.2. Immuno-Oncology

RNAs are critical for gene expression in their multiple forms, whether it's in non-coding forms such as miRNA, siRNA or tRNAs that regulate transcription and translation, or in coding forms such as mRNA [166]. Cancer immunotherapy has been a game changer since the approval of the immune checkpoint inhibitor ipilimumab in 2011 [167,168]. The number of immuno-oncology (IO) drugs in the development pipeline in 2020 has grown 22% compared to 2019, accentuating the high interest of these therapies. Immunomodulators, cell therapies and cancer vaccines represent the most important trends [169]. Actually, these therapies are often given simultaneously, one example of this being TriMiX, whose composition is CD70, CD40 ligand and TLR4 [170]. However, scientists have met a few challenges when it comes to the new development of these immunotherapeutic agents, mostly related to off-target toxicity [171]. Like so, mRNA technology can offer advantages in terms of scalability, cost, and production issues, widening immunotherapy use worldwide. Altogether, immunotherapy combined with nanomedicine could bring a potential solution: engineer the delivery of immunomodulators focusing their action on target tissues such as tumors or tumor-draining lymph nodes, or specific cell types so as to control the timing and location of immunomodulation [171,172]. To achieve so, two main strategies can be performed: the first one, by targeting DCs and taking advantage of their natural immune role; and the second one, by making use of the liver capacity to synthesize proteins.

4.2.1. Cancer vaccines: targeting dendritic cells

The most well-known strategy to treat cancer with mRNA are cancer vaccines[172], in which mRNA is transcribed into a protein which trains the immune system to fight against the tumor. Thus, rather than prophylactic, cancer vaccines seek to stimulate cell-mediated responses. Therapeutically, this technology can be applied 1) by using shared antigens for the same tumor so as to stimulate immune activation or 2) by designing a personalized cancer vaccine which possesses individual's neoantigens, *i.e.*, proteins preferentially expressed in cancerous cells but not in the patients' healthy tissue, such as growth associated factors or antigens [10].

The way the latter strategy 2) is approached involves the most advanced tissue gene sequencing: a patients' tumor and healthy tissue samples are taken and analyzed looking for tumor-associated antigens , as pictured in **Figure 5.**

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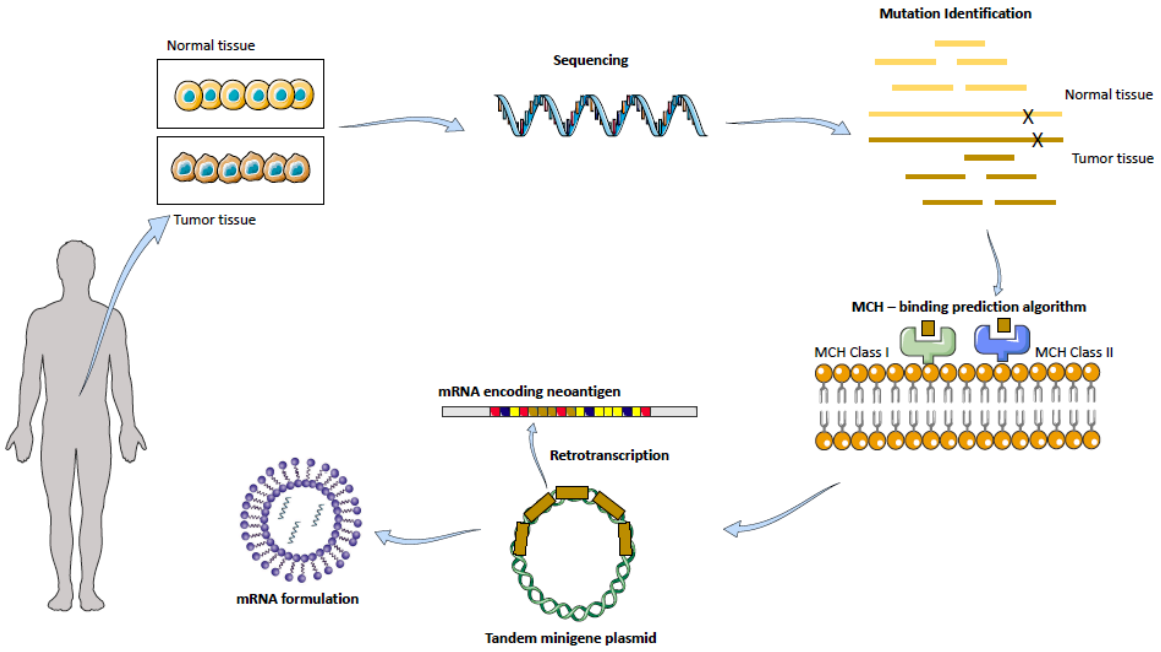


Figure 5. Personalized mRNA cancer vaccines. Healthy and tumor tissue are isolated from the patient sample, analyzed, and compared, in order to find mutations, present in the tumor tissue. Bioinformatic tools are used to predict the MCH Class I and II binding. A tandem gene encoding for several neoantigens is then designed and retrotranscribed to mRNA. Finally, by making use of nanotechnology, the mRNA is formulated and delivered. *Adapted from* (Pastor et al., 2018) [173]. The permission of adaptation can be found in Annexes of this thesis. This figure was previously published[1]. Created with Medical Art.

The technology relies on special algorithms prediction of the binding strength to self-major histocompatibility complex (MHC) receptors. Like so, after mutation identification, neoantigens are ranked based on how well they would bind to this receptor, which will then drive the activation of T-cells, followed by immunological response against that specific neoantigen. Upon administration of the mRNA into a suitable, stable, and efficient formulation, DCs get transfected and, due to their antigen presenting cells nature, they localize the neoantigen onto their MHC receptor located in their surface, which will be then presented to the T-Cells, starting the immune cascade [173,174].

Today, both strategies described 1) and 2) are under clinical evaluation in Phase I or II. Melanoma, prostate cancer, triple negative breast cancer, head and neck cancer are some of the biomedical applications in which this technology is being clinically investigated (**Table 2**). An extensive review on this matter has been recently published [172].

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Table 2. **mRNA Nanomedicines for Oncology in Clinical Trials.** A list of clinical trials for oncology applications, including the subsections described above, is provided in this table. **Abbreviations:** Ab, antibodies; **NSCLC**, non-small cell lung cancer. Clinical trials review searching was based on Sponsor's information (Official website) as well as search in ClinicalTrials.com. Only active trials were included.

Oncology				
Cancer Vaccines				
Shared Tumor Antigens				
BNT111	Advanced Melanoma	Phase II	NCT04526899	BioNTech
BNT113	HPV16+ head and neck cancer	Phase II	NCT04534205	BioNTech
BNT114	Triple Negative Breast Cancer	Phase I	NCT03815058	BioNTech
Cevumeran	Melanoma	Phase II	NCT03815058	Genentech
Personalized cancer vaccines				
mRNA-4157	Melanoma	Phase II	NCT03897881	Moderna
BNT122	Advanced Melanoma	Phase I	NCT03815058	BioNTech
mRNA-4157	Solid Tumors	Phase I	NCT03313778	Moderna
mRNA-4359	Solid Tumors	Phase I/II	NCT05533697	Moderna
mRNA-2752	Solid Tumors and Lymphoma	Phase I	NCT03739931	Moderna
mRNA-2752	Carcinoma	Phase I	NCT02872025	Moderna
BNT122	Colorectal Cancer Stage II / III	Phase II	NCT04486378	BioNTech
MVT-5873	Pancreatic Cancer	Phase I	NCT02672917	BioNTech
mFOLFIRINOX	Pancreatic Cancer	Phase I	NCT04161755[175]	Memorial Sloan Kettering Cancer Center

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GRT-C901, GRT-C902	NSCLC, colorectal cancer, gastroesophageal carcinoma, urothelial cancer	Phase I/II	NCT03639714	Gristone bio
NA	Gastric, oesophageal and liver cancer	NA	NCT05192460	The Affiliated Hospital of the Chinese Academy of Military Medical Sciences
The liver as a bioreactor				
Encoding cytokines				
BNT152, BNT153	Solid Tumor	Phase I	NCT04710043	BioNTech
mRNA-2752	Solid Tumor	Phase I	NCT03739931	Moderna
Encoding antibodies				
BNT142	Solid Tumor	Phase I/II	NCT04503278	BioNTech

4.2.2. The liver as a factory of immunomodulatory proteins

As commented in the previous section, targeting DCs is the main strategy towards the development of cancer vaccines. Nonetheless, this is not the only approach in which mRNA can be used for immunotherapy. The present approach is based on the on the fact that nanoparticles have the intrinsic capacity to accumulate in the liver [176] - which has been, for many years the core center of the research. Thus, targeting the liver can be achieved by exploiting its capacity for protein production so as to build up an endogenous factory of therapeutic proteins, which will then be released into the systemic circulation. In fact, the feasibility of targeting the liver for mRNA delivery for the endogenous production of immunomodulators can be envisioned considering advances in other research areas, such as protein replacement therapies with mRNA, which will also be covered in **Section 4.3**. Furthermore, this strategy also overcomes the main disadvantages of many immunomodulators administration such as their short half-life and, thus, long perfusions and recurrent visits to the medical doctor's office as well as their high production cost [177].

This mRNA application has been studied for two types of recombinant proteins: immunomodulators and antibodies. The production of immunomodulators, such as cytokines, has

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already been proven to be an achievable strategy. For instance, intratumoral administration of LNPs with mRNA encoding cytokines IL-23 and IL-36 and the T-cell costimulatory OX40L was proven to lead to tumor regression in colon cancer and melanoma models, being now under clinical trials (NCT03739931) [178]. In another study using IL-12, a well-known cytokine [179], Lai *et al*. reported mRNA encoding IL-12 LNPs to be a good candidate for slowing down the progression of MYC oncogene-driven human hepatocellular carcinoma, with good biodistribution within the tumor and no toxicity. Treatment reduced liver tumor burden and increased survival of MYC-induced HCC transgenic mice [180].

Interestingly, co-delivery of cytokines and antibodies has proven potential for treating different types of cancer [180–182]. Actually, the use of mRNA to endogenously produce antibodies has also been achieved. Apart from the already commented HIV study in **Section 4.1** [159], another study with trastuzumab aimed to apply this strategy to tackle oncology. Rybakova *et al*. managed to efficiently deliver mRNA encoding for trastuzumab, a monoclonal antibody widely used for breast cancer. They found a dose-dependent increase in trastuzumab protein levels in mouse serum after 24 h administration. When the administration of the monoclonal antibody Herceptin® was compared, results revealed similar half-lives for both antibodies (*i.e.,* endogenously produced and Herceptin) however, the mRNA-based delivery resulted in a 64% higher antibody serum concentration over the course of 30 days. Overall, a single dose of LNPs with the mRNA at 2 mg/kg showed a favorable pharmacokinetic profile when compared to a single injection of Herceptin® at 8 mg/kg [183]. This technology has also proven efficient for the delivery of bispecific antibodies [184]. Stadler *et. al* managed to achieve a promising therapeutic outcome by generating His-tagged bispecific antibodies directed against the T-cell receptor associated molecule CD3 a tumor associated antigen [184].

4.2.3. CAR-T Cells

On a last note on immunotherapy, CAR-T cell field can as well take advantage of mRNA technology. Chimeric antigen receptor (CAR) T-cell therapy is considered by many the next promise of cancer immunotherapy. In August 2017, FDA approved the first CAR-T treatment, Kymriah® (tisagenlecleucel) for the treatment of pediatric and young adults with B-cell acute lymphoblastic leukemia (B-ALL). Several months after that, in October 2017, FDA approved Yescarta® (axicabtagene ciloleucel) for adult patients with certain types of refractory B-cell non-Hodgkin lymphoma (B-NHL) [185]. This approach consists of engineering lymphocyte T surfaces with tumor specific antigens that would bind to tumor specific receptors. Right now, CD19 targeting CAR T-cell therapy is approved for both B-cell acute lymphoblastic leukemia (B-ALL) as well as non-Hodgkin lymphoma (NHL), where it has demonstrated up to 90% complete remission rayed in chemo-refractory B-cell malignancies [185]. Despite the promising

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positive outcomes on liquid tumours, CAR-T therapy has challenges in its application to solid tumours; including limited cancer-specific targets and nonpersistence of the transferred CAR-T cells to allow for low side effects but efficient tumour eradication [186], and research needs to be done so as to tackle solid tumors such as breast, melanoma or ovarian. On solid tumors behalf, on a recent paper published, T-cells were bioengineered with nanoparticle encapsulating mRNA encoding for single chain variable fragment (scFv) that can specifically bind to CLDN6, an oncofetal cell surface antigen for CAR-T cell targeting. Results demonstrated that RNA nanoparticles can efficiently stimulate CAR-T cells [187].

4.3. RNA-based protein replacement therapies

As mentioned before, attempts to deliver therapeutic antibodies as mRNA molecules have been done with success with the aim of overcoming the drawbacks of administering the therapeutic protein. So far, protein replacement therapies are mainly focused on secreted proteins or to proteins of bacterial origin. It is important to understand the processes following the translation of mRNA into proteins, which include folding, post-translational modification, aggregation into secretory granules and transport outside the cell. One of the most important things to consider are the signal peptides, as it takes a great role in directing the protein secretion. Unless the signal sequence is optimized, extracellular mRNA should be transfected ideally into cells that the encoded protein is naturally secreted in [188].

4.3.1. Cystic fibrosis

Cystic fibrosis (CF) is a genetic disease which affects over 90.000 individuals all over the world. The F508del is the most prevalent CF causing mutation, and it affects approximately 82% of the CF population. This mutation leads to cystic fibrosis conductance receptor (CFTR) protein misfolding which is then arrested by the cell machinery and targeted to the proteasome, leading to its premature degradation [189]. Like so, mRNA-mediated replacement therapy can be applied to produce copies of CFTR, which is defective in CF patients. However, when it comes to delivery of mRNA nanoparticles to the lung, these formulations not only need to be endocytosed, escape the endosome, and release the cargo inside the cytoplasm; but they also need to migrate across viscous mucus, avoid clearance and uptake by the macrophages within this mucus and the airways and finally, they need to reach pulmonary epithelial cells [189,190]. Like so, these additional biological barriers make the delivery of mRNA loaded nanoparticles to the lung especially challenging. In 2021, the first human clinical trial involving delivering of LNPs by aerosol revealed well tolerability, nevertheless, no benefit in lung function was observed (NCT03375047) Additionally, safety and tolerability of a nebulized dose of CFTR mRNA loaded into LNPs are being evaluated for ARCT-032 evaluated (NCT05712538).

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4.3.2. Rare metabolic diseases

An important area which would be enormously impacted by this technology are rare metabolic diseases. These disorders lack financial support and thus, effective treatments. From this point of view, mRNA technology can entail an enormous advantage due to the cost-efficient matters already commented. Furthermore, the previous idea and background given on endogenously produced proteins can also be applied in this section.

4.3.2.1. **Acute intermittent porphyria** is a chronic disease caused by the hepatic deficiency of porphobilinogen deaminase (PBGD), the enzyme which catalyzes the transformation of porphobilinogen into protoporphyrinogen during the heme synthesis pathway [191]. This causes the accumulation of the porphyrin precursors γ-aminolevulinic acid (ALA) and porphobilinogen (PBG), which entails acute neurovisceral attacks associated with high production of potentially neurotoxic porphyrin precursors [191]. Treatments so far are composed of hemin replacement therapy, which restores the regulatory heme pool in the liver and suppresses the accumulation of precursors; however, these reductions occur days after the injection and the levels of enzymes should be monitored closely. Although this is an efficacious treatment, 5% of patients suffer recurrent attacks over the years. Prophylactic hemin treatment is being more commonly used for the chronic symptoms; however repeated administrations have been shown to have important side effects [192,193] . RNA therapies seem to give an answer to the treatment of this disease. siRNA drug Givosiran® aims to silence ALAS1 enzyme, stopping the production of ALA and PBG. mRNA therapies are still in preclinical development. In this regard, Jiang et al. evaluated the potential treatment of this disease with mRNA encoding PBGD enzyme into LNPs in two models of acute hepatic porphyria. Furthermore, they also performed a multiple dose study on non-human primates to further prove the translatability to humans. The results showed that administration of hPBGD mRNA led to expression of the therapeutic protein after 2 h post administration, and, most importantly, maintained the activity throughout the entire duration of the attack [192]. Overall, this study reported sustained efficacy and tolerability of the hPBGD mRNA after single and repeated administrations in mice, rabbit, and non-human primates [192], further evidencing the potential of mRNA for the treatment of this disease.

4.3.2.2. **Propionic acidaemia (PA)** is a rare pediatric disorder caused by propionyl-CoA carboxylase (PCC) deficiency, which results in impaired propionate metabolism and accumulation of toxic metabolites, leading to a life-threatening condition[194]. There are two disease subtypes, PCCA deficiency and PCCB deficiency, each of them referring to the different subunits forming PCC, respectively A (of 72 kDa) or B (of 54 kDa). Current treatments for this disease include fluid therapies or actions to remove the excess of toxic metabolites, but liver transplant is a potential outcome for these

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patients. In this term, mRNA therapeutics encoding for PCC enzymes can provide a solution. In this study, Jiang L. et al. report the use of two mRNAs encoding both human PCCA and PCCB. Results showed the restorage of ammonia levels and the function of PCC enzyme in the liver; reducing toxic metabolites associated with this condition up to 6 months upon repeated administrations in mice [195]. Currently, mRNA-3927 from Moderna Therapeutics is under clinical trials (NCT05130437) to assess long term safety.

4.3.2.3. **Fabry disease** is an X-linked lysosomal storage disorder which results from the mutation of the gene GLA encoding for the lysosomal enzyme α-galactosidase A, responsible for glycolipids metabolism. Its loss of activity leads to fat accumulation within the lysosomes of multiple tissues, which in turn leads to clinical disease with fatal renal and cardiac function alteration[196]. Current treatments for this metabolic disease include enzyme replacement therapies named Replagal® and Fabrazyme® [197]and migalastat [198], a small molecule chaperone. While these treatments have shown to slow the progression of the disease, variability of response, immunogenicity, and difficulties with the manufacturing of these therapies are the needs not yet met. In this context, main studies with mRNA have been published so far showing some advancements. Using mRNA encoding for α-galactosidase, DeRosa et al. demonstrated it is possible to achieve sustained delivery in a Fabry model mouse and in a non-human primate with a result in the reduction of relevant biomarkers [199]. Upon delivery, serum protein levels reached as high as 1330-fold over normal physiological values. Another study conducted by Moderna also showed promising results upon intravenous administration of LNPs, with a dose-dependent protein activity and substrate reduction maintained up to 6 weeks in mice. Upon repeated administration, pharmacodynamic response was sustained and the administration to non-human primates confirmed safety and translatability [200].

4.3.2.4**. Methylmalonic acidemia (MMA)** is a rare metabolic disease which is characterized by mutations in the gene coding for the vitamin B12-dependent enzyme methyl-malonyl-CoA (MUT), causing partial or complete loss of activity. This enzyme catalyzes important metabolic reactions linked to the Krebs cycle, playing a central role in intermediary metabolism. Its partial or total lack of activity causes the accumulation of toxic metabolites and the alteration of mitochondrial oxidative phosphorylation. Clinically, patients affected with this disorder experience multisystemic complications which are more common in organs with high metabolic demand such as the brain or heart [194].

There is currently no effective treatment for these patients and this disorder is often treated by carnitine supplementation and protein-limited diets; however, mortality is still around 20% and survivors can develop systemic complications. mRNA therapy encoding for human MUT using LNPs as delivery vehicles was studied by An et al. in two clinically relevant models. In both of them, a decrease in the

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enzyme substrate methylmalonic acid blood levels were achieved, with good pharmacokinetic properties as well. Importantly, none of the clinical models developed any hMUT antibodies upon repeated administration or hepatic inflammation [201]. A new clinical study started in 2022 aims at evaluating long-term safety of the mRNA encoding for the enzyme (NCT05295433).

4.3.2.5. **Ornithine transcarbamylase deficiency (OTD)** affects 6 in 100.000 people, being the most frequent inborn error of the urea cycle. As other urea cycle disorders, it results in ammonia accumulation which clinically translates to fatal neurological disorders. OTD is more specifically caused by the deficiency in ornithine transcarbamylase (OTC). Therapeutic options for these patients include a protein-restricted diet for life, with arginine and citrulline supplementation, but liver transplantation is the only therapeutic option in cases of severe OTC deficiency [202]. In this study, the mRNA administration resulted in increased OTC enzymatic activity up to 10 days after dosing, along with increased mice survival [203]. A recent clinical study has just started to evaluate safety and pharmacodynamics of ARCT-810 treatment (NCT06488313).

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Table 3. mRNA Nanomedicines for Protein Replacement Therapies in ongoing Clinical Trials . A list of clinical trials for protein replacement applications is provided in this table. GSD1a: Glycogen Storage Diseases Type 1a. Clinical trials review searching was based on Sponsor's information (Official website) as well as search in ClinicalTrials.com. Only active trials were included.

Protein replacement therapies				
mRNA-3927	PA	Phase I/II	NCT05130437	Moderna
mRNA-3745	GSD1a	Phase I/II	NCT05095727	Moderna
mRNA-3705	MA	Phase I/II	NCT05295433	Moderna
MRT5005	CF	Phase I/II	NCT03375047	Translate Bio
ARCT-032	CF	Phase I	NCT05712538	Arcturus Therapeutics
ARCT-810	OTC	Phase II	NCT06488313	Arcturus Therapeutics

4.4. Gene editing

Clustered regularly interspaced short palindromic repeat (CRISPR)-related systems have become the main tool when considering therapeutic approaches for the cure of genetic diseases. CRISPR-Cas9 complex is composed of two main elements: Cas9 protein, an endonuclease that is capable of DNA cleaving and a single-guide RNA (sgRNA), which guides Cas9 protein to the site of interest. This gene editing tool is, therefore, based on the recognition of PAM (protospacer-adjacent motif) sequence and a complementary 20-nucleotide genomic sequence that induces DNA brakes, which are repaired by error-prone nonhomologous end-joining or precise homology-directed repair [204]. To date, CRISPR/Cas9-based systems are versatile and have extensively been used for biotechnological purposes such as knock-out/knock-in animal models or cells. In fact, as of 2023, the first CRISPR-based gene therapy was approved. Casegivy® is a cellular gene therapy for Sickle cell disease and β-Thalassemia consisting of CD34+ HSCs cells edited by electroporating the CRISPRR/Cas9 ribonucleoprotein *ex vivo*. [205]

From a gene therapy drug delivery point of view, it is important to understand that intracellular delivery of both components is needed. Like so, various *in vivo* studies addressing differing delivery approaches such as viral vectors, mRNA or recombinant protein have been performed. In this section we will focus solely on mRNA delivery, so as it is the purpose of this thesis [204,206–209].

Of the approaches that have more extensively been researched are hepatic diseases. In 2016, a study on human hereditary tyrosinemia (HHT) showed slightly promising results on correcting the

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fumarylacetoacetate hydrolase (FAH) mutation. This was performed by systemic delivery of Cas9 mRNA encapsulated in LNPs and sgRNA encapsulated in AAV particles. Final results demonstrated an overall gene correction of 6%, suggesting this can be an effective approach for the treatment of a range of metabolic liver diseases. However, authors pointed out that one potential reason for the mild gene correction could be that the nanoformulations were not co-delivered, hypothesizing that the sgRNA suffered degradation during mRNA translation, and thus, decreasing efficacy [210]. Following up on this, another study conducted by the same team aimed to deliver both Cas9 mRNA and sgRNA using LNPs for both components. In order to make it possible for the sgRNA to be efficiently delivered, they conducted several chemical modifications on the sequence, *i.e.,* including the addition of 2' hydroxyl (OH) groups on nucleotides, which resulted in highly improved results with an efficacy of 80%[211]. This study is especially important as it highlights the importance of chemical modifications as well as the possibility of using synthetic nanoparticles, broadening up the industrial applications of gene editing. Similarly, Miller *et al*. reported successful results with a 95% protein knock-down in vitro, by making use of a zwitterionic lipid as delivery vector also by co-delivering the different components [212]. Adding up to this latter study, Finn *et al*. reported a 97% reduction in serum protein levels upon the administration of Cas9 mRNA co-formulated with modified sgRNA into LNPs for gene editing of the mouse transthyretin gene in the liver [213]. To sum up, probably the most important advancement with regards to gene editing is the NTLA-2001 clinical study (NCT04601051), the first clinical trial so far with CRISPR-Cas9 conducted by Intellia Therapeutics for patients with hATTR amyloidosis suffering from polyneuropathy. This gene-editing medicine is based on the CRISPR Cas9 system and it uses mRNA encoding Cas9 protein and the guide RNA encapsulated in a LNPs. Results showed mild adverse effects and the administration led to the decrease of TTR after a single dose [214] . In October 2021, Intellia Therapeutics began a new CRISPR clinical trial, NTLA-2002, for the treatment of hereditary angioedema (HE), a rare, autosomal dominant disorder that causes unexpected inflammatory attacks [215]. Altogether these results present an undeniable precedent for gene editing.

Recently, a new study on CRISPR therapies for cystic fibrosis reported gene correction of CFTR mutations to specific cells using SORT LNPs, providing an important proof-of-concept towards this therapeutic strategy to treat Cystic Fibrosis[216].

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Table 4. mRNA Nanomedicines for in vivo Gene editing in Clinical Trials. A list of clinical trials for gene editing applications is provided in this table. Abbreviations: sg, single-guide. Clinical trials review searching was based on Sponsor's information (Official website) as well as search in ClinicalTrials.com. Only active trials were included.

Gene editing				
NTLA-2001	hATTR	Phase I	NCT04601051	Intellia Therapeutics
NTLA-2002	HE	Phase I/II	NCT05120830	Intellia Therapeutics

4.5. Autoimmune diseases

Autoimmune diseases are chronic and debilitating conditions which are caused by immune response against self-antigens. Disorders such as multiple sclerosis or diabetes type I affect millions of people worldwide. Current treatments available do not target the origin of the disease and are not curative. However, immune tolerance, defined as the state of unresponsiveness of the immune system (IS) to any substance that has the potential to trigger a response, has been described before as a potential therapeutic tool [217,218]. In this term, mRNA technology could potentially be used to induce self-tolerance, by administering a self-antigen encoded in mRNA. In a recent study, Krienke et al. [219] showed that it is possible to deliver myelin oligodendrocyte glycoprotein (MOG) encoded mRNA by making use of lipoplex-type nanocarrier to multiple sclerosis mice models. Upon intravenous administration, modified mRNA was capable of triggering the proliferation of T-reg cells without inducing immune activation, preventing myelin damage and paralysis [219]. This is, to the best of our knowledge, the first proof of concept using mRNA technology for immune tolerance.

4.6. Cardiovascular diseases

Another potential for the development of mRNA therapeutics is treating myocardial infarction (MI) and heart failure (HF), the leading causes of death in many countries. MI leads to a massive loss of cardiomyocytes (CM), which are replaced by highly proliferating fibroblasts, resulting in loss of healthy tissue. These changes are irreversible, and along with oxidative stress and inflammation, the ultimate result is heart failure. Gene therapy with DNA-based or viral vectors has been used to try to induce cardiac regeneration, but it has not had great success due to poor and uncontrolled gene expression. However, mRNA technology could give a plausible response for the treatment of ischemic injury, mainly by three different mechanisms: 1) inducing CM proliferation 2) inhibiting heart cell death and attenuating inflammation and 3) supporting cardiovascular regeneration. It is important to note that although mRNA applications in cardiology are progressing quickly, right now research is focused on intracardiac administration, as it is the most effective delivery method so far; but it causes stress and local injury to the tissue. Like so, it is crucial to develop a delivery vehicle which can target the heart

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and that can be then administered s.c. or i.v. [220,221]. mRNA encoding for vascular endothelial growth factor A (VEGF-A) is currently under clinical phase 2 in a collaboration between AstraZeneca and Moderna Therapeutics after showing the induction of cardiovascular regeneration in several studies. In addition, Magdum *et al*. were able to induce CM proliferation by using mRNA encoding for FSTL1 and Pkm2. [222]

A different approach to treat myocardial infarction has been proposed by the use of regenerative medicine. Using cell reprogramming techniques and Yamanaka Factors, cardiomyocytes could potentially be regenerated to a younger state[223]. In this context, the use of mRNA instead of pDNA therapies would bring important advantages. This novel approach has been subject of study in this thesis and will be presented in Chapter 4.

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Table 5. **mRNA Nanomedicines for cardiovascular diseases in Clinical Trials.** A list of clinical trials for cardiovascular diseases is provided in this table. Clinical trials review searching was based on Sponsor's information (Official website) as well as search in ClinicalTrials.com. Only active trials were included.

Cardiovascular diseases					
AZD8601	Heart Failure	Modified mRNA -LNPs	Phase II	NCT03370887	AstraZeneca

5. Future developments

The development and commercialization of mRNA COVID-19 and the recently approved RSV vaccine have pivoted the way towards future applications of mRNA medicines by finally finding a solution to the delivery problem. After some detractors positioned themselves about the lack of applications for nanotechnology, nanomedicine has now demonstrated it is a translational, relevant, and much needed approach to engineer new genetic drugs. Thus, it is now clear that the full potential of mRNA therapeutics along with its advantages couldn't be envisioned without nanomedicine. As presented in this introduction, mRNA therapeutics are advantageous over other gene therapies in many important ways, such as the improved safety, ease of manufacture and interchangeable mRNA sequence accordingly.

However, despite the importance of having addressed the delivery question, several limitations are still present when considering mRNA therapeutics and nanoparticle formulations. In terms of materials used for nanoparticles, there is still a need on finding new biocompatible and, ideally, biodegradable compounds that would allow repeated administrations, needed for some of the applications reviewed in this introduction, such as protein replacement therapies or gene editing. In this sense, screening of new lipids and polymers that can retain the encapsulation efficiency and performance is a high priority.

In this direction, the aim of this thesis is to advance in the development of novel formulations specifically designed for mRNA delivery for different biomedical applications. Previous work in the group has been focused on the development and assessment of Sphingomyelin Nanoemulsions (SNs, WO 2019/138139A1[224]), composed of sphingomyelin and vitamin E. SNs, modified with DOTAP and stearylamine[134,232,] demonstrated their capacity to effectively deliver short interference RNA (siRNA), micro RNA (miRNA), and plasmid DNA (pDNA) in vitro. More recently, SNs containing putrescine efficiently delivered pDNA encoding apoptotic FasL ligand and inhibited tumor growth in vivo. [233] Sphingolipids represent one of the major types of phospholipids in eukaryotic cells. As such, the study of their structure, function and metabolism has been subject of many reports before [225–228]. Discovered in the XIX century from brain samples[226] , sphingolipids present a common structural feature, the

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sphingosine backbone, which provides bioactive functions to this class of lipids [225], from cell growth regulation to modulators of cell-to-cell interactions. Specifically, sphingomyelin is known to be a major component of lipid bilayers, and in fact, has been previously used in drug delivery for Marqibo®, a vincristine liposomal formulation approved by the FDA in 2012 [229] , and later withdrawn in 2022 [230] upon failure to verify the clinical benefit of the drug. Similarly, vitamin E or α-tocopherol is a lipid-based vitamin, specifically of an oily nature, that has been described as a potent antioxidant. As well, it has been previously used in drug delivery field, and, more importantly is recognized as a safe excipient [231].

Apart from defining novel mRNA formulations, this thesis aims to explore their potential in biomedicine. The restrained delivery to DCs and natural accumulation of nanoparticles in the liver, which will be also studied in this thesis, might be one of the most challenging and important issues to tackle, as it averts the potential of this application to other organs. In this regard, recent studies on selective organ targeting [72] can provide the foundation to design safe and organ targeted nanoparticles. This issue will be also covered in this thesis, with the study of composition adjustments on nanoparticle biodistribution.

Lastly, the therapeutic potential of the designed mRNA nanoparticles will be explored in two different therapeutic areas of interest, which are currently being subject of numerous investigations, oncology, through the development of immunotherapies, and regenerative medicine, to provide reprogramming tools for treating cardiovascular acute diseases.

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Hypothesis

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HYPOTHESIS

This thesis is supported by the main hypothesis (H) that **'it is possible to engineer a new type of nanoparticles specifically designed for mRNA delivery for advanced biomedical applications.'** This full hypothesis is supported by partial hypotheses:

H1. It is possible to design nanoparticles specifically tailored for the association of mRNA.

H2. By optimizing the lipid composition and physicochemical parameters, nanoparticles can have specific features that govern their potency and *in vivo* fate.

H3. It is possible to develop stable and potent biodegradable nanoparticles with PEG alternatives, overcoming the hurdles of such polymers.

H4. It is possible to deliver mRNA loaded nanoparticles to the liver to exploit this organ as an *in-situ* factory of therapeutic proteins with specific applications in immuno-oncology.

H5. It is possible to use mRNA loaded nanoparticles for tissue regeneration purposes, overcoming the disadvantages of DNA therapies.

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Objectives

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OBJECTIVES

Based on such hypothesis, the main objective (O) of this thesis is **to develop a new type of nanoparticles for the design of innovative mRNA therapeutics.**

The main objective is to be accomplished by the establishment of the following partial objectives:

O1. To develop a new type of nanoparticles that can efficiently associate and deliver mRNA.

O2. To explore the influence of composition in the biological behavior of the developed nanoparticles.

