

Development of a Novel Lipopolyplex Platform for Dual mRNA Delivery by Core- and Surface- Loading in the Context of Mucosal Vaccination

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Summary

The recent success of mRNA-based vaccines has stimulated research into their potential use across diverse medical applications. However, as the field is still in its infancy, several limitations of lipid nanoparticles (LNPs), the current gold standard of mRNA delivery, need to be overcome.

This thesis describes the development of a novel lipopolyplex platform to address key challenges of LNPs for mRNA delivery. By combining cationic and anionic polymers with cationic lipids, the core-shell nanoparticle supports independent dual nucleotide loading while achieving improved robustness during pharmaceutical processing. This design expands the potential use of mRNA therapeutics to new fields of applications, including inhalation-based medicine.

As was found by the research described in this thesis, the localization of mRNA within the particle is critical: surface-loaded mRNA exhibited stronger expression compared to core-loaded mRNA, while expression kinetics were unaffected. Importantly, the LPPs demonstrated efficient co-delivery of multiple mRNAs to the same cell, which is particularly relevant for novel technologies such as CRISPR/Cas9.

In this context, the transmembrane receptor CCR7 and chemokine CCL21 were explored as endogenous adjuvants for mucosal vaccination, demonstrating their promise as modulators of immune responses. Finally, the feasibility of LPPs with an oral delivery technology was investigated, showing successful formulation within polymer matrices and subsequent in vitro transfection.

Zusammenfassung

Der Erfolg mRNA-basierter Impfstoffe zeigt das große Potenzial dieser Technologie in verschiedenen medizinischen Gebieten. Da sich die Technologie noch in ihren Kinderschuhen befindet, müssen Einschränkungen von Lipidnanopartikeln (LNPs), dem derzeitigen Goldstandard für mRNA-Transport, überwunden werden.

Diese Arbeit beschreibt die Entwicklung einer neuen Lipopolyplex-Plattform, um zentrale Nachteile von LNPs zu adressieren. Durch die Kombination kationischer und anionischer Polymere mit kationischen Lipiden entsteht eine „Kern-Hülle“-Nanostruktur, die eine unabhängige, doppelte Nukleotid-Beladung ermöglicht und verbesserte Stabilität während pharmazeutischer Prozesse aufweist. Das erweitert die Einsatzmöglichkeiten von mRNA-Therapeutika zu neuen Anwendungsfeldern, einschließlich Inhalationstherapie.

Unsere Untersuchungen zeigten, dass die Lokalisation der mRNA im Partikel entscheidend ist. Oberflächen-beladene mRNA erzielte stärkere Expression als Kern-beladene, bei unveränderter Expressionskinetik. Zudem gelang der Co-Transport von zwei mRNAs in dieselbe Zelle, besonders relevant für neue Technologien wie CRISPR/Cas9.

Weiterhin wurden der Transmembran-Rezeptor CCR7 und das Chemokin CCL21 als potenzielle endogene Adjuvantien für Schleimhaut-Impfungen untersucht und zeigten vielversprechende immunomodulatorische Eigenschaften. Schließlich wurde die Eignung der Kombination von LPPs mit einer oralen Applikationstechnologie demonstriert, wobei die Formulierung in Polymermatrices die anschließende *in vitro*-Transfektion nicht negativ beeinflusste.

List of Abbreviations

AMR	Antimicrobial resistance
APC	Antigen-presenting cell
bPEI	Branched poly(ethyleneimine)
CARPA	Complement activation-related pseudoallergy
CCL21	C-C chemokine ligand 21
CCR7	C-C chemokine receptor type 7
Chit	Chitosan
circRNA	Circular RNA
CLSM	Confocal laser scanning microscopy
cryo-TEM	Cryogenic transmission electron microscopy
CTL	Cytotoxic T cell
D-Lin-MC3-DMA	[(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate]
DC	Dendritic cell
DC-Cholesterol	3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
DLS	Dynamic light scattering
DMG-PEG	1,2-dimyristoyl-rac-glycero-3-methoxypoly(ethylene glycol)
DNA	Deoxyribonucleic acid
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	1,2-di-O-octadecenyl-3-trimethylammonium-propane
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)]

EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration
GDP	Gross domestic product
GIT	Gastrointestinal tract
Hya	Hyaluronic acid
ID	Intradermal
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IM	Intramuscular
IP	Intellectual property
i.v.	Intravenous
kDa	Kilodalton
LAV	Live attenuated virus
LNP	Lipid nanoparticle
IPEI	Linear poly(ethyleneimine)
LPH	Lipid–polymer hybrid particle
LPP	Lipopolyplex
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MWCO	Molecular weight cut-off
NMR	Nuclear magnetic resonance

PBS	Phosphate-buffered saline
PDI	Polydispersity index
pDMAEMA	Poly(2-(dimethylamino)ethyl methacrylate)
pDNA	Plasmid DNA
PE	Phycoerythrin
PEEP	Poly(ethyl ethylene phosphate)
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PLA	Poly-L-arginine
PLGA	Poly(lactic-co-glycolic acid)
PLL	Poly-L-lysine
polyA	Polyadenylic acid
poGA	Poly-L-glutamic acid
Prot	Protamine
P/S	Penicillin/Streptomycin
RNA	Ribonucleic acid
RT	Room temperature
saRNA	Self-amplifying RNA
SC	Subcutaneous
slgA	Secretory immunoglobulin A
S-NHS	N-hydroxysulfosuccinimide
Th	T helper cell
TPP	Triphosphate
U.S.	United States
UTR	Untranslated region
WHO	World Health Organization

1 General Introduction

1.1 Infectious Diseases

Infectious diseases have long posed significant threats to human and animal health. Despite substantial advances in medical science and improvements in the prevention and treatment of infections, these diseases continue to impose a considerable global burden. In the United States (U.S.), for instance, infectious diseases accounted for the third-largest share of healthcare expenditures in 2021, following circulatory and musculoskeletal conditions. They represented approximately 9% of total spending on disease treatment (Peterson-KFF Health System Tracker 2025). However, beyond their impact on global economics, their effects on patient health, quality of life, and mortality remain critical concerns for public health.

In ancient times to the 15th century, western medicine had limited understanding of infectious diseases and lacked effective means to prevent or treat them. This is evidenced by devastating pandemics such as the Antonine Plague (believed to have been caused by smallpox) and the Black Death in the 14th century, which was caused by *Yersinia pestis* and led to the death of up to 30-50% of Europe's population (Sakai and Morimoto 2022; Olea and Christakos 2005). Medical knowledge gradually expanded between the 16th and 18th centuries and eventually the first vaccination against smallpox was developed in 1796. However, it was not until the late 19th century that sterilization, e.g. to prevent surgical infections, was developed (Sakai and Morimoto 2022). A breakthrough in treating bacterial infections was achieved in the early 20th century with the discovery and clinical development of penicillin. Between 1928 and 1941, Alexander Fleming, Howard Florey, and Ernst Chain successfully isolated and purified penicillin from *Penicillium notatum*, a discovery that revolutionized the treatment of bacterial infections and pathed the way for modern medicine (Lobanovska and Pilla 2017).

These advances led to a marked shift in public health, changing the leading causes of death dramatically over the 20th century. In 1900, infectious diseases affecting the lungs and gastrointestinal tract (GIT) were the predominant causes of mortality. By 1997, chronic conditions such as heart disease, cancer, and stroke had become the

leading causes, with only pneumonia and influenza remaining among the top ten, accounting for 3.7% of all deaths (Cohen 2000).

However, proving virologist Sir McFarland Burnett wrong, who famously claimed that “[b]y the end of the Second World War it was possible to say that almost all of the major practical problems of dealing with infectious disease had been solved” in 1962 (Eccleston-Turner and Brassington 2020), new challenges in infectious disease management have continued to emerge in the following decades. Although antimicrobial resistance (AMR) has existed since the onset of the “golden era” of antibiotic discovery in the 1940s to 1960s, it took considerably longer for it to be recognized as a critical global public health threat, earning it the name “the silent pandemic” (Aghamohammad and Rohani 2023; Mahoney et al. 2021). It was not until 2011 that the World Health Organization (WHO) shifted from passive reporting to actively proposing global frameworks for surveillance and stewardship. This shift led to the launch of the WHO Global Action Plan on AMR in 2015 (Kirchhelle and Podolsky 2022), addressing the increasing resistance to critically important antibiotics and the rising number of AMR-attributable deaths among individuals aged five and older between 1990 and 2019 (Naghavi et al. 2024). At the same time, the development pipeline for new antibiotics has been steadily declining since the 1980s, primarily due to limited financial incentives and poor returns in the pharmaceutical sector (Piddock et al. 2024). These combined trends underscore the increasing difficulty of managing infectious diseases and highlight the urgent need for alternative strategies. One of the most effective preventive measures remains vaccination (Rémy et al. 2015).

1.2 Vaccination

Vaccination provides “immunity to a disease by the administration of a preparation of antigenic material (vaccine) to stimulate the formation of appropriate antibodies” (Oxford University Press). As previously mentioned, the first successful vaccine was developed in 1796 against smallpox, a severe infectious disease caused by the variola virus. Edward Jenner inoculated an eight-year-old boy, James Phipps, using pustular material from cowpox lesions on a milker’s hand. Six weeks later, Jenner exposed Phipps to material from a smallpox lesion, and the boy did not develop the disease due to the acquired cross-protection by prior infection with the related orthopoxvirus (Damaso 2018).

This pioneering vaccination laid the foundation for the eradication of the disease, that was declared by the WHO in 1980 (Moore et al. 2006). Today, Jenner's strategy of infecting patients using live, unattenuated viruses is luckily abandoned and has been replaced by safer methods. Modern vaccines achieve protective immunity by application of live attenuated, inactivated, subunit or mRNA-based platforms, designed to minimize risk while effectively stimulating the immune response.

1.2.1 Types of Vaccines

1.2.1.1 Live attenuated vaccines

Live attenuated viruses (LAVs) demonstrated remarkable success in preventing viral infections, with established vaccines against diseases such as smallpox, polio, and measles serving as prominent examples. Traditionally, attenuation has been achieved empirically through serial passaging in non-native cell types, a process that promotes the accumulation of mutations associated with reduced virulence. However, this approach is inherently unpredictable and raises safety concerns due to the potential for reversion to pathogenic forms.

In response, more rational attenuation strategies have been developed. One such approach involves the deletion or mutation of genes essential for viral replication. These viruses can be propagated in helper cell lines that express the missing viral proteins, enabling vaccine production, while the administered virus remains replication-incompetent in the host. Alternatively, deletion of genes required for viral assembly or release results in single-cycle viruses, which can replicate their genomes but cannot generate infectious virus particles. Although these strategies offer promising immunogenicity, they still present safety concerns, particularly due to the high mutation rates characteristic of RNA viruses, which increase the risk of reversion to virulence (Lauring et al. 2010).

Alternatively, viral genes encoding the antigens of interest can be delivered in attenuated viral vectors. This method eliminates the risk of reversion of the pathogen itself but may face limitations due to the development of neutralizing antibodies against the vector upon repeated dosing (McCann et al. 2022). More recent approaches apply codon deoptimization, microRNA- or zinc finger protein-controlled viruses (Tang et al. 2025).

Complementary to LAVs, several vaccines based on live attenuated bacteria, e.g. against tuberculosis and typhoid, have been licensed. They are typically constructed by random chemical mutagenesis resulting in deficiency of crucial virulence factors (Ohtake et al. 2011). While those vaccines bring a very high immunostimulatory capacity resulting in long-term host immunity, they also carry critical safety risks, including the persistence of genetically modified organisms in the host (Ding et al. 2018).

1.2.1.2 Inactivated vaccines

Inactivated vaccines are produced by propagating viruses in substrates such as primary cells, embryonated eggs, or whole organisms. Currently, continuous cell lines are most commonly used for large-scale production. Viral inactivation is achieved through chemical agents – such as ascorbic acid, ethyleneimine derivatives, formaldehyde or β -propiolactone – or through physical methods including heat or UV-treatment. Of these, formaldehyde and β -propiolactone are most frequently used in licensed human vaccines (Sanders et al. 2015).

Inactivated vaccines offer a strong safety profile compared to LAVs and are even suitable for the use in immunocompromised patients (Arvas 2014). However, they often exhibit reduced immunogenicity, making the use of adjuvants necessary to enhance the immune response. Additionally, rare but serious incidents have underscored the risks of incomplete inactivation. A notable example is the Cutter incident in 1955, in which approximately 380,000 doses of polio vaccine containing incompletely inactivated virus were administered to children. As a result, 40,000 children developed abortive poliomyelitis, 51 of which were permanently paralyzed, and five died.

Successful examples of inactivated vaccines include those developed for Hepatitis A, Influenza, and Rabies (Sanders et al. 2015).

1.2.1.3 Subunit vaccines

In subunit vaccines, only specific microbial components required to elicit an immune response – such as protein, peptide or carbohydrate antigens – are administered (Moyle and Toth 2013). Once the antigen of interest has been identified, it is typically produced recombinantly using heterologous expression systems. *Escherichia coli*

often serves as a prokaryotic host, while eukaryotic systems such as *Pichia pastoris* are used when post-translational modifications are required (Wang et al. 2016).

Subunit vaccines offer several advantages over whole cell vaccines. Recombinant production facilitates scalable manufacturing, and the targeted nature of subunit antigens confers a favorable safety profile due to their high specificity. However, identifying antigens that induce protective immunity can be time-intensive. Moreover, because only a portion of the pathogen is presented to the immune system, subunit vaccines often depend on the inclusion of adjuvants to generate a robust and sustained immune response (Perrie et al. 2013; Sanders et al., 2014). Currently licensed subunit vaccines include those for the prevention of tetanus, diphtheria, acellular pertussis and hepatitis B (Josefsberg and Buckland 2012).

1.2.1.4 mRNA vaccines

All of the vaccine types above share a key limitation: their development is relatively time intensive, which restricts their ability to respond swiftly during public health emergencies. This drawback became especially apparent during the outbreak of the COVID-19 pandemic in late 2019. COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a positive-sense RNA virus of the *Coronaviridae* family (V'kovski et al. 2021). While the virus primarily targets the respiratory system – leading to symptoms such as sore throat, fever, cough, and other pneumonia-like manifestations – it also has the ability to infect other tissues. This is made possible by its binding to the angiotensin-converting enzyme 2 (ACE2), a cellular receptor present on a wide range of cell types. As a result, SARS-CoV-2 can also affect the GIT, cardiac muscle, and skeletal muscle, causing additional symptoms such as abdominal pain, chest discomfort, and myalgia (Weng et al. 2021). Moreover, the impact of the virus often extends beyond the acute phase of infection. Approximately 15% of infected adults in the U.S. reported persistent symptoms lasting longer than two months, a condition now known as Long Covid (Perlis et al. 2022).

COVID-19 spreads primarily through respiratory droplets and aerosols and has proven to be highly contagious. Its transmission rate significantly exceeds that of seasonal influenza and varies among different viral variants (Jayaweera et al. 2020; Bai and Tao 2021; Xue et al. 2022). This high transmissibility led to a rapid global spread, with over 4.8 million confirmed cases reported within just five months of the initial outbreak

(Ojokoh et al.). In the absence of a vaccine, governments worldwide implemented drastic containment measures, including contact tracing, school closures, going out bans, and social distancing mandates. While these efforts helped to contain the spread, they also affected peoples mental health and well-being (Rossi et al. 2020). By the end of the first year of the pandemic, COVID-19 had claimed approximately 1.3 million lives globally. In addition to the immense public health burden, the pandemic had severe economic consequences, causing an estimated 3% decline in global gross domestic product (GDP) (Gagnon et al. 2023).

These events highlighted the urgent need for a new class of vaccines – ones that can be developed quickly, produced at scale, and deployed rapidly in response to emerging threats. Nucleotide-based vaccines offer great promise in this regard and had been under investigation for decades, with the first preclinical RNA vaccine being investigated in 1993. However, prior to the COVID-19 pandemic, none had received regulatory approval, largely due to challenges in achieving safe and effective delivery of the nucleotide payload, which limited their immunogenicity (Tregoning and Sanders 2025).

Based on the identified virus and its target antigen, multiple vaccine platforms were developed. These include subunit vaccines such as Nuvaxovid (Novavax), viral vector vaccines such as Vaxzevria (AstraZeneca), and mRNA vaccines such as Comirnaty (Pfizer-BioNTech) and Spikevax (Moderna) (Parums 2022; Kondera-Anasz et al. 2025; Parums 2021). The mRNA vaccines demonstrated several major advantages during the pandemic, including rapid design, good scalability, and robust clinical efficacy. As a result, mRNA vaccines became the dominant modality in large-scale immunization campaigns, accounting for over 84 % of all COVID-19 vaccine doses administered in the EU by November 2021 (Deutscher Bundestag - Wissenschaftliche Dienste 2021).

In mRNA vaccines, synthetic mRNA is delivered into host cells *in vivo*, where it is translated *in situ* into the encoded antigen, thereby eliciting an immune response (Chaudhary et al. 2021). After decades of research, two pivotal advancements accelerated the field: the introduction of TriLink's CleanCap® technology in 2017, which significantly improved mRNA stability and translational efficiency (Pascolo 2023), and the approval of Onpattro® in 2018, the first siRNA-based therapeutic, which validated the clinical viability of RNA-based drug delivery systems. Onpattro® uses a lipid

nanoparticle (LNP) delivery system to transport partisiran, an siRNA that inhibits the synthesis of transthyretin in the liver to treat hereditary transthyretin-mediated amyloidosis (Akinc et al. 2019). The successful use of LNPs revolutionized the field of nucleotide delivery and laid the groundwork for the rapid development of mRNA-based vaccines.

This technology was crucial in the creation of Comirnaty, the first approved COVID-19 vaccine developed by Pfizer and BioNTech. Thanks to LNP-based delivery and prior research in the field, Comirnaty was developed and authorized for emergency use in record time, with the first doses administered on December 11, 2020 – just one year after the pandemic began. In August 2021, the vaccine received its full regulatory approval by the FDA (Parums 2021).

Since then, numerous RNA vaccines targeting SARS-CoV-2 have been licensed. Over the course of three years, billions of doses have been administered and shown remarkable success and safety profiles, saving an estimated 14.4 million lives in 2021 alone (World Health Organization 2024). A systematic review revealed that all vaccinations against COVID-19 pooled together shows significant protection against the diseases. Amongst the different vaccine types, mRNA-based vaccines showed superior efficacy over inactivated vaccines and viral vector-based vaccines (Beladiya et al. 2024). Building on that, an overview by the Verband Forschender Arzneimittelhersteller shows that a large number of vaccines against a variety of infectious diseases are currently undergoing clinical development (Verband Forschender Arzneimittelhersteller 2024).

Figure 1 provides a comprehensive overview over the most common types of vaccines.

Types of Vaccines

Live Attenuated Vaccines



- ➕ Strong, long lasting immunity
- ➕ Fewer dosing required
- ➖ Risk of reversion to pathogenic form
- ➖ Unsuitable for immunocompromised patients
- 📌 Examples: Smallpox, Polio, Measles

Inactivated Vaccines



- ➕ Good safety profile
- ➕ Suitable for immunocompromised patients
- ➖ Often requires adjuvants
- ➖ Risk of incomplete inactivation (Cutter incident)
- 📌 Examples: Hepatitis A, Influenza, Rabies

Subunit Vaccines



- ➕ Highly specific and safe
- ➕ Scalable manufacturing
- ➖ Time-consuming antigen identification
- ➖ Often requires adjuvants
- 📌 Examples: Tetanus, Diphtheria, Hepatitis B

mRNA Vaccines



- ➕ Rapid development
- ➕ Superior efficacy
- ➕ Excellent safety profile
- ➖ Often requires adjuvants
- ➖ Delivery vehicle required
- 📌 Examples: COVID-19

Figure 1: A comprehensive comparison of vaccine types and their characteristics

1.2.2 Routes of Vaccine Administration

In addition to the type of vaccine, the route of administration significantly influences both vaccine safety and efficacy, as well as the nature of the elicited immune response.

The local inflammation caused by the injection recruits innate immune cells, including macrophages, neutrophils, and dendritic cells (DCs), to the muscle tissue. Antigen-presenting cells (APCs), such as DCs, internalize and process the vaccine antigen and migrate to the draining lymph nodes, where they present the processed antigen-derived peptides on major histocompatibility complexes (MHCs) to naïve T cells. Activated CD4⁺ T cells differentiate into T helper (Th) cells, while CD8⁺ T cells become cytotoxic T lymphocytes (CTLs), collectively mediating the cellular immune response. Th cells also provide critical help to B cells, promoting their differentiation into plasma cells, which secrete antigen-specific antibodies and constitute the core of the humoral immune response (Kozak and Hu 2023).

Some administration routes may require specific adjuvants to enhance immunogenicity and ensure a robust immune response. The following section outlines and discusses the advantages and disadvantages of commonly used vaccine administration routes.

1.2.2.1 Intramuscular Administration

Intramuscular (IM) administration is the most commonly used route for vaccine delivery. Vaccines are injected with needle and syringe into deep muscle tissue, typically the deltoid or thigh muscle, potentially eliciting robust humoral and cellular immune responses (Petousis-Harris 2008).

IM vaccination provides a predictable, consistent immune response and is associated with an established safety profile, making it favorable for regulatory approval (Ajana et al. 2008). However, IM administration also comes with a variety of disadvantages. The needle mediated administration requires trained medical staff, rising costs and limiting availability of the vaccine for mass immunizations. Even more importantly, it fails to protect patients from the initial infection with the pathogen due to a lack of production of secretory IgA (sIgA) on mucosal entry sites (Skwarczynski and Toth 2020).

1.2.2.2 Subcutaneous Administration

Subcutaneous (SC) vaccines are administered into the fatty tissue beneath the dermis. SC administration is the second most frequently used administration route, but due to higher local reactogenicity observed with adjuvanted vaccines, the SC route is only used for non-adjuvanted formulations, such as live attenuated viruses, inactivated whole-cell, split-virus, and subunit vaccines (Zhao et al. 2023; Cook 2021). Although SC administration has been associated with lower seroconversion rates and a more rapid decline in antibody levels compared to IM injection (Kozak and Hu 2023), several important vaccines – such as measles, mumps, and rubella (MMR) and varicella – are still routinely administered via the SC route based on their established efficacy and safety profiles (U.S. Centers for Disease Control and Prevention 2021).

1.2.2.3 Intradermal Administration

Intradermal (ID) administration has recently gained attention as a promising route for antigen delivery. In this method, the antigen is introduced directly into the dermal layer of the skin, which is one of the most immunologically active organs, rich in various immune-competent cells (Zhang et al. 2022). Due to its high immunological activity, ID administration has shown potential for dose sparing; for instance, studies have demonstrated that only 20% of the standard IM dose of influenza vaccine can elicit a comparable immune response. However, whether this dose-sparing effect is

generalizable to other vaccines remains yet to be explored. The overall safety profile of ID vaccination is comparable to that of IM and SC routes, though minor local reactions such as erythema and pruritus are more frequently reported following ID delivery (Schnyder et al. 2020). Moreover, the widespread implementation of ID vaccination may necessitate the development and adoption of specialized delivery devices, such as microneedle arrays or tattoo-based injection systems (Kim et al. 2012).

1.2.2.4 Mucosal Administration

Mucosal vaccines are administered orally, intranasally or sublingually. Unlike parenteral vaccination methods, mucosal delivery offers the advantage of needle-free administration, facilitating rapid distribution and enabling more efficient mass immunization. A key immunological benefit of mucosal vaccination is the induction of sIgA at mucosal surfaces. SIgA plays a critical role in immune defense by neutralizing pathogens at their mucosal entry sites, thereby preventing infection at an early stage. In contrast, parenteral vaccines primarily induce systemic immunity, which is more effective at reducing disease severity than blocking initial infection. This limitation became evident during the COVID-19 pandemic, as widespread transmission of SARS-CoV-2 persisted even in highly vaccinated populations (Knisely et al. 2023).

Despite these advantages, relatively few mucosal vaccines are currently licensed for human use, reflecting ongoing challenges associated with this delivery route. For instance, depending on the administration route, antigens must be formulated to withstand degradation in the gastrointestinal tract or nasal passages (Correa et al. 2022a). In addition to biological barriers, such as mucus and the epithelial barrier, that physically hinder administered vaccines from reaching their immunological target (Kirch et al. 2012; Huang et al. 2022), there is mucosal tolerance. Mucosal tolerance is a phenomenon wherein the immune system becomes hyposensitive to antigens administered to mucosal surfaces, necessitating the use of potent yet safe mucosal adjuvants for non-live-attenuated vaccines to elicit robust and durable immune response (Weiner et al. 2011).

To date, four intranasal live-attenuated influenza vaccines have been licensed, all of which are non-adjuvanted. Their immunogenicity depends on limited viral replication in the nasal mucosa. HeberNasvac, a recombinant therapeutic vaccine against chronic

hepatitis B licensed in Cuba, represents the only currently available intranasal vaccine that is non-live-attenuated. It is self-adjuvanted through the inclusion of hepatitis B surface and nucleocapsid antigen, which functions as an endogenous immunostimulant (Kehagia et al. 2023). Nasalflu, an inactivated subunit influenza vaccine adjuvanted with *E. coli* heat-labile toxin was marketed in Switzerland in 2000. However, it was withdrawn from the market just one year later in 2001 following reports of a significantly increased risk of Bell's palsy associated with its use (Mutsch et al. 2004).

The current situation underscores the urgent need for improved adjuvants tailored to mucosal vaccination. Although numerous mucosal vaccine candidates are currently undergoing clinical evaluation, the majority rely on live-attenuated platforms, which are associated with safety concerns. Moreover, recent studies have reported suboptimal systemic and mucosal antibody responses, indicating that viral vectors alone may not fulfill the immunological requirements for effective mucosal immunization (Bai et al. 2024).

In response, several studies are exploring the mucosal application of adjuvants previously validated in parenteral vaccines, including CpG oligonucleotides, monophosphoryl lipid A, and squalene-based oil-in-water emulsions (Ko et al. 2018; Chen et al. 2020). Additional strategies have investigated the use of recombinant cytokines and chemokines, such as IFN- γ , various interleukins (Correa et al. 2022a), and CCL21, which have shown promise as mucosal adjuvants (Sabbaghi et al. 2021). Furthermore, nanotechnology-based antigen delivery systems have been proposed as a means to enhance the efficacy of intranasal vaccines (Bai et al. 2024).

1.3 Nanomedicine and the Evolution of mRNA Delivery Systems

Nanomedicine is a branch of medicine that applies nanotechnology, typically in the range of 1-1000 nm, to diagnose, prevent, monitor, and treat diseases. It employs molecular-scale tools, including nanoparticles, nanodevices, and nanoscale imaging agents, which are engineered to enable targeted and controlled medical interventions (Webster 2006; Astruc 2015). Nanotechnology offers the potential to overcome limitations of potent drug candidates with poor pharmacokinetic profiles by enhancing

solubility, permeability, and overall bioavailability (Thapa and Kim 2023). Moreover, nanoparticle-based formulations can reduce systemic toxicity by altering biodistribution and minimizing off-target effects (Jong and Borm 2008). However, despite their improved safety profiles, the comprehensive assessment of nanoparticles with respect to toxicity, carcinogenicity, and teratogenicity remains challenging due to the absence of standardized and predictive analytical tools and regulatory procedures (Rehan et al. 2024).

Since the first nanomedicine product (Gris-PEG, an ultramicrosized oral antifungal drug) was submitted for approval in 1974, the number of regulatory submissions involving nanomaterials has steadily increased. However, the annual number of FDA-approved nanomedicine products has remained relatively constant, averaging between one and seven approvals per year. Approximately 70% of these submissions involve liposomes, alongside other well-established nanostructures such as nanocrystals and iron colloids, with a primary focus on cancer therapy. About 60% of the approved formulations are designed for intravenous (i.v.) administration, 21% for oral delivery, and the remainder distributed across ophthalmic, respiratory, topical, IM, SC, and other routes of administration (D'Mello et al. 2017).

Recent advancements in the encapsulation of nucleic acids into LNPs, the co-delivery of antigens and adjuvants within a single carrier, and the targeted delivery of nanoparticles to immune cells have opened the path of nanomedicine into emerging fields such as gene therapy, cancer immunotherapy and vaccination (Prasetia et al. 2025; Alshehry et al. 2025; Rehan et al. 2024). Notably, the effectiveness of LNP-based delivery platforms was powerfully demonstrated during the COVID-19 pandemic through mRNA vaccines (Li et al. 2022).

Despite the remarkable initial success, LNP-based mRNA vaccines are still in their infancy. Several limitations associated with LNPs – including their chemical, thermal, and mechanical instability, as well as immunogenicity – remain unresolved. In parallel, there are intrinsic challenges of the mRNA molecule itself, such as susceptibility to degradation and inefficient translation. Addressing these barriers is crucial for enabling new applications of mRNA technology, ranging from cancer immunotherapy to gene editing.

Many of these challenges, and emerging strategies to overcome them, have been analyzed in a recent review article entitled “Exploring the Future of mRNA Delivery: Beyond Lipid Nanoparticles” (see 5.2.1). The author of this thesis was solely responsible for writing sections 2.1, 2.2, and 2.5 of that publication, while the introduction and conclusion were written collaboratively.

1.3.1 Limitations of LNPs in mRNA Delivery and Potential Solutions

1.3.1.1 Advancements in Chemical Modifications for mRNA Therapeutics

Besides necessary delivery technologies, substantial progress has been made in optimizing the design of the mRNA payload itself. Innovations span across every structural component of the mRNA molecule - including the 5' cap structure, the poly(A) tail, untranslated regions (UTRs), modified nucleosides and the overall architecture of the transcript.

In higher eukaryotes, mRNAs are co-transcriptionally capped at the 5' end with a 7-methylguanosine (m7G) cap, often further methylated at adjacent nucleotides. This cap structure is crucial for several biological functions: it enhances mRNA stability by protecting transcripts from 5' to 3' exonuclease degradation, facilitates translation initiation, and helps to prevent innate immune recognition. Cells use cap structures as molecular signatures to regulate gene expression, cell function and to differentiate between endogenous and exogenous mRNAs (Galloway and Cowling 2019). Recent studies have highlighted the potential of synthetic cap analogs to improve mRNA quality and expression. Warminski et al. demonstrated that replacing the native N6,2'-O-dimethyladenosine (m6Am) cap with a trinucleotide N6-benzyl analog enabled more efficient separation of capped and uncapped transcripts during purification, leading to a reduction in double-stranded RNA contaminants (Warminski et al. 2024). This suggests that cap analog selection could be tailored to specific cellular contexts and used as an indirect method for cell-specific targeting.

Chen et al. introduced the concept of “multicapping” - attaching a second capped RNA branch downstream of the +2 position. When combined with poly(A) tail stabilization, these modifications led to a 15-fold enhancement in mRNA translation after 24 hours *in vitro* and a 10-fold enhancement *in vivo* (Chen et al. 2024).

The critical role of 5'capping is underscored by comparisons with circular RNA (circRNA), which lacks both 5' and 3' ends and is therefore inherently resistant to exonuclease-mediated degradation. This contributes to an extended half-life, yet circRNAs typically display lower translation efficiency due to their reliance on internal ribosome entry sites for translation initiation (Koch et al. 2020). To address this limitation, Chen et al. designed a modified construct by covalently attaching their optimized cap to the 3'UTR. The resulting structure, termed QRNA (reflecting its resemblance to the letter "Q"), achieved a remarkable 30-fold increase in translation *in vivo* after 24 hours compared to unmodified circRNA (Chen et al. 2024). Although this performance still fell short of optimized linear mRNA, the inherent stability of circular RNAs may offer advantages regarding challenges in shelf-life and mechanical stability, as well as applications requiring altered expression kinetics.

Another strategy to modulate RNA expression kinetics is the use of self-amplifying RNA (saRNA). SaRNA leverages the replication machinery of alphaviruses to amplify the gene of interest intracellularly. Although the inclusion of the viral replicase, typically comprising 7-8 kb, significantly increases the overall RNA length, saRNA can produce substantially higher protein yields compared to conventional mRNA. Consequently, lower initial RNA doses are sufficient to elicit comparable biological responses, potentially reducing cytotoxicity and minimizing side effects associated with the delivery vehicle (Schmidt and Schnierle 2023). These advantages have recently led to the approval of the first saRNA based COVID-19 vaccine in Japan (Hu et al. 2024). On the downside, the increased length and structural complexity of saRNA make it more susceptible to strand breaks and enzymatic degradation, resulting in a shorter shelf-life compared to standard mRNA constructs (Wayment-Steele et al. 2021).

Unfortunately, it is not possible to combine the approaches of saRNA and circRNA using current technologies, as the viral replicase requires the presence of 5' and 3' ends for recognition and function, features that are inherently absent in circRNA. Nonetheless, the development of engineered replicases capable of initiating replication on circular templates could open the door to a new class of RNA therapeutics that integrates the stability of circRNA with the high expression capacity of saRNA.

1.3.1.2 Thermal and mechanical stability

Not only the chemical susceptibility of nucleic acids but also the limited thermal and mechanical stability of mRNA loaded LNPs remains a significant drawback, particularly for their global distribution and long-term storage. For example, currently approved LNP-based mRNA vaccines, such as those for COVID-19, require storage and transportation at temperatures ranging from -15°C to -90°C, posing logistical challenges. Even when stored at ultralow freezing temperatures, the shelf life remains limited to 18 months (Hermosilla et al. 2023). Consequently, considerable research efforts are focused on enhancing LNP stability under standard storage conditions, with lyophilization emerging as one of the most widely explored strategies to improve shelf life.

In this context, optimization of lyophilization parameters, as well as the selection of appropriate cryoprotectants, buffer systems and LNP compositions are actively investigated. Fan et al. conducted a systematic screening of various cryoprotectants - such as oligosaccharides, polymers and amino acids - in combination with different buffer systems including phosphate-buffered saline (PBS, pH 7.4), Tris buffer (pH 7.5), and acetate buffer (pH 5.0). They found that 10 % sucrose, irrespective of the buffer used, best preserved key parameters including particle size, encapsulation efficiency, mRNA integrity, *in vitro* transfection efficiency and fluorescence intensity (Fan et al. 2024). Similar studies confirm the function of ~9-10% disaccharides partially combined with the stabilizer polyvinylpyrrolidone to optimize the colloidal stability of freeze-dried LNPs for up to 6 months (Wang et al. 2024; Ruppl et al. 2025). Nonetheless, Ruppl et al. observed a substantial decline in transfection efficiency after three months at room temperature. In contrast, lyophilized LNPs stored at 5 °C maintained their functional activity for the full 6-month duration, whereas non-lyophilized formulations lost nearly all transfection capability after only 3 months (Ruppl et al. 2025).

These results suggest that, under standard storage conditions, mRNA chemical stability rather than LNP structural integrity is the critical limiting factor for long-term maintenance of transfection efficiency. The primary mechanism of mRNA degradation under such conditions is intramolecular transesterification, which is influenced by mRNA backbone flexibility, ambient pH and the reactivity of the 2'-hydroxyl group (Oude Blenke et al. 2023; Lönnberg 2011; Li and Breaker 1999). While LNPs offer high

encapsulation efficiency and can partially shield mRNA from water and pH fluctuations, they are less effective at reducing backbone flexibility, leaving mRNA vulnerable to chemical degradation.

In contrast, polymeric condensation of nucleic acids can significantly restrict RNA conformational mobility, which may slow transesterification. However, polymer-based systems typically lack full encapsulation, leaving the mRNA exposed to environmental conditions (Miyazaki et al. 2020; Uchida et al. 2016). Lipopolyplexes (LPPs), in which mRNA is first condensed by polymers and then coated with lipids, represent a promising strategy to combine the benefits of both systems (Vlasova et al. 2025). Alternative approaches, amongst them GEMCOVAC™-19 (IND), a freeze-dried COVID-19 vaccine, utilize electrostatic adsorption of mRNA onto the surface of nanoparticles (Kliesch et al. 2022; Su et al. 2011; Latuski et al. 2025). Although this method offers limited protection against environmental factors as well, the gentle complexation conditions can help preserve the native secondary and tertiary structures of the mRNA. This structural contribution may, in turn, contribute to enhanced chemical stability by limiting intramolecular transesterification (Wayment-Steele et al. 2021). In spite of these theoretical advantages, rigorous studies to comparatively evaluate chemical mRNA stability across different nanoparticle architectures remain lacking. To ensure long-term preservation of mRNA functionality under standard (e.g., 4–25 °C) storage conditions, future research should prioritize strategies that enhance the intrinsic chemical stability of mRNA, in addition to optimizing carrier stability.

