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REVIEW

Exploring the Latest Breakthroughs in Lipid Nanoparticle-Mediated Delivery:

A Deep Dive into Lipid Innovation and Intracellular Discovery

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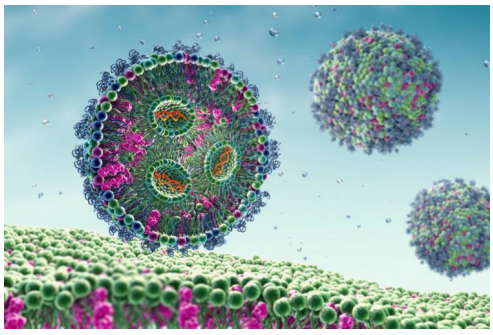
Abstract

Lipid nanoparticles (LNPs) have ushered in transformative progress in the domain of drug delivery, especially for nucleic acid-focused treatments. These expertly crafted nanoparticles, made up of diverse lipid elements, are tailored to efficiently encapsulate and transport therapeutic molecules. Their strategic construction guarantees maximum payload containment, successful delivery, and compatibility with biological systems. Notably, LNPs have been instrumental in the creation of COVID-19 vaccines, demonstrating their capability in transmitting genetic instructions to elicit robust immune defenses. As research in this area evolves, innovation in lipid design and a deeper comprehension of lipid interactions are becoming paramount. The scientific community is zeroing in on enhancing the efficacy of LNPs, tackling hurdles such as enabling selective targeting and minimizing potential adverse effects. The evolution of LNPs, from their initial design in research labs to their practical therapeutic uses, is intricate, yet it offers a bright future for revolutionizing healthcare. This review delves into the latest strides in lipid technology, an in-depth exploration of LNPs' cellular interactions, and the emerging LNP platforms augmented by artificial intelligence and automation.



Abstract figure

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Introduction

Lipid nanoparticles (LNPs) have catalyzed a groundbreaking revolution within the domain of drug delivery, a revolution that has been particularly spotlighted by the rapid development of COVID-19 vaccines (Thi et al., 2021). These LNPs, intricate in their composition, serve as promising vehicles, especially in the context of nucleic acid-based therapies. Comprising ionizable lipid, phospholipid, cholesterol, and polyethylene glycol (PEG)-lipid, these nanoparticles are engineered with precision to encapsulate and convey therapeutic agents efficiently (Marité et al., 2023). The elegance of their design lies in the unique physicochemical structure they exhibit: an outer hydrophilic layer that interfaces with biological environments, coupled with a hydrophobic core adept at encapsulating diverse therapeutic payloads. This architecture ensures optimal payload encapsulation, facilitates effective delivery, and guarantees biocompatibility, factors pivotal for their success (Shi et al., 2022).

The significance of LNPs has been prominently underscored by the role they played in expediting the development and distribution of COVID-19 vaccines. The Pfizer-BioNTech and Moderna vaccines, for instance, utilize LNPs as their delivery vehicles for mRNA encoding the spike protein of the SARS-CoV-2 virus (Thi et al., 2021; Jung et al., 2022). This revolutionary approach allows for the precise delivery of genetic material, prompting cells to generate a protective immune response. In the realm of nucleic acid-based therapies, including mRNA, siRNA, and more, LNPs have garnered widespread recognition for their remarkable versatility and efficiency. The ionizable lipid, crucial for pH-responsive behavior, enables LNPs to respond to the acidic environment of endosomes, facilitating the escape of the therapeutic cargo into the cytoplasm (Carrasco et al., 2021). Simultaneously, the phospholipid component, often a phosphatidylcholine derivative, contributes to the structural stability of the LNP, safeguarding the encapsulated cargo during its journey. The modulation of membrane fluidity by cholesterol further influences LNP stability and interactions with cell membranes. Additionally, the PEG-lipid component, characterized by its hydrophilic nature, prolongs circulation half-life by reducing immune recognition and subsequent clearance (Ermilova et al., 2020; Paloncýová et al., 2021).

As the field of LNPs progresses, the crucial role of lipid innovation becomes increasingly apparent. Beyond introducing novel lipids, researchers are focusing on understanding intricate lipid-lipid and lipid-payload interactions (Li et al., 2022). By customizing lipid structural design, scientists aim to optimize the performance and efficacy of LNPs, addressing challenges such as improving cellular uptake and mitigating potential toxicities (Foroozandeh et al., 2018). This trajectory emphasizes not only the introduction of new lipid components but also the necessity of comprehending the implications of lipid chemical structures on the overall LNP composition.

Exploring the intricacies of LNPs' intracellular behavior adds another layer of complexity. To ensure therapeutic efficacy, LNPs must successfully navigate the intricacies of the intracellular milieu. This involves careful orchestration of events, including endosomal escape and timely payload release (Mazumdar et al., 2021). Innovations in lipid design, such as the incorporation of biodegradable linkages, show potential in enhancing this intricate intracellular journey, underscoring the necessity for ongoing research in this domain (Li et al., 2022). However, the journey from conceptualization to the therapeutic application of LNPs presents its own set of challenges. The methodical pace of lab-based research, while essential for safety and efficacy, sometimes contrasts with the urgency of medical needs. The exploration of alternatives to components like PEG stems from concerns about immunogenicity and long-term effects, spurring further research and innovation (Hoang et al., 2020). In addition, the call for standardized nanoparticle platforms across various therapeutic applications highlights the complexity of translating laboratory achievements into tangible benefits for patients.

As the field of LNPs continues its evolution, the imperative of confronting these challenges head-on remains pivotal. By surmounting these obstacles, the full therapeutic potential of LNPs can be harnessed, solidifying their status as a revolutionary avenue for drug delivery. The journey is complex and demands persistent dedication, but as knowledge grows and collaborative efforts persist, the promise of LNPs reshaping medical treatment becomes increasingly tangible.

LNP composition and structure

Lipid nanoparticles (LNPs) are sophisticated delivery vehicles consisting of an ionizable lipid, phospholipid, cholesterol, and polyethylene glycol (PEG)-lipid (Figure 1). The composition of LNP involves a careful selection and combination of various lipid components to achieve optimal payload encapsulation, payload delivery efficacy, biocompatibility, and stability (Paloncýová et al., 2021; Marité et al., 2023). The molar percentages of each lipid component in LNP are also a critical part of LNP composition, which determines the overall particle structure and nanoparticle perfo rmance. The N/P ratio refers to the ratio of ionizable lipid nitrogen (N) to the negatively charged nucleic acids phosphate (P) groups, which can affect the electrostatic condensation between lipids and the payload, it's an ionizable-LNP exclusive compositional parameter (Hald et al., 2022). Precise adjustments in lipid selection, lipid molar percentages, and N/P ratio allow tailoring LNPs

for specific payload types and desired delivery characteristics. LNPs formulate at signature compositions possess unique physicochemical structures, with a hydrophilic exterior and a hydrophobic core, allowing them to encapsulate a range of therapeutic agents (Hald et al., 2022; Jung et al., 2022). The structural intricacies of LNPs play a vital role in their delivery efficacy. Consequently, the specific composition and resulting structure of LNPs are paramount in determining their delivery activity and overall therapeutic efficacy (Geng et al., 2023).

1. Signature lipid components

LNPs have emerged as versatile and efficient delivery vehicles for various therapeutic applications, particularly in the realm of nucleic acid-based therapies like mRNA and siRNA. These LNPs are composed of several lipid components, each playing a crucial role in ensuring the encapsulation, delivery, stability, and bioactivity of the therapeutic cargo. Among these essential and signature components are the ionizable lipid, phospholipid, cholesterol, and PEG lipid, each contributing distinct functionalities to the overall LNP structure and bioactivity (Hald et al., 2022).

The ionizable lipid, a cornerstone of LNP design, is responsible for the pH-responsive behavior of the nanoparticle. This lipid is typically amphiphilic, featuring both hydrophobic and hydrophilic regions. Its most remarkable attribute lies in its ability to undergo a charge transition in response to changes in environmental pH. At low pH, such as the mildly acidic conditions found in endosomes after cellular uptake, the ionizable lipid carries a positive charge due to protonation. This charge change triggers the destabilization of the LNP structure (Figure 2), facilitating the release of the encapsulated therapeutic cargo into the cytoplasm (Gyanani et al., 2023). This pH-sensitive behavior is pivotal for efficient endosomal escape, a critical step in achieving successful intracellular delivery of therapeutic molecules.

Working in harmony with the ionizable lipid is the

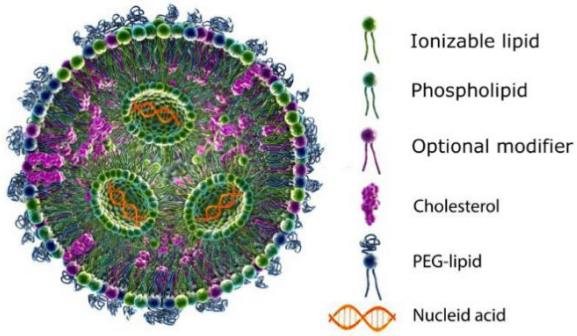

Figure 1. The structure and components of a lipid nanoparticle delivery vehicle encapsulating nucleic acid payload

Notes: Lipid nanoparticles consist of a dynamic assembly of various lipid molecules that collectively form a versatile delivery system. These components typically include ionizable lipid, which aid in both LNP formation and intracellular activity; structural lipids like phospholipid, which supports the nanoparticle's outer structure; cholesterol, which enhances stability and rigidity of the nanoparticle; PEG lipid that promotes circulation in the bloodstream. The resulting LNP structure encapsulates therapeutic payloads within its lipid core, offering a promising avenue for drug delivery and gene therapy applications.

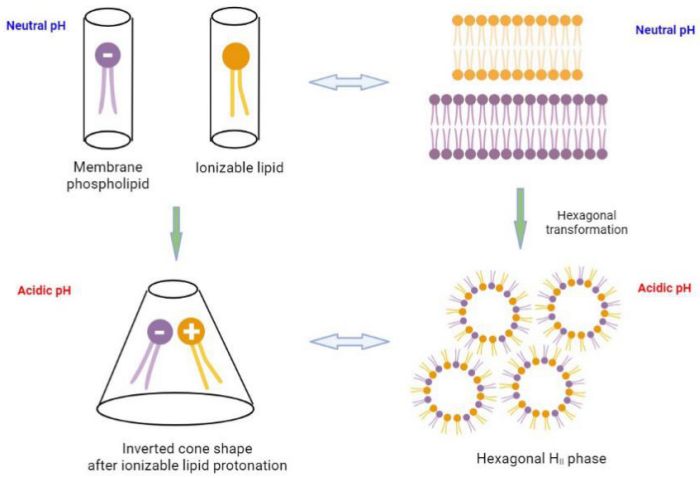

Figure 2. Proposed mechanism of ionizable lipid mediated membrane disruption and endosomal escape

Notes: Ionizable lipids play a key role in aiding lipid nanoparticles' escape from endosomes through pH-responsive membrane changes. These lipids shift from a neutral/negative state at physiological pH to a positively charged state at acidic endosomal pH. This pH-dependent transformation induces structural alterations in the endosomal membrane, potentially leading to hexagonal II phase formation, which facilitates the LNPs' payload release into the cytoplasm and enhances the effectiveness of drug delivery.

phospholipid, as known as the helper lipid. Phospholipids are amphiphilic molecules with a hydrophilic head group and two hydrophobic tails. These lipids serve as the backbone of the LNP structure, forming the lipid shell layer that encapsulates the therapeutic payload. The hydrophilic head groups interact with polar solvents, while the hydrophobic tails create a lipid core that provides a protective environment for the encapsulated cargo. Additionally, the phospholipids contribute to the stability and flexibility of the LNP membrane, allowing it to accommodate changes in shape during cellular uptake and intracellular trafficking (Álvarez-Benedicto et al., 2022). The combination of the ionizable lipid and phospholipid establishes the structural integrity of the LNP, ensuring that it remains intact in circulation, efficiently delivers its payload to the target cells, and facilitates endosomal escape for successful gene silencing or protein expression (Ermilova et al., 2023).

Cholesterol, a naturally existing key component of biological membranes, is another crucial constituent of LNPs. It modulates the physical properties of the LNP structure both in the core and the shell. Cholesterol molecules are interspersed within the phospholipid layer due to their unique chemical structure and length, where they regulate membrane fluidity and stability. By reducing lipid packing and preventing excessive crystallization, cholesterol maintains the LNP membrane in a state that balances rigidity and flexibility (Eygeris et al., 2020). This is crucial for enabling the LNP to maintain its structure during circulation, avoid recognition by the immune system to a certain extent, and withstand the shear forces encountered within the bloodstream. Moreover, cholesterol plays a role in enhancing endosomal escape, as it promotes the fusion of the LNP membrane with the endosomal membrane, facilitating the release of the encapsulated cargo into the cytoplasm. Thus, cholesterol contributes to both the stability and the effective delivery of therapeutic payloads by LNPs.