O3. To develop biodegradable and PEG-free nanoparticles that can efficiently deliver mRNA

O4. To assess the therapeutic potential of the novel mRNA nanoparticles in immuno-oncology

O5. To explore the potential of the novel mRNA nanoparticles in regenerative medicine.

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General Methods

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SNPs and LNPs Formulation

Lipids at the established ratios depending on the formulations, were dissolved in EtOH or in chloroform/ethanol mixtures if needed. mRNA was dissolved in an aqueous phase. Briefly, the ethanol phase was injected to the aq. phase using a 21G syringe (Ref. 10064657543, 0.8mm x 0.25mm, Sterican), and nanoparticles were spontaneously formed.

Control Onpattro® (ONP) Formulation using microfluidics

Lipids (MC3, DMG-PEG-200, DSPC, Cholesterol) were dissolved in EtOH at a concentration of 1 mg/mL. NanoAssemblr® Ignite (Precision Nanosystems, Cytiva, USA) instrument was used at 10 mL/min flow rate ratio, a start waste of 0.25 mL and an end waste of 0.10 mL, with a total final formulation volume of 1.24 mL at pH 3. The aq to EtOH ratio was maintained to 3 and the ionizable lipid/mRNA (% w/w) ratio of 11 or N/P of 6. Buffer exchange was performed using the method described above, in 10 mM DPBS.

Fluorescent formulations TF-SNPs Formulation-and Cy5-eGFP mRNA

Labelled SNPs with TopFluor (TF) (Ref. 810265, Avanti) were formulated with 1µg of TF per formulation in the lipid phase. Buffer exchange was performed as described before. For Cy5-eGFP mRNA (Cy5-mRNA) (Tebubio, Spain) labelled SNPs, the labelled mRNA was loaded in a (1:1) ratio with non-labelled mRNA.

SNs and SNPs characterization by DLS

SNPs were characterized for size and homogeneity using DLS and NTA. SNPs were characterized for size (Z-average, nm) and surface charge (Zeta Potential, mV) using a Nanosizer 2000® (Malvern Instruments, Malvern, UK) or a Zetasizer Lab (Malvern Instruments, Malvern, UK), as stated in each of the experiments. Number of replicates is indicated in the text, according to each experiment. The mean size and its distribution were measured by Dynamic Light Scattering (DLS). For SNPs, measurement was performed using disposable microcuvettes (ZEN0040, Bran), after 1/10 dilution in DPBS (10mM, Gibco) or PBS (10mM) at a final mRNA concentration of ~2µg/mL. For SNs, measurement was performed in MilliQ water. Surface charge was analyzed by measuring zeta potential by Laser Doppler Anemomerty (LDA), after 90-fold dilution in 10mM NaCl (SNPs) or MilliQ (SNs) in Folded capillary cuvettes (DTS 1070, Malvern Instruments).

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SNPs characterization by NTA

For NTA, three (n=3) independent replicates were analyzed per prototype. SNPs were characterized using NTA 3.4 Build 3.4.003, with sCMOS Camera and Blue488 Laser. Each sample was diluted before measurement to allow for good camera resolution. 1SNPs and 2-SNPs were diluted 200-fold, to 0.13mg/mL mRNA concentration. 3-SNPs and 4-SNPs were diluted 500-fold to 0.05 mg/mL. Camera was adjusted from 11 to 13 and each sample run recorded five consecutive measurements.

Encapsulation Efficiency

Encapsulation efficiency for SNs was assessed via Gel Retardation Assay, 1.5% agarose gel. 0.25 µg of mRNA were loaded per line. SybrGold (50X) was used as staining component.

SNPs encapsulation efficiency (%) was assessed by Quant-iT™ RiboGreen™ RNA Assay Kit (Invitrogen) according to manufacturer instructions. Briefly, a calibration curve in Tris-EDTA (TE) buffer was prepared at 100, 50, 25, 12.5, 6.25, 3.12 ng/mL. SNPs were diluted 500-fold in TE and measured in a Black Bottom Greiner P96well plate using VICTOR Nivo Microplate Reader (PerkinElmer). Encapsulation efficiency (%) was calculated by subtracting the amount of mRNA present in TE samples (unencapsulated) to the mRNA amount loaded (total) in the SNPs, following the equation:

$$Encapsulation\ Efficiency\ (\%) = \frac{Loaded\ mRNA - Unencapsulated\ mRNA}{Loaded\ mRNA} x\ 100$$

TEM Microscopy

Morphology and structure of the SNPs were observed by TEM (JEOL JEM-2010) using Copper Carbon Film 400 Mech (Ref. CF400-Cu-50). SNPs were first ultracentrifuged against MilliQ water to remove all the salts. Then, 10 µL of the sample were loaded on the grid, incubated for 10 min at RT and stained in 10 µL of 2% Phosphotungstic acid solution for 1 min. The excess of phosphotungstic solution was removed by pipette and the grid was washed with excess amount of RNAse-free water several times. Samples were air-dried overnight in a desiccator prior to visualization.

mRNA Constructs

CleanCap FLuc mRNA (Ref. L-7602) and CleanCap eGFP mRNA (Ref. L7601) were purchased from TebuBio (Barcelona, Spain). CleanCap® mCherry mRNA (5moU) - (L-7203) was purchased from TriLink Biotechnologies (San Diego, US).

Immunomodulatory mRNA construct was produced from pDNA by In Vitro Transcription (IVT) using the T7 mScript Standard mRNA Production System (Cellscript, Madison, WI, USA) as reported before [25,26]. Briefly, each 100 μg of plasmid were linearized with 100 U HindIII (NEB R0104S, New England Biolabs, Ipswich, MA, USA) for 3 h at 37°C. Linearization was confirmed by 1% agarose gel. The linearized template was purified by double phenol:chloroform:isoamyl alcohol 25:24:1 organic extraction and precipitated overnight with absolute ethanol at –20°C. The pellet was then washed with 70% ethanol and, once the alcohol had evaporated, resuspended in nuclease-free water. 1 µg of the DNA template was subjected to IVT, capped with Cap1 structure at the 5' end and elongated the poly(A) tail with 150 adenines at the 3' end. The *in vitro* RNA was purified with phenol: chloroform: isoamyl alcohol 25:24:1 organic extraction, followed by ammonium acetate precipitation, according to manufacturer's instructions. The purified mRNA was eluted in RNase-free water at 2-3 mg/mL and stored at –80°C. For formulation purposes, mRNA was diluted to 1mg/mL in RNAse-free water.

Oct4, Sox2, Klf4, c-Myc (OSKM) mRNA sequences were synthesized starting from a pDNA encoding each of the factors (Derrick Rossi, Addgene plasmid # 26815, # 26816, # 26817, # 26818, # 26823). Region of interest was amplified using Tail PCR (Reverse Primer encoding for PolyA region) using Phusion™ Plus PCR Master Mix (Thermo Scientific

Cell line models

HEK293 and HEPG2 cells used in Chapter I were obtained from ATCC. HEK293 cell line was cultured in complete DMEM medium: Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (35-075-CV, Gibco), and 1% Penicillin/Streptomycin (PenStrep, 15140-122, Gibco). HEPG2 cells were harvested in complete RPMI medium: RPMI 1640 medium with GlutaMAX (Gibco) supplemented with 10% FBS (35-075-CV, Gibco) and 1% Penicillin/Streptomycin (PenStrep, 15140-122, Gibco). All cells were maintained in a humidified incubator at 37ºC and 5% CO_2. These cell lines were routinely tested for mycoplasma contamination by PCR using Primer MyF (51-2150-1/2, Eurofins Genomics), Primer MyR (51-2150-2/2, Eurofins Genomics), PCR Rnx buffer (P/N Y02028, ThermoFisher Scientific), $MgCl_2$ (P/N Y02016, Invitrogen) dNTPs (18427013, Invitrogen) and Taq DNA Polymerase (100021276, Invitrogen).

The B16-OVA melanoma cell line and MC38 colon carcinoma cell line used in Chapter III were a kind gift from Dr. Lieping Chen (Yale University, New Haven, CT, USA) in November 2001. Both cell lines were maintained in complete RPMI medium: RPMI 1640 medium with GlutaMAX (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 100 IU/mL

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penicillin and 100 mg/mL streptomycin (Gibco) and 50 mM 2-mercaptoethanol (Gibco) and 0.4 mg/mL of G418 (Sigma-Aldrich). B16-OVA cell line was routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). For the tumor models, both B16OVA and MC38 cells were resuspended to a final concentration of 0.5 x10^6 cells and delivered subcutaneously (s.c.) in 100 μL of PBS per mouse.

HFF1, AC-10 and NIH/3T3 used in Chapter IV were obtained from ATCC. HFF-1 cells were harvested in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Waltham, MA) supplemented with 15% fetal bovine serum (FBS) (Omega Scientific, Catalog #: FB-01), and 1% Penicillin/Streptomycin (PenStrep, 15140-122, Gibco). AC-10 were cultured in DMEM:F12 Medium (Gibco) with 12,5% FBS (Omega Scientific, Catalog #: FB-01). NIH/3T3 were cultured in DMEM, 10% FBS (Omega Scientific, Catalog #: FB-01). All cells were maintained in a humidified incubator at 37ºC and 5% CO_2. These cell lines were routinely tested for mycoplasma contamination.

Uptake, transfection, and toxicity Studies

All uptake, transfection and toxicity assays were performed at 1μg/mL concentration. At least three biological replicates (n=3) were performed per experiment. All cells were seeded the previous day of dosing.

Uptake screening assays were performed in P6-well plates (Ref. 10146810, Corning). 1 x 10^6 HEPG2 cells were seeded and allowed to adhere overnight. SM-TopFluor labelled SNPs were dosed and analyzed by Flow Cytometry (Cytek™ Northern Light Northern Lights) after 24h. Briefly, cells were detached using Trypsin-EDTA 0.5% (ThermoFisher Scientific), washed with DPBS (Gibco) and stained with 7-AAD for cytotoxicity for 10 minutes. Then, cells were washed again with DPBS, resuspended in 2% PFA DPBS and incubated at RT in the dark for 10 minutes. FACs analysis was performed immediately after so. For Cy5 mRNA-labelled uptake assays, 100.000 HEK293 cells were seeded the day before dosing. Next day, 500 μg of mRNA (250 μg Cy5-labelled) loaded into SNPs were dosed at 1 μg/mL concentration. After 2h incubation with SNPs, cells were washed with DPBS (1x), detached with trypsin, and analyzed via FACs (Becton Dickinson, FACscalibur). Data analysis was performed using FlowJo. For FLuc mRNA SNPs testing, HEK293 (20.000 cells/well) and HEPG2 (10.000 cells/well) cells were seeded in a white-bottom P96 (Nunc™ MicroWell™ 96-Well, ThermoFisher Scientific) and allowed to adhere overnight prior to SNPs dosing. Next day, SNPs were dosed in triplicates (n=3). After 24h, toxicity was measured using Cell-TiterFluo™ and VICTOR Nivo Microplate Reader (PerkinElmer) as per manufacturer's instructions. Then, bioluminescence produced

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by the encoded protein was measured using 25µL of One-Glo™ Luciferase assay system (Ref. E6110, Promega) in VICTOR Nivo Microplate Reader (PerkinElmer) after 4 minutes incubation in the dark. For eGFP mRNA SNPs testing, 100.000 cells were seeded in a P24 clear-bottom cell plate and allowed to adhere O/N. Next day, 500 µg mRNA loaded into SNPs were dosed. Transfection was analyzed after 24h using Flow Cytometry (Cytek™ Northern Light Northern Lights). Upon reading, cells were detached using Trypsin-EDTA 0.5% (ThermoFisher Scientific), washed with DPBS (1x), centrifuged, and resuspended in 2% FBS DPBS for flow cytometry analysis. Data analysis was performed using FlowJo.

For mCherry mRNA SNPs testing (Chapter IV), HFF1 (10.000 cells/well) cells were seeded in a black-P96 (ThermoFisher Scientific) and allowed to adhere overnight prior to SNPs dosing. Next day, SNPs were dosed in, at least, triplicates (n=3). Messenger Max was used as a control, in at least, two independent replicates (n=2). After 24h, transfection efficiency was assessed with Microplate Reader at λ_{Exc}=587nm and λ_{Em}=610nm. The same plates were visualized under the fluorescent microscope.

eGFP Transfection Assays by Confocal microscopy

For eGFP visualization by confocal microscopy, 50.000 HEK293 cells and 30.000 HEPG2 cells were seeded the day before in microchamber (Nunc® Lab-Tek® II) and allowed to adhere overnight. Next day, dosing was performed with the above-described conditions. After 24h incubation, cells were washed with DPBS (3x) and fixed with 4% PFA for 10 minutes at RT. After washing, nuclei were stained with Hoechst (1:1000) for 10 minutes and washed again. The preparation was then finalized with a coverslip and Mowiol to allow for confocal imaging. Confocal images were acquired using a Confocal Microscope Leica TCS SP5 X.

Animal handling

Animal experiments were performed at two different research centers, as stated in each Chapter. Experiments from Chapter I (Section 3) were approved by the Ethics Committee of the University of Navarra (R-080-19GN), and these mice were purchased from Harlan Laboratories (Barcelona, Spain). Experiments from Chapter I (Section 4) and Chapter II were approved by Ethics Committee of Centro de Medicina Experimental from USC (15012/2023/007 and 15010/14/002). These mice were bred in-house. We have complied with all relevant ethical regulations for animal testing and research.

***In vivo* intravenous or intraperitoneal SNPs and LNPs administrations**

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Female C57BL/6J mice of at least 6 weeks of age were used for all *in vivo* experiments. For experiments from Chapter I (Section 3), mice received IV or IP injection of FLuc mRNA SNPs at a dose of 10 µg mRNA per mice. 6 or 24 hours later (as stated in each experiment), mice were anesthetized with ketamine and xylazine and injected intraperitoneally with 100µL of D-Luciferin (20 mg/mL). 5 minutes later, mice were placed in Photon Imager (Biospace Lab, Paris, France) for bioluminescence acquisition (2 min). At 24h, mice were euthanized via cervical dislocation. Organs were removed and placed on a black cardboard paper. Luminescent signal was measured using Photon IMAGER TM (Biospace Lab, Paris, France). Data were analyzed with M3 Vision Software. For Chapter I (Section 4) and Chapter II experiments, mice received IV injection of FLuc mRNA SNPs at a dose of 10 µg mRNA per mice. 6h later, mice were anesthetized and injected intraperitoneally with 100µL of D-Luciferin (30 mg/mL). 3 minutes later, mice were placed in IVIS® Spectrum System (PerkinElmer) for bioluminescence acquisition. At 24h, mice were euthanized via cervical dislocation. Organs were removed and placed on a black cardboard paper. Luminescent signal was measured using IVIS® Spectrum System (PerkinElmer). Data were analyzed with software Living Image® 4.7 version. Organ specificity ratios were calculated according to the following formula:

$$Organ\ Specificity\ (\%) = \frac{Bioluminiscence\ Specific\ Organ}{Total\ Organs\ Biolumiscence} * 100$$

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Chapter I

Design and development of Nanoparticles for the delivery of Messenger RNA

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Section subjected to Intellectual Property protection

The design and development of a new type of nanoparticles, SNPs, was performed. To this aim, new nanoparticles were developed by using different combinations of lipids and loaded with Firefly Luciferase mRNA (FLuc mRNA). Characterization experiments refer to analysis of the colloidal properties, association efficiencies, and *in vitro* and *in vivo* transfection efficiencies. For reference, SNs previously developed by the research group and efficient for the association of nucleic acids, were also formulated and tested. Results confirmed the superiority of the new nanoparticle formulations.

Next, the proportion of the lipids was adjusted, to understand the influence of the lipid composition on the physicochemical properties and transfection efficiencies. Four different SNPs compositions were formulated and fully characterized (1-SNPs, 2-SNPs, 3-SNPs, 4-SNPs). Physicochemical properties, encapsulation efficiency, colloidal stability and morphology, were determined by Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM) and indirect mRNA fluorescent-based quantification, and are shown in Figure 1. The cell internalization was tested on HEK293 and HEPG2 cell-lines, with fluorescently-labelled nanoparticles, labelled mRNA and double-labelling (nanoparticles and mRNA), via confocal microscopy and flow cytometry. No differences were observed among the four prototypes. Next, *in vitro* transfection efficiency and cytotoxicity were determined. In terms of expression of the encapsulated FLuc mRNA (bioluminescence was measured 24h post-transfection), two of the formulations shown the most promising results (3-SNPs, 4-SNPs), which moreover were as potent as to the positive control, LNPs formulated with Onpattro® composition (ONP). When eGFP-mRNA was encapsulated, the efficient translation of the protein was confirmed via confocal microscopy. In terms of cytotoxicity (studies were carried out by direct quantification of the live-cell protease activity), cells remain ed fully viable upon 24h and 48h incubation with the nanoparticles, as opposed to what it was observed with MessengerMax (Invitrogen), underlaying the cell compatibility of SNPs. Next, SNPs were evaluated *in vivo*. Their potency as well as the functional delivery to main organs (liver, spleen, lungs, kidneys and omenta) was assessed by bioluminescence quantification (ph/s/cm^2/sr) in C57/BL6 female mice. Potency achieved after 6h post IV-SNPs administration was quantified. In agreement with *in vitro* potency results, 3-SNPs and 4-SNPs exhibited higher protein production, and were comparable to the positive control ONP, underlaying the positive prospective of the newly developed mRNA formulations. In a second experimental analysis, 24h post-administration, mice were administered luciferin, sacrificed, and the bioluminescence from each organ was quantified. The percentage of luminescence in each organ was calculated by considering the total organ bioluminescence. As compared to ONP, some SNPs exhibited preferred spleen delivery. In addition, the most potent prototype, 3-SNPs, was compared against Spikevax® LNPs composition (Moderna, MOD) and JetPEI®, a commonly used transfection reagent for *in vivo* dosing. Results

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showed a remarkable overperformance of SNPs as compared to JetPEI®, although they did not reach MOD potency.

To sum up, the first chapter of this thesis dives into the description of a new delivery vehicle for mRNA. By carefully studying from colloidal properties to *in vivo* fate, this chapter provides insights on delivery design, opening new doors for future formulation development works.

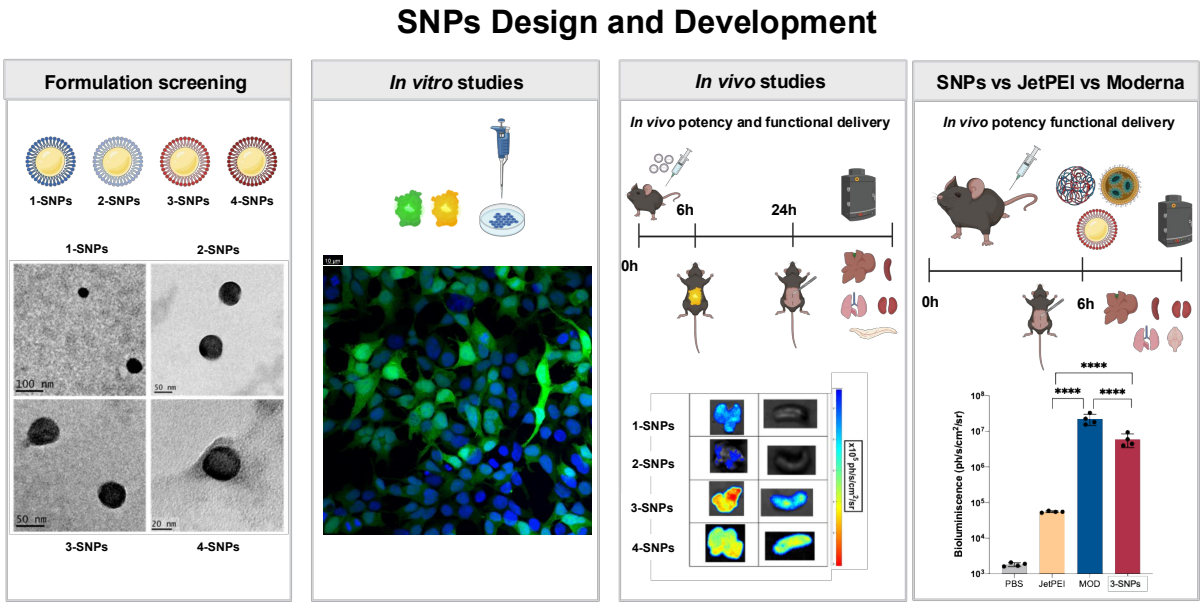


Figure 1. Design and development of the new nanoparticle formulations described in Chapter I. Formulation screening was developed to select the most promising prototypes, and *in vitro* and *in vivo* studies were conducted to assess performance. TEM images and eGFP translated protein upon SNPs administration are shown. Nanoparticles were lastly compared to the commercial reagent JetPEI® and LNPs prepared with the composition of Moderna (MOD). This figure is the author's own design. Created with BioRender.com

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Chapter II

Optimization of nanoparticles for improved biocompatibility and organ delivery

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Section subjected to Intellectual Property protection

In Chapter II, we aimed at providing a response to the current limitations of today's technologies. The need of designing biodegradable and more biocompatible delivery systems as well as the study and understanding of targeted delivery to specific organs were subject of this chapter.

For the design of totally biodegradable SNPs, the removal and substitution of PEG for a biodegradable hydrophilic polymer was performed. In addition, all lipids included in the nanoparticle composition were biodegradable. This is of great advantage as PEG derivatives have been previously described as immunogenic upon IV administration, and even though they have extensively been used for many years thanks to their capacity to prolong circulation time as well as decrease opsonization, the field is currently evolving towards limiting their use. However, their complete removal has been associated with decrease stability, and so, our results demonstrated the possibilities to find alternatives that maintain stability and potency. Formulated nanoparticles resulted stable homogeneous and show activity *in vivo*. Unsurprisingly, these replacements led to a decrease in potency, as observed in transfection experiments in vitro and in vivo, possibly due to rapid degradation upon administration, according to previous reports in the field. Thus, maintaining the potency while providing with biodegradable features remained an important challenge. To overcome this limitation, a helper lipid, was included in the formulation to enhance potency. Indeed, its addition provided a more potent formulation. The study of functional delivery of these biodegradable nanoparticles, BD-SNPs, was also performed. In relation to previous formulations, BD-SNPs exhibited an increase in spleen delivery. For studying and modulating organ distribution, different formulations were prepared, fully characterized and tested. Comparative studies refer to the 4-SNPs formulation, as described in Chapter I, and upon inclusion of a positively charged lipid (P-SNPs) and a negatively charged lipid, (N-SNPs). An increase in lung and spleen delivery was respectively observed (Figure 1), revealing the possibility of modulating biodistribution and defining improved formulations for specific organ targeting. The mechanisms of action should be further explored and can be related to specific functionality of the lipids leading by structure and charge, as well as to specific arrangement of components in SNPs modulating the surface properties and therefore specific features as can be the formation of the protein corona. More studies would be necessary to deepen the understanding of these results. Overall, this chapter provides data on the development of biodegradable SNPs, as well as pivots the study of specific organ delivery for SNPs.

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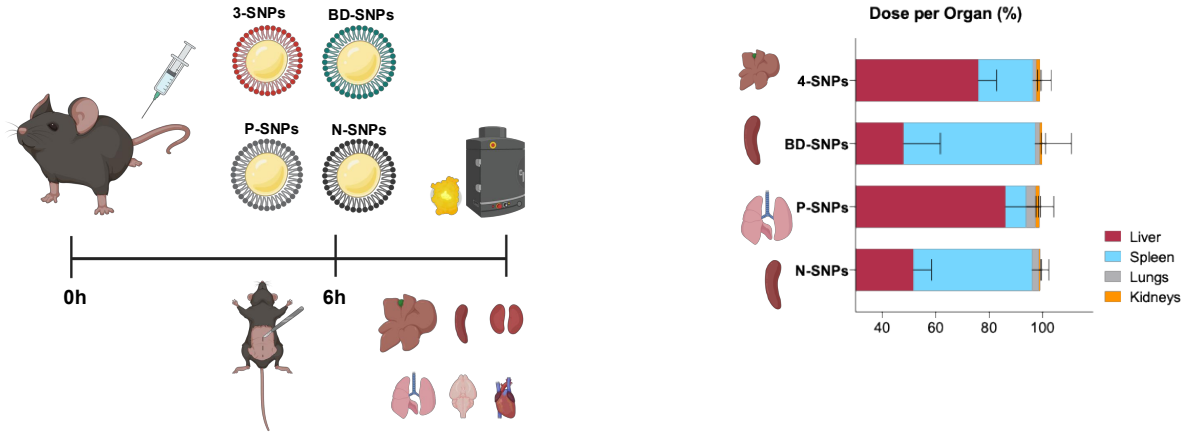


Figure 1. Schematic representation of the work presented in Chapter II. Functional delivery studies were performed with several prototypes. This figure is the author's own design. Created with BioRender.com

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Chapter III

mRNA loaded SNPs for inhibiting distal tumor growth

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ABSTRACT

The safe and efficient delivery of mRNA to target tissues remains a significant challenge in the development of mRNA-based therapies for cancer treatment. In this study, we present the characterization and evaluation of SNPs loaded with mRNA encoding an immunomodulatory protein (namely encoded protein, EP), and the in vivo assessment for their application in immuno-oncology, more specifically after intravenous administration and delivery to the liver, where the EP is expressed and secreted, aiming to interfere in the tumor growth of distal melanoma cancer cells (subcutaneous xenografts). By utilizing the liver as a site for local production of therapeutic molecules, mRNA-loaded SNPs offer a promising strategy to inhibit the growth of distal tumors.

We describe herein the use of the previously developed SNPs presented in Chapter 1, 1-SNPs and 3-SNP, loaded with EP mRNA. Upon intravenous (IV) administration of EP mRNA loaded SNPs in B16-OVA tumor-bearing mice, we observed significant suppression of tumor growth and improved survival outcomes in the treated group. This study highlights the potential of SNPs as a versatile platform for liver-mediated EP mRNA delivery, offering a promising avenue to enhance anti-tumor immune responses and advance cancer therapy. Further exploration of SNPs in preclinical and clinical settings holds the promise of translating these findings into impactful therapeutic interventions for cancer patients.

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INTRODUCTION

The fight against cancer continues to evolve, and the emergence of nucleic acid-based therapies offers promising avenues for developing innovative treatments[1]. Notably, mRNA-based therapies hold important advantages over DNA approaches, such as the increased delivery efficiency and higher safety profiles, as mRNA does not need to reach the nucleus for effective protein translation. Furthermore, the ease in mRNA sequence modification and production improves industrial scalability as compared to recombinant protein engineering [2].

Cancer treatment using mRNA primarily focuses on immuno-oncology (IO) applications and employs two main strategies[3]. The first involves the generation of cancer vaccines against specific tumor-associated antigens, with past or ongoing clinical trials for prostate (NCT04382898), melanoma[4] (NCT03897881), pancreatic cancer [5] (NCT04161755), head and neck (NCT04534205), and solid tumors (NCT03313778). The second strategy utilizes mRNA delivery to trigger the production of therapeutic immunomodulatory proteins[6], as seen in clinical trials for solid tumors or lymphoma (NCT03739931). These strategies can be achieved through passive targeting, where nanoparticles naturally accumulate within tumors or specific tissues due to the enhanced permeability and retention (EPR) effect [7,8]. Alternatively, active targeting utilizes nanoparticles functionalized with ligands to selectively interact with specific cells [9]. Additionally, endogenous targeting, which involves modulating nanoparticle composition to influence its fate within the body, is being currently explored for specific organ delivery [10].

An innovative application within the realm of mRNA therapy in IO involves liver transfection, which induces the secretion of immunomodulatory molecules [6]. This, essentially, harnesses the liver as a factory for generating therapeutic molecules on-site. This strategy has previously been proposed for antibodies such as trastuzumab [11,12], and several clinical trials are currently investigating it for solid tumors (NCT04683939, NCT05262530). The potential advantages of IV mRNA nanoparticle delivery over direct IV administration of recombinant proteins lie in improved controlled release, as proteins have a short circulation half-life and often require multiple administrations. Additionally, mRNA technology offers a better cost-effective profile and streamlined production.

Harnessing the liver as a factory for therapeutic molecules leverages the natural tendency of nanoparticles to accumulate in the liver [10,13] . In fact, the success of Onpattro® hinges on the formation of a protein corona highly enriched in Apo E, facilitating hepatocyte uptake via the Low-Density Lipoprotein Receptor (LDLR) [14,15]. [16–18]

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Our previous work with SNPs, presented in Chapter 1, demonstrated preferential accumulation and potency in the liver. Thus, in this specific application we aim to exploit liver accumulation as a competitive advantage for the development of new immuno-therapeutics in oncology. We present the use of such SNPs for hepatic expression of the EP, a potent immunomodulatory protein, as a strategy to restrain tumor growth. This approach offers several advantages over directly administering recombinant EP protein, which can also cause significant side effects. Our approach will prove the potential of SNPs to transfect the liver and release the EP for a systemic effect, acting on distal tumors, bringing advantages over direct protein administration, and serving as a proof of concept towards systemic mRNA administration for the treatment of metastasized tumors and distal tumors not easily accessible for IT administration. Furthermore, this research could establish the liver's potential as a "biofactory" for producing immunomodulatory proteins using mRNA delivery.

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RESULTS AND DISCUSSION

1. EP mRNA loaded SNPs reduce tumor growth and increase survival of B16-OVA mice model.

In light of the results from Chapter I that confirmed the protein production in the liver, we next aimed at investigating the potential of SNPs in a therapeutic model setting. To this aim, we studied the performance of these SNPs for inhibiting tumor growth upon delivery of EP mRNA to the liver and systemically secrete EP to exert its therapeutic effect. Based on previous results in Chapters I and II, we selected two formulations: 1) 1-SNPs, for their preferential accumulation in the liver; and 2) 3-SNPs, for their superior potency. These results are presented here in Figure 1.A, as well as in Figure 6 in Chapter I.

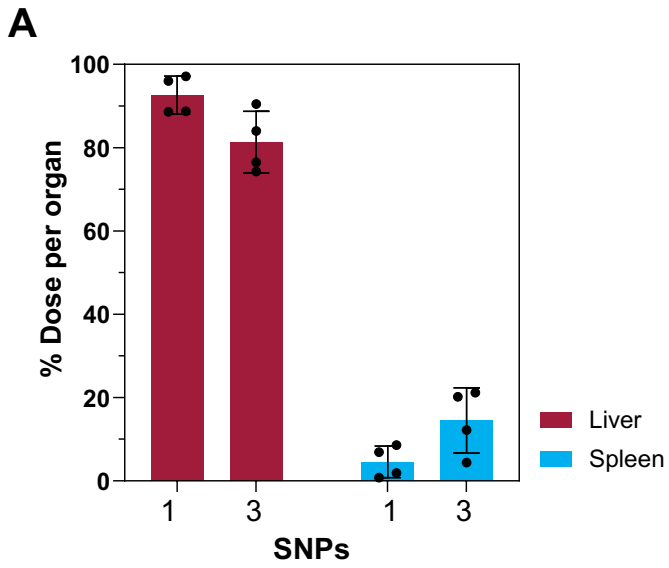


Figure 1. Percentage (%) of dose per organ in Liver and Spleen after 24h SNPs administration of 10μg FLuc mRNA with respect to total organ bioluminescence. Acquisition of bioluminescence was performed after *ex vivo* organ analysis post 24h SNPs administration.

1.2. SNPs produce the Encoded Protein after IV administration

We first studied the *in vivo* kinetics of both prototypes and measured the amount of EP released to the bloodstream via ELISA analysis. A schematic representation of the experiment is presented in Figure 2. A, and experimental results are shown in Figure 2.B. We treated female healthy C57BL/6J mice with 1-SNPs and 3-SNPs loaded with EP mRNA (physicochemical characterization of SNPs with EP mRNA

is presented in Table 1) and the formulations that encapsulate FLuc mRNA as a control. EP levels were quantified at different time points. In addition, we monitored mice weight, extracted blood, and quantified hemoglobin (HGB), white blood cells (WBC), red blood cells (RBC) and platelets (PLT) at 96h (Figure S.1).

Table 1. Colloidal Properties of EP mRNA loaded SNPs. Size and Polydispersity Index (PDI) measured by DLS of EP mRNA SNPs is presented. Data, mean ± SD, of three independent replicates (n=3) is shown.

	Size (nm)	**PDI**
1-SNPs	195 ± 29	0.171 ± 0.071
3-SNPs	90 ± 11	0.165 ± 0.035

Results showed (Figure 2.B) that both formulations were capable of producing the EP, with 3-SNPs (~10^6 ng/mL) overperforming 1-SNPs (~10^4 ng/mL), in alignment with potency results previously observed for FLuc expression. The main peak was obtained after 6h with a significant decrease after 24h for both formulations (Chapter I), suggesting there is no kinetic difference in EP mRNA delivery based on SNPs composition. Of note, 3-SNPs maintained EP levels even after 72h ($6.5 \cdot 10^5$ ng/mL) and 96h ($2.8 \cdot 10^5$ ng/mL) administration. By contrast, 1-SNPs exhibited lower EP levels after 72h ($4.8 \cdot 10^2$ ng/mL) and undetectable levels after 96h administration.

Quantification of HGB, RBC, and PLT after 96h showed no significant differences between FLuc mRNA and PBS controls (Figure S.1.A), further confirming the safety profile of our SNPs. PLT were lower for 3-SNPs group, expected by the increased EP serum levels (Figure S.1.B). Furthermore, we observed a slight decrease in mice weight in EP mRNA treated groups, which was especially significant after 24h of EP administration, in alignment with higher levels of EP and thus, toxicity previously reported. Importantly, mice recovered from the weight loss and were comparable to PBS after 96h (Figure S.1.C).

