IMMUNOWATCH

EDITION N°7 - JUNE 2023

RNA-BASED DRUG PRODUCTS

mabdesign
THE BIOTHERAPY EXPERT

INTRODUCTION

MabDesign's Immunowatch is a one-of-a-kind information monitoring newsletter in the field of biologics. Its aim is to provide members of our association with the most recent and pertinent data gathered or generated through the key expertise of MabDesign and its collaborators in scientific research, business intelligence, market analysis and intellectual property.

Each edition will focus on trending type of biologics. Its general format includes market study research, financial and economic data, invited contributions from scientific teams working in the industry or in academia and a section dedicated to intellectual property. The content of each edition is decided by an editorial composed of two field experts. Decision concerning the theme and conception of each newsletter is done in-house by the permanent members of our editorial team.

Finally, we would like to acknowledge the support of the Ambition Recherche & Développement (ARD) Biomédicaments 2020 Phase II programme, funded by the Centre Val de Loire region during the initial phases of launching this newsletter.

BIOPHARMACEUTICALS
Innovation Dynamics in Health
IN REGION CENTRE-VAL DE LOIRE

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Région Centre-Val de Loire

RD-Biotech

YOUR mRNA STARTING MATERIAL SUPPLIER

PLASMID DNA MANUFACTURING SERVICES
THE MOST COMPLETE SOLUTION

pDNA as starting material for various mRNA applications

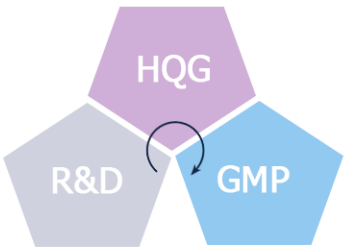
RNA DRUG PRODUCTS

mRNA VACCINES

mRNA BASED THERAPIES

RD-Biotech Expertise:

Custom services dedicated for the production of pDNA for research, preclinical, clinical and commercial applications



QUALITY GRADES

pDNA Engineering

- High expertise in **polyA based constructs**
- Cloning
- Mutagenesis

pDNA manufacturing

- **Purified linearized pDNA** ready to use for IVT appplications
- Circular pDNA
- Several quality grades
- Up to grams scale

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EDITORIAL

Régis Gervier

Sanofi

Regis Gervier has worked in the vaccine industry for over 20 years. Since July 2021, he has led Sanofi's mRNA Center of Excellence. This mRNA Center of Excellence aims to push the boundaries of mRNA science to advance next-generation vaccines and therapies.
The overnight success of mRNA and its potential for use in medicine builds on 60 years of incredible research and groundbreaking work. There were many steps along the way to understand the fundamental biology and science to a point where mRNA could be safely and effectively used as the core of a preventative vaccine targeting a global population.
The potential of mRNA is immense and we're just at the beginning. The next steps in this technology's evolution are already in motion and we're actively shaping our future to deliver on the promise of mRNA. Indeed, there are several elements to address to improve the mRNA technologies used against the SARS-CoV2 virus during the pandemic in order to broaden applications of mRNA even within vaccinology. For example, there are outstanding questions such as the durability of the in vivo expression, the lasting mRNA-stimulated immunity, or the stability of vaccines in the supply chain that must be maintained at sub-freezing temperatures for most of their shelf life. As we overcome these challenges, and many others, we envision a vast field of potential applications to come within prophylactics and therapeutics fields. If you set your eyes on protein production through the mRNA "coding factory" as a goal, you can imagine extending the range of therapeutic areas including rare diseases and oncology. It opens the field to a colossal number of different opportunities.

Chantal Pichon

University of Orléans

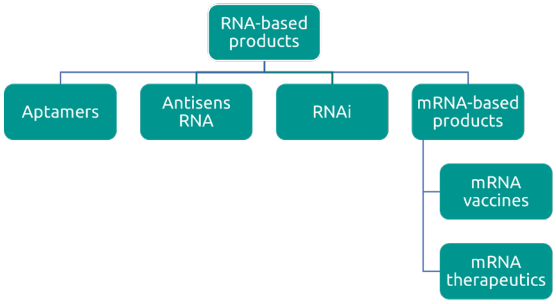
Chantal PICHON is Professor at the University of Orleans (France), senior member of the Institut Universitaire de France holding the chair of Innovation and invited Professor at La Charité Berlin Institute of Health funded by Stiftung Charité. She is conducting interdisciplinary projects based on chemistry and molecular and cell biology with a crosstalk between basic and applied researches. This last decade, she has focused her research interests on messenger RNAs by developing innovative formulations for their delivery for various applications and building a breakthrough approach for mRNA bioproduction. From September 2023, Chantal Pichon will be the director of ART ARNm, a new Inserm lab to support innovations and translation of mRNA-based vaccines and therapeutics (Orléans).
The success of messenger RNA (mRNA) vaccines against COVID-19 has brought biopharmaceuticals and nanomedicine to the forefront of the R&D community, the public and financial investors worldwide. This achievement stems from over twenty years' hard work by a community of researchers from a wide range of disciplines (biology, biotechnology, immunology and nanomedicine), who have worked together to develop the mRNA technology. There is no doubt that we are at the dawn of a revolutionary new era in medicine, as mRNA offers us the possibility of making vaccines against deadly infections, and developing both personalized therapy and more universal strategies for a variety of diseases.
Who would have imagined that mRNA vaccines would win the vaccines race? When BioNtech and Moderna announced that, an mRNA vaccine against Covid 19 would be produced in fast speed, which was revolutionary given that a traditional vaccine requires several years' development. The scientific community working on RNA therapeutics was on tenterhooks, aware of the difficulties we had have to overcome if the results were not up to scratch but the promise was fulfilled! If these biotech companies are in the position they are today, it is because at a given point in time, they were supported in their breakthrough projects. The lesson learned from this situation is also the strength of multi-disciplinary research, and the strong interactions between academic and private laboratories. With the pandemic and the closing of borders, there have been outages of both materials and reagents, showing the limits of dependence. The current situation that we face with regard to some medicines demonstrates how crucial it is to be independent and sovereign in terms of drug production.
This pandemic has brought to light mRNA technology, which for years has lurked in the shadows. This story has revealed the importance of removing the boundaries between basic research and technological or applied research. Bringing these two types of research together enables us to develop innovative projects. There are many forms of excellence, and we urgently need to break down the barriers between disciplines and statuses (private versus public). The European Commission is to be commended for giving us the opportunity to work on high-risk projects, which are the real sources of technological breakthroughs, because even if only a small number of them come to fruition, the payoff is well worth the effort.

GLOBAL RNA market

Discover the marketed products, pipeline drug candidates, major deals and biopharmaceutical companies

RNA market

Scope of RNA-based products



The growing understanding of RNA functions and their crucial roles in diseases promotes the application of various RNAs to target selectively proteins, transcripts and genes, thus potentially broadening the range of therapeutic targets. Here we distinguish coding RNA therapies from non-coding RNAs.

18 Marketed RNA-based products

Drug Name	Brand Name	Companies	Therapy area	Molecule type
nusinersen	Spinraza	Biogen Inc	Central Nervous System	Antisens RNA
eteplirsen	Exondys 51	Sarepta Therapeutics	Genetic Disorders	Antisens RNA
golodirsen	Vyondys 53	Sarepta Therapeutics	Genetic Disorders	Antisens RNA
viltolarsen	Viltepso	Nippon Shinyaku Co	Genetic Disorders	Antisens RNA
patisiran	Onpattro	Alnylam Pharmaceuticals	Metabolic Disorders	RNAi
givosiran	Givlaari	Alnylam Pharmaceuticals	Genetic Disorders	RNAi
lumasiran	Oxlumo	Alnylam Pharmaceuticals	Genito Urinary System And Sex Hormones	RNAi
inclisiran	Leqvio	Novartis Pharmaceuticals	Metabolic Disorders ; Cardiovascular	RNAi
vutrisiran	Amvuttra	Alnylam Pharmaceuticals	Metabolic disorders ; Ophtalmology	RNAi
pegaptanib sodium	Macugen	Pfizer	Metabolic disorders ; Ophtalmology	RNA aptamer
tozinameran	Comirnaty	Pfizer Inc	Infectious disease	mRNA vaccine
elasomeran	Spikevax	Moderna Therapeutics	Infectious disease	mRNA vaccine
davesomeran + elasomeran	COVID-19 Bivalent Vaccine	Moderna Therapeutics	Infectious disease	mRNA vaccine
elasomeran + imelasomeran	Spikevax Bivalent Original/ Omicron BA.1	Moderna Therapeutics	Infectiouse disease	mRNA vaccine
famtozinameran + tozinameran	Comirnaty Bivalent Original/ Omicron BA.4/BA.5	BioNTech/Pfizer	Infectious disease	mRNA vaccine
MIR 19	MIR19	State Organization Institute of Immunology FMBA Russia	Infectious disease	mRNA vaccine
riltozinameran + tozinameran	Comirnaty Original/Omicron BA.1	BioNTech/Pfizer	Infectious disease	mRNA vaccine
SYS-6006	Messenger RNA Vaccine	CSPC Pharmaceutical Group	Infectious disease	mRNA vaccine

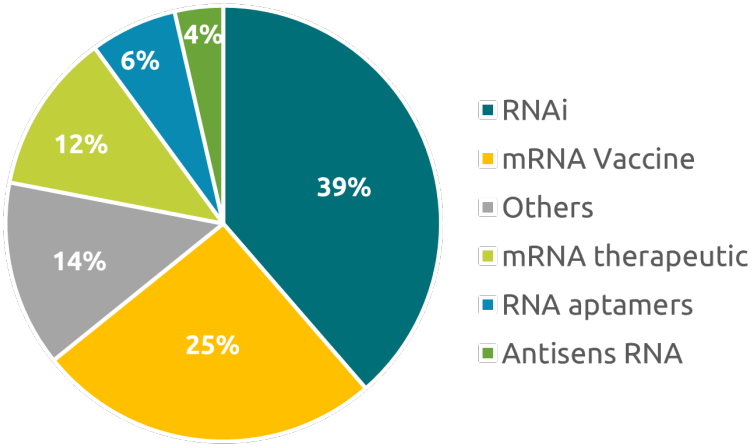
Although mRNA therapies are rather new-comers on the market, exclusively due to the COVID-19 pandemic, they account for 40% of all marketed RNA-based drug products.

** All data has been generated by MabDesign unless stated otherwise*
Source: Globaldata
Data from May 2023

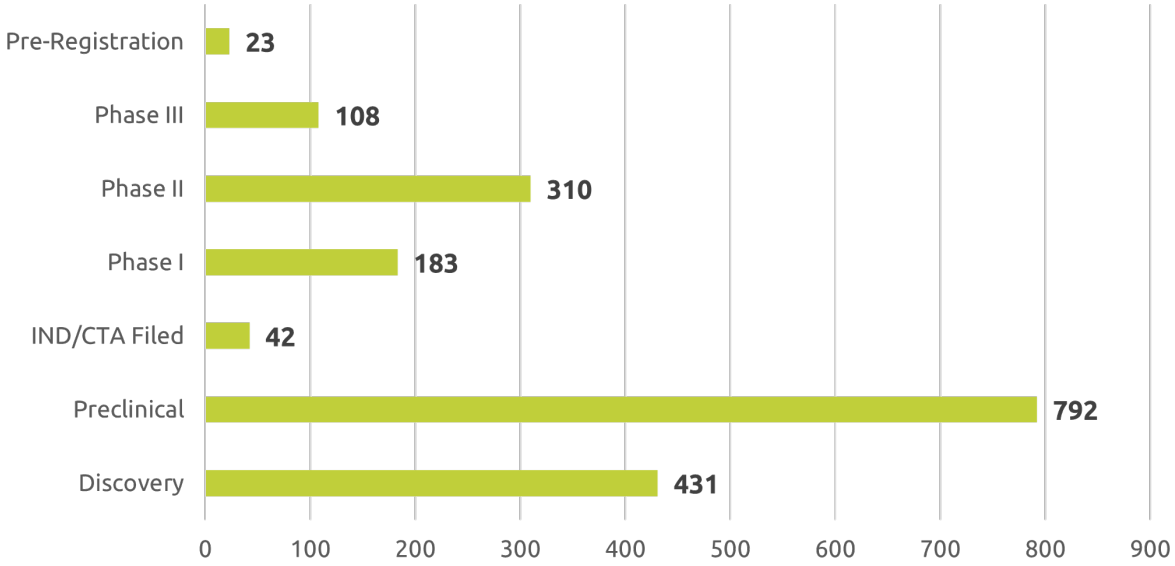
RNA market

Active pipeline (from discovery to phase 3)

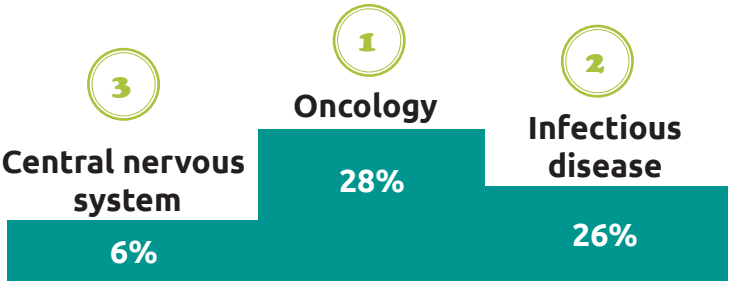
Type of RNA-based products



Phase distribution



Top 3 Therapeutic areas



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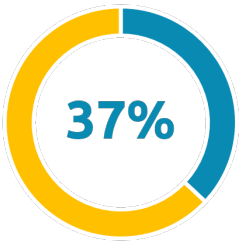
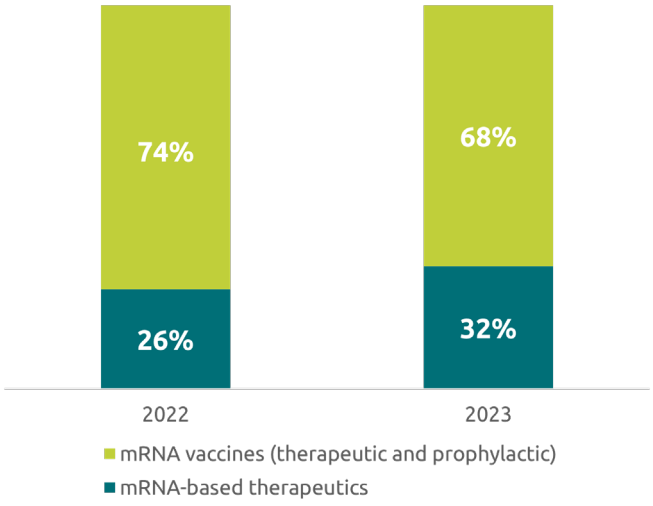
Source: Globaldata
Data from May 2023

Focus on mRNA products in development



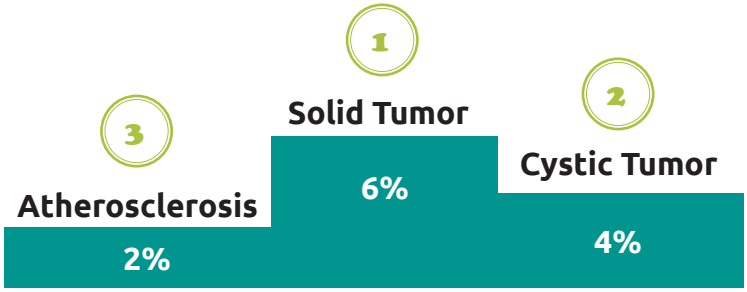
1/3 of pipeline RNA products are mRNA-based products

mRNA-based pipeline

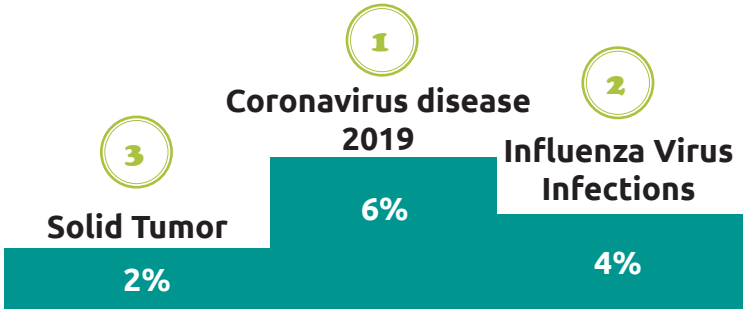


The use of mRNA as vaccinal products has accelerated since the COVID-19 pandemic. This has paved the way for other types of mRNA-based products whose development has also soared. In oncology for example, mRNA therapies do not only include vaccines but also products for targeting and controlling oncogenes.

Top 3 indication mRNA therapeutics



Top 3 indication mRNA vaccines



Main Actors

moderna · BIONTECH · OMEGA THERAPEUTICS · StemRNA · CUREVAC the RNA people® · sanofi

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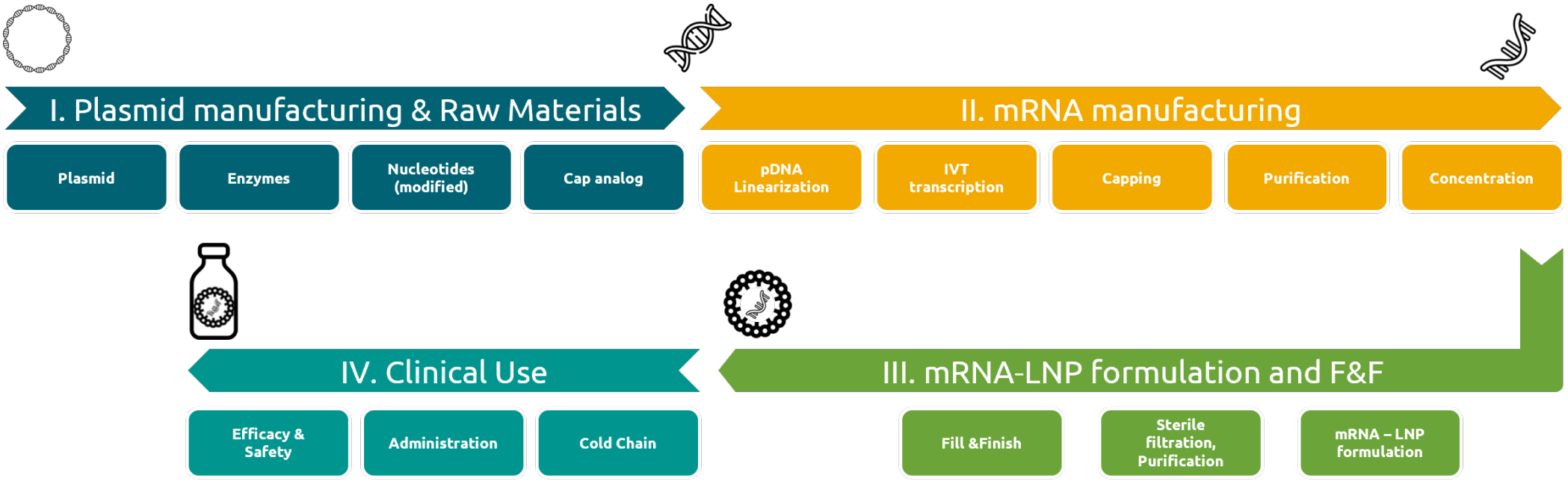
Data from May 2023

Main steps and challenges of mRNA manufacturing



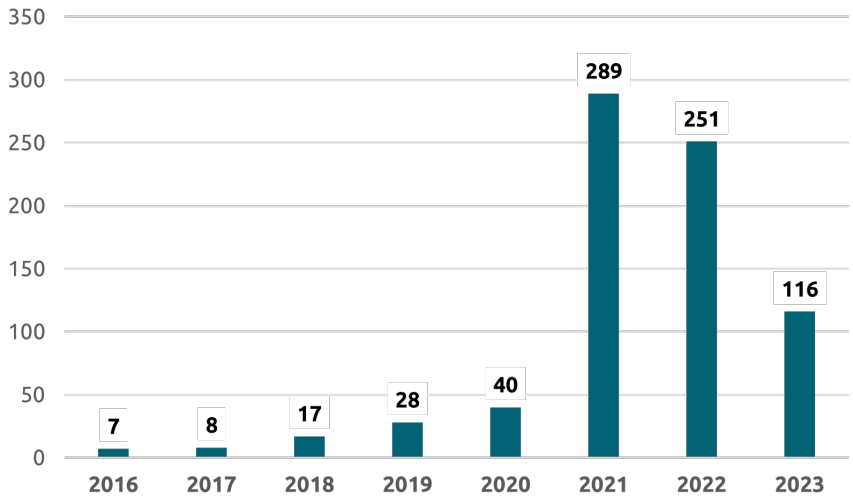
The manufacture of mRNA as therapies includes 4 major steps shown in the diagram above. The challenges and opportunities of this value-chain are numerous.