In addition to extensive research on the thermal and storage stability of mRNA-loaded LNPs, relatively few studies have addressed their mechanical stability. Although mechanical agitation is less relevant during shipping under frozen conditions, it becomes a critical consideration as the field transitions towards transport and storage under standard conditions. Several investigations have reported increases in particle size and decreases in encapsulation efficiency following various forms of mechanical stress, including mixing, vertical and horizontal shaking, inversion and vortexing (Kamiya et al. 2022; Kudsiova et al. 2021; Thaller et al. 2023; Ruppl et al. 2024; Matthessen et al. 2024). Among these, only Ruppl et al. have explored lyophilization as a strategy to mitigate such effects. Their findings showed that while liquid LNPs lost up to 80 % or more of their encapsulated nucleotides following shaking, lyophilized LNPs retained full encapsulation efficiency under the same conditions. However, this

study did not evaluate or compare the post-stress functionality of lyophilized particles with their liquid counterparts (Ruppl et al. 2024).

Overall, lyophilization can be considered as key technology to improve the thermal and mechanical stability of mRNA drug products. While freeze-drying is a pharmaceutically established, well-described process, there is another, less energy-intensive alternative: spray-drying (Merkel 2022). The possibility to achieve dry mRNA formulations does not only inherently increase stability, but may enable alternative application routes.

1.3.1.3 Safety of mRNA LNPs

While immunogenicity is advantageous in the context of vaccination, it poses a significant challenge for the treatment of chronic diseases that require frequent, repeated dosing. The substitution of permanently cationic lipids such as DOTMA and DOTAP with ionizable lipids has helped reduce toxicity associated with the uncontrolled activation of pro-apoptotic and pro-inflammatory pathways (Sharma et al. 2024). However, these ionizable lipids are not entirely immunologically inert (Zhou et al. 2025). To address the inflammatory response elicited by plasmid DNA (pDNA)-loaded lipid nanoparticles (LNPs), Patel et al. incorporated endogenous anti-inflammatory lipids, such as nitro-oleic acid, into LNP formulations. This strategy effectively abolished STING activation *in vitro* and enhanced gene expression by 50-fold (Patel et al. 2024). Similar approaches may be advantageous for improving the safety and efficacy of repeated mRNA delivery.

In addition to their immunogenicity, several widely used ionizable lipids - such as ALC0315 - exhibit poor biodegradability, raising concerns about their accumulation and potential toxicity following multiple administrations (Sharma et al. 2024). Even in the context of vaccination, increased lipid biodegradability has been associated with reduced inflammation at the injection site. Notably, this attenuation of innate immune activation does not compromise the immunogenicity of the vaccine formulations (Hassett et al. 2019). A common strategy to increase biodegradability involves the incorporation of ester bonds, either within the linker between head group and hydrophobic tail, or directly within the hydrophobic tail, replacing double bonds in unsaturated lipids (Maier et al. 2013; Sabnis et al. 2018). Furthermore, lipids with saturated hydrophobic tails tend to undergo more rapid enzymatic degradation than

those with unsaturated tails, as demonstrated by the differing *in vivo* half-lives of ALC-0315 and SM-102 (Sharma et al. 2024).

Building on Paracelsus' foundational principle that “the dose makes the poison”, lipid-polymer hybrid strategies offer an option to minimize the required dose of immunogenic lipids in mRNA delivery systems. By forming a polymeric core that is coated with only a thin layer of functional lipids, these hybrid nanoparticles can achieve efficient delivery with reduced lipid-associated toxicity. Two main classes of such hybrid particles have emerged: LPPs, in which the mRNA is complexed within a cationic polymer core surrounded by a lipid shell (Siewert et al. 2020; Wang et al. 2013; Chen et al. 2023); and lipid-polymer hybrid nanoparticles, typically composed of a hydrophobic polymeric core (e.g., PLGA as approved, generally regarded as safe material), with the nucleic acid encapsulated within or adsorbed onto the surrounding lipid layer (Kliesch et al. 2022; Vencken et al. 2019; Yasar et al. 2018; Ayad et al. 2021). Both approaches have been shown to enhance colloidal stability and reduce cytotoxicity, making them promising platforms for safe and effective mRNA delivery (Karmacharya et al. 2022). Gabelmann et al. integrated both strategies to design a core-shell nanoparticle capable of dual mRNA loading, demonstrating high loading capacity and facilitating the efficient co-delivery of two distinct nucleotides to the same target cell (Gabelmann et al. 2025b).

However, leaving the pro-inflammatory nature of the ionizable lipids aside, the most significant concern associated with the repeated administration of lipid-based nanoparticles lies in a common component across the systems: the PEG-lipid conjugate. PEGylation serves several critical functions, including stabilization of nanoparticles by preventing aggregation, prolongation of circulation half-life by evading immune recognition, and minimizing nonspecific interactions with biological components (Hamidi et al. 2006; Suk et al. 2016). Paradoxically, although initially introduced to reduce immunogenicity, PEG-lipids can provoke immune responses upon repeated administration (“PEG-Dilemma”). One such response is accelerated blood clearance, where subsequent doses of PEGylated nanoparticles are rapidly removed from circulation, decreasing their efficacy (Verhoef and Anchordoquy 2013). More concerning is the risk of complement activation-related pseudoallergy (CARPA), a potentially severe hypersensitivity reaction. CARPA can be triggered by anti-PEG antibodies, which may either develop in response to repeated administration of PEGylated therapeutics or be pre-existing due to widespread exposure to PEG-

containing products such as shampoos or sunscreens (Kozma et al. 2020). Clinical manifestations of CARPA range from mild allergic symptoms to life-threatening anaphylaxis.

To compensate these risks, patients receiving Onpattro®, containing PEG2000-C-DMG, are treated with immunosuppressants to mitigate the likelihood of adverse immune reactions (Sharma et al. 2024). In response to the limitations of PEG, alternative stealth strategies are under active investigation. For example, Yu et al. demonstrated that liposomes coated with poly(ethyl ethylene phosphate) (PEEP) showed extended circulation time, lower hepatotoxicity and minimal induction of anti-PEEP IgM and IgG antibodies upon repeated dosing. Notably, these modifications did not compromise transfection efficiency (Yu et al. 2023). Similar strategies employing various novel polymers, including poly(2-alkyl-2-oxazoline), have shown promise and are progressing into clinical development, such as in the case of a rotigotine prodrug (He et al. 2024; Hassanel et al. 2024; Holick et al. 2025; Huesmann et al. 2015; Olanow et al. 2020).

However, it is important to recognize that all synthetic polymers represent foreign entities to the human immune system. Consequently, the potential for immunogenicity remains, particularly with increased exposure through medical or cosmetic use. An alternative approach involves the use of human serum albumin, as a nanoparticle coating to confer stealth properties and reduce rapid clearance (Bozzer et al. 2024). While these strategies may not fully resolve the underlying issue of antibody formation, it's important to provide options for patients who experience allergic reactions to certain stabilizing components.

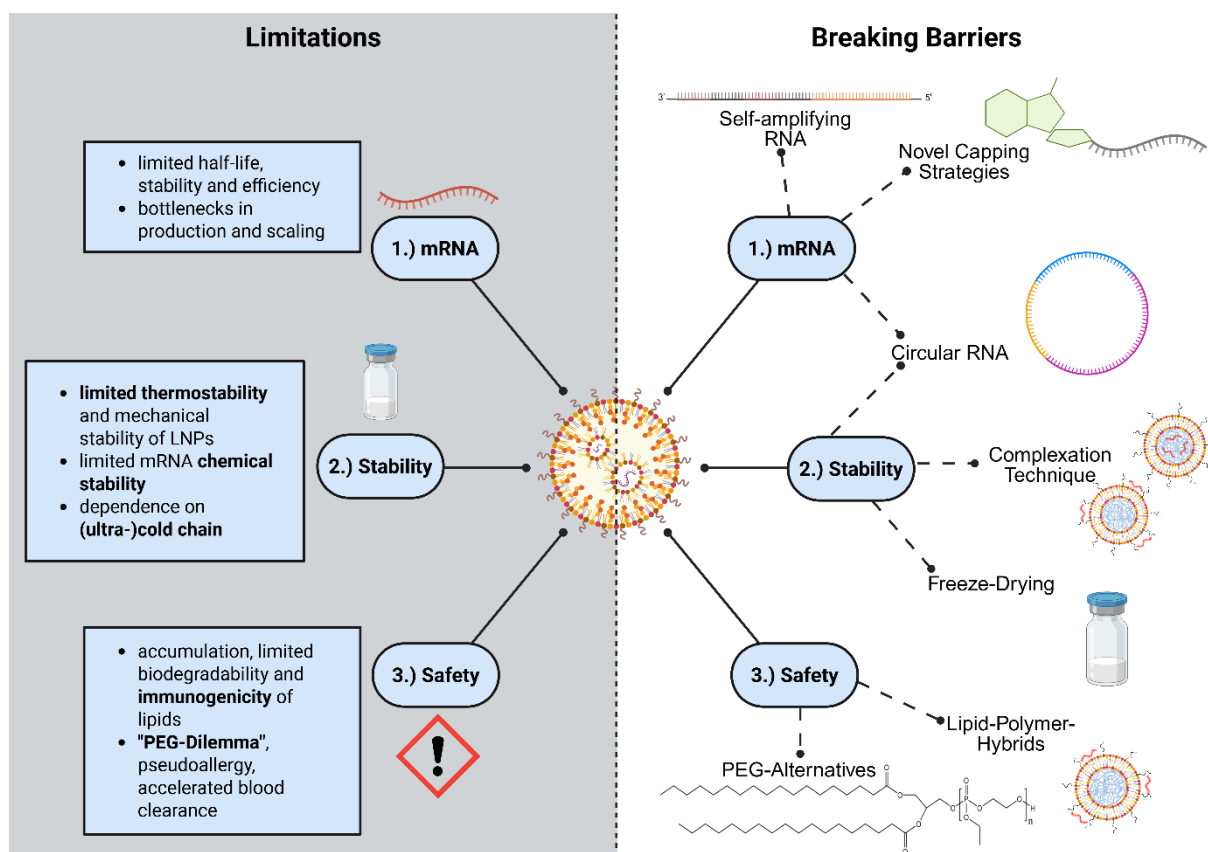


Figure 2: Current limitations of LNPs for mRNA delivery and how to overcome them. Figure was adapted from Gabelmann et al.

1.3.1.4 Further limitations of LNPs

In addition to the three discussed topics mRNA, stability, and safety, the review highlights limitations regarding targeting, regulations and manufacturing of LNP mediated mRNA delivery. To overcome the hurdles of targeted mRNA delivery, three main strategies were identified, comprising novel medical device technology, passive targeting and active targeting mechanisms. Notably, these strategies can be combined, as the case in SORT LNPs decorated with lung targeting antibodies.

Regulatory challenges, such as an increasingly intricate intellectual property (IP) landscape of mRNA technologies and lipid components, could be met by the use of novel polymeric materials. While these offer greater flexibility within the IP landscape, the flexibility comes with the cost of high initial efforts to prove their safety and efficiency.

Key challenges in manufacturing LNPs include limited understanding of nanoparticle architecture, particularly the functional relevance of unloaded particles and the presence of bleb structures, which may either enhance or hinder mRNA delivery.

These uncertainties can be addressed by implementing advanced single-particle and real-time analytical techniques, such as fluorescence spectroscopy and online dynamic light scattering (DLS), to improve characterization and quality control. To further enhance manufacturing flexibility and stability, emerging strategies like surface-loading mRNA onto preformed nanoparticles offer a promising alternative to conventional encapsulation methods.

To conclude, the success of COVID-19 vaccines has accelerated interest and investment in mRNA delivery, with current research focusing on overcoming limitations of conventional LNPs through innovations like saRNA, advanced capping, dry powder formulations, targeted delivery, and novel carrier-types and components. These advances are driving the development of personalized, stable, and broadly applicable mRNA-based therapies (Gabelmann et al. 2025a).

2 Aim of the Thesis

The overall aim of this thesis was to design and develop a novel nanoparticulate platform capable of addressing the key limitations associated with LNPs, the current gold standard for mRNA delivery. While LNPs have proven effective in the context of vaccination against infectious diseases, emerging applications of mRNA therapeutics – including cancer immunotherapy and CRISPR/Cas9-based gene editing – impose new demands on delivery systems. Among these is the need for simultaneous yet distinct delivery of multiple nucleotide species. Although LNPs can, in principle, co-encapsulate several nucleotides, this often results in undesirable nucleotide-nucleotide interactions, binding competition, and reduced or unpredictable encapsulation efficiencies.

To overcome these limitations, this work set out to engineer a structurally defined core-shell nanoparticle platform enabling spatially separated loading: one nucleotide species within the core and another on the surface. This design aimed to offer enhanced stability, modularity and independence from the physicochemical properties of different nucleotide types and sizes. Additionally, the platform was conceived with scalability in mind, with the goal of ensuring feasibility for future translational applications.

Following the establishment of a suitable carrier architecture, a central objective was to validate and optimize its transfection efficiency in cell lines relevant to mRNA-based vaccination. Emphasis was placed on evaluating whether dual-nucleotide delivery offers measurable advantages over conventional single-delivery systems and whether the nucleotide's location influences its stability and expression. It was hypothesized that co-delivery of an mRNA-encoded antigen alongside an mRNA-based endogenous adjuvant – or delivery of mRNA with Cas9 and guide RNA – could benefit from asynchronous release kinetics. Consequently, a further goal was to investigate the kinetics of gene expression from core-loaded versus surface-loaded mRNAs, with the objective of fine-tuning the release behavior of core-loaded nucleotides.

Beyond the use of model mRNAs, a critical part of the research plan involved identifying and characterizing novel adjuvants suitable for mucosal vaccination. To fully leverage the capacity of the newly developed LPPs for co-delivering multiple nucleotides, it was necessary to investigate the adjuvant potential of *in vitro*-

transcribed mRNAs encoding chemokines or chemokine receptors involved in the creation of immune responses. These candidates were selected based on their potential to enhance immunogenicity without compromising safety. Following their custom synthesis by external providers, the first step was to validate protein expression and functionality *in vitro* through specifically designed and improved assays. Finally, performance of the nanoparticle formulations should be tested *in vivo* in collaboration with project partners at the Helmholtz Centre for Infection Research in Braunschweig, which by the time this thesis was submitted is still going on.

3 Outcome of the Thesis

3.1 Development of a Core-Shell Nanocarrier

To guide the development of a suitable core-shell nanocarrier for mRNA delivery, the fundamental design requirements were first defined. Given the intended clinical application, the formulation would need to exhibit low cytotoxicity at therapeutically relevant concentrations. To overcome known stability limitations of mRNA-loaded LNPs, the particles should ideally remain stable for at least four weeks under refrigerated storage conditions. The core-shell architecture was conceptually designed to enable sequential loading of two distinct nucleotides in separate manufacturing steps. This would require both the core and the shell to incorporate cationic components capable of electrostatically binding anionic nucleotides, while maintaining an overall net negative charge of the core to support interaction with the shell.

To ensure efficient cell uptake and robust immune responses, the particles require a size between 125-200 nm (Albarki and Donovan 2020; Stano et al. 2012). In addition to these physicochemical and structural criteria, the system would need to ensure effective transfection for both nucleotide cargos. Although Escalona-Rayó et al. report a limited correlation between *in vitro* transfection and *in vivo* efficacy for certain vaccine candidates (Escalona-Rayó et al. 2023), achieving at least 50% *in vitro* transfection efficiency remains a widely accepted benchmark for predicting *in vivo* performance (Perche et al. 2011).

3.1.1 Methods and Materials

3.1.1.1 Materials

Hyaluronic acid sodium salt (0.2–0.5 million Da) was purchased from BIOZOL Diagnostica (Eching, Bayern, Deutschland). Chitosan hydrochloride (<300 kDa) was purchased from Heppe Medical Chitosan GmbH (Halle, Germany). Sodium triphosphate (TPP), zinc sulfate monohydrate, N-hydroxysulfosuccinimide sodium (S-NHS), N-(3-dimethylaminopropyl)-N-ethyl-carbodiimide (EDC), glutaraldehyde, poly-L-lysine hydrobromide (PLL, 150 kDa), protamine sulfate, poly adenylic acid potassium salt (polyA), and cholesterol were purchased from Sigma-Aldrich (Darmstadt, Germany). CleanCap™ eGFP and mCherry mRNA (5moU) were purchased from TriLink BioTechnologies LLC (San Diego, CA, USA). Lipofectin® transfection reagent

was purchased from ThermoFisher Scientific (Darmstadt, Germany). DOTAP, DOTMA, DOPE, DMG-PEG, and DSPE-PEG were purchased from Avanti Polar Lipids (Birmingham, AL, USA).

3.1.1.2 Particle preparation

Ternary polymeric particles

For core formulation, a solution of a cationic polymer was mixed with an anionic polymer using a w-shaped micromixer with a 90° angle between the two inlets. Two syringe pumps (Standard Infuse/Withdraw PHD ULTRA™ Syringe Pumps, Holliston, MA, USA) were operated simultaneously and set to a total flow rate of 1000 µL/min. The negative polyplexes (PP) were coated with a solution of a second cationic polymer using the same settings.

Lipopolyplexes

LPPs were prepared in a two-step process. In the first step, a cationic polymer was mixed with an anionic polymer in a herringbone micromixer (Fluidic 187, microfluidic Chipshop GmbH, Jena, Germany) to form polyplexes at a positive-to-negative (POS/NEG) charge ratio of 1:2. For these calculations, all ionizable functional groups were assumed to be fully charged. For core-loaded PPs, 25% of the polymer's negative charges were replaced with mRNA.

In the second step, the PPs were combined with an ethanolic lipid solution in a cross-shaped micromixer using hydrodynamic focusing of the lipid stream at a flow rate of 10:1. Based on the total charges of lipids and polymers, the resulting LPPs had a POS/NEG ratio of 2:1. Where applicable mRNA was surface loaded by mixing an mRNA solution (1 µg/µL) with the particles via pipetting.

3.1.1.3 Physicochemical particle characterization

The hydrodynamic particle size, polydispersity index (PDI), and zeta potential of the nanoparticles were measured by dynamic light scattering (DLS; Zetasizer Nano, Malvern Instruments, Malvern, UK). For zeta potential analysis, samples were diluted 10-fold in deionized water.

3.1.1.4 Synthesis of poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA)

pDMAEMA was synthesized via RAFT polymerization as previously described (Yildirim et al. 2017) by Dr. Turgay Yildirim (Helmholtz-Institute for Pharmaceutical Research Saarland, Saarbrücken, Germany). The molecular weight was determined to be 66 kDa by ¹H-NMR-spectroscopy. Unreacted DMAEMA monomers were removed by precipitation in hexane, followed by redissolution of the precipitate in dichloromethane. This purification step was performed four times. Subsequently, the solvent was removed under pressurized air, and the polymer was dried in a vacuum oven. ¹H-NMR analysis confirmed the complete removal of unreacted DMAEMA monomers.

3.1.1.5 Particle visualization

Prior to Cryo-transmission electron microscopy (Cryo-TEM) analysis, particle suspensions were concentrated by centrifugation at 3850 × g using a Vivaspın 20 centrifugal concentrator equipped with a 100 kDa molecular weight cutoff (MWCO) membrane (Sartorius AG, Göttingen, Germany). Centrifugation was carried out in discrete time intervals until the desired concentration was reached (e.g., 1 mL of particle suspension concentrated to a final volume of 100 µL).

For Cryo-TEM, 2 µL of a 10× concentrated particle suspension was applied to a holey carbon support TEM grid (type 147-4; Plano, Wetzlar, Germany), blotted for 2 s, and plunge-frozen in undercooled liquid ethane using a Gatan CP3 plunge freezer (Pleasanton, CA, USA) at -165 °C. The grid was then transferred to a Gatan model 914 cryo-TEM sample holder and imaged with a JEM-2100 LaB6 TEM (JEOL, Akishima, Tokyo, Japan) operated at 200 kV under low-dose conditions. Images were recorded with a Gatan Orius SC1000 CCD camera using an exposure time of 2 s.

3.1.1.6 Cell culture

To assess the ability to transfect immune competent cells *in vitro*, the murine dendritic cell line DC 2.4 (SSC142, Merck Millipore, Darmstadt, Germany) was selected. Cells were cultured in complete medium based on RPMI 1640 (Roswell Park Memorial Institute; 1× Ref. 21875, Gibco/Life Technologies, Paisley, UK), non-essential amino acids (1×, Gibco, Paisley, UK), and β-mercaptoethanol (0.0054×). Cultures were maintained in a humidified incubator at 37 °C with 5% CO₂ and passaged weekly up to passage 17. Routine cultivation was performed without penicillin/streptomycin (P/S)

to avoid potential effects on cellular behavior. For transfection assays, however, 1% P/S was added to reduce the risk of bacterial contamination from non-sterile particle formulations.

3.1.1.7 Transfection studies

A total of 100,000 DC 2.4 cells were seeded in 500 μ L of complete medium or RPMI per well in a 24-well plate (Ref. 662160, Greiner Bio-One GmbH, Frickenhausen, Germany) and cultured for 24 h to reach ~70% confluency. Cells were then washed twice with prewarmed HBSS, and particles diluted in complete medium containing 1% P/S were added at a concentration equivalent to 1 μ g mRNA per well. After a further 24 h of incubation, cells were washed twice with HBSS and detached using 100 μ L Trypsin/EDTA for 5 min at 37 °C. Trypsinization was stopped by adding 900 μ L of complete medium, and the cell suspension was transferred to FACS tubes. Cells were centrifuged at 300 $\times$ g for 5 min, the supernatant was discarded, and the pellet was washed with 1 mL PBS. Following a second centrifugation step, cells were resuspended in 200 μ L PBS and analyzed by flow cytometry (BD LSR Fortessa Cell Analyzer, BD Biosciences, Heidelberg, Germany). Data were processed using FlowJo software (v10.8.1, FlowJo LLC, OR, USA).

3.1.2 Results and Discussion

3.1.2.1 Polymer-Based Nanocarriers

Initial experiments aimed to develop a ternary polymeric nanoparticle system, with a core assembled from a cationic polymer and an excess of anionic polymer. Core formation was carried out using a microfluidic platform, enabling controlled mixing conditions. Where applicable, nucleotides were incorporated into the core by replacing defined percentages of the anionic polymer, normalized based on the number of negatively charged functional groups. In a subsequent step, the overall negatively charged core was coated with a second, structurally distinct cationic polymer, also using the microfluidic system. A schematic representation of the process is shown in Figure 3A.

For the cationic component, chitosan was selected due to its biological origin (chitin-rich shells of crustaceans, fungal cell walls) and proven utility in gene delivery applications (Abo Elsoud and El Kady 2019; Soliman et al. 2020; Lallana et al. 2017;

Karayianni et al. 2023). It was mixed with hyaluronic acid (Hya), a negatively charged biopolymer known for its role in mucosal vaccine delivery (Dedloff et al. 2019), used here in stoichiometric excess to ensure net anionic charge of the core. To enhance particle stability and reduce core size, ionic crosslinkers – specifically zinc sulfate and TPP – were added to induce electrostatic condensation.

Following core formation, surface coating with a secondary cationic polymer, such as PLL, was employed to invert the zeta potential from negative to positive values, a prerequisite for surface loading of a second nucleotide layer. However, initial studies revealed that the ternary nanoparticle system exhibited poor colloidal stability in buffered media (e.g., RPMI). Within 5 minutes, the hydrodynamic diameter of the particles increased dramatically, from approximately 200 nm to about 600 nm, indicating rapid aggregation (Figure 4A).

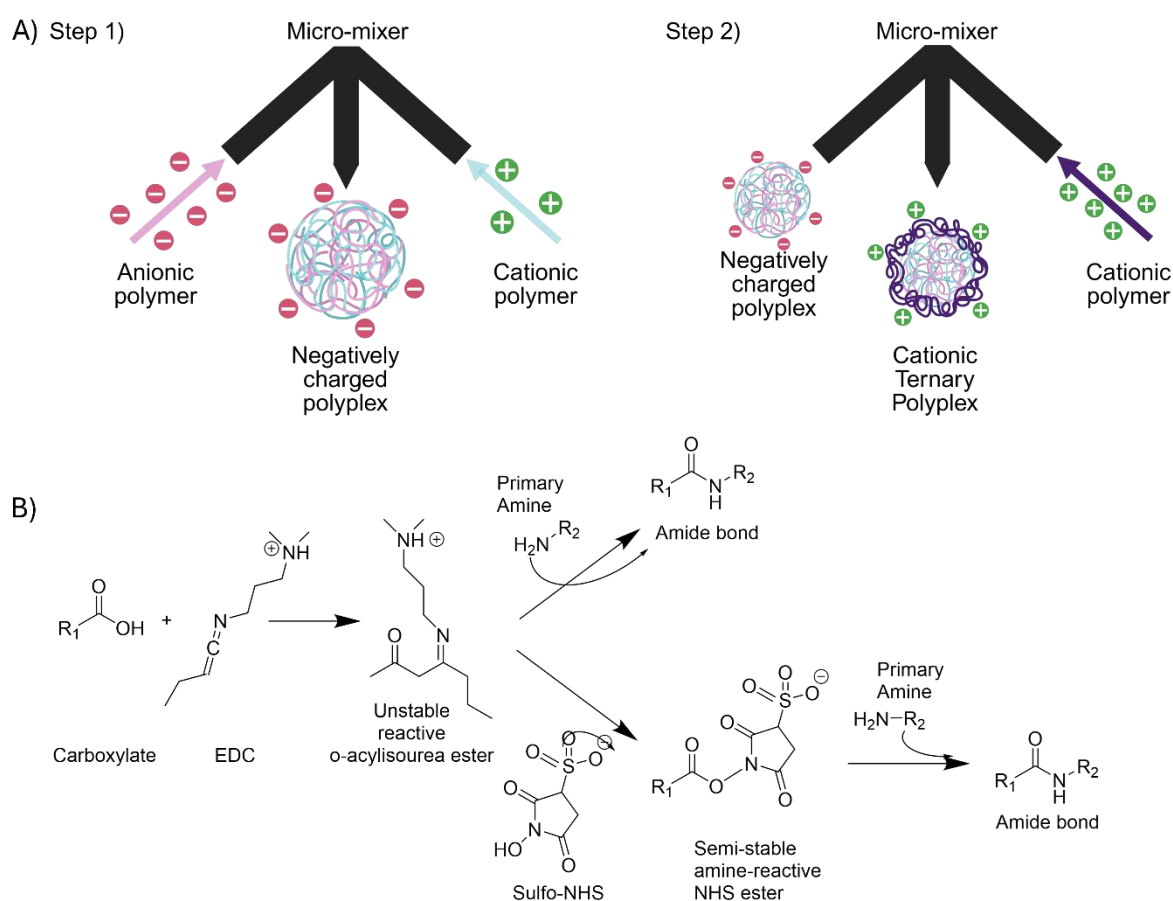


Figure 3: (A) Schematic process of ternary polyplex formation. (B) EDC/S-NHS cross-linking of carboxylic acids and primary amines

To address this issue, a covalent crosslinking strategy was explored. Specifically, EDC/S-NHS chemistry was used to couple primary amines of PLL with carboxylic acid

groups on Hya, forming amide bonds to stabilize the particle shell. This chemistry is commonly used to functionalize Hya with amino acids (Fan et al. 2015). The carboxylic acid groups were activated using EDC in the presence of S-NHS. The crosslinking mechanism is depicted in Figure 3B. This approach resulted in marked improvement in particle stability over a 1-hour incubation period in RPMI; however, longer size retention below 250 nm remained unsatisfactory (Figure 4B).

Alternative crosslinking strategies were subsequently evaluated. Glutaraldehyde-mediated crosslinking of primary amines on the particle shell further improved stability, but introduced significant cytotoxicity in DC 2.4 cells. Efforts to remove unreacted glutaraldehyde via dialysis were ineffective, as the crosslinking reaction is reversible and concentration-dependent, and dialysis ultimately reversed the crosslinking.

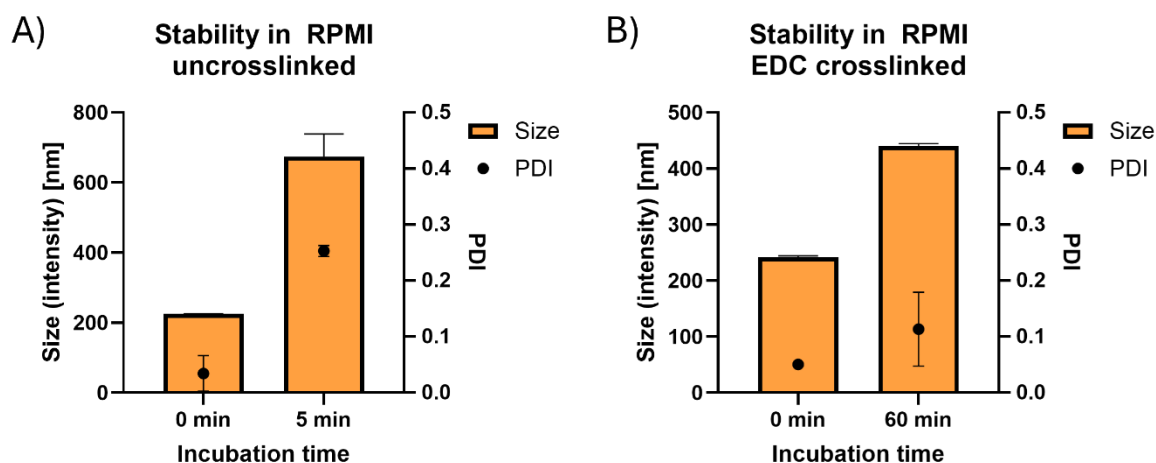


Figure 4: Particle size (intensity) and PDI of (A) non-crosslinked and (B) EDC crosslinked poly-L-lysine coated Chitosan-Hyaluronic acid polyplexes

Given the limitations of covalent crosslinking, a different strategy was pursued. The particle cores were coated with polymers bearing permanently cationic functional groups, designed to provide pH-independent positive charge and enhance colloidal stability in physiological buffers. While this approach yielded some improvements in stability, it was still insufficient to maintain the desired nanoscale particle size over extended incubation periods. Replacing the anionic component by polyA, a nucleotide surrogate with a low pKa value of approximately 1, did not significantly improve the stability either. Therefore, the focus was shifted from a ternary polymeric system towards a lipid-polymer hybrid system.

3.1.2.2 Lipid-Polymer Hybrid Nanocarriers

Lipid-polymer hybrid particles consisting of polyionic polymers surrounded by a lipidic shell are mostly referred to as lipopolyplexes (LPPs). The cationic nature of both the polymeric core and the lipid shell theoretically permits the simultaneous loading of two distinct mRNAs – one within the core and one on the particle surface. However, to date, no published system has successfully demonstrated this dual-loading strategy.

To explore this concept, cores composed of pDMAEMA and Hya were coated with a lipid mixture consisting of DOTAP, DOPE, DSPE-PEG, and cholesterol in a molar ratio of 29.25:29.25:1.5:40. DLS measurements confirmed the formation of homogeneous particles with a mean diameter of ~200 nm and a positive zeta potential of approximately +40 mV. These particles remained fully stable over the tested time interval of 90 minutes at 37 °C (Figure 5A). Cryo-TEM confirmed a well-defined core-shell architecture, with particles ranging from 50-300 nm in diameter (Figure 5B). Additionally, small empty liposomes <50 nm were observed. To evaluate their functionality, the LPPs were loaded with eGFP mRNA in the core and mCherry mRNA on the shell. Transfection experiments were performed in DC 2.4 cells, using 500 ng of each mRNA per well in a 24-well over 21 hours. Lipofectin, a commercially available transfection reagent, served as a positive control. Fluorescence microscopy revealed that the LPPs failed to induce detectable transfection after 21 hours, whereas Lipofectin enabled robust expression of both mRNAs within 4 hours (data not shown).

In response to these initial failures, the LPP formulation was gradually optimized, including variations in the core polymer composition and the lipid coating (e.g., cationic, ionizable, and PEGylated lipids). A notable improvement in transfection efficiency – up to 95 % dual mRNA expression in DC 2.4 cells – was ultimately achieved using a core consisting of protamine complexed with polyA. This was coated with a lipid mixture based on a formulation described by Kliesch et al., comprising DOTMA, DOPE, and DMG-PEG in a molar ratio of 30:70:1.5.

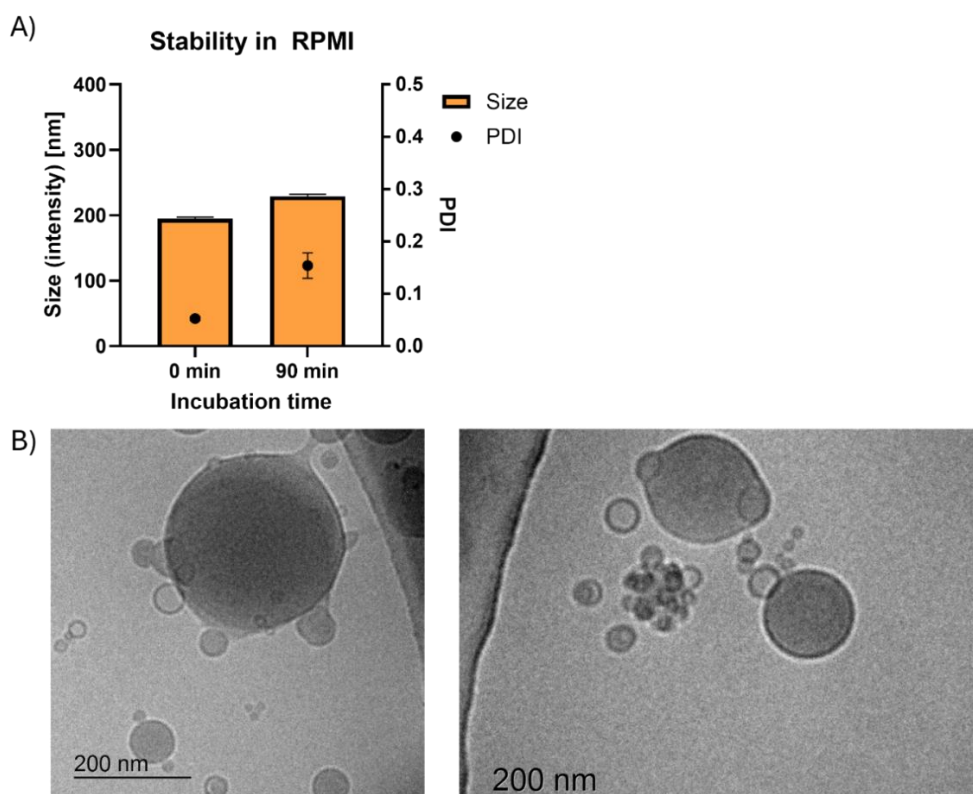


Figure 5: (A) Particle size (intensity) and PDI of lipid-coated pDMAEMA-Hya polyplexes after 0 and 90 min incubation in RPMI, respectively. Error bars indicate standard deviation (B) Cryo-TEM images of lipid coated pDMAEMA-Hya polyplexes at different magnifications.

This optimized formulation was subsequently used to investigate how the spatial distribution of mRNA within the particle (core vs. shell) influences transfection efficiency and expression kinetics. However, cryo-TEM imaging revealed a major structural inconsistency: the expected core-shell structure was absent. Instead, the images showed coexisting populations of polyplexes and liposomes, indicating a failure to assemble into true hybrid particles (Figure 6A).

