LIPID NANOPARTICLES (LNPs)

Guidelines

OZ BIOSCIENCES
The art of delivery systems

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RNA THERAPY WILL CHANGE OUR FUTURE

A plethora of essential breakthroughs has been leading to the implementation of the **RNA therapy field**. Resulting from tens year of research and development, RNA therapeutics hold nowadays strong potential for treating heretofore uncurable diseases through **protein-replacement**, **immunomodulation** and/or **gene editing**. Indeed, the current pandemic unveiled the power of RNA medicine. RNA-based therapies are suitable for a large palette of conditions, ranging from neurodegenerative disorders to infectious diseases and anti-cancer therapies

THE CHALLENGE OF EFFICIENT GENE DELIVERY

The RNA therapeutics have the potential to revolutionize medicinal fields such as **vaccination**, **immuno-oncology**, **chronic** and **genetic diseases' treatment**, because it is safe, easy to reproduce and offers a great deal of versatility. However, naked mRNA is unstable, poorly uptaken by cells due to its negative charges and rapidly degraded into the organism. As a direct consequence, delivery systems are a necessity to efficiently deliver mRNA for in vivo applications. Non-viral gene delivery systems are an efficient approach to deliver nucleic acid payloads (DNA and RNA) into the cells of interest with the aim of a **temporary augmentation** or **knock-down of protein expression**. For this specific purpose, **OZ Biosciences** (**OZB**) provides portfolio and custom **lipid nanoparticles** (**LNPs**) as gene delivery systems. To date, formulation in LNPs represents the most advanced delivery platform for gene therapy and promising candidates to treat manifold diseases.
In addition, LNPs were FDA approved for the treatment of amyloidosis disease by delivery of siRNA (Onpattro™), and more recenly for the widely distributed SARS-CoV-2 vaccines (Comirnaty™ & SpikeVax®), based on mRNA-LNPs.

OZ Biosciences can support every stage of your mRNA-LNP production, from mRNA synthesis to LNP formulation development, manufacturing and fill & finish.

For any of RNA, DNA or APIs encapsulation, you can provide us with your molecule of interest and we will formulateit into LNPs.

WHAT ARE LIPID NANOPARTICLES?

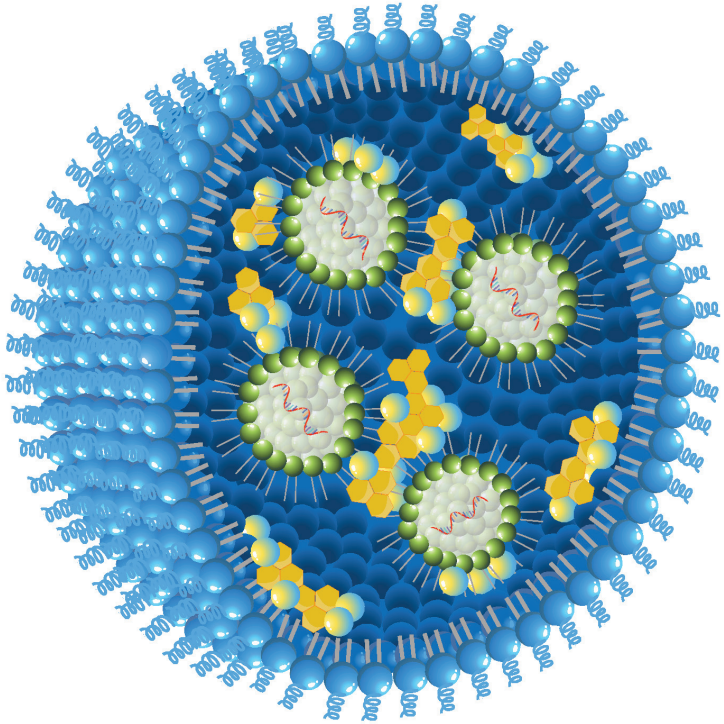
LNPs are liposome-like structures, specially geared towards encapsulating a broad variety of nucleic acids (both, mRNA, miRNA, siRNA, gRNA, lncRNA, circRNA and DNA) or active pharmaceutical ingredients (APIs, e.g. small molecules, proteins); a LNP consists in an **aqueous core surrounded by a lipidic shell based on a combination of different compounds**, each having its own role (**Figure 1**). Generally, LNPs are composed of **four families of chemicals**: a complexing aminated lipid (e.g. ionizable cationic), an helper phospholipid (DSPC, DOPC or DOPE), cholesterol and a pegylated (PEG) lipid at **defined ratio** to potentiate the nucleic acid/APIs activity.