Examples of main challenges linked to the bioprocessing of mRNA-based drug products:

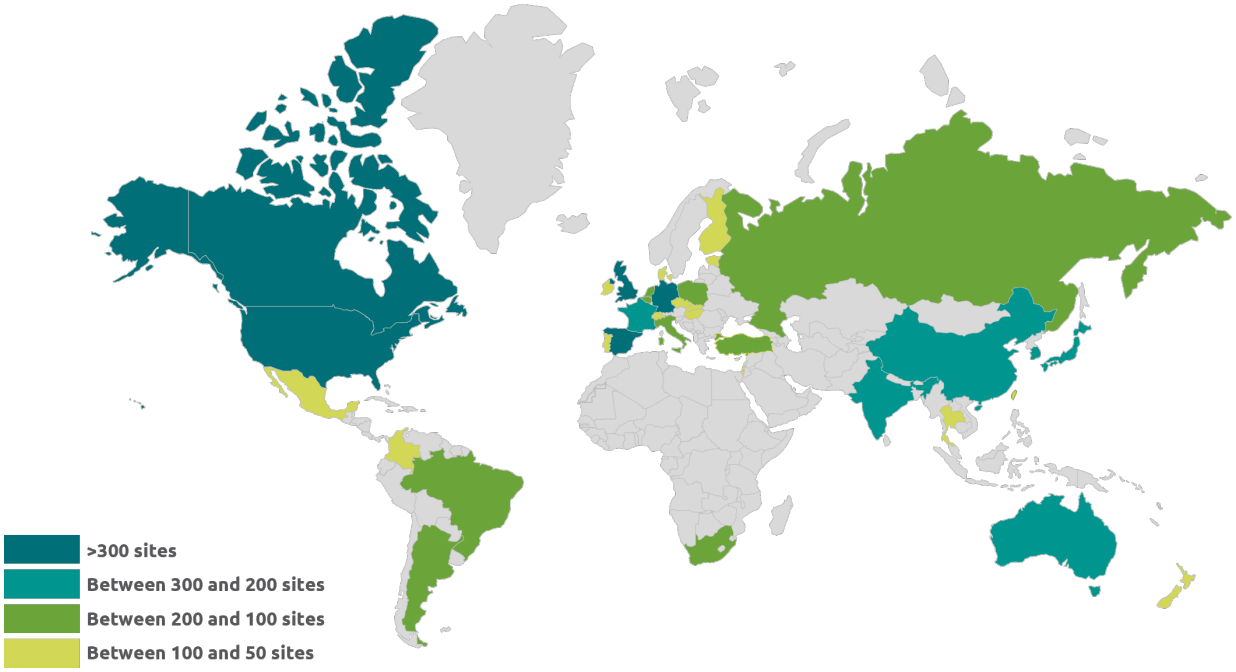
- The strong demand for raw materials resulting from the COVID-19 crisis has led to price increases and shortage in supply.
- The formulation is a step to improve to achieve a robust development and the sufficient quantity of these therapies.
- The control of immunogenicity is at the heart of the development process, particularly for therapeutic products.

RNA clinical trials

Number of clinical trials for RNA by start year



Distribution of clinical trial sites for RNA



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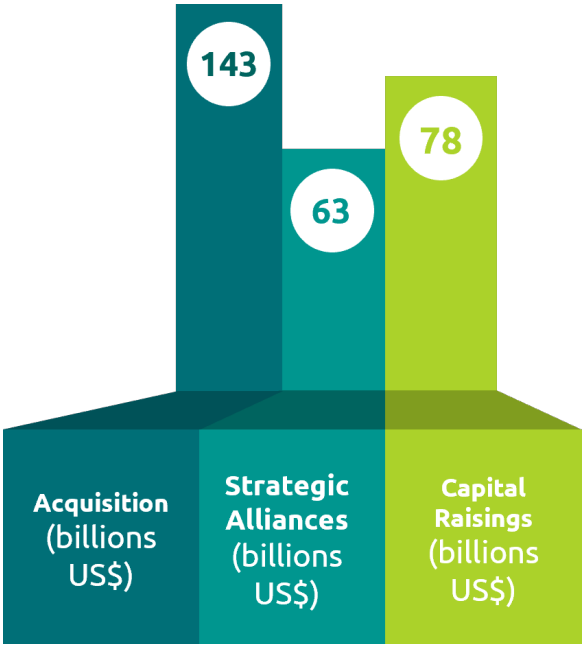
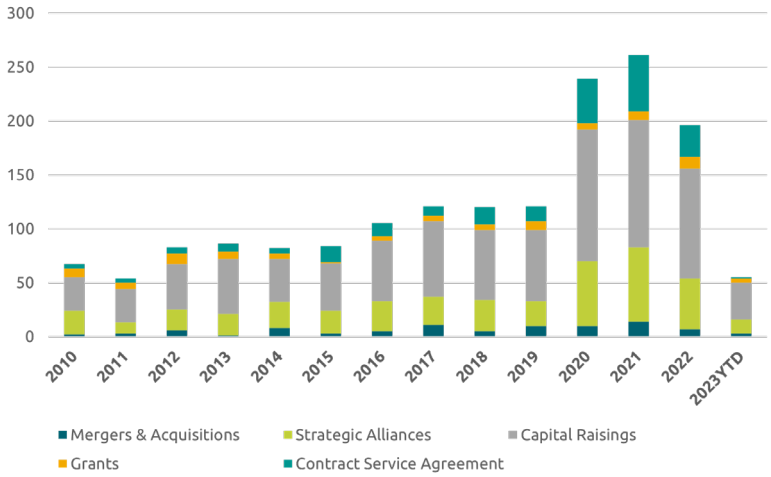
RNA deals

RNA-based therapies deals value



Deals evolution by type (in volume)



The number of deals was constant between 2010 and 2019 to then increase significantly during the COVID-19 crisis. Among the innovators who have raised funds, we find mRNA vaccine leaders such as Moderna and BioNTech.

Strategic Alliances

Partners		Deal value (US$million)	Deal description
Seqirus	Arcturus Therapeutics	4 500	Seqirus (CSL Seqirus) Enters into Licensing Agreement with Arcturus Therapeutics
AstraZeneca Plc	Silence Therapeutics	4 130	Silence Therapeutics Enters into Collaboration with Astrazeneca
Merck Inc	Orna Therapeutics	3 650	Merck and Orna Therapeutics Enter into Co-Development Agreement

Venture Financing

Acquirers	Deal value (US$million)
Abogen Biosciences	700
Moderna Therapeutics	450
BioNTech	325

Acquisition

Partners		Deal value (US$million)
Dicerna Pharma	Novo Nordisk	3 300
Translate Bio	Sanofi	3 200
Sirna Therapeutics	Merck & Co	1 100

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Source: Globaldata
Data from May 2023

scientific articles

Read the different inputs from the scientific community on various aspects of RNA

YEAST CELL FACTORY FOR MRNA BIOPRODUCTION (YSCRIPT)

Chantal Pichon, Fayza Daboussi, Mara Freire, Uhr Cernigoj, Fani Sousa, Tim Beißert, Katarzyna Pachulska-Wieczorek, Michaela Scheid.

Correspondence : chantal.pichon@univ-orleans.fr

The radical vision of a science-enabled technology

More than 180 vaccine platforms have been proposed to tackle the SARS-Cov2 infection. Amongst others, vaccines based on messenger RNA (mRNA) have been developed at unprecedented speed. With a claimed efficacy of ~ 95%, mRNA vaccines represent a giant step in the fight against the COVID-19 pandemic, providing a solution to the global crisis of our age (1). These vaccines are a historic milestone for mRNA therapeutics and nanomedicine. In addition to vaccination, mRNA has a vast array of therapeutic applications, including immunotherapies, replacement of defective proteins in genetic and chronic diseases, gene editing, regenerative medicine, and cell reprogramming (2,3). The potential of mRNA in those applications is undoubtedly verified by significant pre-clinical data and promising clinical trials conducted in different areas (cancer, infection, gene editing, etc.).

Currently, the only existing method to produce mRNA therapeutics is in vitro transcription (**IVT**) (4). Despite the advantage of IVT as being cell-free, the good manufacturing practice (GMP) grade supply chain requires a DNA template, recombinant polymerase, chemically modified nucleotides, and cap analogs, followed by a multi-step and costly RNA purification process. Some of those reagents are difficult to produce, still covered by patents, and expensive. Their supply is may be a real issue and bottle-neck mRNA vaccines manufacturing as happen in COVID-19 pandemic. This problem is even more critical for clinical mRNA applications requiring high doses. Therefore, a robust and more sovereign mRNA production process with simplified manufacturing and reduced cost of goods will help to meet future challenges. In the case of COVID-19 mRNA vaccines, long-term immunity is not guaranteed and the requirement of annual vaccination of the world population is not excluded. Furthermore, future pandemics are unfortunately to be expected. It is, therefore, highly likely that human health will repeatedly or continuously require large-scale mRNA production at low cost.

Yscript's radically new vision lies in the generation of a specific mRNA bioproduction process in yeast, integrating innovative extraction and purification processes. It represents a complete shift of paradigm compared to IVT production and a revolutionary new use of yeast.

Yeasts are unicellular eukaryotic organisms sharing many cellular processes with human cells. They express mRNA in a mature structure that is comparable to higher eukaryotic cells (5). Yeasts combine the advantages of high expression levels, fast growth, easy maintenance and scale-up in low-cost growth media. Furthermore, they are generally recognized as safe (GRAS) for human applications and have been used for decades for the biosynthesis of high-value compounds in medical applications. Accordingly, powerful yeast engineering tools are available, and a robust infrastructure already exists for yeast bioproduction at an industrial scale. Nevertheless, their application is restricted to the production of proteins, toxic compounds and metabolites. Therefore a radical new approach is required to produce and selectively isolate mRNA from yeast.

The challenge is **to express mRNA coding a gene of interest (mRNA-GOI) on demand and to target and accumulate it in a specific compartment, from which mRNA can be extracted and purified through a cost-effective and scalable purification platform.** We envision building an **economically sustainable biotechnology process** for the production of high-quality mRNA therapeutics

Yscript will provide ground-breaking technological improvements by: i) decreasing the mRNA production supply chain; ii) increasing the purity yield of long mRNA by avoiding premature

termination occurring during IVT; and iii) guaranteeing mRNA stability through integrated extraction-purification processes. This will ensure the generation of high-quality mRNA therapeutics, while streamlining production and reducing costs.

To tackle this ambitious project, we built a consortium that brings together the unique skill sets and expertise of seven partners across Europe, ranging from basic RNA biology to clinical translation. CNRS (France) combines world-class expertise in mRNA therapeutics and yeast mRNA processes, to express mRNA in yeast optimally. TRON (Germany) develops advanced novel platforms for individualized therapies and vaccines, and exploits its extensive experience in engineering mRNA vectors, in vitro cell reprogramming and immunological in vivo models. IBCH PAS (Poland) will use its solid experience in viral and retrotransposon RNA to optimize mRNA accumulation in specific compartments. INRAE (France) will leverage its experience as a pre-industrial demonstrator to upscale the process to a pre-industrial scale. Specific purification approaches will be developed by combining the state-of-the-art expertise of UAVR (Portugal), UBI (Portugal) and BIASep (Slovenia). UAVR will bring its unparalleled knowledge on the development of cost-effective integrated extraction-purification platforms based on ionic liquids (ILs), here applied to mRNA purification. UBI will oversee the chromatographic conditions for mRNA purification, building on their world-class experience in nucleic acids purification and stabilization. Furthermore, BIASep, a leading developer of chromatographic columns, will develop ligand-coupling modified mRNA purification columns. Finally, CNRS, TRON and INRAE will perform technology validation and quality control.

Innovation potential

In case of success, Yscript will significantly advance the current state of the art of mRNA manufacturing. It will foster citizens' well-being and health, while decreasing economic and social disparities associated with biopharmaceutical-based therapies, in which mRNA is nowadays a key element. Yscript has many potential impacts: i) decreasing the supply chain for mRNA production and purification processes; ii) increasing the purity yield of long mRNA by avoiding premature termination occurring during IVT; iii) guaranteeyng stability of mRNA through an integrated process (lysis, extraction, clarification, and purification); iv) dramatically reducing production cost (i.e. avoid capping system and nucleotides which together reach 90% of the production costs); and v) ensuring easy scale-up.

Yscript uniquely takes into account industrialization considerations in the project's methodology (e.g. tested yeast strains and downstream processing compatible with industrial scale). This is a key asset to ensure results' exploitability and accelerate their translation from research to innovation. In addition, Yscript relies on yeast cultures, which are a simple, widely used model organism, and necessary infrastructures are already in place, which will foster the diffusion and large adoption of the Yscript technology. The consortium will address global challenges as follows:

Large and agile production of RNA vaccines. While mRNA-based pharmaceuticals have demonstrated their great potential to quickly combat global threats, the complexity and cost-effectiveness of large-scale production are currently major obstacles to mass vaccination. The Yscript methodology could tackle both challenges. Distribution bottlenecks of cold-chain handling are also faced. Yscript process will ease local production of RNA vaccines: the developed yeast clone will be easily distributed among existing yeast production infrastructures worldwide, thereby limiting cold-chain related issues and facilitating rapid distribution of RNA vaccines.

Reduce inequities: affording therapies for low-/mid-income populations. Yscript will allow dramatic cost reduction of mRNA biopharmaceuticals as it will: i) avoid expensive reagents; ii) integrate extraction and purification steps; iii) enable the production of several mRNAs simultaneously; and iv) use yeast as acost-effective cell-factory. Yscript will thus make mRNA therapies widely accessible, which is a major

target of the UN Sustainable Developmental Goal 3.

Pave the way for new therapies. mRNA therapeutics currently target: i) mRNA vaccines against infectious diseases and cancer (> 80% of the market); ii) protein replacement therapies for genetic and chronic diseases; and iii) regenerative medicine. Therapies requiring high doses or frequent administration of mRNA (mRNA-encoded antibodies require up to the gram level of mRNA (6) are not yet established, with mRNAs being mainly restricted to vaccination. Yscript's ground-breaking technology will i) facilitate open access to affordable, high yield and pure mRNA production; ii) enable simple and rapid production of any type of mRNAs; and iii) allow the use of long-size mRNAs (polycistronic or auto-amplifying), which is currently challenging to produce. Thus, it will open mRNA approaches for therapies requiring multiple protein production or long-lasting protein expression.

Greener processes: the processes will be developed under sustainability concepts, allowing reduction of energy consumption, amounts of solvents used, and generated wastes.

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6- Beckert B, Masquida B. 2011. Methods Mol Biol; 703: 29-41;

Partners description

Yscript is performed in the Cell Signalling, Molecular target and Innovative therapies (SCT) of Center for Molecular Biophysics (CBM), a CNRS (National Centre for Scientific Research) research unit affiliated to the University of Orléans (UO, France). The CNRS is a government-funded research organization under the administrative authority of the French Ministry in charge of research. Interdisciplinary programs and actions offer a gateway into new domains of scientific investigation and enable CNRS to address the needs of society and industry. The CBM develops research at the interface of chemistry, biology and physics to study the molecular mechanisms that sustain life or dysfunctions leading to diseases. Built in 1306 by Pope Clement V, the University of Orléans (UO) is one of the oldest European universities, highly renowned across Europe. It was closed during the French Revolution in 1793, like every other university in France and re-opened in the 1960s. The university benefits from very close relations with large national research organizations as well as many European and international research laboratories. The SCT department involved in Yscript aims to decipher molecular biological processes and conceive therapeutic strategies. Their main research is focused on the development of innovative therapies with nucleic acids. They are optimizing both nucleic acids and their delivery by designing innovative formulations. The cellular processes during transfection are studied in particular by imaging and the knowledge acquired is used to implement our approaches. The lab gathers researchers who have a strong experience and skills in non-viral (chemical and physical) nucleic acids delivery, mRNA-based therapies, yeast biology, cell signaling and intracellular trafficking.

Role within Yscript: Pichon's lab has been at the proposition of mRNA bioproduction in yeast. The lab has obtained the proof of concept of mRNA bioproduction in yeast which has been patented. CNRS and the University of Orléans is the coordinator of Yscript with the support of EURICE for the management. CNRS and the University of Orléans is also the lead participants for WP1 dedicated to the expression and accumulation of heterologous mRNA in yeast. With its pivotal role, CNRS and the University of Orléans are involved in different WPs, mainly in WP4 for the biological validation of the bioproduced mRNAs with TRON.

Contact: Prof Chantal Pichon (chantal.pichon@univ-orleans.fr)

INRAE, France's National Research Institute for Agriculture, Food and Environment, aims to carry out science dedicated to life, humans, and the Earth that uncovers solutions to our most pressing concerns. It is participating in the Yscript project via its TWB unit who sets up and conducts R&D projects in the field of industrial biotechnologies in collaboration with public laboratories and industrial players. The expertise of TWB covers a broad scope of industrial biotechnology: synthetic biology, enzyme engineering, metabolic engineering, fermentation, separation and purification processes and state-of-the-art analytical techniques. Microorganisms (bacteria, yeasts, filamentous fungi, micro-algae), enzymes and microbia consortia are the main biological tools. Biosourced plastics & chemicals, cosmetics, food products & biopharmaceuticals are among the fields covered by TWB.

Role within Yscript: In Yscript, INRAE TWB is the leader for Workpackage 2 focusing on development and scale-up of robust bioprocess to produce mRNA in yeast.

Contact: Dr Fayza Daboussi (Fayza.Daboussi@inrae.fr)

UNIVERSITÄTSMEDIZIN DER JOHANNES GUTENBERG-UNIVERSITÄT MAINZ GEMEINNÜTZIGE GMBH. TRON is an independent non-profit translational research organization that develops highly innovative technologies to target diseases with high medical needs such as infectious diseases, autoimmunity and cancer. TRON was founded in 2010 and has already played a transformative role in global health challenges and applied science. Located in the research hub Mainz, the institute has strong ties to both academia and industry and is characterized by a vibrant, cutting-edge and creative research environment, a flat hierarchy and an international and diverse work force. They combine their transdisciplinary competencies in genomics and immunology to develop novel platforms for the identification and validation of 'omics'-based biomarkers to harness and modulate immune system components for use in personalized therapies. Several TRON inventions have already found their way into clinical trials. Collaborating with academia and industry, TRON executes research at the leading edge to support innovative drug design for human health.

Role within Yscript: TRON gGmbH (Germany) brings extensive expertise in engineering mRNA for therapies and vaccines to optimise the features of mRNA produced in yeast (Work package 1). In addition, TRON leads the Quality Control and biological validation of bio-produced mRNA (Work package 4).