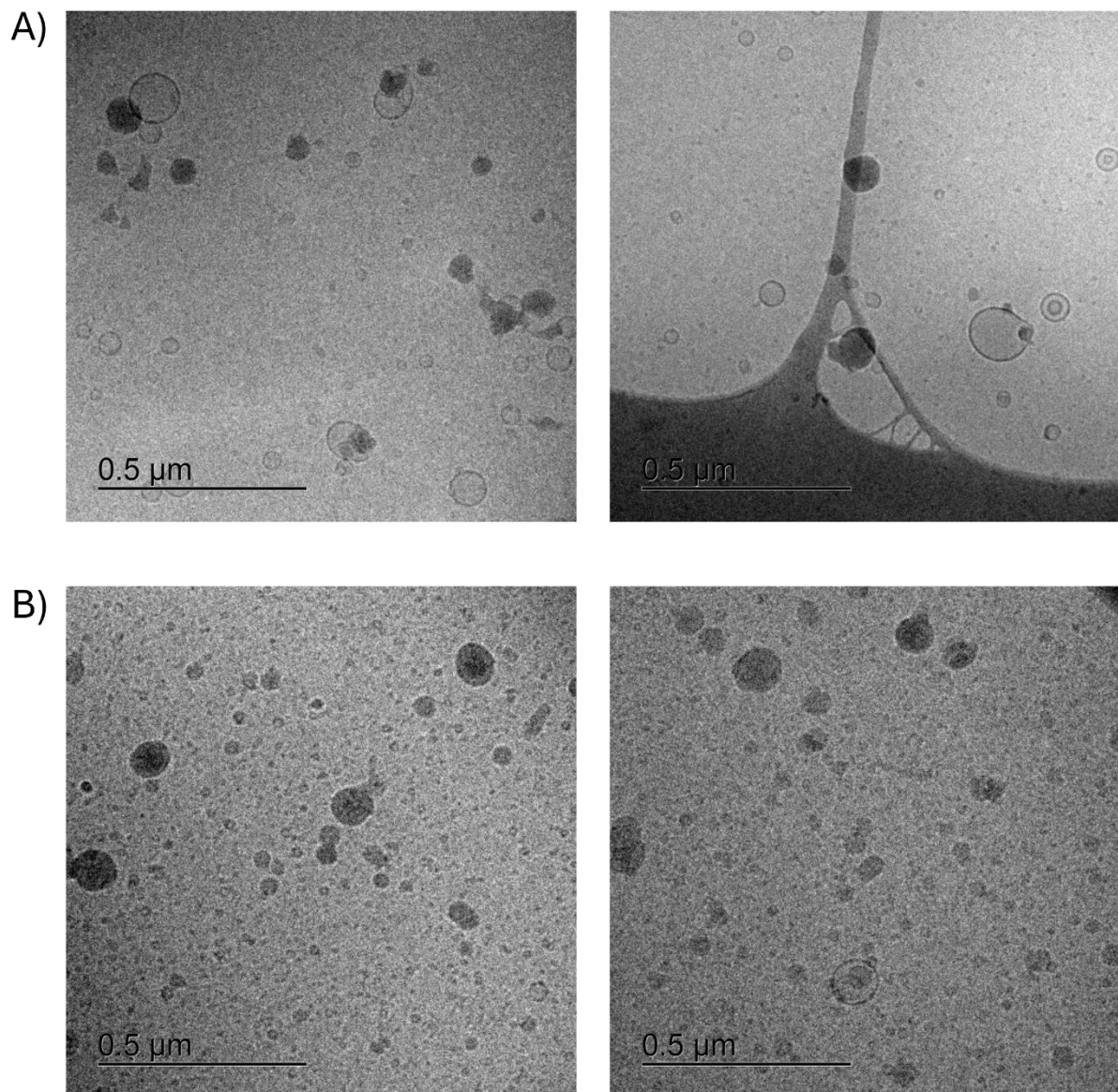


Figure 6: (A) Cryo-TEM images of coexisting liposomes and polyplexes in the DOTMA DOPE DMG-PEG formulation. (B) Cryo-TEM images of lipid-coated polyplexes in the DOTMA DMG-PEG formulation.

This phenomenon was hypothesized to result from electrostatic repulsion between the phosphate groups of polyA and those of DOPE, leading to poor lipid binding and incomplete shell formation (Arnott et al. 2018). To mitigate this, a simplified lipid mixture comprising only DOTMA and DMG-PEG (100:1.5 molar ratio) was used for coating. Although this modification resulted in reduced transfection efficiency, it successfully restored the core-shell architecture, as confirmed by cryo-TEM (Figure 6B). This prototype then served as the basis for a comprehensive optimization and characterization effort involving multiple LPP formulations using the same simplified lipid coating.

3.2 Lipopolyplex Optimization for Dual-mRNA Delivery

To optimize the developed LPP prototype for dual mRNA delivery, the influence of core polymers and their combinations on transfection efficiency was first evaluated in a systematic screening. Based on these results, three promising prototypes were selected for further investigations addressing the impact of mRNA localization within the particle on stability, transfection efficiency, expression kinetics, and performance during nebulization. The complete results of this study were published in the Journal of Controlled Release and are presented in Section 5.2.2 of this thesis. The key findings are summarized below.

3.2.1 A novel lipopolyplex platform for dual mRNA delivery via core and surface loading

3.2.1.1 Essential results

A total of 18 core compositions (six cationic polymers combined with three anionic polymers) were screened. The resulting LPPs exhibited comparable physicochemical, with hydrodynamic diameters of approximately 150 nm and zeta potentials around +45 mV. From each anionic polymer group, the formulation with the highest transfection efficiency was selected for detailed analysis: PLL-polyA, Prot-Hya, and poly-L-arginine (PLA) – poly-L-glutamic acid (poGA).

Under refrigerated storage, all three LPP prospects maintained colloidal stability over 12 weeks. However, transfection efficiency and/or fluorescence intensity declined significantly after 12 weeks for all formulations, irrespective of whether mRNA was loaded in the core or on the surface. Similarly, *in vitro* expression kinetics showed no measurable influence of mRNA localization on the temporal profile of protein expression. However, it influenced the overall expression magnitude, with core-loaded mRNA reaching only 30-50% of the maximum fluorescence intensity achieved by the corresponding surface-loaded mRNA.

A marked difference in the effect of mRNA localization emerged during nebulization studies. While all formulations largely retained their structural integrity after vibrating-mesh nebulization, core-loaded LPPs completely lost their transfection capability. In contrast, surface-loaded LPPs preserved full functionality post-nebulization.

Finally, the capacity of LPPs to deliver two distinct mRNAs to the same cell in a defined ratio was assessed. DC 2.4 cells were transfected with dual-loaded LPPs containing eGFP mRNA in the core and mCherry mRNA on the surface. As a control, cells received a mixture of single-loaded LPPs, each surface-loaded with either eGFP or mCherry mRNA. Quantitative confocal microscopy analysis revealed significantly higher colocalization rates (approaching 100 %) for dual-loaded LPPs in two of the three LPPs. Interestingly, the PLL-polyA formulation also achieved a high colocalization rate in the mixed single-loaded control, resulting in no statistically significant difference from dual delivery, although CLSM images indicated less homogenous transfection.

3.2.1.2 Discussion and impact

Among the three anionic polymers evaluated, Hya was clearly the least effective for *in vitro* mRNA transfection. However, no single formulation emerged as a clear overall winner among the 18 combinations, suggesting combinatorial effects between the two components. Such effects may arise from differences in functional group spacing, which can influence both particle stability and nucleotide binding affinity (Prevette et al. 2010).

The LPPs demonstrated excellent colloidal stability for at least 12 weeks under refrigerated storage; thus, the marked decline in transfection efficiency observed thereafter is most likely due to intramolecular transesterification of the mRNA. This interpretation is supported by the absence of differences between core- and surface-loaded mRNA, making enzymatic degradation an unlikely cause. For surface-loaded particles, this limitation can be mitigated by separate storage of the carrier and mRNA under optimized conditions, followed by on-demand loading. In contrast for core-loaded LPPs, long-term stability would require frozen storage or lyophilization (Khan et al. 2025).

Attempts to alter expression kinetics through polymer choice were unsuccessful, and the lower performance of core-loaded mRNA likely results from incomplete release. Rational, fine-tuned cationic polymer design could address both the expression kinetics and release inefficiency. The most pronounced effect of mRNA localization was observed during nebulization: core-loaded mRNA completely lost functionality, whereas surface-loaded mRNA maintained full transfection efficiency. This difference

is likely attributable to the distinct modes of complexation. Rigid mRNA-polymer complexes in the core are more susceptible to shear-induced strand breaks, whereas the looser surface association may preserve secondary structures, enhancing resistance to mechanical stress.

Importantly, the novel LPP platform enables precise, ratio-controlled co-delivery of two distinct mRNAs to the same target cell. By spatially separating cargos via independent core- and surface-loading, this core-shell architecture overcomes the co-encapsulation challenges faced by conventional LNPs while maintaining carrier stability and functional performance. Such controlled stoichiometry at the single-cell level is particularly valuable for advanced applications such as CRISPR/Cas9 gene editing and multiple antigen vaccination, where synchronized intracellular delivery of multiple nucleic acids is critical to therapeutic success (Lin et al. 2022; Ma et al. 2023).

3.2.2 Resistance of LPPs to Physical and Storage Stresses

As discussed previously, both mechanical and storage stability remains a major limitation of currently approved LNPs for mRNA delivery. To address this issue, we conducted a study investigating the effect of particle type on the stability of both the carrier and its nucleic acid payload during storage, pharmaceutical processing, and agitation. In this study, we compared conventional LNPs, lipid-polymer hybrid particles (LPHs) containing a hydrophobic PLGA core, and LPPs to investigate the hypothetical stabilizing effect of core-shell structures. To ensure comparability, a common lipid mixture (differing from the lipid-coating presented in 3.2.1) was used across the particle types. The complete results of this investigation will be presented in a forthcoming journal publication. The following section summarizes selected, adapted content from the work, focusing on the comparison of LPPs to LNPs formulated with the identical lipid mix. The results were obtained in collaboration with Vesa Kopliku and Achim Biesel and will be published as “Overcoming mechanical stress: Lipopolyplexes show superior stability and mRNA transfection efficiency compared to Lipid nanoparticles” in “Drug Delivery and Translational Research”.

3.2.2.1 Materials and Methods

3.2.2.1.1 Materials

CleanCap™ eGFP mRNA (5moU) was purchased from TriLink BioTechnologies LLC (San Diego, CA, USA). DOTAP, DOPE, DC-Cholesterol and DOTMA were purchased from Avanti Polar Lipids Inc (Alabaster, AL, USA). D-Lin-MC3-DMA and DMG-PEG 2000 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Cholesterol, poly-L-arginine hydrochloride (15-70 kDa), poly-L-glutamic acid sodium salt (50-100 kDa), poly adenylic acid potassium salt and sucrose were purchased from Sigma-Aldrich (Darmstadt, Germany).

3.2.2.1.2 Particle preparation

All particles were prepared with the identical lipid mix consisting of DOTAP, D-Lin-MC3-DMA, DOPE, DC-Cholesterol, and DMG-PEG in a molar ratio of 50:30:10:8.5:1.5.

LPPs were prepared in a two-step process. First, a 69.8 µg/mL solution of poly-L-arginine was combined with a mix of poly-L-glutamic acid (53.6 µg/mL) and eGFP

mRNA (125 µg/mL) in a herringbone micromixer. Polyplexes (PPs) were formed at a positive to negative charge ratio of 1:2. For nucleotide loading, 25 % of the negative charges of poGA were replaced by mRNA. For calculations, all ionizable functional groups were considered charged. A total flowrate of 2000 µL/min was used. In a second step, the PPs were mixed with an ethanolic lipid solution in a cross-shaped micromixer via hydrodynamic focusing of the lipid stream at a flow rate ratio of 10:1 with a total flow rate of 220 µL/min. Following manufacturing, LPPs were dialyzed in ultra-purified water for two hours using Pur-A-Lyzer™ Midi dialysis tubes (Sigma-Aldrich, Darmstadt, Germany).

For the preparation of LNPs, the aforementioned lipids were dissolved in ethanol. Cholesterol was added to the ethanolic mixture at a ratio of 1:2 (w/w) to enhance stability. The organic phase was mixed with a 33.3 µg/mL solution of eGFP mRNA in 50 mM pH 4.0 citrate buffer at a 1:3 ratio (v/v) using two peristaltic pumps (Standard Infuse/Withdraw PHD ULTRA™ Syringe Pumps, Holliston, MA, USA) and a herringbone micromixer (Fluidic 187, microfluidic Chipshop GmbH, Jena, Germany). A total flowrate of 1000 µL/min was used.

3.2.2.1.3 Physicochemical particle analysis

Hydrodynamic particle size, polydispersity index (PDI) and zeta potential of nanoparticles were determined using dynamic light scattering (DLS; Zetasizer Nano, Malvern Instruments, Malvern, UK). To obtain optimal signal strength, particles were diluted 2.5-fold in ultra-purified water prior to measurements.

3.2.2.1.4 Particle visualization

Prior to Cryo-TEM analysis, particle suspensions were concentrated by centrifugation at $3,850 \times g$ using an Amicon Ultra Centrifugal Filter with a 100 kDa molecular weight cutoff membrane (Merck KGaA, Darmstadt, Germany). Centrifugation was performed in discrete time intervals until the target concentration was achieved.

For Cryo-TEM analysis, 2 µL of a 10× concentrated particle solution was applied to a holey carbon support TEM grid (type 147-4; Plano, Wetzlar, Germany), blotted for 2 seconds and plunge-frozen in undercooled liquid ethane using a Gatan CP3 plunge-freezer (Pleasanton, CA, USA) at -165°C. The sample was subsequently transferred to a Gatan model 914 cryo-TEM sample holder and imaged using a JEM-2100 LaB6

TEM (JEOL, Akishima, Tokio, Japan) operated at 200 kV under low dose conditions. Images were acquired with a Gatan Orius SC1000 CCD camera using an exposure time of 2 seconds.

3.2.2.1.5 Cell culture

The human alveolar epithelial cell line A549 (American Type Culture Collection, CCL-185, Rockville, USA) was cultured in complete medium based on RPMI (Roswell Park Memorial Institute; 1640 1x, Ref 21875, Gibco/Life Technologies, Paisley, UK) supplemented with 10% FCS (fetal calf serum, Ref 10270106, Gibco, Paisley, UK) and cultivated in a humidified incubator at 37°C with 5% CO₂. Cells were passaged weekly up to passage 25. Throughout routine cultivation, the culture medium remains free of P/Sto prevent potential effects on cellular behavior. However, 1% P/S is added to the medium during transfection assays to mitigate the risk of bacterial contamination from non-sterile particle formulations.

3.2.2.1.6 Transfection studies

25,000 A549 cells were seeded in 200 µl of cultivation medium in 96-well plates (Ref 650160, Greiner Bio-One GmbH, Frickenhausen, Germany) and incubated for 24 h (70 % confluency) at 37 °C and 5 % CO₂. Then, cells were washed twice with prewarmed HBSS and particles diluted in complete medium (+1 % P/S) were added to the cells at concentrations correlating to 1 µg/mRNA/well. After 24 h, transfection efficiency was assessed via fluorescence imaging using the Tecan SparkCyto. Quantitative image analysis was performed using the Tecan ImageAnalyzer software (Tecan Trading AG, Switzerland).

3.2.2.1.7 Mechanical Robustness

A 200 µL suspension of nanoparticles or an equivalent mass of freeze-dried particles was placed in a 2 mL reaction tube and subjected to mechanical stress by vortexing at 3000 rpm for up to 500 seconds using a vortex mixer (Vortex-Genie 2, Scientific Industries, Bohemia, NY, USA). Following vortexing, particles were analyzed for colloidal stability (3.2.2.1.3) and transfection efficiency (3.2.2.1.6).

3.2.2.1.8 Freeze Drying

Particles were freeze-dried in 2 mL Eppendorf Tubes® using 10% (w/v) sucrose (Sigma-Aldrich, Darmstadt, Germany) as a cryoprotectant. Initially, samples were

snap-frozen in liquid nitrogen and immediately transferred to a Christ Alpha 2-4 LSC freeze dryer (shelf: Lyocube 4-8, Martin Christ Freeze-Dryers, Osterode, Germany). Primary drying was performed overnight at a shelf temperature of -16°C under a pressure of 0.25 mbar. This was followed by a secondary drying phase at 25°C and 0.009 mbar to remove residual moisture. Following freeze-drying, particles were assessed for colloidal stability (3.2.2.1.3), mechanical robustness (3.2.2.1.7), and transfection efficiency (3.2.2.1.6).

3.2.2.2 Results and Discussion

To ensure comparability, all particle types were prepared using the same lipid mixture described in 3.2.2.1.2. The resulting LPPs exhibited an average hydrodynamic diameter of approximately 100 nm and a positively charged surface, with a zeta potential of around +35 mV. LNPs displayed a similar hydrodynamic diameter in DLS measurements but appeared smaller in cryo-TEM images (Figure 7A&C). Cryo-TEM analysis further indicated a core-shell morphology for the LPPs with the newly applied lipid coating (Figure 7B).

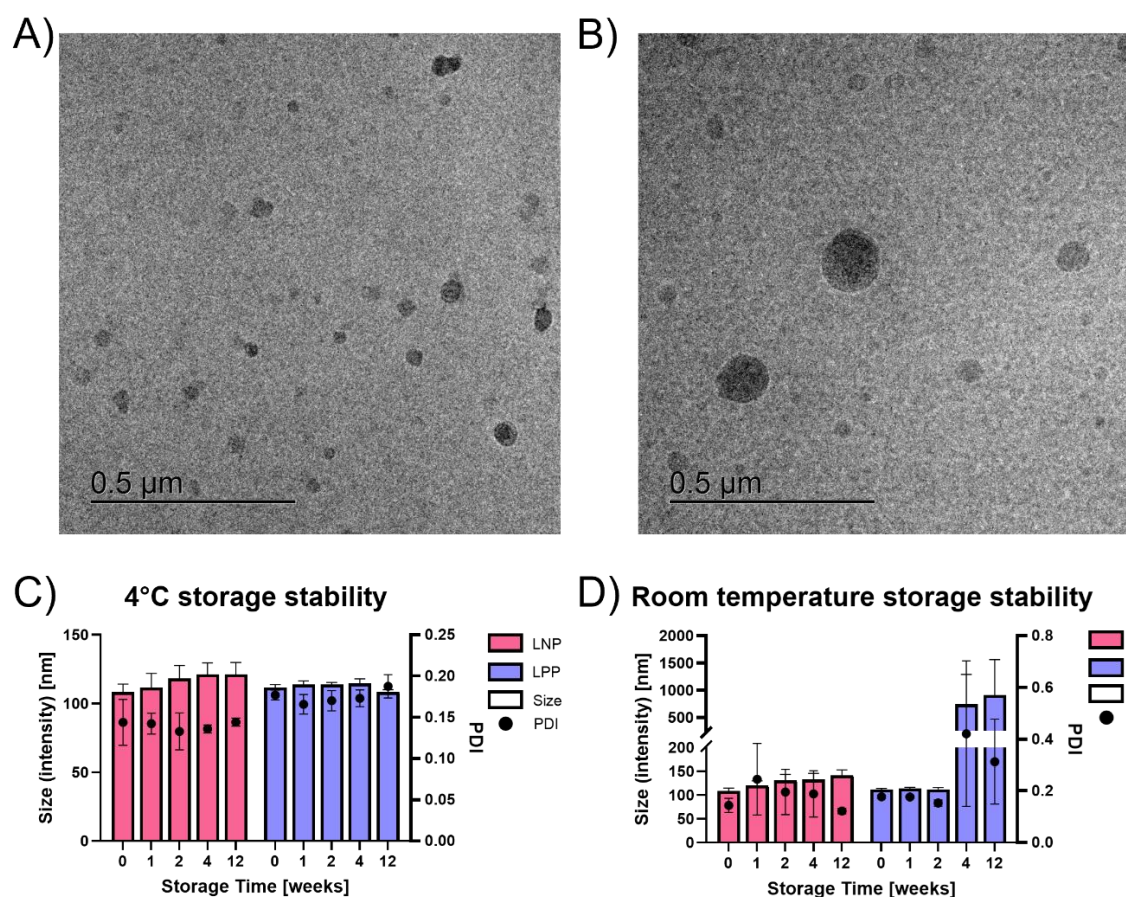


Figure 7: Cryo-TEM images of (A) LNPs and (B) LPPs; Storage stability of LNPs (pink) and LPPs (blue) at 4°C (C) and room temperature (D) over 12 weeks, respectively. Error bars indicate standard deviations.

The altered lipid composition did not compromise storage stability under refrigerated conditions when compared to the LPPs described in section 3.2.1, as the particles remained stable over a 12-week period (Figure 7C). In contrast, LPPs stored at room temperature displayed pronounced aggregation after four weeks, indicating limited stability under ambient conditions (Figure 7D), whereas LNPs remained stable under both refrigerated and ambient conditions throughout the observation period.

Freeze-drying was investigated as a strategy to enhance storage stability. Several sugars and polymers were screened as cryoprotectants for their ability to preserve particle size following lyophilization. Based on preliminary experiments, 10 % (w/v) sucrose was selected, as it effectively limited the increase in particle size after reconstitution to approximately 20 nm (Figure 8A). LNPs, which were included as a comparative control, were processed under identical conditions and showed a more pronounced increase in hydrodynamic diameter, by approximately 60 nm.

LPPs and LNPs encapsulating eGFP mRNA were subsequently evaluated for transfection efficiency in A549 cells before and after lyophilization. After 24 h, transfected cells were analyzed by fluorescence microscopy followed by quantitative image analysis. As shown in Figure 8B & C, lyophilization of LPPs with 10 % sucrose did not reduce transfection efficiency compared to freshly prepared particles. Lyophilized LNPs exhibited a decrease in transfection efficiency from approximately 40% to 25% relative to untreated LNPs. To further assess long-term stability, transfection experiments were repeated after 12 weeks of storage, with particles stored either in suspension at 4 °C or in a lyophilized state at room temperature. Both LPP formulations exhibited reduced transfection efficiency and intensity compared to freshly prepared controls. However, lyophilized samples were more severely affected than those stored in suspension (Figure 8B & C). This reduced performance is most likely attributable to incomplete water removal during lyophilization and accelerated mRNA hydrolysis at room temperature compared to storage in solution under refrigerated conditions (Muramatsu et al. 2022). LNPs, both in solution and freeze dried were affected more substantially than LPPs, with freeze-dried LNPs exhibiting an almost complete loss of function after 12 weeks of storage.

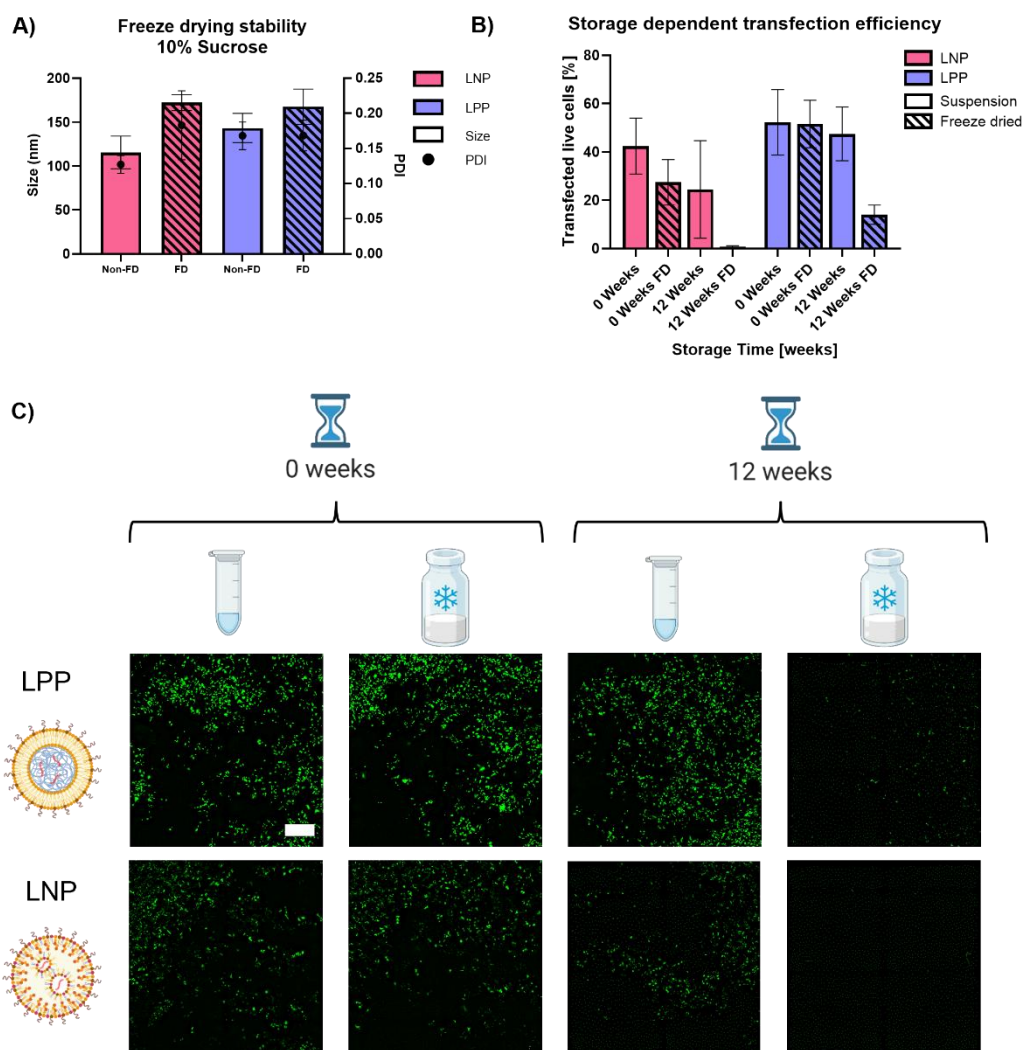


Figure 8: (A) LNP (pink) and LPP (blue) size (intensity) and PDI before and after lyophilization with 10% Sucrose. (B) Transfection efficiency of eGFP-loaded LNPs and LPPs in suspension and freeze-dried in A549 cells, after 0 and 12 weeks, respectively. (C) Fluorescence microscopy images of A549 cells transfected with eGFP-loaded LNPs and LPPs in suspension and freeze-dried, after 0 and 12 weeks, respectively. Error bars indicate standard deviations.

The effect of mechanical stress on particle integrity was also investigated. Particles were vortexed for 30, 100, 300, and 500 s in either suspension or lyophilized states. Both particle types behaved similarly: in suspension, particle size remained stable up to 300 s of vortexing (Figure 9A), whereas lyophilized particles retained stability for at least 500 s (Figure 9B). For functional evaluation, particles, both in suspension and lyophilized, were vortexed for 0, 30, or 100 s prior to transfection. Unlike LNPs, mechanical stress did not impair the transfection efficiency of LPPs in either state, which consistently remained at approximately 50 % (Figure 9C & D). This indicates stronger mRNA encapsulation compared to LNPs. This is in concordance with the findings of Ruppl et al., that showed significant loss of mRNA surrogate in LNPs already after 30 s vortexing (Ruppl et al. 2024). LNPs showed greater variation in

transfection efficiency overall and reduced efficacy upon vortexing, especially in solution.

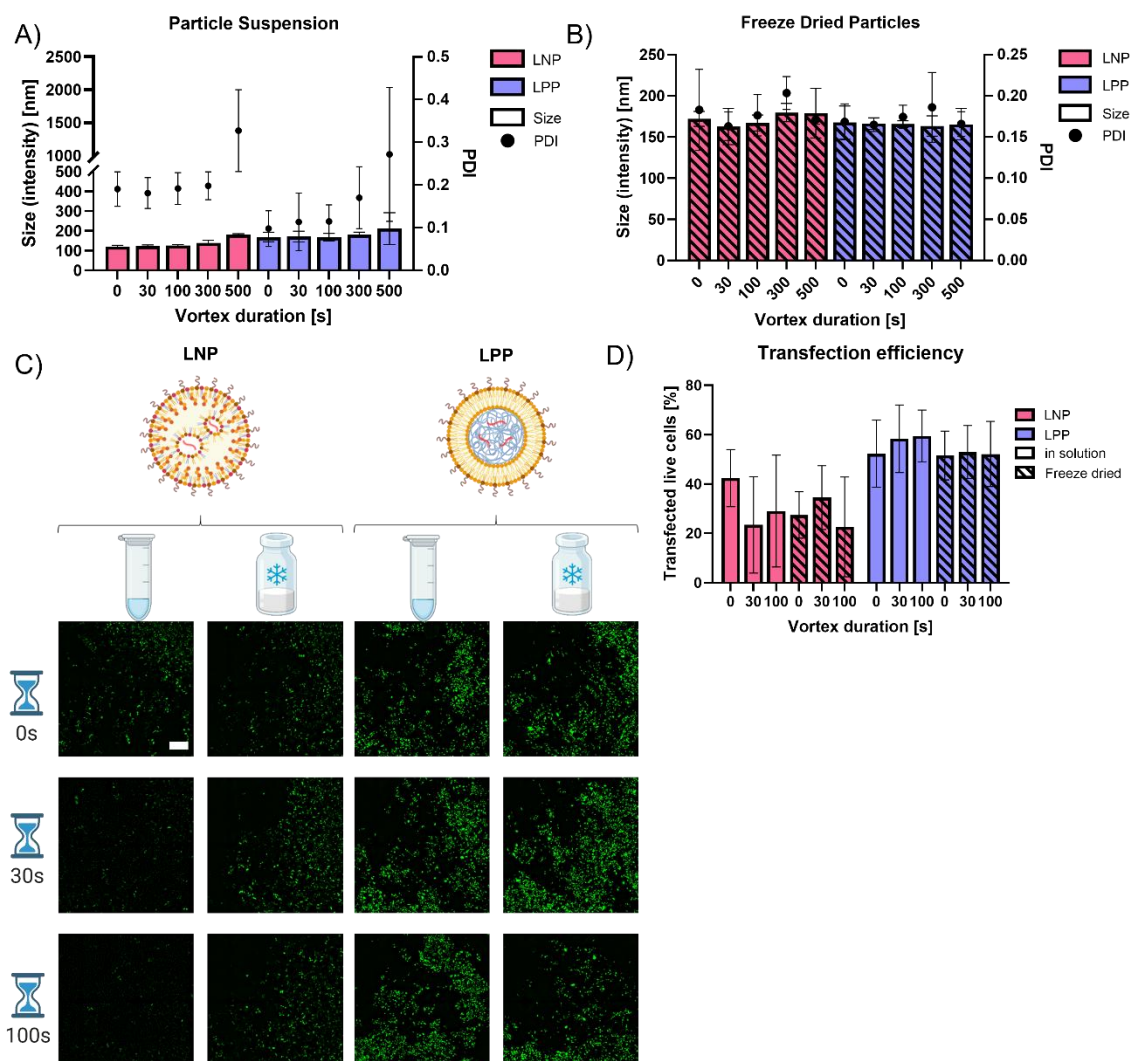


Figure 9: Particle size (intensity) and PDI of (A) LNPs (pink) and LPPs (blue) in suspension and (B) freeze-dried LNPs and LPPs after 0, 30, 100, 300 and 500 s of vortexing, respectively. (C) Fluorescence microscopy images of A549 cells transfected with eGFP-loaded LNPs and LPPs in solution or freeze dried after 0, 30 and 100 s of vortexing, respectively. Error bars indicate standard deviations.

In summary, the LPPs studied here demonstrated high resistance to mechanical stress, tolerance to process stress during freeze-drying, and overall good storage stability under refrigerated conditions. They outperformed LNPs consistently in transfection efficiency under all tested conditions. Unexpectedly, lyophilization with 10% sucrose did not provide an advantage in mid-term stability compared to particles stored in suspension, suggesting that further optimization of lyophilization protocols or excipient selection is required. Additionally, long-term stability studies may be required to point effectively show improved stability of freeze-dried carriers over particles in solution.

3.3 Evaluation of Endogenous Adjuvants for Mucosal Vaccination

The LPPs were designed to deliver both antigen and adjuvant of a vaccine as mRNA. While antigen selection is directly determined by the target pathogen, the use of endogenous immune factors as adjuvants remains insufficiently studied, making their selection more challenging. Having DCs as primary target cells, CCR7 and CCL21 were selected as potential adjuvants based on previously published work. CCR7 is a G-protein-coupled transmembrane receptor naturally present on the surface of maturing DCs. It plays a central role in the homing of peripheral DCs towards lymph nodes. It was previously shown that CCR7-overexpressing DCs accumulate up to 5.5-fold more in draining lymph nodes (Okada et al. 2005). Co-delivery of ovalbumin and CCR7 pDNA increased costimulatory molecule expression and strong inhibition of tumor growth (Yang et al. 2018). CCL21 is a natural ligand of CCR7 whose ability to recruit immune cells towards the site of immunization was proven multiple times (Gunn et al. 1998; Britschgi et al. 2010). Sabbaghi et al. propose it as a strong mucosal adjuvant, albeit only in combination with poly(I:C) (Sabbaghi et al. 2021). In this work, we aimed to identify a suitable adjuvant specifically for a novel delivery approach in which both adjuvant and antigen are encoded as mRNA both within a single carrier.

3.3.1 Materials and Methods

3.3.1.1 Materials

Capped CCR7 mRNA without nucleoside modifications was custom synthesized by Ribopro (Oss, Netherlands) based on the sequence in Table 1. Information about the codon-optimization for expression in mice is proprietary information.

CCR7 and CCL21 mRNA with 100 % N1-methylpseudouridine substitution and Cap1 capping was custom synthesized by GenScript (Rijswijk, Netherlands) based on the codon-optimized sequences displayed in Table 1.

Mouse CCR7 PE-conjugated antibody was purchased from R&D Systems (Minneapolis, Minnesota, USA). Mouse Seroblock FcR was purchased from BIO-RAD (Feldkirchen, Germany). Corning™ Costar™ Transwell™ permeable supports (8 µm pore size, 24-well format) were purchased from fisher scientific (Schwerte, Germany).

CyQUANT™ cell proliferation assay, Mouse CCL21 (Exodus-2) ELISA Development Kit (ABTS), and non-sterile immune 96-well-plates were purchased from ThermoFisher Scientific (Darmstadt, Germany). ABTS™-solution was purchased from Merck (Darmstadt, Germany).