1. **Complexing lipids** are usually comprising a ionizable/positively-charged head group, an hydrocarbon chain or cholesterol derivate, attached via a linker (e.g. glycerol). The charged head of the lipid would interact by electrostatic bindings with anionic nucleic acids to allow their entrapment into LNPs during their formulation. In addition, these lipids can mediate interactions between LNPs and the cellular plasma, and facilitate cell uptake and endosomal release of the cargo. New series of ionizable lipids have highly been developed to potentiate RNA delivery in vivo. Ionizable lipids expose a negative-neutral surface charge under physiological pH (7.4), thereby preventing serum protein opsonization. The pH in the endosome goes acidic, below the pKa of the lipid, inducing protonation and thus the LNPs destabilisation associated with the nucleic acid release.

2. **Helper lipids** such as phospholipids favour colloidal stability and gene delivery efficiency meanwhile increasing systemic circulation of the NPs. 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).

3. **Cholesterol** is commonly used in liposomes and LNPs. It helps stabilizing the lipidic shell and promotes membrane fusion.

4. **PEGylated lipid** into LNPs increases their colloidal stability, resistance to opsonization and reticuloendothelial clearance. Pegylation of the nanoparticles results in long-term biodistribution, better tumour penetration and accumulation by leading to neutral surface charge and by reducing the size of LNPs.



Nucleic acid
DNA, RNA (siRNA, mRNA, saRNA)
Small molecules

Cholesterol
Rigidity and integrity
Help the endosomal release

PEG-lipid
Stealth & increase bio-circulation

Complexing Lipid
Nucleic acid complexation
Membrane fusion & endosomal escape

Helper Lipid
Stability & structure

Figure 1. Schematic representation of lipid nanoparticles (LNPs) composed by a mixture of four chemical compounds: complexing lipid, helper phospholipid, cholesterol and stealth-lipid each with their own specific function and at defined ratio to potentiate nucleic acid activity.

In the last 20 years, OZ Bioscience has developed strong expertise in aminated lipids, which allowed the screening of several dozens formulations in order to develop optimized OZB LNPs referred as **NanOZ-LNPs**.

NanOZ-LNPs have been designed as safe and advanced nanomaterials to potentiate nucleic acids/APIs activity through their effective encapsulation and delivery of payload to specific cell types and tissues.

HOW LIPID NANOPARTICLES ARE PRODUCED ?

Different synthetic methods for RNA-loaded LNPs have been adopted to generate efficient drug carriers. Classical preparation involve a traditional rehydration of a lipidic thin-film with an aqueous buffer containing the RNA drugs. 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. Therefore, **NanOZ-LNPs** are mainly formed throughout our microfluidics technology platform. The microfluidics system, based on a **pressure-driven flow control**, provides tools to manipulate liquids, droplets, cell and particles within micro-channel geometries. To form well-structured lipid nanosystems, typically a stream of **lipid in alcohol solution** is flown by fast mixing against an anti-solvent containing active ingredients (e.g. RNA in an acidic aqueous phase) at the junction of the microfluidic chip (**Figure 2**). The API-loaded LNPs self-assemble at the intersection of the chip by nucleation-growth process before being collected at the output of the system. T**he processing has the advantage to directly encapsulate the molecule of interest into the NP and to quickly form LNPs in various volume range, from hundred µL to several tens of mL**. Once collected, alcohol solvent and free molecules are removed from LNPs by dialysis or tangential flow filtration (TFF). These processes stabilize the LNP structure and adjust the pH to neutral, which is required for further in vitro and in vivo applications. LNPs can be sterilized by filtration and are generally stored at -80°C with the addition of cryoprotectant.