The incorporation of a PEG lipid into the LNP structure further enhances its therapeutic potential. PEGylation is a strategy employed to increase the circulation half-life of nanoparticles by creating a protective shield on the particle surface, minimizing interactions with blood components, and reducing the uptake by the reticuloendothelial system. The PEG lipid extends from the LNP surface, forming a hydrophilic brush-like layer that provides steric stabilization. This steric hindrance effect limits opsonization and prevents rapid clearance by immune cells, thereby allowing the LNPs to circulate in the bloodstream for an extended period (Hoang et al., 2020). PEGylation also contributes to reducing potential immunogenicity and toxicity, which can be concerned with nanoparticle-based drug delivery systems.

In addition to these foundational lipid components, the versatile nature of LNP permits the incorporation of a diverse array of functional lipids tailored to specific objectives. These specialized modifier lipids can be strategically integrated into the LNP formulation to enhance targeting, stability, drug loading, and overall therapeutic efficacy. Ligand-conjugated lipids, such as targeting ligands or antibodies, enable precise recognition and binding to specific cell surface receptors, thereby facilitating targeted delivery to specific cell types or tissues (Kularatne et al., 2022). Fusogenic lipids, like those derived from viral membranes, can be introduced to enhance endosomal escape by promoting membrane fusion with endosomes (Zhang et al., 2022). Additionally, lipids with stimuli-responsive properties, such as temperature or light sensitivity, allow for controlled drug release in response to specific environmental cues (Kularatne et al., 2022; Li et al., 2023).

The essential lipid components and optional modifier lipids contribute to the success of LNP as powerful delivery vehicles for nucleic acid-based therapies. Their orchestrated roles in ensuring structural integrity, pH-responsive behavior, membrane stability, efficient endosomal escape, and prolonged circulation time collectively enable the LNPs to effectively navigate the complex journey from administration to intracellular target. Together, they have expanded the toolbox for designing LNPs that are finely tuned to address diverse therapeutic challenges, ultimately advancing the potential of precision medicine and personalized treatment approaches.

2. Lipid innovation

Innovative lipid design emerges as a crucial driver of progress in LNP development, particularly in optimizing the overall LNP delivery performance and efficacy. Tailored lipids can be meticulously designed to recognize specific receptors on designated cells, ushering in preferential cellular uptake and escalating the therapeutic index (Tenchov et al., 2021). Furthermore, the innovation in lipid chemical structures may address toxicity concerns, translating into heightened LNP safety, shielding against adverse effects, and expanding the breadth of clinical utility. The arena of novel lipid design tackles challenges tied to immune response and elimination kinetics as well, a cornerstone of optimizing LNPs' trajectory in vivo.

By crafting novel lipids with tailored attributes, researchers can surmount existing limitations and elevate LNPs' ability specifically. This proficiency is instrumental in unleashing the maximum therapeutic potential of LNPs while minimizing wastefulness (Liu et al., 2022). The selection and use of novel lipids in LNP formulation represent a substantial alteration in LNP composition, with profound implications for their performance as drug delivery carriers. Each lipid chosen contributes unique properties to the LNP's structure and behavior. For instance, the choice of phospholipids and

cholesterol governs the fundamental structure and certain membrane fluidity (Ermilova et al., 2020; Medjmedj et al., 2022]. Introducing various ionizable lipids alters electrostatic interactions and thus affects payload encapsulation efficiency and cellular interactions (Long et al., 2023). Moreover, modified PEG lipids can significantly modify surface properties, influencing circulation time and immune response. Thus, lipid innovation and utilization require meticulous consideration of the overall physicochemical properties and intracellular behavior of LNPs, as well as comprehensive evaluation to aid in refining lipid design, optimizing LNP composition, and ensuring safe and efficient delivery of therapeutic payloads.

To enhance the LNP endosomal escape and biocompatibility, Li and colleagues completed the design and evaluation of a novel combinatorial library of cholesteryl-based disulfide bond-containing biodegradable cationic lipidoid nanoparticles (Li et al., 2020). These LNPs were specifically crafted for the intracellular delivery of therapeutic biomacromolecules. The rationale behind the design is rooted in the integration of a biodegradable disulfide bond and a cholesteryl moiety into the lipid tail, termed OCholB. This unique design was hypothesized to result in a new class of lipidoids with distinct self-assembly behaviors and varied biological effects, given that the delivery properties of synthetic LNPs are highly sensitive to the chemical structure of the lipidoid. The biodegradable nature of these LNPs was a key focus, with the disulfide bond playing a pivotal role by ensuring that the LNPs are degradable in the highly reductive intracellular environment but remain stable in the blood serum (Li et al., 2020; Li et al., 2023). The study assessed the thiol-triggered degradation of OCholB LNPs in vitro using agents like 1,4-dithiothreitol (DTT) and L-cysteine (Cys) to mimic the reducing environments in the cytoplasm and serum, respectively. The results indicated that DTT treatment led to the disintegration of liposomal structures and the formation of large amorphous aggregates, while Cys treatment had negligible effects on LNP size. Furthermore, the in vitro biocompatibility assessment of the new OCholB-based LNPs revealed that they exhibited significantly higher cell viability compared to other LNPs, especially at increased dosages and exposure durations. This finding supports the hypothesis that incorporating cholesteryl through a biodegradable linkage can enhance the compatibility of lipidoids. The in vitro intracellular delivery of mRNA using OCholB LNPs was studied using GFP mRNA. The delivery conditions were optimized using GFP mRNA/75-OCholB in HeLa cells. Subsequently, the intracellular delivery efficiencies of all OCholB LNPs were tested in various cell lines, including HeLa, B16F10, HEK293, and NIH 3T3. Lpf2k and naked GFP mRNA were used as positive and negative controls, respectively. A subset of the OCholB LNP library consistently delivered mRNA to all cell types screened. Intracellular protein delivery efficiencies were determined using GFP-Cre protein. Six of the nine examined OCholB LNPs showed comparable or even higher transfection efficiency than the positive control Lpf2k. Specifically, 76-OCholB resulted in dramatically higher mean fluorescent intensity (MFI) than Lpf2k and other OCholB LNPs, indicating its high effectiveness in delivering intact GFP-Cre protein per cell. In vivo, studies revealed that individual LNPs might have specific tendencies for delivery to particular organs. For instance, 75-OCholB preferred delivery to the lung, while 76-OCholB showed delivery to the spleen. Systemic mRNA delivery using 76-OCholB LNP was also studied, with positive signals observed in local Cre mRNA delivery and systemic protein delivery (Li et al., 2020). In essence, this study emphasized the importance of thoughtful and innovative lipid design to achieve optimal intracellular delivery of therapeutic agents.

As the key component of the LNP shell, the phospholipid plays a crucial role in the structural support as well as determining LNP surface property. Álvarez-Benedicto et al discovered the intricate relationship between the structural design of phospholipids and the efficacy of LNPs in delivering mRNA (Álvarez-Benedicto et al., 2022). Recognizing that phospholipids play a pivotal role in the LNP delivery process, the study embarked on a systematic exploration of diverse phospholipids with varying headgroups, chain lengths, degrees of saturation, and methyl substitutions. Unique lipids exclusive to organelle membranes, such as Bis-(Monoacylglycerol)-Phosphate and cardiolipin, were also included in the study. The LNPs were formulated with constant components including ionizable cationic lipid (4A3-SC8), cholesterol, and PEG-lipid (DMG-PEG2000), while introducing a variable phospholipid in each formulation. This approach allowed for a focused evaluation of each phospholipid's impact on mRNA delivery efficacy within the LNP context. The study revealed that phospholipids with phosphoethanolamine (PE) head groups likely enhance endosomal escape due to their fusogenic properties. Furthermore, zwitterionic phospholipids predominantly aided liver delivery, while negatively charged phospholipids shifted the LNP tropism from the liver to the spleen. In vitro studies indicated that phospholipids containing hydrophobic chains with unsaturated bonds were particularly effective in mRNA delivery. The study also highlighted that the choice of phospholipid can influence activities. For instance, LNPs formulated with DOPE outperformed those with DSPC in terms of mRNA delivery, suggesting that phospholipids could either enhance cellular uptake or mRNA escape in endosome. Indeed, the study results highlighted the profound influence of phospholipid structure on the delivery efficacy of LNPs, and by

optimizing the choice and design of phospholipids, LNPs can be tailored for improved mRNA delivery (Álvarez-Benedicto et al., 2022).

Patel and colleagues focused on the structural intricacies of cholesterol and its analogues, aiming to optimize LNP formulations for enhanced mRNA delivery (Patel et al., 2020). Recognizing cholesterol's pivotal role in LNP efficacy, the study embarked on a systematic exploration of naturally occurring cholesterol analogues, focusing on their structural domains: the head, body, and tail. The study categorized cholesterol analogues into three groups based on structural resemblances to the cholesterol ring system. Group I comprised Vitamin-D derivatives differing from cholesterol in the body, with or without tail modification. Group II contained alkyl-substituted steroids diverging from cholesterol solely in the tail. Group III featured cholesterol analogues wherein the tail was transformed into a fifth ring. The LNPs were formulated with these cholesterol analogues and assessed for their mRNA encapsulation efficiency and delivery efficacy. The results indicated that LNPs formulated with certain cholesterol analogues, especially those with C-24 alkyl substitutions, outperformed traditional cholesterol-based LNPs. Specifically, LNPs with stigmastanol, a β-sitosterol analogue with a highly flexible body due to the reduction of the Δ5 double bond in the sterol ring, showed significant improvements in transfection. Furthermore, the study utilized Cryo-EM to observe variations in structure and morphology between traditional LNPs and enhanced LNPs (eLNPs). While both exhibited a core-shell structure, eLNPs displayed a highly faceted surface, contrasting with the uniform curvature of traditional LNPs (Eygeris et al., 2020; Patel et al., 2020). This morphological distinction suggests potential differences in surface lipid composition, mRNA packaging, and intracellular trafficking, which could translate into enhanced gene delivery.

Given the observed anti-PEG immune responses that can limit the efficacy of PEGylated therapeutic strategies, Thi et al revealed the importance of identifying polymers that mimic the physicochemical properties of PEG without compromising therapeutic pharmacokinetics (Hoang et al., 2020). One of the highlighted strategies is the modification of PEG into a bottlebrush architecture, presenting poly-oligo ethylene glycol methacrylate (POEGMA) as an alternative to linear PEG. This synthesized POEGMA possesses a 3D hyperbranched structure with many side chains of oligoethylene glycol moieties, ensuring effective stealth properties. The study also touches upon other potential PEG alternatives, such as hyperbranched polymers, which could be utilized to mitigate antigenicity without compromising stealth behavior. For instance, polyglycerols are hyperbranched, non-immunogenic, and highly hydrophilic. They have demonstrated robust blood circulation times and low immunogenicity. However, polyglycerol-based drugs have been shown to accumulate highly within the liver and kidneys (Webb et al., 1998). Another alternative, polyoxazolines, are thermosensitive polymers that have been reported to be both highly resistant to oxidative degradation and do not undergo bioaccumulation. However, challenges with polyoxazolines include the high cost of synthesis, issues with impurities, and difficulty in obtaining FDA approval (Hoang et al., 2020; Hwang et al., 2020).

Overall, these studies emphasized that, by understanding and leveraging the structural nuances of these alternatives, LNPs can be optimized for improved therapeutic delivery, offering promising avenues for therapeutic applications. And the development of a standardized nanoparticle platform that can be easily interfaced with any potential candidate for immunogenicity screening would be optimal for the design and interrogation of alternatives to PEG (Hoang et al., 2020; Mitchell et al., 2020).