Table 1: Sequences of custom-synthesized mRNAs

3.3.1.1.1 CCR7 sequence for Ribopro mRNA
5'UTR+ ATGTATTCTGTCATCTGCTTCGTGGGCCTGCTCGGCAACGGGCTGGTGATACTGACG TACATCTATTTCAAGAGGCTCAAGACCATGACGGATACCTACCTGCTCAACCTGGCCG TGGCAGACATCCTTTTCCTCCTAATTCTTCCCTTCTGGGCCTACAGCGAAGCCAAGTC CTGGATCTTTGGCGTCTACCTGTGTAAGGGCATCTTTGGCATCTATAAGTTAAGCTTC TTCAGCGGGATGCTGCTGCTCCTATGCATCAGCATTGACCGCTACGTAGCCATCGTC CAGGCCGTGTCTGGCTCATCGCCACCGCGCCCGCGTGCTTCTCATCAGCAAGCTGTC CTGTGTGGGCATCTGGATGCTGGCCCTCTTCCTCTCCATCCCGGAGCTGCTCTACAG CGGCCTCCAGAAGAACAGCGGCGAGGACACGCTGAGATGCTCACTGGTCAGTGCCC AAGTGGAGGCCTTGATCACCATCCAAGTGGCCAGATGGTTTTTGGGTTCCTAGTGC CTATGCTGGCTATGAGTTTCTGCTACCTCATTATCATCCGTACCTTGCTCCAGGCACG CAACTTTGAGCGGAACAAGGCCATCAAGGTGATCATTGCCGTGGTGGTAGTCTTCAT AGTCTTCCAGCTGCCCTACAATGGGGTGGTCCTGGCTCAGACGGTGGCCA ACTTCAACATCACCAATAGCAGCTGCGAAACCAGCAAGCAGCTCAACATTGCCTATGA CGTCACCTACAGCCTGGCCTCCGTCCGCTGCTGCGTCAACCCTTTCTTGTATGCCTT CATCGGCGTCAAGTTCCGCAGCGACCTCTTCAAGCTCTTCAAGGACTTGGGCTGCCT CAGCCAGGAACGGCTCCGGCACTGGTCTTCTGCCGGCATGTACGGAACGCGTCCG TGAGCATGGAGGCGGAGACCACCACAACCTTCTCCCCGTAG +3'UTR+150nt Poly(A)
3.3.1.1.2 CCR7 sequence for GenScript mRNA
5'UTR+Kozak+ AUGGACCCCGGCAAGCCUCGCAAGAACGUGCUGGUGGUGGCCUGCUGGUCAUC UUCAGGUGUGCUUUUGUCAGGAUGAGGUGACAGACGAUUACAUCGGAGAGAACA CCACAGUGGACUACACCCUGUACGAGUCCGUGUGCUUCAAGAAGGAUGUGCGCAA CUUCAAGGCUUGGUUUCUGCCACUGAUGUACUCUGUGAUCUGUUUCUGGGCCU GCUGGGCAACGGACUGGUCAUCCUGACAUAUCAUCUACUUUAAGAGGCUGAAGACC AUGACAGACACCUACCUGCUGAACCUGGCCGUGGCUGAUUAUCCUGUUCUGCUGA

UCCUGCCCUUUUGGGCCUACUCUGAGGCUAAGAGCUGGAUCUUCGGCGUGUACCU
GUGCAAGGGCAUCUUUGGAAUCUACAAGCUGAGCUUCUUUCCGGAAUGCUGCUG
CUGCUGUGCAUCAGCAUCGACAGAUACGUGGGCCAUCGUGCAGGCCGUGUCCGCUC
ACAGGCACAGAGCUCGGGUGCUGCUGAUCUCUAAGCUGAGCUGCGUGGGCAUCU
GGAUGCUGGGCCUGUUCUGAGCAUCCAGAGCUGCUGUACUCCGGCCUGCAGAA
GAACUCUGGAGAGGAUACACUGAGAUGUUCCUGGUGUCUGCCCAGGUGGAGGCU
CUGAUCACCAUCCAGGUGGGCCAGAUGGUGUUCGGAUUUCUGGUGCCCAUGCUG
GCUAUGUCCUUCUGCUACCUGAUCAUCAUCCGGACCCUGCUGCAGGCCCGCAACU
UUGAGAGGAACAAGGCCAUCAAAGUGAUCAUCCGCUGUGGUGGUGGUGUUAUCGU
GUUUCAGCUGCCUUACAACGGCGUGGUGCUGGGCCAGACAGUGGCCUAACUUAAC
AUCACAAACAGCUCCUGUGAGACCUCUAAGCAGCUGAACAUCCGUACGACGUGAC
CUACAGCCUGGCUUCCGUGAGAUGCUGCGUGAACCCUUUCCUGUACGCCUUUAUC
GGCGUGAAGUUUCGGUCUGACCUGUUAAGCUGUUUAAGGAUCUGGGAUGCCUGA
GCCAGGAGCGCCUGAGGCACUGGUCUAGCUGUAGACACGUGCGGAACGCCUCCG
UGUCUAUGGAGGCUGAGACCACAACCACAUUCUCCCCAUGA

+3'UTR+100nt Poly(A)

3.3.1.1.3 CCL21 sequence for GenScript mRNA

5'UTR+Kozak+

AUGGCCCAGAUGAUGACCCUGUCUCUGCUGAGCCUGGUGCUGGCUCUGUGCAUC
CCAUGGACACAGGGAUCCGACGGAGGAGGACAGGAUUGCUGUCUGAAGUACAGCC
AGAAGAAGAUCCCCUACUCCAUCGUGCGGGGAUACCGCAAGCAGGAGCCUUCUCU
GGGCUGCCCAAUCCCCGCCAUCCUGUUCUCCCCCGGAAGCACUCUAAGCCUGAG
CUGUGUGCUAACCCAGAGGAGGGCUGGGUGCAGAACCUGAUGAGGAGACUGGACC
AGCCACCUGCUCCUGGAAAGCAGAGCCCAGGCUGCAGGAAGAACAGAGGAACCAG
CAAGUCCGGCAAGAAGGGAAAGGGCUCUAAGGGCUGUAAGAGGACCGAGCAGACA
CAGCCAAGCAGGGGCUG

+3'UTR+100nt Poly(A)

3.3.1.2 Particle formulation

General manufacturing of LPPs was described earlier in section 3.1.1.2. Prot or PLA were used as cationic polymers, polyA and poGA served as the complementary anionic polymers. DOTMA and DMG-PEG were used as coating lipids.

3.3.1.3 Cell culture

Culturing of DC 2.4 cells was described earlier in 3.1.1.6.

A549 cells were maintained in complete medium based on RPMI supplemented with 10 % FCS and cultivated in a humidified incubator at 37 °C with 5 % CO₂. Cells were passaged weekly up to passage 25.

3.3.1.4 Transfection and Flow Cytometry

DC 2.4 cells were seeded at a density of 1×10^5 cells per well in 24-well plates. Cells were treated with Prot-polyA LPPs (Ribopro mRNA) or PLA-poGA LPPs (GenScript mRNA), either unloaded or loaded with CCR7 mRNA (0.5–1 µg per well), and incubated for 24 or 48 hours prior to analysis.

For flow cytometry, cells were washed twice with HBSS and detached with trypsin/EDTA for 5 minutes. Trypsinization was stopped with culture medium, and cells were collected by centrifugation (300 g, 5 min, room temperature). After washing once with PBS, cells were incubated with 1 µg IgG in 100 µl PBS for Fc-block at 37 °C for 15 minutes. PE-conjugated antibodies (anti-CCR7 or isotype control) were then added, and cells were incubated for an additional 15 minutes at 37 °C. Following two washes with PBS, cells were resuspended in 200 µl PBS and vortexed briefly prior to flow cytometric analysis. All centrifugation steps were performed at $300 \times g$ for 5 minutes at room temperature.

3.3.1.5 DC 2.4 migration assay

DC 2.4 cells were seeded at a density of 2×10^6 cells per T25 flask. The following day, cells were transfected in full culture medium containing 1% P/S. PLL-polyA LPPs were used to transfect Flask 1 with 7.5 µg CCR7 mRNA, Flask 2 with 7.5 µg polyA (mRNA surrogate), and Flask 3 with 7.5 µg CCL21 mRNA. Cells were incubated for 24 h post-transfection.

After 24 h, cells in Flasks 1 and 2 were washed once with PBS, detached with trypsin-EDTA for 5 minutes, and trypsinization was stopped with culture medium. Cells were collected, centrifuged at $300 \times g$ for 4 min, and resuspended in culture medium. For Flask 3, the supernatant containing secreted CCL21 was collected and centrifuged to remove any remaining cells. All cells were counted and diluted to 1×10^6 cells/mL.

For migration assays, 100,000 cells were seeded per transwell in 100 µL medium. Corresponding controls included wells without membranes as positive controls and wells without cells as negative controls. Migration was assessed in 24-well plates

containing 600 μ L of either medium, CCL21 at 200 or 400 ng/mL, or CCL21-containing supernatant. Plates were incubated for 4 h at 37°C.

After 5 h, cells adhered to the bottom of the transwells were loosened by dipping the transwell inserts in trypsin-EDTA for 5 minutes, then washed into their respective wells. Plates were centrifuged at 300 $\times$ g for 5 min to collect the cells at the bottom of the wells. To quantify migration, 600 μ L of 2 $\times$ CyQuant solution was added per well, incubated for 1 h at 37°C, and fluorescence was measured using a Tecan SparkCyto plate reader (excitation 500 nm, emission 530 nm).

3.3.1.6 Enzyme-Linked Immunosorbent Assay (ELISA)

DC 2.4 cells were seeded at 8×10^4 cells/well in 96-well plates in 200 μ L of full culture medium. The following day, cells were washed twice with HBSS and 200 μ L fresh medium was added. Cells were then transfected with DD PLL-polyA LPPs loaded with CCL21 mRNA at different doses. Loaded particles containing 4 μ g CCL21 mRNA per 70.8 μ L were added to deliver 100, 200, 300, or 400 ng mRNA per well, in triplicate, while untreated wells served as controls.

After 24 h, 20 μ L of supernatant was collected from each well and diluted 1:20 with ELISA diluent. CCL21 levels were quantified using the PeproTech Mouse CCL21 ELISA Development Kit according to the manufacturer's instructions. Thermo Scientific Nunc MaxiSorp surface plates were used, and absorbance was measured at 5-minute intervals until all readings reached 0.2–1.2, typically after 20–25 minutes.

3.3.2 Results and Discussion

CCR7 mRNA was codon-optimized for expression in murine cells and custom-synthesized by Ribopro with flanking 5' and 3' UTRs. To evaluate its functionality, DC 2.4 cells were transfected with PS-polyA LPPs surface loaded with CCR7 mRNA. Lipofectin, a commercially available transfection reagent, served as a transfection control. Stimulation with 20 ng/mL IFN- γ was included as a positive control, as it has previously been reported to induce CCR7 expression (He et al. 2007). Cells were transfected with either 0.5 μ g/well or 1 μ g/well CCR7 mRNA, harvested after 24 h, stained with a PE-conjugated CCR7 antibody, and analyzed by flow cytometry.

FACS-analysis confirmed a clear separation between stained and unstained untransfected controls (Figure 10A), validating the functionality of the CCR7 antibody.

However, no significant differences in CCR7 expression were detected between stained untransfected and transfected DC 2.4 cells, regardless of whether LPPs (Figure 10B) or Lipofectin (Figure 10C) were used. Furthermore, in contrast to previous findings, IFN- γ stimulation failed to induce CCR7 expression as well. This discrepancy may be explained by intrinsically high basal CCR7 expression in DC 2.4 cells, potentially limiting detectable upregulation.

Additional observations suggested a strong immunostimulatory effect of the CCR7 mRNA. Wells treated with CCR7-loaded particles showed reduced cell numbers and morphological changes, indicating cellular activation or stress. However, these effects were not quantitatively assessed. Such effects were not observed in experiments using base-modified reporter mRNAs, suggesting that the lack of chemical modifications contributed to immunogenicity.

To investigate whether high endogenous CCR7 expression in DC 2.4 cells masked functional mRNA expression, the experiment was repeated in A549 cells, which reportedly express CCR7 at much lower levels (Zhong et al. 2017). FACS analysis confirmed that untransfected A549 cells exhibited minimal CCR7 staining compared to DC 2.4 cells. Transfection of A549 cells with 1 μ g/well CCR7 mRNA resulted in detectable, though relatively modest, CCR7 expression compared to published examples (Figure 10D) (Carlsten et al. 2016).

Taken together, these findings suggest that both the high basal CCR7 expression in DC 2.4 cells and the lack of chemical modifications (such as capping and nucleoside substitutions) contributed to the low apparent functionality of the custom CCR7 mRNA. Therefore, the uncapped, unmodified mRNA design was found to be unlikely to be suitable for use as an adjuvant in mucosal vaccination strategies.

To address this limitation, newly synthesized CCR7 and CCL21 mRNAs were generated incorporating state-of-the-art modifications. In these constructs, all uridines were substituted with N1-methylpseudouridine, a modification also implemented in the well-known mRNA vaccines Comirnaty® and Spikevax® (Morais et al. 2021), and capped with GenScript's Cap1 technology.

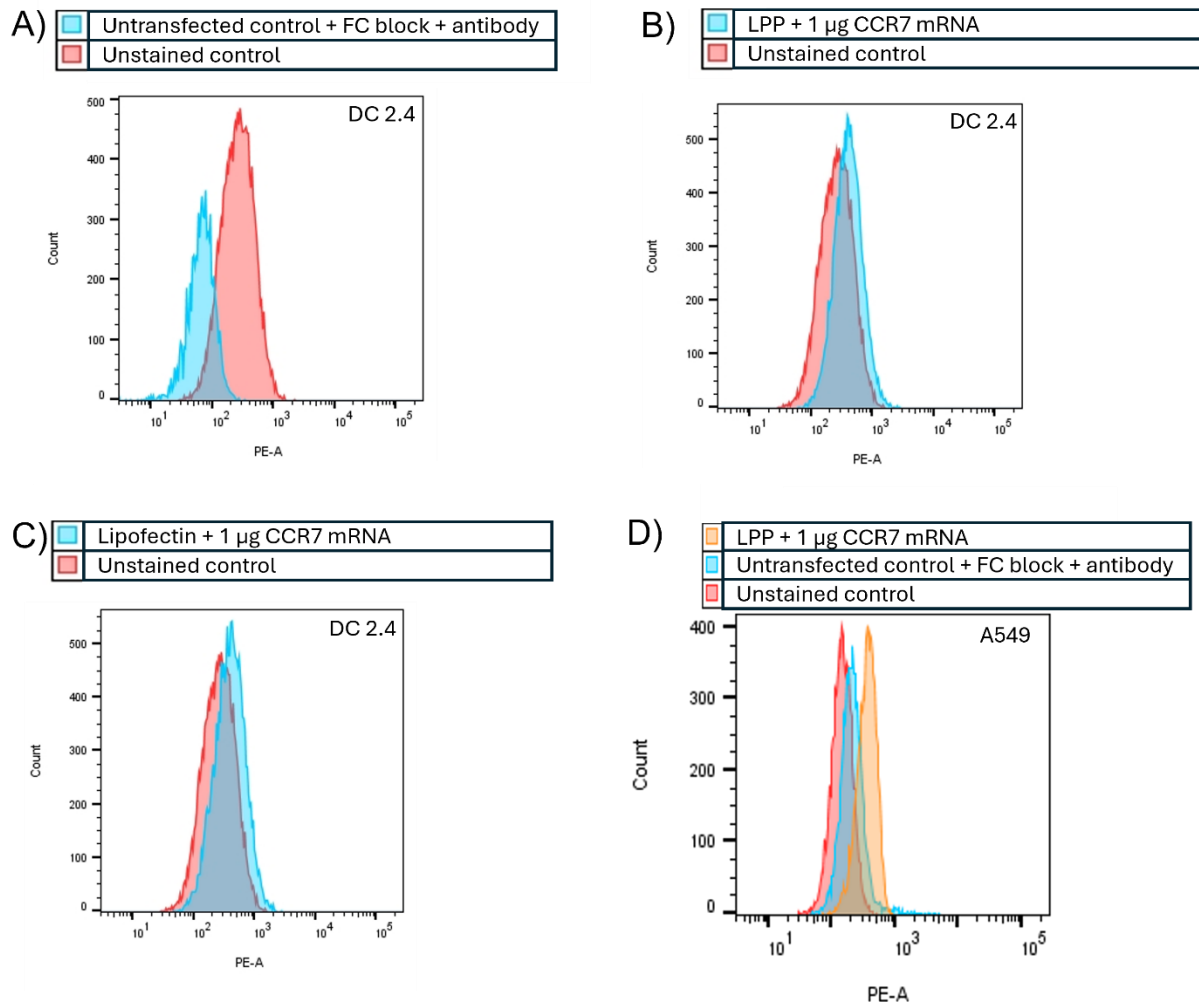


Figure 10: Histograms of DC 2.4 ((A), (B), and (C)) and A549 (D) cells transfected with 1 μ g CCR7 mRNA (Ribopro), unstained and stained controls.

DC 2.4 cells were then transfected with the modified CCR7 mRNA surface-loaded on PLA-poGA LPPs. Transfections were performed with either 0.5 or 1 μ g mRNA per well, followed by staining with a PE-conjugated anti-CCR7 antibody prior to FACS analysis. After 24 h, a significant and dose-dependent increase in CCR7-expressing cells was observed. The proportion of CCR7⁺ cells rose from approximately 25 % in untreated controls to ~60 % and ~80 % at 0.5 and 1 μ g doses, respectively (Figure 11A). Moreover, the MFI within the CCR7⁺ population was markedly increased compared to untreated controls, indicating not only a higher proportion of CCR7-expressing cells but also enhanced receptor density on the cell surface (Figure 11B). Interestingly, cells exposed to unloaded LPPs exhibited reduced CCR7 expression compared to untreated controls, suggesting receptor internalization induced by negative stimulation. At 48 h post-transfection, these effects persisted, albeit at a lower magnitude.

Additionally, CCR7 levels of Untransfected DC2.4 cells rose with longer cultivation time.

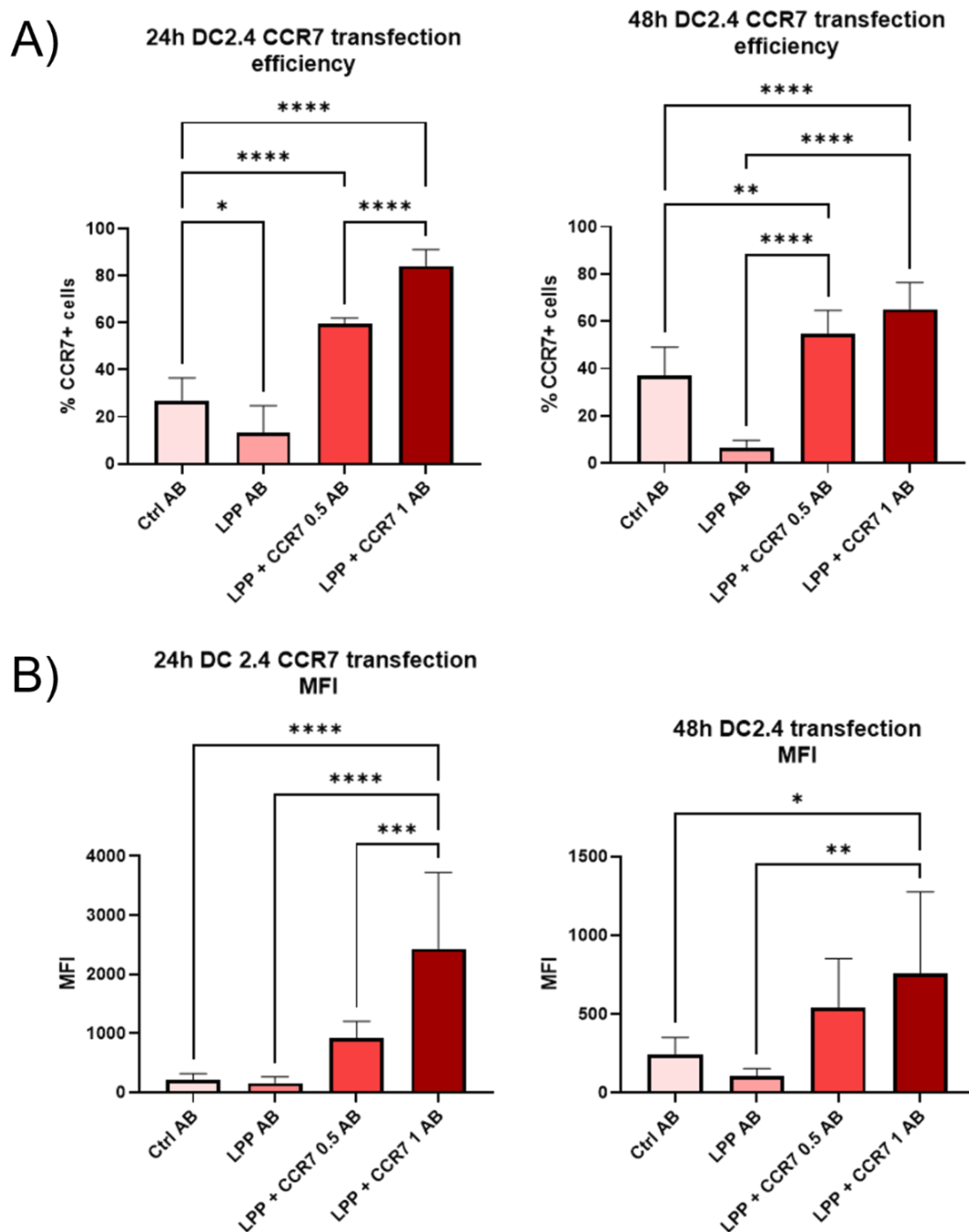


Figure 11: (A) CCR7 transfection efficiency and (B) CCR7 transfection MFI in DC 2.4 after 24 and 48 h, respectively. Error bars indicate standard deviations.

Following verification of mRNA functionality, the biological activity of the expressed CCR7 protein was assessed using a transwell migration assay, adapted from Okada et al (Okada et al. 2005). The experimental workflow is illustrated in Figure 12. Briefly,

CCR7-transfected DC 2.4 cells were harvested and seeded onto the apical compartment of a transwell insert. Cells were incubated for 5 h in the presence of medium containing chemoattractant (recombinant CCL21 or supernatant from CCL21-mRNA-transfected cells) in the basal compartment. Cells that migrated through the transwell membrane into the lower chamber were collected, transferred to a 24-well plate, and quantified using the CyQuant assay. Untransfected cells served as negative controls, while cells seeded directly into wells without a transwell insert served as the 100 % migration control.

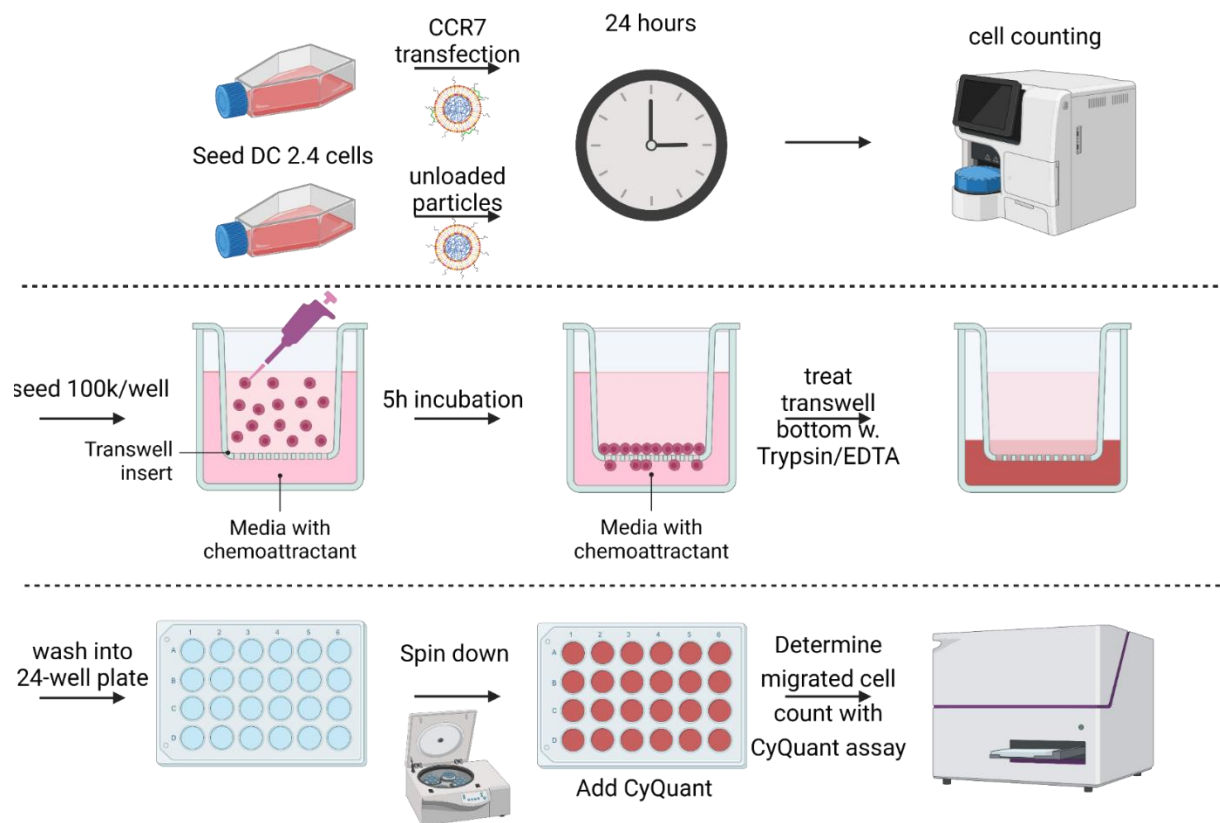


Figure 12: Experimental workflow of the DC 2.4 migration assay.

After 5 h, approximately 15-20 % of CCR7-transfected cells migrated in response to 200 or 400 ng/mL recombinant CCL21. In addition, ~10 % of cells migrated towards the supernatant of CCL21-mRNA-transfected cells, whereas no migration was observed in the absence of chemoattractant. Notably, untransfected cells failed to migrate under all tested conditions.

To quantify CCL21 secretion, an ELISA was performed on the supernatant of CCL21-transfected DC 2.4 cells. A dose-dependent effect was detected, with the highest

mRNA dose (2 $\mu\text{g/mL}$) yielding a CCL21 concentration of approximately 10 ng/mL. Interestingly, despite this comparatively low concentration, the supernatant induced about half the level of cell migration observed with 400 ng/mL recombinant CCL21. This discrepancy suggests that the migration response to CCL21 may be non-linear even in low concentrations, or alternatively, that CCL21-transfected cells secrete additional immunostimulatory factors that enhance cell migration.

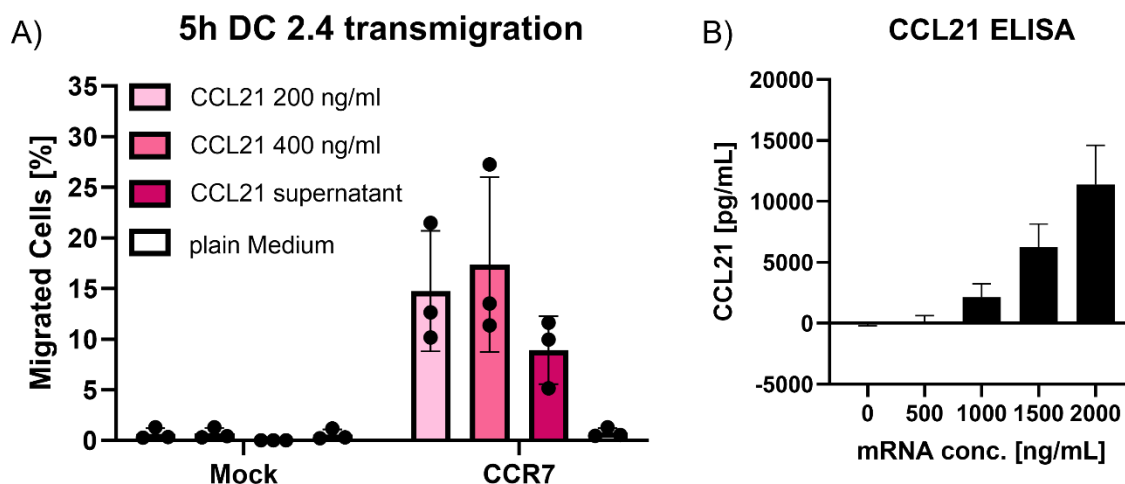


Figure 13: (A) Transmigration assay of CCR7 transfected DC 2.4 cells across a transwell membrane towards chemokines. (B) ELISA quantification of CCL21 in the supernatant of CCL21-transfected DC2.4 cells. Error bars indicate standard deviations.

3.3.3 Conclusion

Evaluation of CCR7 mRNA with and without nucleoside modifications and capping demonstrated that these chemical modifications are essential to achieve a significant upregulation of CCR7 surface expression in DC 2.4 cells. Moreover, transfected cells exhibited markedly enhanced migration towards the chemoattractant CCL21, confirming the functional activity of both custom-synthesized mRNAs *in vitro*. These findings identify the modified CCR7 and CCL21 mRNAs as promising candidates for adjuvant development in mucosal vaccination, requiring further evaluation of their efficacy *in vivo*.

3.4 Feasibility of Lipopolyplex Mediated mRNA Delivery from BIONDD® Vehicle

In precision medicine, biologics are increasingly used to address individual patient needs. However, they are typically administered parenterally, limiting patient convenience and compliance. To overcome this, strategies for oral delivery of biologics have been explored. Biograil™ (Roskilde, Denmark) has developed the proprietary BIONDD® technology, a capsule based delivery platform for biologics, making them orally available by intrawall delivery to the stomach. The functional mechanism of the device relies on a drug-loaded polymeric spike, which is deployed into the stomach wall through a spring-based actuation system within the capsule. Upon dissolution of the polymeric matrix within the gastric tissue, the drug is released into the systemic circulation. While proof-of-concept has been shown for various biologics, mRNA therapeutics present a unique challenge, as they require nanocarrier formulations to enable cellular uptake and protein expression.

In this collaborative study with Biograil™, we investigated the feasibility of incorporating LPPs into the polymer matrix of the BIONDD® capsule for potential oral mRNA delivery. The aim was to demonstrate that LPPs could be formulated within the polymer matrix and subsequently released in a cell culture setting to mediate transfection of a reporter mRNA. LPPs in aqueous solution were used as a reference in the study.

3.4.1 Materials and Methods

3.4.1.1 Materials

CatPure™ mCherry mRNA (N1-Me-Pseudo UTP) was purchased from AmRNA (Dover, Delaware, USA). DOTMA and DMG-PEG were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Poly(ethyleneglycol) 4000 (PEG4000) and Poly(ethylenoxid) (MW 200000 Da) (PEO200k) were kindly supplied by Biograil™. Poly-L-arginine hydrochloride (15–70 kDa) and poly-L-glutamic acid sodium salt (50–100 kDa) were purchased from Sigma-Aldrich (Darmstadt, Germany).

3.4.1.2 LPP formulation

General manufacturing of LPPs was described earlier in section 3.1.1.2. PLA was used as cationic polymer, poGA served as the complementary anionic polymer. DOTMA and DMG-PEG were used as coating lipids.

3.4.1.3 Centrifugal filtration

Particle suspensions were concentrated using centrifugal filtration. For example, 8 mL of the suspension was placed in an Amicon® Ultra centrifugal filter with a 100 kDa MWCO and spun at $3850 \times g$ in several short intervals. Centrifugation continued until the desired concentration was reached, for instance, a 4× concentration corresponds to reducing the original 8 mL volume down to 2 mL.

3.4.1.4 Freeze-drying

For freeze-drying, 500 µL of the respective particle suspension was mixed with 500 µL of a 20 % sucrose (w/v) solution in a freeze-drying vial. The mixture was snap-frozen in liquid nitrogen for 3 minutes and immediately transferred to a CHRIST Alpha 2-4 LSC basic freeze dryer. Primary drying (condenser: -90°C ; pressure: 0.2 mbar) was conducted over night, before a final drying step (condenser: -90°C ; pressure: 0.010 mbar) was applied for 1 h.

3.4.1.5 Cell studies

To evaluate *in vitro* transfection efficiency, the human alveolar epithelial cell line A549 was used. Cells were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS) and maintained in a humidified incubator at 37°C with 5% CO_2 . Cells were passaged weekly and used up to passage 25.

For transfection experiments, cells were seeded into well plates and allowed to adhere for 24 hours (50,000/well for 48-well plates, 100,000/well for 24-well plates and 400,000/well for 6-well plates). Afterward, they were washed twice with HBSS. Complete medium containing 1% Penicillin/Streptomycin (to prevent contamination) was then added (500 µL for 48-well plates, 1 mL for 24-well plates and 3 mL for 6-well plates). Free particles or polymer-formulated particles were added immediately after.

Cells were incubated for 24 hours at 37°C and 5% CO_2 on a shaker at 150 rpm. mCherry transfection efficiency was assessed using the fluorescence imaging mode of a Tecan SparkCyto. Images were captured in both brightfield and the red fluorescence channel at 10× magnification. Quantification was performed with Tecan ImageViewer software and reported as the percentage of red-fluorescent cells relative to the total cell count.

3.4.2 Results and Discussion

mCherry mRNA was selected as reporter mRNA based on prior working experience, its robustness, and its straightforward detectability via fluorescence imaging. PLA-poGA LPPs were chosen as mRNA delivery vehicle. The core-shell particle with the polymeric core was expected to exhibit higher stability during formulation with the polymer matrix than traditional LNPs.

The incorporation of LPPs into two polymer matrices, based on PEG4000 and PEO200k, was investigated. Since the incorporation protocol requires heating to 60 °C and 80 °C, respectively, the colloidal stability and transfection efficiency of LPPs were first evaluated following short-term heat exposure. Specifically, LPP suspensions were incubated at the respective temperatures for 10 min prior to characterization. DLS analysis of particle size and PDI indicated that transient heating did not significantly alter the physicochemical properties of the particles (Figure 14A). To assess functional performance, A549 cells were transfected with 1 µg/well of mCherry mRNA for 24 h using heat-treated particles, with untreated particles serving as controls. Interestingly, LPPs exposed to 60 °C or 80 °C displayed markedly enhanced transfection efficiency compared to the untreated control (Figure 14B). This observation suggests possible thermally induced rearrangements within the lipid shell or modifications in mRNA–lipid interactions. However, the precise molecular mechanisms underlying this effect remain to be investigated and are beyond the scope of this feasibility study.

The dose selected for the study using the BIONDD® vehicle was approximately 5 mg of the particle–polymer construct. Consequently, to achieve therapeutically relevant doses within the construct, pre-concentration of the particles is required prior to construct manufacturing. For that, LPP suspensions were concentrated 10-fold by centrifugal filtration, yielding a final concentration of 5 µg mRNA in 20 µL. The concentrated suspension (20 µL) was subsequently mixed with either 80 mg PEG4000 or 30 mg PEO200k at the respective incorporation temperatures in 2 mL Eppendorf tubes and homogenized for 5 min with a spatula. Upon cooling, the mixtures solidified into soft, gum-like structures. These semi-solid constructs were applied to A549 cells in 6-well plates containing 3 mL of complete medium, with untreated LPP suspensions serving as positive controls (Figure 15A).