Contact: Dr Tim Beißert (Tim.Beissert@TrOn-Mainz.DE)

Founded in the year 2000, Eurice – European Research and Project Office GmbH – is a leading European consultancy firm offering knowledge-intensive business services mainly related to R&I Project Design/ Management, Collaborative Innovation Management and Communication & Training. Eurice provides comprehensive support services for the planning, initiation, and implementation of large international

innovation collaborative projects – with a strong involvement in EU Framework programmes such as Horizon 2020 (54 participations) and Horizon Europe (32 participations until now). Eurice is among Europe's largest R&I project management offices, witha dedicated teams across three locations in Germany and Croatia. In addition to classical project management, Eurice helps to develop and implement coherent IP strategies and supports consortia through communication, dissemination, networking, training, as well as capacity building and sustainability planning activities. Moreover, Eurice is the coordinator of two European-wide IP-flagship initiatives: the European IP Helpdesk and Horizon IP Scan.

Role within Yscript: In Yscript, Eurice is leader of Work Package 6 focusing on the horizontal activities of communication, dissemination and exploitation of project results. A specific focus is put on innovation-related activities. Moreover, Eurice supports the Coordinator in managing the project.

Contact: Mrs Michaela Scheid

INSTITUTE OF BIOORGANIC CHEMISTRY OF THE POLISH ACADEMY OF SCIENCES

the Institute of Bioorganic Chemistry, Polish Academy of Sciences, is a high-profile institution on the Polish stage, covering studies in life sciences. It is a place where interdisciplinary studies encompassing such fields as bioorganic chemistry, biochemistry, molecular biology, systems biology, synthetic biology, and bioinformatics are conducted, particularly research on the synthesis, structure and function of RNA and proteins along with their components. The Institute is one of the leading Polish institutes in attracting European Union funding. It has been awarded numerous prizes, such as Crystal Brussels Prize (five times) for successful participation in European Union Framework Programs. Today, IBCH PAS comprises 32 research departments, supported by 12 specialized laboratories offering high-class infrastructure. The Institute's Ph.D. Program and the Poznan Doctoral School of the Institutes of PAS educate ca. 80 Ph.D. students preparing their theses in the Institute.

Role within Yscript: In Yscript, IBCH PAS is involved in Work Package 1 - Generation of yeast cell factories for efficient mRNA bioproduction, and Work Package 4 - Quality control and biological validation of bioproduced mRNA. In collaboration with CNRS, IBCH PAS will design, based on in vitro and in vivo assays, the best addressing sequence for mRNA of interest trans-targeting to specific cytoplasmic localization (WP1). IBCH will also perform comparative analyses of the secondary structure of bioproduced mRNAs and corresponding in vitro transcripts (WP4).

Contact: Dr Katarzyna Pachulska-Wieczorek (kasiapw@ibch.poznan.pl)

UNIVERSITY OF AVEIRO, AVEIRO INSTITUTE OF MATERIALS

Since its foundation, University of Aveiro (UAVR) has been placed amongst the most active universities in Portugal, being distinguished as one of the best universities worldwide that are 50 years old or younger by the Times Higher Education Young University Rankings. Hosted at UAVR, the Aveiro Institute of Materials (CICECO) is the largest and best-rated Materials Science and Engineering (MSE) institute by FCT, joining ca. 500 chemists, physicists, and materials engineers. Research Lines are Sustainability, Information and Communication Technology, Energy, and Health. CICECO has an international reputation in MSE and is very productive, being engaged in many top EU programs (viz. 9 ERC and 3 EIC grants). CICECO instrumental facilities are the best in the country for research in MSE comprising, among others, SEM, TEM, AFM, X-ray diffraction, NMR, and computer clusters. Researchers are also granted access to CDTM, CICECO interface with companies and external entities assisting

scientists in finding industrial and EU funding, preparing and managing projects, creating startups, dealing with IP issues, and patenting.

Role within Yscript: In Yscript, UAVR is the leader of Work Package 3 focusing on the development of a cost-effective and integrated extraction-purification process of mRNA, feasible of scale-up.

Contact: Prof Mara G. Freire (maragfreire@ua.pt)

UNIVERSIDADE BEIRA INTERIOR

UNIVERSITY OF BEIRA INTERIOR

The University of Beira Interior (UBI) is one of the youngest public universities in Portugal and a member of the UNITA Consortium. UBI is committed to advancing knowledge and technology and developing innovative products and solutions for industry and society. The UBI research ecosystem encompasses 18 research units performing research in all areas of knowledge in alignment with the European, national, and regional priorities and the ONU sustainable development goals. The Health Sciences Research Centre of UBI (CICS-UBI), is located at the Faculty of Health Sciences building, and is part of the Beiras Clinical Academic Centre, enhancing close interactions between researchers and clinicians. The cutting-edge core-facilities include BioImaging and Nuclear Magnetic Resonance units which belong to the roadmap of FCT research infrastructures, an up-to-date animal facility, and laboratories for cell culture, genomics, proteomics, flow-cytometry, electrophysiology, microbiology, chromatography, organic synthesis, and pharmaceutical technology. The innovation and technology incubator/accelerator UBIMedical boosts the desired communication between research and business.

Role within Yscript: In Yscript, UBI will mainly develop activities within Work Package 3, particularly focused on the screening and establishment of the most suitable conditions for mRNA purification and stabilization.

Contact: Prof Fani Sousa (fani.sousa@fcsaude.ubi.pt)

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Role within Yscript: In Yscript, BIASep is part of Work Packages 3 and 4. BIA Sep will develop and produce laboratory and industrial-scale monolith chromatographic columns modified with specific oligonucleotides as well as innovative ion exchanging monolithic columns, all of them applied in the optimized and intensified mRNA downstream processing. A specific focus will be dedicated to the development and utilization of different chromatographic and electrophoretic analytical approaches with the focus on mRNA quality. These methods will be applied in the process analytical technology,

what will be the part of an overall mRNA production platform.

Contact: Urh Černigoj (urh.cernigoj@biaseparations.com)

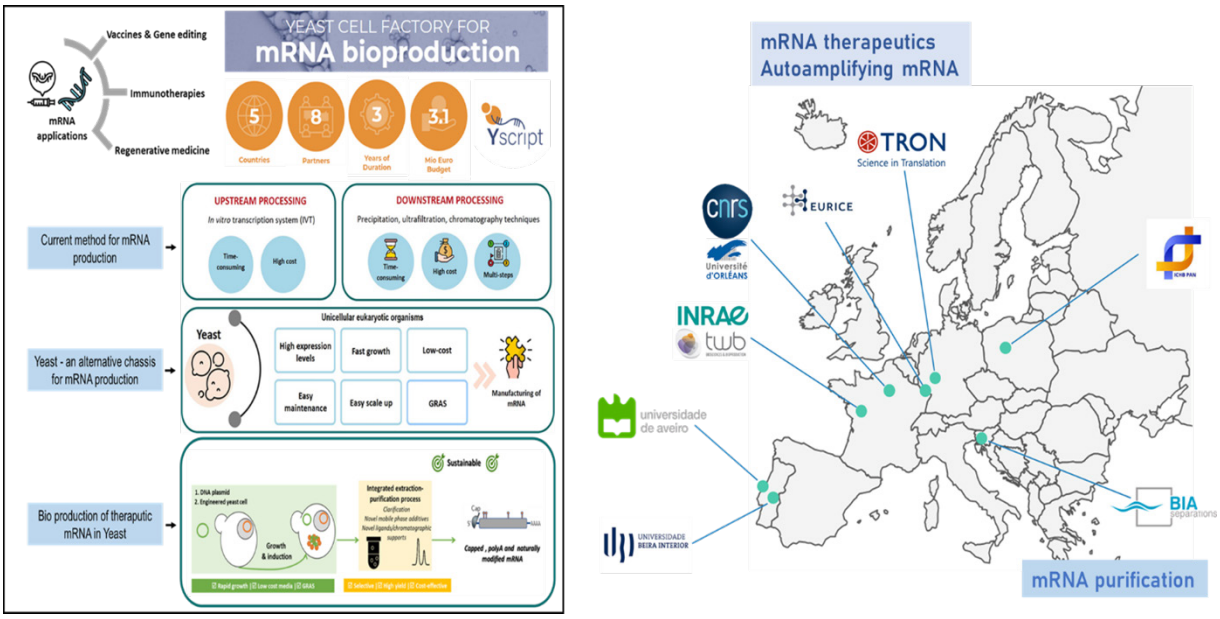

Figure 1: *Yscript at a glance (www.yscript.eu).*

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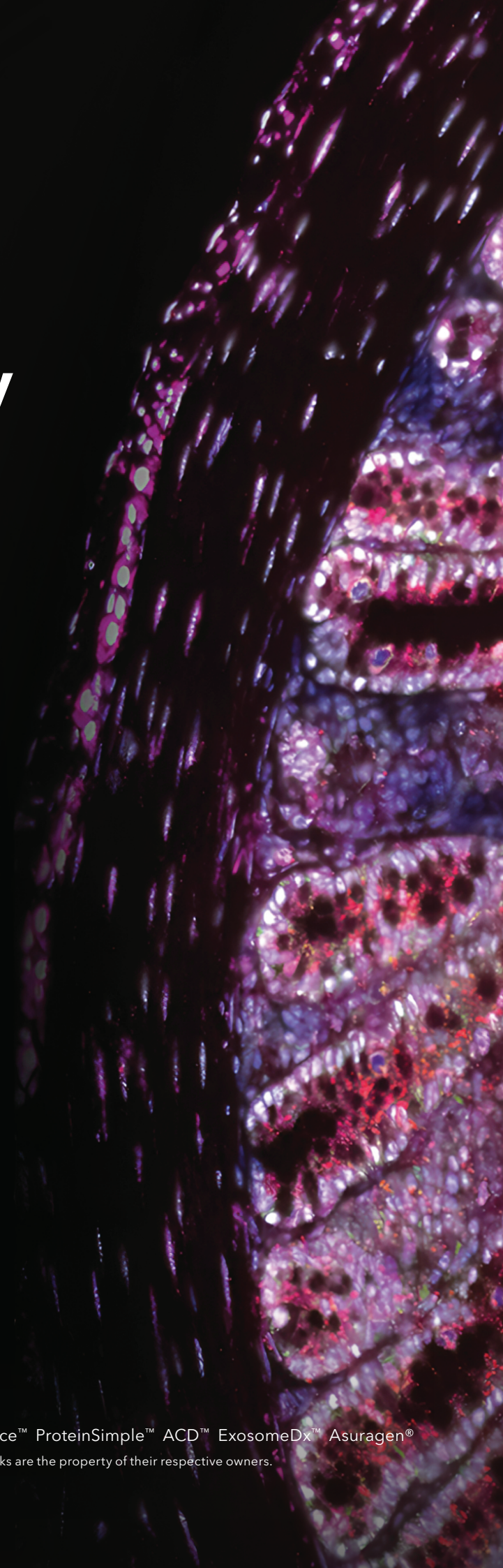

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MINDING YOUR CAPS AND TAILS – CONSIDERATIONS FOR FUNCTIONAL MRNA SYNTHESIS

by Breton Hornblower, Ph.D., G. Brett Robb, Ph.D. and George Tzertzinis, Ph.D., New England Biolabs, Inc

Applications of synthetic mRNA have grown and become considerably diversified in recent years. Examples include the generation of pluripotent stem cells (1-3), vaccines and therapeutics (4-5), and CRISPR/Cas9 genome editing applications (6-8). The basic requirements for a functional mRNA – a 7-methylguanylate cap at the 5′ end and a poly(A) tail at the 3′ end – must be added in order to obtain efficient translation in eukaryotic cells. Additional considerations can include the incorporation of internal modified bases, modified cap structures and polyadenylation strategies. Strategies for in vitro synthesis of mRNA vary according to the desired scale of synthesis. This article discusses options for the selection of reagents and the extent to which they influence synthesized mRNA functionality.

A nascent mRNA, synthesized in the nucleus, undergoes different modifications before it can be translated into proteins in the cytoplasm. For a mRNA to be functional, it requires modified 5′ and 3′ ends and a coding region (i.e., an open reading frame (ORF) encoding for the protein of interest) flanked by the untranslated regions (UTRs). The nascent mRNA (pre-mRNA) undergoes two significant modifications in addition to splicing. During synthesis, a 7-methylguanylate structure, also known as a "cap", is added to the 5′ end of the pre-mRNA, via 5′ □ 5′ triphosphate linkage. This cap protects the mature mRNA from degradation, and also serves a role in nuclear export and efficient translation.

The second modification occurs post-transcriptionally at the 3′ end of the nascent RNA molecule and is characterized by addition of approximately 200 adenylate nucleotides (poly(A) tail). The addition of the poly(A) tail confers stability to the mRNA, aids in the export of the mRNA to the cytosol, and is involved in the formation of a translation-competent ribonucleoprotein (RNP), together with the 5′ cap structure. The mature mRNA forms a circular structure (closed-loop) by bridging the cap to the poly(A) tail via the cap-binding protein eIF4E (eukaryotic initiation factor 4E) and the poly(A)- binding protein, both of which interact with eIF4G (eukaryotic initiation factor 4G), (Figure 1, (9)).

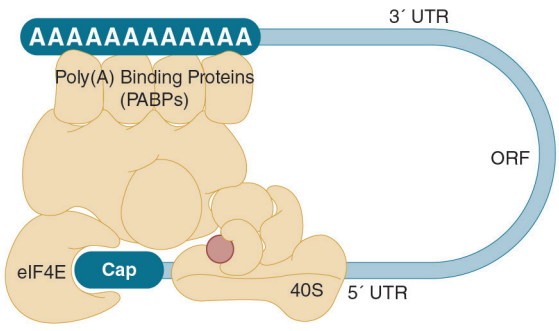

Figure 1. Translation initiation complex.
A mature mRNA, consisting of the 5′ and 3′ untranslated regions (UTRs) and the open reading frame (ORF), forms a "closed loop" structure via interactions mediated by protein complexes that bind the cap structure and the poly(A) tail.

RNA can be efficiently synthesized in vitro (by in vitro transcription, IVT) with prokaryotic phage polymerases, such as T7, T3 and SP6. The cap and poly(A) tail structures characteristic of mature mRNA can be added during or after the synthesis by enzymatic reactions with capping enzymes and Poly(A) Polymerase (NEB #M0276), respectively.

There are several factors to consider when planning for IVT-mRNA synthesis that will influence the ease-of-experimental setup and yield of the final mRNA product. These are discussed in the following sections.

DNA template

The DNA template provides the sequence to be transcribed downstream of an RNA polymerase promoter. There are two strategies for generating transcription templates: PCR amplification and linearization of plasmid with a restriction enzyme (Figure 2). Which one to choose will depend on the downstream application. In general, if multiple sequences are to be made and transcribed in parallel, PCR amplification is recommended as it generates many templates quickly. On the other hand, if large amounts of one or a few templates are required, plasmid DNA is recommended, because of the relative ease of producing large quantities of high quality, fully characterized plasmids. There are different versions of plasmids available that allow for propagation of homopolymeric A-tails of defined length (1).

PCR allows conversion of any DNA fragment to a transcription template by appending the T7 (or SP6) promoter to the forward primer (Figure 2A). Additionally, poly(d)T-tailed reverse primers can be used in PCR to generate transcription templates with A-tails. This obviates the need for a separate polyadenylation step following transcription. Repeated amplifications should, however, be avoided to prevent PCR-generated point mutations. Amplification using PCR enzymes with the highest possible fidelity, such as Q5® High-Fidelity DNA Polymerase (NEB #M0491), reduces the likelihood of introducing such mutations (2).

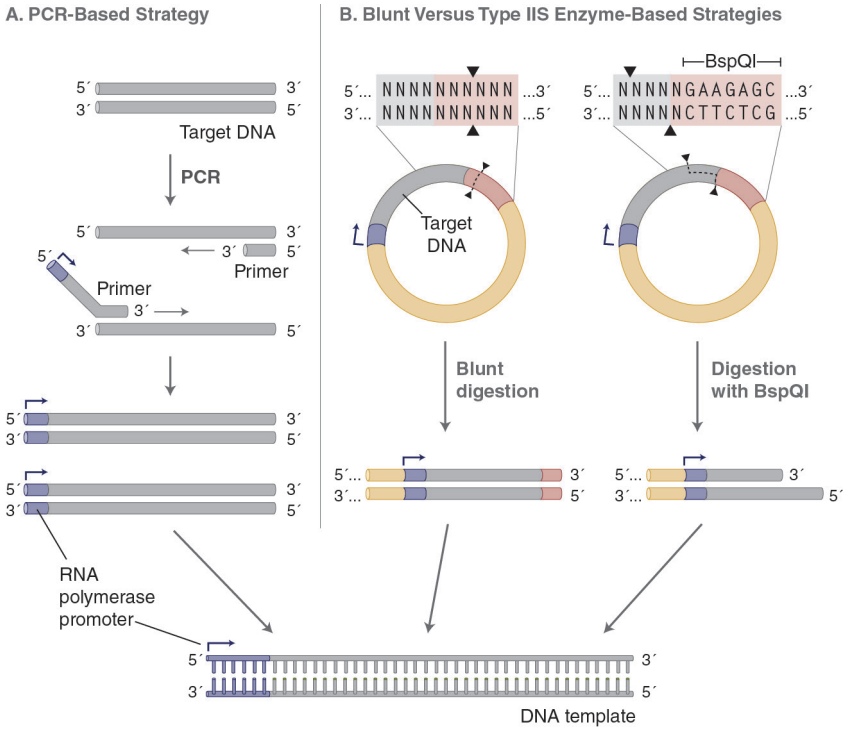

Figure 2. Methods for generating transcription templates.
(A) PCR can be used to amplify target DNA prior to transcription. A promoter can be introduced via the upstream primer. (B) When using plasmid DNA as a template, linearize with an enzyme that produces blunt or 5´-overhanging ends. Using a type IIS restriction enzyme (e.g., BspQI) allows RNA synthesis with no additional 3´-nucleotide sequence from the restriction site.

The quality of the PCR reaction can be assessed by running a small amount on an agarose gel, and DNA should be purified before in vitro transcription using a spin column or magnetic beads (e.g., AMPure® beads). Multiple PCR reactions can be purified and combined to generate a DNA stock solution that can be stored at -20°C and used as needed for in vitro transcription.

Plasmid templates are convenient if the template sequence already exists in a eukaryotic expression

vector also containing the T7 promoter (e.g., pcDNA vector series). These templates include 5´- and 3´-untranslated regions (UTR), which are important for the expression characteristics of the mRNA.

Plasmid DNA should be purified and linearized downstream of the desired sequence, preferably with a restriction enzyme that leaves blunt or 5´ overhangs at the 3´ end of the template. These are favorable for proper run-off transcription by T7 RNA Polymerase (NEB #M0274), while 3´ overhangs may result in unwanted transcription products. To avoid adding extra nucleotides from the restriction site to the RNA sequence, a Type IIS restriction enzyme can be used (e.g., BspQI, NEB #R0712), which positions the recognition sequence outside of the transcribed sequence (Figure 2B, page 2). The plasmid DNA should be completely digested with the restriction enzyme, followed by purification using a spin column (e.g., Monarch® PCR & DNA Cleanup Kit (5 µg) NEB #T1030) or phenol extraction/ethanol precipitation. Although linearization of plasmid involves multiple steps, the process is easier to scale for the generation of large amounts of template for multiple transcription reactions.

In vitro transcription

There are two options for the in vitro transcription (IVT) reaction depending on the capping strategy chosen: standard synthesis with enzyme-based capping following the transcription reaction (post-transcriptional capping) or incorporation of a cap analog during transcription (co-transcriptional capping) (Figure 3). Method selection will depend on the scale of mRNA synthesis required and number of templates to be transcribed.