3. Nano-structure and surface chemistry

The conventional structure of LNPs is often depicted as a spherical assembly, this distinct core-shell structure makes LNP different from other particles, with each region having unique compositions tailored for various in vivo delivery applications. The core is believed to be a distorted hexagonal phase or worm-like phase of ionizable lipids, cholesterol, and nucleic acid payload. The outer shell of LNPs is made up of a hydrated PEGylated layer, while the inner shell comprises cholesterol, phospholipids, and ionizable lipids (Hammel et al., 2023). The design of LNPs is rooted in physicochemical principles, especially the associative phase separation between multivalent, negatively charged nucleic acid and oppositely charged compacting molecules. The structure of the compacted phase or mesophase largely depends on the type of ionizable or permanently cationic lipids used for nucleic acid compaction. The critical packing parameter (CPP) of the lipids plays a significant role in determining the LNP structure. For instance, amphiphiles with a CPP >1 produce inverse hexagonal liquid crystalline phases (Kobierski et al., 2022; Mkam et al., 2022]. The presence of non-lamellar forming phospholipids and cholesterol promotes inverse structures, facilitating membrane fusion and endosomal escape (Abd Elwakil et al., 2023). Advanced characterization techniques are employed to determine the physical properties of LNPs, such as size, shape, morphology, surface charge, lipid organization, internal structure, and stability (Figure 3). Techniques like dynamic light scattering (DLS), cryogenic transmission EM (Cryo-EM) and small-angle X-ray scattering (SAXS) are commonly used for this purpose (Markova et al., 2021).

While the conventional representation of LNP structure depicts ionizable lipids arranged around the nucleic acid payload

in an inverted micellular structure, the exact arrangement of lipids in the core, on the surface, and around the nucleic acid cargo remains to be a controversial topic (Barriga et al., 2022). The structure of a specific LNP formulation is influenced by factors such as the molecular structure of lipid components, molar percentage of each lipid, type of payload and intraparticle heterogeneity. Therefore, a single structural model might not apply to all LNPs (Viger-Gravel., et al, 2018; Janos Szebeni et al., 2022). Various studies have used techniques like DLS, Cryo-EM, SAXS and fluorescence microscopy to investigate the LNP structure and lipid arrangement. These studies have revealed that even minor changes in the molecular structure of a lipid component can significantly impact the overall LNP structure (Viger-Gravel., et al, 2018; Paloncýová et al., 2023]. The term "one does not fit all" is currently the most appropriate description of the LNP structure. Sebastiani and colleagues found that while distearoylphosphatidylcholine (DSPC, a phospholipid) and cholesterol predominantly reside at the surface of the LNPs in buffer, the binding of ApoE instigates a redistribution of these lipids between the shell and the core (Sebastiani et al., 2021). This redistribution also influences the internal structure of the LNP, potentially leading to the release of mRNA.

Carrasco and colleagues delve deep into the surface properties of LNPs and their implications (Carrasco et al., 2021). Cryo-EM imaging revealed that LNPs containing dilinoleic tailed lipids exhibited a homogeneous spherical shape with an exterior bilayer and an electron-dense internal structure. This appearance aligns with a previously proposed model where the peripheral shell consists of the PEG lipid and is rich in phospholipid, while the internal compartment primarily contains the ionizable lipid electrostatically bound to the mRNA, with cholesterol distributed throughout (Leung et al., 2012). In contrast, LNPs containing dioleic-tailed lipids like DODMA and DODAP displayed heterogeneous populations. Specifically, DODAP-LNPs often showed larger segmented multicompartmental structures, with an electron-dense amorphous compartment adjacent to a more electron-lucent compartment with greater internal structure (Kimura et al., 2021). Furthermore, the study found that LNPs with lower N/P ratios exhibited higher TNS binding assay (pKa measurement) fluorescence at low pH, indicating greater surface protonation (Zhang et al., 2013; Kulkarni et al., 2018). Zeta potential (LNP surface charge) measurements revealed that reducing the N/P ratio led to more negatively charged LNPs (Larson et al., 2022). The study also calculated the absolute elemental charge of the LNPs and estimated the LNP dielectric constant by comparing this measured charge at high pH to the expected charge from the calculated number of mRNA copies in the LNP. The LNP dielectric constant ranged from 6 to 24, indicating a balance between lipid and water properties.

As the bio-interactive area of LNPs, the surface chemistry of LNPs can profoundly shape their behavior, making it a pivotal factor to study (Chen et al., 2019). The significance of LNP surface chemistry lies in its capacity to influence critical aspects of drug delivery. By engineering the surface composition, the achievements may include stability enhancement, extended circulation time, selective cellular targeting, modulable immune responses, and magnified payload protection (Sinegra et al., 2021). The most common LNP surface modifications such as the addition of hydrophilic polymers like PEG lipids can confer a stealth-like quality, evading immune recognition and prolonging circulation, leading to improved biodistribution and drug delivery efficiency (Viard et al., 2018; Tenchov et al., 2023). Furthermore, the attachment of targeting ligands to the LNP surface enables precise interaction with specific cells or tissues, minimizing off-target effects (Nobs et al., 2004). Additionally, surface chemistry determined or affected by ionizable lipids may influence how LNPs are internalized by cells, affecting drug release kinetics and therapeutic efficacy by altering LNP surface charge and pKa (Alam et al., 2023).

Moreover, LNP surface properties such as surface charge can affect the formation of protein corona upon in vivo circulation. Pozzi and colleagues employed high-resolution nano LC-MS/MS to characterize and quantify proteins adsorbed onto the nanoparticle surface (Pozzi et al., 2015). The findings suggest that the protein corona absorption is influenced by both the surface charge and chemistry of nanoparticles. Specifically, a total of 224 proteins were consistently detected from Chol-PC/MP complexes, while approximately 280 proteins were identified from DOTAP-Chol-DPPC/MP, DOTAP-Chol-DSPC/MP, and DOTAP-Chol-PC/MP complexes. The study further emphasized that merely classifying nanoparticles based on charge is insufficient to describe the nanoparticle-protein corona composition comprehensively. The chemistry and arrangement of surface lipid functional groups play a pivotal role in regulating liposome- or LNP-protein interactions (Capriotti et al., 2019). Notably, the protein corona that forms around nanoparticles is profoundly influenced by the physiological environment, specifically the type of plasma. This observation suggests that the physiological environment, i.e., the serum type, is a key factor shaping the protein corona (Ren et al., 2022). The team found significant differences in the protein coronas formation after incubation with mouse plasma and human plasma. This underscored the idea that the use of animal models might not guarantee the direct extrapolation of findings to humans (Kalnin et al., 2021). Given the relationship between the biological identity acquired by nanoparticles in vivo and their physiological response, the study suggested that the translation of novel pharmaceutical formulations from

animal models to the clinic should be evaluated on a case-by-case basis.

The pKa value signifies the pH level where a molecule transitions between protonated and deprotonated states, reflecting its acidic or basic nature and its associated change in charge at different pH levels. A recent study by Patel and colleagues focused specifically on the pivotal role of ionizable lipids in determining the efficacy of LNPs for mRNA delivery demonstrated that the primary factor influencing LNP delivery efficiency is the pKa of the LNP containing an ionizable lipid (Patel et al., 2021). The study introduced a method to predict the LNP's pKa based on the structure of the ionizable lipid using various techniques, including NMR and fluorescent-dye binding, the research team comprehensively measured the protonation of both the ionizable lipid and the formulated LNP. Interestingly, the pKa of the ionizable lipid was found to be 2-3 units higher than the LNP's pKa, primarily due to differences in proton solvation energy between the LNP and the aqueous medium (Shobaki et al., 2018). This difference in pKa values has implications for delivery efficiencies both in vitro and in vivo. The study also found that more negatively charged LNPs showed higher off-target systemic mRNA expression in the liver upon intramuscular administration.

In essence, understanding the surface interactions of nanoparticles with proteins in different biological environments is crucial for predicting their behavior and efficacy in therapeutic applications (Saptarshi et al., 2013). The modulation of LNP-mediated delivery efficacy and immune response through structural and surface chemistry modification are crucial, especially for RNA-based therapies, where immune activation can hinder the desired therapeutic effect. Therefore, the comprehensive study and manipulation of LNP surface chemistry can enable a fine-tuning on LNPs' behavior, rendering them versatile vehicles for drug delivery across a spectrum of therapeutic applications.

4. Structure-activity relationship

The efficacy of LNP-mediated drug delivery hinges on its intricate structure, where the core's hydrophobic composition shields encapsulated cargo and the hydrophilic shell dictates interactions with the biological milieu (Eygeris et al., 2022). Understanding the structure-activity relationship of LNPs holds paramount importance as it directly informs the design, optimization, and efficacy of these advanced drug delivery systems. By investigating how various structural features of LNPs influence their interactions with biological systems and therapeutic cargo, LNPs can be strategically tailored to

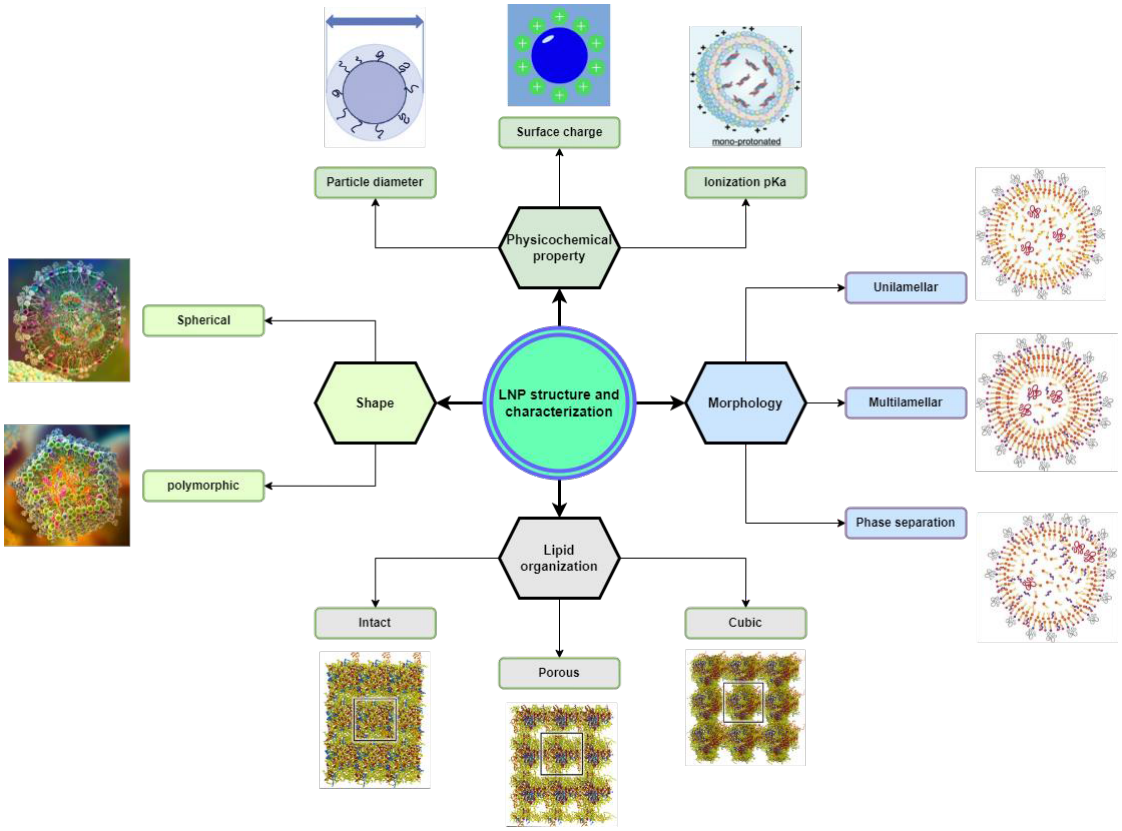

Figure 3. Schematic representation of the main structural characteristics and physicochemical properties of lipid nanoparticles

Notes: Lipid nanoparticles exhibit distinct physicochemical properties crucial to their function. These nanoscale entities typically range from 50 to 200 nanometers in size; surface charge is modulated by components such ionizable lipids; the pKa of ionizable lipids, dictating their protonation state, is critical to intracellular behaviors. LNPs adopt diverse morphologies, and the lipid organization involves shell layer formation aided by structural lipids. These properties collectively affect the payload encapsulation, cellular uptake, and intracellular release of lipid nanoparticles.

enhance their performance and address specific challenges (Sato et al., 2019; Ci et al., 2023). Insights gained from the LNP structure-activity relationship guide the selection of appropriate lipid components, surface modifications, and core-shell configurations, facilitating the translation of LNPs into effective clinical applications (Kulkarni et al., 2019). This knowledge-driven approach not only accelerates the development of novel therapies but also ensures safety and therapeutic success by maximizing the potential of LNPs as versatile and customizable platforms for drug delivery across diverse therapeutic contexts.