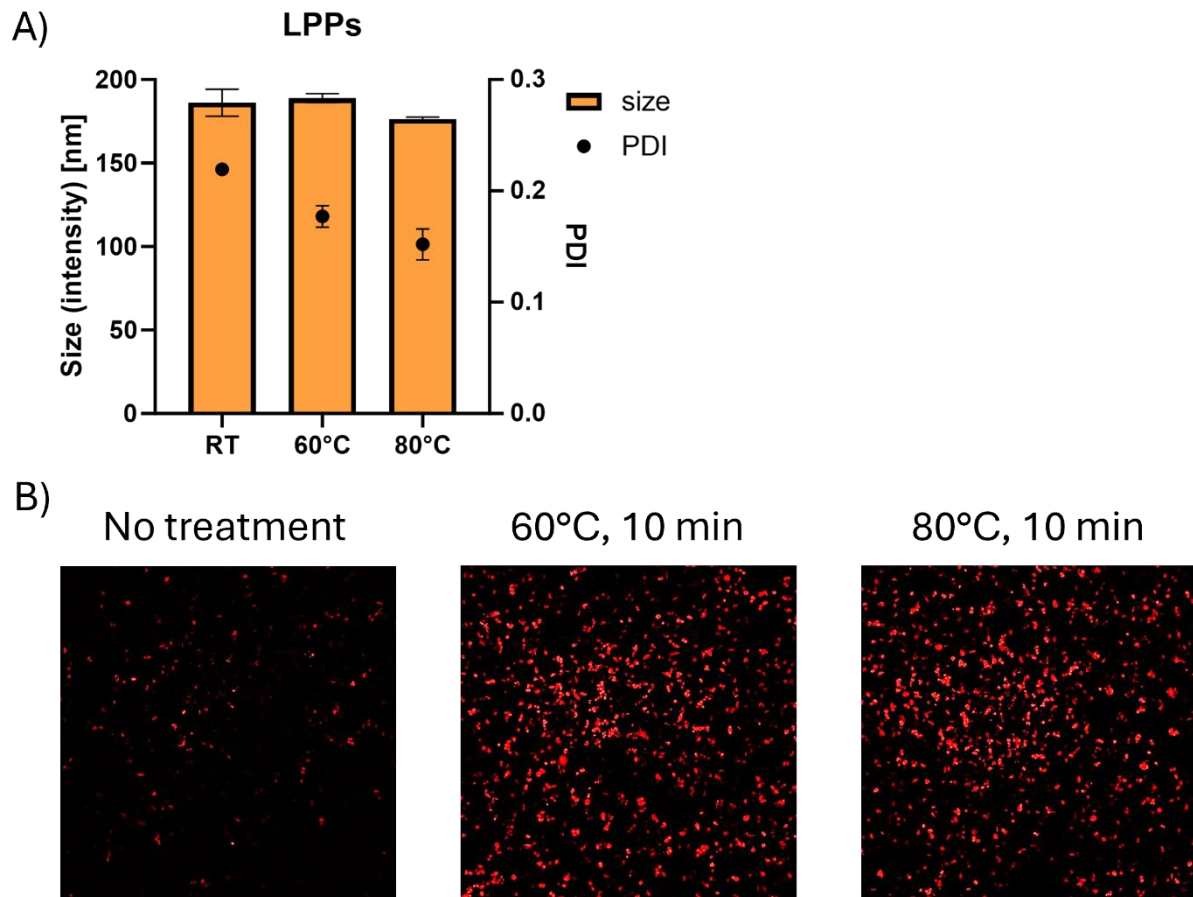
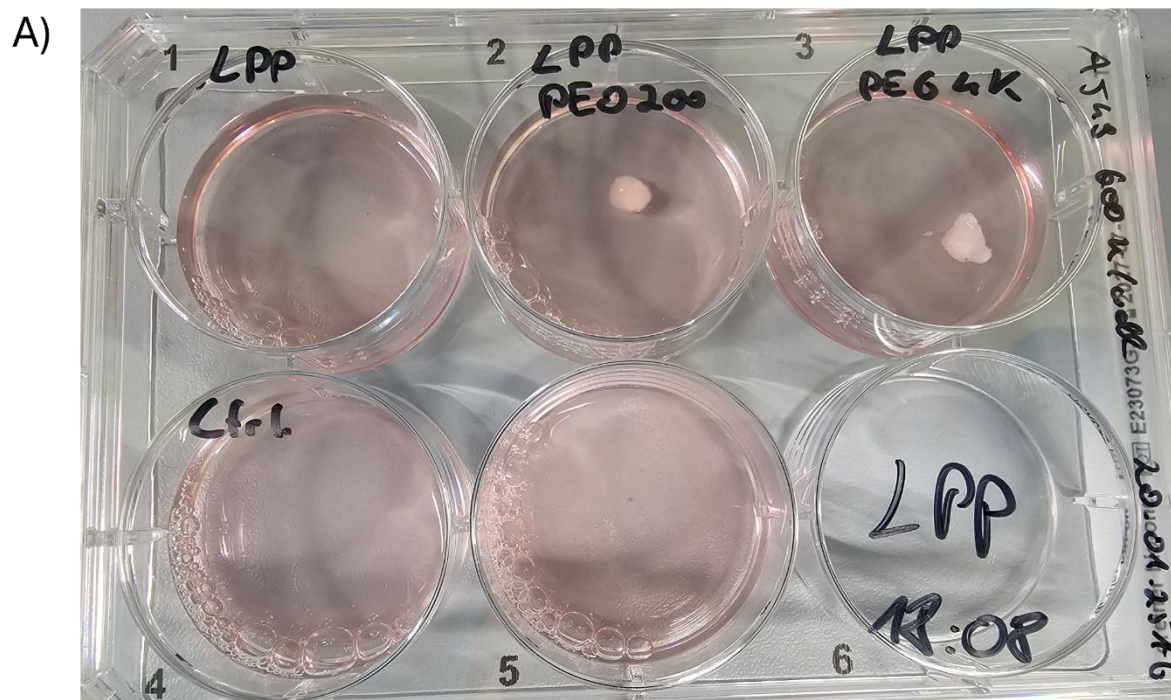


Figure 14: A) Particle size (intensity) and PDI of LPPs measured by DLS before and after heating to 60 °C and 80 °C, respectively; Vertical lines indicate standard deviation (B) Fluorescence microscopy images of A549 cells transfected with mCherry loaded LPPs before and after heating; Cells were imaged using the TecanSparkCyto. Error bars indicate standard deviations.

Fluorescence imaging after 24 h revealed a significant increase in mCherry expression from polymer-formulated particles compared to untreated particles, with a more pronounced effect observed for LPPs incorporated into the PEO200k matrix (Figure 15B).

Despite this strong enhancement of transfection, the resulting constructs lacked the mechanical stiffness required for successful deployment with the BIONDD® device, which requires sufficient hardness to penetrate the stomach wall. Consequently, a lyophilization-based strategy was pursued. LPP suspensions of different concentrations were freeze-dried in the presence of sucrose as a cryoprotectant, after which the lyophilized particles were ground into a fine powder. Lyophilization did not affect LPP size nor PDI (Figure 16A).



B) Free LPPs PEO-matrix PEG-matrix

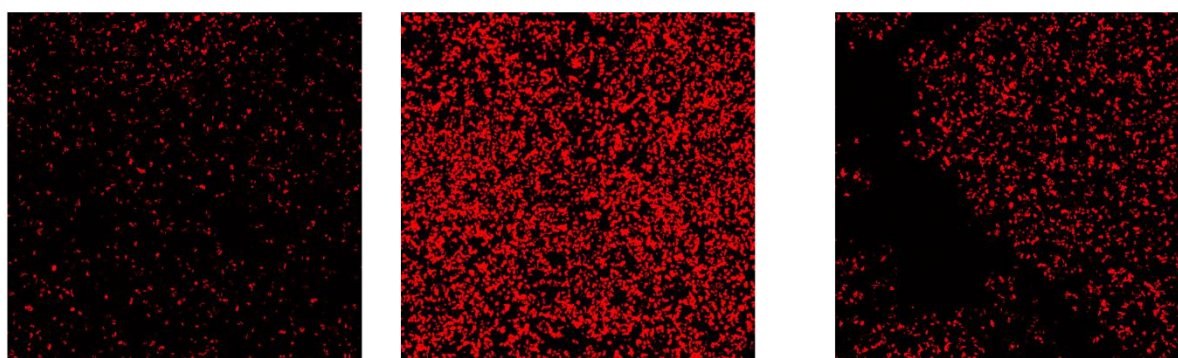


Figure 15: (A) A549 transfection from semi-solid LPP-PEO200k and LPP-PEG4000 matrices in well 2 and 3, respectively. Positive and negative controls in wells 1 and 4 (B) Fluorescence microscopy images of A549 cells transfected with mCherry-loaded LPPs from semi-solid PEO200k and PEG4000 matrices, respectively. Untreated LPPs were used as a positive control.

Approximately 30 mg of this powder was mixed with PEO200k in a 1:1 (w/w) ratio, heated to 80 °C, homogenized, and compressed using a spatula. Upon cooling, the formulation solidified into homogeneous, rigid blocks, which were then cut into pieces of ~5 mg using a scalpel (Figure 16B). LPP–polymer constructs containing 1200 or 2400 ng of mCherry were subsequently tested in A549 cells cultured in 24-well plates with 1 mL of medium. Equivalent doses of LPP suspensions served as controls. Fluorescence imaging after 24 h revealed that transfection efficiency was again improved when LPPs were delivered from the lyophilized polymer matrix compared to untreated particles, though the enhancement was less pronounced than that observed

with suspension-matrix formulations. No significant difference in transfection performance was observed between PEG4000- and PEO200k-based formulations (Figure 17A & B).

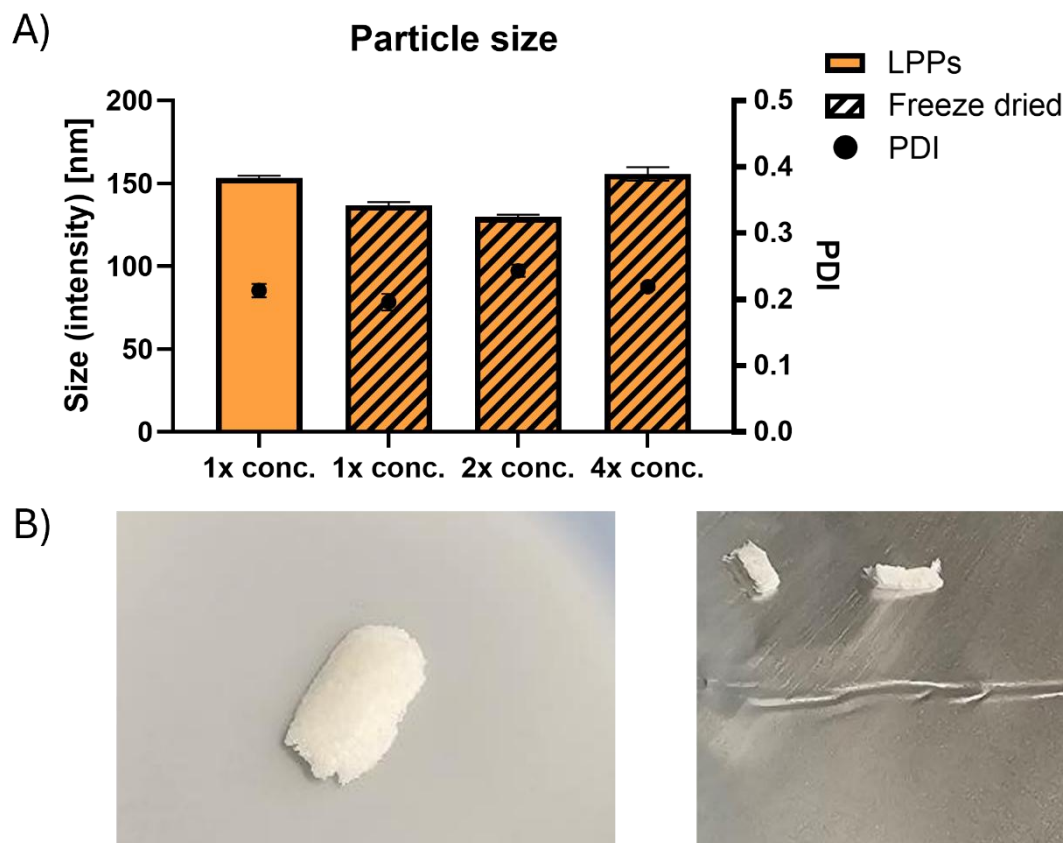


Figure 16: (A) Particle size (intensity) and PDI of LPPs measured by DLS before and after freeze-drying; vertical lines indicate standard deviation (B) Image of the solidified freeze-dried LPP-polymer constructs (left) and 5 mg pieces cut with a scalpel (right). Error bars indicate standard deviations.

Finally, to investigate whether PEO200k itself directly influences transfection, varying concentrations of the polymer (0.3125–20 mg/mL) were added directly to the culture medium during transfection. A549 cells were then transfected with 1 μ g mRNA/well using LPPs. Fluorescence imaging demonstrated a dose-dependent enhancement of transfection efficiency at polymer concentrations up to 10 mg/mL. At higher concentrations, no further improvement was observed (Figure 17C). These findings suggest that the presence of PEO200k may promote transfection through secondary effects such as precipitation phenomena or viscosity-mediated changes in diffusion and uptake. However, further studies are needed to understand the underlying mechanisms responsible for these enhancements.

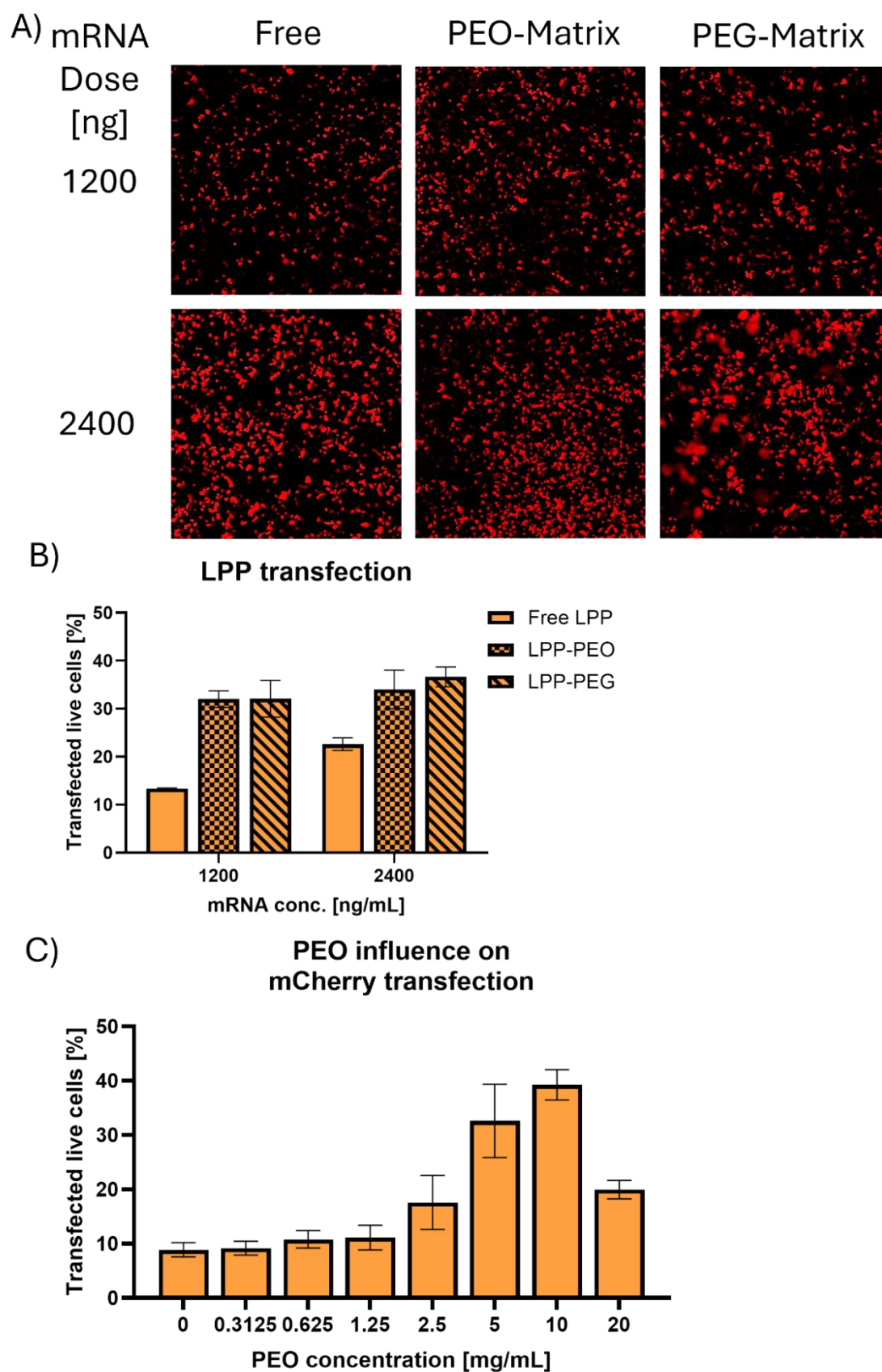


Figure 17: (A) Fluorescence microscopy images of A549 cells transfected with 1200 or 2400 ng of mCherry by solid matrix LPPs in PEO200k or PEG4000, respectively. Untreated LPPs served as a control. (B) Quantitative image analysis of the transfected A549 cells. Vertical lines indicate standard deviation. (C) mCherry transfection efficiency dependent of PEO concentration. Efficiency was determined by quantitative image analysis of images acquired via fluorescence image analysis with the TecanSparkCyto. Error bars indicate standard deviations.

3.4.3 Conclusion

In summary, this feasibility study demonstrated the successful incorporation of LPPs into PEG-based polymer matrices, enabling efficient mRNA delivery *in vitro*. Unexpectedly, the formulation process not only preserved but substantially enhanced transfection efficiency compared to untreated LPPs. Furthermore, lyophilization with sucrose as a cryoprotectant proved to be a suitable approach for generating constructs with sufficient mechanical stiffness for potential application in the BIONDD® device.

While these findings highlight the promise of matrix-formulated LPPs for oral mRNA delivery, the mechanisms underlying the observed improvements following thermal treatment and matrix incorporation remain to be elucidated. Future work should therefore aim to investigate these mechanistic aspects and extend the evaluation to *ex vivo* models, such as pig stomach, to assess the functionality of the BIONDD® device with particle–polymer constructs under physiologically relevant conditions.

4 Conclusion and Outlook

In this work, a novel nanoparticle platform was developed to address several key limitations of LNPs, the current gold standard for mRNA delivery. While the initial aim of generating a stable ternary polymeric core–shell system could not be realized, the integration of cationic and anionic polymers with cationic lipids yielded a new hybrid design. This system enables dual nucleotide loading, within the polymeric core and on the lipidic shell, while maintaining essential physicochemical properties such as particle size and PDI for at least 12 weeks under refrigerated conditions. Importantly, the polymer–lipid combination enhanced stability during critical pharmaceutical processes, including nebulization and freeze-drying, thereby broadening the potential application of mRNA-based medicines to inhalable formats.

The evaluation of transfection efficiency revealed that performance was not dictated by single polymer species alone but by the interplay of anionic and cationic polymers. While hyaluronic acid generally showed inferior results compared to polyA and poly-L-glutamic acid, specific polymer combinations demonstrated synergistic effects. This highlights the importance to closely investigate the mechanistic aspects behind, such as molecular interactions and spatial arrangement of functional groups.

The localization of mRNA within the nanoparticle was found to critically influence expression outcomes: surface-loaded mRNA consistently outperformed core-loaded mRNA, most likely due to incomplete release from the core. Although this currently limits the full exploitation of the dual-loading concept, the issue could be addressed by designing polymers that degrade rapidly in endosomal or cytosolic environments (Zhuo et al. 2020; Iyer et al. 2020). Even under these constraints, the platform successfully enabled the co-delivery of two mRNAs to the same cell with markedly higher efficiency than separately loaded particles. This capability potentially improves functional applications such as CRISPR systems, where coordinated delivery of mRNA and guide RNA is essential.

A complementary objective of this thesis was to assess endogenous adjuvants for mucosal vaccination. CCR7 mRNA required base modifications to achieve significant receptor expression in dendritic cells. With (N1-pseudouridine) modification, high

expression was observed after 24 hours, and receptor functionality was validated by a 5-hour migration assay, where dendritic cell migration across a transwell membrane increased from 0% to 20% compared to untransfected DC 2.4 cells. Similarly, CCL21 mRNA induced dose-dependent expression and functional chemotaxis, approximating the effect of recombinant protein. Together, these results demonstrate that both CCR7 and CCL21 hold promise as endogenous adjuvants, warranting *in vivo* evaluation and comparative studies to determine their relative effectiveness.

Finally, the feasibility of combining lipid–polymer particles (LPPs) with a novel oral delivery technology (BIONDD®) was demonstrated. Freeze-dried LPPs were incorporated into a solid polymer matrix without compromising transfection efficiency, establishing proof of concept for oral nucleic acid delivery. Further studies in advanced models will be required to validate this approach.

In summary, this thesis establishes a versatile nucleotide delivery platform that advances the field beyond conventional LNPs. Although some initial objectives, such as achieving controlled expression kinetics, remain unresolved, the work generated valuable insights into polymer–lipid interactions, mRNA release mechanisms, and adjuvant design. These findings not only highlight the challenges still to be addressed but also provide clear directions for future research. By refining the platform and deepening mechanistic understanding, this work lays the foundation for safer, more effective, and more broadly applicable mRNA therapeutics.

5 Appendix

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5.2 Original publications

5.2.1 Exploring the future of mRNA delivery: Beyond lipid nanoparticles

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Exploring the future of mRNA delivery: Beyond lipid nanoparticles

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ABSTRACT

Over six decades of fundamental research and technological innovation have culminated in the emergence of lipid nanoparticles (LNPs) as the gold standard for mRNA delivery. In the COVID-19 pandemic, they have been pivotal for the rapid development of mRNA-based vaccines. Despite their clinical success, LNPs possess several intrinsic limitations. In this work, we aim to showcase these weak spots but going beyond, we intend to point out solutions and current trends.

We discuss strategies to improve mRNA stability and translational efficiency, including formulation stabilization through lyophilization. Furthermore, we analyze novel materials such as poly (beta-amino esters) (PBAEs) and alternative lipid components to replace PEG-lipids, as well as hybrid (e.g. lipid-polymer) nanoparticle systems designed to enhance delivery efficiency, reduce toxicity, and enable tissue-specific targeting. Different targeting strategies, including passive and active nanoparticle-mediated delivery are compared to approaches based on novel medical devices as inhalers or microneedles. Finally, we address advancements in manufacturing technologies tailored for personalized medicine, alongside analytical, regulatory, and intellectual property considerations critical for the development and commercialization of next-generation mRNA formulations.

1. Introduction

The onset of the COVID-19 pandemic in 2020 coincided with recent progress in nucleic acid delivery technologies, notably following the approval of the first siRNA-based therapeutic Onpatro® in 2018 [1]. This convergence accelerated the development and emergency authorization of Comirnaty®, the first mRNA-based vaccine, which employed lipid nanoparticles (LNPs) as a delivery platform [2]. Since then, LNPs have gained significant attention across academic and industrial research sectors due to their modular design, broad applicability and the accessibility of microfluidic-based manufacturing methods, which are cost-effective and require relatively low technical complexity.

Despite their undoubtedly demonstrated clinical success, particularly in vaccination and hepatic delivery, LNPs continue to face critical challenges that limit their broader therapeutic potential. Ongoing efforts aim to enhance their stability, to reduce off-target effects and immunogenicity [3,4], and to enable efficient targeting of extrahepatic tissues [5]. Moreover, issues related to large-scale manufacturing,

reproducibility and regulatory compliance remain non-trivial.

This review will not illustrate the current mRNA drug pipeline in detail, but will instead present and discuss the technical challenges in mRNA delivery and potential solutions. We propose some strategic approaches and emerging alternatives to address these limitations and support the development of more effective and widely applicable mRNA delivery platforms.

2. Current limitations and strategies for resolution

The current limitations of LNPs as delivery vehicles for mRNA therapeutics are categorized into six distinct groups, as illustrated in Fig. 1, which also provides the structure of this review.

2.1. Advancements in chemical modifications for mRNA therapeutics

Besides necessary delivery technologies, substantial progress has been made in optimizing the design of the mRNA payload itself.

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Innovations span across every structural component of the mRNA molecule - including the 5' cap structure, the poly(A) tail, untranslated regions (UTRs), modified nucleosides and the overall architecture of the transcript.

In higher eukaryotes, mRNAs are co-transcriptionally capped at the 5' end with a 7-methylguanosine (m^7G) cap, often further methylated at adjacent nucleotides. This cap structure is crucial for several biological functions: it enhances mRNA stability by protecting transcripts from 5' to 3' exonuclease degradation, facilitates translation initiation, and helps to prevent innate immune recognition. Cells use cap structures as molecular signatures to regulate gene expression, cell function and to differentiate between endogenous and exogenous mRNAs [6]. Recent studies have highlighted the potential of synthetic cap analogs to improve mRNA quality and expression. Warminski et al. demonstrated that replacing the native $N^6,2'O$ -dimethyladenosine (m^6Am) cap with a trinucleotide N^6 -benzyl analog enabled more efficient separation of capped and uncapped transcripts during purification, leading to a reduction in double-stranded RNA contaminants [7]. This suggests that cap analog selection could be tailored to specific cellular contexts and used as an indirect method for cell-specific targeting.

Chen et al. introduced the concept of "multicapping" - attaching a second capped RNA branch downstream of the +2 position. When combined with poly(A) tail stabilization, these modifications led to a 15-fold enhancement in mRNA translation after 24 h *in vitro* and a 10-fold enhancement *in vivo* [8].

The critical role of 5' capping is underscored by comparisons with circular RNA (circRNA), which lacks both 5' and 3' ends and is therefore inherently resistant to exonuclease-mediated degradation. This contributes to an extended half-life, yet circRNAs typically display lower translation efficiency due to their reliance on internal ribosome entry

sites for translation initiation [9]. To address this limitation, Chen et al. designed a modified construct by covalently attaching their optimized cap to the 3'UTR. The resulting structure, termed QRNA (reflecting its resemblance to the letter "Q"), achieved a remarkable 30-fold increase in translation *in vivo* after 24 h compared to unmodified circRNA [8]. Although this performance still fell short of optimized linear mRNA, the inherent stability of circular RNAs may offer advantages regarding challenges in shelf-life and mechanical stability, as well as applications requiring altered expression kinetics.

Another strategy to modulate RNA expression kinetics is the use of self-amplifying RNA (saRNA). SaRNA leverages the replication machinery of alphaviruses to amplify the gene of interest intracellularly. Although the inclusion of the viral replicase - typically comprising 7–8 kb - significantly increases the overall RNA length, saRNA can produce substantially higher protein yields compared to conventional mRNA. Consequently, lower initial RNA doses are sufficient to elicit comparable biological responses, potentially reducing cytotoxicity and minimizing side effects associated with the delivery vehicle [10]. These advantages have recently led to the approval of the first saRNA based COVID-19 vaccine in Japan [11]. On the downside, the increased length and structural complexity of saRNA make it more susceptible to strand breaks and enzymatic degradation, resulting in a shorter shelf-life compared to standard mRNA constructs [12].

Unfortunately, it is not possible to combine the approaches of saRNA and circRNA using current technologies, as the viral replicase requires the presence of 5' and 3' ends for recognition and function, features that are inherently absent in circRNA. Nonetheless, the development of engineered replicases capable of initiating replication on circular templates could open the door to a new class of RNA therapeutics that integrates the stability of circRNA with the high expression capacity of

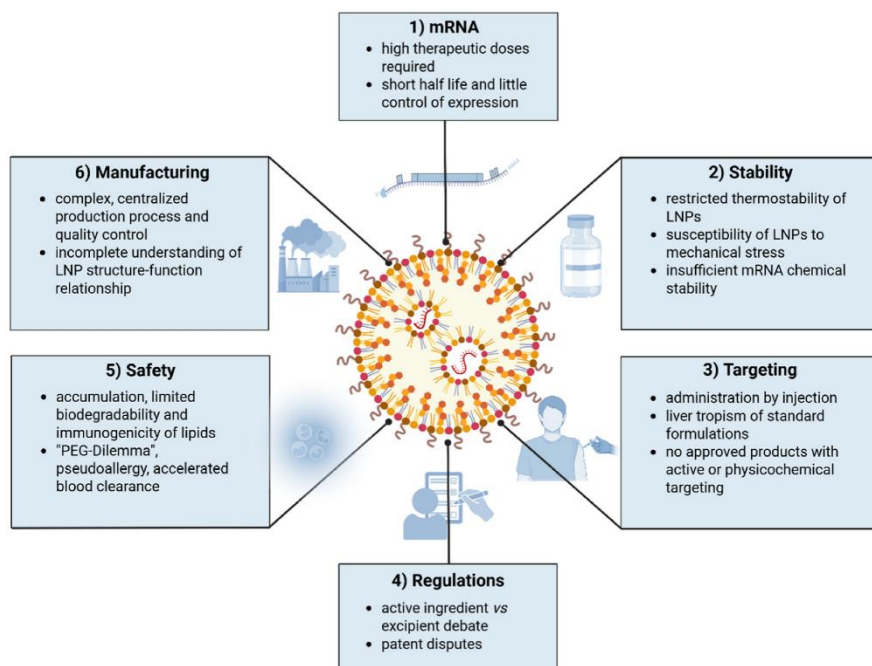


Fig. 1. Current main limitations and challenges in LNP mediated mRNA delivery. Created with [Biorender.com](https://www.biorender.com).

saRNA.

2.2. Thermal and mechanical stability

Not only the chemical susceptibility of nucleic acids but also the limited thermal and mechanical stability of mRNA loaded LNPs remains a significant drawback, particularly for their global distribution and long-term storage. For example, currently approved LNP-based mRNA vaccines, such as those for COVID-19, require storage and transportation at temperatures ranging from -15°C to -90°C , posing logistical challenges. Even when stored at ultralow freezing temperatures, the shelf life remains limited to 18 months [13]. Consequently, considerable research efforts are focused on enhancing LNP stability under standard storage conditions, with lyophilization emerging as one of the most widely explored strategies to improve shelf life.

In this context, optimization of lyophilization parameters, as well as the selection of appropriate cryoprotectants, buffer systems and LNP compositions are actively investigated. Fan et al. conducted a systematic screening of various cryoprotectants - such as oligosaccharides, polymers and amino acids - in combination with different buffer systems including phosphate-buffered saline (PBS, pH 7.4), Tris buffer (pH 7.5), and acetate buffer (pH 5.0). They found that 10 % sucrose, irrespective of the buffer used, best preserved key parameters including particle size, encapsulation efficiency, mRNA integrity, *in vitro* transfection efficiency and fluorescence intensity [14]. Similar studies confirm the function of ~9–10 % disaccharides partially combined with the stabilizer polyvinylpyrrolidone to optimize the colloidal stability of freeze-dried LNPs for up to 6 months [15,16]. Nonetheless, Ruppl et al. observed a substantial decline in transfection efficiency after three months at room temperature. In contrast, lyophilized LNPs stored at 5°C maintained their functional activity for the full 6-month duration, whereas non-lyophilized formulations lost nearly all transfection capability after only 3 months [16].

These results suggest that, under standard storage conditions, mRNA chemical stability - rather than LNP structural integrity - is the critical limiting factor for long-term maintenance of transfection efficiency. The primary mechanism of mRNA degradation under such conditions is intramolecular transesterification, which is influenced by mRNA backbone flexibility, ambient pH and the reactivity of the 2'-hydroxyl group [17–19]. While LNPs offer high encapsulation efficiency and can partially shield mRNA from water and pH fluctuations, they are less effective at reducing backbone flexibility, leaving mRNA vulnerable to chemical degradation.

In contrast, polymeric condensation of nucleic acids can significantly restrict RNA conformational mobility, which may slow transesterification. However, polymer-based systems typically lack full encapsulation, leaving the mRNA exposed to environmental conditions [20,21]. Lipopolyplexes (LPPs) - where mRNA is first condensed by polymers and then coated with lipids - represent a promising strategy to combine the benefits of both systems [22]. Alternative approaches, amongst them GEMCOVAC™-19 (IND), a freeze-dried COVID-19 vaccine, utilize electrostatic adsorption of mRNA onto the surface of nanoparticles [23–25]. Although this method offers limited protection against environmental factors as well, the gentle complexation conditions can help preserve the native secondary and tertiary structures of the mRNA. This structural contribution may, in turn, contribute to enhanced chemical stability by limiting intramolecular transesterification [12]. In spite of these theoretical advantages, rigorous studies to comparatively evaluate chemical mRNA stability across different nanoparticle architectures remain lacking. To ensure long-term preservation of mRNA functionality under standard (e.g., $4-25^{\circ}\text{C}$) storage conditions, future research should prioritize strategies that enhance the intrinsic chemical stability of mRNA, in addition to optimizing carrier stability.

In addition to extensive research on the thermal and storage stability of mRNA-loaded LNPs, relatively few studies have addressed their

mechanical stability. Although mechanical agitation is less relevant during shipping under frozen conditions, it becomes a critical consideration as the field transitions towards transport and storage under standard conditions. Several investigations have reported increases in particle size and decreases in encapsulation efficiency following various forms of mechanical stress, including mixing, vertical and horizontal shaking, inversion and vortexing [26–30]. Among these, only Ruppl et al. have explored lyophilization as a strategy to mitigate such effects. Their findings showed that while liquid LNPs lost up to 80 % or more of their encapsulated nucleotides following shaking, lyophilized LNPs retained full encapsulation efficiency under the same conditions. However, this study did not evaluate or compare the post-stress functionality of lyophilized particles with their liquid counterparts [29].

Overall, lyophilization can be considered as key technology to improve the thermal and mechanical stability of mRNA drug products. While freeze-drying is a pharmaceutically established, well-described process, there is another, less energy-intensive alternative: spray-drying [31]. The possibility to achieve dry mRNA formulations does not only inherently increase stability, but may enable alternative application routes.

2.3. Targeting other organs than the liver

An organ of interest is the lungs, which can be reached by inhalable aerosols, in particular dry powders which are typically produced by spray-drying. However, this production process is critical as it exposes mRNA and nanoparticles to thermal stress, mechanical shear and increased air-liquid interfaces [32,33]. Although still in its early stages, this field is advancing steadily in both academic research and industry. For example, the company Ethris (DEU) announced a collaboration with Lonza (CHE) in April 2025 to develop room-temperature-stable dry powder mRNA vaccines. A further example is RNhale, a spin-off from Ludwig Maximilian University of Munich, that aims to enable dry powder inhalation of siRNA to the lungs [31,34].

Similar advances are being made in the pulmonary delivery of liquid mRNA formulations by nebulization. Standard vibrating mesh nebulizers exert significant shear stress, which can disrupt conventional LNPs and drastically reduce delivery efficiency. To overcome this, researchers have modified nanoparticle formulations by using poly (beta-amino ester) (PBAE) based particles or highly adapted lipid nanoparticles [35–44]. A complementary approach is to develop low-shear nebulization devices, which rely on different physical mechanisms. These include surface acoustic wave nebulizers (e.g. Nebu ~ flow®, GBR) [45], soft mist inhalers based on Rayleigh breakup (e.g. Resyca, NLD) [46,47], and a microfluidic aerosolization platform for aerosol droplet generation, developed at Oregon State University [48].

In spite of the need for such further improvements, the relative simplicity of nebulization - compared to dry powder delivery - has enabled first clinical programs, targeting conditions such as cystic fibrosis or primary ciliary dyskinesia [49]. The aforementioned technical innovations in inhaler technology might further extend the lead of nebulized liquid mRNA formulations.

Innovative medical technologies and devices can offer new targeting solutions for other organs but also systemic delivery. For instance, patients generally prefer oral dosage forms as tablets or capsules [50]. However, nucleic acids are vulnerable to degradation in the gastrointestinal tract (GIT) [51]. Recent studies aim to address this by optimizing LNP design, for example through the use of archaeal tetraether lipids or ginger-derived lipid systems, which improve nanoparticle stability under the harsh, acidic conditions of the GIT [52,53]. Alternatively, a formulation-device hybrid strategy was employed by designing capsules capable of injecting poly (beta-amino ester) mRNA particles directly into the gastric submucosa after orienting towards the stomach wall, thus enabling both gastric and systemic delivery [54].

The skin is another attractive target for local and systemic mRNA delivery. The Hedtrich lab demonstrated successful gene editing in

human skin tissues using mRNA or ribonucleoprotein (RNP)-loaded LNPs, achieving a ~5 % base correction efficiency in cells derived from patients with congenital ichthyosis [55]. Even such modest editing rates might be sufficient to alleviate severe disease symptoms. In this work, microneedles or an ablative laser (P.L.E.A.S.E. system®) were used to create epidermal pores through which LNPs were topically administered.

An alternative skin delivery strategy involves microneedle patches that embed mRNA-loaded nanoparticles within a polymeric matrix for intradermal release - an advantageous way to perform mass vaccination [56,57]. These patches are easy to apply and well-suited for use in low-resource settings due to their room-temperature stability and minimal requirements for trained healthcare personnel [57]. The company Vaxxas (AUS) has developed a proprietary high-density microneedle array patch ("HD-MAP") for self-administration and announced efforts in early 2025 to adapt this technology for mRNA delivery [58]. Such technologies could enhance pandemic preparedness and contribute to the democratization of mRNA vaccines, including low-income regions.

In an effort to eliminate the need for any nanocarrier, microneedle electrodes have been combined with piezoelectric electroporation, resulting in a device called Piezopen. This system enables intracellular delivery of naked mRNA or DNA by creating transient pores in the cell membrane using electrical pulses [59–61]. Another intradermal delivery method involves jet injection technology, as exemplified by the Indian COVID-19 booster vaccine GEMCOVAC-OM. Here, a high-velocity fluid jet penetrates the skin to deliver mRNA LNPs in a needle-free and reproducible way [62].

These examples of oral, pulmonary, and intradermal delivery demonstrate how technological innovations at device level enable targeted extra-hepatic delivery or improved systemic distribution by circumventing hepatic metabolism. Additionally, efforts have been made to improve delivery to the brain, nasal cavity, eyes, ears, placenta, spleen and lymphatic system, tumors, bone, heart and vasculature, and kidneys, which have already been discussed elsewhere [63–65].