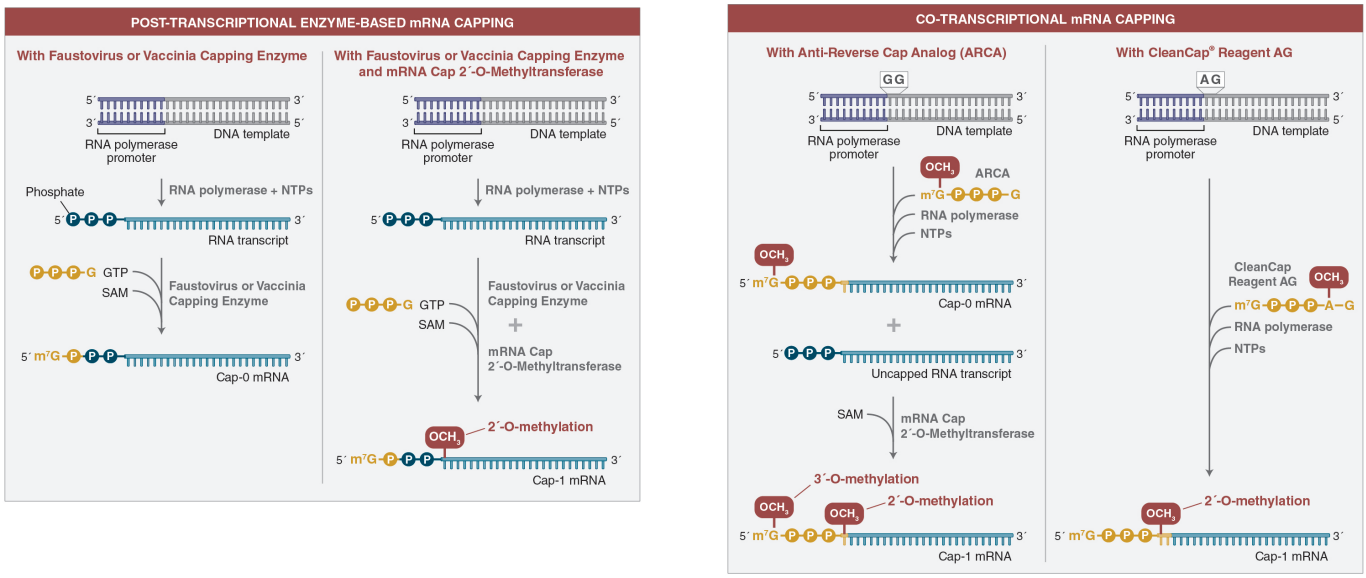

Figure 3. In vitro transcription options based upon capping strategy.

Enzyme-based capping (top) is performed after in vitro transcription using 5´-triphosphate RNA, GTP, and S-adenosyl-methionine (SAM). Cap-0 mRNA can be converted to Cap-1 mRNA using mRNA cap 2´-O-methyltransferase (MTase) and SAM in a subsequent or concurrent reaction. The methyl group transferred by the MTase to the 2´-O of the first nucleotide of the transcript is indicated in red. Conversion of ~100% of 5´-triphosphorylated transcripts to capped mRNA is routinely achievable using enzyme-based capping.

Co-transcriptional capping (bottom) uses an mRNA cap analog, shown in yellow, in the transcription reaction. For ARCA (anti-reverse cap analog) (left), the cap analog is incorporated as the first nucleotide of the transcript. ARCA contains an additional 3´-O-methyl group on the 7-methylguanosine to ensure incorporation in the correct orientation. The 3´-O-methyl modification does not occur in natural mRNA caps. Compared to reactions not containing cap analog, transcription yields are lower. ARCA-capped mRNA can be converted to cap 1 mRNA using mRNA cap 2´-O-MTase and SAM in a subsequent reaction. CleanCap Reagent AG (right) uses a trinucleotide cap analog that requires a modified template initiation sequence. A natural Cap-1 structure is accomplished in a co-transcriptional reaction.

Transcription for enzyme-based capping (post-transcriptional capping)

Standard RNA synthesis reactions produce the highest yield of RNA transcript (typically ≥100 µg per 20 µl in a 1 hr reaction using the HiScribe®; Quick T7 High Yield RNA Synthesis Kit, NEB #E2050S). Transcription reactions are highly scalable, and can be performed using an all-inclusive kit (e.g., HiScribe kits), or individual reagents. More information on the HiScribe kits can be found later in the article.

Following transcription, the RNA is treated with DNase I (NEB #M0303) or DNase I-XT (#M0570) to remove the DNA template, and purified using an appropriate column, kit or magnetic beads, prior to capping. This method produces high yields of RNA with 5´-triphosphate termini that must be converted to cap structures. In the absence of template-encoded poly(A) tails, transcripts produced using this method bear 3´ termini that also must be polyadenylated in a separate enzymatic step, as described below in "A-tailing using E. coli Poly(A) Polymerase".

Transcription with dinucleotide co-transcriptional capping

In co-transcriptional capping, a cap analog is introduced into the transcription reaction, along with the four standard nucleotide triphosphates, in an optimized ratio of cap analog to GTP 4:1. This allows initiation of the transcript with the cap structure in a large proportion of the synthesized RNA molecules. This approach produces a mixture of transcripts, of which ~80% are capped, and the remainder have 5´-triphosphate ends. Decreased overall yield of RNA products results from the lower concentration of GTP in the reaction (Figure 4).

There are several cap analogs used in co-transcriptional RNA capping (3,4). The most common are the standard 7-methyl guanosine (m7G) cap analog and anti-reverse cap analog (ARCA), also known as 3´ O-me 7-meGpppG cap analog. ARCA is methylated at the 3´ position of the m7G, preventing RNA elongation by phosphodiester bond formation at this position.

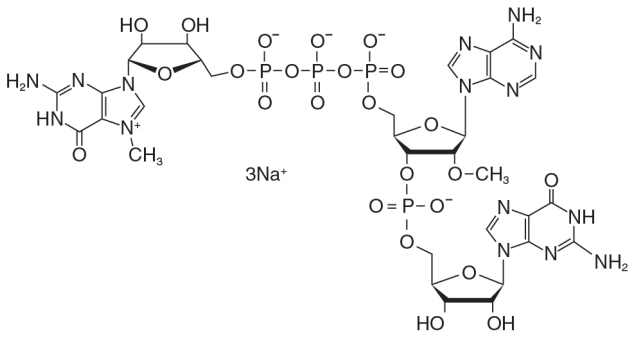

Figure 4. Structure of CleanCap Reagent AG.

Thus, transcripts synthesized using ARCA contain 5´-m7G cap structures in the correct orientation, with the 7-methylated G as the terminal residue. In contrast, the m7G cap analog can be incorporated in either the correct or the reverse orientation.

HiScribe T7 ARCA mRNA Synthesis kits (NEB #E2060 and #E2065) contain reagents, including an optimized mix of ARCA and NTPs, for streamlined reaction setup for synthesis of co-transcriptionally capped RNAs.

Transcription with CleanCap® reagent AG co-transcriptional capping

The use of CleanCap reagent AG results in significant advantages over traditional dinucleotide co-

transcriptional capping. CleanCap Reagent AG is a trinucleotide with a 5´-m7G joined by a 5´-5´ triphosphate linkage to an AG sequence. The adenine has a methyl group on the 2´-O position (Figure 4). The incorporation of this trinucleotide in the beginning of a transcript results in a Cap-1 structure.

In order to use CleanCap Reagent AG in an in vitro transcription reaction the template must contain an AG in place of a GG following the T7 promoter in the initiation sequence.

Unlike traditional co-transcriptional capping, reduction of GTP concentration is not required and therefore yield is higher and high capping efficiencies, >95%, are achieved (Figure 5).

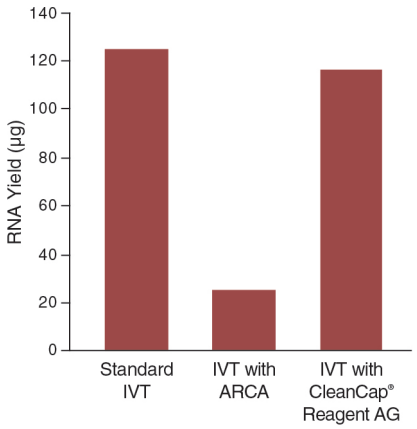

Figure 5. Comparison of RNA yields from in vitro transcription reactions.

All reactions were performed with 5 mM CTP, 5 mM UTP and 6 mM ATP. Standard IVT reactions included 5 mM GTP and no cap analog. ARCA reactions contained a 4:1 ratio of ARCA:GTP (4 mM:1 mM). IVT with CleanCap Reagent AG contained 5 mM GTP and 4 mM CleanCap Reagent AG and was performed as described below (Standard mRNA Synthesis). Reactions were incubated for 2 hours at 37°C, purified and quantified by NanoDrop®.

Transcription with complete substitution with modified nucleotides

RNA synthesis can be carried out with a mixture of modified nucleotides in place of the regular mixture of A, G, C and U triphosphates. For expression applications, the modified nucleotides of choice are the naturally occurring 5´-methylcytidine and/or pseudouridine in the place of C and U, respectively. These have been demonstrated to confer desirable properties to the mRNA, such as increased mRNA stability, increased translation, and reduced immune response in the key applications of protein replacement and stem-cell differentiation (1). It is important to note that nucleotide choice can influence the overall yield of mRNA synthesis reactions.

Fully substituted RNA synthesis can be achieved using the HiScribe T7 mRNA Kit with CleanCap Reagent AG (NEB #E2080), HiScribe T7 High-Yield RNA Synthesis Kit (NEB #E2040) or HiScribe SP6 RNA Synthesis Kit (NEB #E2070) in conjunction with NTPs with the desired modification. Transcripts made with complete replacement of one or more nucleotides may be post-transcriptionally capped (see next section), or may be co-transcriptionally capped by including CleanCap Reagent AG, ARCA or another cap analog, as described previously.

If partial replacement of nucleotides is desired, the HiScribe T7 ARCA mRNA Synthesis Kits (NEB #E2060 and #E2065), may be used with added modified NTPs, to produce co-transcriptionally capped mRNAs, as described above. Alternatively, the HiScribe T7 Quick RNA Synthesis Kit (NEB #E2050) may be used to prepare transcripts for post-transcriptional capping.

Post-transcriptional capping and Cap-1 methylation

Post-transcriptional capping is often performed using the mRNA capping enzyme from Vaccinia virus or

Faustovirus. These enzymes complex converts the 5´-triphosphate ends of in vitro transcripts to m7G-cap (Cap-0) required for efficient protein translation in eukaryotes. The Faustovirus capping enzyme (NEB #M2081) comprises three enzymatic activities (RNA triphosphatase, guanylyltransferase, guanine N7-methyltransferase) that are necessary for the formation of the complete Cap-0 structure, m7Gppp5´N, using GTP and the methyl donor S-adenosylmethionine. As an added option, the inclusion of the mRNA Cap 2´ O-Methyltransferase (NEB #M0366) in the same reaction results in formation of the Cap-1 structure (m7Gppp5´Nm), a natural modification in many eukaryotic mRNAs responsible for evading cellular innate immune response against foreign RNA. This enzyme-based capping approach results in a high proportion of capped message, and it is easily scalable. The resulting capped RNA can be further modified by poly(A) addition before final purification.

A-tailing using E. coli Poly(A) Polymerase

The poly(A) tail confers stability to the mRNA and enhances translation efficiency. The poly(A) tail can be encoded in the DNA template by using an appropriately tailed PCR primer, or it can be added to the RNA by enzymatic treatment with E. coli Poly(A) Polymerase (NEB #M0276). The length of the added tail can be adjusted by titrating the Poly(A) Polymerase in the reaction (Figure 6).

The importance of the A-tail is demonstrated by transfection of untailed vs. tailed mRNA. When luciferase activity from cells transfected with equimolar amounts of tailed or untailed mRNAs were compared, a significant enhancement of translation efficiency was evident (Figure 6). HiScribe T7 ARCA mRNA Synthesis Kit (with tailing) (NEB #E2060) includes E. coli Poly(A) Polymerase, and enables a streamlined workflow for the enzymatic tailing of co-transcriptionally capped RNA.

For mRNA synthesis from templates with encoded poly(A) tails, the HiScribe T7 ARCA mRNA Synthesis Kit (NEB #E2065) provides an optimized formulation for co-transcriptionally capped transcripts.

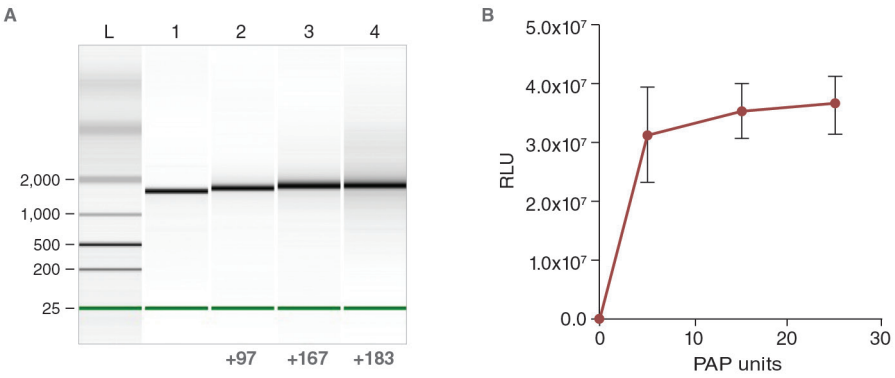

Figure 6. Analysis of capped and polyadenylated RNA.

(A) Agilent® Bioanalyzer® analysis of capped and polyadenylated RNA. Longer tails are produced by increasing the enzyme concentration in the reaction. Calculated A-tail lengths are indicated over each lane. Lanes: L: size marker, 1: No poly-A tail, 2: 5 units, 3 :15 units, 4 : 25 units of E. coli Poly(A) Polymerase per 10 µg CLuc RNA in a 50 µl reaction.

(B) Effect of enzymatic A-tailing on the luciferase reporter activity of CLuc mRNA.

Analysis of capped RNA function in transfected mammalian cells

The effect of capping can be studied by delivering the mRNA to cultured mammalian cells and monitoring its translation. Using RNA encoding secreted luciferases (e.g., Cypridina luciferase, CLuc) the translation can be monitored by assaying its activity in the cell culture medium (Figure 7A).

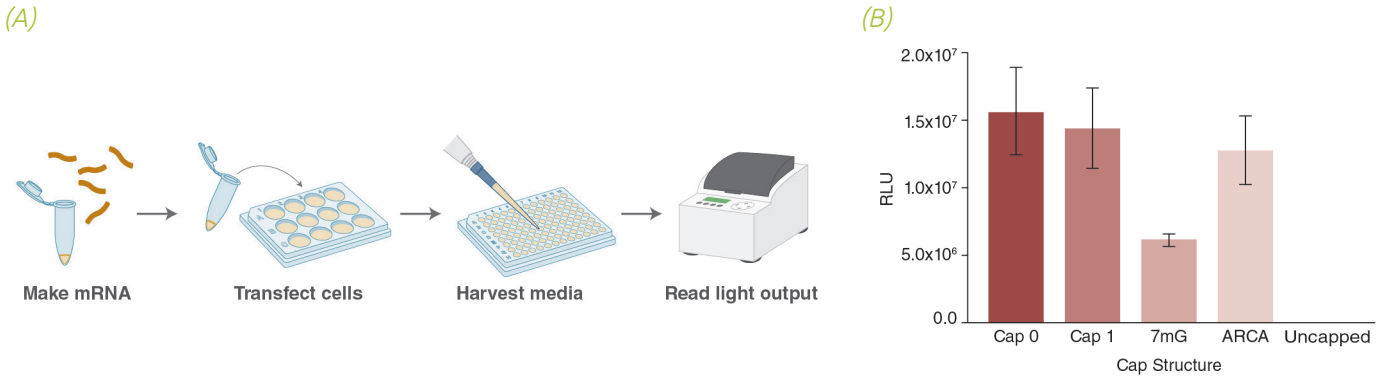

Figure 7. Analysis of capped RNA function.

(A) Schematic representation of reporter mRNA transfection workflow.

(B) Expression of Cypridina luciferase (CLuc) after capping using different methods. High activity from all capped RNAs is observed.

CLuc mRNA was synthesized and capped post-transcriptionally (Cap 0 or Cap 1) or co-transcriptionally (as described above) using standard (7mG) or anti-reverse cap analog (ARCA). For consistency, the mRNAs were prepared from templates encoding poly-A tails of the same length.

After capping, the mRNA was purified using magnetic beads and quantified before transfection into U2OS cells using the TransIT® mRNA transfection reagent following the manufacturer's protocol. CLuc activity was measured 16 hrs after transfection using the BioLux® Cypridina Luciferase Assay Kit (NEB #E3309).

Virtually no luciferase reporter activity was observed in conditions where uncapped RNA was transfected (Figure 7B). In contrast, robust activity was detected from cells transfected with RNA capped using the methods described above. As anticipated, lower activity was observed from cells transfected with mRNA capped using the 7mG cap analog as compared to ARCA-capped mRNA.

Summary

In summary, when choosing the right workflow for your functional mRNA synthesis needs, you must balance your experimental requirements for the mRNA (e.g., internal modified nucleotides) with scalability (i.e., ease-of-reaction setup vs. yield of final product).

In general, co-transcriptional capping of mRNA with template encoded poly(A) tails or post-transcriptional addition of poly(A) tail is recommended for most applications. This approach, using the HiScribe T7 mRNA Kits with CleanCap Reagent AG (NEB #E2080), enables the quick and streamlined production of one or many transcripts with typical yields of ≥90 µg per reaction, totaling ~1.8 mg per kit.

Post-transcriptional mRNA capping with Faustovirus Capping Enzyme or Vaccinia Capping System is well suited to larger scale synthesis of one or a few mRNAs, and is readily scalable to produce gram-scale quantities and beyond. Reagents for in vitro synthesis of mRNA are available in kit form or as separate components to enable research and large-scale production.