Patel et al delved deep into the intricate relationship between the structural components of LNPs and their efficiency in gene delivery (Patel et al., 2020). A standout observation was the transformative influence of cholesterol and its natural counterparts, phytosterols, on LNP morphology and structure. When β-sitosterol, a specific phytosterol, was integrated into the LNP formulation as an alternative to commonly used cholesterol, the resultant structure exhibited a distinct faceted surface (Patel et al., 2020; Medjmedj et al., 2022). This is a marked departure from the conventional smooth structure observed in traditional cholesterol-based LNPs. It's believed that this morphological shift can be attributed to lipid phase separation and the potential formation of two-dimensional lipid crystals (Kim et al., 2022). Furthermore, the study explored LNPs formulated with C24 alkyl derivatives of cholesterol. These LNPs showcased a rich diversity in their morphologies and levels of multi-lamellarity. Intriguingly, specific structural modifications amplified the multi-lamellarity or lipid partitioning within these particles. Notably, LNPs with a multilamellar and faceted structure demonstrated a heightened capability for gene transfection (Pratsinis et al., 2023). This observation is particularly significant, as it suggests a direct correlation between LNP structure and its functional efficacy.

Hammel and colleagues underscored the LNP structure-activity relationship and highlighted that LNPs can possess varied internal structures, from monolayers enveloping a densely packed core to multilamellar particles (Hammel et al., 2023). Notably, the core's organization, whether it's an inverse hexagonal lipid phase (HII) or an amorphous oil phase, significantly influences the LNP's delivery efficiency and cellular uptake. Techniques including SAXS and Cryo-EM were employed in elucidating these structures, offering insights into the nuanced interplay between structure and function (Goldman et al., 2023; Hammel et al., 2023). The study also emphasizes the role of PEG lipids in governing LNP particle size and core organization, with a direct correlation between the core's ordered structure and gene knockdown efficacy. This research underscored the potential of tailoring LNP structures to optimize gene delivery, emphasizing the importance of understanding these structures for effective therapeutic applications.

The intricacies of mRNA packaging and localization within LNP structure are also significant. Li et al introduced a novel quantification method based on the multi-laser cylindrical illumination confocal spectroscopy (CICS) technique to examine mRNA and lipid contents at the single-nanoparticle level (Li et al., 2022). This method was able to differentiate between unencapsulated mRNAs, empty LNPs, and mRNA-loaded LNPs, providing insights into the payload distribution within LNPs. Notably, a benchmark formulation using DLin-MC3 as the ionizable lipid was found to contain an average of two mRNA molecules per loaded LNP, with a presence of 40–80% empty LNPs, depending on assembly conditions. The study further reveals a kinetically controlled assembly mechanism governing the payload distribution and capacity in LNPs. This mechanism is influenced by the relative molar ratio of mRNA to lipids, which determines the rate of lipid precipitation and mRNA-ionizable lipid complexation (Li et al., 2023). The research underscores the importance of understanding the molecular assembly and mRNA localization in LNP structure, as it forms the foundation for optimizing LNP designs for therapeutic applications.

Inspired by payload distribution and localization in LNPs, Cheng and colleagues revealed that mRNA-LNPs, which are formulated with certain ionizable lipids, often display distinctive mRNA-rich "bleb" structures (Cheng et al., 2023). Interestingly, these structures can be induced in LNPs containing less active ionizable lipids when formulated in the presence of high concentrations of acidic buffers, such as sodium citrate. And this unique "bleb" compartment containing drug substance resulted in enhanced transfection potencies both in vitro and in vivo. This enhanced transfection potency of mRNA-LNP displaying the "bleb" structure can be attributed, in part, to the heightened integrity of the encapsulated mRNA (Brader et al., 2021). The research posits that the optimization of ionizable lipids to achieve enhanced potency might lead to improvements in mRNA integrity through the formation of the "bleb" structure rather than enhanced intracellular delivery. The presence of high-concentration acidic buffer is believed to cause the formation of "blebs" via mRNA-induced segregation of the positively charged ionizable lipid from the core of the LNPs (Kloczewiak et al., 2022). This study demonstrated that delivery efficacy can be enhanced by manipulating the payload-LNP structure to bolster payload stability.

LNP structure can also be affected by formulation process alterations such as buffer pH and buffer ionic strength (Ball et al., 2017; Hassett et al., 2021). Li and colleagues focused on the significance of the LNP formulation process to structure-activity relationship utilized techniques including SAXS and small-angle neutron scattering (SANS) to investigate the

structural phase transitions that occur upon the encapsulation of nucleic acids in LNPs (Li et al., 2023). A notable observation was the acidification-induced structure evolution (AISE) of LNPs. During AISE, LNPs undergo a volume expansion process, leading to a redistribution of LNP components and nanostructures. This structural transformation is evident across different length scales. The study also highlights the impact of the payload molecular size on LNP structure. LNPs encapsulating different-sized plasmids, such as pUC19 and pLuc, were examined in this study. And it was found that the size of the plasmid payload had minimal impact on the LNP structure (Li et al., 2023; Yu et al., 2023). Another key finding is the role of phospholipids in determining LNP structure and AISE. Different phospholipids were explored, and it was observed that phospholipids with unsaturated acyl chains could enhance the extent of AISE, leading to higher transfection efficacy.

A similar study from Koitabashi et al focused on the AISE effects on LNPs delved into the physicochemical properties of LNPs containing ionizable cationic lipids under acidic conditions, simulating the environment of maturing endosomes (Koitabashi et al., 2021). The study highlighted the significance of the interaction between LNPs and endosomes for the efficient release of encapsulated nucleic acid payload into the cytosol of targeted cells. A key observation was the change in the lipid packing of LNPs in response to varying pH levels. Using the Laurdan-Generalized-Polarization-Values (LGPV), the study found that the lipid packing of the LNP membrane decreased as pH values dropped, suggesting that the membrane becomes less ordered under acidic conditions. This decrease in lipid packing is believed to promote the protrusion of the cationic lipid, potentially facilitating the fusion of LNPs with endosomal membranes (Aliakbarinodehi et al., 2022). The study also shed light on the electrostatic repulsion between lipid molecules at acidic pH which reduced the packing density of the lipids in the LNP membrane. The findings provide valuable insights into the mechanisms underlying the endosomal escape of LNPs which is a crucial factor to effective payload delivery, paving the way for the development of more efficient LNP delivery vehicles.

To comprehensively demonstrate the correlations between lipid chemistry and LNP structure, as well as LNP structure-activity relationships, Goldman et al introduced a novel family of ionizable lipids synthesized from a piperazine core derived from the HEPES Good buffer (Goldman et al., 2023). These lipids possess unique asymmetric tails and two different degradable moieties within their structure. The research evaluated lipid tails with varying lengths, degrees of unsaturation, branching, and the inclusion of additional ester moieties to understand their impact on protein expression. Key findings revealed specific structure-activity relationships, such as lipid tails of 12 carbons on the ester side and the influence of carbon spacing on the disulfide arm of the lipids, which correlated with enhanced protein production in vivo. Differences in LNP physical characteristics, especially in terms of LNP structure and lipid bilayer packing, were observed through Cryo-EM. These characteristics influenced the amount of protein produced in vivo. In non-human primates, the Good HEPES LNPs, when formulated with an mRNA encoding an influenza hemagglutinin (HA) antigen, generated functional HA inhibition (HAI) antibody titers. These titers were comparable to industry standards MC3 and SM-102 LNPs, showcasing their potential as effective vaccines and highlighted the importance of understanding the structural intricacies of LNPs to optimize their efficacy in mRNA delivery for vaccine applications (Willis et al., 2020).

Overall, the structural nuances of LNPs have always been of paramount importance in the realm of drug delivery. The ability to manipulate and tailor these structures can pave the way for targeted and more efficient drug delivery mechanisms. LNPs, given their biocompatibility and versatility, have been at the forefront of revolutionary mRNA therapies, especially in the wake of emerging diseases. The potential to customize their structure, as highlighted in recent studies, can significantly enhance the delivery and efficacy of mRNA-based therapies and vaccines (Sato et al., 2019; Cheng et al., 2023; Hammel et al., 2023). As the medical community continues to grapple with evolving health challenges, such research underscores the promise and potential of LNPs in ushering in a new era of targeted and effective treatments.

LNP delivery pathway

Once introduced into the bloodstream, LNPs circulate and often leverage specific surface modifications to target desired cell types. Upon reaching the target cells, they are taken up primarily through endocytosis (Sayers et al., 2019). Within the cell, LNPs are initially contained in endosomes. However, their unique composition, especially the ionizable lipids, facilitates the timely release of their payload into the cytoplasm, a process known as endosomal escape (Maugeri et al., 2019). This step is vital to prevent the degradation of the encapsulated therapeutic agent within lysosomes. Despite their sophisticated design, LNPs can elicit immune responses, with their potential immunogenicity being a consideration in therapeutic applications, as they can influence efficacy and safety profiles.

1. Cell and tissue targeting

LNPs have a natural tendency to interact with certain cell types and tissues due to their unique physicochemical properties. While they do not specifically target a particular

cell or tissue without intervention, some common interactions occur passively based on the characteristics of LNPs and the biological environment. These interactions are influenced by factors such as particle size, surface charge, morphology and surface chemistry. Generally, LNPs tend to interact with cells and tissues that have a natural affinity for lipid-based structures (Eygeris et al., 2022). For instance, the mononuclear phagocyte system, which includes cells like macrophages and dendritic cells, can interact with LNPs circulating in the bloodstream. Macrophages in the liver and spleen often recognize and clear nanoparticles from circulation. This phenomenon can influence the biodistribution of LNPs, leading to their accumulation in organs of the reticuloendothelial system. In terms of tissue interactions, LNPs may show some preference for tissues rich in lipid membranes, such as the liver and adipose tissue (Li et al., 2022). The liver, being a central organ for lipid metabolism, is a common site of accumulation for lipid-based nanoparticles. Adipose tissue, which is abundant in fat cells containing lipid-rich organelles, can also have interactions with LNPs. However, it's important to note that these interactions are generally non-specific and may not lead to targeted delivery in a therapeutic context.

One significant challenge in LNP-based targeting is achieving optimal selectivity while maintaining efficient drug delivery. While the use of targeting ligands enhances specificity, ensuring that the LNPs reach their intended targets in sufficient quantities remains a hurdle. The complexities of biological variability and the dynamic nature of cellular environments contribute to the need for rigorous optimization and validation. Moreover, the potential for target downregulation or alteration over time poses a risk to sustained efficacy, necessitating strategies that accommodate adaptability (Shobaki et al., 2018). Addressing these challenges requires a deep understanding of cellular behavior, interplay, and potential resistance mechanisms. As such, researchers are continually refining LNP formulations, incorporating insights from both computational modeling and experimental data to tailor nanoparticles for optimal pharmacokinetics and biodistribution.

Recent target-specific LNP development employs both passive and active targeting strategies to enhance the precision and efficacy of therapeutic cargo delivery (Alavi et al., 2019). Passive targeting takes advantage of the natural physiological characteristics of LNPs and the tumor microenvironment. LNPs are designed with specific physicochemical attributes that enable them to exploit the enhanced permeability and retention (EPR) effect often observed in tumors. This effect arises from the leaky vasculature and impaired lymphatic drainage commonly found in cancerous tissues. The leaky blood vessels allow LNPs to extravasate into the tumor interstitium, where they can accumulate due to restricted lymphatic clearance (Dilliard et al., 2023). This passive accumulation of LNPs within tumors increases the concentration of therapeutic payloads in the target area, potentially leading to improved treatment outcomes. While passive targeting is valuable, its efficacy can vary, and it is often combined with other strategies to achieve optimal results.

Active targeting, on the other hand, involves the deliberate modification of LNPs with ligands or antibodies that bind to specific cell surface receptors overexpressed on target cells (Alavi et al., 2019). This strategy enables precise delivery to desired cell types and tissues, enhancing therapeutic effects while minimizing off-target effects. The ligands or antibodies attached to the LNP surface guide the nanoparticles to their intended destination, increasing the likelihood of cellular internalization and payload release. Active targeting offers a level of specificity and selectivity that passive targeting alone cannot achieve. By customizing the surface chemistry of LNPs with ligands that interact specifically with tumor markers or receptors, researchers can enhance drug accumulation in tumors or certain targets, bypassing healthy tissues and minimizing side effects (Dilliard et al., 2023). The synergistic combination of passive and active targeting strategies holds immense promise for tailoring LNP delivery systems to the unique characteristics of different diseases, ultimately advancing precision medicine and revolutionizing therapeutic interventions.