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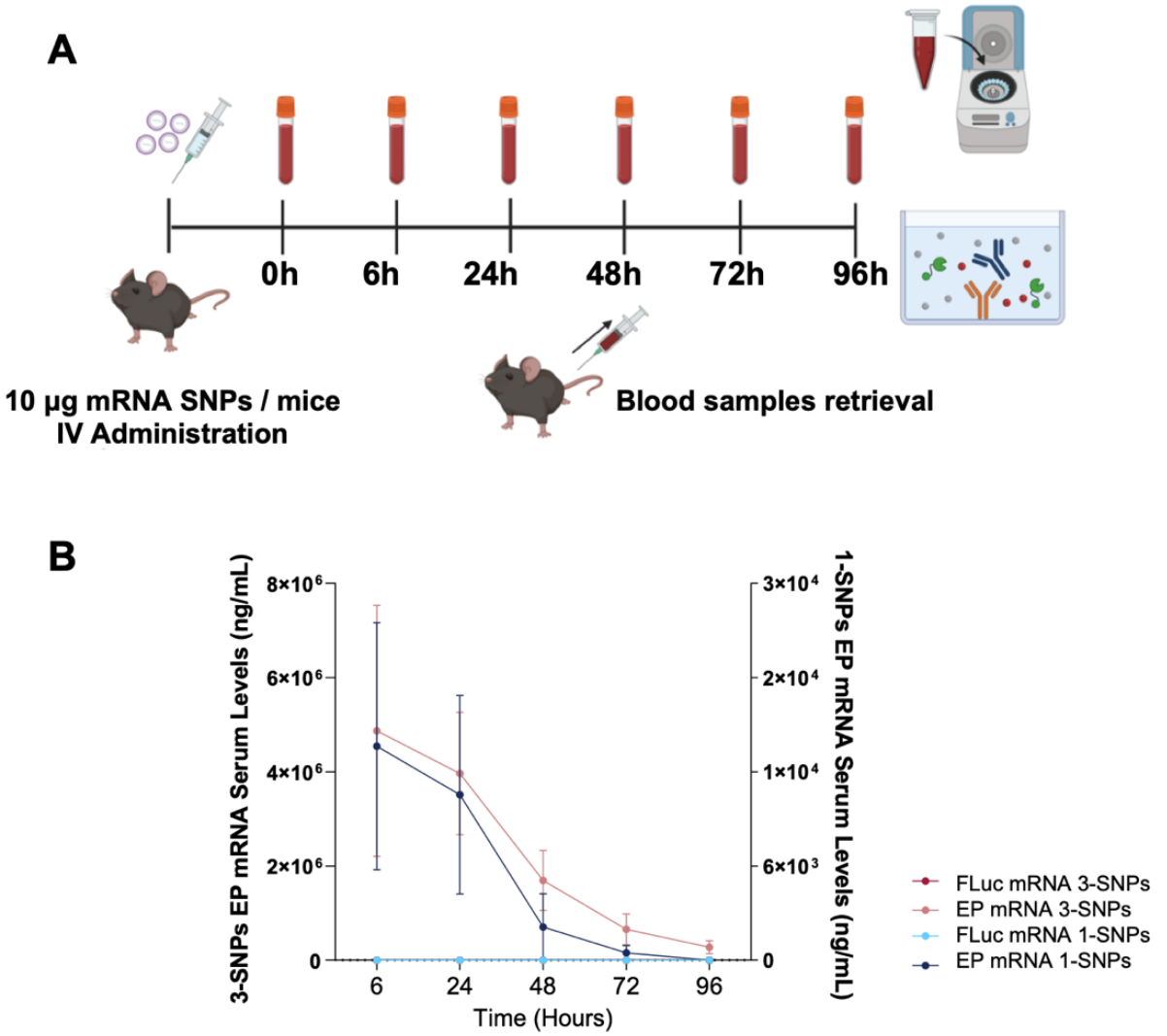


Figure 2. Serum EP levels after EP mRNA SNPs IV administration. A) Schematic representation of *in vivo* dosing. 10μg/mRNA-SNPs were administered per mice. B) Mean ± SEM of EP serum levels (ng/mL) after 6, 24, 48, 72 and 96h. Levels of 3-SNPs are represented in left Y axis, levels of 1-SNPs are represented in right Y axis to improve data visualization.

1.3. EP mRNA loaded into 3-SNPs decrease tumor size with controlled weight loss and improve survival in B16-OVA tumor model

To study the translational potential of our approach, we tested EP mRNA-loaded SNPs in a therapeutically relevant model, B16-OVA tumor C57BL/6J bearing mice. Two doses of EP mRNA were IV administered at day 10 and day 14 after tumor inoculation with $5{\cdot}10^5$ B16-OVA cells/tumor. Mice were monitored for weight loss, tumor growth, and survival. A schematic representation of the procedure is shown in Figure 3.A.

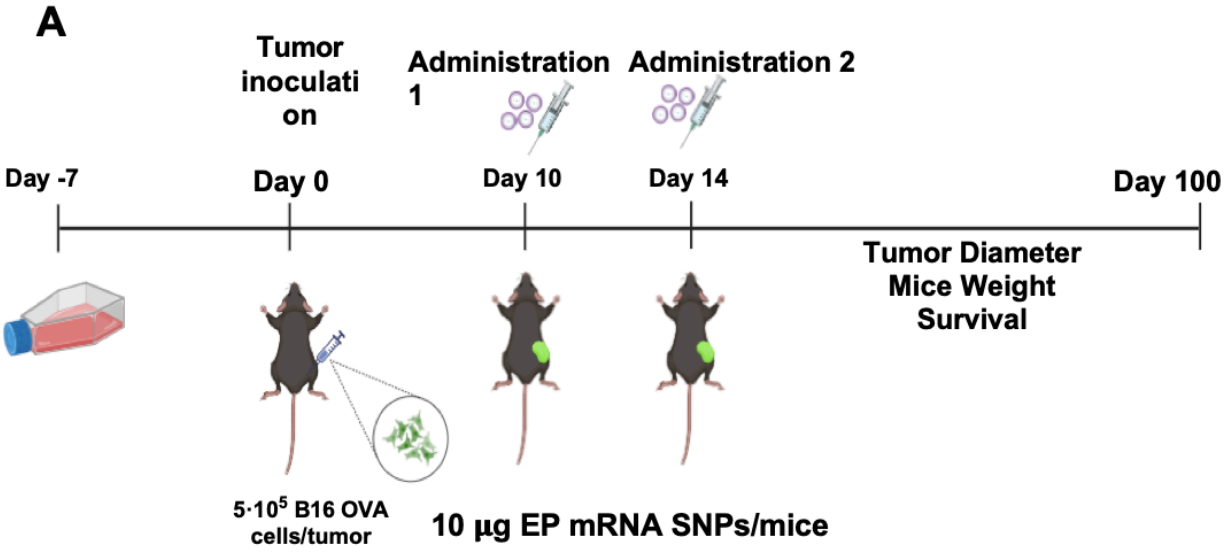
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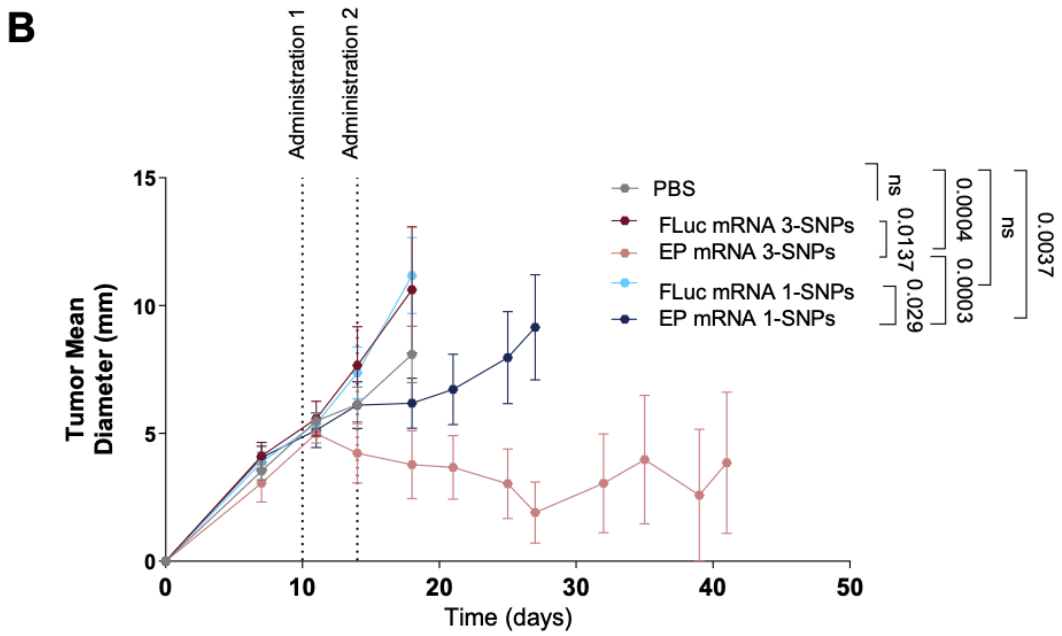
Results showed relevant differences between both SNPs formulations, with 3-SNPs exhibiting the most potent therapeutic effect (Figure 3.B). The individual follow-up of each group is presented in Figure S.2. Successfully, 3-SNPs exhibited a potent antitumoral effect as compared to PBS (p=0.0004), FLuc mRNA 3-SNPs (p=0.0137) and EP mRNA 1-SNPs (p=0.0003) (Figure 3.B). Of note, in this group, 50% of the cases presented tumor remission (Figure S.2). Overall, this effect translated to a 100% survival rate after 35 days and 50% after 100 days, as compared to controls, PBS (0%) and FLuc mRNA 3-SNPs groups (16%) (Figure 2.C), confirming the success of the treatment. Important weight loss was observed in EP mRNA 3-SNPs treated mice, with 25% after 18 days. Nonetheless, all mice recovered their initial weight after 30 days (Figure 3.D). This agrees with results obtained with previous kinetic experiments, after which the mice recovered after 96h (Figure S.1). As stated before, this weight loss is expected as EP is a potent antitumoral agent.

Conversely, EP mRNA 1-SNPs induced a slight tumor growth delay, as compared to PBS (p=0.0037) and the control formulation containing FLuc mRNA (p=0.029) (Figure 2.B). However, this did not translate into an overall improved survival rate (Figure 3.C), as only 14% of the individuals survived. Furthermore, no significant weight loss was observed with 1-SNPs, EP mRNA, or FLuc mRNA (Figure 3.C), probably due to the fact that 1-SNPs were not as potent as 3-SNPs. Interestingly, 1-SNPs did show a slight statistically significant decrease in tumor growth when the study was performed in MC38 tumor models, nevertheless this was not translated to any difference in survival (Figure S.3.). In fact, these results are in alignment with previous formulation performance results all of which showed the underperformance of this formulation, underlying the importance of formulation development to achieve a good therapeutic effect.

Altogether, our data provide evidence of the potential of SNPs as a therapeutic tool for IV mRNA administration to use the liver as a platform for systemic immunomodulatory protein delivery as a strategy to develop anticancer strategies for distal tumors. In this regard, our approach could expand beyond melanoma tumors. [24,25]

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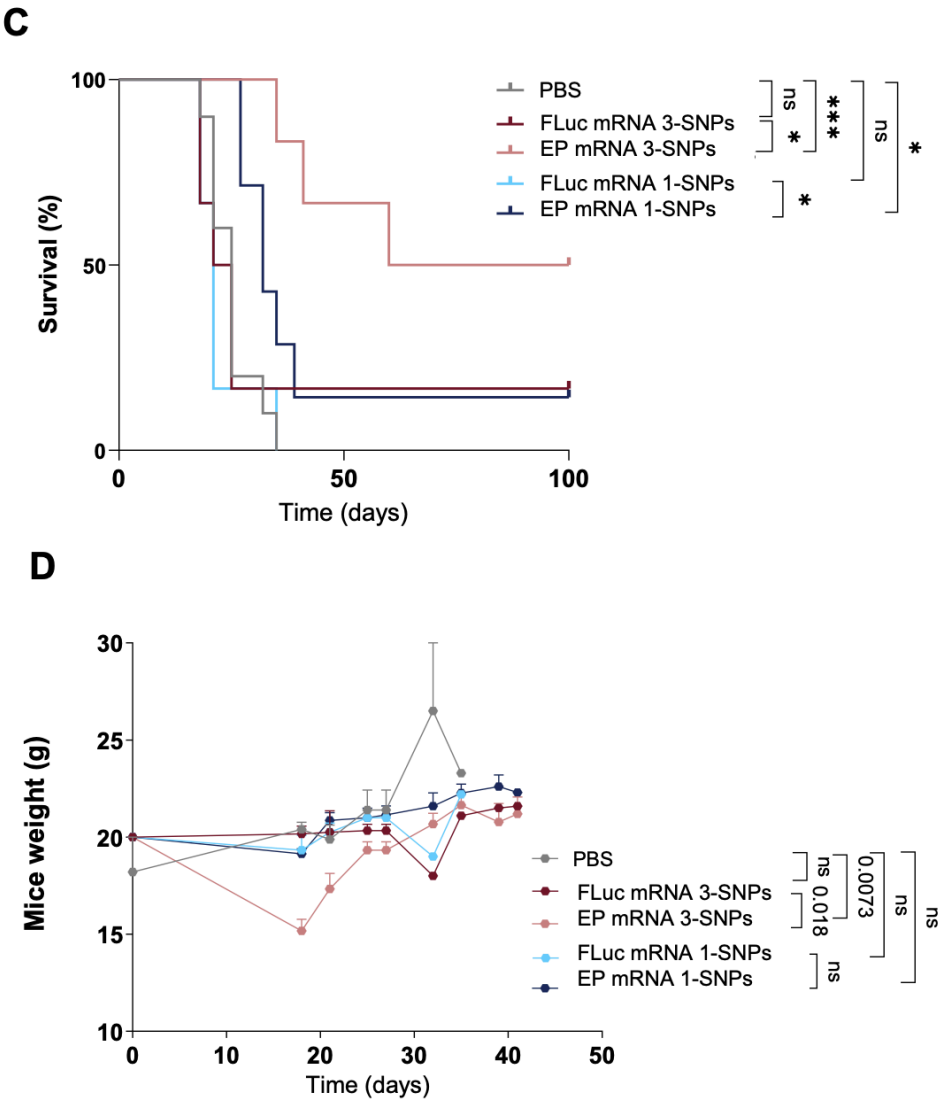


Figure 3. **EP mRNA 3-SNPs exhibit a potent antitumoral effect, which translates to increased survival** A) Schematic representation of the procedure. Each administration was performed with 10μg of mRNA-SNP per mice. B) Average data of tumor mean diameter (mm) over time and statistical analysis. Representation ends when the first individual of each group dies. C) Mice survival data follow-up is presented (N≥6/group). D) Mice weight over the course of the experiment. Mean and SEM is presented in each graph. Longitudinal data were fitted to a third-order polynomical equation and compared using an extra sum-of-squares F test for A). Survival data B) was analyzed using Log-rank (Mantel-Cox) tests. Nonlinear regression was applied for D). n≥6. (ns=0.1234; ****p<0.0001; ***p=0.0002; **P=0.0021; *p=0.033).

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CONCLUSION

Anticancer immunomodulatory proteins regimens are commonly used in the clinical practice. Nevertheless, some of them still face challenges related to systemic toxicity. Furthermore, the low half-life and difficult scalability of recombinant proteins make mRNA therapies a great candidate to advance such treatments.

In this work, we hypothesized that by encapsulating EP, an immunomodulatory protein in the form of mRNA into SNPs, we can use the liver as a factory of proteins, treating distal tumors. Thus, we present herein, the most promising prototypes of previously developed SNPs to test the potential of these nanosystems as a platform for EP mRNA delivery to treat B16-OVA distal tumors. EP mRNA loaded SNPs were capable of inhibiting tumor growth. Specifically, 3-SNPs showed higher potency throughout, achieving tumor remission in 50% of the cases as well as increased survival.

This study opens up new doors towards the possibility of delivering therapeutic proteins in the form of mRNA to treat distal, difficult-to-access as well as metastasized tumors. Furthermore, co-delivery of different agents, as well as different immunomodulatory proteins such as neoantigens, cytokines or antibodies, are envisioned in future work.

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MATERIALS AND METHODS

SNPs Formulation

SNPs were formulated as reported in detail in Chapter 1.

mRNA Constructs

CleanCap FLuc mRNA (Ref. L-7602) were purchased from TebuBio (Barcelona, Spain).

EP mRNA construct was produced from pDNA by In Vitro Transcription (IVT) using the T7 mScript Standard mRNA Production System (Cellscript, Madison, WI, USA) as reported before. Briefly, each 100 μg of plasmid were linearized with 100 U HindIII (NEB R0104S, New England Biolabs, Ipswich, MA, USA) for 3 h at 37°C. Linearization was confirmed by 1% agarose gel. The linearized template was purified by double phenol:chloroform:isoamyl alcohol 25:24:1 organic extraction and precipitated overnight with absolute ethanol at –20°C. The pellet was then washed with 70% ethanol and, once the alcohol had evaporated, resuspended in nuclease-free water. 1 µg of the DNA template was subjected to IVT, capped with Cap1 structure at the 5' end and elongated the poly(A) tail with 150 adenines at the 3' end. The *in vitro* RNA was purified with phenol: chloroform: isoamyl alcohol 25:24:1 organic extraction, followed by ammonium acetate precipitation, according to manufacturer's instructions. The purified mRNA was eluted in RNase-free water at 2-3 mg/mL and stored at –80°C. For formulation purposes, mRNA was diluted to 1mg/mL in RNAse-free water.

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SNPs characterization by DLS

SNPs were characterized for size and homogeneity using DLS and NTA. For DLS, at least five (5) biological replicates were analyzed per prototype. SNPs were characterized for size (Z-average, nm). The mean size and its distribution were measured by Dynamic Light Scattering (DLS). Measurement was performed using disposable microcuvettes (ZEN0040, Bran), after 1/10 dilution in DPBS (Gibco) at a final mRNA concentration of ~2μg/mL.

Tumor mouse models

The B16-OVA melanoma cell line and MC38 colon carcinoma cell line were a kind gift from Dr. Lieping Chen (Yale University, New Haven, CT, USA) in November 2001. Both cell lines were maintained in complete RPMI medium: RPMI 1640 medium with GlutaMAX (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin and 100 mg/mL streptomycin (Gibco) and 50 mM 2-mercaptoethanol (Gibco) and 0.4 mg/mL of G418 (Sigma-Aldrich). B16-OVA cell line was routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). For the tumor models, both B16OVA and MC38 cells were resuspended to a final concentration of 0.5 x10^6 cells and delivered subcutaneously (s.c.) in 100 μL of PBS per mouse.

***In vivo* intravenous SNPs administrations**

Female C57BL/6J mice of at least 6 weeks of age were used for all *in vivo* experiments. Animal experiments were approved by (R-080-19GN). 10 μg EP mRNA or FLuc mRNA loaded into SNPs were IV administered (retro-orbitally). *In vivo* experiments were performed at Centro Investigación Médica Aplicada (CIMA) from Universidad de Navarra, with the kind help of Virginia Belsúe and Dr. Pedro Berraondo.

Kinetic studies

Mice received IV injection of FLuc mRNA SNPs and EP mRNA SNPs at a dose of 10µg mRNA per mice. Blood samples were extracted at 6, 24, 48, 72 and 96h after administration. After each extraction, blood samples were centrifuged at maximum speed for 10minutes. EP protein levels in the sera were assayed by ELISA, following the manufacturer's instructions.

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Anti-tumor efficacy

Experiments with mice were performed in Centro de Investigación Médica Aplicada (CIMA, Pamplona, Comunidad Foral de Navarra) and approved by the Ethics Committee of the University of Navarra (R-080-19GN). We have complied with all relevant ethical regulations for animal testing and research. Anti-tumor efficacy experiments were performed in 6-week C57BL/6J mice that were purchased from Harlan Laboratories (Barcelona, Spain) and bred in CIMA's facilities (CIMA, Pamplona, Spain). B16-OVA and MC38 cells were thawed one week before injection. On day 0, cells detached with pre-warmed trypsin, washed, and counted. Then they were suspended in a final concentration of $5x10^5$ cells in 100μL of PBS per mouse for subcutaneous (s.c.) injection, for both cell types. 10 μg EP mRNA or FLuc mRNA loaded into SNPs IV administration was performed at days 10 and 14 for B16-OVA and MC38 cells bearing mice. Control mice were injected with same volume of PBS. Mice were observed for 100 days, twice a week. Mice were sacrificed when their tumor size was above 15mm or when they presented signs of distress (e.g., piloerection, lethargy, poor feeding) following the Ethics Committee for Animal Experimentation guidelines.

Acknowledgments

Experiments were performed at Centro Investigación Médica Aplicada (CIMA) from Universidad de Navarra, with the kind help of Virginia Belsúe, Nuria Marín Ardáiz and Dr. Pedro Berraondo.

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SUPPLEMENTARY

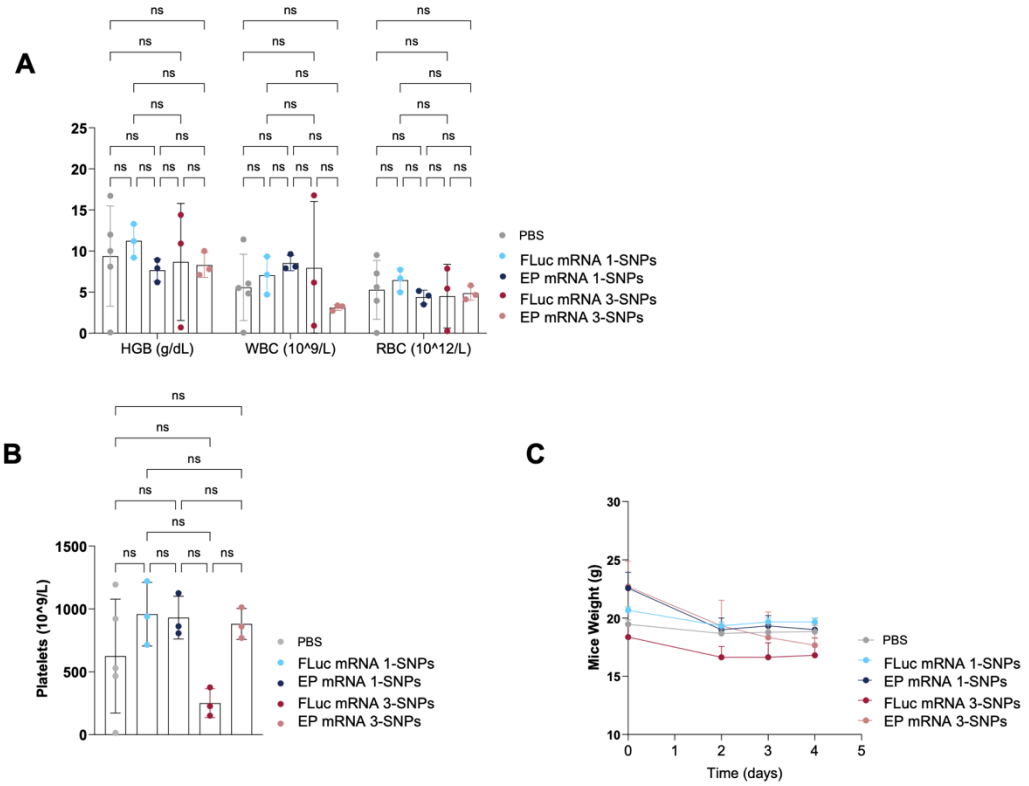


Figure S. 1. A) Hemoglobin (HGB), White Blood Cells (WBC) and Red Blood Cells (RBC) counting after 96h of SNPs administration**.** B) Levels of Platelets after 96h administration. C) Mice weight (g) monitored during kinetics experiment. Statistical Analysis: Nonlinear regression, n≥3. (ns=0.1234; ****p<0.0001; ***p=0.0002; **P=0.0021; *p=0.033).

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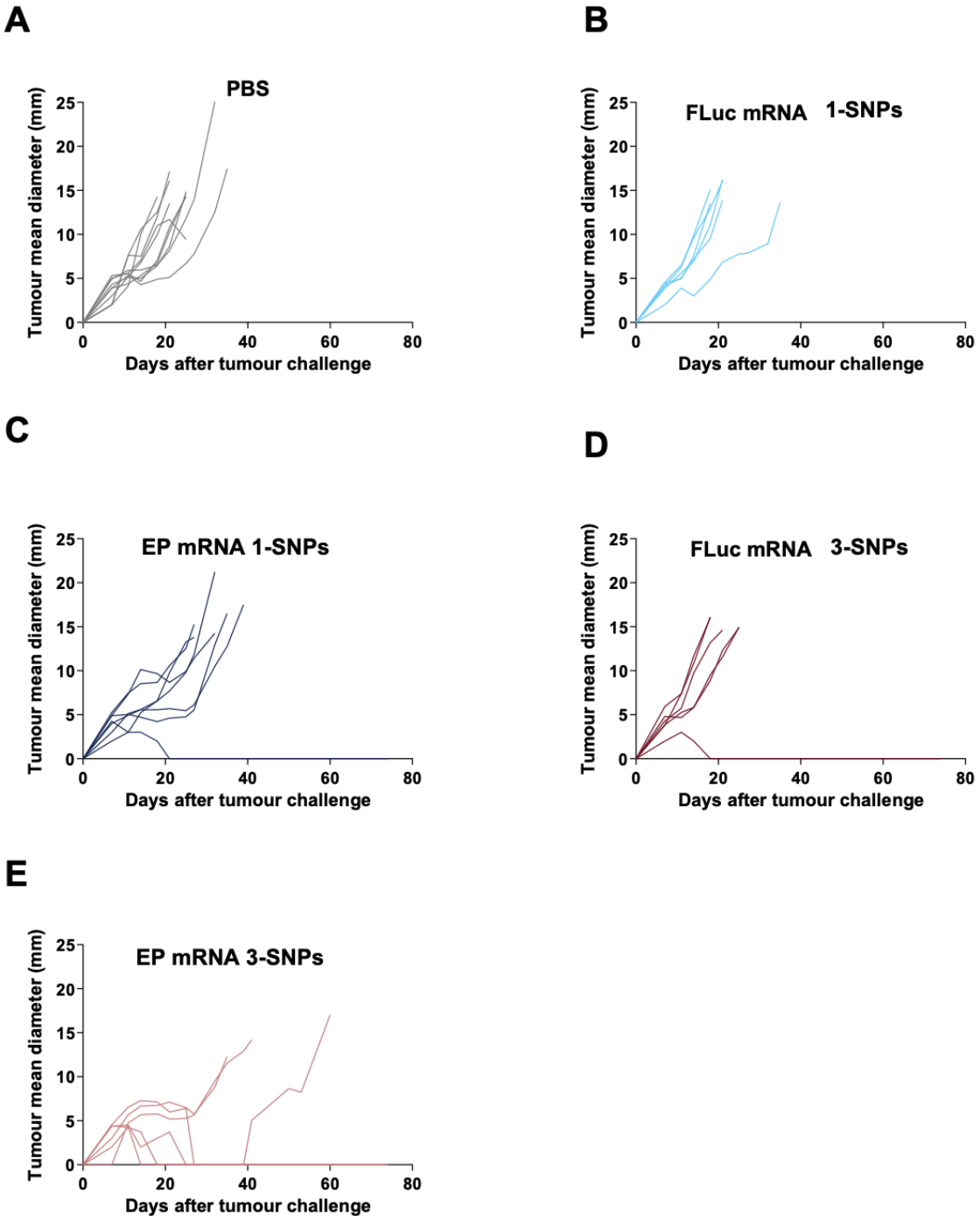


Figure S. 2. A) Mice weight monitorization in B16-OVA tumor models (N=4). B-F) Individual Follow Up for B16-OVA bearing mice. Statistical Analysis: Nonlinear regression, n≥3. (ns=0.1234; ****p<0.0001; ***p=0.0002; **P=0.0021; *p=0.033).

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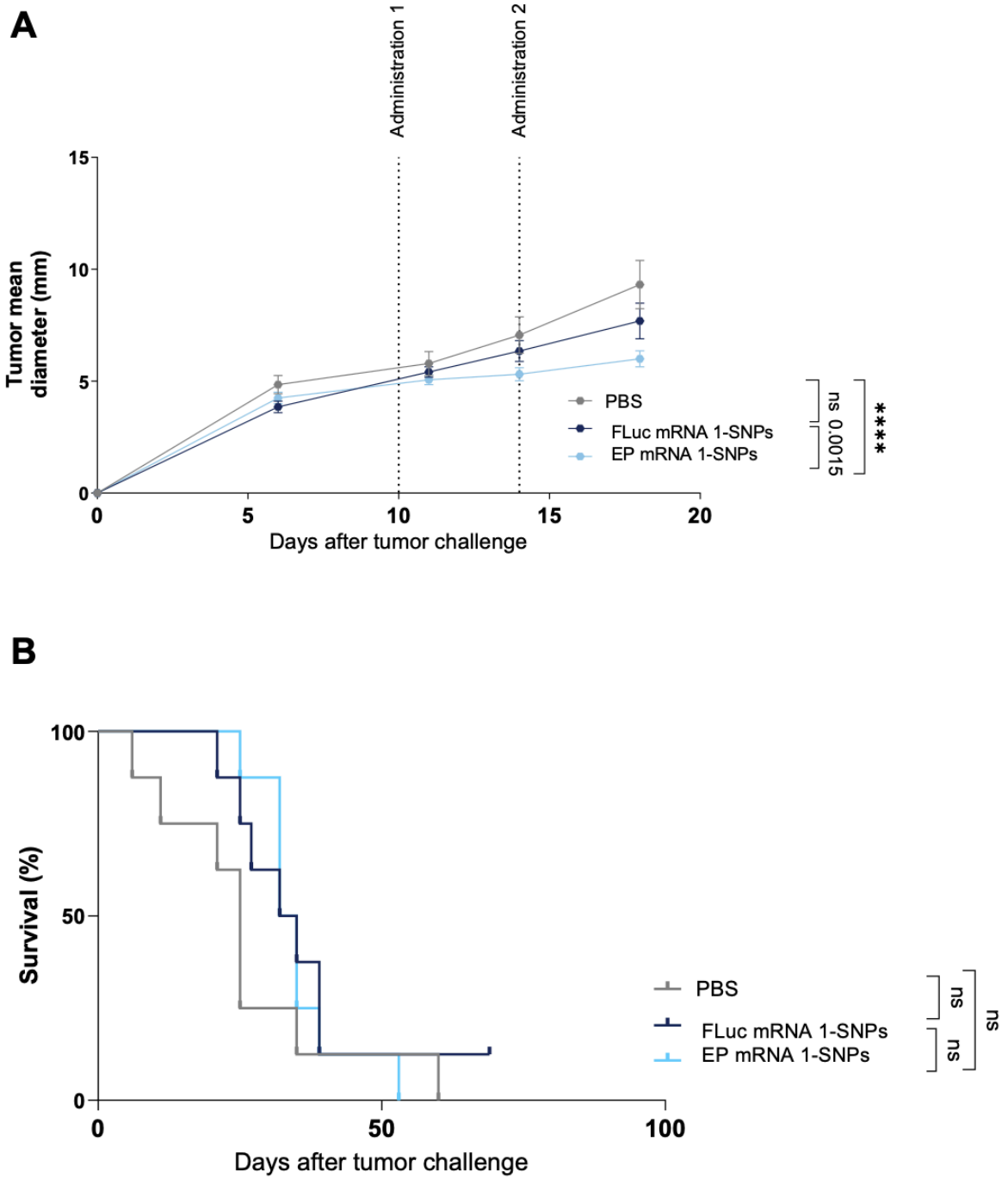


Figure S. 3. EP mRNA 1-SNPs exhibit a slight antitumoral effect in MC38 tumor bearing mice. A) Average data of tumor mean diameter (mm) over time and statistical analysis. Representation ends when the first individual of each group dies. B) Mice survival data follow-up is presented (N≥6/group). Mean ± SEM is presented in each graph. Longitudinal data were fitted to a third-order polynomial equation and compared using an extra sum-of-squares F test for (A). Survival data (B) were analyzed using Log-rank (Mantel-Cox) tests. (ns=0.1234, *p=0.0332, **p=0.0021, ***p=0.0002, ****p< 0.0001).

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Chapter IV

The potential of Biodegradable SNPs for tissue reprogramming

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ABSTRACT

Ageing is a complex process which is described as loss of functionality due to the accumulation of cellular damage, in a time-dependent way. Anti-ageing treatments have been studied for long, aiming to provide a solution to degenerative or cardiovascular diseases. In this space, tissue reprogramming has emerged in the last decades as a potential strategy for controlling age. Since the discovery of Yamanaka factors (Oct4, Sox2, Klf4 and cMYC), multiple laboratories have proven that it is possible to revert somatic cells to pluripotent cell-like states (iPSCs) by overexpression of such using plasmid DNA or viral vectors.

Nevertheless, the real translational approach of these therapies cannot be envisioned without the use of tools that can effectively control doses or expression time. In this sense, mRNA can be used as a tool to 1) express such factors transiently, 2) provide higher safety profiles, 3) control doses of every factor. However, mRNA needs to be engineered and deliver into vehicles so as to be available for intracellular delivery and *in vivo* administration.