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mRNA SYNTHESIS WORKFLOW
EXAMPLE & AVAILABLE NEB PRODUCTS

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Q5® High-Fidelity DNA Polymerase	HiScribe® T7 mRNA Kit with CleanCap® Reagent AG		*E. coli* Poly(A) Polymerase	Monarch® RNA Cleanup Kit (10 µg)
	HiScribe T7 ARCA mRNA Synthesis Kit (with tailing)			
dNTP solution mixes	HiScribe T7 ARCA mRNA Synthesis Kit			Monarch RNA Cleanup Kit (50 µg)
BspQI* (GMP)	HiScribe T7 High Yield RNA Synthesis Components (GMP)	Faustovirus Capping Enzyme (GMP COMING SOON)		Monarch RNA Cleanup Kit (500 µg)
		Vaccinia Capping System (GMP)		
DNA Assembly • NEBuilder HiFi DNA Assembly • Golden Gate Assembly	HiScribe T7 Quick High Yield RNA Synthesis Kit	mRNA Cap 2´-O-Methyltranferase (GMP)		Lithium Chloride
	HiScribe SP6 High Yield RNA Synthesis Kit	ARCA and other mRNA cap analogs		
	T3 & SP6 RNA Polymerases			
	T7 RNA Polymerase (GMP)			
	Hi-T7 RNA Polymerase			
	Companion Products			**Companion Products**
	RNase inhibitor (Murine) (GMP)			Monarch RNA Cleanup Binding Buffer
	RNase Inhibitor (Human Placental)			Monarch RNA Cleanup Wash Buffer
	Pyrophosphatase, Inorganic (*E. coli*) (GMP)			Nuclease-free Water
	Pyrophosphatase, Inorganic (Yeast)			
	DNase I (RNase-free) (GMP)			
	DNase I-XT			
	NTPs			

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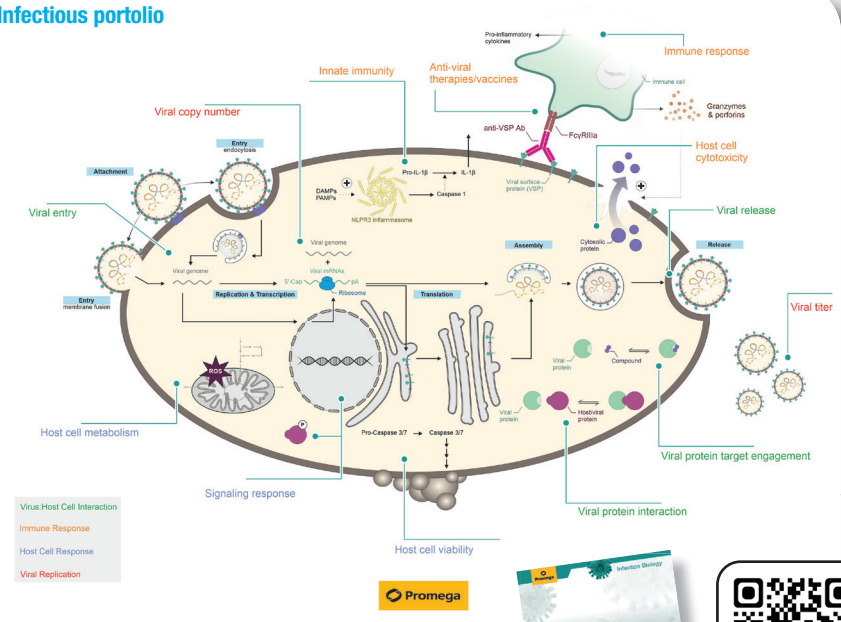

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RNA SYNTHESIS AND NANOPARTICLES DELIVERY SYSTEMS PLATFORMS

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Interest in RNA use to cure untreatable diseases is not novel, research goes on for decades, but its potential became striking since the Covid-19 pandemic. In France, research and develop-ment on this topic is active not only in academic and universities labs, but in small, medium- and large size companies as well. Our lab is actively taking part on the RNA adventure. Herein, we present the overall process for RNA production, the different types of RNA that can be gen-erated through an RNA platform with length varying from a few hundred of nucleotides to 10 kilobases, with emphasis on the versatility of RNA modifications and corresponding usage. Na-noparticle-based delivery technologies used for increasing RNA, bioavailability, stability and efficacy are also presented.

Keywords: RNA synthesis, Transfection, Lipid Nanoparticles, RNA drug, Gene therapy, Gene silencing, Regenerative medicine, Vaccines, RNA/LNP tracking.

A nascent mRNA, synthesized in the nucleus, undergoes different modifications before it can be translated into proteins in the cytoplasm. For a mRNA to be functional, it requires modified 5´ and 3´ ends and a coding region (i.e., an open reading frame (ORF) encoding for the protein of interest) flanked by the untranslated regions (UTRs). The nascent mRNA (pre-mRNA) undergoes two significant modifications in addition to splicing. During synthesis, a 7-methylguanylate structure, also known as a "cap", is added to the 5´ end of the pre-mRNA, via 5´ 5´ triphosphate linkage. This cap protects the mature mRNA from degradation, and also serves a role in nuclear export and efficient translation.

The second modification occurs post-transcriptionally at the 3´ end of the nascent RNA molecule and is characterized by addition of approximately 200 adenylate nucleotides (poly(A) tail). The addition of the poly(A) tail confers stability to the mRNA, aids in the export of the mRNA to the cytosol, and is involved in the formation of a translation-competent ribonucleoprotein (RNP), together with the 5´ cap structure. The mature mRNA forms a circular structure (closed-loop) by bridging the cap to the poly(A) tail via the cap-binding protein eIF4E (eukaryotic initiation factor 4E) and the poly(A)- binding protein, both of which interact with eIF4G (eukaryotic initiation factor 4G), (Figure 1, (9)).

1. Introduction

RNAs such as messenger RNA (mRNA) or self-amplifying RNA (saRNA) and non-coding RNAs such as microRNA (miRNA), small interfering RNA (siRNA), antisense oligonucleotide (ASO), long non-coding RNA (lncRNA), and genome editing systems containing RNA components like guide RNA (gRNA), represent useful tools in research and powerful strategies in clinic. RNA-based therapies have played a huge role in the fight against cancer, infectious diseases, allergies, and in protein replacement therapy, cell reprogramming, and genome editing (U. Sahin 2014).

RNA use presents important limitations such as unfavorable pharmacokinetics, and pharmacodynamics when administered without a delivery agent. Indeed, naked mRNA is un-stable, poorly uptaken by cells due to its negative charge, hydrophilic nature, high molecular weight and is rapidly degraded by enzymes in the serum. As a direct consequence, delivery systems are a necessity to efficiently deliver RNAs for in vivo applications. Their incorporation in nanoparticles can overcome these limitations by protecting them from enzymatic degrada-tion and enabling their uptake and release into the cytosol of target cells.

Innovations in both the chemical modification of nucleic acids and the synthesis of vectors with novel properties have been instrumental in driving RNA therapies into the clinic. The first approved therapy using small nucleic acid by the FDA was Fomivirsen™ in 1998, an antisense oligonucleotide drug which was administered locally into the eye by intravitreal injection to treat cytomegalovirus retinitis. Since

then, innovations in science and technology have over-come some major hurdles and hastened the development of oligonucleotide therapeutics that led to the FDA/EMA approval in the last decade of not only several new ASO-based therapies, but siRNA, mRNA and other types as well. ONPATTROTM is a prescription medicine for adults, treating the polyneuropathy caused by an illness called hereditary ATTR (hATTR) amyloidosis. This therapy was approved by the FDA in 2018 and was the first siRNA and Lipid Nanoparticle (LNP)-based drug in its kind to reach the market. Experience gained in the clinical development of LNP for the delivery of small molecules, combined with an understanding of the physical properties of lipids, can be applied to rationally design LNP systems for in vivo delivery of RNA. In addition to implementation on RNA vehicle such as lipids, huge efforts in mRNA quality and technique of production have brought the mRNA-LNP system as one of the more potent and promising therapy that can be broadly applied in clinics, ranging from applications in regenera-tive medicine, oncology to gene therapy. Recently, vaccine towards Covid-19 has paved the way for usage of mRNA-LNP formulation in clinics. Appropriate mRNA structure modifications and formulation methods have been investigated to limit naked RNA instability, innate immuno-genicity, and inefficient in vivo delivery. Here, we present the mRNA manufacturing process by highlighting some critical RNA features and functions. In the second part, we highlight li-pid-based nanoparticles delivery systems platforms.

2. RNA features and functions – mRNA synthesis platform

2.1. RNA-required qualities

Mature mRNA produced in higher eukaryotes is composed of an open reading frame (ORF) region that encodes the sequence to be translated, flanked by five-prime (5') and three-prime (3') untranslated region (UTR), and further stabilized by 5'cap and 3' poly (A) tails (Figure 1).

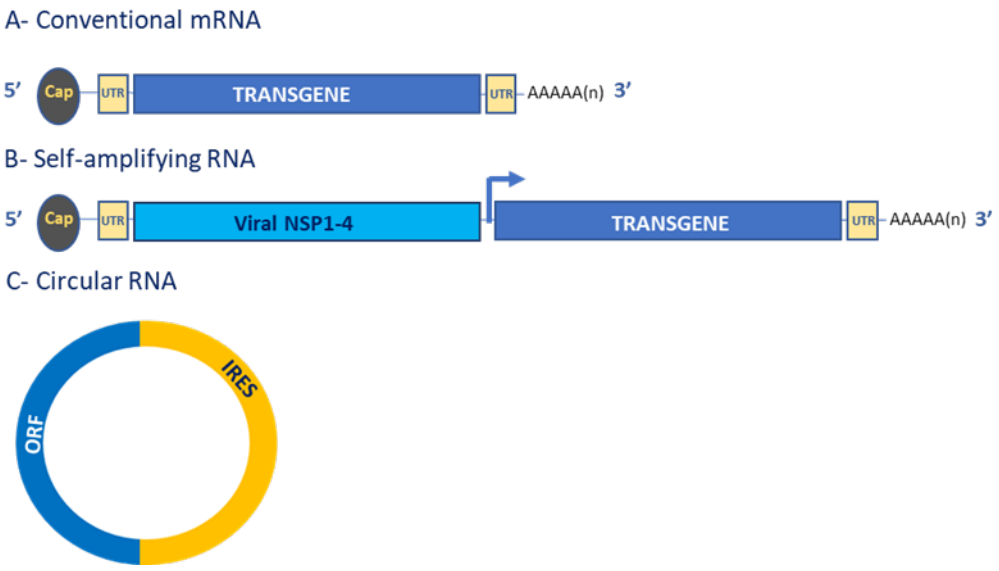

Figure 1. *Structure of synthetic conventional mRNA (A), saRNA (B) and circular RNA (C) with ei-ther/or Cap1, UTRs, Replicase (viral non-structural protein 1 to 4), IRES (Internal Ribosome Entry Site), transgene (ORF) and poly-A tail.*

In the eukaryotic cell, capping of mRNA 5' end is an essential structural modification that allows efficient mRNA translation, directs pre-mRNA splicing and mRNA export from the nucleus, limits mRNA degradation by cellular 5'–3' exonucleases and allows recognition of foreign RNAs (including viral transcripts) as 'non-self'. Almost all endogenous eukaryotic mRNA con-tains a 5' cap structure and a 3' chain of adenosine nucleotides (poly(A) tail) added during RNA processing. The 5' cap (an N7-methylated guanosine linked to the first nucleotide of the RNA via a reverse 5' to 5' triphosphate linkage (the 5' m7G cap)) is critical in initiating protein syn-thesis, but also functions as a protective group against 5' to 3'

exonucleases; Higher eukaryotes can further modify this minimal cap structure with the addition of a methyl group on the ribose 2'-O position of the first transcribed nucleotide (Cap1) and sometimes at the adjoining nucleo-tide (Cap2). The poly(A) tail increases translational efficiency and improves message stability by protecting against 3 to 5 exonucleases.

2.2. mRNA synthesis

RNA can be synthesized in vitro by the so-called in vitro transcription (IVT) technique (Figure 2 and 3). Linearized plasmid DNA, PCR products or cDNA can be used as templates for transcription if they contain a double-stranded RNA polymerase promoter region in the correct orientation with consensus promoter sequences of either T7, T3 or SP6 prokaryotic phage polymerases. The cap1 can be added enzymatically after initial IVT (using guanylyl transferase and 2-O-methyltransferase to yield a Cap1 (N7MeGpppN2-OMe) structure) or Cap analog such as ARCA or cleanCAPTM can be incorporated into mRNA during IVT. In a similar manner, poly(A) tail can be encoded in the DNA template or added by enzymatic method (by action of poly-A polymerase). The first strategy largely favor the reproducicility of the tail length whereas the enzymatic addition is difficult ot control and leads to some length variability. Both the capping and the addition of the long poly(A) tail are critical for proper function and stability of the transcribed mRNA, but both slow production and add cost. The common size of IVT mRNAs range between 500 to 5000 nucleotides, although smaller or larger RNAs can be synthesized upon protocol adaptation.

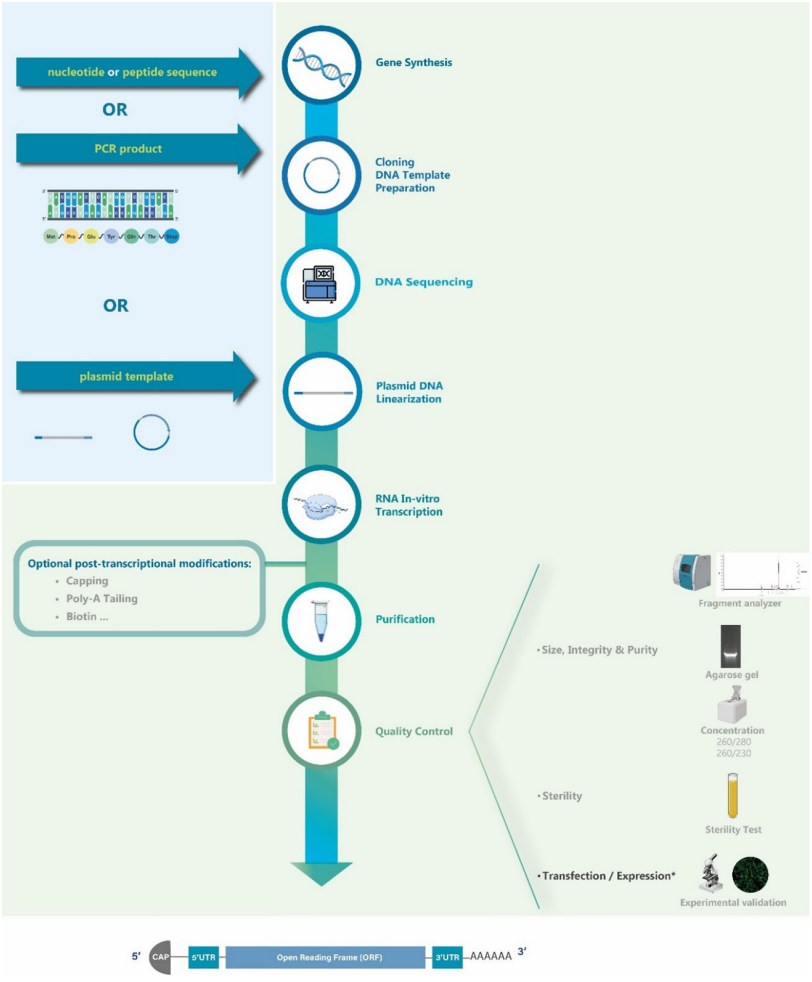

Figure 2. *OZ Biosciences RNA synthesis workflow.*

2.3. Reduction of immunogenic reaction

"Native" (unmodified) mRNAs are immunogenic. Altering the mRNA's base composition is an established method to reduce immunogenicity for mRNA therapeutics and enhancing its stability. Since uridine-rich sequences of RNA trigger the innate immune response, depleting Uridine triphosphate (UTP) from the final mRNA sequence can improve evasion (M. B. Katalin Karikó 2005). Uridine depletion, for which a U-containing codon are substituted during se-quence optimization for one codon without U will result in the same downstream protein while reducing the mRNA immunogenicity (Sriram Vaidyanathan 2018).

Similarly, modified nucleoside triphosphates (NTPs) such as pseudoUridine triphosphate, thioUridine triphosphate, N1-methylpseudoUridine triphosphate (N1-mψ), and 5-methoxyUridine (moU) triphosphate can substitute the UTP base to evade innate immune detection (Callum J C Parr 2020). Recent advances in high-throughput RNA-sequencing tech-niques have resulted in the elucidation of the important roles played by mRNA chemical modi-fications and mRNA nucleotide sequences in regulating mRNA stability and immunogenicity reduction (H. M. Katalin Karikó 2008). To date, hundreds of different RNA modifications have been characterized. Among them, several RNA modifications, including N6-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), 8-oxo-7,8-dihydroguanosine (8-oxoG), pseudouri-dine (Ψ), 5-methylcytidine (m5C), and N4-acetylcytidine (ac4C), have been shown to regulate mRNA stability, consequently affecting diverse cellular and biological processes (Figure 3). This approach has spread quickly in the past years and led to a globally accepted replacement of Uridine by N1-methylpseudouridine (Ψ) (Weissman 2015). Indeed, replacement of only 25% of Uridine and Cytidine with 2-thioUridine and 5-methyl-Cytidine synergistically decreased mRNA binding to pattern recognition receptors, such as TLR3, TLR7, TLR8 and RIG-I, in human pe-ripheral blood mono-nuclear cells (PBMCs) (Michael S D Kormann 1 2011).

In addition, double stranded RNA (dsRNA) impurities formed during IVT significantly contribute to the innate immune activity of mRNA therapeutics. Purification of IVT mRNA by means of high-performance liquid chromatography (HPLC), or alternative methods using cellu-lose columns or selective digestion of dsRNA fragments using RNAse III have been established and proven to greatly reduce immune reaction (Markus Baiersdörfer 2019). Though this effect was mainly observed for wild type (unmodified nucleotides) mRNA, purification of chemically modified mRNA did not decrease inflammation markers. Overall, mRNA chemically modified with moU, N1-methyl pseudo-U, or mC/pseudo–U do not necessarily need to be HPLC purified before use in vitro or in vivo which is of great interest in production process.

2.4. Enhancement of mRNA duration and expression levels

Due to the propensity of cells to limit the duration of expression of mRNAs, only a transient and low level of expression can be achieved in vivo, necessitating the administration of high doses of mRNA. Stability, durability and expression levels of mRNA-based therapy have been achieved by several means such optimization of specific sequence elements or use of self-replicating RNAs (saRNAs or Replicons).

2.4.1. Optimization of specific sequence elements

As mentioned above, gene expression upon RNA transfection in mammalian hosts can be significantly enhanced by substituting rare codons with more frequent ones (Liu 2020). As a result, the strategy of using synonymous codons while maintaining the original protein se-quence proved to be particularly successful and nowadays most of the DNA templates are co-don optimized for a specific host.

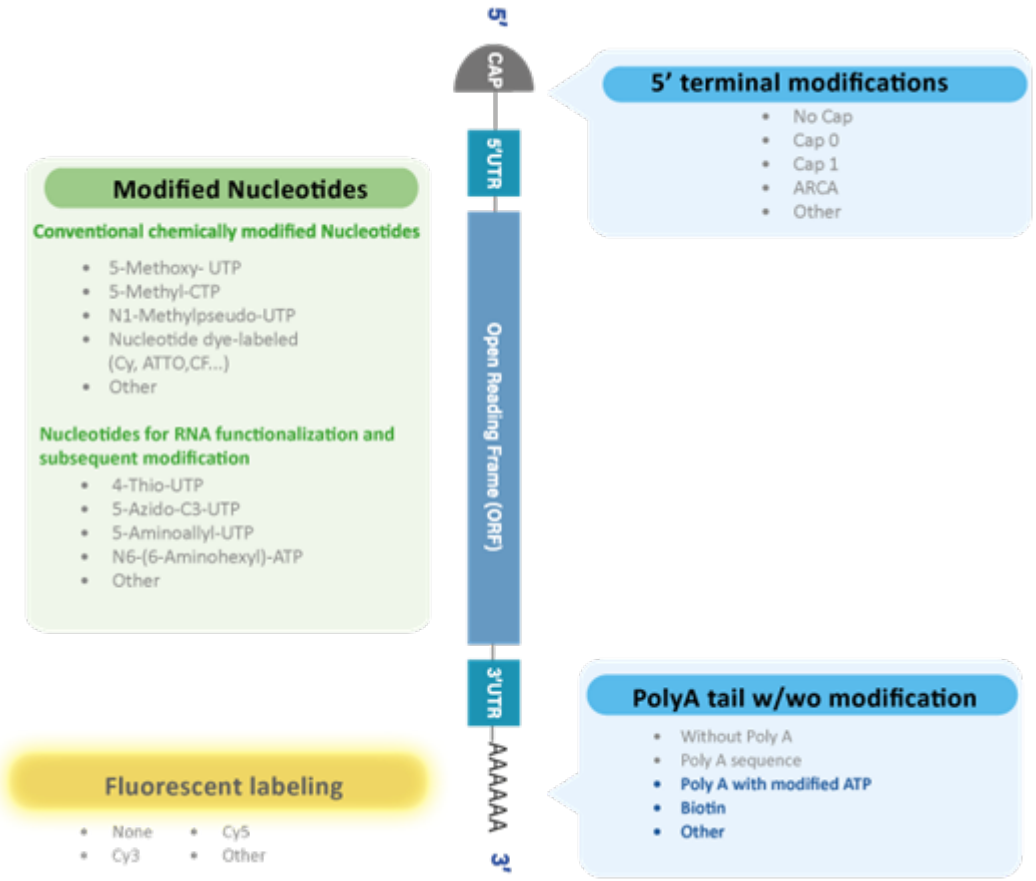

Figure 3. *OZ Biosciences RNA synthesis workflow - modifications.*

2.4.2. Self-amplifying RNAs

Actually, single-stranded RNA viruses such as alphaviruses, flaviviruses, measle viruses and rhabdoviruses are characterized by their capacity of highly efficient self-amplification of RNA in host cells, which make them attractive vehicles for vaccine development (Lundstrom 2020). saRNA replicates and translates without producing infectious progeny, has shown extraordi-nary properties to induce immune response (C Smerdou 1999). Most saRNAs used in vaccina-tion studies derive from alphaviruses, including Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), and Sindbis virus. They have been dedicating to high level expres-sion of viral and tumor antigens, which in immunization studies have elicited strong cellular and humoral immune responses in animal models. Compared to “classic” mRNA, replicons are considerably larger (~9-12 kb); they contain all the basic elements of mRNA but have a large ORF at the 5’end that encodes four non-structural proteins (nsp1-4) and a sub-genomic pro-moter (Anna K Blakney 2021) (Figure 1). The interest here resides in the fact that genes in the viral genome normally downstream of the sub-genomic promoter are replaced by gene(s) en-coding the vaccine antigen(s); the deletion of the viral structural proteins rendering the RNA not able to produce an infective virus. The self-amplification properties thus enable use of a much lower dose of saRNA compared to messenger RNA (mRNA), typically 100-fold lower (Annette B Vogel 2018) with a production level at least ten-fold higher than those of genomic RNA leading to high production of antigen in vivo. Novel and efficient non-viral delivery sys-tems have arisen that can have broad application to vaccinate against human pathogens, in-cluding emerging ones. Among them, cationic lipid formulations (Englezou PC 2018) or lipid nanoparticle formulations of saRNA are currently the most potent, requiring as little as 10 ng of nucleic acid to induce a robust immune response (Paul F McKay 2020) that make them candi-dates of choice for the design and development of saRNA vaccine.

