Lipid nanoparticle technology for mRNA delivery: bridging vaccine applications with fundamental insights into nano-bio interactions

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Chapter 6

Summary and Outlook

Lipid nanoparticles (LNPs) enabled the breakthrough of mRNA vaccines, such as those used for COVID-19; nevertheless, their broader therapeutic application remains limited by challenges in delivery efficiency, targeting, and mechanistic understanding [1,2]. The main aim of this thesis was to advance the rational design and functional optimization of LNP-based mRNA delivery systems for therapeutic and vaccine applications by systematically evaluating how LNP composition influences delivery performance and immune activation, developing innovative immunization strategies to boost antigen-specific responses, and investigating how LNP chemistry affects biodistribution and interactions with macrophages, including uptake and intracellular trafficking, using both cell culture and zebrafish models. Finally, advanced imaging tools—such as expansion microscopy combined with bioorthogonal click chemistry—were established with the intent to resolve the subcellular localization of LNP-ionizable lipids at super resolution.

1. From lipid chemistry to immune response in mRNA-LNPs

mRNA vaccines formulated in LNPs have transformed vaccinology, offering a versatile platform for rapid, scalable, and effective immunization [3–5]. However, a major limitation is the weak correlation between *in vitro* potency and *in vivo* efficacy, which complicates the rational design of LNP-mRNA formulations [6,7]. In addition, the inherently poor immunogenicity of many tumor antigens restricts the effectiveness of mRNA-based cancer vaccines[8,9]. The work presented here addresses these challenges by examining how lipid chemistry influences mRNA delivery across distinct biological contexts and by exploring new vaccination strategies aimed at enhancing immune responses.

Ionizable lipids are regarded as the central drivers of the mRNA-LNP functions, yet their precise roles in modulating biological performance—such as encapsulation efficiency, endosomal escape, biodistribution, and immunogenicity—remain incompletely understood [10–12]. In **Chapter 2**, we systematically compared different ionizable lipids and found that while all generated nanoparticles with comparable physicochemical properties and efficient mRNA encapsulation, their biological activity diverged depending on the experimental context. Certain ionizable lipids appeared more effective *in vitro* at driving protein expression and T cell proliferation, but these differences did not translate consistently *in vivo*, where protein expression and immune responses followed different patterns. Immunization studies further demonstrated that all clinically relevant ionizable lipids elicited strong antigen-specific T cell responses, regardless of their *in vitro* profile. These results underscore the limited predictive

power of cell culture assays for *in vivo* performance and highlight the importance of evaluating mRNA-LNPs in physiologically relevant models.

Building on this, **Chapter 3** addressed one of the central challenges in mRNA-based cancer vaccine development: the weak immunogenicity of tumor antigens [13,14]. Here, we introduced a heterologous prime–boost regimen that combines antigen-encoding mRNA-LNPs with costimulatory agonist-based boosters. This approach markedly amplified antigen-specific T cell responses, improved their durability, and revealed that lipid composition could shape tissue-specific immune outcomes. LNP-mediated delivery of mRNA-encoding costimulatory molecules provided an alternative to antibody-based stimulation and enhanced effector T cell activation, though without generating durable memory. Together, these findings show that both lipid chemistry and vaccination strategy are key parameters of potency and quality of LNP-mRNA vaccines.

2. Rethinking LNP design: Lessons from nano–bio interactions

The second part of this thesis highlights the importance of studying nano–bio interactions to better understand how LNP composition shapes the biological performance of mRNA vaccines and therapeutics. Since processes such as cellular uptake and endosomal escape remain poorly understood, new tools are needed to track LNP behavior inside cells [15,16]. To address this, we developed a chemical strategy to visualize ionizable lipids directly within cells, providing a platform that can be applied to elucidate the mechanisms underlying endosomal escape and other critical steps in mRNA delivery mediated by LNPs.