Using Biodegradable SNPs reported in Chapter 2 (BD-SNPs), the work reported herein aims at providing a tool for delivering OSKM factors. First, the transfection capacity of these particles in three relevant cell models was evaluated with a reported mRNA: human fibroblasts and cardiomyocytes, and mice fibroblasts. Then, upon mRNA IVT synthesis, the encapsulation, physicochemical properties as well as *in vitro* capacity of transfection of SNPs for tissue reprogramming is evaluated. Results showed Oct4 and Sox2 are efficiently delivered by SNPs, however, such efficiency does not translate to efficient reprogramming. Future work aims to provide deeper insights on SNPs application to tissue reprogramming.

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INTRODUCTION

Ageing is a multifactorial process which entails the accumulation of cellular damage in a time-dependent manner, leading, thus, to a progressive loss of functionality and increased risk to death[1]. Nevertheless, ageing might not be as inevitable as previously thought [2–4], and several strategies, including manipulation of dietary intakes or administration of several drugs have been described to have an anti-ageing effect [5–7].

In principle, age-related cell phenotypes can be reversed, including in non-dividing and differentiated cells[8–10]. In fact, in 2012, Yamanaka's laboratory was awarded with Nobel Prize for indeed providing evidence of the possibility of turning somatic cells into pluripotency stem cells, namely induced pluripotency stem cells (iPSCs), in a process called dedifferentiation[11]. This study showed that the overexpression of four transcription factors using retroviral vectors, *i.e*. Oct4, Sox2, Klf4 and cMyc (OSKM factors or Yamanaka factors) was enough to induce somatic cells to a pluripotency stem-like state [11] . Indeed, this breakthrough opened the door to other works, showing that both *in vivo* and *in vitro* overexpression of such transcription factors (along with others) can convert multiple cell types to iPSCs [12–14]. The possibility of generating iPSCs *ex vivo* envisions therapeutics for regenerative medicine for diseases that are currently untreatable such as neurodegenerative, cardiovascular diseases or even cancer, as well as surpasses the ethical concerns of using embryonic cells for such treatments.

Nevertheless, while effective, translational applications of *in vivo* dedifferentiation remain challenging as sustained OSKM expression has been shown to trigger tumorigenesis [15–17] . In this scenario, cellular reprogramming has emerged as a recent strategy [4] for controlling age. Cellular reprogramming refers to the modulation of cell age and fate without function alteration, and it has been proposed as a novel strategy to target different diseases [1,4]. Thus, *in vivo* partial reprogramming (IVPR) aims to ameliorate the signs of aging while still preventing the loss of cell identity and the risks of induced pluripotency (dedifferentiation) [18] . This hypothesis states that by looking at intermediate states of dedifferentiation, a viable rejuvenation therapeutic application might exist [4,18,19] .

In fact, since the first IVPR proof of concept [20] , this strategy has been proven effective to extend lifespan and decrease hallmarks of ageing in multiple tissues and organs such as liver, skeletal muscle, skin or heart. For example, the reduced scar formation [21], the regeneration of skeletal muscle tissue [22] , the restoration of vision in old mice [23], the improved memory in mice [24] or the partial reprogramming of cardiomyocytes [25] .

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Yet, translational applications of such treatments can only be envisioned with tools that can effectively control doses, expression time and specific organ delivery. In this regard, majority of the work that has been performed so far has looked at overexpression of transcription factors using pDNA, with little control over specific transcription factors doses and ratios. In this space, messenger RNA (mRNA) technology could provide a much safer strategy, with transient expression of factors, as well as avoid the risk of intersectional mutagenesis that DNA therapies present. mRNA therapies have recently strengthened their translational application, with three marketed vaccines and many clinical trials ongoing [26–30]. Despite this, mRNA is a highly unstable and immunogenic molecule which needs to be engineered to allow for therapeutic potential. In this sense, two strategies have been proposed: 1) the chemical modification of the sequence, to allow for decreased immunogenicity and improved stability and potency[31–34]; and 2) the use of delivery vehicles to transport mRNA .

Actually, *in vitro* reprogramming using mRNA has successfully been performed before [25,35], nevertheless it has always been achieved with transfection reagents that cannot successfully be translated into relevant animal models or higher species. One study found that it was possible to reprogram old patient derived fibroblasts to a younger state [35]. Similarly, cardiomyocyte reprogramming was achieved with chemically modified mRNA [25].

Despite the huge advances in the field, lipid-based nanoparticles that could effectively and safely be used in tissue reprogramming field need to further comply with safety issues, as these treatments are envisioned with multiple administrations. As such, this Chapter aims to explore the use of BD-SNPs presented in Chapter 2 as an alternative for the development of the tissue regeneration field. By studying the transfection capacity of BD-SNPs on relevant cell models and the later transfection with OSKM factors, we report the effective delivery of Oct4 and Sox2 factors, pioneering the use of mRNA delivery approaches for this specific application.

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RESULTS AND DISCUSSION

1. BD-SNPs render great transfection capacity in both human fibroblasts and cardiomyocytes

To the purpose of this Chapter, these experiments were carried out at a specialized tissue reprogramming laboratory (NanoBioEngineering, Assistant Professor Irene de Lázaro, New York University) in a 4-month research stay.

The biodegradable formulation reported in Chapter 2, BD-SNPs, was selected for this specific application, due to the need to perform repeated administration, and the key aspect of fully preserving cell viability in this specific area or research. To this aim, the transfection capacity of 5moU-mCherry mRNA (997bases) loaded into BD-SNPs was assessed in several cell lines which have interest for tissue regeneration purposes, specifically human (HFF1) and mice (NIH/3T3) fibroblasts as well as human cardiomyocytes (AC10). For reference, cells were also transfected with alternative SNPs presented in Chapter 2 that are not biodegradable but show the highest transfection efficiencies.

BD-SNPs and SNPs (4-SNPs) were formulated following the same method reported in Chapter 2, and briefly described in the General Methodology Section. Both formulations exhibited similar sizes (~ 120 nm) and PDI (<0.15). Surface change measurement revealed neutral particles for both systems, nevertheless, BD-SNPs exhibited a slightly more negative zeta potential, in alignment with previous experiments (Chapter 2). Encapsulation efficiencies (EE %) revealed greater values for 4-SNPs , with > 85% for both prototypes, in alignment with previous works [36,37]. BD-SNPs exhibited slightly lower encapsulation capacity, also in alignment with previous experiments shown in Chapter 2, where the development and full characterization of both formulation is fully disclosed.

Next, the performance of BD-SNPs in delivering mCherry mRNA (Figure 2.D, 2.E) was studied. Representative pictures of transfections with 4-SNPs and the commercial reagent Messenger Max® are also presented in Figure S.2).Interestingly, BD-SNPs showed the most promising results in relation to 4 -SNPs and the control Messenger Max® in human cells, HFF-1 and AC10 (although no statistical significance was obtained, ns=0.1234). These results are remarkable, as cardiomyocytes are reported to be difficult-to-transfect cells [38].

On the contrary, 4 -SNPs overperformed BD-SNPs in mice fibroblasts (p=0.033), fact that could be associated with different selectivity and performance of SNPs across species. In fact, the performance of nanoparticles for mRNA delivery has been described to change depending on the cell type metabolism[39] as well as on species[40]. A high-thruput study comparing different nanoparticles across

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human, murine and non-human-primate's species, identified differences in delivery, highlighting the importance of choosing the correct models for translational applications[40].

Given these results, next reprogramming studies were performed with BD-SNPs. Similarly, HFF-1 cells were chosen due to the increased fluorescence signal observed, correlated with the increased amount of protein produced, and thus, with potency.

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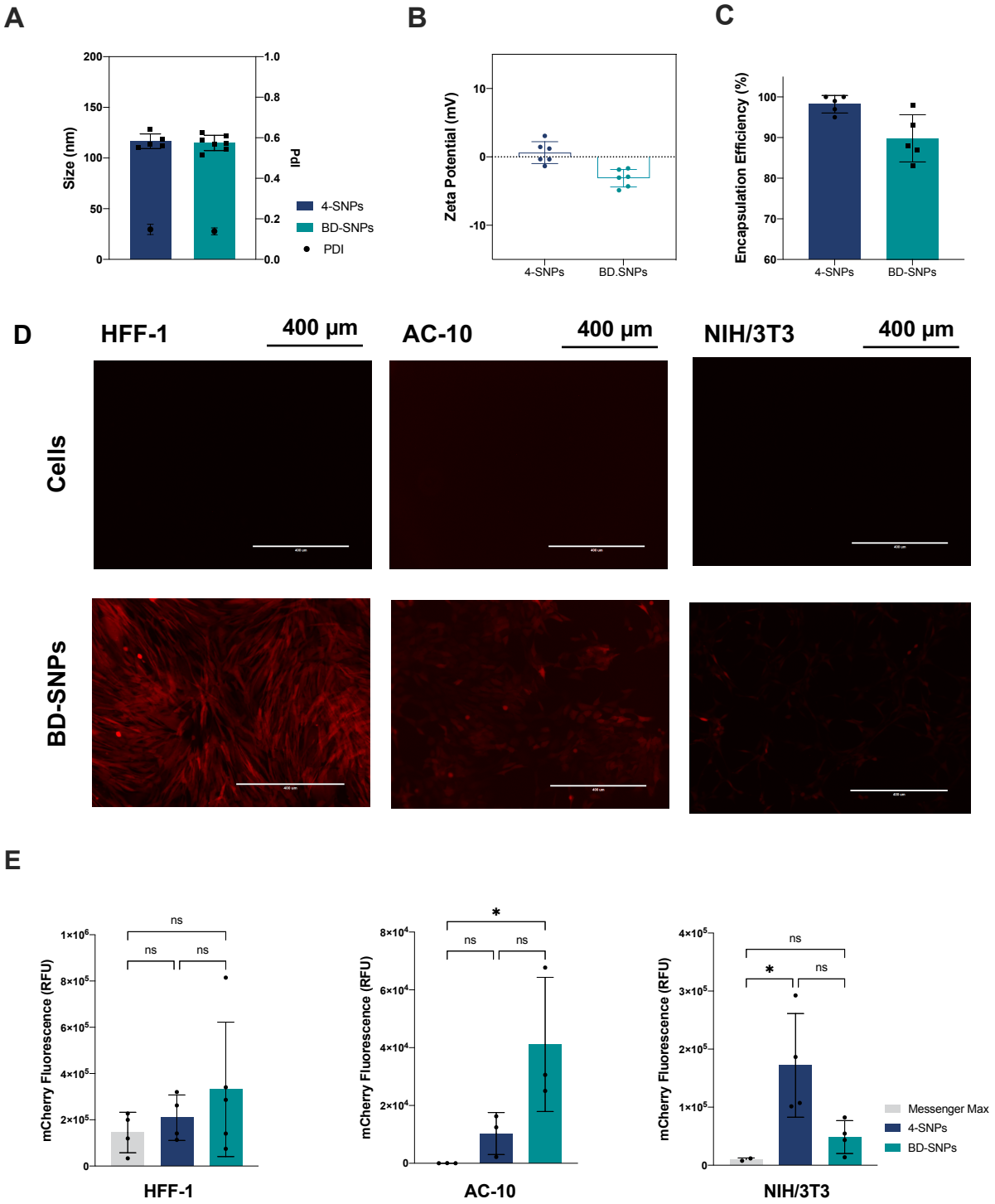


Figure 1. Physicochemical properties and *in vitro* performance of Biodegradable SNPs (BD-SNPs) in relevant cell models, compared to 4 -SNPs. A) Colloidal properties in terms of Size (nm) and Polydispersity Index (PDI) of at least five independent replicates (mean ± SD, n≥5); B) Zeta potential (mV) (mean ± SD, n≥5); C) Encapsulation efficiency (EE%) measured by Ribogreen Assay (mean ± SD, n=5); D) Representative microscope images of mCherry protein produced after 24h incubation with 100 ng of mRNA loaded BD-SNPs (1μg/mL mRNA concentration in a P96 well-plate). AC-10 images were adjusted with ImageJ post-acquisition to facilitate visualization in both control (cells) and treatment. E) Transfection efficiency (n≥2) in HFF1, NIH/3T3

and AC10 cells measured as fluorescence (Relative Fluorescence Units, RFU) produced after 24h incubation with 100 ng of mRNA loaded-SNPs (1µg/mL mRNA concentration in a P96 well-plate) and normalized to non-treated cells. Statistical analysis performed with One-way ANOVA ± SD of Log (Y) was followed by Tukey's multiple comparison test. (ns=0.1234; ****p<0.0001; ***p=0.0002; **P=0.0021; *p=0.033)

2. SNPs efficiently encapsulate OKSM-mRNA

The delivery of OKSM factors so far has been proven effective using plasmid DNA (pDNA) [22] as well as mRNA [25,35] . Nevertheless, the latter works have used either naked nucleic acids or commercial transfection reagents as delivery vehicles, which are known to be more toxic and untranslatable to higher species.

Based on previous cell transfection results, next experiments were aimed at validating whether the *in vitro* results with BD-SNPs (from now on, SNPs) for mCherry mRNA would be further translated to OKSM mRNAs, which were synthesized via IVT reaction (Figure 2.A). Briefly, the region of interest from each of the factors pDNA (Addgene) was amplified by PCR, using forward and reverse primers previously optimized. PCR product (Figure 2.B) was then cleaned-up and the *in vitro* transcription (IVT) reaction with the respective nucleotides was followed, after which the ethanol precipitation of the mRNA rendered the final product (Figure 3.B). Final quantification of mRNA was performed via UV absorbance using NanoDrop One (Thermofisher). Sequences were chosen based on previous experiments in the laboratory (data not shown) which aimed at screening different chemical modifications and study their transfection potential in HFF-1 cells for all mRNAs: Oct4 mRNA (O-mRNA) and Klf4 (K-mRNA), Sox2-mRNA (S-mRNA) and c-MYC (M-mRNA).

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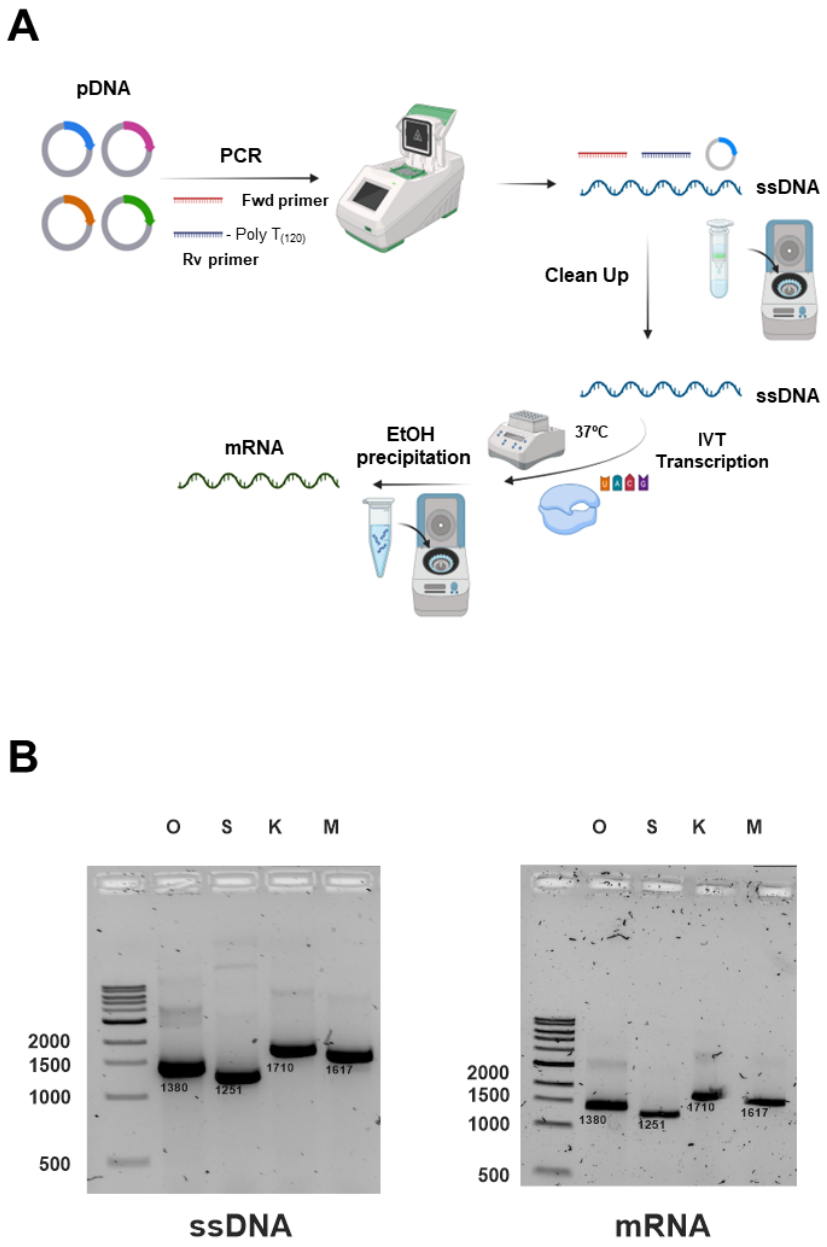


Figure 2. mRNA *In Vitro* Transcription (IVT) Synthesis. A) Schematic representation of the procedure is shown. B) Post-PCR product, ssDNA, and final mRNA product loaded in 1% agarose gel. OSKM stands for Oct4, Sox2, Klf4, c-Myc, respectively. The molecular weight of nucleic acid is shown in number of nucleotides.

SNPs loaded with OSKM factors (OSKM-SNPs, O-SNPs, S-SNPs, K-SNPs and M-SNPs) were formulated following the same procedure, ethanol injection method. All SNPs rendered similar colloidal properties, both in terms of size and PDI (Table 2). This is not surprising given previous results with mCherry and FLuc mRNA (Chapter 2), highlighting the versatility of these particles. Surface charge was slightly negative for all, in agreement with previous results with reporter mRNA sequences (Chapter 2, Figure 1.B), and encapsulation efficiencies surpassed 80% in all cases (Table 2). Similarly, no size

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changes were observed upon incubation at 37ºC, indicating stability of SNPs in the presence of serum proteins (Figure S.3). This is an important factor to study before *in vitro* experiments. Upon incubation, the proteins present in the FBS might drive the destabilization of the particles, or form the called protein corona, a phenomenon related to stability. As well, it has been reported to be crucial in controlling circulation time and extrahepatic targeting [41].

Table 2. Physicochemical characterization of OSKM-SNPs, loaded with OSKM. Mean and Standard Deviation (SD) of * 4 independent replicates (n=4). **Zeta Potential (ZP) of at least 3 independent replicates (n=≥3). #Encapsulation Efficiency (EE %) determined by Ribogreen® Assay (n=7).

	Size*		Zeta Potential**	EE%#
	0h	48h		
O-SNPs	96 ± 7	97 ± 10	-3.7 ± 1.8	87 ± 10
S-SNPs	96 ± 8	101 ± 8	-3,7 ± 6.9	86 ± 7
K-SNPs	104 ± 17	114 ± 20	-1.6 ±2.8	86 ±6
M-SNPs	104 ± 12	111 ± 12	-1.4 ± 4.2	80 ± 15

3. Reprogramming Experiments

Prior to the start of reprogramming experiments, we sought to assess the potential of SNPs in delivering each of the factors. We chose Oct4 and Sox2 for immunofluorescence and western blot analysis.

In vitro immunofluorescence performed after 24h with O-SNPs and S-SNPs revealed encouraging results, with both factors being expressed at a nuclear level, confirmed by the colocalization of blue (Hoechst, nuclei) and green (Antibody, factor) channels. Thus, the particles would efficiently be uptake by cells, delivered the mRNA in the cytoplasm, available for protein translation by the ribosomes. Then, the transcription factors would migrate to the nuclei, where they can be seen after 24h (Figure 3.A, 3.B). To further confirm the effective protein production, western blotting analysis using the same conditions was carried out. Again, the effective protein translation was confirmed (Figure 3.C) when O-SNPs and S-SNPs were incubated with the cells for 24h.

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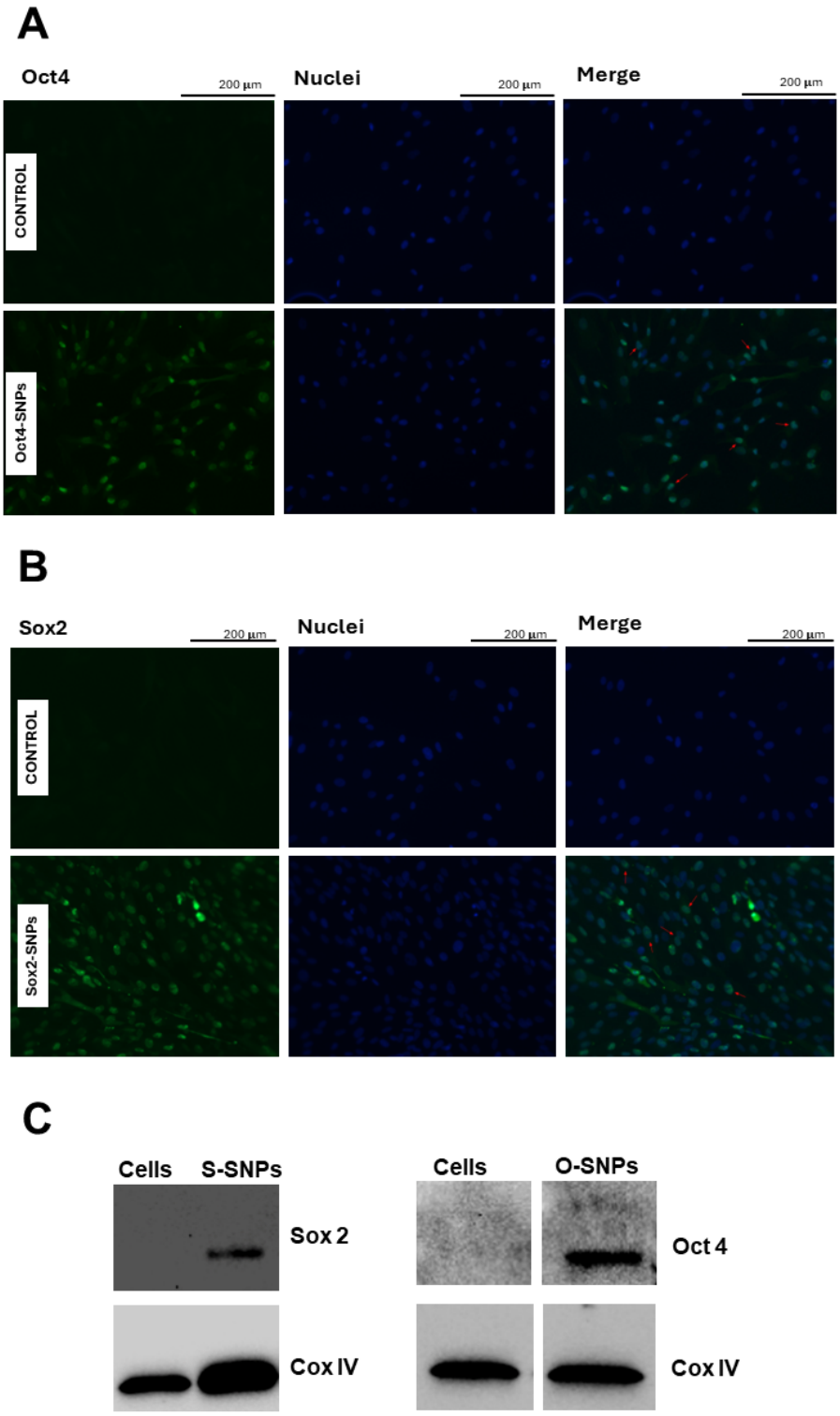


Figure 3. SNPs efficiently deliver Oct4 and Sox2 mRNA. A) Fluorescence microscopy images of A) Oct4 or B) Sox2 levels assessed by immunofluorescence after 24h of mRNA loaded SNPs dosing (1μg mRNA, 1mg/mL). mCherry loaded SNPs were used as negative controls. Nuclei was stained with Hoechst (Blue, 1:1000, 10 min). Green signal (secondary antibody) successfully confirms the presence of the transcription factors upon efficient translation. C) Western Blot Analysis confirming the successful formation of the proteins. COX IV was used as house-keeping gene. 40 µg of protein were loaded per line for O-SNPs and cells sample. 100 µg of protein were loaded per line for S-SNPs and cells sample.

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These results are particularly encouraging as, to the best of our knowledge, there is no reported study using nanoparticles to deliver Yamanaka Factors. As mentioned earlier, this is of vital importance as tissue reprogramming using mRNA cannot be envisioned without the use of a delivery vehicle. As such, upon overall positive results from physicochemical data with mRNAs and having proved the ability of the particles to transfect HFF-1 cells with mCherry mRNA, as well as having provided evidence of the Oct4 and Sox2 translation in such cells, reprogramming experiments were performed to assess the real potential of SNPs for tissue regeneration.

To this aim, a 16-day experiment was designed, with 8 administrations entailing five treatments (Table 3). We divided such treatments by ratios between factors and cell to mRNA ratio of OCT4. Finally, we also included a treatment with no cMYC, as it has been shown to be oncogenic and translational approaches of reprogramming would require its absence [42].

Table 3. Reprogramming conditions for 5 different conditions or treatments (T). Different ratios between factors as well as different doses (ng) were used.

	Ratios between factors	**Cell/mRNA Ratio of OCT4**	**OCT4 (ng)**	**SOX2 (ng)**	**KLF4 (ng)**	**cMYC (ng)**
T1	1:1:1:1	100	100	100	100	100
T2	1:1:1:1	400	25	25	25	25
T3	3:1:1:1	100	100	33	33	33
T4	3:1:1:1	400	25	8	8	8
T5	1:1:1	400	25	25	25	0

Over the course of the 16 days, we treated the cells every other day with fresh SNPs (up to 48h, as shown to be colloidally stable, Table 2) and monitored the cells morphology. Each of these treatments were performed with the respective controls, *i.e.,* the respective mRNA dose with a mock sequence (mCherry mRNA loaded SNPs). Results showed no differences in cell morphology over the course of the experiments (Figure 4.A). On day 13, 24h after the sixth administration, we observed morphological changes only in Treatment 3, nevertheless, they were also present in the mCherry controls (Figure 4.B). Such morphological changes were also present at day 16, on the last day of treatment. Fluorescence staining with Anti-SEEA1 antibody, a receptor present in iPSCs cells, was found negative. (Figure 4.C). Cytotoxicity evaluation post 8 administrations was also performed (Figure 4.D), with over 90% viability for all the treatments, highlighting the potential for repeated administrations of these biodegradable SNPs, in contrast with lipofectamine treatments which encompass really high toxicities even with small

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number of administrations. On day 18, western blot analysis was performed, with no protein production visualization (Figure 4.E).

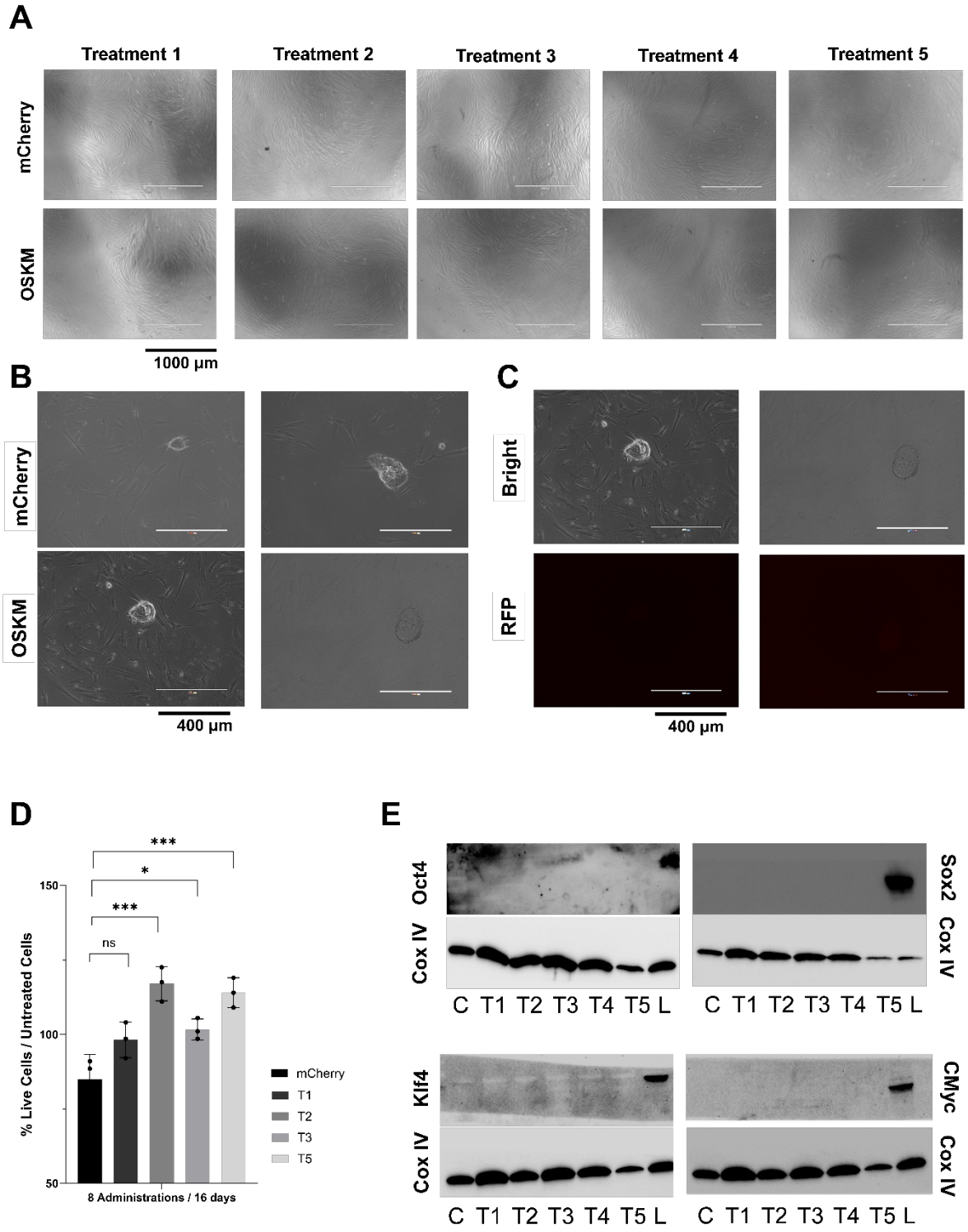


Figure 4. Reprogramming experiments using OSKM-SNPs. A) HFF-1 pictures after OSKM-SNPs administrations, where no morphological changes can be observed. B) Morphological changes observed at day 13 in T3 after 6 administrations. C) Anti-SEEA1 antibody staining negative signal. D) Lactate-dehydrogenase (LDH)

cytotoxicity assay of T1, T2, T3 and T5. E) Western Blotting analysis from samples at day 16. Messenger Max Lipofectamine (L) transfection reagent was used as a control, upon incubation of 24h with the cells. 80 μg of protein were loaded per line for all the OSKM-SNPs (T1, T2, T3, T4 and T5) and cells samples. For L, 100 μg of protein were loaded for Oct4, Klf4 and cMyc factors; and 50 μg for SOX2 factor. COX IV was used as house-keeping gene.

These negative results arise several questions. First, it remains to be understood if transfection with a reporter gene such as mCherry provide a good or enough correlation to what happens with other mRNA sequences. According to our experiments, mCherry loaded BD-SNPs efficiently deliver and translate mCherry protein in HFF-1 cells, comparable to Messenger Max (Figure 1). By contrast, we were able to see protein production with all the factors using this transfection reagent after 24h incubation (Figure 4.E). This would indicate that the secondary structure of mRNAs, at the same time presenting chemical modifications, could also impact formulation development.

Secondary, another question is brought up when considering that if the expression of the reprogramming factors is not maintained after the point-of-no-return (PNR), the cells would return to a somatic state [19]. Thus, according to that, even if the factors are indeed delivered, this treatment wouldn't be providing the factors often enough. In fact, our treatment encompassed 8 administrations over the course of 18 days. Nevertheless, other works with fibroblasts have reported everyday administrations over the course of four days [35]. More experiments need to be performed so as to drive conclusions of the real potential of SNPs to reprogram cells.

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CONCLUSIONS

Tissue reprogramming is a novel mRNA application which can surpass the drawbacks of pDNA therapies in tissue regeneration space. However, little has been performed with mRNA sequences, and nothing has been reported yet using lipid-based nanoparticles.

In this work, the use of a totally biodegradable nanoparticle, BD-SNPs, for delivery of Yamanaka Factors is presented. We proved that BD-SNPs exhibited great colloidal properties and encapsulation efficiencies with OSKM factors and that could efficiently lead to the expression of Oct4 and Sox2 factors in HFF1 cells. Upon reprogramming experiments, results showed no signals of reprogramming or morphology changes, underlaying the small correlation between transfection experiments with the reporter gene and reprogramming experiments. Next experiments will need to be designed to study in depth how such factors are delivered using BD-SNPs as well as different administrations treatments.