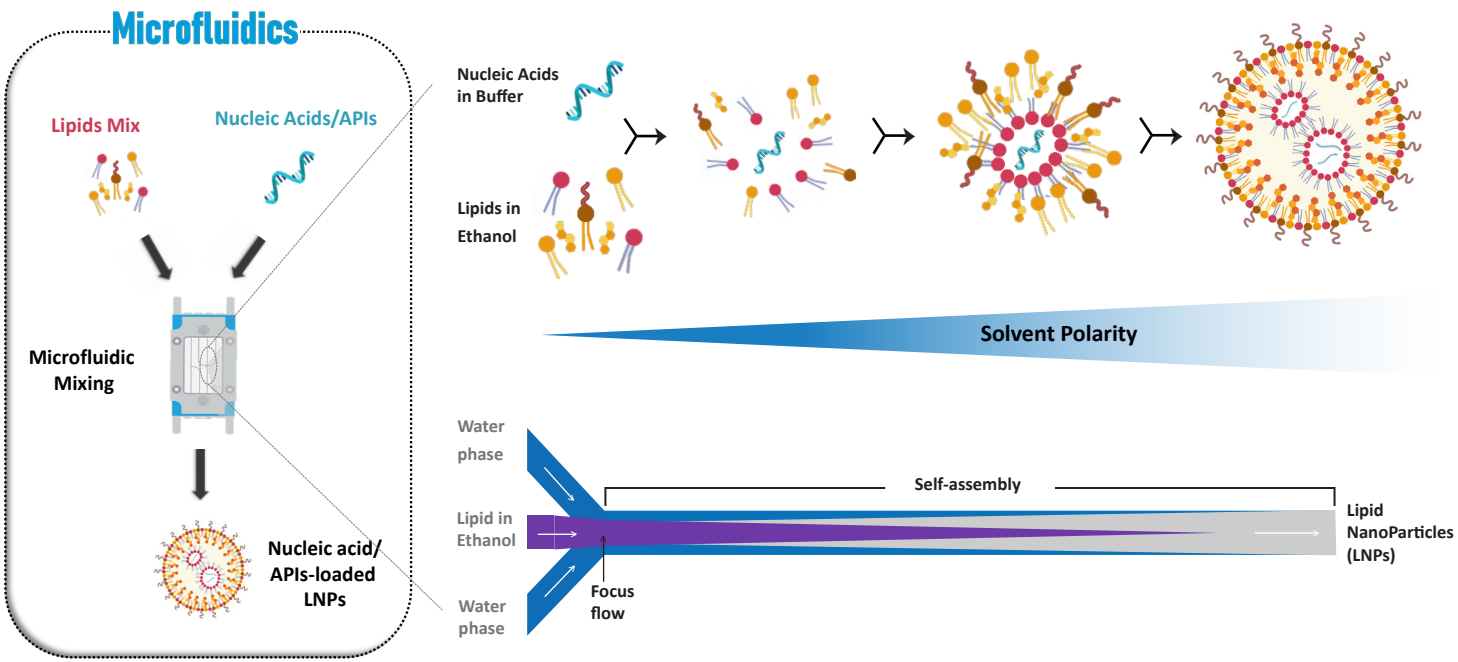


Figure 2. LNPs manufacturing with precise control and high reproducibility based on fast-mixing microfluidics technology

- LNPs are formed through nucleation/growth process
- The particule size can be tuned by modulating TFR and FRR
- API can be brought by continous (aqueous) phase or inner (lipophilic) phase