Gallud and colleagues focused on the LNP stealth from undesired immune clearance utilizing the passive targeting strategy to escape from lymphatic system attack. The lymphatic system is a vital consideration when designing nanomedicines, especially for activating immune responses against threats like pathogens and cancers. The lymphatic system plays a crucial role in enabling immune cells to detect external entities and activate defense mechanisms. The design of LNPs, considering their physicochemical properties such as size and charge, significantly influences their interaction with the lymphatic system. In the lymph nodes, strategies that modify particle size and dispersibility can optimize lymph node targeting. For instance, increasing particle size and introducing a cationic charge can enhance targeting of antigen-presenting cells (APCs) (Basha et al., 2011). Conversely, smaller particle sizes and high dispersibility might be more effective for T cell targeting within the lymph nodes. It emphasized that understanding the dynamics of nanoparticles both at the administration site and within the lymph nodes is essential. This knowledge can guide the design of new strategies for cancer immune therapy using LNP passive targeting strategy.

Selective Organ Targeting (SORT) is a groundbreaking methodology developed by Siegwart groups that facilitates the systematic and predictable targeting of LNPs to deliver mRNA

into specific organs. By adding a supplemental component, known as a SORT lipid, to traditional LNP compositions, the in vivo delivery profile can be systematically altered, enabling tissue-specific delivery based on the percentage and biophysical properties of the SORT lipid (Cheng et al., 2020). The discovery of SORT is anticipated to revolutionize the development of protein replacement and gene correction therapeutics. The methodology's versatility means it can be applied to existing LNPs and other nanoparticle systems, potentially allowing for the rational design of carriers for various cargoes and organ targets.

Preliminary data suggests that the inclusion of SORT molecules modifies the biodistribution of SORT LNPs to different tissues, changes the global apparent pKa, and endows distinct protein coronas (Dilliard et al., 2021). This discovery positions SORT LNPs as a promising tool for treating a range of diseases with high precision. To validate the hypothesis that internal charge adjustment could mediate tissue-specific delivery, a strategy was conceived to adjust efficacious LNP formulations without altering the core component ratios essential for RNA encapsulation and endosomal escape. By systematically increasing the percentage of an additional permanent cationic lipid, luciferase protein expression was observed to move progressively from liver to spleen and then to lung, showcasing a clear organ-specific delivery trend. Moreover, the inclusion of negatively charged 1,2-dioleoyl-sn-glycero-3-phosphate (18PA) as a SORT molecule led to selective delivery to the spleen (Cheng et al., 2020). This indicates that the selection of SORT lipids in terms of inherent charge and their molar percentage in the LNP composition are both critical factors in determining tissue-specific delivery.

Furthermore, SORT LNPs have demonstrated their ability to target specific organs and apply findings to tissue-specific gene editing via IV injection. The CRISPR/Cas technology can edit the genome in a precise and sequence-dependent manner, and with the aid of SORT, there's potential for targeted gene editing in specific organs. For instance, SORT LNPs enabled tissue-specific Td-Tomato activation by Cre mRNA delivery, with specific fluorescence observed in the liver, lung, and spleen, depending on the SORT LNP formulation used (Wang et al., 2023). This SORT methodology offers a modular and generalizable strategy for achieving tissue-targeted delivery, opening new avenues for the development of gene correction therapeutics and other applications.

A follow-up study to further reveal the mechanism of SORT lipids has identified that the chemical nature of the added SORT molecule controls various factors like biodistribution, global/apparent pKa, and serum protein interactions of the nanoparticles. A proposed mechanism suggests that organ targeting occurs through a sequence of events: desorption of PEG lipids from the LNP surface, binding of specific proteins due to the exposure of SORT molecules, and subsequent interactions between these proteins and their corresponding receptors in specific tissues (Dilliard et al., 2021). This mechanism provides a crucial link between the molecular composition of SORT nanoparticles and their precise organ-targeting properties.

In addition to the passive and active targeting strategies, other methods have been proposed such as stimuli-responsive LNPs, co-delivery systems and biodistribution modifiers (Lin et al., 2023). Stimuli-responsive LNPs are ingeniously designed to react to specific triggers in their target environment, such as changes in pH, temperature, or the presence of enzymes. This adaptability ensures that the LNPs release their therapeutic cargo precisely where and when it's needed. For instance, certain LNPs can be tailored to discharge their contents in the acidic milieu of tumor tissues or within cellular endosomes, maximizing therapeutic impact while minimizing side effects (Rahim et al., 2021) Co-delivery Systems represent another innovative approach, where LNPs are engineered to transport multiple therapeutic agents simultaneously. This can be a combination of different drugs or a pairing of a drug and a gene. Such co-delivery can lead to synergistic therapeutic outcomes, especially beneficial in scenarios like overcoming multi-drug resistance in cancer treatments (Carvalho et al., 2021). Lastly, biodistribution modifiers are molecules added to LNPs to alter their distribution profile within the body. By doing so, these modifiers ensure that LNPs accumulate predominantly in the target tissue, reducing their presence in off-target areas and thereby enhancing therapeutic efficacy and safety (He et al., 2022). More studies need to be conducted to validate and improve the efficacy of these selective targeting strategies.

2. Cellular uptake

The cellular uptake of LNPs describes the mechanism of nanoparticles entering the cells and predominantly hinges on the endocytic pathway, a complex process that encompasses various mechanisms. One of the primary routes is clathrin-mediated endocytosis, where specific molecules are internalized into cells. In this mechanism, ligands in the extracellular fluid bind to receptors on the cell membrane, forming a ligand-receptor complex. This complex then migrates to regions of the cell membrane rich in clathrin, leading to the formation of clathrin-coated vesicles. Once these vesicles are inside the cell, their clathrin coatings are shed before they fuse with early endosomes (Sousa de Almeida et al., 2021). Different nanoparticles have their preferred uptake pathways. For instance, nanoparticles made of poly lactic-co-glycolic acid and D, L-polylactide, and polyethylene glycolco-lactide are internalized via the clathrin-mediated endocytic pathway.

Similarly, lipid-based nanoparticles, due to their structural similarity to the cell membrane, also utilize the clathrin-mediated endocytosis pathway. Another significant route is caveolae-mediated endocytosis, which involves flask-shaped membrane invaginations known as caveolae. These structures, typically ranging from 50 to 80 nm, are found in various cell types, including endothelial, epithelial, and muscle cells (Hassett et al., 2021) Caveolae-mediated endocytosis differs from clathrin-mediated endocytosis in that it doesn't typically direct its cargo to lysosomes, thereby avoiding potential degradation. This makes it a particularly relevant pathway for the delivery of therapeutic agents using LNPs. Beyond these primary mechanisms, there are also clathrin- and caveolae-independent endocytic pathways. These mechanisms can be activated by certain nanoparticles depending on their size, charge, and surface modifications.

Studies toward the understanding and examination of the LNPs' cellular uptake and intracellular trafficking pathway are critical to the prediction and analysis of LNPs' in vitro and in vivo behavior, as well as the design and optimization of potent LNPs. However, generalizing the current findings remains challenging due to variations in endocytic mechanisms dependent on cell types and different LNPs (Degors et al., 2019). When LNPs interact with physiological fluids, they can associate with various biomolecules, including opsonin, which facilitates cellular recognition and clearance by the mononuclear phagocyte system (MPS). Factors like efflux pumps, overexpression of specific transporters on the cell membrane, and mitosis can reduce LNP accumulation in target cells. Hence, a minimal number of LNPs reach the target cells, which might not be sufficient for effective disease treatment (Sousa de Almeida et al., 2021). To address this, strategies have been developed to enhance LNP-based targeted delivery while avoiding unwanted internalization by the MPS. Various stimuli, including inflammatory cytokines and LNP functionalization with ligands, have been described to increase LNP uptake in target cells. Conversely, certain substances have been identified that decrease LNP internalization, preventing accumulation in non-target cells or organs where they could induce toxicity (Sayers et al., 2019). Such endocytosis interferon or inhibitor allows the identification of LNP internalization pathway by comparing the payload delivery efficacy with and without the presence of these endocytosis inhibitors.

LNP surface chemistry and structure can also play a critical role in the interactions with the biological milieu. A study by Sato et al emphasized how the structural and biochemical properties of LNPs influence their tissue distribution, cellular uptake, and intracellular trafficking, which subsequently determine the activation of antiviral humoral and cellular immunity (Sato et al., 2019). Specifically, LNPs were found to be primarily internalized by myocytes, avoiding lysosomal degradation, leading to a humoral-biased immune response. In contrast, cationic nanoemulsions (CNE) and cationic liposomes (Lipo) induced a cellular-preferred immunity. The enhanced cellular immunity from CNE was attributed to better lysosomal escape in dendritic cells, while Lipo's superior biodistribution in secondary lymphoid organs contributed to its cellular immunity preference. The study utilized techniques like structure illumination microscopy (SIM) to confirm the successful loading of nucleic acids by these nanoparticles.

LNPs undergo a dynamic interaction with biological fluids upon administration, leading to the formation of a protein corona on their surface. This protein corona also plays a pivotal role in determining the cellular uptake and subsequent intracellular trafficking of LNPs. A study from Gallud and colleagues underscored the significance of protein corona maturation in unlocking the potential of LNPs for cellular entry and efficient mRNA delivery (Gallud et al., 2021). When LNPs are exposed to serum, certain serum proteins are absorbed onto their surface, influencing their interaction with cells. The study results emphasized that a considerable duration of approximately 3-4 hours is essential for LNPs to attain their most uptake-competent state. This maturation process of the protein corona is crucial for enhancing the cellular uptake of LNPs. Furthermore, pre-incubating LNPs in serum facilitates rapid uptake during short cell exposures, enabling detailed studies on the intracellular trafficking of LNPs. The research also highlights the effects of temperature and serum heat-inactivation on the cellular uptake and delivery efficiency of serum pre-incubated LNPs. Live cell imaging revealed variations in LNP uptake and protein expression based on temperature and the state of fetal bovine serum (FBS) used for pre-incubation (Liu et al., 2023). In essence, the protein corona's formation and maturation are integral to the cellular uptake dynamics of LNPs, and understanding these interactions can provide valuable insights for optimizing LNP-based therapeutic delivery.

3. Intracellular activity

Endosomal escape is one of the most significant intracellular LNP activities which describes the process of LNPs releasing the encapsulated payload from their core to achieve therapeutical efficacy, LNPs are initially housed within early endosomes, which serve as primary sorting hubs. These early endosomes transport their ingested cargos to specific cellular destinations, with a portion of cargos being recycled back to the plasma membrane via recycling endosomes. As the early endosomes mature, they transform into late endosomes through a series of differentiation processes. These late endosomes eventually fuse with lysosomes, forming endolysosomal vesicles. Within these vesicles, hydrolytic enzymes work to degrade the entrapped

nanoparticles (Gilleron et al., 2013). For LNPs to effectively deliver their therapeutic cargo, they must evade this degradation process, for the successful delivery of sensitive cargoes, such as nucleic acids, which would otherwise be degraded within the lysosomal environment (Paramasivam et al., 2022). Another significant intracellular degradation pathway that impacts the fate of nanoparticles is autophagy. In this process, cytoplasmic contents are enveloped by an autophagosome and subsequently delivered to the lysosome for degradation and recycling. This pathway is essential for cellular homeostasis, as it degrades aggregated proteins and dysfunctional organelles (Li et al., 2019). Recent studies have highlighted that various nanoparticles can induce autophagy, further emphasizing the importance of understanding this endosomal pathway in the context of nanoparticle-based therapies. In essence, the journey of LNPs within cells is a complex interplay of endocytic, endosomal, and autophagic pathways, with the endosomal escape being pivotal for the therapeutic efficacy of the delivered cargo.