Besides by the device, targeted delivery is often achieved by modifying the drug carrier itself. After intravascular administration, conventional LNPs tend to accumulate in the liver due to their interaction with apolipoproteins such as ApoE, followed by binding to low-density lipoprotein (LDL) receptors expressed on hepatocytes [66]. The inherent liver tropism of LNPs has led to a growing demand for nanoparticles that can be engineered to selectively target specific cell types, tissues or organs. As a result, two principal strategies have emerged: 1) passive targeting based on biophysical or biochemical phenomena (e.g. organ distribution driven by blood supply and capillary properties, as well as opsonization or corona formation guiding the clearance route) and 2) active targeting based on specific receptor-ligand interactions [67–69]. For passive targeting, adjusting the lipid composition and thus surface charge and chemistry of LNPs allows to direct the particles to specific organs as spleen or lungs. This principle has gained popularity within the academic LNP community but has also been picked up by biotechnology companies, such as ReCode Therapeutics (USA) with their Selective Organ Targeting ("SORT") Platform or Panthera Therapeutics (DEU, tissue-specific PTXΔLNP® technology) [70–76]. By incorporating permanently cationic lipids like *Diioleoyl-3-trimethylammonium propane* (DOTAP), it is possible to produce "SORT"-LNPs that primarily target the lungs after intravenous administration. While the exact mechanism behind this lung tropism is not yet fully understood, the formation of an altered protein corona on the particle surface (compared to LNPs without permanently cationic lipids) and interactions with the blood and vascular system appear to play key roles. Regarding the latter, the Brenner Lab reported a high risk of thrombosis and inflammatory exacerbations associated with lung-targeting LNPs. This risk can be nevertheless reduced by decreasing the size of the LNPs or by administering systemic anticoagulants [72]. Recently, Dan Peer's group investigated a formulation containing 30 mol% of Distearoylphosphatidylcholine (DSPC), which showed a high

affinity towards the inflamed murine colon [77]. In this case, the lipid composition was adjusted by increasing the phospholipid content, rather than adding an additional "SORT" molecule. It is important to note that most studies on physicochemical targeting of mRNA-LNPs have been conducted in murine models, which generally limits their translatability to human physiology.

To achieve active targeting, nanoparticles have been functionalized with molecules such as antibodies, aptamers, vitamins or carbohydrates, which specifically bind to target epitopes [64]. Although promising, this method faces challenges in scalability due to the inherent complexity of combining nanoparticles with additional ligands, as well as issues in production, bioconjugation and purification [67,78].

To summarize, there are three strategies for targeted mRNA delivery: 1) by novel medical device technology, 2) passive targeting, and 3) active targeting mechanisms. Notably, these strategies can also be combined, e.g. by decorating SORT LNPs with lung targeting antibodies [67].

2.4. Regulatory and patent related challenges

In addition to the technical challenges discussed above, the success of mRNA vaccines has led to regulatory demands. While excipients do not exert therapeutic effects per definition, all lipid components significantly influence the efficacy and safety of the drug product, and the mRNA as the active pharmaceutical ingredient cannot function independently. Therefore, leading experts have proposed that the entire nanoparticle formulation - including both the lipid components and mRNA - should be considered the active substance, as it was the case for the Moderna COVID-19 vaccine Spikevax® [79]. Beyond regulatory hurdles, biotechnology companies face major constraints due to an increasingly intricate intellectual property (IP) landscape, which includes both, mRNA technologies (e.g. chemical modifications) and lipid components [80]. Among the latter, ionizable lipids are particularly heavily patented, owing to their crucial role in facilitating endosomal escape of the mRNA payload [81]. Polymeric delivery systems may offer a promising alternative to navigate current IP constraints. Materials like PBAEs have been used for gene delivery for approximately 25 years. [82, 83]. However, since no polymer-based mRNA delivery systems have received regulatory approval to date, their implementation requires extensive initial efforts to prove safety, efficacy and quality. Moreover, although the field is still emerging, several polymer compositions are already protected by patents, suggesting that the chemical space may also become rapidly saturated with IP claims.

To conclude, we consider novel polymeric materials not only as technically promising alternatives to LNPs, but also as offering greater flexibility within the IP landscape – albeit at the cost of substantial initial efforts to prove their safety.

2.5. Safety of mRNA LNPs

While immunogenicity is advantageous in the context of vaccination, it poses a significant challenge for the treatment of chronic diseases that require frequent, repeated dosing. The substitution of permanently cationic lipids such as DOTAP with ionizable lipids has helped reduce toxicity associated with the uncontrolled activation of pro-apoptotic and pro-inflammatory pathways [84]. However, these ionizable lipids are not entirely immunologically inert [85]. To address the inflammatory response elicited by plasmid DNA (pDNA)-loaded lipid nanoparticles (LNPs), Patel et al. incorporated endogenous anti-inflammatory lipids, such as nitro-oleic acid, into LNP formulations. This strategy effectively abolished STING activation *in vitro* and enhanced gene expression by 50-fold [86]. Similar approaches may be advantageous for improving the safety and efficacy of repeated mRNA delivery.

In addition to their immunogenicity, several widely used ionizable lipids - such as ALC-0315 - exhibit poor biodegradability, raising concerns about their accumulation and potential toxicity following multiple

administrations [84]. Even in the context of vaccination, increased lipid biodegradability has been associated with reduced inflammation at the injection site. Notably, this attenuation of innate immune activation does not compromise the immunogenicity of the vaccine formulations [87]. A common strategy to increase biodegradability involves the incorporation of ester bonds, either within the linker between head group and hydrophobic tail, or directly within the hydrophobic tail, replacing double bonds in unsaturated lipids [88,89]. Furthermore, lipids with saturated hydrophobic tails tend to undergo more rapid enzymatic degradation than those with unsaturated tails, as demonstrated by the differing *in vivo* half-lives of ALC-0315 and SM-102 [84].

Building on Paracelsus' foundational principle that "the dose makes the poison", lipid-polymer hybrid strategies offer an option to minimize the required dose of immunogenic lipids in mRNA delivery systems. By forming a polymeric core that is coated with only a thin layer of functional lipids, these hybrid nanoparticles can achieve efficient delivery with reduced lipid-associated toxicity. Two main classes of such hybrid particles have emerged: LPPs, in which the mRNA is complexed within a cationic polymer core surrounded by a lipid shell [90–92]; and lipid-polymer hybrid nanoparticles, typically composed of a hydrophobic polymeric core (e.g., PLGA as approved, generally regarded as safe material), with the nucleic acid encapsulated within or adsorbed onto the surrounding lipid layer [23,93–95]. Both approaches have been shown to enhance colloidal stability and reduce cytotoxicity, making them promising platforms for safe and effective mRNA delivery [96]. Gabelmann et al. integrated both strategies to design a core-shell nanoparticle capable of dual mRNA loading, demonstrating high loading capacity and facilitating the efficient co-delivery of two distinct nucleotides to the same target cell [97].

However, leaving the pro-inflammatory nature of the ionizable lipids aside, the most significant concern associated with the repeated administration of lipid-based nanoparticles lies in a common component across the systems: the PEG-lipid conjugate. PEGylation serves several critical functions, including stabilization of nanoparticles by preventing aggregation, prolongation of circulation half-life by evading immune recognition, and minimizing nonspecific interactions with biological components [98,99]. Paradoxically, although initially introduced to reduce immunogenicity, PEG-lipids can provoke immune responses upon repeated administration ("PEG-Dilemma"). One such response is accelerated blood clearance, where subsequent doses of PEGylated nanoparticles are rapidly removed from circulation, decreasing their efficacy [100]. More concerning is the risk of complement activation-related pseudoallergy (CARPA), a potentially severe hypersensitivity reaction. CARPA can be triggered by anti-PEG antibodies, which may either develop in response to repeated administration of PEGylated therapeutics or be pre-existing due to widespread exposure to PEG-containing products such as shampoos or sunscreens [101]. Clinical manifestations of CARPA range from mild allergic symptoms to life-threatening anaphylaxis.

To compensate these risks, patients receiving Onpattro™, containing a PEGylated myristoyl diglyceride derivative (PEG₂₀₀₀-C-DMG), are treated with immunosuppressants to mitigate the likelihood of adverse immune reactions [84]. In response to the limitations of PEG, alternative stealth strategies are under active investigation. For example, Yu et al. demonstrated that liposomes coated with poly (ethyl ethylene phosphate) (PEEP) showed extended circulation time, lower hepatotoxicity and minimal induction of anti-PEEP IgM and IgG antibodies upon repeated dosing. Notably, these modifications did not compromise transfection efficiency [102]. Similar strategies employing various novel polymers, including poly (2-alkyl-2-oxazoline), have shown promise and are progressing into clinical development, such as in the case of a rotigotine prodrug [4,103–106].

However, it is important to recognize that all synthetic polymers represent foreign entities to the human immune system. Consequently, the potential for immunogenicity remains, particularly with increased exposure through medical or cosmetic use. An alternative approach

involves the use of human serum albumin, as a nanoparticle coating to confer stealth properties and reduce rapid clearance [107]. While these strategies may not fully resolve the underlying issue of antibody formation, it's important to provide options for patients who experience allergic reactions to certain stabilizing components.

2.6. Manufacturing and quality control

The efficacy and safety of mRNA formulations depend not only on their molecular composition and design, but also on robust manufacturing processes. A wide array of microfluidic mixing techniques has been developed, incorporating various chip/mixing architectures, coating materials and scalability strategies [108–112]. While the production methodologies are generally well-documented, there remains limited insight into the internal architecture of LNPs, particularly regarding how lipid components self-assemble with mRNA to form core-shell structures. For instance, the number of mRNA molecules encapsulated per LNP remains a topic of ongoing research. Using advanced single-particle fluorescence spectroscopy, Li et al. demonstrated that 40–80 % of LNPs are empty, and those that are loaded generally contain only two mRNA molecules [113]. In contrast, Münter et al. reported more uniform mRNA loading by using single particle fluorescence confocal microscopy, with no more than 16 % of particles empty [114]. It is important to note that the two studies differ in methodology (e.g. mRNA length or analytical method); nevertheless, these conflicting results highlight the need for adequate and standardized analytical techniques. Although density gradient centrifugation can be employed to separate empty LNPs from loaded ones, this approach would significantly increase manufacturing complexity [115]. As it remains unclear whether empty LNPs have some function, indeed, or only elevate the risk of adverse reactions [116], single-particle analysis techniques might be valuable to detect the frequent occurrence of empty particles.

Beyond the role of empty LNPs, a comprehensive understanding of these nanocarriers is further complicated by the presence of "bleb" structures, which are phase-separated domains featuring an aqueous core enclosed by a lipid bilayer. These structures can modulate transfection efficiency, either positively or negatively depending on the specific manufacturing process, and are thus sometimes regarded as undesirable artifacts. Currently, blebs are most commonly detected by cryo-transmission electron microscopy, though emerging assays using nuclear magnetic resonance (NMR) or fluorescence-based techniques may enable more simple identification of bleb-containing populations [115]. However, implementing such cost- and work intensive methods as standardized, validated quality control procedures might remain challenging. Powerful analytical platforms allow to simultaneously measure size distribution, encapsulation efficiency and structural features. For example, Graewert et al. combined asymmetrical flow field-flow fractionation (AF4) with small-angle X-ray scattering (SAXS), while Li et al. paired single-nanoparticle free solution hydrodynamic separation (SN-FSHS) with confocal fluorescence spectroscopy [117, 118]. Still, the versatility and power of such methods must be weighed against their technical complexity, including the need of labeling RNA or lipids for the SN-FSHS method. A more simple method that can be implemented as process analytical technology is the use of an online dynamic light scattering (DLS) system, controlling particle size and polydispersity in real time [119].

At present, the manufacturing of mRNA-LNPs is centralized in specialized facilities equipped with dedicated production lines, quality control laboratories and trained personnel [120]. However, given that LNPs serve as a platform technology, where the lipid composition remains largely consistent and only the mRNA payload varies, a decentralized "plug-and-play" manufacturing approach appears feasible. One example is the modular production of mRNA vaccines in resource-limited regions, utilizing container-based production units such as e.g. BioNTech's (DEU) "BioNTainer", which integrate cleanroom

facilities and on-site manufacturing equipment [17]. Another promising application is the local production of personalized nucleic acid therapies, including anti-cancer vaccines and treatments for rare diseases. The Dutch project NANOSPRESSO aims to implement such on-site manufacturing directly in hospitals - as industrial production is per se inflexible and slow [121]. Taking this idea further, one might think of mRNA LNPs as a *Formula magistralis*, where a tailored, personalized mRNA is “prescribed” and encapsulated into standardized nanoparticles. Many hospital pharmacies already have decades of experience preparing sterile, sensitive formulations, such as monoclonal antibodies (e.g. reconstitution and individual dosing).

To produce personalized therapies, it can be useful to separate nanoparticle production from mRNA loading. Tanaka et al. recently described a “post-encapsulation” approach by mixing mRNA solution with an aqueous suspension of (empty) LNPs instead of mRNA encapsulation during the initial microfluidic particle assembly [122]. In contrast to encapsulation, an alternative strategy involves surface-loading mRNA onto preformed nanoparticles. This can be achieved using a two-chamber system, where one chamber contains mRNA and the other contains pre-fabricated “off-the-shelf” particles [17]. Upon mixing, mRNA adsorbs electrostatically on the particle surface [25]. Specialized formulations for surface-loading often incorporate permanently cationic lipids combined with squalene, to create stable nano-emulsions [17]. One successful clinical implementation is the already mentioned above Indian COVID-19 vaccine GEMCOVAC™-19, containing the quaternary amine DOTAP, which benefits from simplified production processes and freedom from current patent constraints [25]. However, the use of quaternary cationic lipids remains controversial. While they offer strong RNA binding capacity and provide nuclease protection, they are also associated with cytotoxicity and pro-inflammatory responses [72,123]. Fortunately, the positive zeta-potential of such formulations typically decreases after mRNA adsorption [23,94,124], and can be further reduced by incorporating

zwitterionic lipids such as Dioleoylphosphatidylethanolamine (DOPE) [23]. Alternatively, ionizable lipids - or a combination of ionizable and permanently cationic lipids - can be used [124]. By applying a slightly acidic buffer, the ionizable lipids are protonated, thereby enabling efficient mRNA complexation [124,125]. Nevertheless, ionizable lipids can undergo N-oxidation, leading to impurities that can form lipid-mRNA adducts, which should not be the case for quaternary amines [126]. Finally, the flexibility of the surface-loading strategy is further enhanced through the use of hybrid delivery systems (compare 2.2) [23,94,124,127–129].

To summarize, understanding the exact nanoparticle structure, enabling decentralized production and improving analytical methods remains challenging. Emerging strategies such as modular manufacturing, point-of-care production or loading “off-the-shelf” particles with mRNA (e.g. by surface-loading) show promise in enhancing flexibility, individualization and overall accessibility.

3. Conclusion

The success of COVID-19 vaccines has brought strong momentum to mRNA delivery. The interest and resulting investments are now focusing on the key challenges in the field. Fig. 2 provides an overview for current research topics and enabling approaches aiming to mitigate limitations of conventional LNPs. Emerging approaches like saRNA and advanced capping technologies are improving the efficacy of the nucleic acid and prolonging its effects, making protein replacement therapies more feasible. Besides the cargo, the success of mRNA vaccination would not have been possible without nanotechnology. To improve stability and simplify storage of mRNA therapeutics, dry powder formulations produced by lyophilization or spray-drying may gradually replace liquid forms, eliminating the need for ultra-cold storage. Topical or targeted delivery is also becoming more achievable through the use of specialized medical devices such as inhalers or microneedles, as well as through

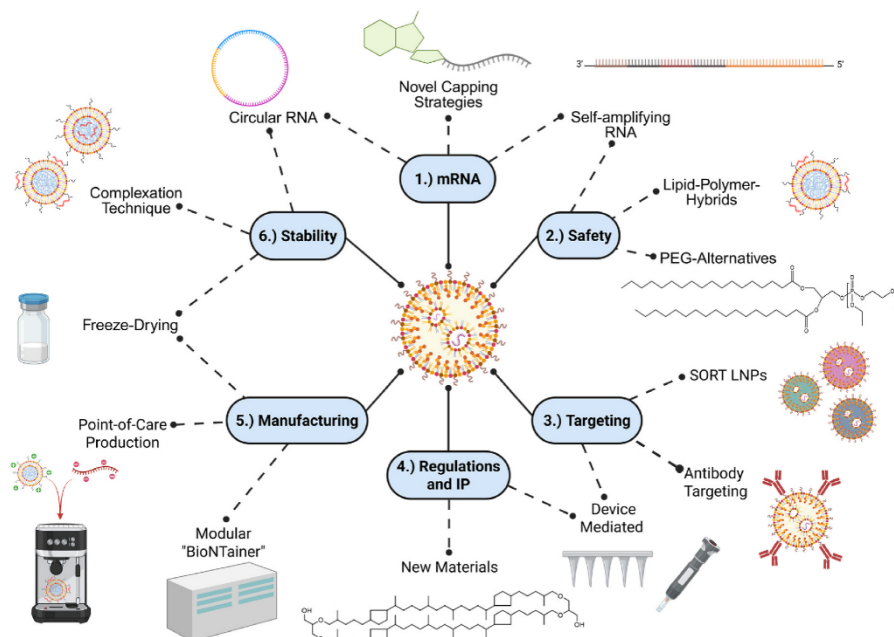


Fig. 2. Current trends, innovations and key technologies to overcome the main limitations in LNP mediated mRNA delivery. Created with Biorender.com.

nanoparticle engineering via passive and active targeting. While nanoparticles based on new polymers show promise for special applications as inhalation, their clinical adoption might be hampered by stringent regulatory demands regarding their safety and quality. Additionally, the repertoire of structurally new lipids, especially ionizable lipids and PEG-lipid alternatives is being expanded. Finally, decentralized and flexible manufacturing solutions are emerging, offering both broader access to therapies and opportunities for personalization.

Taken together, these advances are paving the way for a future where mRNA-based treatments can be personalized, safely delivered, and applied across a wide range of medical conditions - unlocking the full potential of mRNA technology step by step.

CRedit authorship contribution statement

Aljoscha Gabelmann: Writing – original draft, Visualization, Conceptualization. **Achim Biesel:** Writing – original draft, Visualization, Conceptualization. **Brigitta Loretz:** Writing – original draft, Supervision, Conceptualization. **Claus-Michael Lehr:** Writing – original draft, Supervision, Conceptualization.

Declaration of generative AI in scientific writing

During the preparation of this work the authors used DeepL and ChatGPT in order to improve readability and language. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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5.2.2 A novel lipopolyplex platform for dual mRNA delivery via core- and surface-loading

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A novel lipopolyplex platform for dual mRNA delivery via core- and surface-loading[☆]

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ABSTRACT

The approval of Onpatro® (2018) and Comirnaty (2020) has driven interest in nanoparticulate nucleotide delivery. Newer concepts in gene therapy however, require not only the delivery of one, but multiple nucleotides. Examples are CRISPR/Cas9 gene editing and cancer immunotherapy. However, the current gold standard for nucleotide delivery – lipid nanoparticles – faces significant challenges, including limitations for co-encapsulation and nucleotide-nucleotide interactions. Aim of this study was to design a core-shell system featuring separate encapsulation of two nucleotides via a two-step formulation process. Six distinct cationic polymers were combined with three anionic polymers, resulting in 18 core compositions. Screening of these formulations identified three potent lipopolyplexes (LPPs), which were further evaluated and compared in terms of transfection efficiency, expression kinetics, storage stability, and nebulization performance. Among them, the combination of poly-L-arginine and poly-L-glutamic acid demonstrated the highest overall performance. Our systems enabled precise co-delivery of two model mRNAs in a controlled ratio, demonstrating potential for advanced therapeutic applications. Additionally, the role of mRNA localization within the LPP was investigated. Surface-loaded mRNA demonstrated superior transfection efficiency and shear resistance compared to core-loaded mRNA, which lost functionality under nebulization.

1. Introduction

Ushered in by the approval of the siRNA-based therapeutic Onpatro® in August 2018 [1] and reinforced by the first mRNA-based vaccine approved late 2020 against COVID-19 [2], the field of nucleotide-based therapeutics has experienced significant advancements and interest. Both of these groundbreaking therapeutics use lipid nanoparticles (LNPs) to encapsulate and safely deliver their payload. These LNPs consist of ionizable lipids, helper lipids, cholesterol and pegylated (PEG)-lipids. While effectively serving their purpose in these applications, LNPs present notable limitations in certain contexts. Specifically, achieving organ targeting beyond the liver, spleen and lung – enabled by SORT-LNPs – and maintaining stability during pharmaceutical processes such as spray drying and nebulization remain challenging [3] [4,5]. Furthermore, there are applications and diseases where the delivery of

more than one nucleotide is required, such as in cancer immunotherapy, innovative vaccination strategies and gene editing with CRISPR/Cas9 [6–8]. For the latter the activity of the Cas9 nuclease relies on the presence of guide RNA (sgRNA), making co-delivery indispensable. In the context of vaccine development, emerging strategies involve co-delivery of endogenous adjuvants alongside antigens to enhance immune responses. For some of them, e.g. CCR7, a chemokine receptor that plays a key role in homing of immune cells, it is crucial that they are delivered to the same cell as the antigen to fully spoon out their immunological potential [9]. Several studies have demonstrated that encapsulation of both cargos in one particle is often more effective than administration in separate nanocarriers. This approach ensures a controlled delivery to the same cell in a predictable ratio-metric manner and further could reduce costs and regulatory complexity compared to a mixture of two separately loaded particles [10–13].

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However, due to the inherent differences in size, charge and structural properties between various nucleotides, co-encapsulation in a single LNP often requires compromises in formulation, as different nucleic acids show different complexation mechanisms based on electrostatic and hydrophobic interactions [14]. A study of Schober et al. has recently shown that RNA encapsulation efficiency highly depends on nucleotide morphology and net charge [15]. While Ball et al. demonstrated that co-encapsulation of siRNA targeting luciferase and mRNA coding for mCherry in one LNP increased the efficacy of luciferase gene silencing, it came along with a decrease of mCherry expression compared to the single delivery [10]. Similarly, in a different study, co-delivery of an AmCyan encoding plasmid DNA (pDNA) and mCherry entrapped in the Onpatro®-LNP was successful for the mRNA expression, but was lacking efficiency of pDNA transfection [16]. The growing range of applications, combined with the previously mentioned limitations, clearly highlights the need for the development of new, precisely tailored nanocarriers.

To overcome the challenges associated with the co-encapsulation of distinct nucleotides, we propose the use of a core-shell system enabling independent loading of nucleotides via a two-step formulation process. Although there are many examples on core-shell nanoparticles loaded either on the shell [17–20] or in the core [19,21–24], to date only one core-shell system, developed by Nasr et al., has successfully demonstrated simultaneous nucleotide loading in both compartments [16,25]. While successfully co-delivering mRNA on the shell and pDNA in the core, the system relies on a coacervation of gelatin with pDNA, constraining both type and concentration of the core nucleotide.

In response to these limitations, we developed a novel lipopolyplex (LPP) system that allows high loading of nucleotides in the polymeric core as well as on the surface formed by the cationic lipid DOTMA. Up until now, a large variety of LPP formulations has been reported, typically encapsulating only one nucleotide within the core, complexed by an excess of cationic polymer followed by a coating with neutral or charged (phospho-)lipids [24,26–29]. In contrast to that, our formulation incorporates an anionic polyelectrolyte in addition to the nucleotide in the core, which ensures an overall negative charge, and allows flexibility in the type and concentration of the nucleotide payload without affecting the overall polyplex (PP) characteristics. The negative charge is hypothesized to facilitate the enclosure by the cationic lipids, which are necessary for loading of a second cargo on the particle shell.

A large variety of cationic polymers has been evaluated for their ability to transfect various nucleotides. Some of the most featured polymers include polyethyleneimine (PEI), protamine and polyamino acids [30–36]. While they have been extensively tested in combination with varying compositions of lipidic shells, they rarely have been put head-to-head with each other using identical lipid coatings for mRNA transfection efficiency. Therefore, this study aimed to conduct a comprehensive screening of the polyelectrolytes and their influence on the mRNA transfection efficiency of LPPs.

Once delivered, the kinetics of mRNA expression have a major impact on the therapeutic success. In CRISPR/Cas9 applications, rapid release expression of Cas9 mRNA may enhance gene-editing efficiency by ensuring concurrent availability of the Cas9 protein and its associated sgRNA. Delayed expression can lead to premature degradation of the sgRNA before sufficient Cas9 protein synthesis has occurred, causing reduced effectiveness of the gene-editing system [37]. This latency can be circumvented by a delayed release of the sgRNA. In the context of vaccination, the sequential expression of antigens and biological adjuvants ensures the proper activation of the adaptive immune response towards the antigen. This temporal order helps maximizing efficacy and minimizing the risk of immune tolerance or unintended autoimmune reactions, which may occur if immune cells are stimulated with a wrong kinetics or sequence, as well as in a protracted manner [16,38]. Hence, this study examines how the localization of mRNA in the core-shell system influences its expression in response to interactions with different cationic polymers. The most promising candidates arising from

the initial screening are further investigated for their ability to co-deliver mRNA and their stability during nebulization, as needed for pulmonary aerosol delivery.

2. Methods & materials

2.1. Materials

DOTMA and DMG-PEG 2000 were purchased from Avanti Polar Lipids (Birmingham, AL, USA). CleanCap™ eGFP and mCherry mRNA (5moU) were purchased from TriLink BioTechnologies LLC (San Diego, CA, USA). Hyaluronic acid sodium salt (0.2–0.5 million Da) was purchased from BIOZOL Diagnostica (Eching, Bayern, Deutschland). Poly adenylic acid potassium salt, poly-L-ornithine hydrobromide (30–70 kDa), poly-L-arginine hydrochloride (15–70 kDa), poly-L-lysine hydrobromide (150 kDa), poly-L-glutamic acid sodium salt (50–100 kDa), branched polyethyleneimine (25 kDa), linear PEI (60 kDa) and protamine sulfate were purchased from Sigma-Aldrich (Darmstadt, Germany).

2.2. Particle preparation

LPPs were prepared in a two-step process. First, a cationic polymer was mixed with an anionic polymer in a herringbone micromixer (Fluidic 187, microfluidic Chipshop GmbH, Jena, Germany). Polyplexes (PPs) were formed at a positive to negative (POS/NEG) charge ratio of 1:2. For calculations, all ionizable functional groups were considered charged. For core loaded PPs, 25 % of the negative charges of the polymer were replaced by mRNA.

In a second step, the PPs were mixed with an ethanolic lipid solution in a cross-shaped micromixer via hydrodynamic focusing of the lipid stream at a flow rate ratio of 10:1. The lipid solution contains DOTMA (3.71 mg/mL) and DMG-Peg (208 µg/mL). Considering all charges of lipids and polymers, the POS/NEG ratio of the lipopolyplexes is 2:1. If applicable, mRNA can be surface loaded by mixing mRNA solution (1 µg/µL) with particles via pipetting.

2.3. Physicochemical particle characterization

Hydrodynamic particle size, polydispersity index (PDI) and zeta potential of nanoparticles were determined using dynamic light scattering (DLS; Zetasizer Nano, Malvern Instruments, Malvern, UK). For zeta potential measurements, samples were diluted 10-fold in deionized water (exception: PPs formed with IPEI and bPEI. Measured undiluted due to signal strength).

2.4. Particle visualization (Cryo-Transmission Electron Microscopy (Cryo-TEM))

Prior to Cryo-TEM analysis, particle suspensions were concentrated by centrifugation at 3850 ×g using a Vivaspin 20 centrifugal concentrator with a 100 kDa molecular weight cutoff (MWCO) membrane (Sartorius AG, Göttingen, Germany). Centrifugation was performed in discrete time intervals until the target concentration was achieved (e.g. 1 mL particle suspension was concentrated to a final volume of 100 µL).

For Cryo-TEM analysis, 2 µL of a 10× concentrated particle solution was applied to a holey carbon support TEM grid (type 147-4; Plano, Wetzlar, Germany), blotted for 2 s and plunge-frozen in undercooled liquid ethane using a Gatan CP3 plunge-freezer (Pleasanton, CA, USA) at −165 °C. The sample was subsequently transferred to a Gatan model 914 cryo-TEM sample holder and imaged using a JEM-2100 LaB6 TEM (JEOL, Akishima, Tokyo, Japan) operated at 200 kV under low dose conditions. Images were acquired with a Gatan Orius SC1000 CCD camera using an exposure time of 2 s.

2.5. Cell culture

For in vitro testing of LPPs, two cell lines were selected. The ability to transfect immune competent cells was assessed with the murine bone-marrow derived dendritic cell line DC 2.4 (SSC142, Merck Millipore, Darmstadt, Germany). The cells were maintained in complete medium based on RPMI (Roswell Park Memorial Institute; 1640 1×, Ref 21875, Gibco/Life Technologies, Paisley, UK) supplemented with 10 % FCS (fetal calf serum, Ref 10270106, Gibco, Paisley, UK), HEPES buffer (4-2 (2-hydroxyethyl)-1-piperazineethanesulfonic acid; 0.01 M, Gibco, Paisley, UK), non-essential amino acids (1×, Gibco, Paisley, UK) and β -mercaptoethanol (0.0054×) and cultivated in a humidified incubator at 37 °C with 5 % CO₂. DC 2.4 cells are maintained in culture and passaged weekly up to passage 17. Throughout routine cultivation, the culture medium remains free of Penicillin/Streptomycin (P/S) to prevent potential effects on cellular behavior. However, 1 % P/S is added to the medium during transfection assays to mitigate the risk of bacterial contamination from non-sterile particle formulations.

To assess transfection of an epithelial cell line, we selected the human alveolar epithelial cell line A549 (American Type Culture Collection, CCL-185, Rockville, USA). The cells were maintained in complete medium based on RPMI supplemented with 10 % FCS and cultivated in a humidified incubator at 37 °C with 5 % CO₂. Cells were passaged weekly up to passage 25.

2.6. Cytotoxicity assessment

Lactate dehydrogenase cytotoxicity assays (LDH) were used to assess cytotoxicity of LPPs. 20,000 DC 2.4 cells were seeded per well in a 96-well plate (Ref 650160, Greiner Bio-One GmbH, Frickenhausen, Germany) and cultivated for 24 h. Cells are washed twice with prewarmed HBSS (Hank's Balanced Salt Solution, Gibco, Paisley, UK) and particles diluted in complete medium (+ 1 % Penicillin/Streptomycin) are added to the cells. After 24 h, 100 μ L supernatant of each well were transferred to a 96-well plate. The LDH assay is performed according to the manufacturer's protocol with the Cytotoxicity Detection Kit (LDH, Ref 11644793001, Roche Diagnostics GmbH, Mannheim, Germany). Cells cultivated in complete medium without particles was used as negative control, a 1 % Triton X solution in HBSS is used as positive control.

2.7. Transfection studies

100,000 DC 2.4 cells were seeded in 500 μ L complete medium in each well of a 24-well plate (Ref 662160, Greiner Bio-One GmbH, Frickenhausen, Germany) and cultivated for 24 h (70 % confluency). Then, cells were washed twice with prewarmed HBSS and particles diluted in complete medium (+ 1 % P/S) were added to the cells at concentrations correlating to 1 μ g/mRNA/well. After 24 h, cells were washed twice again with HBSS and detached with 100 μ L Trypsin/EDTA for 5 min at 37 °C. Trypsinization was stopped by addition of 900 μ L complete medium and cells were transferred to FACS tubes. Cells were centrifuged for 5 min at 300 g before the supernatant was decanted and cells were washed with 1 mL PBS. After a second centrifugation step, cells were resuspended in 200 μ L PBS and measured using flow cytometry (BD LSR Fortessa Cell Analyzer, Biosciences, Heidelberg, Germany). Obtained data was analyzed with the FlowJo Software (FlowJo 10.8.1, FlowJo, OR, USA).

2.8. Storage stability

To assess the effect of mRNA localization on the storage stability of LPPs at 4 °C, batches of LPPs were core or surface loaded with mCherry mRNA prior to storage in a fridge for up to 12 weeks in Eppendorf tubes. Their transfection efficiency in DC 2.4 cells was measured after 0, 4 and 12 weeks according to method 2.6.

2.9. mRNA expression kinetic

50,000 DC 2.4 cells were seeded per well in 500 μ L complete medium in a 48-well plate (Ref 677180, Greiner Bio-One GmbH, Frickenhausen, Germany) and cultivated for 24 h. Then, cells were washed twice with prewarmed HBSS and particles diluted in complete medium (+ 1 % P/S) were added to the cells at concentrations correlating to 1 μ g/mRNA/well. Cells were incubated in a humidity cassette at 37 °C with 5 % CO₂ for 60 h in a Tecan SparkCyto (Tecan Trading AG, Switzerland). Fluorescence intensity was measured every hour. Excitation wavelength (eGFP): 480 $\pm$ 15 nm; Emission: 510 $\pm$ 10 nm; Excitation wavelength (mCherry): 570 $\pm$ 15 nm; Emission: 610 $\pm$ 10 nm.

2.10. Simultaneous mRNA delivery

100,000 DC 2.4 cells or A549 cells were seeded in 500 μ L complete medium in each well of a 24-well glass bottom plate (Ref P24-1.5H-N, Cellvis, Mountain View, U.S.) and cultivated for 24 h. After washing the cells twice with HBSS, core (mCherry) and surface (eGFP) loaded particles diluted in complete medium (+ 1 % P/S) are added to the cells at concentrations correlating to 1 μ g/mRNA/well. For single mRNA control, PLA-poGA LPPs are surface loaded with one of the respective mRNAs and added at the same concentration. For mixed separate delivery, particles were individually surface-loaded with one respective mRNA and combined in the well at 1 μ g/mRNA per well. For dual delivery controls, mCherry and eGFP mRNA were mixed prior to surface loading onto LPPs and added to the cells at 1 μ g/well concentration for each mRNA. After 48 h incubation, cells were washed once with PBS and then fixed with 4 % paraformaldehyde (PFA) in PBS for 15 min. Then, the PFA was aspirated and cells were stored in PBS protected from light until they were imaged with confocal laser scanning microscopy (CLSM, Leica DMi8, Germany), using a 10× objective (HC PL Fluotar 10×/0.3 Dry, Leica, Germany) and the LAS X software. All samples were imaged at two different random positions. All images were collected using equal parameters.

2.11. Nebulization

Particle suspensions were nebulized with a vibrating mesh nebulizer (Aeroneb® Lab Nebulizer, standard particle size, Aerogen Ltd., Galway, Ireland) by adding 100–200 μ L of particle suspension on top of the mesh. Nebulization was stopped after the nebulizer reservoir was empty, and the liquid was collected in a sterile 1.5 mL micro tube placed directly under the mesh. The nebulizer was cleaned in-between with 3 $\times$ 500 μ L PBS. Following nebulization, particles were evaluated for colloidal stability (Method 2.2), mRNA integrity (Method 2.11) and transfection efficiency, using the Tecan SparkCyto (Tecan Trading AG, Switzerland) for fluorescence imaging.

2.12. mRNA extraction and characterization

mRNA was extracted from the core and surface of LPPs an isopropanol-ammonium acetate extraction method. Briefly, 100 μ L of the particle solution was mixed with 900 μ L of a 60 mM ammonium acetate solution in isopropanol to disrupt the lipid shell and precipitate nucleotides and polymers. The sample was centrifuged at 14,000 $\times$ g for 5 min at 4 °C, before the supernatant was discarded and the pellet was washed with 1 mL isopropanol, followed by another centrifugation step under the same conditions. The supernatant was discarded again, and the pellet was resuspended in 20 μ L of a solution of 2 mg/mL heparin (Sigma Aldrich, Darmstadt, Germany) dissolved in 0.1 M NaOH, to release the mRNA loaded in the polymeric core.

Gel electrophoresis was performed using a 0.7 % agarose gel (1000–20,000 bp; SERVA, Heidelberg, Germany) containing HDGreen Plus DNA Stain (0.005 %; Intas Science Imaging Instruments GmbH, Göttingen, Germany) and a 1 $\times$ Tris-Borate-EDTA (TBE) buffer. Samples

were mixed with 6× DNA loading dye (Thermo Fisher Scientific, Waltham, MA, USA) and approximately 1 µg mRNA was loaded into each pocket of the gel. Electrophoresis was conducted at 70 V for 40 min. Nucleic acids were visualized using a Gel Doc EZ imager (Bio-Rad Laboratories GmbH, Feldkirchen, Germany).