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MATERIALS AND METHODS

mRNA Sequences

CleanCap FLuc mRNA (Ref. L-7602) was purchased from TebuBio (Barcelona, Spain). CleanCap® mCherry mRNA (5moU) - (L-7203) was purchased from TriLink Biotechnologies (San Diego, US). OSKM mRNA sequences were synthesized starting from a pDNA encoding each of the factors (Derrick Rossi, Addgene plasmid # 26815, # 26816, # 26817, # 26818, # 26823). Region of interest was amplified using Tail PCR (Reverse Primer encoding for PolyA region) using Phusion™ Plus PCR Master Mix (Thermo Scientific™). The PCR reaction steps are detailed in next Table Extension length was dependent on sequence length, with 30s/1000 bases.

Cycle step	Temperature	Time	Cycles
Initial Denaturation	98°C	5 min	1
Denaturation	98°C	10 s	35
Annealing	67°C	10 s	
Extension	72°C	33 s	
Final extension	72°C	5 min	1
	4°C	Hold	Hold

The reaction yield was checked by 1% agarose gel. The ssDNA obtained was then purified using DNA Clean and Concentrator (Zymo Research) according to manufacturer's instructions. Final concentration was quantified using Nanodrop (Thermofisher). In Vitro Transcription (IVT) Reaction was performed using HiScribr^R T_7 mRNA Kit with CleanCap^R Reagent AG (New England Biolabs) according to manufacturer's instructions. For sequence modification, modified nucleotides 5-Methylcytidine-5'-Triphosphate (5-mC) or/and 5-Methoxyuridine-5'-Triphosphate (5-moU) were incorporated to the reaction instead of the unmodified ones. The reaction was stopped after 4h with DNAse-I incubation for 15 minutes. The final mRNA product was quantified using Nanodrop and sequence integrity was assessed by 1% agarose gel.

SNPs Formulation

BD-SNPs and 4-SNPs are formulated as previously described (Chapter 2).

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Encapsulation Efficiency

Encapsulation efficiency (%) was assessed by Quant-iT™ RiboGreen™ RNA Assay Kit (Invitrogen) according to manufacturer instructions. Briefly, a calibration curve in Tris-EDTA (TE) buffer was prepared at 100, 50, 25, 12.5, 6.25, 3.12 ng/mL. SNPs were diluted 500-fold in TE and measured in a Black Bottom Greiner P96well plate using VICTOR Nivo Microplate Reader (PerkinElmer). Encapsulation efficiency (%) was calculated by subtracting the amount of mRNA present in TE samples (unencapsulated) to the mRNA amount loaded (total) in the SNPs, following the equation:

$$Encapsulation\ Efficiency\ (\%) = \frac{Loaded\ mRNA - Unencapsulated\ mRNA}{Loaded\ mRNA} x\ 100$$

Nanoparticle characterization by DLS

SNPs were characterized for size and homogeneity using DLS and NTA. SNPs were characterized for size (Z-average, nm) and surface charge (Zeta Potential, mV) using a Zetasizer Pro (Malvern Instruments, Malvern, UK) using disposable microcuvettes (ZEN0040, Bran), after 1/10 dilution in PBS. Number of replicates is indicated in the text, according to each experiment. Surface charge was analyzed by measuring zeta potential by Laser Doppler Anemomerty (LDA), after 90-fold dilution in 10mM NaCl in Folded capillary cuvettes (DTS 1070, Malvern Instruments). For cell culture media stability, SNPs were diluted 1/10 to 2 μg mRNA/mL and incubated with 15% FBS DMEM culture media at 37ºC for one hour.

Cell lines and tumor mouse models

HFF1, AC-10 and NIH/3T3 were obtained from ATCC. HFF-1 cells were harvested in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Waltham, MA) supplemented with 15% fetal bovine serum (FBS) (Omega Scientific, Catalog #: FB-01), and 1% Penicillin/Streptomycin (PenStrep, 15140-122, Gibco). AC-10 were cultured in DMEM:F12 Medium (Gibco) with 12,5% FBS (Omega Scientific, Catalog #: FB-01). NIH/3T3 were cultured in DMEM, 10% FBS (Omega Scientific, Catalog #: FB-01). All cells were maintained in a humidified incubator at 37ºC and 5% CO_2. These cell lines were routinely tested for mycoplasma contamination.

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Transfection experiments

For mCherry mRNA SNPs testing, HFF1 (10.000 cells/well) cells were seeded in a black-P96 (ThermoFisher Scientific) and allowed to adhere overnight prior to SNPs dosing. Next day, SNPs were dosed in, at least, triplicates (n=3). Messenger Max was used as a control, in at least, two independent replicates (n=2)After 24h, transfection efficiency was assessed with Microplate Reader at λ_{Exc}=587nm and λ_{Em}=610nm. The same plates were visualized under the fluorescent microscope.

Western Blotting

Cells were detached using 0,25% Trypsin (Gibco), pelleted and then incubated in RIPA Buffer (RIPA containing protease inhibitor) for 20 minutes on ice. Then, cells were centrifuged at 14000g 30 min at 4ºC, and the supernatant was collected. Total protein quantification was performed using BCA assay (Pierce™ BCA Protein Assay Kits, Thermofisher). Respective amount of total protein (stated in each experiment), Laehmli Buffer (Biorad) and 1X DDT was loaded onto Precast-Gels (Mini-PROTEAN® TGX™ Precast Gels, BioRad) and ran at 200V for 25 minutes using 1x Tris/Glycine/SDS running buffer (10x Tris/Glycine/SDS, BioRad). Gel transfer to nitrocellulose membrane was performed using Trans-Blot Turbo RTA transfer kit (BioRad) and TransBlot Turbo (BioRad) for 30 minutes. Membrane blocking was performed with 5% Milk Powder in PBS for 1h at RT. Primary Antibody (Cell Signaling, #2750, #3579, #4038, #5605) incubation was performed for 2h at RT and Secondary Antibody linked to HRP (Cell Signaling, Anti-Rabbit IgG, HRP-linked #7074), was done for 1h at RT. Post washing, ECL (Clarity Western ECL Substrate, BioRad) was incubated for 5 minutes with the membrane and read using Chemiluminescence (Chemidoc, BioRad).

Immunofluorescence

Cells were seeded on a P8 well chamber (Thermofisher) and left to adhere O/N. Next day, cells were treated with O-SNPs and S-SNPs. After 16h incubation, cells were washed with DPBS (3x5 min), fixed with 4% PFA (10min) and then washed with DPBS again (2x5min). After drying, cells were blocked with 10% Rabbit Serum-DPBS for 1h 30 min. After incubation, the serum was removed, the primary antibodies (Cell Signaling, #2750, #3579) was added in 10% Rabbit Serum-DPBS and left O/N at RT. Next day, cells were washed with 0.1%Tween-PBS (3x5min) and the Secondary Antibody (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488) was incubated in 0.1%Tween-PBS for 1h at RT. After incubation, cells were washed again with 0.1%Tween-PBS, stained

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with DAPI (1:1000, 5 minutes) and then washed again (2x10min). Upon drying, the preparation was mounted using a coverslip and visualized under fluorescent microscope.

Reprogramming Experiments

10.000 HFF-1 cells were seeded in a P24-well plate (Thermofisher) on day 1. SNPs administration was performed every other day for 16 days, with a total number of administrations of 8. On day 5, cells were transferred to P6 well-plates when they reached confluency, where they were kept until the end of the experiment. Doses and mRNA ratios are depicted in Table 4. At the end of the experiment, cells were detached, and western blot was performed. LDH cytotoxicity assay was also performed using the culture media as per manufacturer instructions.

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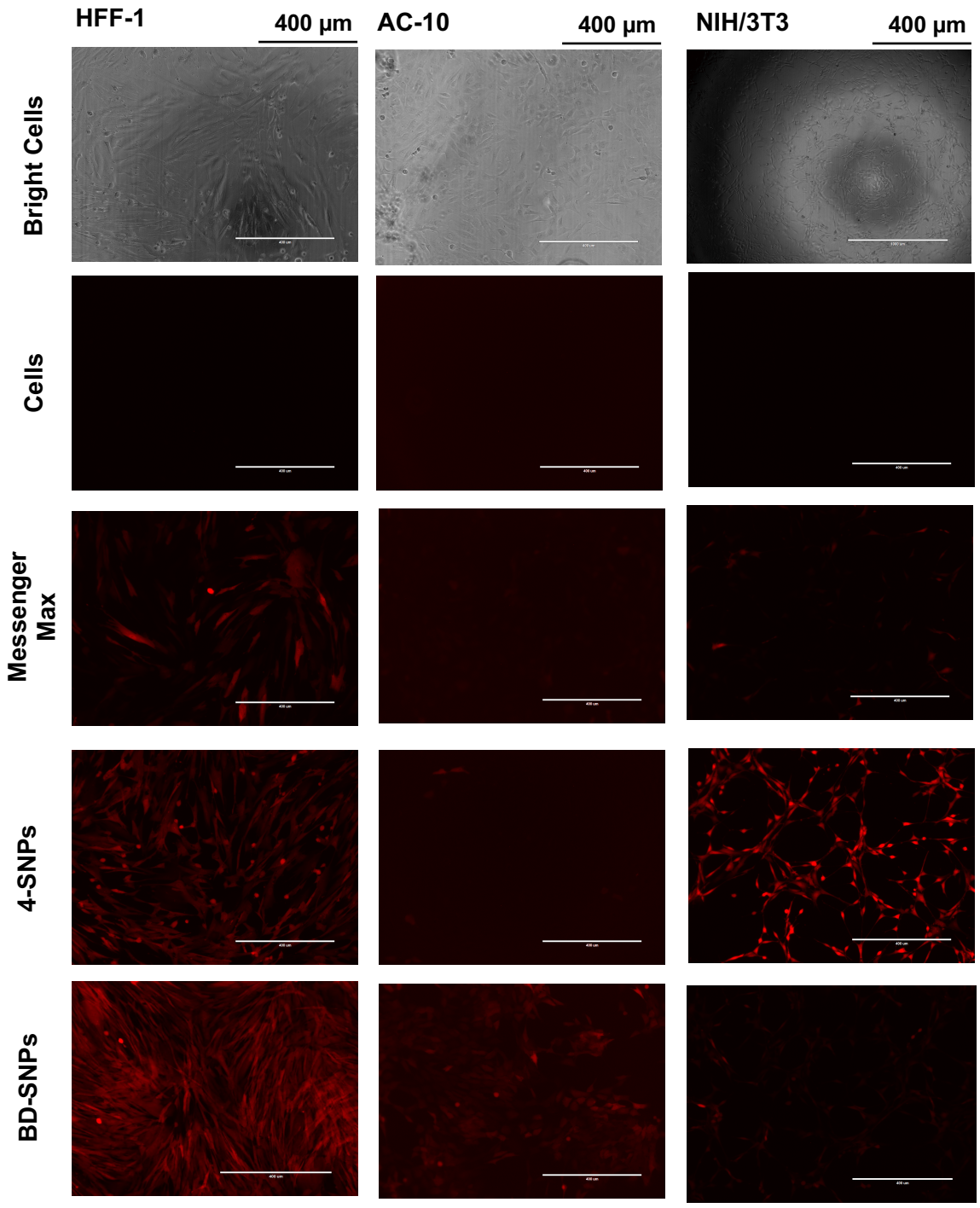


Figure S.2. Representative microscope images of mCherry protein produced after 24h incubation with 100 ng of mRNA loaded-SNPs (1μg/mL mRNA concentration in a P96 well-plate). BD-SNPs and 4-SNPs were used for transfection in human fibroblasts (HFF1), human cardiomyocytes (AC-10) and mice fibroblasts (NIH/3T3). Messenger Max® (Invitrogen) was used as a positive control. AC-10 images were appropriately adjusted with ImageJ post-acquision to facilitate mCherry visualization. Such changes were also performed in Control samples.

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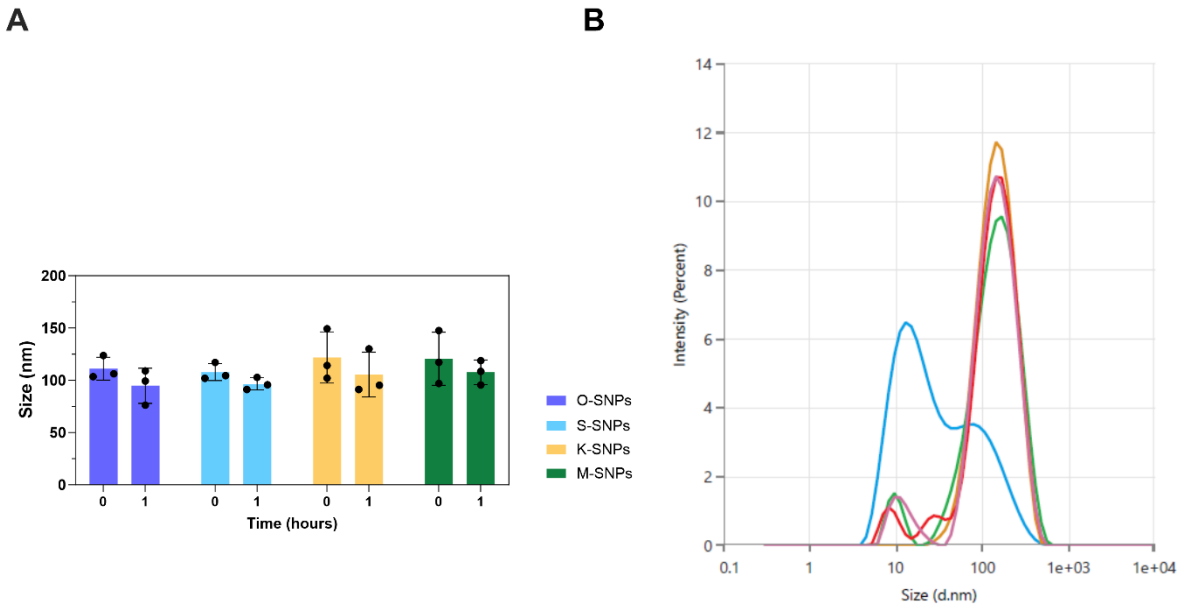


Figure S.3. Cell culture media stability of OSKM mRNA SNPs at 37ºC for 1h. Size was assessed by DLS measurement, considering only SNPs peak and not media alone (B, blue line).

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Overall Discussion

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OVERALL DISCUSSION

Messenger RNA (mRNA) is a single strand biopolymer which is responsible of translating the information encoded in the DNA, enabling protein synthesis. Its use as a therapeutic agent relies on its cellular location and function, providing important advantages over other genetic therapies. Its higher safety profile, easier delivery, high versatility and improved industrial scalability has converted mRNA to one of the modalities with higher potential to tackle future challenges in modern medicine[1]. Nevertheless, its pharmaceutical use is limited by its physicochemical properties. Large and negatively charged, exogenously and naked administered mRNA triggers important immunological reactions, it is not capable of crossing cell membranes and presents a short half live.

Since its discovery in 1961[2], researchers have proposed two main strategies to address these important disadvantages. Such approaches consist of chemically modifying mRNA sequences in order to avoid immune activation and increase *in vivo* potency [3,4], and designing vectors which can effectively transport mRNA to the target site[5,6].

Lipid Nanoparticles (LNPs) have evolved from liposomes to become the most clinically advanced delivery vehicle to date. In fact, all the marketed products rely on their use[7,8]. LNPs success has been strongly driven by the discovery of ionizable lipids[9], lipids which are protonated only at acidic pH, allowing for the formation of neutral particles which can circulate uncharged in the bloodstream, but which become protonated at acidic environments (like the one in the endosome), permitting the release of encapsulated mRNA. As such, most studied LNPs are composed of an ionizable lipid, in charge of encapsulation and potency; cholesterol and phospholipids (typically DSPC or DOPE), which provide rigidity and structural functionalities, and a PEG-lipid derivative, which stabilizes the particles [10]. Despite the clinical and preclinical success of LNPs, there is still room for studying new components *en route* to develop innovative delivery vehicles. Furthermore, the existence of patents and intellectual property considerations, limit their use.

In this context, this thesis aimed at developing an innovative type of nanoparticle, SNPs, able to efficiently deliver mRNA. This work was built upon the previous work carried out in the laboratory with SNs, which demonstrated to be a versatile delivery vehicle[12–14], easy to formulate and efficient for pDNA [15] and miRNA delivery[16,17]. Thus, SNPs development and characterization, optimization and evaluation of their efficacy, is fully described in the present dissertation.

The use of novel materials that have been only marginally explored for nucleic acid delivery, is of vital importance for the field, as a way to deepen the understanding on mRNA delivery systems. As well, there is is a need to find solutions for improved tolerability and biodegradability. In this context,

Chapter I aimed at exploring the study of new lipids and how variations in composition modify performance.

Interestingly, SNs, used as a reference, exhibited higher *in vitro* potency but SNPs remarkably overperformed SNs- *in vivo*, both after intraperitoneal (IP) and intravenous (IV) administration, underlaying the poor *in vitro* in vivo (IVIV) correlation that exists in mRNA nanomedicines, which has been previously reported[23].

These promising results with SNPs highlighted the potential of the follow-up strategy. Changes in components' ratios and amounts that drive SNPs performance were next explored, and four initial SNPs prototypes were fully characterized and assesed: 1-SNPs, 2-SNPs, 3-SNPs and 4-SNPs.

Physicochemical properties, *in vitro* and *in vivo* potency as well as functional delivery (*i.e.* production of the encoded protein in specific organs) of the prototypes highlight how the SNPs properties can be tuned by carefully modifying composition. Colloidal properties (size and particle concentration) were found to be directly impacted, some SNPs rendering larger nanoparticles (~190nm) with a much lower concentration in the nanoparticle suspension (~10^{11} particles/mL) (1-SNPs). As opposed, different SNPs (3-SNPs and4-SNPs) showed tremendous improvement in physicochemical properties (100 nm) and particle concentration (~6x10^{12} particles/mL). Additionally, in the case of ,1-SNPs, NTA data also showed higher heterogeneity. These results highlight the fact that not only choosing appropriate materials is important, but the ratios and formulation processes can definitely have an impact on formulation properties.

Aiming to study how such changes might impact *in vitro* studies, both uptake and transfection experiments were carried out. Uptake results with flow cytometry showed more than 90% of cells were positive, further confirmed by confocal microscopy. By contrast, *in vitro* studies in HEK293 and HEPG2 cells showed a remarked improvement in transfection for 3-SNPs and 4-SNPs prototypes, as opposed to what was observed for cell uptake. Nevertheless, cargo delivery from uptake to protein translation is a complex process which entails endosomal escape. This process, which is responsible for the sequestration of more than 90% of the encapsulated cargo [26], is the main bottleneck for nucleic acid delivery using nanoparticles. In this experiment, flow cytometry analysis showed that all prototypes are equally uptaken by cells after 24h, nevertheless, this study was performed with labelled-lipids and it only looks at SNPs entry to the cell, without properly assessing the endosomal escape and release of the cargo to the cytoplasm. Differences in endosomal escape might explain differences in protein levels translation, however, specific trafficking experiments should be carried out to further elucidate the reasons behind [27–29].

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With these results, *in vivo* potency after intravenous (IV) was next assessed, showing again a dramatical overperformance of 3-SNPs and 4-SNPs.. Importantly, these two prototypes successfully achieved protein levels compared to Onpattro® LNPs composition, marketed for liver delivery. Additionally, SM-SNPs overperformed JetPEI (*in vivo* JetPEI*,* Polyplus*)* protein levels, a commonly used transfection reagent and a slight decrease as compared to Moderna LNPs..

Ex vivo studies demonstrated the successful protein production in the liver for all of the prototypes, in alignment with previous SNs [31,32] data and most of LNPs [6]. However, one of the main focus of the field nowadays is the delivery to other organs other than the liver. In this sense, all SNPs showed preferential liver protein production, especially 1-SNPs (93%). Nevertheless, results also showed a shift in functional delivery towards the spleen for 2-SNPs, 3-SNPs and 4-SNPs, as compared to Onpattro® and 1-SNPs. Interestingly, despite their differences in size, 2-SNPs and 4-SNPs exhibited 30% of protein production in this organ. This data elucidates the possibility of achieving lower liver delivery without the inclusion of a fifth component, as suggested before[33] . Interestingly, important changes in functional delivery upon intraperitoneal (IP) administration of 2-SNPs and 3-SNPs were observed, with 70% liver protein production for 2-SNPs and 40% for 3-SNPs. Indeed, this might be explained by the differences in extracellular fluids surrounding peritoneum area versus the blood circulating proteins[34], which could potentially change biomolecular corona and, thus, SNPs fate, as reported for LNPs[35].

Overall, these data highlight the changes that can occur when lipids are used in different ratios as well as the differences in delivery upon different administration routes, describing new insights for future formulation development.

Expanding on these encouraging results, the development of enhanced formulations that are able to overcome current drawbacks in the field was presented in Chapter 2: the removal of PEG-lipids and development of vehicles with higher safety profiles, and the targeted delivery to different organs other than the liver. The systematical substitution of the lipids used so far for biodegradable alternatives rendered fully biodegradable SNPs (BD-SNPs), which were then tested *in vitro* and *in vivo*. With decreased potency than original SNPs, BD-SNPs exhibited a 49% spleen protein production. suggests that .

Furthermore, the addition of a fifth component to SNPs demonstrated that it is possible to achieve greater protein production in desired organs. When a positive cationic lipid, DOTAP, was included to SNPs (P-SNPs), the bioluminescence achieved in the lungs remarkably improved, with 3-times more percentage of total dose in this organ. When negatively charged phospholipid was included (18:1PA, N-SPs), the organ delivery to the spleen shifted away from the liver to achieve a 44%, with no significant

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increase in potency in this organ, highlighting spleen specificity. Remarkably, improved SNPs containing achieved potencies as high as Moderna LNPs (Spikevax® formulation).

So far, the work from Chapter I and II underlines the fact that SNPs are a versatile platform which can be tuned in terms of size, composition, potency and organ delivery, proving their potential as an approach for mRNA delivery.

Next Chapters, Chapter III and IV, aimed at exploring efficacy of SNPs in the context of specific biomedical applications. As such, the work presented in Chapter III aimed at investigating the potential of SNPs for an innovative approach in mRNA cancer therapy. This strategy involves the administration of immunomodulatory proteins in the form of mRNA and their secretion to the systemic circulation after liver transfection [38], using the liver as a factory for generating therapeutic molecules on-site [39,40]. For this work, SNPs which demonstrated preferential liver accumulation in Chapter I were chosen. Results from a first kinetic experiment with 1-SNPs and 3-SNP showed the production of the immunomodulatory encoded protein (EC), with serum levels detectable after 96h post-administration for 3-SNPs and 72h 1-SNPs. These levels are in alignment with potency described by FLuc mRNA SNPs in Chapter I, showing a good correlation with therapeutic mRNA.

Upon efficacy studies on B16-OVA tumor bearing mice, results showed relevant differences between formulations. 3-SNPs exhibited dramatically better results, both in terms of antitumoral effects and survival. 3-SNPs induced an important antitumoral effect as compared to both PBS, mock mRNA sequence and therapeutic mRNA loaded 1-SNPs. Importantly, this formulation induced tumor regression in 50% of the individuals. By contrast, 1-SNPs induced tumor growth delay, nevertheless, this did not translate to improved survival, as it did for 3-SNPs, with 100% survival after 35 days.

The differences in formulation efficacy among the two prototypes tested can indeed be explained and correlated to previous data. First, colloidal NTA and DLS data showed better physicochemical properties of 3-SNPs, with increased number of particles, and thus, potentially, delivering more mRNA molecules [44,45]. Nevertheless, other experiments such as confocal spectroscopy should be carried out in order to understand SNPs loading capacity [46]. Second, *in vitro* transfection potency showing 3-SNPs overperformance was effectively translated to *in vivo* potency, both with FLuc mRNA and therapeutic mRNA, contrary to reports reporting low *in vitro-in vivo* correlation[23]. Overall, these efficacy results highlight the potential of SNPs for the delivery of immunomodulatory proteins, with future work envisioning co-delivery of these type of proteins or other therapeutic molecules by using the liver as a factory[38]. Furthermore, they highlight the importance of formulation preclinical development to achieve good therapeutic outcomes, especially important in the field of nanomedicine[47].

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In Chapter IV, we aimed at investigating the use of BD-SNPs for tissue regeneration, a novel mRNA application[48]. First, the transfecti on efficiency capacity of BD-SNPs in three relevant cell models was assessed, mice and human fibroblasts cardiomyocytes and mice fibroblasts. BD-SNPs were able to transfect all cell types, with improved results in human fibroblasts. Next, by effectively encapsulating Yamanaka Factors (Oct4, Sox2, Klf4, cMyc mRNA, OSKM-mRNA sequences), the first evidence of the use of biodegradable nanoparticles for tissue reprogramming was reported. All formulations formed successfully, with no major differences in colloidal properties between factors. Immunofluorescence and western blotting transfection experiments showed the effective protein translation and effective transport to the nucleus, shown by colocalization of factor and nuclei staining signals. Final reprogramming experiments with 8 administrations over the course of 16-days showed no cytotoxicity, proving the low toxicity of our approach. Nevertheless, no reprogramming effect was achieved, with further experiments to be performed to fully elucidate the causes of such. Overall, this chapter provides the fully development of biodegradable SNPs and their *in vitro* and *in vivo* transfection capacity, opening new doors for future applications. Moreover, the negative results of reprogramming experiments arise several questions important to tackle when designing new lipid vectors, such as the correlation of reporter genes with actual therapeutic molecules, the use of the same delivery vehicle with different sequences and whether this is the right strategy to follow for efficacious and translational approaches.

Altogether, this thesis provides the following general achievements, 1) the successful development of a new type of delivery particle for mRNA, SNPs, with improved performance as compared to the previously reported and patented technology (SNs); 2) the enhanced performance of SNPs obtained when varied components,; 3) the effective modulation of targeted delivery upon intravenous and intraperitoneal administration; 4) the effective proof of an immunomodulatory protein biosynthesis following mRNA loaded SNPs administration, providing proof of hepatic delivery of immunomodulatory proteins; 5) the success of efficacy experiments for loaded SNPs for inhibiting tumor growth in B16 OVA mice models; 6) the development of totally biodegradable SNPs with high potencies *in vitro* and *in vivo*; 7) the effective SNPs formulation with Yamanaka factors mRNA sequences, and effective production of Sox2 and Oct4 transcription factors, not reported before.

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Conclusions

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CONCLUSIONS

The experimental data obtained from this thesis led us to withdraw the following conclusions:

1. A new type of nanoparticles was successfully developed and fully characterized. It has been proven the ability of these nanoparticles to associate and efficiently deliver a wide variety of mRNA sequences.

2. Physicochemical properties, *in vitro* and *in vivo* performance of the developed nanoparticles can be modulated by adjusting the composition without increasing the complexity.

3. *In vivo* functional delivery of the developed nanoparticles can be tuned to different organs, providing important insights towards targeted therapeutics.

4. The substitution of the PEG-derivative together with the inclusion of bioreducible and biodegradable lipids led to the development of biodegradable nanoparticles with high potencies *in vivo*.

5. The proved therapeutic efficacy of the nanoparticles loaded with mRNA and delivered to the liver, confirmed the potential of this novel formulation and the described strategy for the development of immunomodulatory therapies to treat distal tumors.

6. The developed nanoparticles have demonstrated ability to transfect human fibroblast and cardiomyocytes, and to deliver mRNA encoding transcription factors to human fibroblasts, opening new opportunities for tunning cell reprograming in tissue regeneration.

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Glossary

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GLOSSARY

A.U.	Arbitrary Units
AAV	Adeno-Associated Virus
Ab	Antibody
ADC	Antibody Drug Conjugate
APCs	Antigen Presenting Cells
ApoE	Apolipoprotein E
ASOs	Antisense Oligonucleotides (ASOs),
BD	Biodegradable
CAR	Chimeric antigen receptor
CF	Cystic fibrosis
CFTR	Cystic fibrosis conductance receptor
COVID 19	Coronavirus Diseases 2019
CRISPR	Clustered regularly interspaced short palindromic repeat
CVM	Cytomegalovirus
DLS	Dynamic Light Scattering
DPBS	Dulbecco's Phosphate Buffer Saline
dsDNA	Double Stranded DNA
EE %	Encapsulation Efficiency
eGFP	Enhanced Green Fluorescence Protein
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EPR	Enhanced Permeation and Retention Effect
EtOH	Ethanol
FACs	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
FLuc	Firefly Luciferase
GMP	Good Manufacturing Practices
hATTR	Hereditary transthyretin-mediated amyloidosis
HE	Hereditary angioedema
HGB	Hemoglobin

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HMPV	Human Metapneurovirus and Parainfluenza Virus
ID	Intradermal
IM	Intramuscular
IN	Intranasal
IP	Intraperitoneal
iPSCs	Induced pluripotent cell-like states
IT	Intratumoral
IV	Intravenous
IVIV correlation	*In vivo-In vitro* correlation
IVPR	*In vivo* partial reprogramming
LDH	Lactate dehydrogenase
LDLR	Low Density Lipoprotein Receptor
LNPs	Lipid Nanoparticles
LPX	Lipoplexes
MCH	Major histocompatibility complex (MHC)
mCherry	Monomeric Cherry Protein
MPS	Macrophage Phagocytic System
mRNA	Messenger RNA
N/P	Nitrogen / Phosphate
NEs	Nanoemulsions
NIAID	National Institute of Allergy and Infectious Diseases
NK Cells	Natural Killer Cells
nm	Nanometer
NTA	Nanoparticle Tracking Analysis
O/N	Overnight
ORF	Open Reading Frame
OSKM	Oct4, Sox2, Klf4, cMyc
OVA	Ovoalbumin
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDI	Polydispersity Index
pDNA	Plasmid DNA
PLT	Platelets

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PNR	Point-of-no-return
RBC	Red Blood Cells
RES	Reticuloendothelial System
RFP	Red Fluorescence Protein
RNAses	Ribonucleases
RSV	Respiratory Syncytial Virus
RT	Room Temperature
saRNA	self-amplifying RNA
SAXS	Small Angle X-Ray Scattering
SD	Standard Deviation
sgRNA	single-guide RNA
siRNA	Small Interfering RNAs
SORT	Selective Organ Targeting
w/w	Weight / weight ratio
WBC	White Blood Cells

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Agradecimientos

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'Every voyage is the unfolding of a poetic. The departure, the crossover, the fall, the wandering, the discovery, the return, the transformation[1*]*'*. Con esta cita me topé yo en el MoMA varios días después de llegar a Nueva York con una maleta llena de ganas de explorar. Eso, precisamente, las ganas de explorar, fue lo que me llevó a querer realizar esta tesis. Hoy, cuatro años después, puedo decir que uno de los aprendizajes más importantes que este (mi) camino por Santiago me ha dado es el de aceptar que más allá de nuestras hipótesis, teorías y experimentos, existe el amor por la ciencia, que es precisamente eso, el amor por la exploración.

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Durante esta tesis he tenido además la posibilidad de trabajar con científicos que me han inspirado y han dejado huella en mi carrera investigadora.

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[1*] *Cada viaje es el desarrollo de una poética. La partida, el cruce, la caída, el deambular, el descubrimiento, el regreso, la transformación.* ***(Trinh T. Minh-ha, 2010; Elsewhere, Within Here: Immigration, Refugeeism and the Boundary Event).***

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Ad maiora.

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Annexes

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ANNEXES

- **ANNEX I –** PATENT SUBMISSION CONFIRMATION
- **ANNEX II –** PERMISSION TO REPRODUCE PAPERS
- **ANNEX III –** PERMISSION TO REPRODUCE IMAGES
- **ANNEX IV –** ETHICAL ISSUES
- **ANNEX V –** FULL PUBLICATION INCLUDED IN THIS THESIS

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ANNEX I. PATENT SUBMISSION CONFIRMATION



Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

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Application number	EP24382805.0
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Date of receipt	23 July 2024
Your reference	909138
Applicant	DIVERSA Technologies S.L.
Country	ES
Title	Lipid nanosystems

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5.1 The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2)) ☐

Procedural language: en

Description and/or claims filed in: en

6 Applicant's or representative's reference: 909138

Filing Office: ES

Applicant 1

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14.1 The/Each applicant requests a reduction of fees under Rule 7a(1) EPC and is a natural person or entity under Rule 7a(2) EPC. ☐

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Representative 1

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Form 1002 - 1: Public inventor(s)

Designation of inventor

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ANNEX II. PERMISSION TO REPRODUCE PAPERS

The following publication has been adapted and included in the Introduction of this PhD Thesis.

The potential of nanomedicine to unlock the limitless applications of mRNA

Laura Taina-González, María de la Fuente.

Pharmaceutics **2022**, *14*(2), 460.

https://doi.org/10.3390/pharmaceutics14020460

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Contribution to this work:

I, Laura Taina González, has been involved in Conceptualization, Methodology, Investigation, and Writing the original draft and reviews.

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ANNEX III. PERMISSION TO REPRODUCE IMAGES

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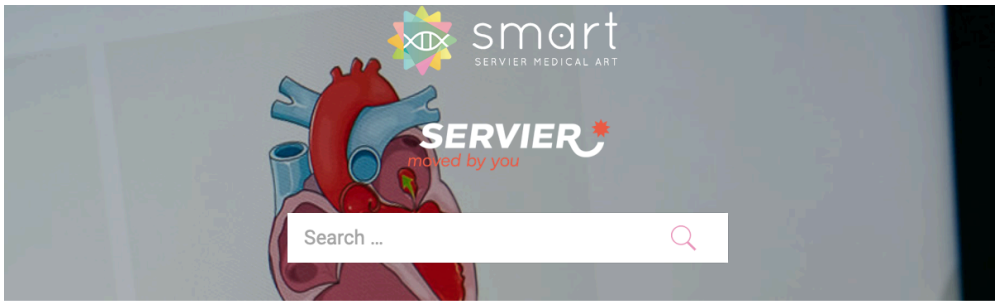
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Publication name:	*Universidade Santiago de Compostela*
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ANNEX IV. ETHICAL ISSUES

1. HUMAN CELL CULTURE

All cell lines used in this work, unless specified, were acquired from commercially available resources (American Tissue Culture Collection, ATCC) and cultured in the conditions recommended by the manufacturers and only used for research purposes specifically described in the present thesis.