A deeper understanding of nano–bio interactions is essential for the advancement of mRNA-LNP therapeutics, as the physicochemical properties of nanocarriers strongly influence their biological fate [17,18]. These interactions determine cellular uptake, intracellular trafficking, and ultimately mRNA delivery efficiency [18]. Among the many cell types involved, macrophages are of particular interest due to their central role in immunity, their ability to phagocytose nanocarriers, and their function as both biological barriers and therapeutic targets [19]. **In Chapter 4**, we systematically varied helper lipid chemistry across anionic, neutral, and cationic types to assess how charge influences macrophage targeting and transfection. Cationic helper lipids consistently produced the highest mRNA delivery efficiency, correlating with enhanced endosomal escape and with distinct changes in the LNP nanostructure; higher cationic lipid concentration may promote the formation of cubic-phase lipid arrangements, known to

facilitate membrane disruption and improve transfection [20,21]. Biodistribution studies further confirmed that LNP formulations containing cationic helper lipids achieved higher levels of macrophage transfection, but their use at high concentrations raises concerns about potential toxicity and immunostimulation, pointing to the need for careful balance between efficacy and safety in future mRNA-LNP designs.

Chapter 5 explores a chemical modification strategy to create clickable ionizable lipids by introducing a terminal alkyne group into the hydrophobic tail. This subtle modification preserved the physicochemical properties and biological performance of LNPs, while enabling efficient bioorthogonal conjugation with azide-linked fluorescent dyes through click chemistry [22]. With this approach, the modified ionizable lipids allowed sensitive and specific visualization of mRNA-LNPs inside cells, revealing uptake kinetics, vesicular localization, and progressive disassembly prior to endosomal escape and mRNA release. Co-localization studies validated them as reliable markers for intact LNPs, and additional headgroup substitutions with reactive groups enhanced lipid retention during cell permeabilization, improving the accuracy of localization analysis. Overall, multifunctional ionizable lipids establish a versatile imaging platform for tracking LNP behavior to potentially uncover the mechanisms that govern mRNA-LNP delivery *in vitro* and in *vivo*.

3. Conclusions and perspectives

First, systematic evaluation of ionizable lipids revealed how compositional variations critically shaped physicochemical properties, cellular uptake, protein expression, and immune activation, thereby providing design principles for improved mRNA-LNP formulations. Second, innovative immunization strategies, such as heterologous prime–boost regimens with costimulatory mRNA-LNP boosters, were shown to enhance antigen-specific cellular immunity. Third, the biological interactions between LNPs and macrophages were investigated in depth, using advanced analytical techniques in both cell culture and zebrafish models to elucidate how biodistribution, uptake, and intracellular trafficking create barriers to efficient delivery. Finally, new imaging approaches—combining bioorthogonal click chemistry with expansion microscopy—enabled direct visualization of LNP-ionizable lipid trafficking and localization inside cells. Together, these findings linked formulation design with immune outcomes and nano–bio interactions, advancing both fundamental understanding and practical strategies for next-generation mRNA-LNP therapeutics and vaccines.

Chapters 2 and 3 together highlight the need to rethink how mRNA-LNP vaccines are evaluated and optimized. The weak predictive power of assays *in vitro* underscores the urgency of developing experimental frameworks that better capture *in vivo* complexity, which will be critical for accelerating translation and avoiding misleading conclusions during formulation screening. At the same time, the limited immunogenicity of tumor antigens points to the necessity of designing vaccination regimens that strategically integrate lipid chemistry with immunological cues. Looking ahead, advancing mRNA cancer vaccines will require coupling improved preclinical models with rationally engineered prime–boost strategies to unlock stronger, more durable antitumor immunity.

Chapters 4 and 5 demonstrate that progress in mRNA-LNP delivery depends not only on formulation design but also on mechanistic insight into nano–bio interactions. The pronounced effects of helper lipid chemistry on macrophage targeting suggest that tuning composition can be a powerful strategy to direct delivery, though this must be balanced against potential toxicity at high content of cationic lipids. Moreover, the persistent bottleneck of endosomal escape demands innovative tools to probe intracellular fate of mRNA-LNPs. The clickable ionizable lipids introduced here provide such a platform, enabling direct visualization of LNPs within cells, opening new opportunities to investigate intracellular trafficking pathways and mechanisms, that in turn, could support the rational design of next-generation mRNA-LNPs. Future research should integrate mechanistic insight with engineering design to develop safer, more effective, and tissue-targeted mRNA-LNPs for diverse therapeutic applications.

6.4. References

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