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

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Chapter 1

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

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1. The concept of mRNA-based medicine

Nucleic acids are biomacromolecules essential for life that play crucial roles in the evolution and health of living organisms. According to the central dogma of molecular biology, nucleic acids—deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)—carry and regulate expression of genetic information, which is read in cells to produce RNAs (transcription) and proteins (translation) [1–3]. (Figure 1a). In eukaryotic cells, DNA is first transcribed into a messenger (m)RNA precursor, which is then spliced to generate a mature mRNA. This mature mRNA transcript is subsequently translated by ribosomes to produce functional proteins responsible for nearly every task of cellular life [4,5]. The better understanding of the mechanisms involved in the flow of genetic information within biological systems has opened new avenues in modern medicine [6–8]. In this scenario, nucleic acid-based therapeutics have emerged as a new class of drugs that hold promise for combating multiple diseases. They are generally divided into two classes: small nucleic acid drugs and mRNA-based drugs; Figure 1b-e shows their mechanisms of action. The former includes small antisense oligonucleotides (ASOs), siRNA (small interfering RNA), shRNA (short hairpin RNA), miRNA (microRNA), which are engineered to target specific (abnormal) genes and modulate their expression or function [6,9,10]. On the other hand, mRNA-based drugs rely on the production of desired proteins using the body's natural protein synthesis machinery [11,12]. Unlike DNA that needs to cross the nuclear membrane, mRNA only needs to reach the cytoplasm [4], and it is unable to integrate into the host genome due to its natural degradation during translation; as a result, the protein expression is intrinsically transient avoiding unwanted long-term effects, desirable in many gene therapy approaches [3,13].

After the first demonstration *in vivo* of the mRNA-based drug efficacy in which naked mRNAs were intramuscularly injected into mice to induce the expression of functional proteins [14], the field of mRNA-based medicine has rapidly expanded, prompting the development of multiple therapeutic modalities. These mainly include: a) prophylactic and therapeutic vaccines, where synthetic mRNAs designed to encode specific protein antigens are administered to trigger humoral and cellular immune responses against pathogens in patients, in order to prevent or treat diseases [13,15,16]; b) protein replacement therapies, where mRNAs are delivered to compensate desired proteins inside the target cells or to supply therapeutic proteins [17–19]; c) cancer immunotherapies, where mRNAs encoding either tumor-associated antigens, tumor-specific antigens, or immunostimulatory factors, such as costimulatory ligands,

receptors, enzymes, or cytokines, enhance or suppress the host immune response against cancer cells [20–23]; d) cell-based therapies, where cells of interest are transfected *ex vivo* with synthetic mRNAs to induce specific phenotypic or functional changes, and subsequently administered to the patient. For example, chimeric antigen receptor T (CAR-T) cells and chimeric antigen receptor-macrophage (CAR-M) cells can be produced *ex vivo* using synthetic mRNAs [24,25]; and e) gene editing, which involves the use of mRNAs to deliver gene-editing tools, particularly, the clustered regularly interspaced short palindromic repeats associated nucleases (CRISPR-Cas), allowing for highly precise and efficient targeting of defined DNA regions of the genome (Figure 1e) [26,27]. CRISPR-Cas gene editing can be achieved using mRNAs encoding Cas proteins and guide (g)RNA sequences [28].