2. ANIMAL STUDIES

All animal procedures were conducted in accordance with the institutional and national guidelines for the animal care and welfare, the ARRIVE guidelines. Procedures were approved by the internal ethical research and welfare committee (as stated in each of the chapters) and by the Local Authorities (Comunidad Foral de Navarra and Xunta de Galicia), according to the Spanish Government rules (Real Decreto RD53/2013) and European Union legislation (86/609/CEE, 2003/65/CE and 2010/63/EU). Experimental procedures were executed in the animal facilities of Centro de Investigación Médica Aplicada (CIMA, Pamplona, Navarra) and Centro de Biomedicina Experimental, (CEBEGA, Santiago de Compostela, Galicia). The author has complied with all regulations according to the experiments that were carried out, including obtaining the Animal Experimentation Diploma (Animalaria S.L.).

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RESOLUCIÓN 337/2019, de 23 de octubre, de la Directora Gerente del Instituto de Salud Pública y Laboral de Navarra, por la que se autoriza al centro usuario Universidad de Navarra-CIMA, el proyecto "Evaluación terapia antitumoral con citoquinas".

La Resolución 647/2006, de 7 de abril, del Director General de Salud, autorizó el funcionamiento como centro usuario para la utilización de animales de experimentación y otros fines científicos del establecimiento, titularidad Universidad de Navarra, NIF R3168001J, con domicilio en Pío XII, 55, 31008 Pamplona y código de identificación ES312010000132.

El día 16 de octubre de 2019, Don Juan José Lasarte, de la Universidad de Navarra, presentó la documentación Técnica del proyecto de experimentación animal "Evaluación terapia antitumoral con citoquinas", procedimiento dirigido por Don Pedro Berraondo López, responsable de su ejecución. La documentación incluye los elementos del Anexo X del Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. En la documentación se incluye la descripción y el diseño experimental del proyecto de investigación aplicada, las actividades a realizar, un análisis del balance de daño y beneficio, la justificación de que la finalidad del procedimiento no puede conseguirse utilizando animales diferentes de los propuestos, la aplicación de los principios de reemplazo, reducción y refinamiento incluyendo métodos de eutanasia, anestesia y analgesia, las referencias bibliográficas de referencia en la aplicación de dichos principios y que avalan el fundamento científico del proyecto y el "Resumen no técnico del proyecto".

El proyecto ha sido revisado, corregido y finalmente evaluado y aprobado, por el Comité de Ética para la Experimentación Animal del centro Universidad de Navarra, en su reunión del 4 de octubre de 2019. Se acompaña certificado de la Secretaria del Comité de los acuerdos adoptados en relación con el proyecto. Este Comité dispone de habilitación para la evaluación de proyectos a partir del 15 de octubre de 2013, de acuerdo con lo dispuesto en Real Decreto 53/2013, de 1 de febrero.

Puede verificar su autenticidad en:
https://administracionelectronica.navarra.es/validarCSV/default.aspx
2019-10-30 14:32:05

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CSV: **4d005fcc714d45b3**

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La Sección de Seguridad Alimentaria del Instituto de Salud Pública y Laboral de Navarra informa favorablemente la realización del procedimiento solicitado por cumplirse los requisitos mínimos establecidos en el Real Decreto 53/2013, de 1 de febrero.

De conformidad con lo expuesto, y en ejercicio de las atribuciones que me confiere el artículo 32.1 d), de la Ley Foral 11/2019, de 11 de marzo, de la Administración de la Comunidad Foral de Navarra y del Sector Público Institucional Foral,

RESUELVO:

1. Autorizar por un periodo máximo de cinco años el proyecto "Evaluación terapia antitumoral con citoquinas", a los efectos de lo dispuesto en el artículo 33 Real Decreto 53/2013 de 1 de febrero, por el que se establecen las normas básicas para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia, con las siguientes condiciones:

- Usuario que llevará a cabo el proyecto: Don Pedro Berraondo López.
- Responsable del proyecto: Don Juan José Lasarte.
- El procedimiento se realizará en las instalaciones del centro registrado con número ES312010000132, de titularidad Universidad de Navarra y autorizado mediante Resolución 647/2006, de 7 de abril, del Director General de Salud.
- Cualquier cambio del proyecto que pueda tener un impacto negativo en el bienestar de los animales implicará una nueva evaluación y, cuando proceda, autorización del proyecto.
- Las condiciones generales del desarrollo del proyecto serán las que se determinan en el Real Decreto 53/2013.

2. Se asigna al proyecto el código de identificación 080-19.

Puede verificar su autenticidad en:
https://administracionelectronica.navarra.es/validarCSV/default.aspx
2019-10-30 14:32:05

Helbide honetan haren benetakotasuna egiaztatzen ahal duzu:
CSV: **4d005fcc714d45b3**

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3. Trasladar esta Resolución a la Sección de Seguridad Alimentaria del Instituto de Salud Pública y Laboral de Navarra, a los efectos oportunos.

4. Notificar esta Resolución a la Universidad de Navarra, advirtiendo que contra la misma, que no agota la vía administrativa, se podrá interponer recurso de alzada ante la Consejera de Salud, en el plazo de un mes a contar desde el día siguiente al de su notificación.

Pamplona, veintitrés de octubre de dos mil diecinueve

LA DIRECTORA GERENTE DEL INSTITUTO DE SALUD PÚBLICA Y LABORAL DE NAVARRA

Nafarroako Osasun Publikoaren eta Lan Osasunaren Institutua
Instituto de Salud Pública y Laboral de Navarra

Kudeaketako Zuzendaritza
Dirección Gerencia

Mª Ángeles Nuin Villanueva

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XUNTA DE GALICIA
DELEGACIÓN TERRITORIAL DA CORUÑA
Xefatura Territorial da Consellería do Medio Rural e do Mar

Edificio administrativo Monelos
Vicente Ferrer, 2
15071 A Coruña

galicia

REXISTRO XERAL DA XUNTA DE GALICIA
REXISTRO DO EDIFICIO ADMINISTRATIVO DA CORUÑA
A CORUÑA
Data 21/07/2014 09:45:39
SAÍDA 77426 / RX 648375

José Antonio Costoya Puente
Centro de Investigación en Medicina Molecular y Enfermedades Crónicas (CIMUS
Avda. Barcelona, 22
15782 Santiago de Compostela (A Coruña)

Ref.: JLPV/lld

NOTIFICACIÓN DE RESOLUCIÓN DUNHA AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 15010/14/002
Interesado: José Antonio Costoya Puente
Procedemento: resolución de autorización
Data de inicio: 15-06-2014
Forma de inicio: solicitude do interesado

Notifícolle que con data 17 de xullo de 2014, o xefe territorial da Consellería do Medio Rural e do Mar emitiu unha resolución de autorización de proxectos de experimentación animal, cuxo texto íntegro é o seguinte:

ANTECEDENTES

O interesado, como representante do centro CIMUS (Universidade de Santiago de Compostela), presentou con data 10-04-2014 e rexistro de entrada 43279 RX 329898, unha solicitude para a realización do proxecto de experimentación animal, cuxos datos se detallan a continuación:

Denominación do proxecto: Dinámica espazo-temporal e regulación de oncoproteínas como base para o desenvolvemento de novas abordaxes terapéuticas no cancro
Nome do centro usuario: CIMUS (USC)
Persoa responsable do proxecto: José Antonio Costoya Puente
Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): CIMUS (USC)
Clasificación do proxecto : Tipo I ☐ Tipo II ☒ Tipo III ☐

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1. O Real decreto 53/2013, de 1 de febreiro (BOE núm. 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.
2. O artigo 89 da Lei 30/1992, de 26 de novembro, do réxime xurídico das administracións públicas e do procedemento administrativo común (BOE núm. 285, 27 de novembro de 1992), modificada pola Lei 4/1999, de 14 de xaneiro, establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

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XUNTA DE GALICIA
DELEGACIÓN TERRITORIAL
DA CORUÑA
Xefatura Territorial da Consellería do
Medio Rural e do Mar

Edificio administrativo Monelos
Vicente Ferrer, 2
15071 A Coruña

galicia

3. O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto, realizada polo órgano habilitado Sección de Experimentación animal do Comité de Bioética da Universidade de Santiago de Compostela.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co artigo 11 do Decreto 245/2009 de 3 de abril, polo que se regulan as delegacións territoriais da Xunta de Galicia e o Decreto 46/2012, de 19 de xaneiro, polo que establece a estrutura orgánica da Consellería do Medio Rural e do Mar e do Fondo Galego de Garantía Agraria.

De acordo con todo o indicado, RESOLVO:

1. Autorizar o proxecto solicitado.
2. Notificarlle esta resolución ao interesado.

O mencionado proxecto non deberá someterse a unha avaliación retrospectiva.

A autorización deste proxecto terá unha duración de 5 anos, transcorridos os cales, deberá ser renovada esta autorización.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida, no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpor recurso de alzada ante a conselleira de Medio Rural e do Mar da Xunta de Galicia no prazo dun mes contado a partir da recepción da notificación da presente resolución, conforme coa Lei 30/1992, do 26 de novembro, (BOE núm.: 285, 27 de novembro de 1992), de réxime xurídico das administracións públicas e do procedemento administrativo común na súa redacción dada pola Lei 4/1999, do 13 de xaneiro.

A Coruña, 17 de xullo de 2014
O xefe do Servizo de Gandaría

Eugenio Romero Sanande

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XUNTA DE GALICIA | CONSELLERÍA DO MEDIO RURAL

RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTO DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 15012/2023/007
Data de inicio do expediente: 28.3.2023
Persoa solicitante: Anxo Vidal Figueroa
Procedemento: resolución de autorización
Forma de inicio: solicitude da persoa interesada

ANTECEDENTES

A persoa solicitante, como representante do Centro de Biomedicina Experimental CEBEGA, presentou con data 28.3.2023 unha solicitude para a realización do proxecto de experimentación animal (entrada no Rexistro Electrónico da Xunta de Galicia 2023/1001168), cuxos datos se detallan a continuación:

Denominación do proxecto: Avaliación de terapias innovadoras contra o cancro
Nome do centro usuario: Centro de Biomedicina Experimental CEBEGA
Persoa responsable do proxecto: Anxo Vidal Figueroa
Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): Centro de Biomedicina Experimental da USC (CEBEGA) (ES1507800292901)
Clasificación do proxecto: Tipo I ☐ Tipo II ☐ Tipo III ☒

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1 O Real decreto 53/2013, de 1 de febreiro (BOE 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

2 O artigo 88 da Lei 39/2015, de 1 de outubro, do procedemento administrativo común das administracións públicas (BOE 236, do 2 de outubro de 2015) establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

CONSELLERÍA DO MEDIO RURAL
XEFATURA TERRITORIAL
SERVIZO DE GANDARÍA
Edificio Administrativo Monelos
R/ Vicente Ferrer, 2, 4º andar
15071 A Coruña
T: 981 184 565
servizo.gandaria.a.coruna@xunta.gal
mediorural.xunta.gal

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3 O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto realizada polo órgano habilitado, o Comité de Bioética de Experimentación Animal da Universidade de Santiago de Compostela.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co Decreto 149/2018, do 5 de decembro, polo que se establece a estrutura orgánica da Consellería do Medio Rural e se modifica parcialmente o Decreto 177/2016, do 15 de decembro, polo que se fixa a estrutura orgánica da Vicepresidencia e das consellerías da Xunta de Galicia (DOG 235, do 11 de novembro).

De acordo con todo o indicado, RESOLVO:

1 Autorizar o proxecto solicitado.

2 O proxecto precisa someterse a unha avaliación retrospectiva aos tres anos dende a súa aprobación.

3 A autorización deste proxecto terá unha duración de cinco anos e unha vez transcorrido este tempo, esta autorización deberá ser renovada.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpoñer un recurso de alzada ante o conselleiro de Medio Rural. O prazo comezará a contar dende o día seguinte ao da recepción desta resolución. Todo isto, segundo o disposto nos artigos 121 e 122 da citada Lei 39/2015.

Mediante este escrito notifícaselle a Anxo Vidal Figueroa esta resolución segundo o esixido no artigo 40.1 da antedita Lei 39/2015.

A Coruña, 4 de abril de 2023
O xefe territorial
Jose Manuel Santos Maneiro

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Animalaria
Formación y Gestión S.L.

Nº de Matrícula 2022481

Diploma del Curso

Función de Realización de los Procedimientos: Función C

Animalaria Formación y Gestión, S.L. informa que

Laura Taina González

47412924B

Ha superado el curso de 50 horas de duración para la obtención de la Función C de experimentación animal, para todas las especies del Anexo II, celebrado de forma no presencial del 10 de enero al 7 de marzo de 2022 y que ha sido reconocido por la Dirección General de Agricultura y Ganadería de la Comunidad de Madrid para toda España, con fecha 4 de abril de 2016 (Referencia CAM O566-7).

Y para que conste, se expide en Madrid, a 7 de marzo de 2022

La Dirección del Curso

Firmado digitalmente por Aurora María Solares, DNI 05263281F, representante legal de Animalaria S.L., B84624535

Animalaria, Formación y Gestión S.L.

De acuerdo con la Orden Ministerial ECC 566/2015 de 1 de abril de 2015

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28015 Madrid

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Animalaria
Formación y Gestión S.L.

De acuerdo con la Orden Ministerial ECC 566/2015 de 1 de abril de 2015

Programa del curso para la Función C	
	Módulos Troncales
1	Legislación nacional
2	Ética, bienestar animal y las Tres Erres. Nivel 1
3	Biología básica. Nivel 1
4	Cuidado, salud y manejo de los animales. Nivel 1
5	Reconocimiento del dolor, el sufrimiento y la angustia
6	Métodos incruentos de sacrificio (eutanasia). Nivel 1
	Módulos de Función
2	Biología básica. Nivel 2
6	Procedimientos mínimamente invasivos realizados sin anestesia. Nivel 1
7	Procedimientos mínimamente invasivos realizados sin anestesia, Nivel 2
8	Anestesia para procedimientos menores
9	Anestesia para intervenciones quirúrgicas o procedimientos prolongados
10	Principios de cirugía

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ANNEX V. PUBLICATION INCLUDED IN THIS THESIS

pharmaceutics

MDPI

Review

The Potential of Nanomedicine to Unlock the Limitless Applications of mRNA

Laura Taina-González [1,2] and María de la Fuente [1,3,4,*]

[1] Nano-Oncology and Translational Therapeutics Group, Health Research Institute of Santiago de Compostela (IDIS), SERGAS, 15706 Santiago de Compostela, Spain
[2] Universidad de Santiago de Compostela (USC), 15782 Santiago de Compostela, Spain
[3] Cancer Network Research (CIBERONC), 28029 Madrid, Spain
[4] DIVERSA Technologies, 15782 Santiago de Compostela, Spain
* Correspondence: maria.de.la.fuente.freire@sergas.es; Tel.: +34-9-8195-5704

Abstract: The year 2020 was a turning point in the way society perceives science. Messenger RNA (mRNA) technology finally showed and shared its potential, starting a new era in medicine. However, there is no doubt that commercialization of these vaccines would not have been possible without nanotechnology, which has finally answered the long-term question of how to deliver mRNA in vivo. The aim of this review is to showcase the importance of this scientific milestone for the development of additional mRNA therapeutics. Firstly, we provide a full description of the marketed vaccine formulations and disclose LNPs' pharmaceutical properties, including composition, structure, and manufacturing considerations Additionally, we review different types of lipid-based delivery technologies currently in preclinical and clinical development, namely lipoplexes and cationic nanoemulsions. Finally, we highlight the most promising clinical applications of mRNA in different fields such as vaccinology, immuno-oncology, gene therapy for rare genetic diseases and gene editing using CRISPR Cas9.

Keywords: mRNA; LNPs; vaccine; COVID-19; nanomedicine; nanotechnology; gene therapy; CRISPR; cancer; protein replacement

check for updates

Citation: Taina-González, L.; de la Fuente, M. The Potential of Nanomedicine to Unlock the Limitless Applications of mRNA. *Pharmaceutics* **2022**, *14*, 460. https://doi.org/10.3390/pharmaceutics14020460

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1. Prospects and Limitations of mRNA

The first isolation of messenger RNA (mRNA) was published in *Nature* in 1961 [1,2], starting the path towards a full understanding of this molecule [3]. The potential to synthetically produce mRNA from DNA in the 1980s, i.e., in vitro transcribed mRNA (IVT mRNA), opened a broad spectrum of preclinical applications [4]. Since then, multiple efforts have been performed to understand its mechanism of action and the path towards developing mRNA-based drugs. Therapeutic mRNA's potential lies in its capacity to induce the expression of proteins [4,5] for preventing or altering a particular disease state. mRNA therapeutics hold many opportunities, which can depend on the targeted cells, organ selective accumulation and encoded protein of interest. Nowadays, however, two main approaches are considered when using mRNA, both of which will be reviewed and explained in depth. The first is dendritic cell (DC) targeting, so as to achieve immune activation. The second is to exploit the natural capacity of nanoparticles to accumulate in the liver and use it to endogenously produce therapeutic proteins.

Importantly, mRNA has several advantages over other gene therapies such as DNA or pDNA which make this molecule more translational in terms of pharmaceutical properties: (i) mRNA does not need to reach the nucleus of the cell as opposed to DNA, resulting in more efficient delivery; (ii) mRNA does not integrate into the genome of the host cell, a fact associated with risk of mutagenesis; (iii) mRNA can be synthesized in the lab with fairly easy protocols, following scalable processes in agreement with GMP regulations; and (iv)

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mRNA sequences can be easily modified and updated, which is an important fact to consider in vaccinology (i.e., when mutations of the target protein occur). These advantages are also important when comparing mRNA technology to protein delivery technology, which normally comes with short half-lives and expensive and tedious industrial processes [4–6].

However, despite all these important advantages, two major drawbacks had been holding up the clinical development of this technology: its high instability and its potential immunogenicity. On one hand, if naked mRNA were administered, ribonucleases (RNA-ases) present in extracellular fluids would quickly degrade it; on the other hand, exogenous RNA molecules would be recognized by activating toll-like receptors (TLR) and trigger immunological responses [7]. Moreover, naked mRNA is not capable of crossing lipid bilayers to reach its target. To overcome these limitations and to improve the pharmacokinetic and pharmacodynamic properties of naked mRNA, two main strategies have been proposed to date: the introduction of chemical modifications on the sequence and the use of a delivery vehicle, which will be discussed in more detail in the following sections.

1.1. Structure and Chemical Modifications of mRNA

From a chemical point of view, mRNA is a single-strand biopolymer composed of nucleotide subunits linked by phosphodiester bonds. Each nucleotide is composed of a ribose sugar, a phosphate group and a nitrogenous base (cytosine, C, guanine, G, adenine, A and uracil, U). The mRNA sequence has a 7′methyl guanosine residue (m7G) at the 5′ cap and a poly(A) tail at the 3′ end. As other oligonucleotides, mRNA forms secondary structures thanks to the hydrogen bonding between complementary nucleotides, which is important for its stability [8]. Similarly, another promising technology, self-amplifying RNA (saRNA), is presented when the mRNA molecule is engineered to include an RNA virus genome [4], acquiring the capability to self-replicate and encode for multiple protein copies. This platform allows not only for the maintenance of the aforementioned mRNA's advantages, but also shows a further benefit by requiring much lower doses [9]. For instance, its pharmacological activity can be maintained for up to two months, further increasing the potency and reducing manufacturing costs of both excipients and RNA molecules, potentially allowing for even higher-speed development processes. The fact that the saRNA molecule is longer, however, could result in a loss of stability and increased risk of immunogenicity [10]. A recently published review on saRNA vaccines further explains and comments the potential of these platforms [11].

As previously mentioned, one of the main advantages of mRNA is that it can be easily synthesized in a laboratory. This process can be performed following two main strategies. The first, which is very commonly used, is based on the in vitro transcription of a linearized pDNA. The second is by making use of PCR, using a template with a bacteriophage promoter, a 5′ UTR, an open reading frame or ORF, a 3′ UTR and a poly[d(A/T)] sequence [5]. For saRNA, the introduction of a replicase prior to the antigen sequence would allow the self-replication of the RNA once administered [11,12].

As introduced, mRNA can be subjected to chemical modifications to achieve increased in vivo stability. Some reported modifications refer to changes in the m7G residue which would prevent the mRNA from being rapidly degraded by RNA-ases and its recognition via the immune system [13,14]. In this sense, recent advances in 5′cap analogues for enhancing mRNA expression include ARCAs (anti-reverse cap analogues) and Beta-S-ARCAs, as well as the incorporation of a 2S analogue [15]. Other modifications to enhance stability are related to the incorporation of longer PolyA tails, which would lead to a change in the secondary structure of mRNA [16,17]; substitutions using synthetic nucleotides such as N1-methyladenosine or N6-methyladenosine instead of natural adenosine, cytidine with 5-methylcytidine or uridine with pseudouridine [18,19]. Improvements in the purification processes and removal of secondary products from the in vitro transcription process have shown to reduce immunogenicity [20]. For further information regarding the chemical modification of RNA, we invite the reader to consult specialized reviews [21,22].

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In line with the subject of this review, it should be mentioned that marketed COVID-19 vaccines, described in detail in Section 2.1, have modified RNA sequences [23,24]. However, it is worth noting that this first strategy is not always used, as it can be seen in the available data published for ongoing clinical trials (Table 1 and Tables 2–5). Furthermore, considering the disappointing recently published CureVac mRNA vaccine results [25], described in Section 2.3, which uses unmodified mRNA, it seems plausible that this strategy is potentially needed for achieving good and translational in vivo results. As will be further detailed in Section 2.3, unmodified mRNA sequences are often related to higher reactogenicity, increasing tolerability issues as compared to modified sequences. The second strategy—referring to the use of a delivery vehicle able to efficiently associate, protect and deliver the intact mRNA molecule to its target—is discussed next.

Table 1. mRNA Nanomedicines for Infectious Diseases in Clinical Trials. A list of clinical trials for infectious diseases other than COVID-19 is provided in this table.

Infectious Diseases Other than COVID-19					
Therapeutic	**Clinical Application**	**Formulation**	**Phase**	**NCT Number**	**Sponsor**
mRNA-1653	hMPV/PIV3	Modified mRNA-LNPs	Phase I	NCT04144348 NCT03392389	Moderna
mRNA-1345	RSV	Modified mRNA-LNPs	Phase I	NCT04528719	Moderna
mRNA-11777	RSV	Modified mRNA-LNPs	Phase I	Unregistered	Moderna, Merck
mRNA-1172	RSV	Modified mRNA-LNPs	Phase I	Unregistered	Moderna, Merck
mRNA-1893	Zika	Modified mRNA-LNPs	Phase I	NCT04064905	Moderna
mRNA-1325	Zika	Modified mRNA-LNPs	Phase I	NCT03014089	Moderna
mRNA-1647	CMV	Modified mRNA-LNPs	Phase II	NCT04232280	Moderna
mRNA-1647	CMV	Modified mRNA-LNPs	Phase II	NCT03382405	Moderna
mRNA-1443	CMV	Modified mRNA-LNPs	Phase I	NCT03382405	Moderna
MRT5400	Influenza A (H3N2)	Not disclosed	Phase I	Unregistered	TranslateBio, Sanofi
MRT5401	Influenza A (H3N2)	Not disclosed	Phase I	Unregistered	TranslateBio, Sanofi
mRNA-1010	Influenza A, Influenza B	Not disclosed	Phase II/III	NCT04956575	Moderna
mRNA-1851	Influenza A (H7N9)	Modified mRNA-LNPs	Phase I	NCT03345043	Moderna
mRNA-1440	Influenza A (H7N8)	Modified mRNA-LNPs	Phase I	NCT03076385	Moderna
mRNA-1388	Chikungunya	Modified mRNA-LNPs	Phase I	NCT03829384	Moderna
CV7201	Rabies	Non-modified mRNA- in RNActive	Phase I	NCT02241135	CureVac
CV7202	Rabies	Unmodified mRNA-LNPs	Phase I	NCT03713086	CureVac
GSK3903133A	Rabies	saRNA–cationic nanoemulsion	Phase I	NCT04062669	GSK

Abbreviations: hMPV/PIV3—human metapneumovirus/parainfluenzavirus type 3; RSV—respiratory syncytial virus; CMV—cytomegalovirus; HPV—human papillomavirus.

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2. Delivery Vehicles for mRNA

2.1. Lipid Nanoparticles (LNPs), the Delivery Vehicles behind COVID-19 mRNA Vaccines

The Moderna and BioNTech/Pfizer mRNA vaccines were first marketed under emergency authorization in December 2020. Both vaccines were developed in less than a year, proving the potential of mRNA technology to be quickly adapted to emergency situations. These vaccines are composed of SARS-CoV-2 spike protein (S) [23,24] loaded into LNPs and have not only meant a breakthrough in terms of design and manufacturing speed, but also in terms of efficacy. The BioNTech/Pfizer vaccine exhibited a 95% efficacy in phase 3 of clinical trials [26], and more recently, another study was released showing that efficacy was maintained over a period of 6 months, irrespective of previous SARS-CoV-2 infections [27]. For its part, the Moderna vaccine's phase 3 clinical trial results showed an efficacy of 94.1% [28]. A recent published paper compared the immune response kinetics between mRNA vaccines with the Janssen vaccine, concluding that mRNA vaccines elicit a high initial peak antibody response which decreases after 8 months, while the Janssen vaccine had stable but lower antibody levels over the same period of time [29]. With regards to this, clinical data on vaccine safety and efficacy in children from 5 to 11 [30] and in adolescents [31,32] is available now, increasing the application of this vaccine technology to the younger population as well. Regarding variants, up until the appearance of Omicron, available data suggested that mRNA vaccines protect against all the other variants, including Delta [33–35]. However, in regard to this new variant, contradictory data have been shown. On one hand, this study—not yet peer-reviewed—showed a decrease in antibody levels against Omicron [36]. Similarly, in another study which used antibodies from vaccinated and convalescent people to study the effectiveness against all variants, found that the neutralization of Omicron was much lower than of any other variant [37]. On the other hand, there are also data supporting the claim that T-cell immunity is still effective against Omicron [38].

None of these achievements would have been possible without nanotechnology, defined as the production and application of structures, devices and systems by controlling the shape and size of materials at a nanometer scale. In recent years, nanotechnology applied to medicine has been referred to as nanomedicine [39,40]. The term "nanomedicine" thus comprises formulations in the nanometer scale (i.e., nanoformulations) composed of organic materials such as lipids or polymers, or inorganic materials such as metals, that can be used to deliver small molecules or biomolecules, namely proteins, peptides or nucleic acids. Nanomedicine can be used for therapeutic purposes [41], but it also has an application in diagnostics and regenerative medicine [42] With respect to the specific application of nanomedicine in gene delivery, all the research performed in the last 30 years has resulted in safe and effective delivery platforms for nucleic acids, such as antisense oligonucleotides (ASOs), small interfering RNAs (siRNA), DNA and more recently mRNA, using different types of strategies such as Gal-NAc conjugates, and different delivery vehicles such as Adeno-Associated Virus (AAD) and more recently LNPs, as recently reviewed in this article [43]. Despite this great effort, at present there is only one nanoformulation in the market for siRNA delivery, Onpattro®; however, many others are undergoing clinical trials, and more genetic nanomedicines are expected to be seen in the near future [43]. In this term, the approval of Onpattro® for hereditary transthyretin-mediated (hATT) amyloidosis in 2018 meant a huge breakthrough in nanoparticle design; LNPs, later used for the development of mRNA vaccines, were marketed for the first time. Therefore, these nanoparticles have been key for the development of the two marketed COVID-19 vaccines and are today the most advanced technology in mRNA delivery. The origin of LNPs lies in the discovery of liposomes, bilayer lipid structures which were firstly described in 1965 by Banghman, setting up the basis of model membrane systems [44]. A few years later, Gregoriadis et al. established the idea that these lipidic vesicles could be used for drug-delivery purposes, opening a door towards a full development of the liposome field [45–47], and later LNPs [48]. Other lipidic-based delivery vehicles have also been a matter of research, whose most recent advancements will be discussed in Section 2.2. In this next section, we

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will describe in depth the composition, structure, manufacture and stability considerations of LNPs.

2.1.1. Unraveling LNP Composition

LNPs used today have a very well-established composition based on a mixture of lipids whose ratios, types and structures are carefully defined. Major constituents are (i) an ionizable lipid, (ii) structural and helper lipids and (iii) PEG-lipid.

For each of the vaccines, detailed compositions are described here. The BioNTech/Pfizer LNP composition (ionizable lipid: phospholipid: cholesterol: PEG-lipid) is ALC-0315:DSPC:Chol:ALC-0159 (molar ratio 46.3:9.4:42.7:1.6), with 30 µg of mRNA dose. Moderna LNP composition is SM-102:DSPC:Chol:DMG-PEG (molar ratio 50:10:38.5:1.5) with 100 µg mRNA dose [49]. Each of these constituents possesses a crucial function in the structured nanoparticle, described in detail below. A schematic representation of the composition of LNPs for both vaccines is shown in Figure 1, and chemical structures of lipidic components are shown in Figure 2. The detailed structure hypothesis is explained in Section 2.1.3 and represented in Figure 3.

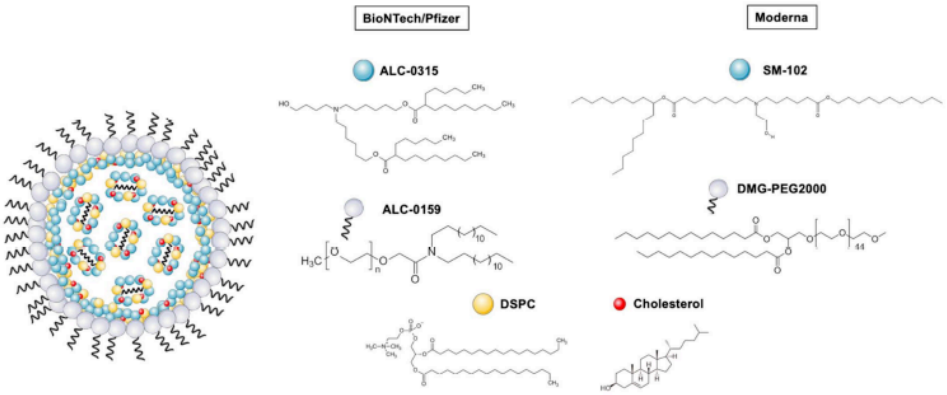


Figure 1. Structure and composition of the authorized COVID-19 mRNA formulations.

(i) Ionizable lipids are possibly the most innovative component of LNPs, having represented a huge breakthrough in RNA delivery. Their mechanism of action is based on complexing the nucleic acid by electrostatic interactions. Several properties, such as the charge or lipid shape, as well as the protein corona formation, have been described as important factors to consider when understanding structure–activity relationship studies, and thus, the design of new ionizable lipids.

On behalf of the charge, prior to the use of ionizable lipids, permanently positively charged lipids, such as DOTAP, DOTMA or DDAB, were of preference. However, the positive surface charge contributed to low stability due to aggregation phenomena, and had toxicity issues [50]. By contrast, today the most commonly used lipids are ionizable lipids: pH-titratable lipids which are positively charged only at acidic pH, allowing the formation of complexes with nucleic acids during the formulation process, but are neutral at pH7'4, overcoming the inconveniences of cationic lipids, and as a result, allowing for longer circulation times, increasing accumulation in targeted tissues and promoting better tolerability [51,52]. Their use, together with the optimization of manufacturing processes, as described in Section 2.1.2, allows the mRNA to be encapsulated with high efficiency inside the nanoparticle, in contrast to what is reported for conventional cationic lipoplexes or cationic nanoemulsions, described in Section 2.2. Today, although the use of these lipids

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is predominant for new LNP design and SAR studies, permanently positive cationic lipids are still widely used, especially when considering other lipid-based technologies.

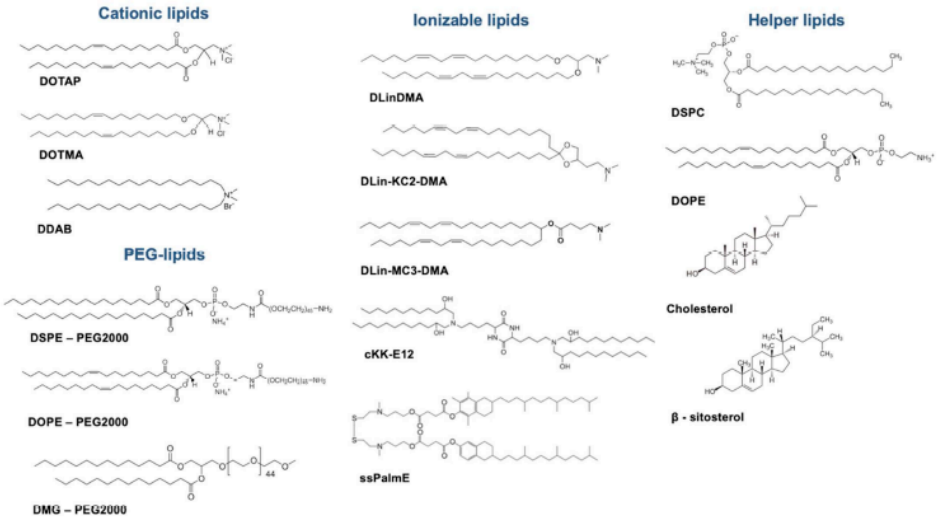


Figure 2. Chemical structure of some LNPs' components.