2.13. Statistical analysis

Statistics were calculated with the GraphPad Prism 9 software, using one-way ANOVA followed by a Tukey's multiple comparison test. A p value below 0.05 was considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

3. Results and discussion

3.1. Lipopolyplex design and physicochemical characterization

LPPs were prepared in a two-step process to enable individual loading of core and shell with different nucleotides (Fig. 1A). To ensure comparability between LPPs formed with different cores, the absolute number and ratios of positive and negative charges were maintained constant, resulting in the concentrations shown in Table 1. For core-loaded particles, 25 % of the negative charges provided by the anionic polymer were replaced with mRNA. The anionic mixture was combined with the cationic polymer in a herringbone micromixer at a 1:1 volumetric ratio.

Here, PP size and PDI can be controlled by adjusting the mixing speed (Fig. 1B). In a second step, the negatively charged PPs were coated with the cationic lipid DOTMA and DMG-PEG through microfluidic

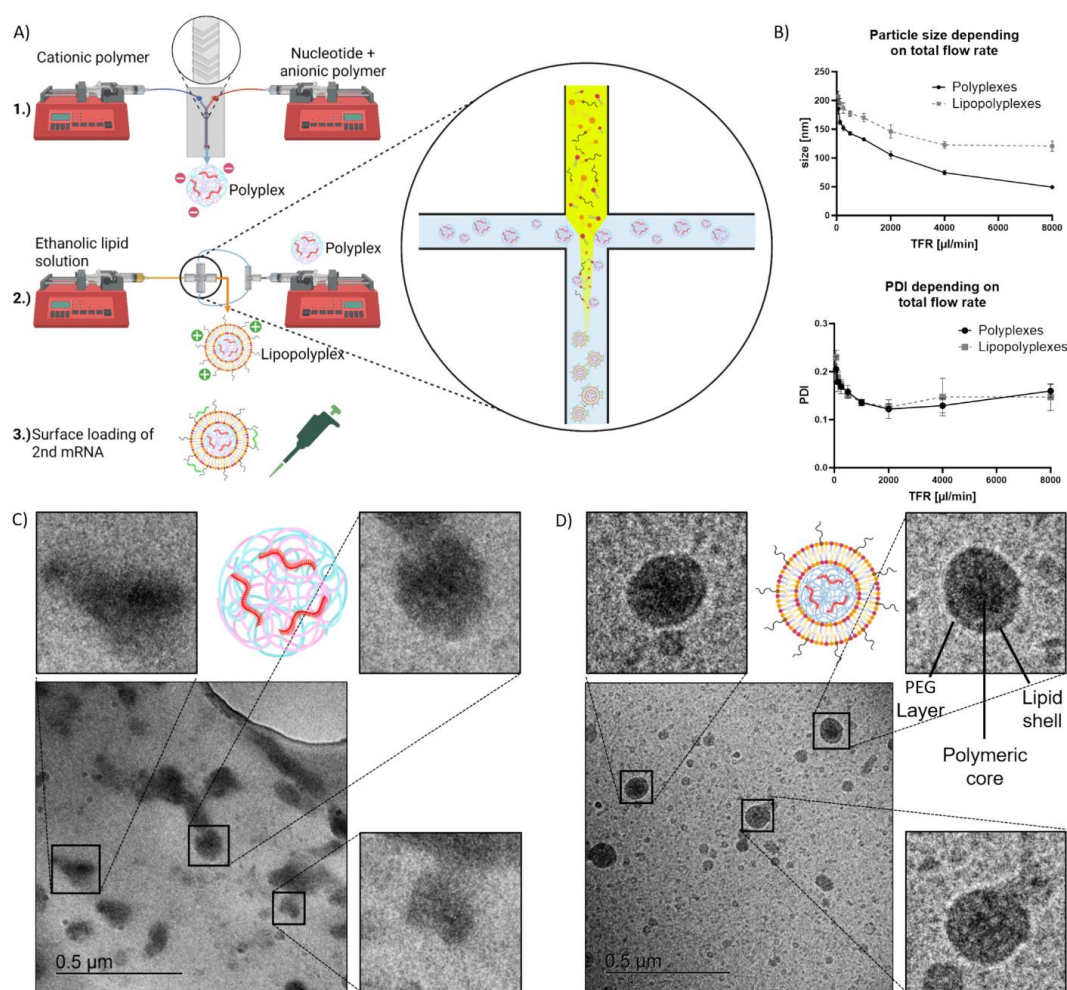


Fig. 1. (A) schematic workflow of LPP preparation via a two-step microfluidic mixing process. As a third, optional step, mRNA can be surface-loaded via pipetting. Created with [Biorender.com](https://www.biorender.com) (B) Particle size and PDI of Prot-polyA PP and LPPs depending on the mixing speed; TFR = total flow rate; Cryo-TEM image of (C) uncoated Prot-polyA PP and (D) Prot-polyA LPPs.

Table 1
Polymer concentrations used for core formulation.

Polymer	Concentration [μg/mL]
Protamine (Prot)	100.6
Linear polyethyleneimine (IPEI)	15.6
Branched polyethyleneimine (bPEI)	17
Poly-L-Lysine (PLL)	75.8
Poly-L-Arginine (PLA)	69.8
Poly-L-Ornithine (PLO)	70.6
Poly adenylic acid (PolyA)	250
Hyaluronic acid (Hya)	274
Poly-L-glutamic acid (poGA)	107.2

mixing in a cross-shaped micromixer via hydrodynamic focusing of the ethanolic lipid stream. Optionally, the anionic mRNA can be electrostatically adsorbed onto the surface of the cationic LPP by pipetting in a third step.

The underlying PP size dictates the size of the resulting LPPs. At a total flow rate of 2000 μL/min an optimal balance between size and PDI was achieved. Higher mixing speed was correlated to smaller PP sizes; however, further increase of the mixing speed from 4000 to 8000 μL/min had influence on the PP size, while the size of the LPPs remained consistent.

Cryo-TEM imaging provided further insights into the structural features of both particle types. Prot-polyA PP appeared as pale, roundish particles with irregular shapes and poorly defined edges (Fig. 1C). In

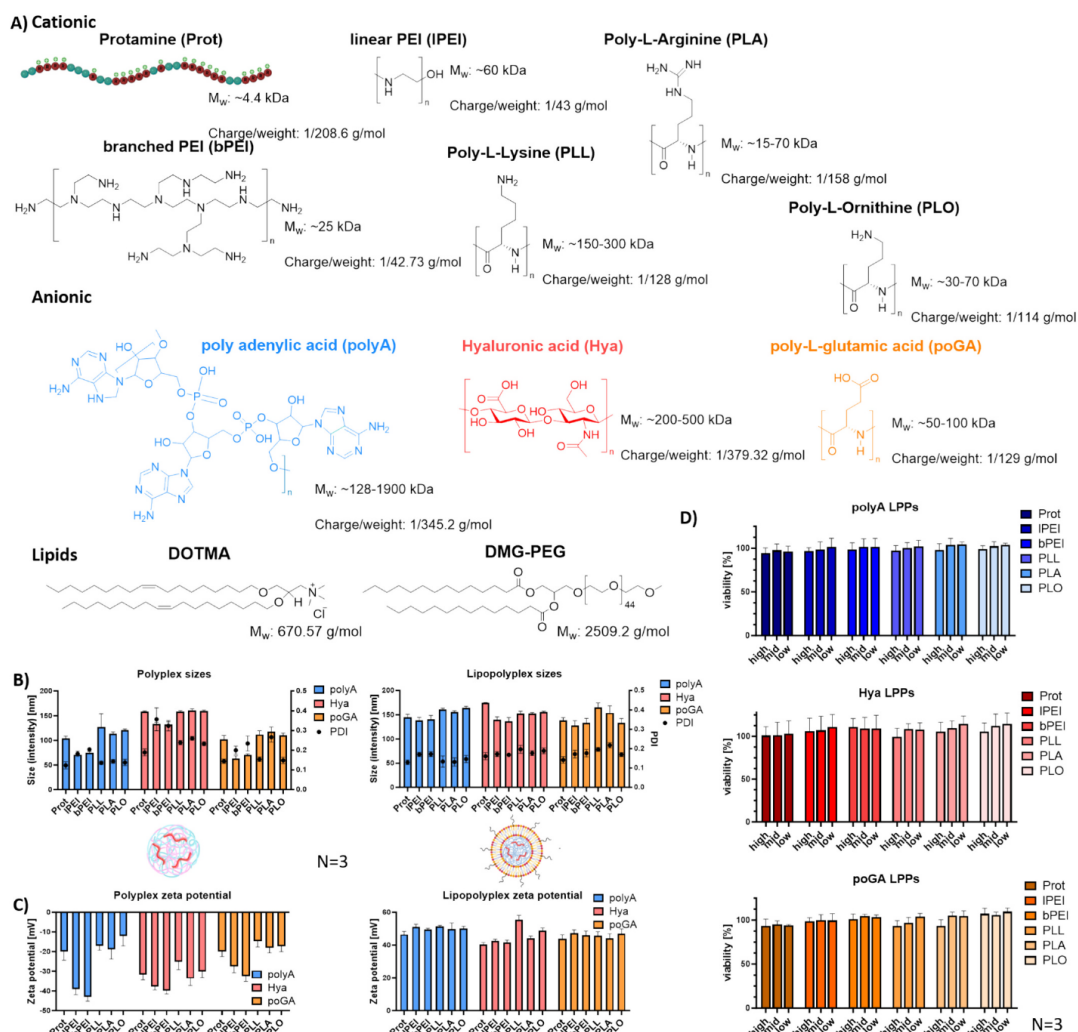


Fig. 2. A) Chemical structures, molecular weights and charge densities of the polymers and lipids used for LPP formation; B) Particle size (intensity) of PPs and LPPs as determined by DLS, depending on the polymers used; C) Zeta potential measurements of PPs and LPPs; vertical lines indicate standard deviation. D) 24 h cytotoxicity evaluation of unloaded LPPs on DC 2.4 cells using LDH assay to assess membrane integrity using high, mid and low concentrations equal to DOTMA concentrations of 23.74 μg/mL, 11.87 μg/mL and 5.94 μg/mL, respectively.

contrast, the respective LPPs exhibited a distinct core-shell structure characterized by a dense, polymeric core surrounded by a round, dark and well-defined lipid shell. The light outer phase is formed by the PEG-layer (Fig. 1D). Overall, particles appeared slightly smaller than expected by DLS measurements. This is a known phenomenon caused by a DLS bias towards larger particles and the measurement of hydrodynamic particle size rather than the physical size [39].

To examine the impact of core polymers on the general properties of LPPs, a library of 18 PPs and their corresponding LPPs was generated. This library comprised six distinct cationic polymers combined with three different anionic polymers depicted in Fig. 2A. To ensure comparability, all PPs were uniformly coated with the same lipid mixture of 98.5 % (mol/mol) DOTMA and 1.5 % DMG-PEG. The particles were categorized into three groups based on their anionic polymer. Determined by their significantly higher charge density, complexes formed with IPEI or bPEI were notably smaller within each group, whereas the size differences between the other four cationic polymers were negligible (Fig. 2B). This is in line with the results of Schatz et al.,

who observed an increase in particle size upon reducing the charge density of dextran sulfate [40]. As expected, all PPs exhibited a negative zeta potential varying from -10 mV to -35 mV. On average, PPs formed with Hya displayed a more negative charge compared to those formed with polyA and poGA. Following lipid coating, the zeta potential of the resulting LPPs shifted to positive values ranging from $+40$ mV to $+50$ mV, with only minor variation between the three groups (Fig. 2C).

To evaluate the potential cytotoxicity of LPPs, LDH assays were conducted. A dilution series of three relevant concentrations – high, mid and low – was prepared. The mid concentration corresponded to the particle amount used in transfection studies, while the high and low concentrations were set at double and half of this amount, respectively. Since the DOTMA concentration remained constant across all LPPs, it was used for standardization. The tested concentrations were equal to $23.74 \mu\text{g/mL}$, $11.87 \mu\text{g/mL}$ and $5.94 \mu\text{g/mL}$ DOTMA, respectively. As depicted in Fig. 2D, a slight dose dependent trend was visible although none of the formulations caused a significant drop in viability. Hypothetically, up to $4 \mu\text{g}$ mRNA can be delivered using the highest particle

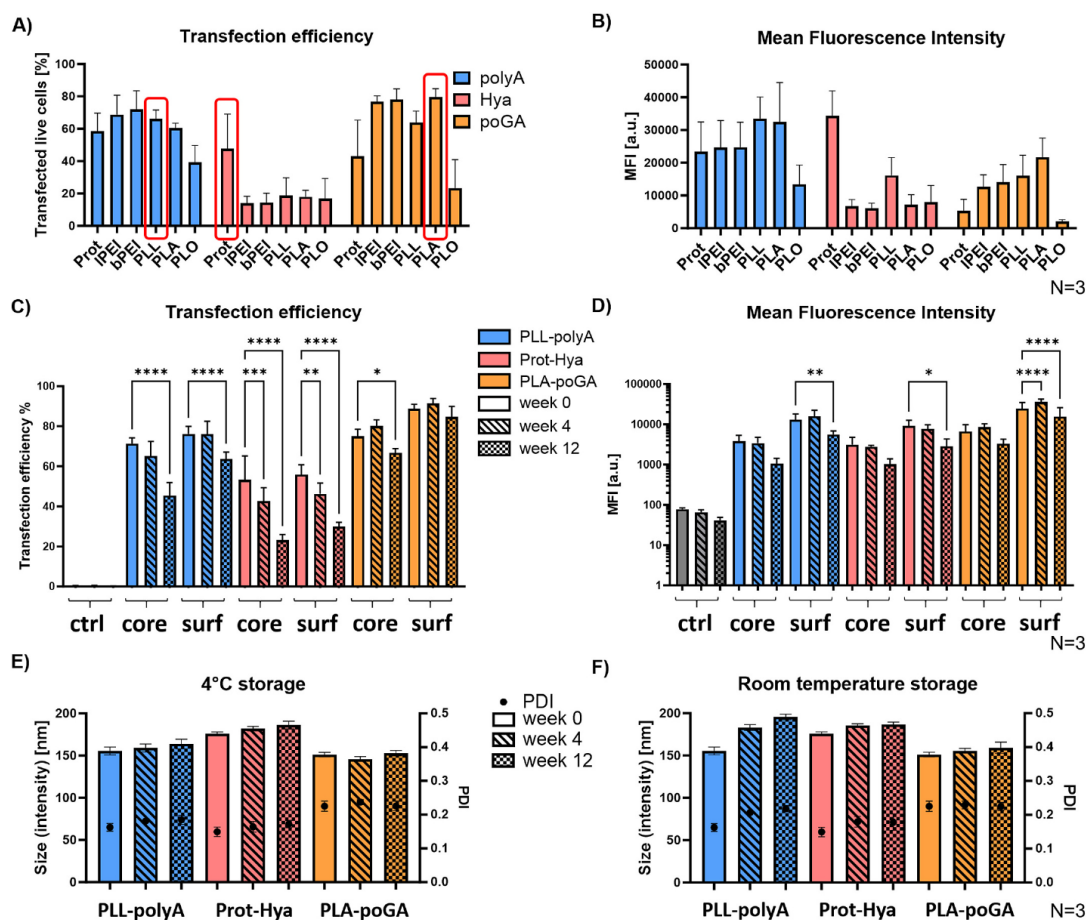


Fig. 3. A) Transfection efficiency and (B) MFI of DC 2.4 cells transfected with various mCherry surface-loaded LPP formulations; Storage stability of mCherry core- and surface loaded LPPs assessed by (C) transfection efficiency and (D) MFI over a 12-week storage period at 4°C ; untreated cells served as a control to assess the background signal. Colloidal stability of LPPs stored at (E) 4°C and (F) room temperature for up to 12 weeks. Vertical lines indicate standard deviation. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

concentration (Fig. S1). Therefore, all particles can be regarded as safe with the immunogenicity of mRNA most likely being the limiting factor.

3.2. Functional analysis with focus on transfection kinetics

Next, the impact of core polymers on the particle transfection efficiency was assessed. All LPPs were surface-loaded with mCherry mRNA, and cells were transfected with 1 μ g mRNA per well for 24 h. Transfection efficiency and the mean fluorescence intensity (MFI) were then measured using FACS. Notably, LPPs formulated with Hya demonstrated significantly lower transfection efficiency and fluorescence intensity compared to those formulated with polyA or poGA (Fig. 3A and B). Given that there were no substantial differences in particle size or zeta potential, we hypothesize that Hya negatively affects the endosomal escape of LPPs. Due to Hya's low pKa of approximately 3 [41] and its high molecular weight, PPs formed with Hya exhibit increased stability at the endosomal pH range of 4–6 [42]. This leads to reduced osmotic pressure and less disruption of the endosomal membrane, hindering the release of mRNA into the cytosol [43]. In line with this hypothesis is the fact that the Prot-Hya complex exhibited the highest transfection efficiency within the group, while both PEI-Hya complexes were the least effective. Prot has the lowest charge density among the six cationic polymers evaluated, while both IPEI and bPEI possess the highest. This difference in charge density likely contributes to the formation of more stable complexes, as higher charge density typically results in stronger electrostatic interactions [44]. In contrast, polyA with an even lower pKa (1–2), should form similarly acid-stable particles as Hya but does not exhibit the same limitations in transfection efficiency. This suggests that pKa values alone cannot explain the observed differences. In a dedicated stability study, PPs formed by PLA with polyA, Hya and poGA were diluted 1:2 in 100 mM citrate buffer at pH 4 to simulate endosomal conditions. DLS analysis of particle size after 30 min of incubation revealed that PLA-Hya PPs remained stable, whereas PLA-polyA and PLA-poGA exhibited significant size increases, likely due to polymer swelling or dissociation (Fig. S2). This indicates that despite its low pKa, PPs from polyA can be degraded under endosomal conditions, probably as a result of high ion concentrations [45]. Additionally, it is possible that polyA has a destabilizing effect on the endosomal membrane, while Hya does not appear to affect phospholipid bilayer stability [46]. However, the specific mechanisms underlying these differences require further investigation.

From each group, the best performing candidate regarding transfection efficiency and MFI was selected for further analysis. Prot-Hya and PLA-poGA demonstrated the highest values for both parameters within their respective groups, making their selection straightforward. In the polyA group, bPEI achieved the highest transfection efficiency (~72% of live cells were mCherry positive), whereas the PLL-polyA exhibited superior MFI. Since PLL also achieved a high transfection efficiency of 65 %, it was selected over bPEI, which, despite its higher transfection efficiency, raises greater concerns regarding cytotoxicity and biodegradability [47].

Before proceeding further, the selected candidates were used to evaluate the cytotoxicity of single- and dual-loaded LPPs through two complementary assays: LDH and a colorimetric assay based on cellular reductase activity, which reflects metabolic function. The LDH assay indicated cell viability above 80 % for unloaded, core-loaded and dual-loaded LPPs, suggesting that the encapsulated mRNA had minimal effects on membrane integrity at a concentration of 2 μ g/mRNA/mL (Fig. S3A). In contrast, the cellular reductase-based assay revealed that dual-loaded PLA-poGA LPPs reduced the metabolic activity of DC 2.4 cells to approximately 70 % relative to the untreated control, suggesting a greater impact of mRNA loading on cellular metabolism than on membrane integrity (Fig. S3B).

Then, we used the three selected core-shell systems to investigate the role of mRNA localization within the particles in greater detail. Specifically, we sought to determine whether mRNA encapsulated in the core

was better protected from degradation compared to surface-loaded mRNA and whether the choice of polymer influenced this stability.

To address this, all three LPPs were loaded with equal amounts of mCherry mRNA, either within the core or on the surface. Transfection efficiency and MFI were assessed immediately after preparation, as well as after 4 and 12 weeks of storage at 4 °C.

Notably, transfection efficiency, and particularly the MFI, were consistently lower at all time points when mRNA was loaded in the core. After 12 weeks, all tested formulations exhibited a significant decline in transfection efficiency, regardless of mRNA localization (Fig. 3C). The only exception was surface-loaded PLA-poGA, whose transfection efficiency remained statistically unchanged, although its MFI decreased significantly (Fig. 3D). Notably, PLA-poGA initially exhibited the highest transfection efficiency and MFI among all tested particles. We hypothesize that despite a decline in fluorescence intensity over time, it remained above the detection threshold required for transfection efficiency calculations, thereby masking any potential reduction in transfection efficiency.

In contrast, both core- and surface-loaded Prot-Hya particles exhibited a significant decline in transfection efficiency already after 4 weeks. Given that Prot-Hya initially had the lowest MFI, even a small reduction in fluorescence intensity may have been sufficient to fall below the detection threshold, leading to a substantial drop in the measured transfection efficiency. These findings highlight the importance of considering both transfection efficiency and fluorescence intensity when evaluating the stability of mRNA-based delivery systems over time.

We conclude that mRNA loaded on the surface is not more susceptible to degradation than mRNA protected by complexation in the core. This aligns with the observations of Kliesch et al., who demonstrated that surface complexation of mRNA with DOTMA effectively protects the nucleotide from enzymatic degradation by RNase [17].

Analysis of the colloidal stability of LPPs over the 12-week storage period at 4 °C revealed only minor increases in particle size for all three formulations (Fig. 3E). A slightly more pronounced increase was observed when particles were stored at room temperature; however, even under these conditions, all formulations remained below 200 nm in diameter (Fig. 3F). Given the minimal changes in particle size, we propose that non-enzymatic mRNA hydrolysis is the primary factor contributing to the observed decline in transfection efficiency [48]. To combat this drawback, unloaded particles could be stored at 4 °C for at least 12 weeks and retain their functionality, allowing the possibility of long-term separate storage of the particle and nucleotide payload until needed. However, due to the constraints of the formulation process, surface loading would be the only viable option following delayed mRNA incorporation.

Furthermore, our mRNA-loaded LPPs demonstrated remarkable robustness, both in their ability to withstand shipment across different locations and in their successful application by the involved collaborating researchers. Despite the potential challenges associated with transportation, like physical stress [49], and handling [50], the LPPs protected the mRNA integrity and maintained their functional efficacy. Fluorescence microscopy revealed successful co-transfection of DC 2.4 cells with core-loaded mCherry (pre-shipment) and surface loaded eGFP (after shipment). In concordance with the results gained at the providers lab, core-loaded mRNA resulted in lower expression compared to surface loaded eGFP mRNA. The versatility of the LPPs was further validated by their ability to efficiently adsorb and transfect a larger mRNA molecule, specifically firefly luciferase mRNA (Fig. S5). This mRNA, at 1913 nucleotides in length, is approximately twice the size of mCherry and eGFP mRNAs (996 nucleotides). Larger mRNA molecules show different adsorption kinetics, likely due to formation of secondary structures [51].

Discovering the weaker performance of core loaded mRNA after 24 h of transfection, we hypothesized that the discrepancies could be attributed to a delayed release of the nucleotide from the polymeric core. To investigate this hypothesis, we designed an experiment in

which either mCherry mRNA was encapsulated within the LPP core while eGFP was attached to the surface, or vice versa, and monitored the expression kinetics of both reporter proteins over 72 h using fluorescence intensity measurements.

As shown in Fig. 4A, absolute fluorescence intensity for mCherry revealed an initiation phase of approximately 3 h post transfection, followed by a near linear increase between 12 and 36 h, reaching a peak at approximately 60 h. Contrary to initial expectations, the discrepancies between core- and surface-loaded mRNA became even more pronounced beyond 24 h. While at first glance, core-loaded mRNA appeared to exhibit delayed expression kinetics, a closer examination of the relative fluorescence intensity over time (Fig. 4B) demonstrated that, for all three LPP formulations, core-loaded mRNA followed the same kinetic profile as its surface-loaded counterpart. Similar trends were observed with eGFP mRNA as the second model transcript (Fig. S3). However, the discrepancy between core and surface-loaded mRNA was less pronounced for eGFP, which can likely be attributed to a lower maximum expression capacity for eGFP.

These findings suggest that the observed differences are primarily due to incomplete mRNA release from the particle core rather than delayed translation. We hypothesize that particle cores are not completely degraded within the endosome, and the remaining PP released into the cytosol remains stable within the intracellular environment. Beyond the scope of this study, we propose that these limitations could be addressed by incorporating a disulfide-crosslinked polymer, which would undergo reductive degradation in the response to the high glutathione concentrations present in the cytosol. This mechanism would potentially release the remaining core-loaded mRNA [52].

3.3. Evaluation of LPPs for nebulization and co-delivery

As we discovered that mRNA storage stability is not affected by its localization and core-loaded LPPs transfect worse than surface-loaded LPPs, we aimed to investigate whether core loading would be beneficial in a physically challenging process like nebulization. Nebulization is an attractive technology for delivering therapeutic nucleotides to the lungs [53]. However, due to the high shear forces generated during the

nebulization process, nebulizing mRNA vaccines with common nebulization techniques led to mRNA fragmentation and subsequent loss of function in LNP formulations [54]. In response, various efforts have focused on optimizing lipid compositions to enhance both LNP and mRNA stability during nebulization [55,56], but up to date, no study has investigated the role of mRNA localization during nebulization.

We first examined the colloidal stability of LPPs post-nebulization using a vibrating mesh nebulizer (Aeroneb® Lab Nebulizer). While PLL-polyA and PLA-poGA maintained their structural integrity, showing only minor increases in particle size and PDI, Prot-Hya exhibited significant aggregation (Fig. 5A). This was evident from a doubling in size and an increase of the PDI above 0.3. These differences may be caused by the higher viscosity of the Prot-Hya formulation, resulting in prolonged nebulization times and increased shear stress [57,58].

Analysis of the mRNA-integrity via gel electrophoresis revealed a significant reduction of intact core-loaded mCherry mRNA post nebulization, while the vibrating mesh caused no degradation of surface-loaded mRNA (Fig. 5B). This observation was also reflected by a loss in transfection efficiency. Again, equal amounts of mCherry mRNA were loaded either in the core or on the surface of LPPs. 200 μ L particle suspension was nebulized into an Eppendorf tube and subsequently pipetted onto DC 2.4 cells cultured in a 24-well plate. Non-nebulized particles served as a control. The results defied common expectations, as it was initially assumed that core-loaded mRNA would be better protected than mRNA located on the surface. However, nebulization resulted in a complete loss of function of core-loaded LPPs, while surface-loaded particles retained 100 % of their transfection efficiency (Fig. 5C, Table S1). We propose that these significant differences can be attributed to the mechanism of mRNA complexation. Rigid encapsulation within the polymeric core could make the large nucleotide susceptible for strand breaks upon particle deformation, while loose attachment to the surface could leave the mRNA with sufficient flexibility to withstand the shear stress during nebulization.

TEM analysis of both surface-loaded and core-loaded PLA-poGA LPPs confirmed that the structural integrity of the nanoparticles was preserved following nebulization (Fig. S7). Notably, surface-loaded particles appeared slightly larger and exhibited a greater tendency to

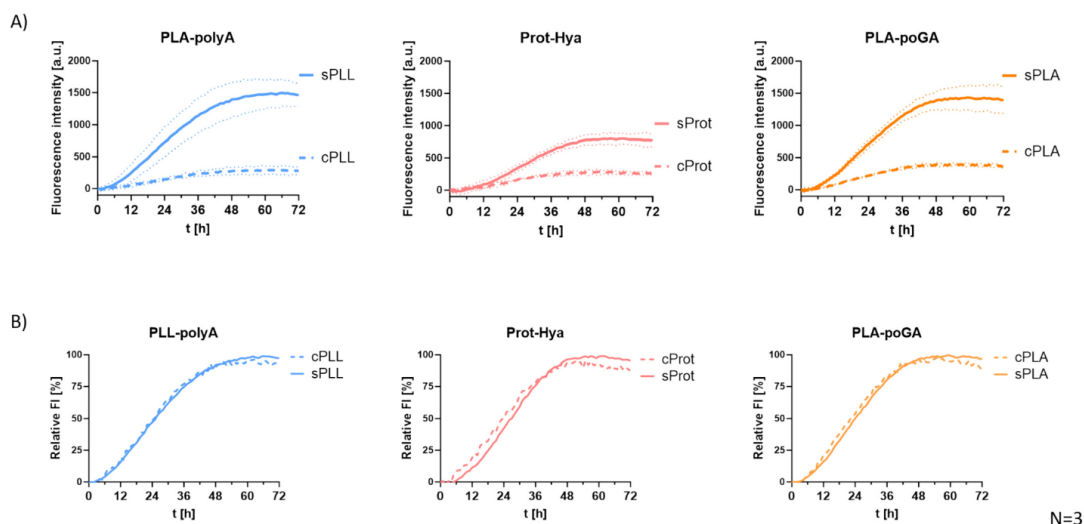


Fig. 4. (A) Expression kinetic of core-loaded (dashed line) versus surface-loaded (solid line) mCherry mRNA from PLL-polyA, Prot-Hya and PLA-poGA LPPs, presented as absolute fluorescence intensity values. Dotted lines indicate standard deviation; (B) Expression kinetics normalized to the respective maximum expression levels, shown as relative values.

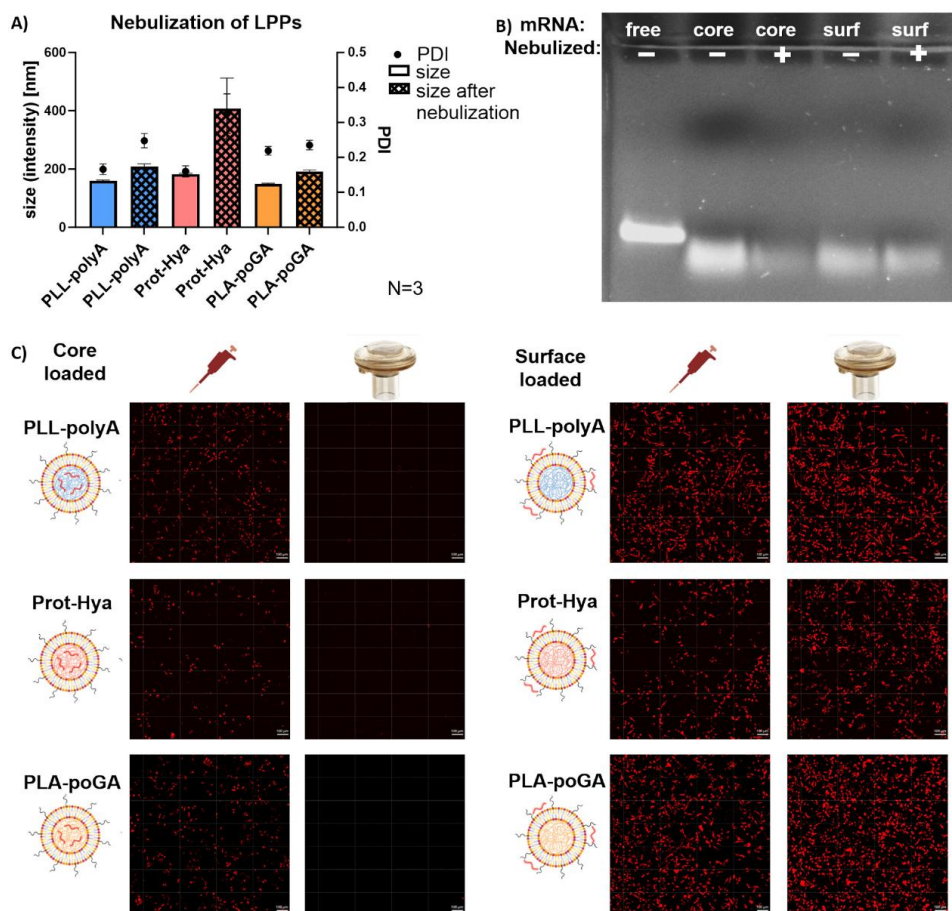


Fig. 5. (A) Particle size (intensity) and PDI of LPPs measured by DLS before and after nebulization; Vertical lines indicate standard deviation; (B) Gel electrophoresis analysis of mCherry mRNA released from PLA-poGA core- and surface-loaded LPPs before and after nebulization. (C) Fluorescence microscopy images of DC 2.4 cells transfected with mCherry using core-loaded and surface loaded LPPs before and after nebulization; Cells were imaged using the TecanSparkCyto.

aggregate compare to core-loaded counterparts, irrespective of nebulization. This observation is supported by DLS data, which revealed a minor peak in the micrometer range for concentrated, surface-loaded samples, while core-loaded particles displayed no such peak. This increased aggregation tendency of surface-loaded particles is likely due to the reduced zeta potential resulting from mRNA adsorption on the particle surface. We hypothesize that this effect, together with the 10-fold sample concentration required for TEM imaging, contributes to the observed aggregation behavior.

While few studies have reported successful delivery of pDNA and siRNA from nebulized LPPs [59,60], there have been no documented cases of successful mRNA delivery using this approach. This limitation may be a consequence from the conventional LPP formulation process, which typically does not incorporate an additional anionic polymer. Consequently, particle assembly relies on the electrostatic complexation of the cationic polymer with the nucleotide cargo, necessitating core-loading for effective particle formation.

This data effectively demonstrated that surface-loading is superior to core-loading for the delivery of a single nucleotide cargo. However,

given the diverse applications requiring dual nucleotide delivery [6–8], we were eager to demonstrate that LPPs can co-deliver to mRNAs in a defined ratio to the same cell. For that, LPPs were formulated with mCherry mRNA in the core and eGFP mRNA on the surface. DC 2.4 cells were transfected for 48 h in a 24-well glass-bottom plate and subsequently imaged via CLSM. To demonstrate the importance of co-delivery, a control condition was included in which cells were treated with a mixture of LPPs, each loaded individually on their surface with either mCherry or eGFP mRNA. Additionally, to verify the absence of fluorescence crosstalk, separate controls containing only mCherry or eGFP were installed using PLA-poGA.

Cells transfected with dual-loaded LPPs exhibited a uniform yellowish/light green fluorescence, indicative of simultaneous mCherry and eGFP expression within the same cell. In contrast, cells transfected with the mixed single-loaded particles displayed a heterogeneous distribution of red, yellow and green fluorescence, resulting from a mix of mono- and randomly co-transfected cells (Fig. 6A). This trend is further supported by the corresponding histograms, which depict the fluorescence intensity distribution of red and green signals at the individual

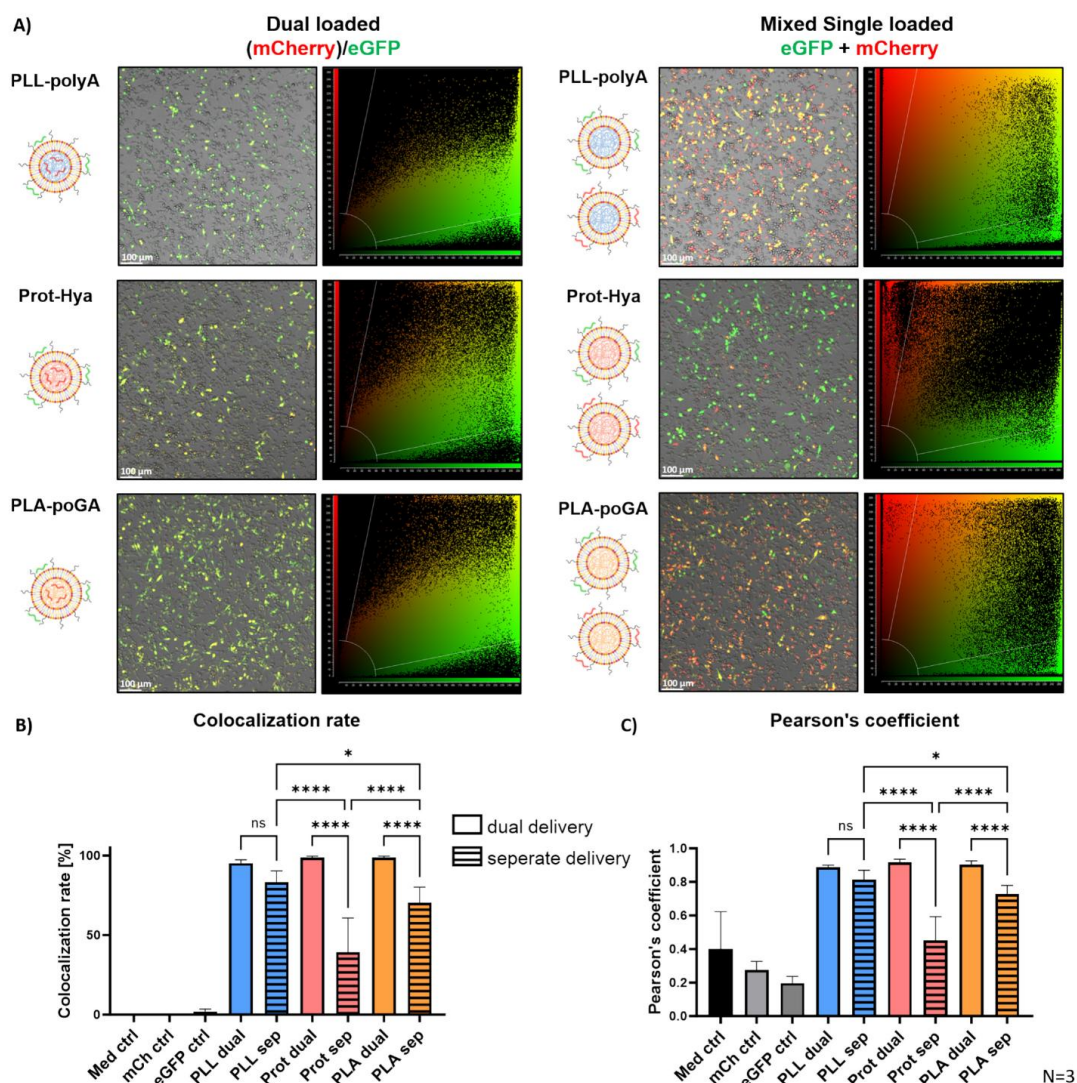


Fig. 6. (A) Representative CLSM images of DC 2.4 cells transfected for 48 h with eGFP and mCherry using dual-loaded or mixed single-loaded LPPs. Corresponding histograms display the fluorescence intensity distribution of red and green signals at individual pixels; Cells were imaged using the Leica DMi8 fluorescence microscope. (B) Average colocalization rates of mCherry and eGFP fluorescence in DC 2.4 cells; (C) Average Pearson's correlation coefficient of mCherry and eGFP fluorescence in DC 2.4 cells. Vertical lines indicate standard deviation. (* $p < 0.05$; **** $p < 0.0001$).

pixel level. Dual-transfected cells exhibit a relatively narrow, ratio-metric intensity distribution, whereas cells transfected with mixed single-loaded particles display a broader distribution spanning the entire spectrum (PLL-polyA) or a distinct distribution shifted towards the extremes (Prot-Hya).