2.4.3. Circular RNAs

Circular RNAs (circRNAs) are covalently closed, single-stranded RNA species that are prev-alent in eukaryotic transcriptomes and have recently gained widespread interest for their roles in disease regulation and as therapeutic modalities for gene delivery and protein production (Wesselhoeft R.A 2018) (L. Chen 2020). Still, much is still unknown about their function, bio-synthesis, degradation, and associated cellular machinery. Circular RNAs are formed through 'back-splicing' and can be composed of exons, introns, or both. Indeed, endogenous circRNAs lack the free ends necessary for exonuclease-mediated degradation, rendering them resistant to several mechanisms of RNA turnover and granting them extended lifespans as compared to their linear mRNA counterparts. General translation of mRNA begins when the translational machinery recognizes the end 5' CAP and scans the UTR region until the start codon is recog-nized. Translation can also occur independently of the 5'CAP and requires an internal ribosome entry site (IRES) (Dawood B. Dudekula 2016) (Figure 1). Since in vitro synthesized circRNAs can be translated in cap-independent fashion and most circRNAs are localized in cytoplasm, it is highly possible that many circRNAs function as mRNAs to direct protein synthesis. Methods to produce synthetic circRNA from in vitro transcription reactions using enzyme-mediated liga-tion or permuted autocatalytic introns have been described (Wesselhoeft 2019) (Y. Chen 2017). New methodology to prepare purified synthetic circRNAs could be developed into a safe and effective vaccine platform. Overall, the promise of synthetic circular RNAs in the production of recombinant proteins and as RNA-based therapies is only now coming into focus (Costello 2019).

3. RNA delivery

High doses of nucleic acid are required when naked siRNA is injected intravenously as de-scribed by Thompson, J. D. et al. The authors demonstrated that when ≤200 mg/kg siRNA was administered, the plasma concentration 30 min after injection was ~90% lower and two hours after injection, less than 2% siRNA was found is the plasma due to rapid clearance by kidneys (James D Thompson 2012). Effective delivery of RNA molecules into cells is a crucial step for successful RNA-based therapeutics and thus an ideal RNA delivery system should have low tox-icity as well as high cell specificity and high delivery efficiency. Therefore, there is a need to protect RNA molecules from degradation and renal clearance and ionizable lipid forming com-plexes with nucleic acids have been used to this end. Initially developed as carriers for in vivo siRNA delivery (Srinivas Ramishetti 2016) (S. M. Muthusamy Jayaraman 2012) (Semple 2010) the same ionizable lipid nanoparticles have, later on, been used to deliver large RNA molecules such as mRNA (Nuphar Veiga 2018).

3.1. Vehicles for RNA delivery. Cationic and ionizable lipids

Among the different types of delivery systems, cationic and ionizable lipids-based vehicles have been extensively studied due to their properties such as simple chemical synthesis or wide packaging capabilities. They use their positive charges to counterbalance the inherent instabil-ity of negatively charged RNA molecules, to overcome biological barriers and to release RNA cargo into the cell. Based on these compounds, lipid nanoparticles or LNP count among the most widely used delivery systems for RNA-based therapeutics, as evidenced by the clinical approvals of three LNP-RNA formulations, Patisiran, BNT162b2 and mRNA-1273. Recently, the latter two formulations of LNP encapsulating mRNA, BNT162b2 and mRNA-1273, have ob-tained emergency use authorizations (EUA) from the FDA and EMA as SARS-CoV-2 vaccines for the prevention of coronavirus disease 2019 (COVID-19).

Cationic lipids are permanently positively charged and chemically stable even in the envi-ronment involving strong oxidants and acids. However, the positive charges might lead to po-tential cytotoxicity, e.g., hemolytic, and undesired immunostimulation (M C Filion 1997). Cyto-toxicity of cationic lipids may be related to the generation of reactive oxygen species (ROS) and the increase of cellular calcium

levels (S Dokka 2000). As mentioned before, the positive charges of the cationic or ionizable lipid-based liposomes can improve the RNA encapsulation efficiency as the result of electrostatic interactions between the negatively charged phosphate backbone of RNA molecule and the positively charged head group of cationic lipids under a favorable formulation envi-ronment. The key point here is to modulate the nitrogen/phosphate ratio (N/P) in such a way that the liposome presents a net positive charge that (1) neutralizes, complexes, and condenses RNA molecule, (2) prevents aggregation and (3) facilitates the binding of liposomes to the neg-atively charged cell membrane. Early developments have used permanently charged cationic lipids to formulate liposomes, which complex with RNAs and escort them to the site of action [28].

Geometrically, cationic liposomes usually show a unilamellar structure of a single lipid bi-layer surrounding an aqueous core with a diameter of 100–200 nm. Structurally, synthetic li-pids usually contain three parts: (a) cationic or ionizable polar head group, (b) linker and (c) hydrophobic tail. Chemically at last, according to the structures of their head groups, they are organized into four categories: quaternary ammonium, guanidinium, pyridinium, and imidazo-lium lipids. The positive charges are involved in the interactions with RNA and cell membrane while the hydrophobic tail can affect their pKa, transition temperature, lipophilicity and most of all, the potency of RNA delivery (the hydrocarbon tail generally counts between 8 to 18 carbon units in length with various unsaturation degrees – such as linoleoyl or oleoyl groups). Finally, the linker (e.g., ethers and esters, phosphate, or phosphonate…) is of high importance as its cleavability gives the cationic lipids its biocompatibility characteristics and its stability to sur-vive in a biological environment. To stabilize the lipid-based RNA delivery system, many helper lipids can also be included as formulation components, such as cholesterol, 1,2-dioctadecanoyl-sn-glycero-3- phosphocholine (DSPC), or 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE). These helper lipids may not only stabilize the particles but also enhance RNA delivery efficiency (Cheng 2016) A large variety of cationic lipids have been developed over the years such as DOTMA, DOTAP, DOGS, DOSPA, DDAB, DC-Chol and GL67, to name a few (Mahato 2010). We have focused on the design of a new library of biodegradable cationic lipids that were formulated as transfection reagents and used as lipoplexes in a large variety of models to successfully transfect siRNA (Shanks 2014), mRNA (S Guan 2017), miRNA (S E Jalava 2012), saRNA (Pavlos C Englezou 2018) just to name a few examples since currently they are more than >1000 publications using the transfection reagents based on this lipid library. The efficacy of these amino acid-based lipids to deliver a large variety of RNAs reveals also the requirement of optimizing the lipid structure and formu-lations in function of the RNAs to be delivered and the cell types targeted.

In recent years a new generation series of ionizable lipids have been developed for deliv-ery of nucleic acid therapeutics to overcome the limitation of cationic lipids with improved in vivo transfections efficiency (Kelsey L. Swingle 2023). The ionizable lipid head is decorated with a characteristic tertiary amine/amines polar functionality having a characteristic pKa, ide-ally in the range of 6 to 6.5. The surface pKa of these tertiary amine functionality plays two im-portant roles. Firstly, it becomes positively charged at acidic environment and suitable for complexation with nucleic acids with high encapsulation efficiency. Second these tertiary amine gets protonated at acidic environment of endosome facilitate their endosomal escape by inter-acting anionic endosomal membrane (Martijn J. W. Evers 2018). This ionizable nature makes them neutral at physiological pH is beneficial for nucleic acids delivery in vivo because it has less interactions with the anionic membranes of blood cells and, thus, improve the biocompati-bility as well as prevent rapid sequestration by the mononuclear phagocyte system, less im-mune stimulation, and toxic effects (Yulia Eygeris 2022). Extensive research has been devoted since last decade for fine tuning of the properties for better efficient ionizable lipids to target specific tissues by altering their headgroups, linkers and hydrophobic tails (Hou 2021).

These ionizable lipids makes 30–50% of the total lipids in a formulations, various ioniza-ble lipids have

been tested over the years especially for siRNA delivery such as 1,2- dilinoleyloxy- N, N-dimethyl-3-aminopropane (DLin-DMA), 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA), and (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino) butanoate (DLin-MC3-DMA; MC3) etc (Hou 2021). MC3 showed successful siRNA delivery and it is a major component in FDA-approved siRNA drug Onpattro for the treatment of polyneuropathy in peo-ple with hereditary transthyretin-mediated amyloidosis (Akinc 2019). Further research in this area resulted in finding better ionizable lipids for mRNA delivery which paved crucial role in countering the COVID-19 pandemic in quick time. Ionizable lipids SM102 and ALC-0315 (Hou 2021) which has better delivery efficacy and pharmacokinetics than MC3 became successful components in the mRNA-1273 of Moderna and BNT162b BioNTech COVID-19 vaccines re-spectively to deliver nucleoside modified Spike CoV2 mRNA. Various ionizable lipids apart from MC3, SM102 and ALC-0315 are under clinical development for treatment of infectious disease and cancer to deliver mRNA vaccines and other RNA therapeutics (Hou 2021).

3.2. Lipid Nanoparticles (LNPs)

Since the first report of gene transfer activity of the cationic lipid DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane) in 1987, the challenge to efficiently deliver RNA drugs is more topical than ever before. To date, formulation in lipid nanoparticles (LNPs) represents the most advanced delivery platform for gene therapy and promising candidates to treat manifold diseases. LNPs were FDA approved for the treatment of amyloidosis disease by delivery of siRNA, and more recently for the widely distributed SARS-CoV-2 vaccines, based on mRNA-LNPs. Despite the current LNPs breakthrough, there are still some concerns about the inherent offtargerted toxicity of these nanocarriers. Notably, the biodistribution and cellular uptake of LNPs are affected by their surface composition, size as well as by their colloidal stability. Therefore, a deep knowledge of the LNPs physico-chemical features is still needed.

3.2.1. Composition

LNPs carrying nucleic acids are lipid shell structures encapsulating nucleic acids by organising them in inverse lipid micelles or intercalated between the lipid bilayers inside the nanoparticles (Rumiana Tenchov 2021).

LNPs consist in an aqueous core surrounded by a lipidic shell based on a combination of different lipids, each having distinct functions. Generally, LNPs are composed of four families of chemicals: a complexing aminated lipid (either ionizable or cationic), a helper phospholipid (DSPC, DOPC or DOPE), cholesterol and a pegylated lipid (PEG) that allow the encapsulation of the nucleic acid drug within their core (Figure 4).

Complexing lipids are usually amphiphilic molecules, comprising an ionizable and/or cationic / head group, a hydrocarbon chain or cholesterol derivate, attached via a linker (e.g. glycerol). The positively charged head group allow their entrapment into LNPs during their manufacturing. In addition, these lipids can mediate the interactions between LNPs and the cellular plasma, and facilitate cell uptake and endosomal release of the cargo (Xinwei Cheng 2016). As described above, cationic lipids and/or ionizable lipids are in charged of this task. Some cationic lipids such as DOTAP (Gustavo Lou 1 2020) and our biodegradable amino ac-id-based lipids (Patent US9107931B2) (Englezou PC 2018) and ionizable lipids have even shown their potential in delivering large saRNA molecules (Anna K. Blakney 2019) (Aliahmad 2022). Nonetheless, the in vivo efficiency of cationic lipid formulation is limited by low efficiency and some toxicity. Accordingly, new series of ionizable lipids have highly been developed to potentiate LNPs efficiency, especially for RNA delivery in vivo (Genc Basha 2011).

Helper lipids such as phospholipids are usually part of LNP formulation and favour colloidal stability and gene delivery efficiency meanwhile increasing systemic circulation of the nanoparticles.

Phosphatidylcholine lipids (PCs) compose cell membranes. Saturated PCs, such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), are the most representative helper lipid used to form highly stable LNPs due to its high melting temperature (Tm). Cholesterol is commonly used in liposomes and LNPs. It helps stabilizing the lipidic shell and promotes membrane fusion. At high ratio (>35mol %), cholesterol has been proved to potentiate the activity of cationic lipids and thus gene transfer, presumably by increasing bilayer destabilization [48]. It is now well known that incorporation of a PEGylated lipid into LNPs increases their colloidal stability and resistance to opsonization and reticuloendothelial clearance. Among the pegylated lipid, methoxy PEG2000-DSPE is the most used in LNPs. It has been shown that pegylation of the nanoparticles result in long-term biodistribution, better tumour penetration and accumulation (Yanjie Bao 2013). In the range of 1 to 5 mol%, PEG-lipids lead to neutral surface charge and reduce the size of LNPs.

3.2.2. Methods for LNP synthesis, screening, quality control

Different synthetic methods for RNA-loaded LNPs have been adopted to generate efficient drug carriers. Classical methods for LNP preparation involve the traditional rehydration of a lipidic thin-film with an aqueous buffer containing the RNA. The major drawback to this method is the random nature of the complexes, reproducibility and the polydispersity of the resulting nanoparticles, requiring the use of an additional extrusion. Accordingly, the development of microfluidic techniques in the early 2000s allows reproducible, relevant and scalable LNP production (Andreas Jahn 2004). Microfluidics provides tools to manipulate liquids, droplets, cell and particles within micro-channel geometries. Typically, a stream of lipid in alcohol solution is flown by fast mixing with an anti-solvent (e.g. RNA in acidic aqueous phase) at the junction of the microfluidic chip. The RNA-loaded LNPs self-assemble at the intersection by nucleation-growth process prior to being collected at the end of the microfluidic chip. Then, alcohol solvent and free molecules are usually removed by dialysis or diafiltration. These processes stabilize the LNP structure and adjust the pH to neutral, which is required for further in vitro and in vivo application. LNPs can be sterilized by filtration and are generally stored at -80°C with the addition of cryoprotectant (Figure 5). Resulting formulations are characterized by different analytical techniques including dynamic light scattering (DLS) to monitor their hydrodynamic size, polydispersity index (PdI) and zeta potential, cryogenic transmission electronic microscopy (cryoTEM) to evaluate their morphologies (as shown in Figure 4).

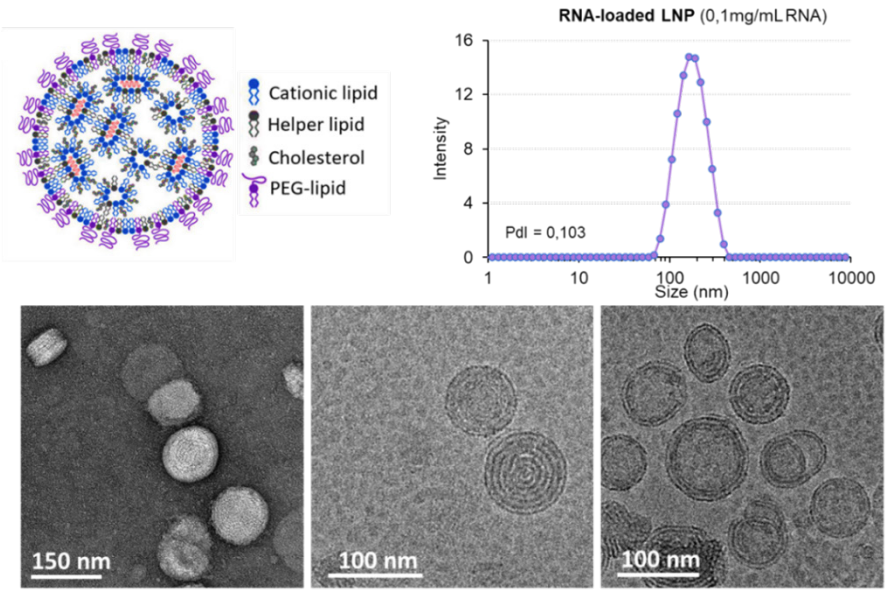

Figure 4. *Schematic representation of lipid nanoparticles (LNPs). Dynamic Light Scattering (DLS), nega-tive stain and Cryo-TEM micrographs of around 100nm RNA-loaded LNPs.*

The nucleic acid entrapment rate is usually determined by spectroscopy techniques while using tagged-RNA or reporter genes enables to easily monitor the biodistribution of the LNPs delivery in tissues and organs. Microfluidics brings the possibility to work with a wide range of nucleic acids (e.g., PolyA, ssDNA, mRNA) and to rapidly screen a large library of lipids in order to optimize the formulation efficacy for gene delivery. Once the LNP formulation optimized, microfluidic technology provides the huge benefit to easily reproduce and scale-up the manufacturing of the delivery systems.