However, LNPs are often trapped within the endo/lysosomal pathway after internalization, where they face degradation and result in poor delivery and therapeutic efficacy. To ensure effective delivery, these nanoparticles must escape this pathway. There are several mechanisms through which this escape can occur such as membrane fusion, proton sponge effect and membrane rupture (Wu et al., 2021). Membrane fusion has been employed by some viruses and liposomal delivery systems to achieve endosomal escape by fusing with the lipid cell membrane. This fusion-based mechanism has been observed in nano-assemblies of polymer-functionalized gold nanoparticles, which can fuse with the cell membrane to directly deliver their cargo into the cytosol. The challenge lies in controlling this fusion to target specific cells or ensuring it occurs only within the endo/lysosomal compartment (Álvarez-Benedicto et al., 2022). Incorporating pH-responsive polymers can help control this fusion, allowing it to occur within a specific pH range, such as the endo/lysosomal environment. Osmotic pressure and the proton sponge effect suggest that during the acidification of the endosome, certain polymers with buffering capacities can inhibit the pH drop. This leads to the cell continuing to pump protons into the endosome, resulting in an influx of chloride counterions and water molecules. The increased pressure eventually causes the endosome to rupture (Vermeulen et al., 2018). However, the proton sponge effect, which has been a standard explanation for endosomal escape for over two decades, has faced scrutiny due to inconsistencies in its predictive power and observations. Particle swelling and membrane rupture rely on nanoparticles which can swell or induce other physicochemical property shifts within the acidic environment of the endosome, exerting mechanical strain that ruptures the endosomal membrane. The liberated payload from LNPs, whether it's gene-editing agents, vaccine instructions, or other therapeutic molecules, can then effectively traverse into the cell's cytoplasm. Once in the cytoplasm, these molecules can interact with their designated targets, be it for gene silencing, protein production, or immune response initiation, thereby significantly amplifying the therapeutic impact of the delivered payload (Paramasivam et al., 2022). While significant progress has been made, the endosomal escape efficiency of non-viral vectors remains a challenge. Combining advanced techniques and insights into endosomal escape mechanisms can pave the way for more effective therapeutic delivery using LNPs, the commonly used strategy is the incorporation of ionizable lipids in LNP structure to initiate the membrane rupture and fusion mechanism.

A closer look at the endosomal escape mechanism which involves the protonation of ionizable lipid in LNPs: The early endosomes that engulf the LNPs have a pH ranging between 5.5 and 6.5; As these vesicles mature, their pH progressively drops to 5.0-5.5, characterizing the late endosomes; Eventually, fusion with lysosomes further reduces the vesicle's pH to 4.5-5.5 (Aliakbarinodehi et al., 2022). Lysosomes are equipped with various enzymes capable of dismantling the LNP structure and degrading the nucleic acids. To ensure effective nucleic acid delivery, a significant portion of the functional molecules must escape this endosomal compartment before the degradation cascade initiates (Gilleron et al., 2013). Ionizable lipids, which can adjust their charge based on the surrounding pH, are identified as a crucial LNP component for facilitating endosomal escape. As the endosomal maturation commences, the heads of these ionizable lipids acquire a positive charge, binding to the negative lipids on the endosomal membrane. This interaction disrupts the original endosomal membrane structure, leading to the formation of a non-bilayer, hexagonal structures that induce membrane fusion and endosomal disruption, allowing the release of the entrapped nucleic acid.

Studies and innovative design of ionizable lipids toward the objective of improving endosomal escape become the popular topic in LNP development and have shown significant progress. Shen et al embarked on the development of these lipids by integrating disulfide bonds, which are known for their biodegradability and have been previously used in prodrugs and fluorescent probes (Shen et al., 2023). They initiated their synthesis from dimercaprol, a commercially available compound, and constructed a library of lipids that contained these disulfide bonds. The lipids were then used to prepare LNPs using a standard four-component formulation, which included DSPC, Cholesterol, and DMG-PEG 2000. When these disulfide bonds containing LNPs enter the cellular interior and encounter this reducing milieu, the disulfide bonds undergo

a redox reaction. This involves the interaction of the sulfur atoms in the disulfide bond with thiol groups (-SH) present on molecules like glutathione. In the presence of reducing agents like glutathione, the thiol groups can catalyze the cleavage of the disulfide bonds. This cleavage results in the breaking of the sulfur-sulfur linkage, leading to the formation of two thiol-containing products (Tanaka et al., 2020). This redox reaction changes the LNP's structure, rendering it more dynamic and flexible. And the structural rearrangements triggered by the disulfide bond cleavage contribute to the destabilization of the endosomal membrane, a critical step in enhancing endosomal escape. By disrupting the endosomal membrane, these modified LNPs overcome a major barrier and facilitate the release of their therapeutic cargo from the confines of endosomes.

The preliminary screening of these disulfide bonds containing lipids was based on the size, polydispersity index (PDI), and stability of the resulting LNPs. The researchers found that lipids with hydrophobic tails that included a double bond were more effective than those with saturated alkyl chains. Two formulations, C4S18A and C4AS18A, which both incorporated linoleoyl tails, emerged as leaders in the in vitro screening process. These LNPs demonstrated stability at 4°C for up to 40 days and exhibited minimal cytotoxicity towards 293T and HepG2 cells. In vivo experiments with these LNPs, loaded with firefly luciferase mRNA, showcased impressive luminance intensity when injected into mice (Shen et al., 2023). By successfully developing and synthesizing a library of 16 ionizable lipids with disulfide bonds through a cost-effective and efficient three-step synthetic procedure, these biodegradable LNPs have presented promising potential as biodegradable and highly efficient nanocarriers for mRNA delivery.

Contrarily, a recent study by Paramasivam et al revealed that LNPs may not predominantly escape from mid-to-late endosomes and proposed a novel model of LNP structure and lipid localization (Paramasivam et al., 2022). The research team suggested that the distribution of LNPs within various subcellular compartments plays a pivotal role and only specific compartments are conducive to macromolecule escape. Instead of late endosome escape under the pH-responsive membrane rupture theory, early endocytic and recycling compartments presented the highest probability for mRNA escape. This finding may challenge the traditional view of endosomal escape mechanisms, emphasizing that the precise sites and mechanisms facilitating this escape remain elusive. A breakthrough in the research was the use of super-resolution microscopy, which allowed the visualization of individual LNP-mRNA within sub-endosomal compartments and the capturing of mRNA escape events from endosomal recycling tubules. The research offers a paradigm shift in understanding endosomal escape, highlighting the importance of the endosomal distribution of LNPs and the specific compartments that are conducive for mRNA escape. Live cell imaging has previously shown that lipoplexes, another type of delivery system, release siRNAs through endosome bursting. However, this mechanism does not seem to apply to LNPs (Gilleron et al., 2013). The study leverages single-molecule techniques to visualize the escape of a small number of mRNAs, a feat not achievable with conventional microscopy. Through a systematic comparison of six mRNA-containing LNPs with diverse cationic lipids, the research identifies variations in uptake and endosomal distribution. Interestingly, some LNPs demonstrated high eGFP expression despite moderate uptake, suggesting that factors other than uptake efficiency play a role in delivery efficacy. The study also highlights the potential of subcutaneous administration in adipose tissue, which could pave the way for patient self-administration and long-term mRNA-based treatments (Paramasivam et al., 2022). Together, it provided a comprehensive exploration of LNP-mediated mRNA delivery and emphasized the significance of comprehensively understanding the LNP endosomal escape mechanisms to enhance the therapeutic potential of mRNA formulations.

Sebastiani et al focused on the significance of understanding the structural dynamics of LNPs, especially when they encounter extracellular proteins like ApoE (Sebastiani et al., 2021). ApoE plays a pivotal role in the LNP's plasma circulation time and is known to bind to LNPs, leading to their accumulation in the liver. The research employed small-angle neutron scattering (SANS) combined with selective lipid, cholesterol, and solvent deuteration to elucidate the structure of the LNP and the distribution of lipid components in the presence and absence of ApoE. The findings reveal that while DSPC and cholesterol are primarily located at the LNP surface in buffer, the binding of ApoE induces a redistribution of the lipids in the shell and core of the LNP (Dehghani-Ghahnaviyeh et al., 2023). This interaction also impacts the LNP's internal structure, potentially causing the release of mRNA. The study hypothesizes that the rearrangement of LNP components upon ApoE incubation might have implications for LNP endosomal escape. The methodology employed in the study involved the use of SANS in conjunction with selective deuteration, enabling the researchers to determine the exact composition and distribution of cholesterol, DSPC, and ionizable lipids across the LNP shell and core. This study provided valuable insights into the structural and compositional dynamics of mRNA-LNPs, shedding light on their relationship with the intracellular behavior of LNPs.

To reveal the LNP-endosome interaction more visibly, a study by Spadea and colleagues employed various reflectometry

techniques to investigate the interactions of nucleic acid-loaded LNPs with models of early and late endosomal membranes to simulate and demonstrate the fate of LNPs upon endocytosis (Spadea et al., 2022). The endosomal monolayer models were designed to mimic in vivo endosomal compositions. Specifically, two types of model endosomal monolayers were prepared: the early endosomal membrane (EEM) and the late endosomal membrane (LEM). The EEM was composed of 40 mol % POPC, 20 mol % DOPE, 6 mol % SM, and 34 mol % cholesterol, while the LEM consisted of 61 mol % POPC, 16 mol % DOPE, 6 mol % BMP18:1, and 17 mol % cholesterol. For the preparation of these monolayers, the lipid components were weighed separately, dissolved in chloroform, and mixed to create a spreading solution. This solution was then aliquoted, dried, and stored. The research revealed that the insertion of lipids from the LNPs into the model membrane was most pronounced at pH levels of 6.5 and 5.5. At higher pH levels, lipid insertion was suppressed, emphasizing the critical role of pH in the fusion of LNPs during the endosomal pathway. The study also found that the nature of the nucleic acid cargo can influence the extent of interaction. Specifically, LNPs containing mRNA exhibited a greater extent of interaction than those containing Poly A. The significance of this study lies in its potential to provide insights into the physicochemical processes occurring between LNPs and endosomal membranes, LNP binding and the possible migration of ionizable lipid-nucleic acid complexes (Alam et al., 2023). By understanding these interactions, the study aims to establish a robust foundation for future research that seeks to optimize the design of LNPs for enhanced nucleic acid delivery to the cytosol. The combination of surface pressure measurements and ellipsometry data was particularly beneficial in distinguishing different types of processes and understanding the underlying mechanisms. The findings from this research have important implications for the efficacy prediction and bioactivity analysis of LNPs.

Interestingly, another study from Maugeri and colleagues has disclosed a hidden path of LNP trafficking out of endosomal cycle by delving into the intricate mechanisms of LNP-mediated mRNA delivery and its subsequent fate after cell entry and endosome formation by proposing a hypothetical mechanism explaining the fate of LNP endosomes (Maugeri et al., 2019). After endocytosis and subsequent lysosomal fusion, the endosomal environment becomes acidic and the positive charge on the LNP surface draws it closer to the negatively charged inner membrane of the endosomes, enabling the lipid components of LNPs to fuse with the endosomal membrane. This fusion allows the mRNA to translocate to the water phase outside the endosomes. However, only mRNA that is neutrally charged by ionizable cationic lipids can cross the endosomal membrane. Part of the LNP-mRNA that escapes the endosomal membrane localizes to the cytoplasm and could be dissociated from the ionizable lipids. Conversely, when LNP-mRNA is transported to the cytoplasmic side of the endosomal membrane, intraluminal vesicles are formed by invagination of the endosomal membrane, and a portion of the LNP-mRNA could be incorporated into these vesicles. These vesicles are then released into the extracellular environment upon the fusion of multivesicular endosomes with the plasma membrane, suggesting that part of the mRNA delivery is achieved by such endosome-derived extracellular vesicles (Endo-EVs). This groundbreaking research offers a deeper understanding of the cellular mechanisms underpinning LNP-mediated mRNA delivery (Figure 4).

LNP structure and topology were also considered as key players in determining intracellular behavior. Zheng's group hypothesized that the nanostructure of LNP-RNA complexes could influence the energy dynamics of LNP fusion with endosome membranes (Zheng et al., 2022). This led to the introduction of "cuboplexes", a novel form of bicontinuous cubic RNA-LNPs, believed to be more efficient than traditional lamellar LNPs. The methodology was anchored in controlling the nanostructure of these complexes. Glycerol monooleate, a neutral lipid known for stabilizing into various bicontinuous cubic phases was chosen and formulated into "cubosome LNPs" encapsulating siRNA, and further enriched with a cationic lipid. To validate their design, a series of techniques were employed such as SAXS to provide structural insights, while cell cultures elucidated the biological implications. Endosomes were isolated to conduct membrane fusion studies to understand the LNP's behavior within cellular environments. Live cell imaging was pivotal in visualizing the real-time interactions of these LNPs and the findings were revelatory. The cubic structures of the complexes, as opposed to other forms, were adept at promoting membrane fusion between LNPs and endosomal membranes. This was attributed to their intrinsic ability to reduce the elastic cost of topological transformation, ensuring RNA's seamless transport into the cytosol. Notably, the cuboplexes showcased superior RNA delivery capabilities compared to their lipoplex counterparts.