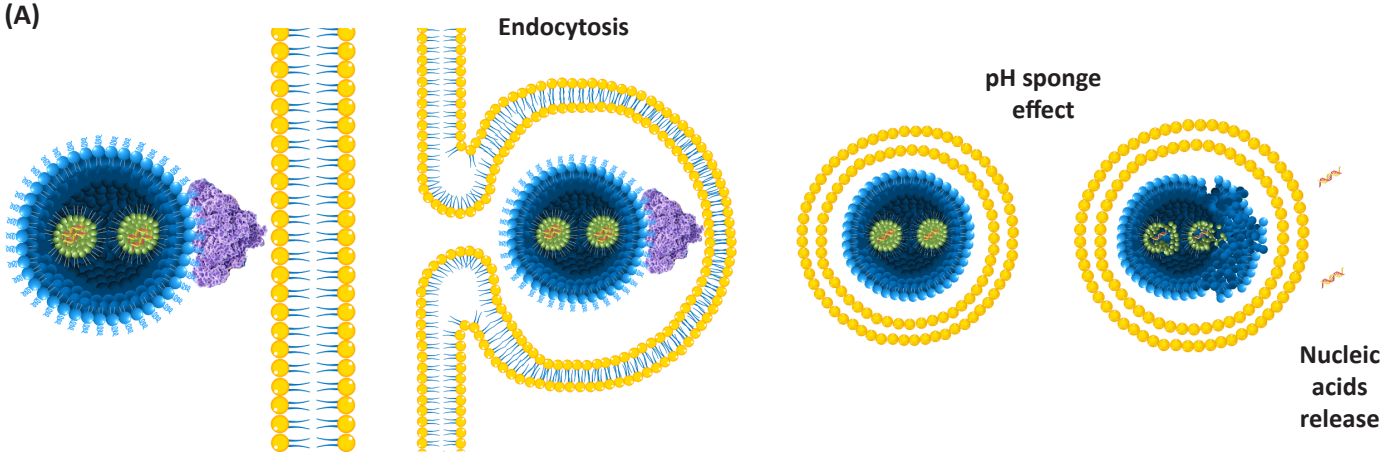
Microfluidics brings the possibility to work using a **wide range of nucleic acids** (e.g. mRNA, siRNA, saRNA, DNA) and to rapidly screen a large library of lipids in order to optimize the formulation efficacy for gene delivery. Once the LNP formulation optimized, microfluidic technology provides the huge benefit to easily reproduce and scale-up the manufacturing of the delivery systems. Given all these aspects, microfluidics technology offers **unprecedented precision** and **control** over the production of nanoparticles.

HOW DOES IT WORKS ?

LNPs are lipid-based drug delivery systems that carry APIs payload within their inner core (**Figure 3**). For nucleic acids, the ionizable cationic lipids sequester the genetic material mainly through **electrostatic interactions**. Consequently, nucleic acids are **protected from nuclease degradation**.

Once administrated into the organism, LNPs allow the delivery of payload within the cell mainly following endocytosis phenomenon. Indeed, in the endosomes, LNPs' integrity is destabilized by "**pH sponge effect**", leading to the release of nucleic acids into the cytosol. Finally, the cellular machinery enables to translate the delivered genetic material into a cellular response. LNPs are applied in numerous medicinal application through protein-replacement, immunomodulation and/or gene editing.

(A)



APPLICATIONS

(B)

Immunization
i.m. or s.c.
Immune response
RNA: OVA, Spike…

Biodistribution
i.v. or i.p.
Fluo/Bioluminescences
RNA: GFP, mCherry or Luc

Gene Editing
parenteral
Treat Disease
RNA: Cas9, Cre, other

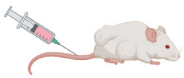


Figure 3. **(A)** Simplified schematic representation of cellular uptake and intracellular trafficking of LNP encapsulated mRNA **(B)** Lipid Nanoparticles after injection in mouse for different R&D applications with preferential mode of administration (including intramuscular (i.m.), subcutaneous (s.c.), intraperitoneal (i.p.) or parenteral) and associated mRNA.

RESULTS

Our resulting **NanOZ-LNPs** are characterized by different analytical techniques including **dynamic light scattering** (DLS) to monitor their hydrodynamic size, polydispersity index (PdI) and zeta potential and **electronic microscopies** (cryoTEM, negative stain EM) to evaluate their morphologies (**Figure 4**). The nucleic acid entrapment rate is determined by DNA- or RNA-**quantitation kit assay** and proved to be very high (typically ≥90%). Meanwhile, the use of tagged-RNA or reporter genes enables to easily monitor the nucleic acids delivery in tissues and/or organs. In addition, **the biodistribution** of the LNP itself can be directly observed by using fluorescent lipid incorporation (e.g. Lissamine Rhodamine B) into the lipidic formulation. Finally, sterility is assessed using Thioglycolate and Caso assays for at least 7 days. A certifi-