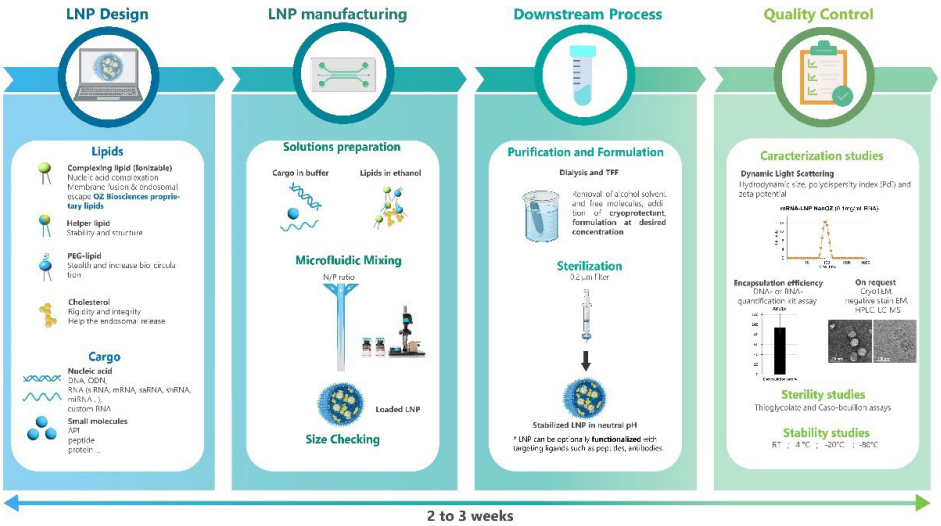

Figure 5. *OZ Biosciences LNP design, manufacturing, and QC workflow.*

LNPs represent a revolutionary treatment in nanomedicine as mRNA vaccines (Rein Verbeke 2019), but also in diverse medicinal applications including cancer therapy (Margaret M. Billingsley 2020), gene editing (Pieter R Cullis 2017) and rare diseases (Timofei S Zatsepin 2016). Therefore, an efficient screening platform allowing numerous LNP formulations to be produced in a reproducible manner for physical characterization and biological testing ap-peared as a necessity (Figure 5). As an illustration, our group generated mRNA-LNPs by en-trapping Firefly Luciferase mRNA (0.10mg/mL) and evaluated the nanoparticle delivery system in mice model. The Luc-RNA localization and expression was monitored by IVIS® imagery sys-tem (PerkinElmer). The optimal formulation was selected by examining properties of tens of LNPs including size, surface charge, N/P ratio, lipid nature as well as injection site and RNA dose in nude mice. As observed, a single intra-peritoneal (i.p.) injection of Luc-LNPs enables high bioluminescence signal and luciferase expression into surrounded organs for at least 48h (Figure 6).

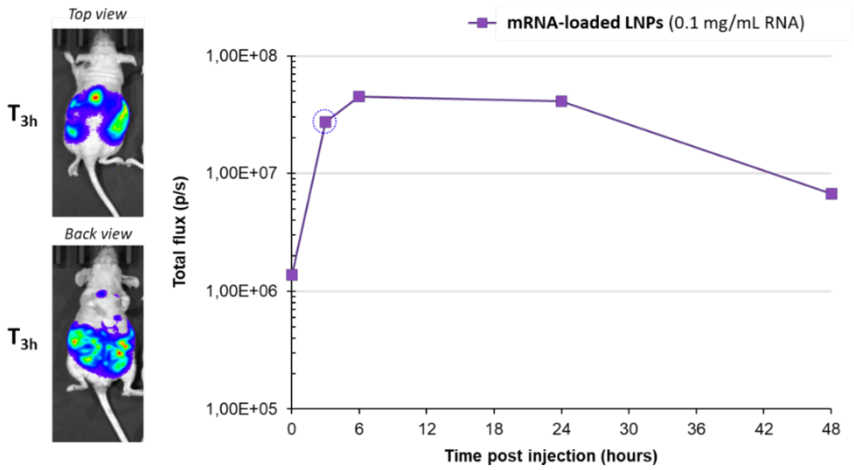

Figure 6. *Bioluminescence signal 3h after i.p. administration of Luc-LNPs in nude mice. Kinetics of biolumi-nescence signal over 48h after i.p. administration of Luc-LNPs (dose equivalent to 10µg RNA).*

4. Vector-free RNA delivery

Vector-free delivery strategy in the development of mRNA drugs is getting attention. In this direction, several research groups have proposed a concept of mRNA delivery by (1) bun-dling mRNA strands with RNA oligonucleotide linkers ((Naoto Yoshinaga 2019) or using the RNA origami technology. Indeed, as a highly programmable self-assembling nanotechnology, DNA or RNA origami make it possible to construct scalable nucleic acid nanostructure which holds the characteristics of high stability for a large range of applications including drug deliv-ery. Studies have shown that staple-assisted mRNA origami nanolization could promote cellular endocytosis, while the relatively flexible structure allows the mRNA to still be recognized and translated. The obtained structure, called “mRNA nano-lantern” exhibited better biocompatibil-ity, lower toxicity, and a similar level of mRNA delivery ability (Hu 2023). Such alternative strategies for mRNA-based therapy are at early days and require improvement and confirma-tion of their potential performance.

5. Conclusions and Perspectives

Nowadays, we can foresee RNA as drugs for personalized medicine and immunotherapy as well as to address genetic, infectious, and chronic diseases. This forecast will ensure the con-tinued development of RNA therapeutics for years to come. Since mRNA-lipid nanoparticle formulations should be designed according to biomedical demand (vaccines, therapeutics...) and site targeting (specific cells or organs), the continuous development of both RNA and de-livery vehicles is needed. Consequently, we have designed and developed two genuine plat-forms: one for mRNA synthesis and the other for LNP manufacturing. RNA expression can fur-ther be improved by upgrading the RNA engineering and the delivery efficacy (e.g. rational de-sign of lipids, through modulation of head groups and hydrophobic tails, lipid structure, making hybrid nanoparticles...). Research in the RNA/nano delivery field will most likely continue to expend and to give rise to the next-generation of mRNA-based therapies to improve health care.

Author Contributions: All authors equally contributed to writing of this review. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: Bonvin, E, Dhumal, D. and Zelphati, O. are employed by OZ Biosciences, which manufactures and commercializes research reagents and technologies such as RNA, lipid- and polymer-based technologies.

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THE FUTURE OF MRNA BASED THERAPEUTICS AND THEIR MAIN CHALLENGES

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Introduction

mRNA is a ribonucleic acid used by cells as an intermediate carrier of the genetic information contained in the DNA to synthesize proteins and corresponds to the copy of one of the two strands of DNA. Over the past years, major technological innovation has allowed mRNA to become an increasingly used tool for therapeutic purposes. The recent Covid-19 epidemic has demonstrated the effectiveness of mRNA vaccines against infectious diseases. Indeed, these are faster to develop, simpler, cheaper to manufacture, and pose fewer health risks compared to conventional vaccine approaches1, 2, 3, 4. In addition, the use of mRNA is further being considered as a therapeutical solution against non-infectious diseases such as cancer5, to produce chimeric antigen receptor T cells (CAR-T) used in immunotherapy, as well as for therapies based on the administration of proteins or antibodies.

Despite recent technological advances in terms of mRNA modifications and delivery solutions, several important challenges remain for mRNA-based therapeutics to be effective: mRNA Immunogenicity and stability, improvement of the transcription and translation rate, level of antigen production.

In this article we will discuss the future of mRNA vaccines, the importance of mRNA sequence optimization to maximize the impact of production, delivery, and administration of therapeutic mRNAs, and how Tebubio's research grade mRNA production service can support you in responding to your challenges and enable you to quickly advance your projects to the next steps.

The importance of UTRs for high translation efficiency

mRNA comprises 3 regions: a 5' untranslated region (5'-UTR), a coding region for a protein (CDS), and a 3' untranslated region (3'-UTR). The 5'-UTR region contains the translation signals allowing the recruitment of the ribosome for the translation of the coding region into protein. A cap structure, a highly methylated modification at the 5' end of the RNA, protects it from degradation, marking it as "self" to avoid recognition by the innate immune system. At the 3' end, poly(A) tail and additional elements make the RNA molecule more stable and prevent its degradation.

The amount of protein produced from any given mRNA depends on its in-cell translational rate (how well the translational machinery initiates and elongates the CDS) as well as of the mRNA half-life (how long it remains intact inside cells). The 5'- and 3'-UTRs flanking the coding sequence profoundly influence mRNA translation and stability. In particular, they are the elements with the greater impact on ribosome load, which corresponds to the average number of ribosomes associated to a given mRNA 6.

Historically, only a handful of natural UTR elements, derived from either cellular or viral genomes, have been tested and characterized. Among them are the 5'- and 3'-UTRs from human hemoglobin subunit beta (hHBB), which is one of the most efficiently expressed mammalian mRNAs and is commonly used in studies on mRNA translation and stability. Viruses have evolved regulatory elements to take over the host translation machinery and effectively promote translation of their own mRNAs. For example, internal ribosome entry sites (IRESs) can recruit ribosomes to initiate translation without the need for the eukaryotic initiation factors.

More recent work has focused on systematic analysis of libraries of natural, mutated or rationally designed UTRs for their potential to enhance or fine-tune mRNA translation or stability 6.

Tebubio has developed a proprietary pDNA template with optimized 5'- and 3' -UTRs to ensure optimal transcription and translation of the target protein in mammalian cells. Expression tests in mammalian

cells (fig.1) show that the protein expression level of the eGFP mRNA produced with our proprietary vector, is comparable to the eGFP mRNA prepared byTriLink, our CDMO partner and leader in mRNA production, and superior to that developed by a competitor.

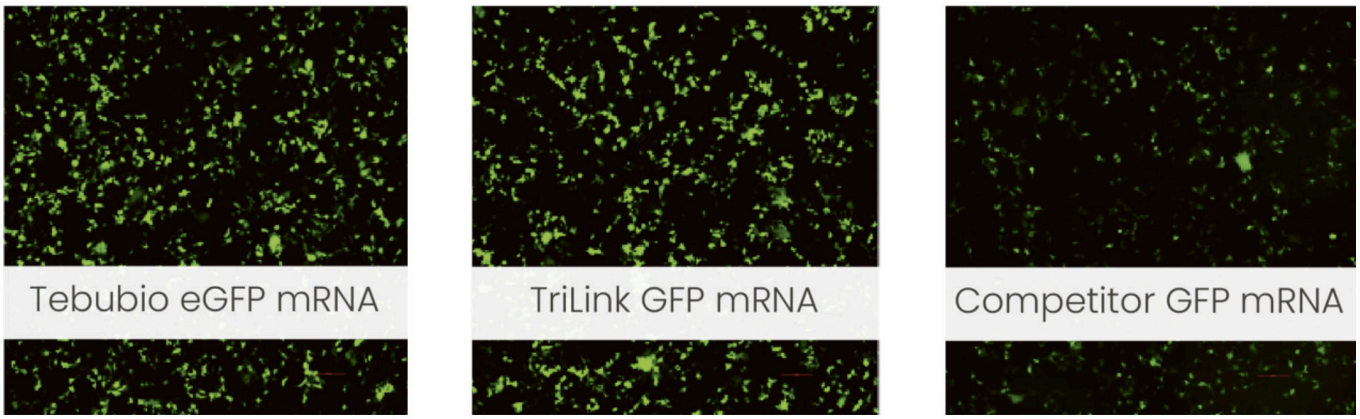


Figure 1. *Comparison of EGFP protein expression in HEK293 by fluorescence microscopy using FITC (510 nm) filter, after transfection with mrna produced by Tebubio, TriLink BioTechnologies, or a competitor mRNA supplier*

In addition, in our plasmid, the poly-A tail has also been optimized to guarantee you a constant tail length, for better mRNA stability, and batch-to-batch production reproducibility.

The role of secondary structure in transcription and translation efficiency

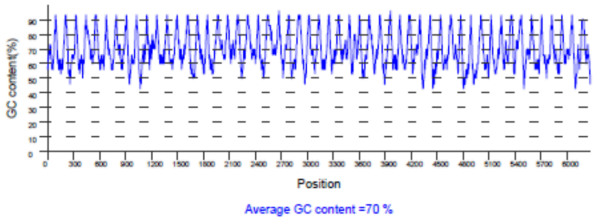
Besides the nature of the UTRs, both translational efficiency and half-life are influenced by multiple factors in primary nucleotide sequence, including GC content, codon usage, codon pairs, and secondary structure. While a low secondary structure in the 5'-UTR and in the first ~10 codons of the CDS is required for high translation efficiency, mRNAs containing highly structured CDS and 3'-UTR have been found to give the best protein output 6. This is due to the longer half-life of highly structured mRNA. Indeed, mRNA length and structure are the main drivers of in-cell and in-solution mRNA stability.

Whereas highly structured mRNAs can be efficiently translated thanks to the strong intrinsic helicase activity of the ribosome while translocating along mRNA, secondary structures may pose significant issues to the transcription efficiency. Indeed, during in vitro transcription, the RNA polymerase can be slowed down or even blocked by sequences rich in G and C and hairpin-shaped secondary structures, leading to low yields of production or to the generation of truncated forms of RNA.

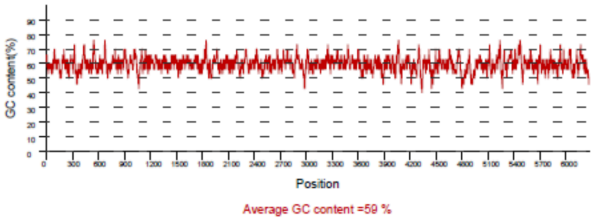
For this reason, Tebubio proposes a series of optimizations in order to maximize the transcription efficiency whilst ensuring high protein expression in the species of choice.

First, with a codon optimization of the CDS that includes a reduction of the G and C content (fig.2) as well as of the secondary structures such as hairpins.

GC Content Analysis



Plot of GC content of wild-type gene. Y and X axis represent local GC content (window size=30bp) and base position, respectively



Plot of GC content of optimized gene. Y and X axis represent local GC content (window size=30bp) and base position, respectively

Hairpin Analysis	Wild-type	Optimized
Small Hairpins	27	5
Medium Hairpins	14	1
Large Hairpins	3	0

Figure 2. *GC content and hairpin analysis in an example sequence, before (blue) and after (red) codon optimization*

Some sequences, especially those of several thousands of nucleotides, are of such complexity that they cannot be sufficiently improved only by codon optimization. For difficult-to-transcribe sequences, the production and purification conditions can be tuned to increase mRNA quality. For example, the interactions at the origin of the secondary structures are based on hydrogen bonds, which can be weakened by modifying the temperature of the transcription reaction.

Moreover, RNA purification with silica gel is based on the ability of the latter to absorb nucleic acids reversibly depending on the salt concentration, pH, and temperature. Modulating one or more parameters, based on the complexity of your sequence often results in fewer truncated forms and better homogeneity of your mRNA produced (fig.3).

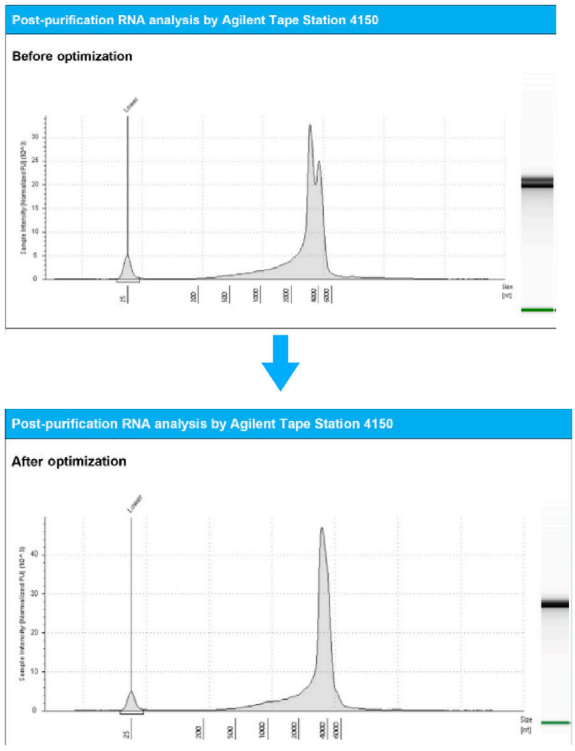


Figure 3. *mRNA size and homogeneity analysis, by tape station, of difficult-to-transcribe sequences before and after optimization. Presence of 2 spikes before optimization (mRNA truncated forms) vs. A unique and homogeneous spike and mRNA produced after optimization*

Reducing immunogenicity and improving half-life by uridine depletion and modification

Potential immunogenicity of mRNA transcripts is a major issue for some mRNA-based medicines. Indeed, unmodified exogenous mRNA activates Toll-like receptors (TLRs), resulting in upregulation of proinflammatory cytokines such as IFN-I, IL-6, IL-12, TNF-a and chemokines, which hamper mRNA translation through eIF2 activation by protein kinase R (PKR) and promote RNA degradation 8, 9. To overcome this, therapeutic mRNA can be modified by replacing nucleotides with analogues that make it less immunogenic and more resistant to degradation (fig. 5). For example, the first mRNA vaccines to be approved, BNT162b2 and mRNA-1273 against SARS-CoV-2, have all uridines replaced by N1-methylpseudouridine, a naturally occurring analogue of uridine present in various types of RNA in eukaryotic cells.

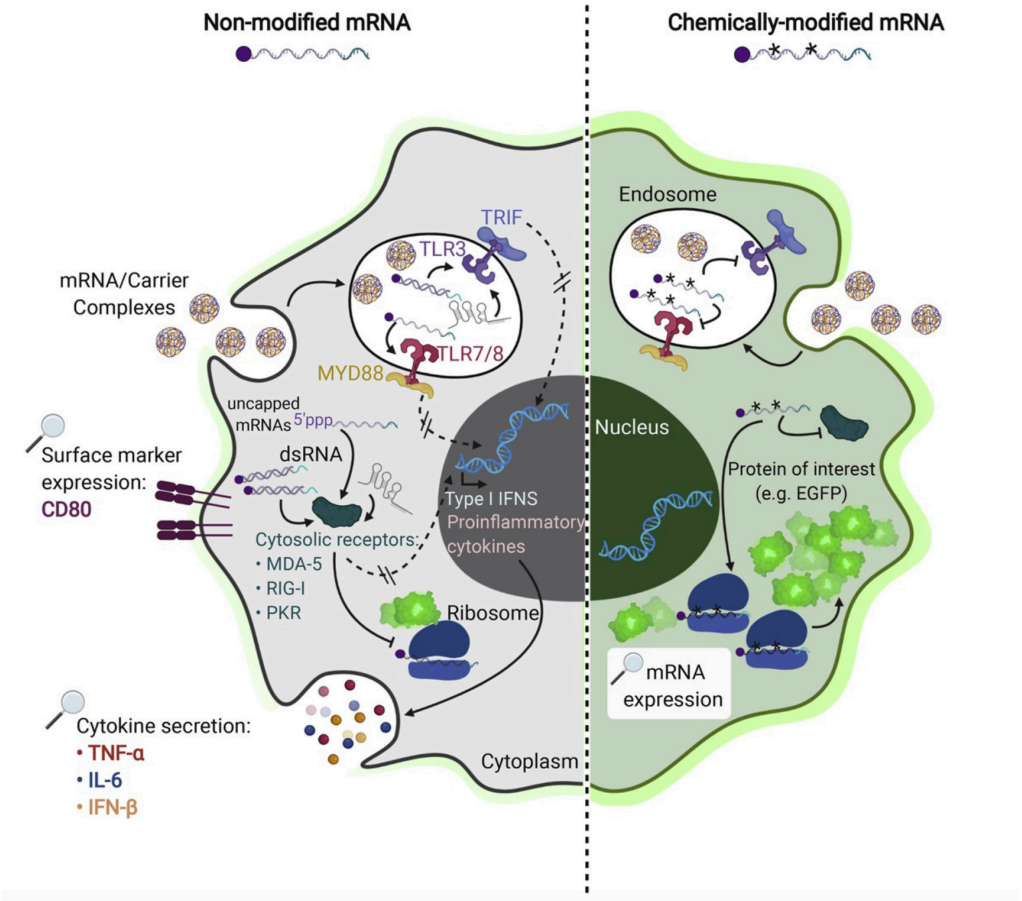


Figure 4. *Intracellular pathways of innate immune response in cells transfected with non-modified (left) versus chemically modified exogenous mRNA (right) (Moradian et al, 20229)*

In addition to reducing the immunogenicity, the replacement of uridine with pseudouridine has been found to stabilize mRNA in-solution 6, 7. Indeed, the RNA linkages 5' of uridine residues are particularly susceptible to degradation, which can be alleviated through the inclusion of uridine analogues.