Given the significance of efficient LNP endosomal escape in enabling the successful delivery of therapeutic payloads, the quantification of endosomal escape can provide invaluable support to the prediction of LNP efficacy prior to in-vivo experiment. Two popular methodologies employed for this purpose are leakage assays and membrane lysis assays (Xu et al., 2021). Leakage assays are based on the concept of entrapping a fluorescent dye or probe within a delivery system, such as a LNP or liposome, which would then be internalized by cells. Upon successful endosomal escape, the encapsulated

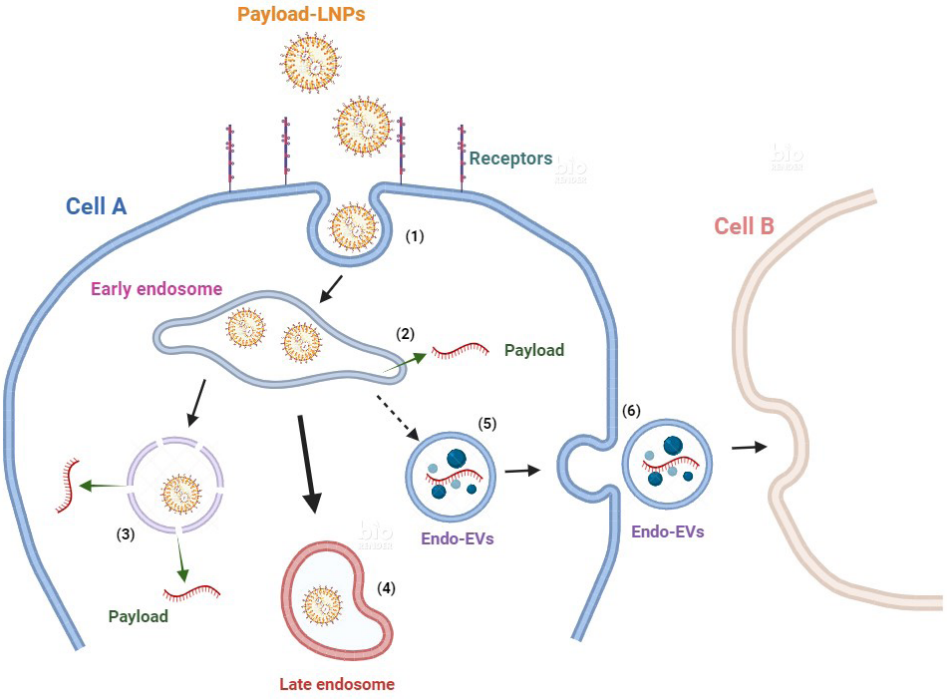

Figure 4. The intracellular activities of lipid nanoparticle mediated payload delivery

Notes: (1) Upon cellular uptake, LNPs are internalized through endocytosis, leading to endosome formation. (2) A fraction of payload may escape from recycling tubules in early endosome (3) Else must overcome endosomal entrapment, often achieved via ionizable lipid mediated membrane disruption. (4) Otherwise, the trapped cargo within the endosomes might undergo lysosomal degradation due to the endosomal maturation pathway. (5) A fraction of these endosomes can fuse with extracellular vesicles, potentially enabling wider cargo dissemination. (6) These extracellular vesicles take another delivery pathway.

probe is released into the cytosol, altering its fluorescence properties due to changes in the environment, like pH or ionic strength. This can be quantified using fluorescence microscopy or flow cytometry, providing a direct measure of the fraction of the delivered payload that has evaded the endosomal pathway (Beach et al., 2022). The selection of the fluorescent probe is crucial: some probes, when entrapped, are quenched and exhibit no fluorescence but regain their fluorescence upon release, while others might interact with other cellular components, producing fluorescence upon such interactions.

On the other hand, the membrane lysis assay takes a slightly different approach. It exploits the premise that endosomal escape often involves the disruption of the endosomal membrane. To quantify this, a self-quenching fluorescent dye is incorporated into the lipid bilayer of synthetic vesicles that mimic the endosomal membrane. When these vesicles are intact, the fluorescence is quenched due to the proximity of the dye molecules. However, upon interaction with a membrane-disrupting agent or delivery vehicle such as LNP, the vesicles lyse, dispersing the dye molecules and thus relieving the quenching. This results in an increase in fluorescence intensity which can be quantified spectroscopically (Xu et al., 2021). The advantage of this assay is that it offers a direct measure of the membrane-disrupting potential of a given agent, a proxy for its ability to facilitate endosomal escape.

Both assays have certain strengths and limitations. While the leakage assay is more cell-centric and provides data on actual cellular escape, its interpretation can be complicated by multiple intracellular events that might alter probe fluorescence. The membrane lysis assay, although a more simplified and direct measure of membrane disruption, is conducted in a cell-free system, and hence might not fully capture the complexities of intracellular endosomal escape. As with all methodologies, the results from both assays need to be interpreted in the context of other biological and physicochemical data to paint a comprehensive picture of the endosomal escape process and thus lead to a demand of more efficient and precise endosomal escape quantification methods.

To enable more precise and efficient endosomal escape quantification, Wensley and colleagues presented a method to quantitatively track the endolysosomal escape of a fluorescently labeled saporin toxin using flow cytometry (Wensley et al., 2019). The rationale behind this work was to address the challenge of delivering macromolecules, such as protein therapeutics or nucleic acids, into cells. After these molecules are internalized into early endosomes, they progress to late endosomes and eventually fuse with lysosomes, where they are degraded similarly to the fate of unescaped LNPs. To be effective, these macromolecules must escape this endolysosomal pathway before degradation. This novel endosomal escape quantification assay employs flow cytometric measurements of fluorescent pulse width and height to monitor

the endocytic uptake of the toxin into Daudi cells and its subsequent escape from the endolysosomal compartment into the cytosol. The study demonstrated that the use of triterpenoid saponin could enhance the endolysosomal escape of the internalized toxin. This assay was found to be more sensitive than confocal microscopy in detecting endolysosomal escape, especially at lower concentrations of the enhancing agent, saponin. The flow cytometric method offers a direct, quantitative assessment of endolysosomal escape, allowing for a more specific measurement of the efficacy of escape enhancers without the need for complex microscopic image analysis. It provided a direct and quantitative endosomal escape assessment that could be applied to LNPs but requires specific adaptations and validations to fit unique properties of LNPs.

To address the same challenge associated with the intracellular behavior of non-viral RNA delivery, Munson and colleagues introduce a novel assay that can be translated onto high throughput platform (Munson et al., 2021). This imaging-based endosomal escape assay employs a Galectin-9 (GAL9) reporter and fluorescently labeled mRNA, it is designed to probe the correlations between nanoparticle-mediated uptake, the frequency of endosomal escape, and the subsequent mRNA translation. Galectin-9 is a member of the galectin family, which is known to play roles in various cellular processes, including cell-cell adhesion, cell-matrix interactions, and intracellular signaling (Nag et al., 2022). When LNPs or other agents induce endosomal escape, Galectin-9 is recruited to the site of damaged endosome and this recruitment can be visualized as the formation of punctate structures or spots in the cell, which are indicative of endosomal escape events that can be detected and quantified using imaging techniques. The fluorescently labeled mRNA allows for simultaneous monitoring of mRNA uptake and translation and this dual monitoring system provides a comprehensive view of the intracellular processing of the nanoparticles and their payload.

The validation and evaluation of this assay used various cell lines Iding HeLa, HepG2, NCI-H358, and Huh7. Stable cells expressing mCherry-GAL9 were generated by knock-in at the AAVS1 locus. This was achieved by transfecting cells with mCherry-GAL9 reporter and AAVS1 zinc-finger nuclease. After incubation, Puromycin was added to select cells with stable integration. These cells were then sorted by flow cytometry to ensure similar expression levels. Cells were then seeded into plates and stained with Hoechst 33342 to visualize nuclei. LNPs or small molecules were dispensed into a source plate containing media and Hoechst. The imaging captured fluorescence from various lasers corresponding to different fluorophores and for time-course measurements, the same fields of view were imaged over time. Within individual cell regions of interest, spot populations (indicative of endosomal escape events) were quantified (Munson et al., 2021). The study demonstrated that Galectin-9 recruitment is a robust and quantitative indicator of endosomal escape events. The assay was also integrated into a high throughput screening platform for the optimization of LNP formulations. This integration allowed for the rapid assessment and optimization of various LNP formulations in terms of their endosomal escape capabilities.

The use of Galectin-9 as a reporter offers several advantages compared to traditional assays and probes. Firstly, it provides a direct and quantitative means to assess endosomal escape, eliminating the need for indirect or labor-intensive methods. Secondly, the recruitment of Galectin-9 is a rapid and robust response to endosomal damage, making it a reliable indicator of endosomal escape events. Lastly, by combining the Galectin-9 reporter with fluorescently labeled mRNA, the researchers were able to simultaneously monitor nanoparticle uptake, endosomal escape, and mRNA translation, providing a comprehensive view of the intracellular processing of RNA delivery vectors. Overall, traditional methods for assessing endosomal escape are often labor-intensive and lack the granularity provided by this new method. By offering a direct, quantitative, and high-throughput means to assess endosomal escape, this assay can accelerate the development and optimization of RNA delivery vectors such as LNPs.

Overall, understanding the LNP intracellular pathway, encompassing selective targeting, cellular entry, and especially endosomal escape, is crucial for the efficient delivery of therapeutic payloads, particularly for next-generation mRNA-based therapeutics and gene therapies. LNPs function as delivery vehicles, ensuring that therapeutic agents reach their intended cellular destinations without degradation. The endosomal escape is particularly vital, as failure to exit the endosome results in the degradation of the therapeutic payload within the lysosome. The development of assays to quantify payload escape efficiency is imperative to optimize the design and functionality of LNPs, ensuring the maximal therapeutic effect and facilitating the discovery of more nanoparticle relevant intracellular behaviors.

Advanced techniques in LNP research and development

LNP research and development have seen groundbreaking advancements due to the integration of cutting-edge techniques, ensuring enhanced delivery and therapeutic potential of encapsulated molecules, especially in the realm of mRNA vaccines and gene therapies. Among these, the Design of Experiments method has revolutionized the optimization process. Rather than examining one factor at a time, the Design

of Experiments allows simultaneous evaluation of multiple factors, systematically understanding their interactions and determining the optimal conditions for LNP formulation and performance (Nag et al., 2022). Meanwhile, machine learning has emerged as a pivotal tool in LNP R&D, harnessing computational power to predict, model, and optimize LNP properties based on vast datasets. Machine learning algorithms can correlate structural attributes with functional outcomes, enabling the forecasting of LNP behavior in various biological environments, thus mitigating potential challenges in the development phase (Fan et al., 2021). Complementing these techniques, high-throughput screening has facilitated rapid evaluation of thousands of LNP formulations in parallel. It allows the swift identification of lead candidates with desired attributes from immense combinatorial libraries (Harrison et al., 2021). This accelerated selection process reduces the timeline for hit-to-lead and lead optimization phases in drug development. Collectively, the implementation of advanced techniques in LNP research not only expedites the discovery and optimization process but also paves the way for more effective, tailored, and safer therapeutic agents.

1. Method of DOE

The Design of Experiments (DOE) method is a systematic and efficient approach used to investigate and optimize complex processes by strategically planning and conducting experiments. In the context of LNP optimization, DOE holds remarkable significance. The multifaceted nature of LNPs, influenced by intricate interactions among various lipid components, encapsulated cargoes, and environmental factors, demands a methodical exploration of variables to achieve optimal performance (Nag et al., 2022). DOE allows researchers to methodically vary these factors, such as lipid types, ratios, and formulation techniques, while quantifying their impact on critical outcomes like LNP characterization, targeted delivery and stability. This approach provides a structured framework to comprehensively understand the intricate relationships within LNP formulations, identify influential factors, and pinpoint their optimal levels for maximal efficacy. By efficiently evaluating a multitude of factors in a limited number of experiments, DOE expedites the optimization process, reduces guesswork, and facilitates the development of highly effective LNPs with improved drug delivery capabilities, bringing precision and efficiency to LNP design and development.