The lipid shape—which comes hand in hand with the degree of the lipid chain's degree of unsaturation—also seems to play an important role in promoting endosomal escape. Heyes et al. proved that when increasing the number of instaurations, the efficiency of delivery increases dramatically due to the formation of H11 hexagonal phase in the particle that promotes fusogenicity with the cell membrane [53]. Semple et al. further discussed this by hypothesizing that once in the endosome, lipids would be protonated thanks to the acidic environment. This in turn would facilitate the interaction with the anionic phospholipids of the endosomal membrane, disrupting it and liberating the cargo [53,54].

The design of new lipids has been evolving ever since the first proof of concept for ApoB gene silencing using DLinDMA lipid in hepatocytes was achieved [55]. This research led to the discovery of DLin-KC2-DMA, which demonstrated promotion of increased in vivo activity at siRNA doses of 0.01 mg/kg in rodents and 0.1 mg/kg in non-human primates in silencing factor VII [54]. On its part, DLinMC3DMA (i.e., MC3) lipid, considered even today a gold standard for LNP formulation development, was first discovered after carrying out a systematic screening to improve gene silencing in vivo, which also concluded that the optimum pKa for hepatic delivery should stand between 6.2 and 6.5 [56]. This lipid would later be commercialized with Onpattro® in 2018 [57].

As opposed to cationic lipids, a specific uptake mechanism has been postulated for these ionizable lipids closely related to the formation of a protein corona enriched in Apolipoprotein E (ApoE), a well-known phenomenon driving the nanoparticle's fate and biodistribution upon administration [58], as firstly disclosed by Akin et al. [59]. This work, which compared LNPs formulated with the ionizable lipid DLinKC2DMA (iLNPs) versus cationic-lipid LNPs (cLNPs), refers to an ApoE-dependent mechanism by which iLNPs would be efficiently endocytosed by hepatocytes via LDL receptor, mediating accumulation in this organ. Further studies suggested that the main mechanism driving the endocytosis for ionizable DLinMC3DMA-based LNPs were both clathrin-mediated endocytosis as well as macropinocytosis [60]. On this note, the most recent publication on LNP structure indicated that upon binding DLinMC3DMA-based LNPs with ApoE,

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a rearrangement is induced in the structure of the nanoparticle, which could drive the nanoparticle's fate [61]. More recently, other studies with mRNA showed that efficient LNP–mRNA transfection could also rely on an early narrow endosomal escape window before lysosomal sequestration or exocytosis [62]. Furthermore, another study showed a link between exogenously administered mRNA and extracellular vesicles (EVs) after observing mRNA present in these EVs at 1:1 ratio, concluding that mRNA should be neutrally charged by the ionizable lipid to promote endosomal escape [63].

As a step forward in lipid design, a subtype of ionizable lipids was presented in 2008, commonly called lipidoids, with a dendrimer-like structure [64] and with the advantage of a straightforward synthesis protocol. Later, Dong et al. reported the synthesis of cKK-E12, a lipidoid capable of silencing hepatocytes with the highest potency in rats, mice and non-human primates reported until that point [65].

Research on new ionizable lipids has also been focused on obtaining more biocompatible and biodegradable lipids, classified as new-generation lipids. In this term, Maier et al. showed that including an ester bond in the alkyl chains improved tolerability and retained potency, as compared to MC3 [66]. Other studies have also reported better cell viability in vitro by including disulfide bonds [67], which led Akita et al. to firstly synthesize ssPalmE, a lipid which incorporates a tocopherol-like moiety in its structure [68].

(ii) Structural and helper lipids are also constituents of LNPs, playing an important role in terms of stability and fusogenicity [69]. These lipids are mainly phospholipids (such as DOPE, DSPC, DEPC, DSPE) and cholesterol, which forms the main skeleton of LNPs and might aid in endosomal escape.

Phospholipids are a key component, possibly due to their nature mimicking biological membranes. However, not all phospholipids seem to be equally efficient. It has been reported that the type of structures formed by phospholipids is an important factor driving fusogenicity. In fact, it seems that lipid species such as unsaturated phosphatidylethanolamines (PEs) adopt non-bilayer structures, which in turn are less stable, driving the disruption of the LNPs and inducing fusion with the cell membrane and the consequent release of the cargo inside the endosome [54,70]. Therefore, the structure and unsaturation of phospholipids also seem crucial to improve endosomal escape. When in vivo biodistribution is considered, the helper lipid structure also seems to play a major role. In this regard, a recent study which aimed to systematically screen a library of 96 LNP compositions found that DOPE-containing LNPs preferentially accumulated in the liver, as opposed to the DSPC-containing LNPs. Furthermore, authors reported that DOPE-containing LNPs also exhibited higher ApoE-binding affinity, which also correlated to higher liver-protein expression [71]. On the other hand, cholesterol has been shown to give stability to LNPs and improve efficacy by enhancing membrane fusion [72,73]. Patel et al. have also investigated different cholesterol analogues and their function in enhancing intracellular delivery of mRNA, concluding that the type and flexibility of the alkyl tail present in the sterol ring can affect transfection efficiency [74].

(iii) Polyethilenglycol (PEG) is a widely known polymer that has been described and previously used in liposomes and other types of nanoformulations. It seems to play a main role in prolonging circulation time by reducing macrophage phagocytic system (MPS) interactions, improving colloidal stability and preventing the formation of the protein corona [53,75,76]. PEGylated lipids typically included in these formulations consist of two domains, a hydrophilic PEG polymer conjugated to a hydrophobic lipid branch. In LNPs, the hydrophilic domain is located towards the exterior surface of the particle and the lipid branch towards the interior. Additionally, LNP properties can be tuned by modulating the PEG-lipid/total lipid content ratio. For instance, it is known that smaller LNPs are generated at higher PEG-lipid ratios—a matter that is further described in Section 2.1.3. PEG-lipids can additionally balance the half-life and cellular uptake of LNPs [54]. For this reason, the PEG amount is today kept to a minimum of 1.5 mol% of the total lipid content, after Semple et al. reported a 5-fold potency improvement when decreasing PEG content from 10% to 1.5% [54]. Considering that PEG can also affect cell–nanoparticle

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interactions by preventing a close approach between the two and impeding endosomal escape by preventing fusion [77]; some studies suggest that causing PEG lipids to diffuse from the LNPs upon administration would allow for intracellular delivery promotion [78].

Despite the well-characterized and studied molar compositions, little is known about how changing the overall composition might affect the LNPs' biodistribution. Apart from the above-described study where DOPE and DSPC LNP formulations were compared [71], another important study was recently published in *Nature* about selective organ targeting of LNPs [79]. In this study, the authors concluded that it is possible to include additional lipids to tune LNPs' base composition, and thus modify their surface charge to target specific organs such as the spleen and lungs. To achieve this, they performed several experiments with different ionizable lipid LNPs (5A2-SC8, MC3 and C12-200) which they tuned with positive or anionic lipids. Results showed that increased amounts of DOTAP achieved an increase in protein expression in the spleen and lungs, and results were reproduced when other cationic lipids were used. Meanwhile, negative lipids drove the protein expression to the spleen. Furthermore, authors described the enhancement in liver delivery when supplementing established LNPs with more ionizable lipids [79].

Similarly, it was also described that mannosylation of LNPs may enhance the uptake of nanoparticles by antigen-presenting cells (APCs). By incorporating a mannose-cholesterol amine conjugate to LNPs, an enhanced in vitro uptake in dendritic cells was observed as compared to non-decorated LNPs, a fact that translates into a faster onset of the antibody response upon administration by both intradermal (i.d.) and intramuscular (i.m.) routes [80]. Authors further investigated how the length of this LNP-surface decoration may influence antibody response, observing that vaccine priming response was improved with a higher chain length [81].

2.1.2. Manufacturing Processes for LNPs

The production of LNPs is based on the ethanol dilution technique first described in the early 2000s. This method consists of lipid dissolution in an ethanolic phase, followed by rapid mixing using a T tube mixer with an aqueous buffer solution containing the nucleic acid at acidic pH. As these two solutions are mixed, the ionizable lipid becomes positively charged and complexes with the negatively charged nucleic acids at the same time as the overall lipid solubility is reduced by the dilution of ethanol. This forms unstable LNPs in terms of size, which will then need pH adjustment to raise their value to physiological pH, normally achieved by dialysis processes [51,52]. The use of continuous microfluidics has revolutionized the bench-top formulation and manufacturing process of LNPs, allowing for the accurate formation of LNPs with defined sizes, by altering and optimizing the mentioned parameters as well as reducing manufacturing time [82]. This technique involves the mixture of the lipids with the aqueous phase using a micromixer, and is highly reproducible, scalable and robust [82,83]. Recently, manufacturing considerations for the production of LNPs using both cationic and ionizable lipids have been extensively reviewed in this article [84].

When considering bench-top formulations, important factors need to be considered to obtain LNPs with desired characteristics. In a recent study, Terada et al. investigated the effect of bench-top siRNA-LNP preparation parameters, i.e., total lipid content, flow rate ratio and total flow rate, by systematically comparing formulations using the design of experiments (DoE) platform, providing a statistically relevant study [85]. Conclusions were driven with respect to the lipid concentration and flow rate ratio, stating that these two factors might be critical for size dispersion when formulating and manufacturing LNPs. Furthermore, this study points to the fact that the presence of nucleic acids—in this case siRNA—dramatically impacts the LNPs behavior within the whole formulation process as compared to empty LNPs [85], which is also in line with the conclusions driven by Sebastiani et al. [61]. This issue will be further described in the next section.

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2.1.3. Disclosing the LNPs' Structure

The structure of LNPs has not been fully deconvolved yet and is still a matter of debate. The two main structures proposed, which are explained in this section, can be pictured in Figure 3. The first studies on LNP structures have been conducted mainly for siRNA-loaded LNPs, and more recently, several studies have also reported the structural organization of mRNA-loaded LNPs.

Starting with siRNA LNPs, various studies have been carried out since Leung et al. first proposed that LNPs have an electron-dense core in which nucleic acid would be located inside inverted micelles [83]. According to this hypothesis, the initial association of the ionizable lipid with the nucleic acid would form such types of micelles, which in turn would associate with more empty micelles, altogether structuring the core. This core would then be coated with polar lipids, i.e., DSPC and PEG-lipids [83]. Later, other studies suggested that components would be located forming a layered structure, where the phospholipids and the PEG-lipids would be located at the surface, and the cholesterol and ionizable lipids would form the core of the nanoparticle, reporting differences in the structure depending on the cargo [86]. Subsequently, experiments using cryo-TEM and small-angle X-ray approaches suggested that LNPs would be formed upon fusion of smaller particles occurring after rapid mixing [87]. Later, the same authors performed experiments with larger cargoes such as pDNA, mRNA and gold nanoparticles [88]. Results obtained supported the initial idea that the formation of LNPs would happen through fusion of smaller particles in a pH-dependent manner after the rapid-mixing process, and that this fusion would be highly dependent on the amount of PEG-lipid accumulated in the surface. Thus, PEG content would dictate LNP size, with a higher PEG content leading to smaller LNPs [88]. Recently, the same group published a study hypothesizing that the entrapment of siRNA into LNPs occurs through inversion of preformed vesicles [89].

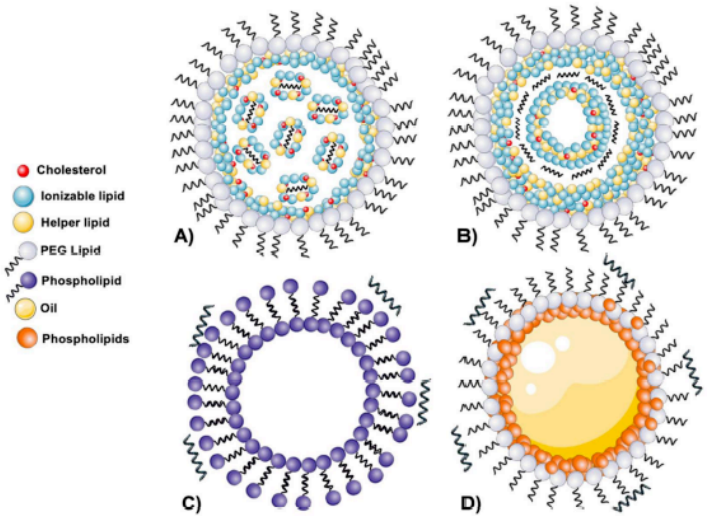


Figure 3. Lipid delivery vehicles for mRNA delivery. (**A**) Lipid nanoparticles (LNPs), structure proposed [87]; (**B**) Lipid nanoparticles (LNPs), structure proposed. Reproduced from Viget et al. [86] which is licensed under a Creative Commons Attribution-(CC BY 4.0) International License (http://creativecommons.org/licenses/by/4.0/); (**C**) Lipoplexes (LPX) and (**D**) cationic nanoemulsions (.NEs).

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Studies performed with mRNA formulations firstly suggested that the mRNA would be located inside water cylinders surrounded by ionizable lipids [90]. In a later study using MC3 LNPs, authors concluded that the shell of the particle would indeed be enriched with DSPC, MC3 and cholesterol, the latter being mainly present here. The core would be composed of MC3, where it would be present in double the quantity as compared to the shell [61]. In the same study, LNPs and ApoE-interaction analysis concluded that this protein corona could mediate an irreversible rearrangement of components, affecting the LNPs' fate and protein expression efficacy [61]. Furthermore, recent evidence showed that cholesterol analogues can also change the LNPs' structure, by switching spherical particles to polyhedral [91].

Despite these recent advancements and hypothesis, more studies are still needed to better understand the structural organization of LNPs. In this regard, a study performed by Blakney et al. [92] aimed to describe how the association strategy of the nucleic acid to the LNPs would dictate the LNPs' colloidal properties, in vitro and in vivo behavior. To achieve this, authors compared two different approaches: encapsulation and complexation (i.e., interior vs. exterior RNA localization). For this purpose, they used two different types of LNPs: LNPs incorporating a cationic lipid (cLNPs, DDA or DOTAP) or an ionizable lipid (iLNPs, C12-200). In terms of physicochemical characterization, results showed that saRNA localization did not seem to dictate colloidal properties, i.e., no difference was seen when comparing LNPs of the same composition but different saRNA localization. In regard to the transfection efficiency, authors showed that for iLNPs it was higher when the RNA was encapsulated, as opposed to cLNPs, where higher transfection rates were achieved when the RNA was complexed [92]. This study is important as it gives an alternative approach to the paradigm of encapsulation RNA for efficient delivery. The possibility of complexing the saRNA onto the LNP surface could have the potential advantage of having a ready-to-use blank formulation for association of RNA when needed, allowing shorter RNA working times, easy updating of the sequence, and so on.

2.1.4. Stability Considerations

Undoubtedly, one of the biggest challenges when it comes to mRNA formulation is the stability of the biomolecule. As mentioned before, mRNA is extremely unstable and needs to be stored at −80 °C. The importance of stability lies in the fact that even a small change in structure can stop mRNA translation into proteins. Physical instability comprises denaturation and the loss of secondary structure, which is related to aggregation and precipitation and linked to poor translation efficiency [49]. However, according to Pogocki et al., the main stability issue of mRNA is chemical degradation, which occurs via oxidation and hydrolysis via the phosphodiester bonds that form the backbone of the molecule. In vivo, there is a major challenge due to the presence of RNA-ases, converting protection of mRNA into a key issue to guarantee its efficacy [8].

From a pharmaceutical perspective, achieving a formulation that can be stored at higher temperatures while preserving biomolecular integrity is in fact one of the biggest challenges. To this end, studies evaluating pH, temperature and lyophilization techniques had been previously conducted with siRNA-containing LNPs, showing that when stored in aqueous media, refrigeration kept LNPs stable for the longest time, as compared to −20 °C or room temperature (RT). Interestingly, pH seemed to not be a factor influencing stability. Remarkably, authors showed that stability was protected upon freeze-thaw cycles when cryoprotectants such as sucrose or trehalose [93]—two excipients used in the marketed vaccines—were used. In this regard, more recently, a novel class of mRNA reactivity that leads to loss of activity has been reported: the formation of mRNA-LNP adducts though the covalent union between some reactive lipids and the nucleobase [24,94]. It is also worth noting that mRNA could potentially have more stability issues than siRNA, as it is a longer and more complex molecule. Indeed, Onpattro® siRNA-LNP formulation has a shelf life of three years at 4 °C [95], while the BioNTech/Pfizer vaccine is reported in the technical documents to be stable for just 6 months between −90 °C and −60 °C [24]. However, Pfizer

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and BioNTech have submitted data on increased stability, proposing that the vaccine can be stored at −25 °C to −15 °C, and later in refrigerator storage, for up to one month once unfrozen. On the other hand, the Moderna vaccine can be stored frozen for up to 7 months and it is stable in refrigeration for up to 3 months [23].

2.2. Additional Lipid-Based Delivery Vehicles for mRNA

As introduced in Section 2.1, LNPs are today the most-researched delivery vehicle for mRNA delivery. Nevertheless, LNPs are not the only vehicle under investigation, and it is worth noting the importance of others. Both lipids and polymer materials hold tremendous potential, especially because it seems apparent that the idea of having one delivery system that fits all kinds of nucleic acids is no longer accepted. As such, polymeric nanoparticles and materials such as polyethyleneimine, polyacrylates, polyesters and polyamines, among others, are currently evolving as an alternative for lipid carriers. Nonetheless, this review will be focusing only on the newest advances in lipidic nanoformulations, leaving aside polymeric materials as they have been extensively covered in recent reviews [96,97].

2.2.1. Lipoplexes

It was in the 1970s when Dimitriadis et al. succeeded in efficiently delivering encoded rabbit globin mRNA to mouse spleen lymphocytes for the first time, using large unilamellar liposomes [98]. At the same time, Dray et al. published results on protein translation after treating HEp-2 cells with mRNA encapsulated in liposomes [99]. However, due to the physicochemical characteristics of nucleic acids, traditional approaches used for small molecule encapsulation in response to pH changes had not been successful [52]. As mentioned in Section 2.1, LNPs also evolved from liposomes; however, also using approaches relying on the use of complexing agents, different delivery vehicles emerged, and are typically disclosed as lipoplexes (LPX, Figure 3C).

Regarding the composition of LPX, the most commonly described materials for mRNA delivery include several lipids such as DOPE, DOPC, DSPC and cholesterol, as well as complexing agents such as cationic lipids such as DOTAP, DOTMA and DC-Cholesterol; ionizable lipids such as MC3 or C12-200; or other positively charged peptides or polymers. Formulation methods described in the literature include the traditional approach of hydration lipid film [100], pre-complexation of the cationic component with the nucleic acid [101,102] and the simple mixture of lipids dissolved in ethanol with an aqueous phase containing the nucleic acid [103,104].

As mentioned, the use of complexing agents to link the mRNA to the nanocarrier is a commonly used strategy and normally involves cationic lipids. For instance, Sahin et al. showed in 2018 that an intravenously (i.v.) administered DOTMA/DOPE LPX formulation was capable of triggering INF-γ and T-cell memory upon administration of antigen-encoding mRNA [105]. On the same note, DC-Cholesterol/DOPE LPX have been studied for α-1-antitrypsin-encoding mRNA-protein production, showing minimal animal toxicity [106]. Van Hoecke et al. also used cationic LPX to study how the route of administration could impact T-cell activation [107]. More recently, Salomon et al. carried out a study with LPX containing DOTMA and DOPE, showing the potential of a CD4 neoantigen vaccine to increase the antitumor activity of local radiotherapy in mice [108]. Regarding SARS-CoV-2, a vaccine investigation using LPX has also been carried out. In a very recent study [109], the potential approach of using cholesterol/DOTAP LPX was evaluated preclinically resulting in a 30 μg mRNA dose, achieving immune activation after three doses. Interestingly, some studies have also been conducted for cationic LPX for other routes of administration other than i.v. and i.m. Interestingly, Dhaliwal et al. have shown the potential of DPPC/DOTAP/cholesterol LPX for mRNA delivery to the brain via intranasal (i.n.) administration. Results showed dose-dependent and region-specific distribution of mRNA when LPX are used, as opposed to naked mRNA administration [110].

Despite the fact that cationic lipid usage is still a main approach when formulating LPX, another approach has been described by Zhang et al. [102]. This strategy involves the

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use of a positively charged polymer, i.e., protamine, which complexes the nucleic acid prior to the formation of the nanoparticle. In this study, they succeeded in efficiently delivering T34A mRNA locally and systemically, improving the results on their DNA counterparts and also with a better safety profile on colon-cancer gene therapy [102]. The same team also proposed the use of this delivery vehicle for immunotherapy when they succeeded in delivering IL-15 cytokine mRNA in vitro and in vivo [101]. Later on, they also reported the use of this strategy to efficiently deliver a neoantigen mRNA vaccine using a positively charged peptide as complexing agent, i.e., DP-7 [111]. More recently, LPXs using KALA peptide as a complexing agent have also been reported [100] further evidencing the fact that other complexing agents can be used.

An interesting study regarding the complexing lipid type and localization aimed to characterize how this component affects the cellular uptake and mRNA expression in different cell lines in vitro and after intradermal (i.d.) administration. For this purpose, they used LPX composed of cholesterol, DOPE and different ionizable (C12-200, MC3), cationic (DDA, DOTAP) and zwitterionic (cephalin) lipids. The most relevant conclusion was that the resulting overall LPX-surface charge was the most important predictive factor for efficient protein expression [112].

In addition to complexing-agent modification, tuning the composition of LPX has also been studied. For instance, Ziller et al. published results on how EPC/DOPC/DOTAP LPX could be further modified to achieve controlled-release effects [104]. Furthermore, similarly to that which Goswami et al. [80,81] studied for LNPs, and as previously commented in Section 2.1.1, the development of mannosylation strategies through the incorporation of mannose-modified lipids has also been proposed for LPX for specific targeting to macrophages, achieving positive results in the induction of a stimulatory immune response upon mice vaccination [113,114].

2.2.2. Cationic Nanoemulsions

Another form of lipid nanoformulations that are currently under preclinical and clinical investigation are cationic nanoemulsions (NEs), represented in Figure 3D. These delivery vehicles have been extensively used in nanomedicine for different kinds of drugs and applications thanks to their versatility and ability to encapsulate different types of therapeutic molecules [115–117] Emulsions are by definition a mixture of two immiscible phases that are stabilized by an emulsifier or surfactant, normally added to the continuous or external phase to stabilize the internal phase. Depending on the nature of the phases, emulsions can be categorized as oil-in-water (O/W) or water-in-oil (W/O) emulsions. Furthermore, emulsions can be classified depending on their droplet size as coarse emulsions or nanoemulsions, and there has in fact been some debate on nomenclature when considering nanoemulsions or microemulsions [118]. In this term, nanoemulsions are exclusively formed by spherical nanosized oil droplets; however, they are not thermodynamically stable, as microemulsions are [119]. Although microemulsions can exist in the nanosized ranges [119], we will refer to nanoemulsions here when considering only kinetically nanosized emulsions.

When considering formulation methods, NEs can be obtained by different methods, such as high-pressure homogenization, microfluidization or sonication. The technique employed in the manufacturing process and the type of lipids and formulation parameters have a huge impact on the physicochemical properties of the final formulations [115,117]. Undoubtedly, the simplest approach to formulate these nanoemulsions is the ethanol-injection method [120].

Specifically for gene-delivery purposes, NEs usually contain a hydrophobic oily core surrounded by phospholipids and other surfactants, and similar to what was proposed for LPX, cationic NEs typically contain a cationic component which complexes the nucleic acid. On this behalf, recent modeling of a siRNA-loaded nanoemulsion has been performed, finding it to have a compact shape in which the hydrophobic domains of the phospholipids and the oil are placed in the core, while the polar groups form a hydrophilic shield on

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the exterior of the particle. Moreover, ethanol molecules would be placed throughout the system, not contributing to the nanoemulsion's size [121]. The siRNA would be attached to the nanoemulsion via non-covalent interactions with the cationic lipid. Interestingly, the physical radius of the nanoemulsion did not increase after the addition of the siRNA [121]. Apart from siRNA [122], NEs have been proposed for pDNA [123–125], miRNA [126] and—of the interest of this review—saRNA delivery [127].

Major advancements when considering preclinical and clinical development for cationic nanoemulsion technology have been achieved with saRNA [127,128]. The same formulation, composed of Tween80, Span 85, DOTAP and squalene, which initially showed prolonged levels of saRNA protein expression [127], was chosen to be preclinically tested in different animal models and for different applications. Notably, this formulation provided the first evidence in a non-human primate species that HIV vaccination with saRNA technology is safe and immunogenic [129]. Later, Brazzoli et al. studied the potential application of this formulation against the influenza virus, demonstrating good immunogenicity in ferrets and mice, exhibiting both T-cell and antibody responses [130]. Furthermore, it was also investigated for the Venezuelan equine encephalitis virus (VEEV) [131] and rabies virus [132]. A recent study described the development of a Zika virus vaccine using this same technology, and when comparing two different vaccine candidates, both elicited potent neutralizing antibody responses in mice and non-human primates [133]. Today, there is one cationic nanoemulsion in clinical trials for rabies virus (NCT04062669), as can be seen in Table 1.

As the reader can acknowledge by the information given so far, several delivery strategies are being proposed for RNA delivery. However, until recently, no study was conducted to make a head-to-head comparison of different formulations. A recently published study by Blakney et al. evaluated how the delivery vehicle might impact saRNA protein expression and vaccine immunogenicity [112]. To achieve this, authors compared pABOL, a polyplex formulation, which has previously shown its potential in saRNA delivery [134], with several LNP compositions, varying ionizable lipids and phospholipids (DOPE vs. DSPC). Formulation characterization results showed no major differences between colloidal properties and encapsulation efficiency, irrespective of their composition. The main difference was the surface charge; positive for pABOL and neutral for LNPs. However, when in vivo studies were performed, results showed an increased protein expression upon i.m. administration with the pABOL formulation. Within LNP formulations, DOPE-containing nanoparticles showed higher protein expression. Interestingly, when vaccine immunogenicity was investigated, LNPs exhibited higher IgG titers after the second shot, despite pABOL exhibiting higher protein expression levels upon i.m. administration. Higher antibody titers were also translated to higher protection against influenza virus with LNPs. When routes of administration were compared (i.m. vs. i.n.), i.m. administration of 1 ng of saRNA LNPs exhibited similar antibody titers to 1 µg of saRNA formulated in pABOL nanoparticles. An important factor describing vaccine immunogenicity is the capacity to achieve mucosal antibodies upon i.m. administration. In this study, mucosal antibodies were found in the majority of the doses given by LNPs, but not with pABOL. When given i.n., LNP formulations exhibited systemic antibody titers; however, these were lower than the same dose i.m. Overall, this study shows that the selection of the delivery vehicle can drive the fate and the therapeutic application and effect [112].

When considering the nanoparticle's physicochemical properties, surface charge has been previously reported to be involved in the nanoparticle's biodistribution [106], as well as the phospholipid selection [105]. Furthermore, the fact that LNPs exhibited a higher immunogenicity can be explained by increasing evidence that LNPs can act as adjuvants [135]. In this study, Alameh et al. compared three different treatments: first, rHA mRNA-loaded LNPs; second, empty LNPs (e-LNPs) with hemagglutinin recombinant protein (rHA); and third, a well-known adjuvant (Addavax) with rHA. Results showed that the first and second groups exhibited similar antibody titers, demonstrating the adjuvant capacity of e-LNPs [135].

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2.3. Additional COVID-19 Vaccines Based on mRNA and Delivery Vehicles

Apart from the Moderna and BioNTech/Pfizer mRNA approved vaccines based on LNPs, it is worth mentioning that other companies are developing additional mRNA vaccines worldwide. That which has caused the most expectation so far is the vaccine from CureVac, named CVnCov, which was also expected to be stable at 4 °C, making it available in low-income countries; a very relevant and potential advantage over the Moderna and BioNTech vaccines. However, clinical data were not as good as expected. Results of their 40,000-participant clinical trial (NCT04652102) unexpectedly resulted in only 47% efficacy, as opposed to over 90% efficacy reported by the approved vaccines [136]. Several hypotheses have been described, such as the presence of new SARS-CoV-2 variants in the countries in which the study was held. However, Moderna and BioNTech/Pfizer (as well as different-technology vaccines) have shown to be efficacious against the new variants, including the Delta variant, which was first described in India [33–35]. In terms of delivery, to the best of our knowledge, CureVac LNPs have a similar composition to the BioNTech/Pfizer vaccine, but contain a lower mRNA dose, only 12 µg [49]. At the beginning of the clinical studies, Kremsner et al. compared different dosage regimens for CVnCoV and found that higher doses triggered too many side effects [137]. The level of neutralizing antibodies found in participants dosed with 12 µg was comparable to people who had had the infection; however, it was lower than recipients of the Moderna and BioNTech/Pfizer vaccine [137]. Of an important note, the CureVac vaccine uses unmodified mRNA, without introducing chemical modifications that can be fundamental for its efficacy and safety, as commented in Section 1.1. It is soon to conclude the reasons behind these results; however, CureVac's clinical trial is still ongoing. CureVac has announced their work in optimizing these results as well as updating the vaccine for the new variants [138].

Also using LNPs, Translate Bio and Sanofi are developing MRT5500, currently under phase 1/2 (NCT04798027) clinical trials. In vivo preclinical results showed that it produces a strong immune response [139]. Other mRNA vaccine candidates refer to ARCT-021, from Arcturus Therapeutics, currently under phase 2 clinical trials. This vaccine contains saRNA encapsulated into a lipid-based LUNAR® patented delivery system—the structure and composition of which are not disclosed—and it has shown to produce an immune response similar to the one produced by the people who are infected (NCT04668339). On a similar note, Gennova Biopharmaceuticals and HDT Bio are also working on a saRNA vaccine, which links the RNA in the exterior of 'LIONS' (lipid inorganic nanoparticles) and is currently under phase 1/2 after showing promising results in preclinical models [140]. Other companies and research institutes such as Daiichi Sankyo, Elixirgen Therapeutics (NCT04863131), Walvax Therapeutics (NCT04847102) Chulalongkorn University (NCT04566276), Providence Therapeutics (NCT04765436) and Stemirna (ChiCTR2100045984) are also working on the development of more efficacious and safe mRNA vaccines, with nondisclosed formulations.

3. Biomedical Applications of mRNA Using Nanomedicine

The approval of mRNA vaccines using LNPs can indeed be considered the biggest milestone to foster the clinical translation of many other mRNA nanoformulations that can address current clinical needs. Indeed, the technology can be applied to a broad spectrum of applications and therapeutic areas. Vaccines for infectious diseases might be the first application that has reached the market, but nanoformulated mRNA has the full potential to answer clinical demands related to the needs of therapeutic proteins. In the next sections, we aim to provide a full overview of the most promising developments to date. Biomedical applications for mRNA therapeutics are pictured in Figure 4.

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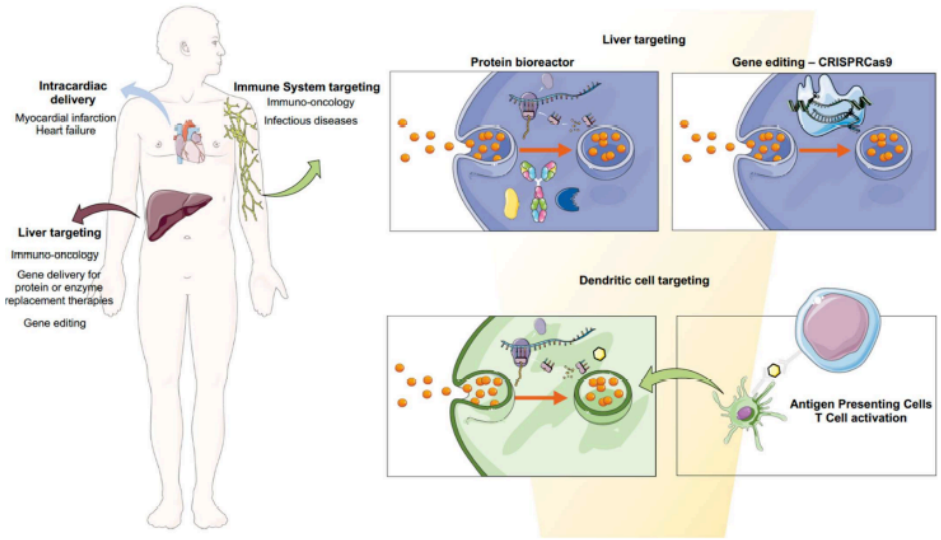


Figure 4. Biomedical applications for mRNA therapeutics. Immuno-oncology treatments can be envisioned using the liver as a factory of therapeutic proteins such as neoantigens or antibodies, as well as by using the antigen-presenting cells' physiological function to induce immune activation towards cancerous cells. The latter can also be applied for the development of safe and efficacious mRNA and saRNA vaccines against infectious diseases. Gene delivery for therapeutic protein synthesis or restoration of a particular enzymatic function as well as gene editing using CRISPR cas9 technology can be achieved by targeting the liver. Cardiovascular regeneration can be achieved by intracardial delivery of mRNA encoding for growth factors.