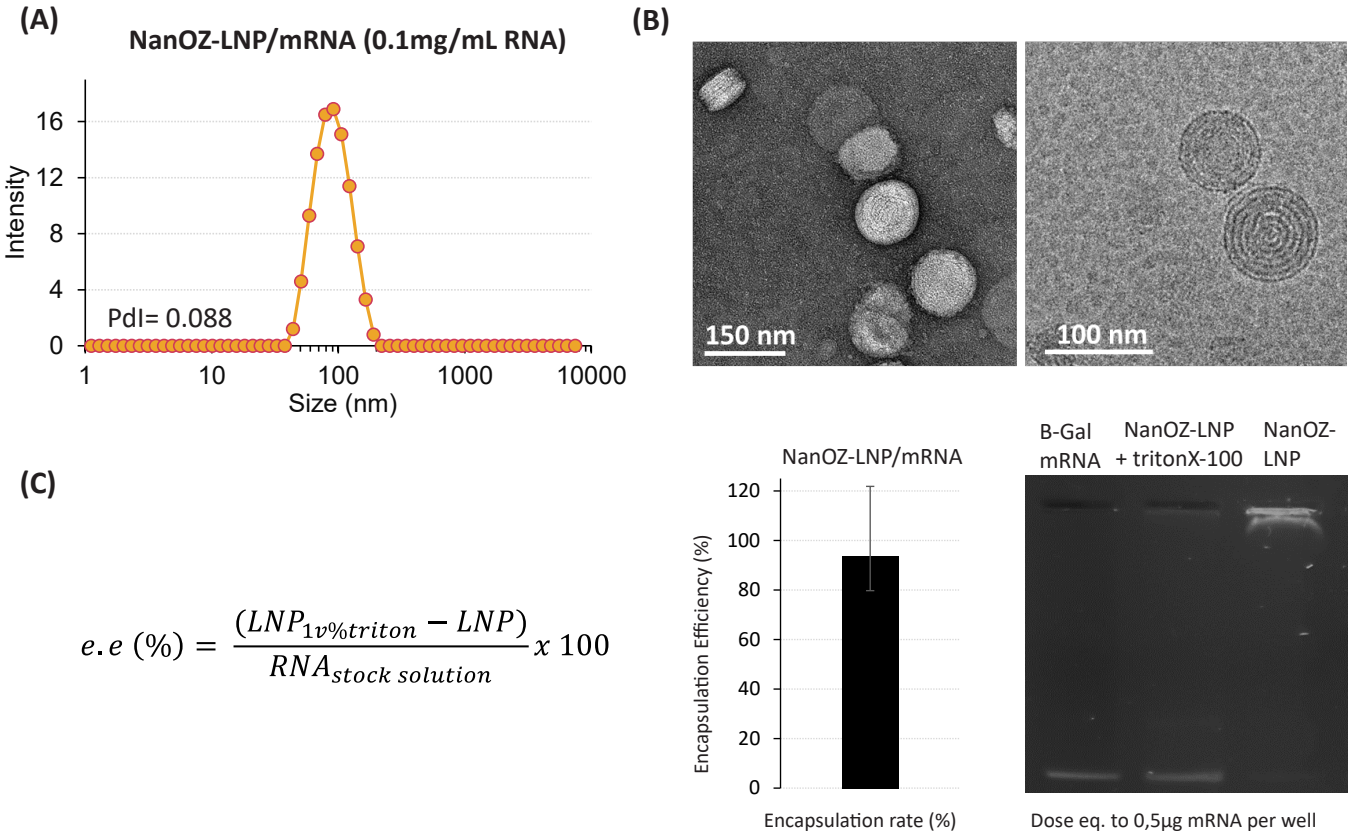


Figure 4. (A) Dynamic Light Scattering (DLS), and **(B)** negative stain and Cryo-TEM micrographs of around 85nm mRNA-loaded LNPs. **(C)** Encapsulation efficiency (e.e.) of NanOZ-LNP encapsulated β-Gal mRNA determined by Sybr-Gold® and gel electrophoresis (at 0.5µg mRNA dose). Samples that have or not been destabilized by surfactant (± 1v% TritonX-100) reveal the integrity of the mRNA payload.

The successful entrapment and delivery of RNA using LNPs relies on the RNA itself (length, format and chemical modifications…) as well as the nanoformulation.