To customize DOE method for the implementation in the realm of LNP development, Qin and colleagues focused on the application of the DOE method to optimize LNP formulations for the delivery of both pDNA and siRNA (Qin et al., 2023). The DOE approach is a powerful formulation optimization technique that allows for the systematic study of multiple experimental parameters simultaneously. By establishing a mathematical model based on limited input data, DOE predicts the outputs of any given experiment, thereby reducing labor and minimizing the use of animals in vivo studies. The study utilized DOE customize design to achieve three primary objectives: to optimize a Stable Nucleic Acid Lipid Particle (SNALP) formulation for the delivery of pDNA and siRNA payloads, to identify a SNALPs formulation that can potentially deliver nucleic acids at the extreme ends of the payload size spectrum to cancer cells, and to determine whether DOE models based on in vitro data can be used to predict in vivo efficacy. The DOE approach employed a four-factor mixture D-optimal design to construct polynomial models for the SNALPs lipid composition optimization for siRNA or pDNA delivery. The total molar percentage of four factors (ionizable lipid, phospholipid, cholesterol and C16-PEG2000 lipid) was kept constant at 100%, with each factor having adjusted values. The results indicated that the particle size, and in vitro and in vivo transfection efficiency of both pDNA- and siRNA-SNALP formulations were significantly affected by lipid compositions. Notably, the encapsulation efficiency of pDNA-SNALPs was influenced by the lipid composition, but this was not observed for siRNA-SNALPs. The study also revealed that the optimal lipid compositions of SNALPs for pDNA and siRNA delivery were distinct. Furthermore, it was found that in vitro transfection efficiency could not be used as a direct predictor for promising LNP candidates in vivo. This study has confirmed the potential of the DOE approach in the comprehensive and efficient optimization of LNPs for various applications.

2. Machine learning

Machine learning, a branch of artificial intelligence, offers a transformative approach to iterative lipid design and LNP development. In the intricate landscape of lipid interactions, encompassing structure-function relationships and intricate physicochemical properties, machine learning emerges as a potent tool. Its capacity to sift through vast datasets and discern complex patterns equips researchers to unravel optimal lipid compositions, architectures, and interactions. By establishing predictive models that bridge the gap between lipid chemical structures, LNP characterization and performance, machine learning expedites the iterative design process, reducing the need for exhaustive trial-and-error experimentation (Fan et al., 2021). Furthermore, machine learning-driven insights into cellular interactions, immune responses, and biocompatibility enable the selection of lipids that enhance targeted delivery, minimize adverse effects, and optimize therapeutic payloads.

To expedite the LNP development process for mRNA vaccines with the support from machine learning, Wang et al collected 325 data samples of mRNA-LNP formulations with IgG titer (Wang et al., 2022). They employed the machine

learning algorithm, light-GBM, to construct a predictive model which exhibited commendable performance with an R^2 value exceeding 0.87. The algorithm identified critical substructures of ionizable lipids in LNPs, aligning with previously published findings. Experimental results demonstrated that LNPs using DLin-MC3-DMA as the ionizable lipid at an N/P ratio of 6 were more efficient in mice compared to LNPs with SM-102, corroborating the 'odel's predictions. Molecular dynamic modeling was also utilized to explore the molecular mechanisms of LNPs in experiments, revealing that lipid molecules aggregate to form LNPs while mRNA molecules wrap around them. The 'odel's predictions were validated through in vivo experiments, and molecular dynamic simulations were employed to investigate interactions at the molecular level.

To revolutionize the traditional LNP development process which is relatively more laborious, costly, and heavily reliant on trial and error, Xu and colleagues have developed the AI-Guided Ionizable Lipid Engineering (AGILE) platform, which synergistically combines machine deep learning and combinatorial chemistry (Xu et al., 2023). This platform streamlines the iterative development of ionizable lipids, which are essential for LNP-mediated mRNA delivery. AGILE offers three key features: efficient design and synthesis of combinatorial lipid libraries, comprehensive lipid screening using deep neural networks, and adaptability to various cell lines. By employing AGILE, the team rapidly designed, synthesized, and evaluated new ionizable lipids for mRNA delivery in muscle and immune cells, selecting from a vast library of over 10,000 candidates. The platform revealed cell-specific preferences for ionizable lipids, emphasizing the need for different tail lengths and head groups for optimal delivery to different cell types. The AGILE platform operates in three stages. First, it constitutes a virtual library and undergoes initial self-supervised model training. Second, it acquires empirical data from an experimental library, refining the pre-trained 'odel's accuracy. Lastly, it performs analysis on ionizable lipids in a candidate library using refined deep learning algorithms. The pla'form's deep learning model is trained on a vast collection of unlabeled lipid molecules, extracting insights from observed molecules, and extrapolating to a broader range of unobserved molecules. This approach transforms the discovery of chemical compounds from a trial-and-error process to an intelligent, data-driven strategy. The AGILE pla'form's predictions were validated in the wet lab, leading to the identification of a lipid "H9", which showed superior mRNA transfection potency compared to FDA-approved LNPs. In essence, the success of AGILE platform demonstrated the power and potential of machine deep learning in the development and optimization of LNPs.

3. High-throughput screening platform

High throughput screening (HTS) in LNP development is an avant-garde approach aimed at expeditiously evaluating a myriad of lipid formulations for drug delivery potential. Central to accelerated drug development, these platforms can scrutinize hundreds of LNP compositions simultaneously by bulk preparation, formulation and evaluation of various LNPs. The typical frame of an HTS system for LNP starts with a preparation and formulation unit while utilizing robotic mechanisms designed for meticulous mixing of lipids and therapeutic payloads to produce qualified LNP samples (Cui et al., 2022). Once LNPs are formulated, automated spectroscopy and assay stations outfitted with high throughput accessories can generate LNP characterization data set including size, zeta potential, pKa and encapsulation efficiency. An ideal control nerve center of the platform can orchestrate the synchronized operations across different segments to ensure that tasks are not just executed but are followed by an immediate preliminary analysis. By amalgamating the extensive data generated by HTS with the prowess of machine learning, the results can be enlightening. This integrated HTS platform for LNP development can enable pattern discernment and predictive modeling, propelling the precision and efficacy of LNP development to unprecedented heights.

However, the challenge of HTS platform construction and implementation lies in the multitude of formulation parameters that can influence the quality of LNPs. Fan et al developed an automated HTS workflow that can prepare and analyze LNPs loaded with antisense oligonucleotides (ASOs) in a 96-well plate within 3 hours (Fan et al., 2021). The LNPs were formulated using an automated solvent-injection method facilitated by a robotic liquid handler. The LNPs were then evaluated based on particle size distribution, encapsulation efficiency, and stability across different formulation compositions and ASO loadings. Key findings revealed that the content of PEGylated lipid significantly influenced the particle size distribution. In contrast, the ratio of ionizable lipid to ASO charge affected the encapsulation efficiency of ASOs. The results from the HTS method correlated well with those from a state-of-the-art scale-up method using a microfluidic formulator. This new approach offers a more efficient avenue for formulation development, reducing material usage by tenfold and increasing analytical outputs by a hundredfold. Sarode and colleagues also introduced an automated, low-volume HTS workflow to prepare, characterize, and assess LNPs loaded with therapeutic ASOs. A library of 54 ASO-LNP formulations with varying PEG-lipid compositions was created using a robotic liquid handler. These LNPs were then evaluated for their physicochemical properties and gene silencing efficacy in murine cortical neurons. The study found that the molar ratio

of anionic PEG-lipid in LNPs influenced particle size, while the PEG-lipid carbon tail length affected the gene silencing activity of ASO-LNPs. This advancement supports the use of HTS workflows during the early stages of LNP formulation development, offering significant time and material savings, and holds potential for broader applications in identifying optimal formulations for various cellular targets.

High throughput method can be applied to not only lipid and LNP screening, but also in vitro and in vivo evaluation for selective targeting and efficacy enhancing purposes. Nelson et al put efforts into creating combinatorial synthetic (CS) libraries of LNP structures optimized for siRNA delivery (Nelson et al., 2022). These CS techniques, which encompass solid-phase synthesis, Michael addition, and click chemistry, can be adapted to generate a vast array of LNP structures. By leveraging automated robotic systems, LNPs can be prepared using solvent injection or microfluidic techniques in high-throughput formats like 96- or 384-well plates. A pioneering method to simultaneously evaluate biodistribution and biofunctionality involves encapsulating DNA or mRNA molecular barcodes into the LNPs during the initial CS phase. This allows for the HTS of LNPs upon binding to their specific cellular and tissue targets. The subsequent quantification of LNP biodistribution is based on target DNA sequence amplification via PCR, followed by next-generation sequencing. This innovative approach, combining molecular barcoding with HTS, offers a paradigm shift in rapidly screening and pinpointing optimal LNP formulations for targeted nucleic acid delivery, effectively bridging the often-divergent results of in vitro and in vivo performance evaluations.

More toward the HTS evaluation of LNPs, Sago and colleagues introduced a system named Fast Identification of Nanoparticle Delivery (FIND) that can simultaneously quantify the functional delivery of mRNA by over 100 LNPs in vivo (Sago et al., 2018). The traditional approach to LNP selection involves in vitro screening, followed by in vivo testing of a few selected LNPs. However, this method often leads to LNPs that target the liver due to the assays used for selection. The authors believed that testing a large number of distinct LNPs in vivo would increase the chances of identifying nanoparticles that deliver mRNA to new cell types. Using DNA barcodes, they previously quantified how over 350 LNPs were distributed in vivo. However, biodistribution alone 'oesn't guarantee functional RNA delivery, as a significant portion of RNA delivered into a'cell's endosome can be degraded. The FIND system addresses this by measuring cytosolic mRNA delivery by over 100 LNPs in vivo to various cell types. The study identified two LNPs, named "7C2" and "7C3", which can efficiently deliver RNA to endothelial cells. Notably, LNP "7C3" delivered Cas9/sgRNA to splenic endothelial cells as effectively as hepatocytes, distinguishing it from other LNPs. This high-throughput approach, combined with the innovative use of DNA barcoding, offers a promising method for rapidly screening and pinpointing optimal LNP formulations for targeted nucleic acid delivery, bridging the gap between in vitro and in vivo evaluations.

Conclusion and perspectives

Lipid nanoparticles (LNPs) have undoubtedly ushered in a revolutionary era in the realm of drug delivery, offering an exceptionally promising avenue, especially in the context of nucleic acid-based therapies. Their intricate and deliberate design, characterized by the integration of signature and innovative lipids, has unlocked a realm of possibilities. These nanoparticles are not just passive carriers, they are masterfully engineered to ensure optimal encapsulation of therapeutic payload with precision in delivery and seamless biocompatibility (Hald Albertsen et al., 2022). The ionizable lipid in these LNPs ensures the encapsulation and safe transportation of fragile mRNA molecules. This innovation demonstrates the real-world impact and transformative potential of LNPs in driving medical advancements. The dynamic behavior of ionizable lipids, capable of responding to pH changes, synergizes with the structural integrity provided by phospholipids, ensuring the successful traversal of barriers within cells. The role of cholesterol extends beyond its molecular simplicity, and it dynamically fine-tunes the physical properties of LNPs, enhancing stability and therapeutic efficacy. Meanwhile, the strategic incorporation of PEG-lipids contributes to extended circulation half-life by mitigating immunogenic and toxic responses (Chen et al., 2019).

The adaptability of INPs is further highlighted by the customization of lipid components to serve specific purposes. These customized lipids can range from ligand-conjugated lipids that target specific cell types to fusogenic lipids that enhance endosomal escape. The evolution of lipid design is pivotal for advancing LNP-mediated drug delivery. Tailored lipid compositions can optimize cellular uptake, mitigate potential toxicity, and address challenges related to endosomal escape. Additionally, the research on cholesterol analogues offers insights into the potential of structural alterations to enhance mRNA delivery efficiency. Gazing ahead, the forthcoming generation of LNPs holds the promise of even more profound breakthroughs in drug delivery. As the field continues to evolve, there will be an intensified focus on tailoring LNPs with precision to cater to specific therapeutic demands. This endeavor transcends mere lipid component refinement and extends to the meticulous optimization of LNP architecture and surface properties (Pozzi et al., 2015). The

quest for targeted drug delivery will occupy a central position in this evolution. This entails the integration of ligands or antibodies capable of recognizing and binding to specific cell receptors, amplifying therapeutic precision and minimizing collateral effects.

The persistent challenge of immune responses, particularly those associated with PEGylated LNPs, will propel the search for alternative materials. Another avenue ripe for exploration is the realm of stimuli-responsive LNPs. These ingenious nanoparticles can release their therapeutic cargo in response to distinct environmental cues, such as variations in pH, temperature, or light exposure. Such controlled release mechanisms hold the potential to elevate therapeutic efficacy while curtailing undesirable side effects and can be magnified with a thorough understanding of the LNP behaviors and interactions at the cellular level. As the journey of LNP research unfolds, the translation of laboratory insights into real-world clinical applications will emerge as a pivotal endeavor. This transition necessitates not only the refinement and optimization of LNPs but also rigorous testing to ascertain their safety and efficacy in human subjects. Collaboration among researchers, clinicians, and industry partners will be paramount to fully unlock the potential of LNPs in shaping the next chapter of drug delivery innovation.

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