3.1. Infectious Diseases

Despite the fact that COVID-19 vaccines have been the first marketed mRNA formulations, this technology has been researched for decades using different delivery approaches, from LPX to cationic nanoemulsions to polymeric nanoparticles [9]. Since mRNA loaded into liposomes demonstrated for the first time that it could trigger a specific T-cell response against influenza virus in mice [141], the field has enormously advanced. In fact, only in 2019, 15 mRNA candidates entered clinical trials [10]. Because this area has very recently been covered in an extensive review [142], we will only showcase some of the most promising approaches, redirecting the reader to this article for further information. Nonetheless, all the clinical trials for infectious diseases other than COVID-19 are shown in Table 1. In this regard, clinically, respiratory syncytial virus (RSV) and influenza virus are amongst the most advanced clinical candidates.

Furthermore, preclinically, one of the most important applications is the HIV vaccine. HIV currently affects 38 million people; these numbers are expected to increase by 2030 [143]. This virus has been extensively researched for decades; however, its retroviral nature, along with the fact that it possesses a very high antigen diversity in its envelope, make it very difficult to achieve good outcomes [144]. In recent years, several studies have tried to deliver mRNA-vaccine encoding for HIV proteins; however, their success has not been as expected [127,129,145]. Nevertheless, a recently published study has shown broad neutralization and reduction in the risk of infection in rhesus macaques upon the administration of mRNA encoding for both HIV-1 envelope protein and simian immunodeficiency virus Gag proteins, which would be responsible for forming virus-like particles (VLP) [146]. A different approach based on targeting hepatocytes, which will be further

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discussed for immuno-oncology applications in Section 3.2.2, has achieved good results. This study involved the delivery of mRNA encoding the light and heavy chains of VRC01, a neutralizing antibody against HIV-1. Using LNPs, they were able to achieve robust protein expression in the liver. Furthermore, prophylactic immunization was also achieved at a dose of 0.7 mg/kg as opposed to the 10–20 mg/kg needed if the recombinant protein is administered [147].

Another important preclinical advancement was achieved with the Ebola vaccine. Ebola virus (EbolV) caused the deaths of more than 11,000 lives across West Africa from 2014 to 2016 [148]. This lethal virus causes a hemorrhagic fever that is fatal in up to 50% of the patients [148]. In 2019, one viral vector-based Ebola vaccine was approved by the FDA; however, clinical trials reported some safety concerns. As explained in the introduction, from this point of view, mRNA vaccination presents an advantage. In a study on guinea pigs, 20 µg of glycoprotein-encoding mRNA was administered using LNPs as a delivery vehicle, resulting in good antibody titers and protection from death [149].

Lastly, it is important to note the advancements achieved in other infectious diseases other than viruses. In this regard, *Plasmodium*, the causal agent of Malaria, is highlighted next. Very recently, the first malaria vaccine was approved by WHO [150]. The RTS, S malaria vaccine is a recombinant protein vaccine which has shown to prevent lethal disease, and is especially efficacious when given together with pharmacological treatment [150]. Several studies have also been conducted towards the development of an mRNA vaccine against malaria. The first is an saRNA-based [151] encoding for *Plasmodium*-secreted cytokine macrophage migrating inhibitory factor, which had previously shown efficacy [152]. In this study, anti-*Plasmodium* antibodies and protective T-cell memory were achieved [151]. In another study, *Plasmodium falciparum* acid-rich protein (PfGARP) mRNA-encoded LNPs were dosed to infected aotus monkeys, showing reduced levels of the parasite after three doses [153].

Altogether, these advancements provide strong evidence of the possibilities ahead regarding mRNA vaccinology, which seems to provide necessary advantages as the next vaccine technology, especially when considering future pandemics.

3.2. Immuno-Oncology

RNAs are critical for gene expression in their multiple forms, whether in non-coding forms such as miRNA, siRNA or tRNAs that regulate transcription and translation, or in coding forms such as mRNA [154]. Cancer immunotherapy has been a game changer since the approval of the immune checkpoint inhibitor ipilimumab in 2011 [155]. The number of immuno-oncology (IO) drugs in the development pipeline in 2020 grew by 22% compared to 2019, accentuating the high interest in these therapies. Immunomodulators, cell therapies and cancer vaccines represent the most important trends [156]. In fact, these therapies are often given simultaneously, with one example of this being TriMiX, whose composition is CD70, CD40 ligand and TLR4 [157]. However, scientists have met a few challenges when it comes to the new development of these immunotherapeutic agents, mostly related to off-target toxicity [158]. Thus, mRNA technology can offer advantages in terms of scalability, cost and production issues, widening immunotherapy use worldwide. Altogether, immunotherapy combined with nanomedicine could bring a potential solution: to engineer the delivery of immunomodulators, focusing their action on target tissues such as tumors or tumor-draining lymph nodes, or specific cell types, so as to control the timing and location of immunomodulation [158]. To achieve this, two main strategies can be performed: the first, by targeting DCs and taking advantage of their natural immune role; and the second, by making use of liver capacity to synthesize proteins. In the following sections we will describe both mechanisms in depth.

3.2.1. Cancer Vaccines: Targeting Dendritic Cells

The most well-known strategy for treating cancer with mRNA is cancer vaccines, in which mRNA is transcribed into a protein which in turn trains the immune system to fight

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against the tumor. Thus, rather than being prophylactic, cancer vaccines seek to stimulate cell-mediated responses. Therapeutically, this technology can be applied (1) by using shared antigens for the same tumor to stimulate immune activation, or (2) by designing a personalized cancer vaccine which possesses the individual's neoantigens, i.e., proteins preferentially expressed in cancerous cells but not in the patients' healthy tissue, such as growth-associated factors or antigens [9].

The way the latter strategy (2) is approached involves the most advanced tissue gene sequencing: a patients' tumor and healthy tissue samples are taken and analyzed looking for tumor-associated antigens, as pictured in Figure 5.

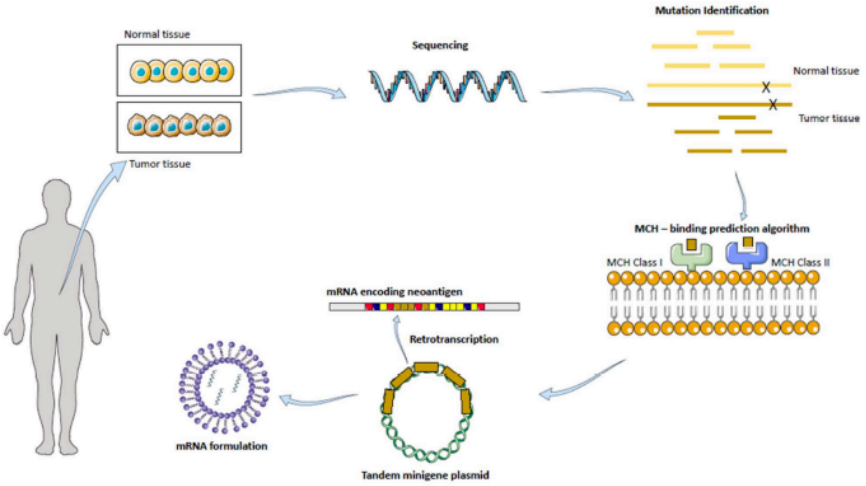


Figure 5. Personalized mRNA cancer vaccines. Healthy and tumor tissue are isolated from the patient sample, analyzed and compared in order to find mutations present in the tumor tissue. Bioinformatic tools are used to predict the MCH Class I and II binding. A tandem gene encoding for several neoantigens is then designed and retrotranscribed to mRNA. Finally, by making use of nanotechnology, the mRNA is formulated and delivered. Adapted with permission from [159], published by Nature Reviews Drug Discovery, 2018.

The technology relies on a special algorithm prediction of binding strength to self-major histocompatibility complex (MHC) receptors. After mutation identification, neoantigens are ranked based on how well they would bind to this receptor, which will then drive the activation of T cells, followed by the immunological response against that specific neoantigen. Upon administration of the mRNA into a suitable, stable and efficient formulation, DCs are transfected, and due to their antigen-presenting cells' nature, they localize the neoantigen onto their MHC receptor located in their surface, which will be then presented to the T cells, starting the immune cascade [159,160].

Today, both strategies described, (1) and (2), are under clinical evaluation in phase 1 or 2. Melanoma, prostate cancer, triple-negative breast cancer and head and neck cancer are some of the biomedical applications in which this technology is being clinically investigated (Table 2). An extensive review on this matter has been recently published [161].

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Table 2. mRNA Nanomedicines for Oncology in Clinical Trials. A list of clinical trials for oncology applications, including the subsections described above, is provided in this table.

Oncology					
Cancer Vaccines					
Shared Tumor Antigens					
BNT111	Advanced Melanoma	Modified mRNA-LPX	Phase II	NCT04526899	BioNTech
BNT112	Prostate Cancer	Modified mRNA-LPX	Phase I/II	NCT04382898	BioNTech
BNT113	HPV16+ head and neck cancer	Modified mRNA-LPX	Phase II	NCT04534205	BioNTech
BNT114	Triple-Negative Breast Cancer	Modified mRNA-LPX	Phase I	NCT03815058	BioNTech
BNT115	Ovarian Cancer	Modified mRNA-LPX	Phase I	NCT04163094	BioNTech
mRNA-5671	Carcinoma, NSCLC, Pancreatic and colorectal neoplasms	Non-disclosed	Phase I	NCT03948763	Merck/Moderna
Personalized cancer vaccines					
mRNA-4157	Melanoma	Modified mRNA-LNPs	Phase II	NCT03897881	Moderna
BNT122	Advanced Melanoma	Modified mRNA-LPX	Phase I	NCT03815058	BioNTech
mRNA-4157	Solid Tumors	Modified mRNA-LNPs	Phase I	NCT03313778	Moderna
mRNA-2752	Solid Tumors	Modified mRNA-LNPs	Phase I	NCT03739931	Moderna
CV8102	Solid Tumors	Not disclosed	Phase I	NCT03291002	CureVac
V941	Solid Tumors	Not disclosed	Phase I	NCT03948763	Merck Sharp & Dohme Corp.
Medi1191	Solid Tumors	Modified mRNA-LPNs	Phase I	NCT03946800	MedImmuneLC
mRNA-2416	Solid Tumors and Lymphoma	Modified mRNA-LNPs	Phase I/II	NCT03323398	Moderna
mRNA-2752	Solid Tumors and Lymphoma	Modified mRNA-LNPs	Phase I	NCT03739931	Moderna
mRNA-2752	Carcinoma	Modified mRNA-LNPs	Phase I	NCT02872025	Moderna
BNT122	Colorectal Cancer Stage II/III	Modified mRNA-LPX	Phase II	NCT04486378	BioNTech
MVT-5873	Pancreatic Cancer	Not disclosed	Phase I	NCT02672917	BioNTech
BNT131	Metastatic Neoplasm	Not disclosed	Phase I	NCT03871348	BioNTech
The Liver as a Bioreactor					
Encoding Cytokines					
BNT152, BNT153	Solid Tumor	Not disclosed	Phase I	NCT04710043	BioNTech
BNT151	Solid Tumor	Not disclosed	Phase I/II	NCT04455620	BioNTech
mRNA-6231	Autoimmune Disorders	Modified mRNA-LNPs	Phase I	NCT04916431	Moderna
Encoding Antibodies					
BNT142	Solid Tumor	Encoding Ab	Phase I/II	NCT04503278	BioNTech
mRNA-1944	Chikungunya	Modified mRNA-LNPs	Phase I	NCT03829384	Moderna

Abbreviations: Ab—antibodies; NSCLC—non-small-cell lung cancer.

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3.2.2. The Liver as a Factory of Immunomodulatory Proteins

As commented in the previous section, targeting DCs is the main strategy towards the development of cancer vaccines. Nonetheless, this is not the only approach in which mRNA can be used for immunotherapy. This approach is based on the on the fact that nanoparticles have the intrinsic capacity to accumulate in the liver [162], which has been for many years the core center of the research. Thus, targeting the liver can be achieved by exploiting its capacity for protein production in order to build up an endogenous factory of therapeutic proteins, which will then be released into systemic circulation. In fact, the feasibility of targeting the liver for mRNA delivery for the endogenous production of immunomodulators can be envisioned by considering advances in other research areas, such as protein replacement therapies with mRNA, which will also be covered in Section 3.3. Furthermore, this strategy also overcomes the main disadvantages of many immunomodulator administrations, such as their short half-life and subsequent long perfusions and recurrent visits to the medical doctor's office, as well as their high production cost [163].

This mRNA application has been studied for two types of recombinant proteins: immunomodulators and antibodies. The production of immunomodulators, such as cytokines, has already been proven to be an achievable strategy. For instance, intratumoral administration of LNPs with mRNA-encoding cytokines IL-23 and IL-36 and the T-cell costimulatory OX40L was proven to lead to tumor regression in colon cancer and melanoma models and is now under clinical trials (NCT03739931) [164]. In another study using IL-12, a well-known cytokine [165], Lai et al. reported mRNA-encoding IL-12 LNPs to be a good candidate for slowing down the progression of MYC oncogene-driven human hepatocellular carcinoma, with good biodistribution within the tumor and no toxicity. Treatment reduced liver-tumor burden and increased survival in MYC-induced HCC transgenic mice [166].

Interestingly, co-delivery of cytokines and antibodies has proven to have potential for treating different types of cancer [166–168]. In fact, the use of mRNA to endogenously produce antibodies has also been achieved. Apart from the already-commented HIV study in Section 3.1 [147], another study with trastuzumab aimed to apply this strategy to tackle oncology. Rybakova et al. succeeded in efficiently delivering mRNA encoding for trastuzumab, a monoclonal antibody widely used for breast cancer. They found a dose-dependent increase in trastuzumab protein levels in mouse serum after 24 h administration. When the administration of the monoclonal antibody Herceptin® was compared, results revealed similar half-lives for both antibodies (i.e., endogenously produced and Herceptin); however, the mRNA-based delivery resulted in a 64% higher antibody serum concentration over the course of 30 days. Overall, a single dose of LNPs with the mRNA at 2 mg/kg showed a favorable pharmacokinetic profile when compared to a single injection of Herceptin at 8 mg/kg [169]. This technology has also been proven to be efficient for the delivery of bispecific antibodies [170]. Stadler et al. succeeded in achieving a promising therapeutic outcome by generating his-tagged bispecific antibodies directed against the T-cell receptor-associated molecule CD3, a tumor-associated antigen [170].

3.2.3. CAR-T Cells

On a last note on immunotherapy, the CAR-T-cell field can also take advantage of mRNA technology. Chimeric antigen receptor (CAR) T-cell therapy is considered by many the next promise of cancer immunotherapy. In August 2017, the FDA approved the first CAR-T treatment, Kymriah® (tisagenlecleucel), for the treatment of certain patients, both pediatric and young adults with B-cell acute lymphoblastic leukemia (B-ALL). Several months after that, in October 2017, the FDA approved Yescarta® (axicabtagene ciloleucel) for adult patients with certain types of refractory B-cell non-Hodgkin lymphoma (B-NHL) [171] This approach consists of engineering lymphocyte T surfaces with tumor specific antigens that would bind to tumor-specific receptors. Right now, CD19-targeting CAR-T-cell therapy is approved for both B-cell acute lymphoblastic leukemia (B-ALL) as well as non-Hodgkin lymphoma (NHL), where it has demonstrated up to 90% complete remission when rayed in chemo-refractory B-cell malignancies [171]. Despite the promising positive outcomes on

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liquid tumors, CAR-T therapy has challenges in its application to solid tumors, including limited cancer-specific targets and non-persistence of the transferred CAR-T cells, allowing for low side effects but efficient tumor eradication [172], and research needs to be carried out to tackle solid tumors such as breast, melanoma or ovarian. With regard to solid tumors, in a recent paper published, T-cells were bioengineered with nanoparticle-encapsulating mRNA encoding for a single-chain variable fragment (scFv) that can specifically bind to CLDN6, an oncofetal cell-surface antigen for CAR-T-cell targeting. Results demonstrated that RNA nanoparticles can efficiently stimulate CAR-T cells [173].

3.3. RNA-Based Protein Replacement Therapies

As mentioned before, attempts to deliver therapeutic antibodies as mRNA molecules have been carried out with success with the aim of overcoming the drawbacks of administering the therapeutic protein. So far, protein replacement therapies are mainly focused on secreted proteins or proteins of bacterial origin. It is important to understand the processes following the translation of mRNA into proteins, which include folding, post-translational modification, aggregation into secretory granules and transport outside the cell. One of the most important things to consider are the signal peptides, as they take a great role in directing the protein secretion. Unless the signal sequence is optimized, extracellular mRNA should be transfected ideally into cells that the encoded protein is naturally secreted in [174]. A table with current clinical trials on going is provided in this section (Table 3).

Table 3. mRNA Nanomedicines for Protein Replacement Therapies in Clinical Trials. A list of clinical trials for protein replacement applications is provided in this table.

mRNA Delivery for Protein Replacement Therapies					
mRNA-3927	Propionic Acidemia	Modified mRNA-LNPs	Phase 1/2	NCT04159103	Moderna
MRT5005	Cystic Fibrosis	Modified mRNA-LNPs	Phase 1/2	NCT03375047	Translate Bio

3.3.1. Cystic Fibrosis

Cystic fibrosis (CF) is a genetic disease which affects over 90,000 individuals all over the world. The F508del is the most prevalent CF-causing mutation, and affects approximately 82% of the CF population. This mutation leads to CFTR-protein misfolding, which is then arrested by the cell machinery and targeted to the proteasome, leading to its premature degradation [175]. Thus, mRNA-mediated replacement therapy can be applied to produce copies of the cystic fibrosis conductance receptor (CFTR), which is defective in CF patients. However, when it comes to delivery of mRNA nanoparticles to the lung, these nanoformulations not only need to be endocytosed, escape the endosome and release the cargo inside the cytoplasm, but they also need to migrate across viscous mucus, avoid clearance and uptake by the macrophages within this mucus and the airways and finally reach the pulmonary epithelial cells [175,176]. These additional biological barriers make the delivery of mRNA-loaded nanoparticles to the lung especially challenging. To date, the most advanced mRNA therapy for cystic fibrosis comes from Translate Bio and it is currently in Phase 1/2 (NCT03375047) [177].

3.3.2. Rare Metabolic Diseases

An important area which would be enormously impacted by this technology are rare metabolic diseases. These disorders lack financial support, and thus, effective treatments. From this point of view, mRNA technology can entail an enormous advantage due to the cost-efficient matters already commented. Furthermore, the previous idea and background given on endogenously produced proteins can also be applied in this section.

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Acute Intermittent Porphyria

Acute intermittent porphyria is a chronic disease caused by the hepatic deficiency of porphobilinogen deaminase (PBGD), the enzyme which catalyzes the transformation of porphobilinogen into protoporphyrinogen during the heme synthesis pathway [178]. This causes the accumulation of the porphyrin precursors γ-aminolevulinic acid (ALA) and porphobilinogen (PBG), which entails acute neurovisceral attacks associated with a high production of potentially neurotoxic porphyrin precursors [178]. Treatments so far are composed of hemin replacement therapy, which restores the regulatory heme pool in the liver and suppresses the accumulation of precursors; however, these reductions occur days after the injection and the levels of enzymes should be monitored closely. Although this is an efficacious treatment, 5% of patients suffer recurrent attacks over the years. Prophylactic hemin treatment is being more commonly used for chronic symptoms; however, repeated administrations have been shown to have important side effects [179] RNA therapies seem to give an answer to the treatment of this disease. The siRNA drug Givosiran® aims to silence the ALAS1 enzyme, stopping the production of ALA and PBG. mRNA therapies are still in preclinical development. In this regard, Jiang et al. evaluated, along with the Moderna, the potential treatment of this disease with mRNA-encoding PBGD enzyme into LNPs in two models of acute hepatic porphyria. Furthermore, they also performed a multiple-dose study on non-human primates to further prove its translatability to humans. The results showed that administration of hPBGD mRNA led to expression of the therapeutic protein after 2 h post-administration, and most importantly, maintained its activity throughout the entire duration of the attack [180]. Overall, this study reported sustained efficacy and tolerability of the hPBGD mRNA after single and repeated administrations in mice, rabbit and non-human primates [180], which further evidenced the potential of mRNA for the treatment of this disease.

Propionic Acidaemia (PA)

Propionic Acidaemia (PA) is a rare pediatric disorder caused by propionyl-CoA carboxylase (PCC) deficiency, which results in impaired propionate metabolism and accumulation of toxic metabolites, leading to a life-threatening condition. There are two disease subtypes, PCCA deficiency and PCCB deficiency, each of them referring to the different subunits forming PCC, respectively A (of 72 kDa) or B (of 54 kDa). Current treatments for this disease include fluid therapies or actions to remove the excess of toxic metabolites, but liver transplant is also a potential outcome for these patients. In this term, mRNA-therapeutic encoding for PCC enzymes can provide a solution. In this study, Jiang L. et al. report the use of two mRNAs encoding both human PCCA and PCCB. Results showed the restoration of ammonia levels and the function of the PCC enzyme in the liver; reducing toxic metabolites associated with this condition up to 6 months upon repeated administrations in mice [181]. Currently, mRNA-3927 from Moderna Therapeutics is under clinical trials (NCT04159103).

Fabry Disease

Fabry Disease is an X-linked lysosomal storage disorder which results from the mutation of the gene GLA encoding for the lysosomal enzyme α-galactosidase A, responsible for glycolipids' metabolism. Its loss of activity leads to fat accumulation within the lysosomes of multiple tissues, which in turn leads to clinical disease with fatal renal and cardiac function alteration [182]. Current treatments for this metabolic disease include enzyme replacement therapies named Replagal® and Fabrazyme® [183]; and migalastat [184], a small molecule chaperone. While these treatments have shown to slow the progression of the disease, the variability of response, immunogenicity and difficulties with the manufacturing of these therapies are needs which have not yet been met. In this context, the main studies with mRNA have been published so far, showing some advancements. Using mRNA encoding for α-galactosidase, DeRosa et al. demonstrated it is possible to achieve sustained delivery in a Fabry model mouse and in a non-human primate with a resulting reduction of relevant biomarkers [185]. Upon delivery, serum protein levels

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reached as high as 1330-fold over normal physiological values. Another study conducted by Moderna also showed promising results upon intravenous administration of LNPs, with a dose-dependent protein activity and substrate reduction maintained for up to 6 weeks in mice. Upon repeated administration, the pharmacodynamic response was sustained and the administration to non-human primates confirmed safety and translatability [186].

Methylmalonic Acidemia (MMA)

Methylmalonic Acidemia (MMA) is a rare metabolic disease which is characterized by mutations in the gene coding of the vitamin B12-dependent enzyme methyl-malonyl-CoA (MUT), causing partial or complete loss of activity. This enzyme catalyzes important metabolic reactions linked to the Krebs cycle, playing a central role in intermediary metabolism. Its partial or total lack of activity causes the accumulation of toxic metabolites and the alteration of mitochondrial oxidative phosphorylation. Clinically, patients affected with this disorder experience multisystemic complications which are more common in organs with high metabolic demand, such as the brain or heart [187].

There is currently no effective treatment for these patients, and this disorder is often treated by carnitine supplementation and protein-limited diets; however, mortality is still around 20%, and survivors can develop systemic complications. mRNA therapy encoding for human MUT using LNPs as delivery vehicles was studied by An et al. in two clinically relevant models. In both, a decrease in the enzyme substrate methylmalonic acid blood levels were achieved, with good pharmacokinetic properties. Importantly, none of the clinical models developed any hMUT antibodies upon repeated administration or hepatic inflammation [188].

Ornithine Transcarbamylase Deficiency (OTD)

Ornithine transcarbamylase deficiency (OTD) affects 6 in 100,000 people, and is the most frequent inborn error of the urea cycle. As other urea-cycle disorders, it results in ammonia accumulation which clinically translates to fatal neurological disorders. OTD is more specifically caused by a deficiency in ornithine transcarbamylase (OTC). Therapeutic options for these patients include a protein-restricted diet for life, with arginine and citrulline supplementation, but liver transplantation is the only therapeutic option in cases of severe OTC deficiency [189]. In this study, the mRNA administration resulted in increased OTC enzymatic activity up to 10 days after dosing, along with increased mice survival [190].

3.4. Gene Editing

Clustered regularly interspaced short palindromic repeat (CRISPR)-related systems have become the main tool when considering therapeutic approaches for the cure of genetic diseases. CRISPR-Cas9 complex is composed of two main elements: Cas9 protein, an endonuclease that is capable of DNA cleaving; and a single-guide RNA (sgRNA), which guides the Cas9 protein to the site of interest. This gene-editing tool is, therefore, based on the recognition of PAM (protospacer-adjacent motif) sequence and a complementary 20-nucleotide genomic sequence that induces DNA brakes, which are repaired by error-prone nonhomologous end-joining or precise homology-directed repair [191]. To date, CRISPR/Cas9-based systems are versatile and have extensively been used for biotechnological purposes such as knockout/knock-in animal models or cells. From a drug-delivery point of view, it is important to understand that intracellular delivery of both components is needed. Therefore, various in vivo studies addressing differing delivery approaches such as viral vectors, mRNA or recombinant protein have been performed. In this section we will focus solely on mRNA delivery, as it is the purpose of this review [191–195].

One of the approaches that have been most extensively researched are hepatic diseases. In 2016, a study on human hereditary tyrosinemia (HHT) showed slightly promising results on correcting the fumarylacetoacetate hydrolase (FAH) mutation. This was performed by the systemic delivery of Cas9 mRNA encapsulated in LNPs and sgRNA encapsulated in

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AAV particles. The final results demonstrated an overall gene correction of 6%, suggesting that this could be an effective approach for the treatment of a range of metabolic liver diseases. However, authors pointed out that one potential reason for the mild gene correction could be that the nanoformulations were not co-delivered, hypothesizing that the sgRNA suffered degradation during mRNA translation, and thus decreasing efficacy [195]. Following up on this, another study conducted by the same team aimed to deliver both Cas9 mRNA and sgRNA using LNPs for both components. In order to make it possible for the sgRNA to be efficiently delivered, they conducted several chemical modifications on the sequence, i.e., including the addition of 2' hydroxyl (OH) groups on nucleotides, which resulted in highly improved results with an efficacy of 80% [196] This study is especially important as it highlights the importance of chemical modifications as well as the possibility of using synthetic nanoparticles, broadening up the industrial applications of gene editing. Similarly, Miller et al. reported successful results with a 95% protein knockdown in vitro, by making use of a zwitterionic lipid as a delivery vector by also co-delivering the different components [197] Adding to this latter study, Finn et al. reported a 97% reduction in serum protein levels upon the administration of Cas9 mRNA co-formulated with modified sgRNA into LNPs for gene editing of the mouse transthyretin gene in the liver [198]. To sum up, probably the most important advancement with regards to gene editing is the NTLA-2001 clinical study (NCT04601051), the first clinical trial so far with CRISPR-Cas9, conducted by Intellia Therapeutics for patients with hereditary ATTR amyloidosis suffering from polyneuropathy. This gene-editing medicine is based on the CRISPR Cas9 system and it uses mRNA-encoding Cas9 protein and the guide RNA encapsulated in a LNPs. Results showed mild adverse effects, and the administration led to a decrease in TTR after a single dose [199]. In October 2021, Intellia Therapeutics announced the beginning of a new CRISPR clinical trial, NTLA-2002, for the treatment of hereditary angioedema (HE), a rare autosomal dominant disorder that causes unexpected inflammatory attacks [200]. Altogether, these results present an undeniable precedent for gene editing. A current landscape on CRISPR-Cas9 clinical trials is provided next (Table 4).

Table 4. mRNA Nanomedicines for Gene editing in Clinical Trials. A list of clinical trials for gene editing applications is provided in this table. Abbreviations: sg, single-guide.

Gene Editing					
NTLA-2001	Hereditary Transthyretin Amyloidosis	Cas9 mRNA and sg mRNA-LNPs	Phase 1	NCT04601051	Intellia Therapeutics
NTLA-2002	Hereditary Angioedema	Cas9 mRNA and sg mRNA LNPs	Phase 1/2	Not registered yet	Intellia Therapeutics

3.5. Autoimmune Diseases

Autoimmune diseases are chronic and debilitating conditions which are caused by immune response against self-antigens. Disorders such as multiple sclerosis or diabetes type I affect millions of people worldwide. Current treatments available do not target the origin of the disease and are not curative. However, immune tolerance—defined as the state of unresponsiveness of the immune system (IS) to any substance that has the potential to trigger a response—has been described before as a potential therapeutic tool [201,202]. In this term, mRNA technology could potentially be used to induce self-tolerance, by administering a self-antigen encoded in mRNA. In a recent study, Krienke et al. [203] showed that it is possible to deliver myelin oligodendrocyte glycoprotein (MOG)-encoded mRNA by making use of the lipoplex-type nanocarrier to multiple-sclerosis mice models. Upon intravenous administration, modified mRNA was capable of triggering the proliferation of T-reg cells without inducing immune activation, preventing myelin damage and paralysis [203]. This is, to the best of our knowledge, the first proof of concept using mRNA technology for immune tolerance.

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3.6. Cardiovascular Diseases

Another potential for the development of mRNA therapeutics is treating myocardial infarction (MI) and heart failure (HF), the leading causes of death in many countries. MI leads to a massive loss of cardiomyocytes (CM), which are replaced by highly proliferating fibroblasts, resulting in loss of healthy tissue. These changes are irreversible, and along with oxidative stress and inflammation, the ultimate result is heart failure. Gene therapy with DNA-based or viral vectors has been used to try to induce cardiac regeneration, but has not had great success due to poor and uncontrolled gene expression. However, mRNA technology could give a plausible response for the treatment of ischemic injury, mainly by three different mechanisms: (1) inducing CM proliferation; (2) inhibiting heart cell death and attenuating inflammation; and (3) supporting cardiovascular regeneration. It is important to note that although mRNA applications in cardiology are progressing quickly, right now research is focused on intracardiac administration, as it is the most effective delivery method so far; however, it causes stress and local injury to the tissue. Therefore, it is crucial to develop a delivery vehicle that can target the heart and that can be then administered s.c. or i.v. [204,205]. mRNA encoding for vascular endothelial growth factor A (VEGF-A) is currently under clinical phase 2 (Table 5) in a collaboration between AstraZeneca and Moderna Therapeutics after showing the induction of cardiovascular regeneration in several studies. In addition, Magadum et al. were able to induce CM proliferation by using mRNA encoding for FSTL1 and Pkm2 [204].

Table 5. mRNA Nanomedicines for cardiovascular diseases in Clinical Trials. A list of clinical trials for cardiovascular diseases is provided in this table.

Cardiovascular Diseases					
AZD8601	Heart Failure	Modified mRNA-LNPs	Phase II	NCT03370887	AstraZeneca

4. Highlights and Conclusions

The development and commercialization of mRNA COVID-19 vaccines have pivoted the way towards future applications of mRNA medicines by finally finding a solution to the problem of delivery. Despite concerns from detractors about a lack of applications for nanotechnology, nanomedicine has now demonstrated that it is a translational, relevant and much-needed approach to engineer new genetic drugs. Thus, it is now clear that the full potential of mRNA therapeutics, along with its advantages could not be envisioned without nanomedicine. As presented in this review, mRNA therapeutics are advantageous over other gene therapies in many important ways, such as improved safety, ease of manufacture and interchangeable mRNA sequence.

However, despite the importance of having addressed the question of delivery, several limitations are still present when considering mRNA therapeutics and nanoparticle formulations. In terms of materials used for nanoparticles, there is still a need to find new biocompatible and—ideally—biodegradable compounds that would allow repeated and recurrent administration, which is needed for some of the applications reviewed in this article, such as protein replacement therapies. In this sense, a high-throughput screening of new lipids and polymers that can retain encapsulation efficiency is a high priority. Moreover, understanding, minimizing or even taking advantage of the pro-inflammatory response to LNPs is crucial for the future development of new mRNA-based drugs. Additionally, the COVID-19 pandemic has proven that new materials capable of maintaining mRNA stability at higher temperatures in order to achieve an easy-to-transport technology is crucial, especially considering equitable access to vaccines.

In regard to the mechanism of action, assays to investigate endosomal escape and release of the cargo once endocytosed, as well as endosomal trafficking, should be considered as relevant assays to undertake during the preclinical development of mRNA medicines. This matter is of particular interest, as endosomal escape has not been fully

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understood yet, and is one of the main problems regarding the transfection efficiency of mRNA nanoparticles.

Considering biomedical applications, the restrained delivery to DCs and natural accumulation of nanoparticles in the liver might be one of the most challenging and important issues to tackle, as it averts the potential of this application to other organs. In this regard, recent studies on selective organ targeting [80] have shown that by correctly bioengineering nanoformulations it is possible to address this inconvenience. Recently, however, this mechanism was proposed based on the formation of the protein corona, by which nanoparticles interact with blood proteins, redirecting them towards different organs. In this way, a new paradigm—endogenous delivery—starts, in which the carriers' composition is designed to interact with specific and known proteins to overcome the hepatic barrier [206].

Today, after millions of doses have been administered worldwide, the available data on mRNA COVID-19 vaccines showcase a great safety profile together with high real-world effectiveness, providing more evidence on how this technology can reshape the future of modern medicine for many therapeutic applications, from cancer vaccines to gene editing.

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