Quantitative analysis of red and green fluorophore co-occurrence in individual pixels revealed a significantly higher colocalization rate in cells treated with dual-loaded LPPs compared to those transfected with mixed single loaded LPPs (Fig. 6B). Notably, colocalization was consistently high across all dual-loaded formulations. However, significant differences were observed among the three LPP formulations in the

mixed single-loaded condition. Specifically, Prot-Hya demonstrated a markedly lower colocalization rate (~40 %) compared to the two other candidates (~80 % and ~70 %, respectively), which can be attributed to its lower overall transfection efficiency. This relationship aligns with the expectation that a lower transfection rate reduces the probability of incidental co-transfection within the same cell. Theoretically, if the transfection efficiency of individually loaded particles were 100 %, all cells would express both fluorescent proteins, resulting in a colocalization rate of 100 %.

To further quantify colocalization, Pearson's correlation coefficient (PCC) was calculated. The PCC ranges from -1 (perfect negative

correlation) to +1 (perfect correlation), with 0 indicating no correlation. Additionally to the co-occurrence of fluorophores, it examines the relationship between their intensities [61]. In this study, the PCC values presented in Fig. 6C closely mirrored the co-occurrence data in Fig. 6B. However, contrary to the assumption of purely random uptake in the mixed single-loaded condition, PCC values were greater than zero. This deviation likely reflects intrinsic biological variability, as some cells exhibit a greater propensity for nanoparticle uptake and protein expression than others, leading to an apparent, yet biologically driven, positive correlation [62,63]. These results are in line with the findings of Zhang et al., who investigated ratiometric mRNA co-delivery to HeLa cells using lipoplexes [11]. Additionally, DC 2.4 cells exhibit a tendency to grow in clumps and on top of each other rather than forming a uniform monolayer, which can lead to an overestimation of colocalization in 2D imaging due to signal overlapping. To overcome this limitation, the experiment was repeated using A549 cells, a human lung carcinoma cell line derived from respiratory epithelium, which is known to form consistent monolayers [64]. As anticipated, transfecting a monolayer culture instead of multilayered cells resulted in lower colocalization rates and PCC values for mixed single-loaded mRNA delivery, whereas both high co-localization rates and PCCs were preserved for dual mRNA delivery (Fig. S4).

4. Conclusion

In summary, our findings demonstrate that the choice of polymer had only a minor influence on the physicochemical properties of LPPs. However, our screening revealed that both cationic and anionic polymers significantly affect mRNA transfection efficiency, even when they are not directly involved in nucleotide complexation. Stability assessments over 12 weeks confirmed the overall colloidal stability of LPPs, but also highlighted a marked decline in transfection efficiency and MFI after more than four weeks of storage at 4 °C. The observed lack of influence of mRNA localization within the particle on transfection stability suggests that mRNA hydrolysis is the primary factor contributing to this decline.

Further investigation into the expression kinetics of core- and surface-loaded mRNA demonstrated that, with the polymers used in this study, it was not possible to achieve delayed expression of core-loaded mRNA. However, the incomplete release of core-loaded mRNA presents an opportunity for controlled cytosolic release by employing alternative cationic polymers with lower intracellular stability.

Additionally, our study demonstrated that nebulized delivery of mRNA via LPPs was only successful when the mRNA was attached to the surface, whereas core-loaded mRNA resulted in complete functional loss, regardless of the polymer used. We hypothesize that the more rigid complexation within the core leads to increased strand breaks compared to the more flexible surface attachment. However, further studies are needed to dissect this phenomenon in greater detail and elucidate the precise mechanisms governing mRNA integrity in different loading configurations.

Despite the identified limitations of core-loading, we demonstrated that dual-loading strategies offer a clear advantage for simultaneous delivery of two mRNAs. They led to significantly higher co-transfection and the expressed proteins are present in defined ratios within cells, as compared to mixtures of single-loaded LPPs. Encouraged by these promising in vitro results using model mRNAs, we aim to translate our LPP system into more clinically relevant studies.

CRediT authorship contribution statement

Aljoscha Gabelmann: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Elham Mansouri-Ghahnavieh:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Marcus Koch:** Writing –

review & editing, Investigation, Data curation. **Prashant Shinde:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Carlos A. Guzmán:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Brigitta Loretz:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Claus-Michael Lehr:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

Statement: During the preparation of this work, the authors used ChatGPT based on GPT-4-turbo in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2025.113875>.

Data availability

Data will be made available on request.

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5.2.2.1 Supporting information to “A novel lipopolyplex platform for dual mRNA delivery via core- and surface-loading”

5.2.2.1.1 Supplementary methods:

S 1.1 Cytotoxicity evaluation

Cell viability was assessed using LDH assay (Ref C20301, Thermo Fisher Scientific Inc., Waltham, USA) and a colorimetric assay based on cellular reductase activity (Ref ab112118, Abcam, Cambridge, UK). DC2.4 cells were seeded at a density of 20,000 cells per well in flat bottom 96-well plates (Ref 92096, Techno Plastic Product, Trasadingen, Switzerland) and incubated at 37°C and 5% CO₂ for 24 h. Following incubation, cells were treated with LPP formulations with each formulation assessed in unloaded, single loaded (mCherry mRNA in the core) and double loaded with mCherry (core) and eGFP mRNA (surface) (CleanCap™ eGFP and mCherry mRNA (5moU), TriLink BioTechnologies LLC, San Diego, USA). Cells were treated with LPPs equal to 1 µg/mRNA/mL. After a further 24 h incubation at 37°C and 5% CO₂, cytotoxicity was determined using two assays according to the manufacturer's protocols. For the LDH assay, 50 µL of each sample was transferred to a flat bottom 96-well plate, and 50 µL of the kit's reaction mixture was added. After a 30 min room temperature incubation, the reaction was terminated with the stop solution, and absorbance was measured at 490 nm and 680 nm. This slightly modified version of the LDH assay is the in-house method used by our involved collaborators. Data obtained with this method can be found in Figure S3. For the cellular reduction-based assay, 20 µL of the reagent compound provided in the kit was added to each well, and the plates were incubated for 3 h at 37°C and 5% CO₂. Absorbance was subsequently measured at 570 nm and 605 nm using a microplate reader.

S 1.2 Fluorescence microscopy imaging

DC2.4 cells were seeded at a density of 100,000 cells per well in flat bottom 24-well plates (Ref 92024, Techno Plastic Product, Trasadingen, Switzerland) and incubated at 37°C and 5% CO₂ for 24 h. Subsequently, cells were washed with pre-warmed HBSS (Hank's Balanced Salt Solution, Sigma-Aldrich, Darmstadt, Germany), and then transfected with LPPs formulations loaded with mCherry mRNA in the core and eGFP mRNA on the surface at concentrations of 2 µg/mRNA/mL. After a further 24 h incubation at 37°C and 5% CO₂, cells were imaged using Agilent BioTeK Cytation 7

(Agilent Technologies Inc., Santa Clara, California). Data obtained with this imaging method can be found in Figure S4.

S 1.3 Luciferase assay

Luciferase activity was measured using the Luciferase Assay kit (ref E1500, Luciferase assay system, Promega, Madison, USA), where luminescence correlates with luciferase protein translation from delivered mRNA in transfected cells. DC2.4 cells were seeded at a density of 100,000 cells per well in flat bottom 96-well plates and incubated at 37°C and 5% CO₂ for 24 h. Following incubation, cells were transfected with LPPs loaded with luciferase mRNA (Firefly luciferase mRNA (N1-Me-PseudoUTP), CATUG Inc, Cambridge, USA) on the surface. After a further 24 h incubation at 37°C and 5% CO₂, cells were washed once with 1X phosphate-buffered saline (PBS, PH 7.4), ensuring minimal disturbance of the cell monolayer. Cells were lysed with 20 µL of 1X lysis reagent provided in the kit. The lysed cells were then transferred to a black flat-bottom 96-well plate (Ref 655077, Greiner Bio-One GmbH, Frickenhausen, Germany) and mixed with 100 µL of Luciferase Assay Reagent. The resulting luminescence was measured immediately.

S 1.4 Transmission Electron Microscopy

Prior to TEM analysis, particle suspensions were concentrated by centrifugation at $3,850 \times g$ using a Vivaspin 20 centrifugal concentrator with a 100 kDa molecular weight cutoff (MWCO) membrane (Sartorius AG, Göttingen, Germany). Centrifugation was performed in discrete time intervals until the target concentration was achieved (e.g. 1 mL particle suspension was concentrated to a final volume of 100 µL).

For TEM analysis, 2 µL of a 10× concentrated particle solution was applied to a holey carbon support TEM grid (type 147-4; Plano, Wetzlar, Germany) and dried for 1 minute on a filter paper. The samples were imaged using a JEM-ARM 200F TEM (JEOL, Akishima, Tokyo, Japan) operated at 200 kV.

5.2.2.1.2 Supplementary figures

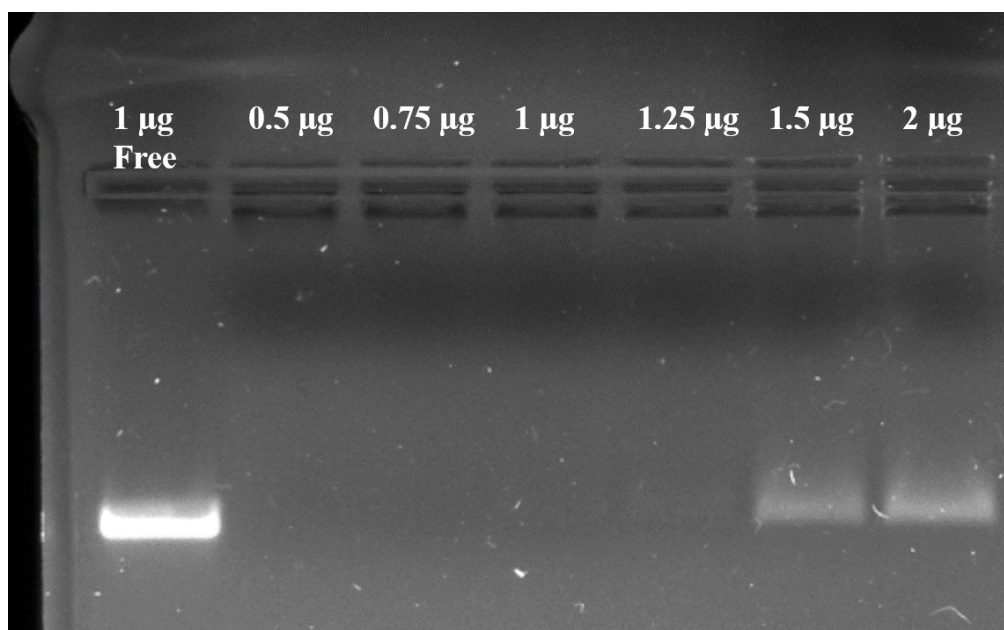


Figure S 1: Gel electrophoresis of mRNA-loaded PLA-poGA LPPs. 1 µg of free mCherry mRNA was used as a control. 17.6 µl LPPs were loaded with the respective amount of mRNA and applied to the gel wells.

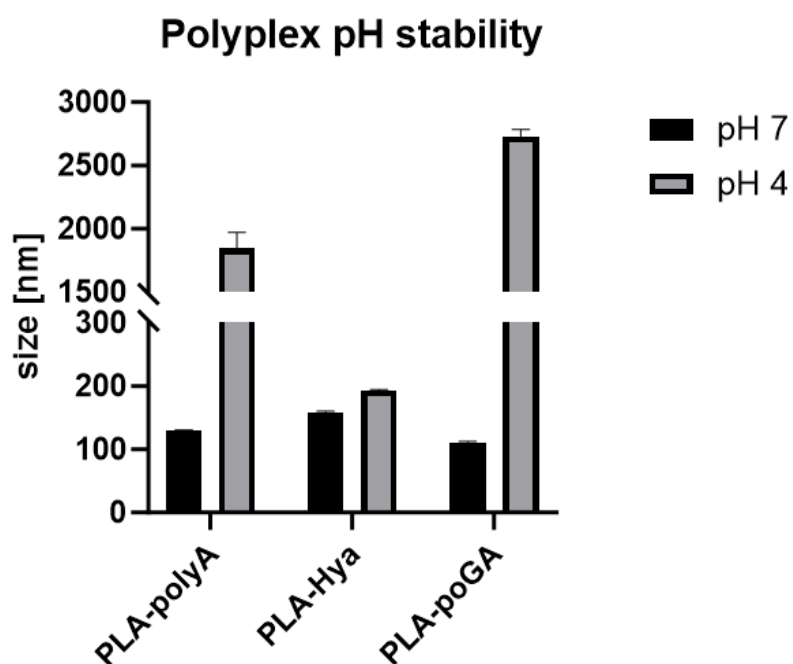


Figure S 2: PP size before and after dilution with 100 mM citrate buffer pH 4 (1:2 v/v); vertical lines indicate standard deviation.

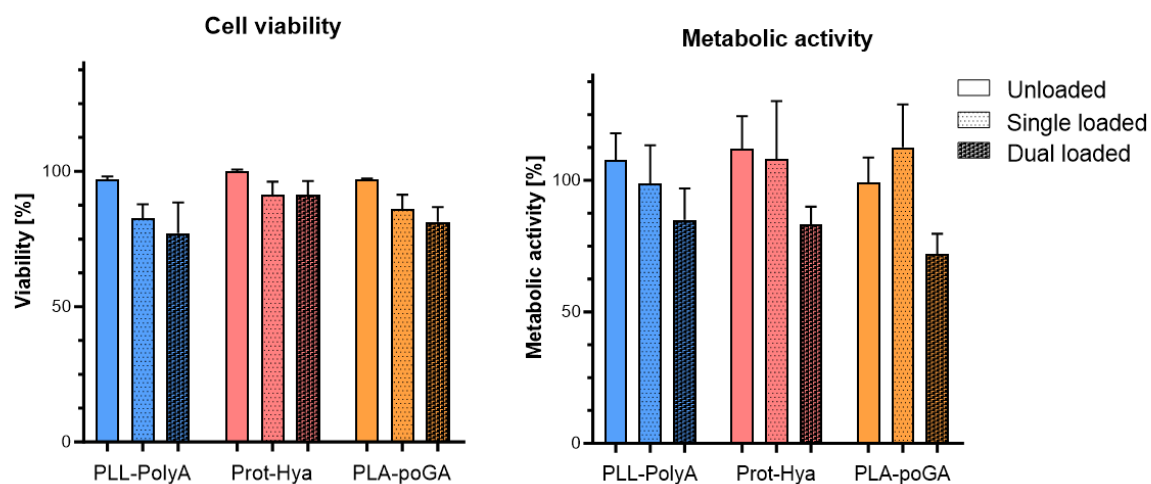


Figure S 3: Cytotoxicity evaluation of unloaded, single-, and dual-loaded LPPs on DC 2.4 cells using (A) LDH assay to assess membrane integrity and (B) metabolic activity assay (Abcam). Vertical lines indicate standard error of mean.

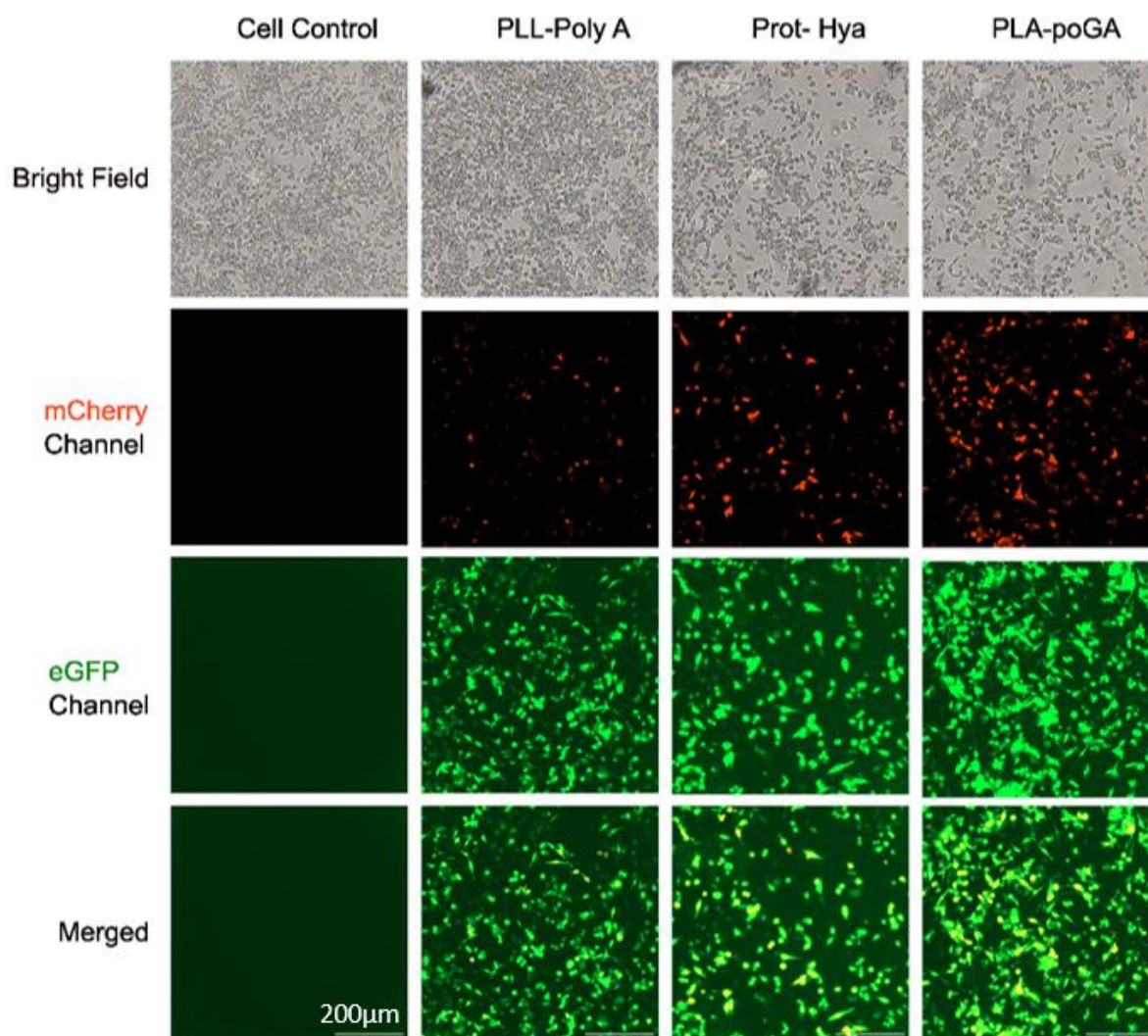


Figure S 4: Transfection of DC 2.4 cells dual-loaded LPPs carrying mCherry in the core (loaded pre-shipment) and eGFP on the surface (loaded post-shipment). Scale bars: 200 µm. Cells were imaged using Agilent BioTeK Cytation 7.

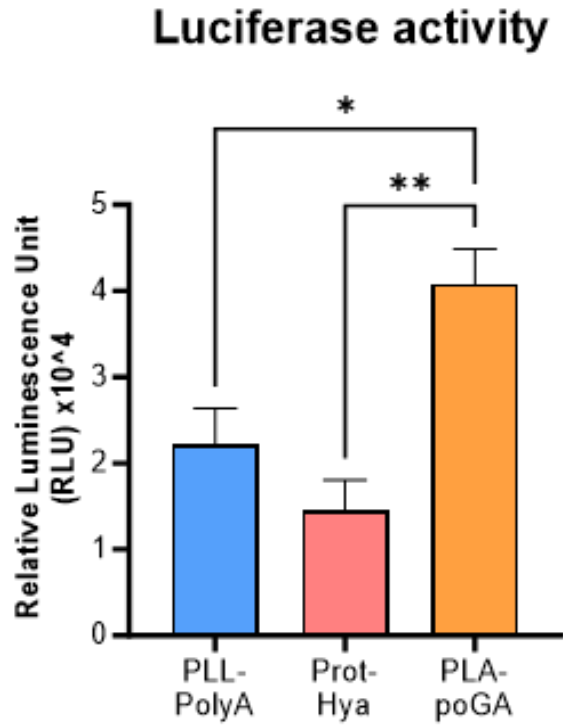


Figure S 5: Transfection of adsorbed firefly luciferase mRNA by LPPs. Data represents mean luciferase activity $\pm$ standard error of mean. (* $p < 0.05$; ** $p < 0.01$).

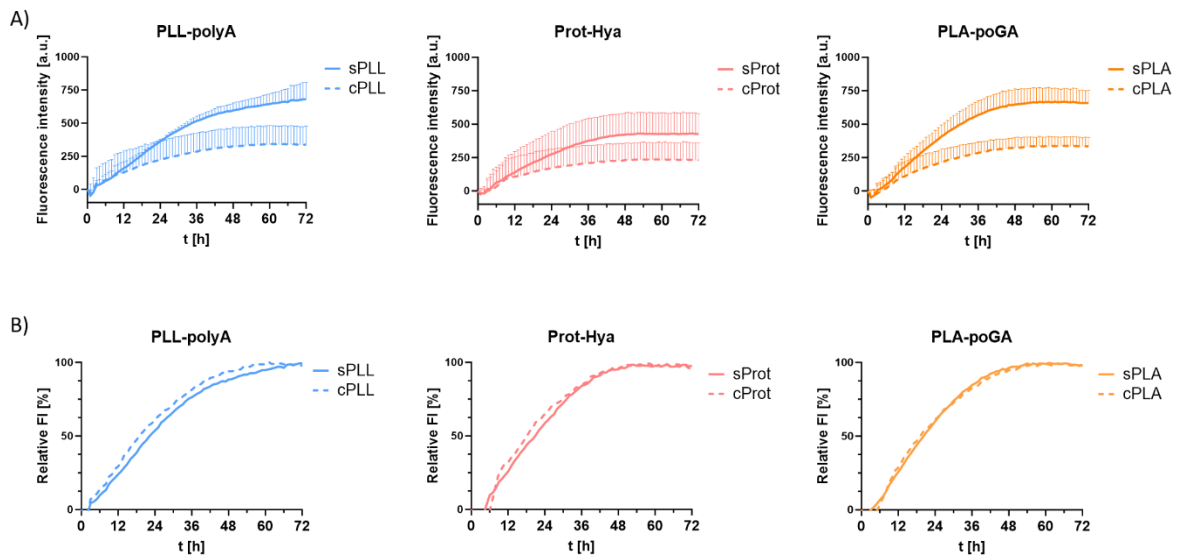


Figure S 6: (A) Expression kinetic of core-loaded (dashed line) versus surface-loaded (solid line) eGFP mRNA from PLL-polyA, Prot-Hya and PLA-poGA LPPs, presented as absolute fluorescence intensity values. Dotted lines indicate standard deviation; (B) Expression kinetics normalized to the respective maximum expression levels, shown as relative values.

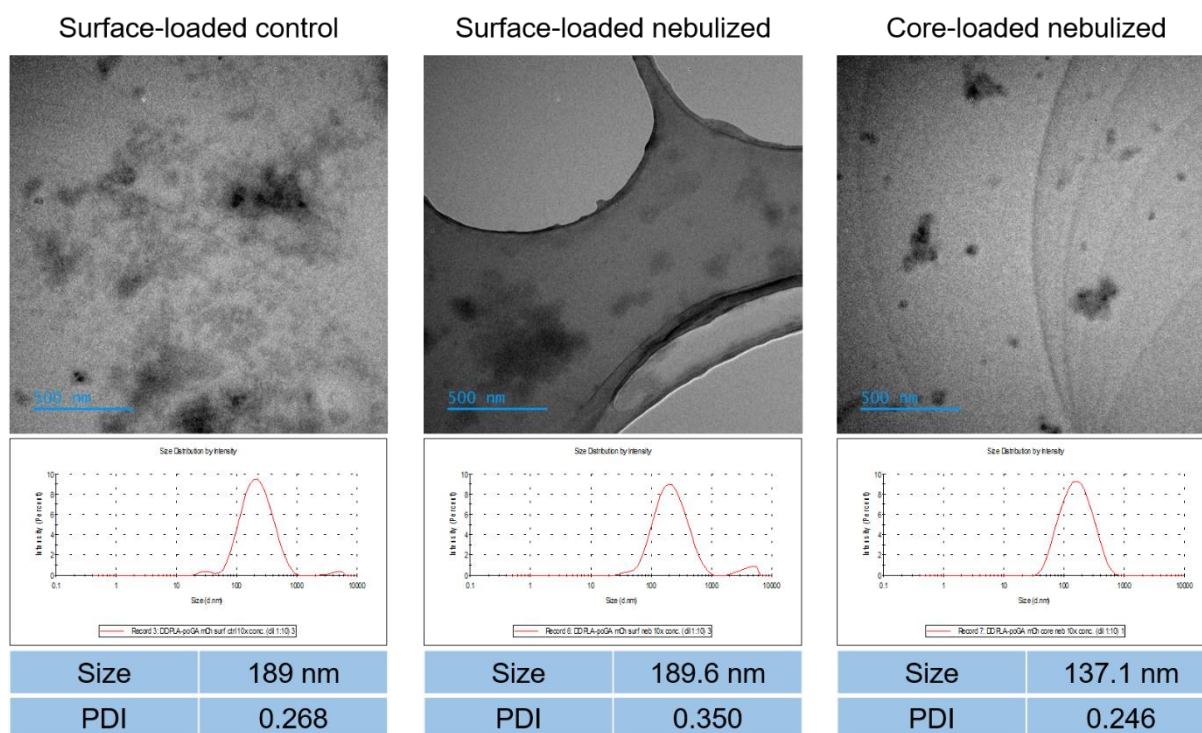


Figure S 7: Representative TEM images and respective DLS analysis of surface-loaded non-nebulized, surface-loaded nebulized and core-loaded nebulized PLA-poGA LPPs.

Formulation	mRNA localization	Transfection efficiency	
		Pre-nebulization	Post-nebulization
PLL-polyA	Core	21.5±0.1%	0.4±0.0%
	Surface	56.7±0.1%	60.8±1.0%
Prot-Hya	Core	27.0±0.3%	1.3±0.2%
	Surface	41.8±4.5%	32.6±0.2%
PLA-poGA	Core	30.6±0.5%	0.9±0%
	Surface	62.8±1.3%	64.7±0.5%

Table S 1: LPP transfection efficiency before and after nebulization assessed by quantitative image analysis using Tecan Image Analyzer software.

Note: The differences in transfection efficiencies between Table S1 and Figure 3C are due to the distinct quantification methods used. Transfection efficiency in Figure 3C was determined by the more sensitive FACS method, whereas the values in Table S1 were obtained through less sensitive fluorescence microscopy using the Tecan SparkCyto combined with quantitative image analysis.

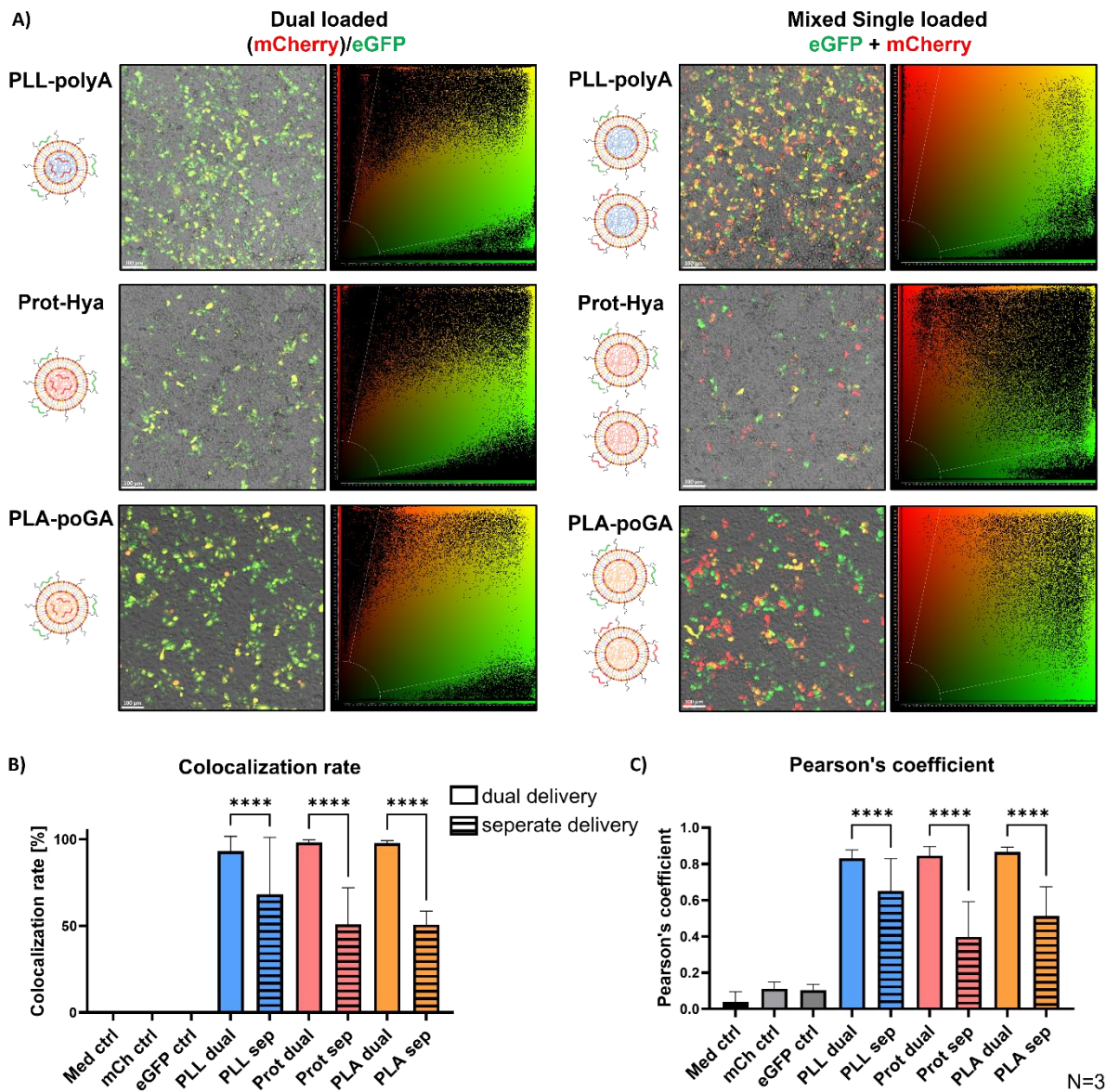


Figure S 8: (A) Representative CLSM images of A549 cells transfected for 48 h with eGFP and mCherry using dual-loaded or mixed single-loaded LPPs. Corresponding histograms display the fluorescence intensity distribution of red and green signals at individual pixels; Cells were imaged using the Leica DMI8 fluorescence microscope. (B) Average co-localization rates of mCherry and eGFP fluorescence in A549 cells; (C) Average Pearson's correlation coefficient of mCherry and eGFP fluorescence in A549 cells. Vertical lines indicate standard deviation. (**** $p < 0.0001$)

5.3 Scientific output

5.3.1 Publications in Peer-Reviewed Journals

- Gabelmann, Aljoscha; Mansouri-Ghahnavieh, Elham; Koch, Marcus; Shinde, Prashant; Guzmán, Carlos A.; Loretz, Brigitta; Lehr, Claus-Michael (2025b): A novel lipopolyplex platform for dual mRNA delivery via core- and surface-loading. In: *Journal of controlled release : official journal of the Controlled Release Society* 384, S. 113875. DOI: 10.1016/j.jconrel.2025.113875
- Gabelmann, Aljoscha; Biesel, Achim; Loretz, Brigitta; Lehr, Claus-Michael (2025a): Exploring the future of mRNA delivery: Beyond lipid nanoparticles. In: *Biochemical and biophysical research communications* 778, S. 152347. DOI: 10.1016/j.bbrc.2025.152347.
- Kliesch, Lena; Delandre, Simon; Gabelmann, Aljoscha; Koch, Marcus; Schulze, Kai; Guzmán, Carlos A. et al. (2022): Lipid-Polymer Hybrid Nanoparticles for mRNA Delivery to Dendritic Cells: Impact of Lipid Composition on Performance in Different Media. In: *Pharmaceutics* 14 (12). DOI: 10.3390/pharmaceutics14122675.
- Bassil, Justine; Kamal Mohamed A.M.; Gabelmann, Aljoscha; Christoulaki, Anastasia; Koch, Marcus; Hamed, Mostafa M; Loretz, Brigitta; Gallei, Markus; Buhler, Eric; Lehr, Claus-Michael; Hirsch, Anna K.H.; Lee, Sangeun (2025): Direct monitoring of intracellular polymer degradation via BODIPY dynamic dequenching. In: *Materials and Design* 256 DOI: 10.1016/j.matdes.2025.114240.
- Kopliku, Vesa; Gabelmann, Aljoscha; Biesel, Achim; Koch, Marcus; Loretz, Brigitta; Lehr, Claus-Michael (*in preparation*): Overcoming mechanical stress: Lipopolyplexes show superior stability and mRNA transfection efficiency compared to Lipid nanoparticles.

5.3.2 Oral Presentations

- “Development of an advance vaccine platform to promote protective mucosal immunity”, CRS Germany local chapter 2024, Bad Dürkheim, Germany

- “Development of an advance vaccine platform to promote protective mucosal immunity”, HIPS Symposium 2024, Saarbrücken, Germany

5.3.3 Poster Presentations

- “Development of an advance vaccine platform to promote protective mucosal immunity”, GPEN 2024, Copenhagen, Denmark

5.4 Curriculum vitae

Personal information

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Education

01/2022 – 05/2025 PhD Student
Helmholtz-Institute for pharmaceutical Research Saarland
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08/2007 – 06/2016 High school diploma
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07/2025 – 12/2025 Formulation Scientist
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I am especially grateful to my supervisor, Dr. Brigitta Loretz, for her inspiring ideas, her support in all aspects of my PhD, and her constant availability whenever I needed advice.

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5.6 Declaration of the use of Generative AI

During the preparation of this work, the author used ChatGPT based on GPT-5 in order to improve readability and language. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the thesis.