As an illustration, our group generated **NanOZ-LNPs** loaded with Firefly Luciferase mRNA (0.10µg/µL) and evaluated the NP delivery system in a murine model. To monitor the delivery efficacy of our novel NanOZ-LNP, the formulation was evaluated in comparison with classical formulations based on either DLinMC3DMA or DOTAP complexing lipids as well as free mRNA. The Luc-RNA localization and expression was monitored by IVIS® imagery system (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 wild type mice. As observed, a single intra-peritoneal (i.p.) injection of Luc-LNPs at 10µg mRNA dose enables high bioluminescence signal and luciferase expression into surrounded organs for at least 25h (**Figure 5**).

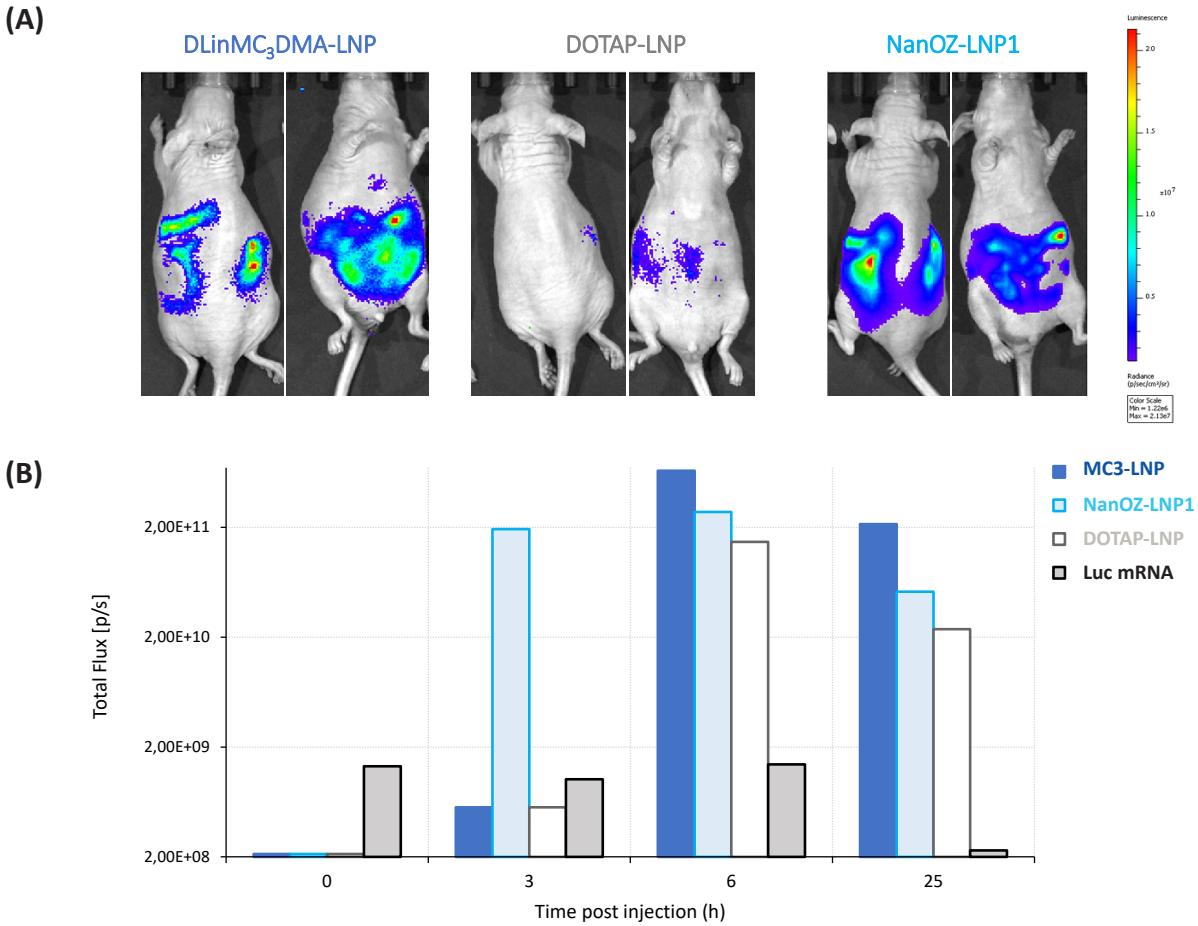


Figure 5. (A) IVIS imaging of the bioluminescence signal 3h after intra-peritoneal administration of Luc-mRNA-LNPs in nude mice (dose equivalent to 10µg RNA) of 3 differents LNPs: DLinMC3DMA-, NanOZ- and DOTAP-based LNPs compared to free Firefly Luciferase mRNA used as control. **(B)** The corresponding bioluminescence intensity kinetics over a period of time of 25h. Interestingly, microfluidics brings the possibility to work with a wide range of nucleic acids (e.g., DNA, ssDNA, mRNA, saRNA, siRNA) and to rapidly screen a large library of lipids in order to optimize the formulation efficacy for nucleic acids delivery.

OUR LIPID NANOPARTICLES CUSTOM SERVICE

Our company has developed a LNP platform using a highly performant pressure-driven flow microfluidic technology to screen our **patented library of cationic and ionizable amino acid-based lipids**. We aim to design safe and efficient nanomaterials able to potentiate nucleic acids activity. We design, develop and manufacture **custom-based LNP formulations** to pharmaceuticals and biotechnology institutes engaged in gene therapeutics with several advantages:

- High loading efficiency (>90%)
- Concentration and Protection of the cargo from degradation
- High permeability through the membranes and release within the cell
- Most Advanced Nucleic acid delivery systems for Gene Therapy
- Flexible production scale from microliter to tens mililiters
- Custom-tailored service & support to meet specific application or project needs
- Competitive & affordable prices.