To both limit immune sensing and enhance protein production, Tebubio proposes codon optimization that includes uridine depletion. Moreover, the remaining U in the mRNA sequence, as well as other nucleotides, can be replaced with modified analogs. While the best modifications depend on your cell type, N1-methylpseudouridine is the most used, as it provides a high protein expression in many cellular systems (fig.6).

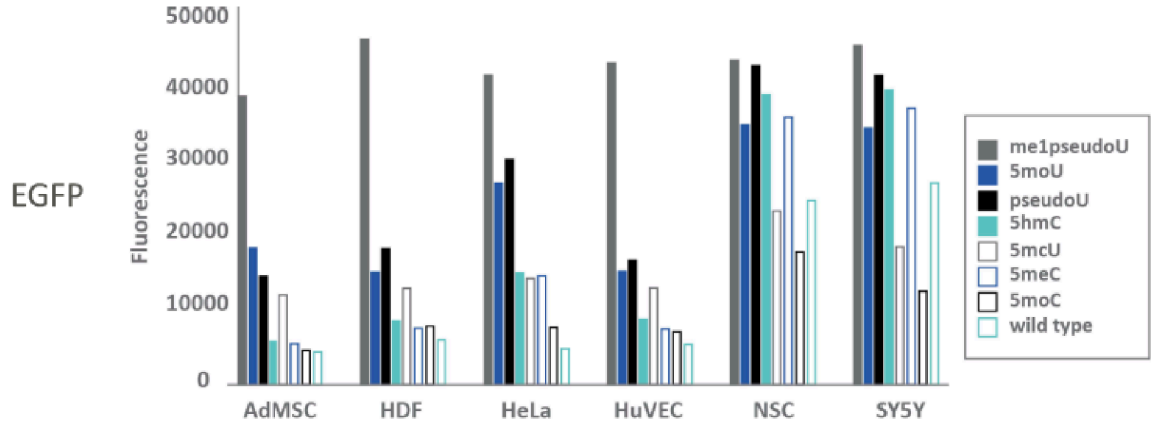


Figure 5. *Comparison of EGFP expression level in 6 cell types, using unmodified or modified mRNA with different nucleotides analogues (TriLink BioTechnologies)*

Self-amplifying RNA - the future of RNA vaccines

saRNA is an mRNA molecule that codes for four additional proteins in addition to the antigen of interest. These four additional proteins are non-structural viral proteins encoding a replicase, which is able to amplify the same saRNA inside cells. As it does not contain viral structural protein, it is unable to produce infectious virus.

Since the time that the mRNA remains functional inside the cells is a major driver of the corresponding protein expression, self-amplifying RNA (saRNA) is regarded as the solution to induce the production of proteins over long periods of time from a single dose of RNA (fig.6). With mRNA vaccines currently on the market, expression of the antigen of interest is proportional to the number of mRNA molecules successfully delivered into the cells. Achieving a sufficient level of expression for protection or immunomodulation may thus require high doses or repeated administrations, as in the case of vaccines against SARS-COVID-19. Vaccines based on saRNA could remedy this limitation, enabling reducing of the dose of RNA to be administered, with consequent benefits in terms of cost and speed of production, as well as a reduction in side effects. In a pandemic context, a saRNA vaccine would allow the production of 10 to 1,000 times more doses compared to an mRNA vaccine (Blakney, Vaccine Strategies, 2021).

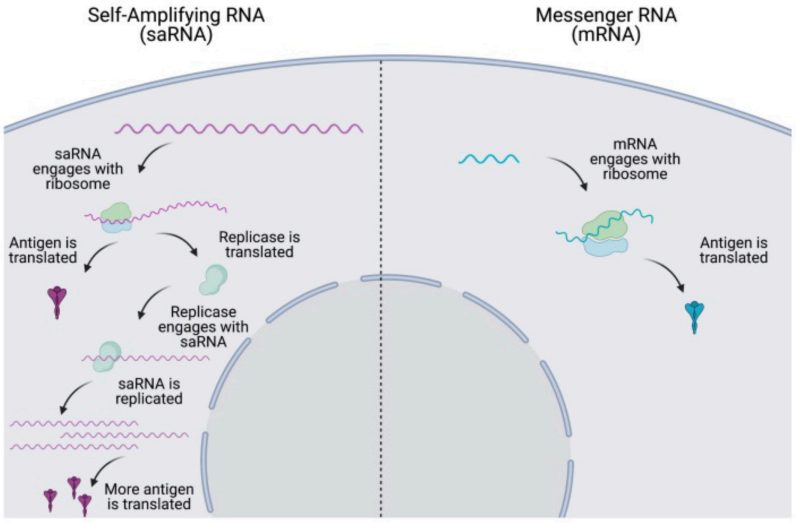


Figure 6. *The difference between mRNA and saRNA (Blakney et al; 202113)*

The saRNAs currently under development are derived from alphaviruses, such as the Venezuelan equine encephalitis virus (VEEV), the Semliki forest virus (SFV) or the Sindbis virus. A first phase I/II clinical trial was carried out in 2020 for a saRNA vaccine against SARS-COVID-19 and others are in development for infectious diseases such as influenza (influenza virus), rabies (rabies virus), malaria (Plasmodium parasite), chlamydia (chlamydia trachomatis bacteria), viruses such as HIV-1, Ebola, RSV and Zika, as well as for oncology applications such as melanoma and colon carcinoma10.

Two different approaches are currently being studied for the generation of saRNA therapeutics:

1 - Use of a single DNA template containing the gene of interest and those of the four replicase-coding proteins (Fig.7B).

2 - Use of 2 DNA templates containing respectively the gene of interest and the four additional proteins (Fig.7C).

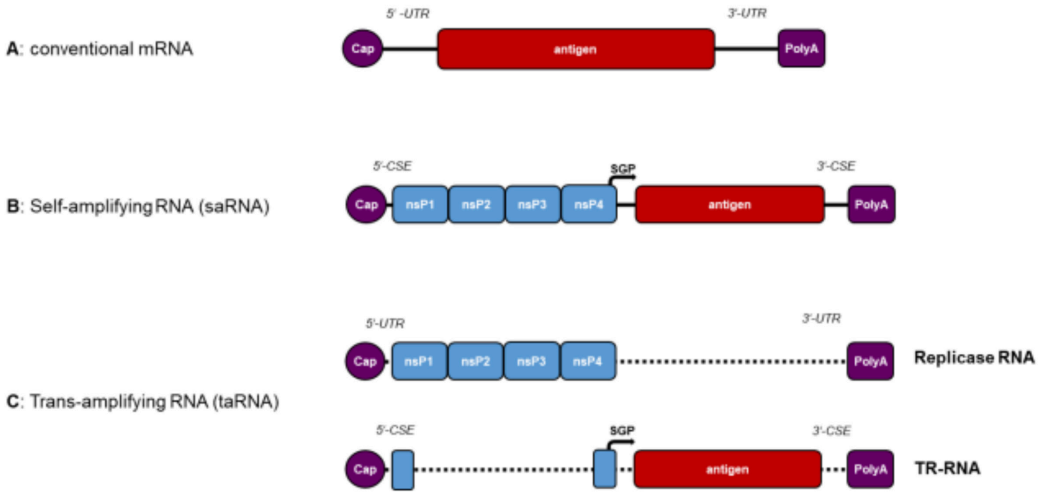


Figure 7. *Figure showing different saRNA approaches vs conventional mRNA (Schmidt et al, 202311)*

A recent study 12 has demonstrated a double interest in the second approach. On one hand, the separation of the system into 2 RNAs simplifies its delivery into the cells, since the saRNA coding for all the proteins is difficult to deliver because of its very large size (~ 10,000 nt). On the other hand, it would make it possible to vary only the DNA matrix coding for the gene of interest without having to modify the replicase matrix. Moreover, the RNA coding for the replicase could be combined with several RNAs coding for different antigens, making it possible to generate multivalent RNA vaccines.

At Tebubio, our project managers are already working on the production of saRNA and on the optimization of the best approach based on researchers needs.

Conclusion

Even if the use of mRNA as a therapeutic solution has proved it efficacy, numerous challenges remain to be overcome in order to improve the efficiency of mRNA in the treatment of many diseases.

At Tebubio, with our mRNA production service, our goal is to facilitate your research by quickly providing optimised research grade sequences (µg production in as little as 5 days), to enable you to validate your PoCs and to advance your research projects as quickly as possible.

To learn more about our mRNA production service and our optimization capacities, we invite you to read our latest Application Note and to visit our webpage dedicated to our small scale mRNA services.

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INNOVATIVE LIPIDS FOR YOUR NUCLEIC ACID DELIVERY RESEARCH

by Kyle Black, Sales and Marketing Manager, Croda Pharma

The history and evolution of Nucleic Acid Delivery systems

mRNA and liposomes were both first discovered in the 1960s, and liposomes were used to deliver mRNA into eukaryotic cells in 1978.[1,2] Even with the ability to inject mRNA into cells, there were several technical challenges to overcome before mRNA could be used as a therapeutic, primarily the rapid degradation of mRNA once injected into the body. In 1990, naked mRNA was injected into the muscles of rats to prove that in vivo direct gene transfer was possible.[3] As early as 1995 the first mRNA vaccine carrying cancer antigens was reported.[4] And more recently, **lipid nanoparticles (LNPs) have been used to deliver mRNA capable of fighting SARS-CoV-2**, as well as CRISPR/Cas-9 gene editing technologies.

Our understanding of mRNA and its delivery has evolved substantially in the past few decades - from naked mRNA delivery to liposomal delivery and then LNPs. We are far from where we started and probably equally as far away, if not farther, from where we are headed.

Not only do delivery systems change, but their individual components are constantly evolving to meet current challenges and demands. To date, LNPs have primarily been formulated using the following components:

- Cationic Lipids - DOTAP, ALC-0315, DLin-MC3-DMA, SM-102
- Phospholipids - DSPC, DOPE
- PEG (Polymer) - Lipid Conjugates - DSPE-PEG2000, DMG-PEG2000, DSG-PEG2000, ALC-0159
- Cholesterol

Recent research has been directed at finding **new, innovative ways to improve encapsulation and transfection efficiency**, and stability, as well as reduce systemic toxicity of LNP therapeutics. Keep reading to see how LNP components are evolving and where LNP technology is headed!

Considerations for future cationic lipids

In LNP formulations, cationic ionizable lipids are used to complex negatively charged mRNA. Aside from playing a major role in the **LNP formulation** itself, cationic lipids also serve a major role in the **biological administration of mRNA to target cells**. The RNA-loaded LNP fuses with the cell membrane and is then delivered into the cytosol. To be able to play these roles efficiently, a cationic ionizable lipid must be engineered with a suitable apparent acid dissociation constant (pKa). The apparent pKa of a cationic ionizable lipid is the likely pKa at the LNP surface. Currently, the cationic ionizable lipids in FDA-approved therapeutics all have an apparent pKa between 6-7. This is crucial for the cationic ionizable lipid to maintain a neutral charge while in systemic circulation (pH above the pKa of the lipid, pH ~7.5), as well as its ability to become positively charged in the endosome (pH ~6.5) and facilitate membrane fusion and subsequent cytosolic release.[5]

As crucial components in LNPs, new cationic ionizable lipids for nucleic acid delivery are being investigated to **optimize mRNA complexation**, endosomal membrane fusion and cargo release into the cytosol.

PEGylated lipids and the future of stealth agents in LNPs

Historically, polyethylene glycol (PEG)-lipids have been the polymer-lipid conjugate of choice for LNP formulations. **PEG-lipids are used to prevent proteins from binding to LNPs** and **increase systemic circulation times**, mediate indirect targeting capabilities of LNPs, promote LNP self-assembly, and control LNP size and stability. The ability of LNPs formulated with PEG-lipids to bypass the reticulo-endothelial clearance system and stay in systemic circulation gives them their nickname - "stealth agents".[5]

As great as PEG-lipids are, they also present a few challenges. These challenges are commonly referred to as the "PEG dilemma":[6]

- Steric hindrance of long PEG chains hinders the ability of a cell to uptake the therapeutic
- PEGylation also hinders endosomal escape of nanoparticles, leading to decreased activity of the delivery system
- Repeated administration of PEGylated systems can result in a phenomenon called accelerated blood clearance (ABC).

Other alternatives to PEG-lipids in LNPs are being explored, and recent work has identified polysarcosine (pSar)-lipids as a promising alternative. pSar-lipids, a polymer-lipid conjugate based on the amino acid sarcosine, have demonstrated similar stealth properties to PEG-lipids. Previous studies have also shown polysarcosine lipids to induce a lower immunogenic response than PEG-lipids when administered to rabbits, zebrafish embryos, and mice. And most recently, pSar-lipids were evaluated in mRNA-LNPs and displayed high RNA transfection ability and an improved safety profile.7 Avanti is now offering pSar-lipids with varying polymeric chain lengths and lipid chain lengths for your PEG-free mRNA delivery research!

Cholesterol and its structural analogs

Another major component of LNPs, making up roughly 35-45% of the final formulation, is cholesterol. **Cholesterol works as a stability enhancer** by filling gaps in the lipid layer and assists in the transfection of RNA. Cholesterol must be present to have an effective lipid-based delivery system but using too much cholesterol can prevent particle formation.

Since cholesterol makes up such a large portion of an LNP formulation, it makes sense that current research efforts would attempt to find more effective alternatives. With so many naturally occurring analogs of cholesterol available, evaluating the substitution of cholesterol with its structural analogs was a great starting point for a collaboration between Moderna and Oregon State University. They evaluated how three groups of steroids, 9,10-secosteroids, C-24 alkyl, and pentacyclic steroids, affected the particle size, mRNA encapsulation efficiency, and transfection efficiency of the resultant LNP formulations.[7]

Of the evaluated cholesterol analogs, β-sitosterol proved to be head and shoulders above the rest. β-sitosterol LNPs had a comparable particle size (diameter ~100 nm) and encapsulation efficiency (~95% using 200 ng mRNA) as the cholesterol control. Where the β-sitosterol-substituted LNPs stood out was their transfection efficiency which was several-fold higher than the cholesterol control. So, comparable size and encapsulation efficiency, coupled with markedly improved transfection efficiency makes β-sitosterol an intriguing alternative to cholesterol in future RNA-LNPs.[8]

LNPs are being evaluated in research labs across the globe as therapeutic delivery systems to diagnose, prevent, or fight a number of diseases. As you encounter new delivery challenges, Croda Pharma will be here to offer innovative lipids to tackle those challenges head on.

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MRNA DOWNSTREAM PROCESS DEVELOPMENT: CHALLENGES AND INSIGHTS

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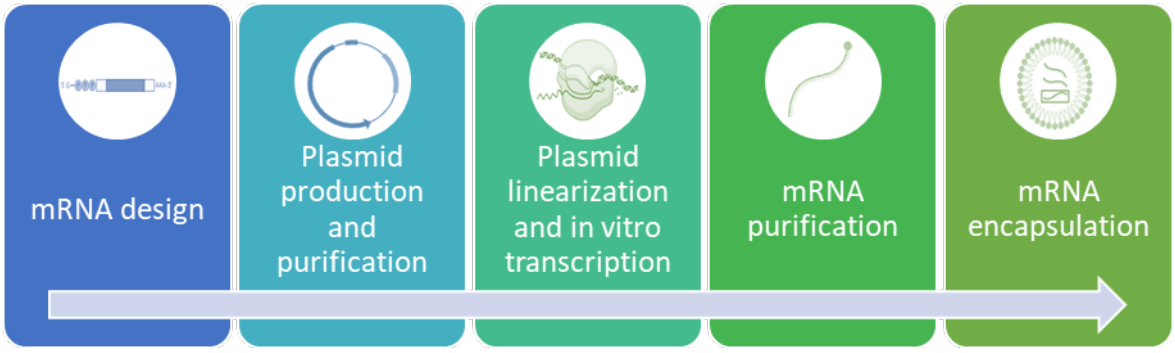
The Covid-19 pandemic in 2020 made it possible to expand the use of mRNA to vaccines and infectious diseases. Moreover, beyond this, new therapeutic strategies based on mRNA in cancer, immune-mediated diseases, and rare diseases are on development.

Currently, the goal is to enhance and optimize the industrial manufacturing process of mRNA, which was created to address a critical demand. Certain steps were inspired by knowledge of molecular biology at the laboratory scale and transposed to the industrial scale, whereas some others, such as the purification of plasmid DNA or mRNA, were inspired by what was known in protein purification processes.

What is the process?



The mRNA therapeutic workflow begins with the DNA template preparation corresponding to the target requirements. The plasmid should then be produced, purified and linearized before in vitro transcription. The resulting mRNA should then be purified before encapsulation in Lipid Nano Particules (LNP).

Focus on the purification steps

The purification steps concern both the plasmid DNA and the mRNA:

- Plasmid DNA must be purified prior to in vitro transcription. Ion exchange or hydrophobic interaction chromatography techniques are currently used.
- The mRNA must be purified before its encapsulation. The techniques used are based on different types on chromatography and tangential filtration.

Unlike the purification of mAbs, there is no consensus process at the moment, and each company has its own specificities, mainly based on its “history” and previous knowledge. The main trend is to apply what is done for protein purification using chromatography beads.

However, nucleic acids differ from proteins in their intrinsic characteristics. mRNA is a negatively charged,

moderately hydrophobic and a fragile molecule. mRNA size is estimated to be around ten times of the protein it codes for. So the hydrodynamic radius around ten times greater leads to a poorer diffusion in chromatographic beads. The size of the pores, of the order of a hundred nm, seems to exclude large molecules such as mRNA or plasmid DNA. They will only be able to reach a fraction of the binding sites in the pores of most resins. It is therefore appropriate to question the relevance of the use of this type of bead supports for the purification of nucleic acids. Supports like membrane or monolith have a geometry which appears, a priori, to be more suitable. The pores, of the order of a µm, are accessible to large molecules. Another useful feature is that the flow through the bed is predominantly laminar. This offers milder conditions for molecules, such as mRNA, which are particularly sensitive to shear forces.

Our research activities

Our team aims to explore different types of supports (resins, monoliths, membranes) and to compare their performance with respect to nucleic acids (pDNA and mRNA). Different chromatography techniques are tested with particular attention to mixed mode chromatography. Mixed mode chromatography is a powerful technique with a high selectivity for adsorption and elution under different conditions. This allows a wide variety of applications and reduces the number of steps in a purification process. The development is more complex with mixed mode chromatography resins than traditional resins. Different ligands and multiple binding and elution conditions must be screened. Each manufacturer provides its own mixed mode chromatography ligand with a particular structure. Moreover, the interactions involved are complex and different conditions must be tested.

Column experimentation involves “One Factor At a Time” (OFAT) based on a very time-consuming trial/error approach. Our approach is based on high throughput screening in microplates. It allows a large number of parameters to be tested simultaneously. The development time is reduced and a very large amount of information is obtained. Microplates or miniaturized columns are the tool of choice for high throughput screening. Recently multi-well plate with Convective Interaction Media (CIM) monolith technology has been developed.

The cost and efficiency of the process is decisive for the accessibility of therapies to the greatest number. DSP optimization is especially crucial for the rise of mRNA vaccines and therapies.

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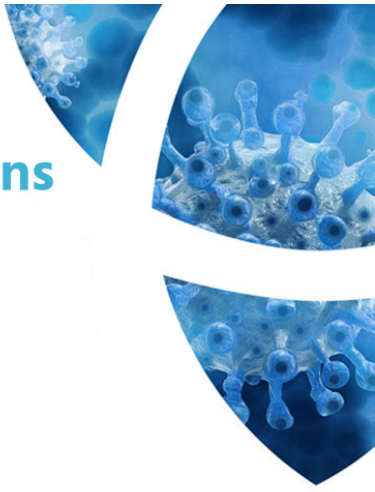

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