Furthermore, our company is currently developing, in collaboration with several partners, **efficient RNA-LNP carriers** for **cancer**, **regenerative medicine** and **vaccine applications**. Therefore, we offer the opportunity to evaluate RNA therapeutics through reproducible nanoparticle production and thus minimizing the use of APIs, which are usually very expensive and scarce. Notably, formulation parameters including nature of the cationic/ionizable lipid, N/P ratio, PEG-lipid amount and mRNA type and length were independently examined for their effect on nanoparticle attributes. We believe that screening and subsequent optimization of complex RNA-LNPs therapeutics would be essential to streamline future design of nanomedicines.

OUR SERVICES INCLUDE

- Design of LNPs suitable for your biological model and their formulation process using microfluidics for highly monodisperse nanoparticles and reproductible method.
- Complete physico-chemical characterization including: size distribution, polydispersity index, zeta potential, nucleic acid encapsulation efficiency (RNA kit assay) and concentration, sterility…. Other specification can be provided upon request (e.g. CryoTEM, HPLC, LCMS, RMN…)
- Downstream processing and quality control; All our LNPs are checked for their quality.

After defining the formulation and processing, **OZ Biosciences** provides a detailed quotation and deadline for the desired LNP's manufacturing. Usually, 2-3 weeks if RNA is provided or at our catalogue or 2-3 months for LNPs prepared with custom RNA from **OZ Biosciences**.

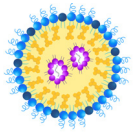
Our expert product support team is at your service for your special requests- you are invited to reach out to **tech@ozbiosciences.com**.

OUR CUSTOM SERVICES

mRNA Synthesis

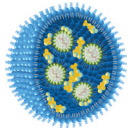
- Gene synthesis, Cloning & DNA template production.
- *In vitro* Transcription.
- Purification & Quality control.

NanOZ-LNP™ Design Platform



- Lipid Chemistry & Functionalization.
- Formulation Design & Manufactring.
- NanOZ-LNPs™ Custom.

Customer DNA, RNA, API



- Provide us with your molecule of interest and we will formulate it into LNPs

BIOMEDICAL APPLICATIONS

Europe / Asia OFFICE
OZ Biosciences SAS
Parc Scientifique de Luminy, zone entreprise, case 922, 13288 Marseille Cedex 09, FRANCE
Ph : +33 486 94 85 16
Fax : +33 486 94 85 16
E-mail: contact@ozbiosciences.com

OZ BIOSCIENCES
The art of delivery systems

US OFFICE
OZ Biosciences Inc.
4901 Morena Boulevard, Suite 901
San Diego, CA 92117,
USA
Ph : +1 858 246 7840
Fax : +1 855 631 0626
E-mail : contactusa@ozbiosciences.com