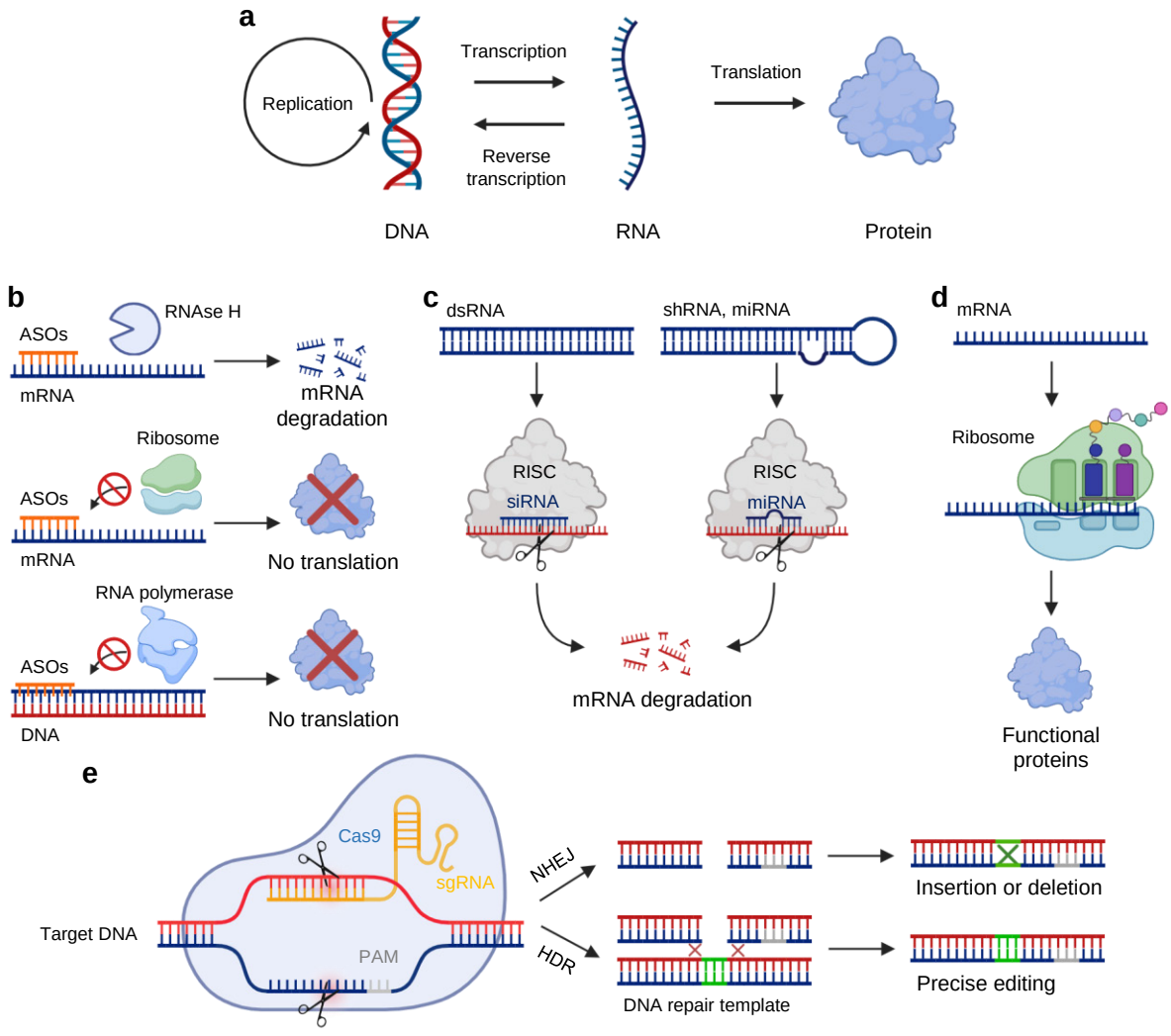

Figure 1. Classification and mechanisms of nucleic acid-based therapeutics. **a** Schematic representation of central dogma of molecular biology that illustrates the flow of genetic information from DNA to RNA to protein, providing the conceptual framework for nucleic acid-based therapeutics [1,2]. **b** Antisense oligonucleotides (ASOs) regulate gene expression

by blocking translation, inducing RNase H-mediated degradation, or modulating pre-mRNA splicing [8,9]. **c** RNA interference strategies employ double-stranded RNA precursors—including siRNAs, shRNAs, and miRNAs—which are processed and loaded into RISC to mediate sequence-specific mRNA cleavage, degradation, and/or translational repression [29,30]. **d** mRNA act as transient templates for protein expression, enabling applications such as vaccination, protein replacement, and cell reprogramming [18,20,21]. **e** CRISPR-Cas systems mediate targeted genome editing by guiding the Cas nuclease to specific DNA sequences through RNA-DNA base pairing, leading to precise cleavage and enabling gene disruption, correction, or insertion [31,32].

2. Design of mRNA-based drugs

mRNA-based drugs are typically synthesized in cell-free systems via *in vitro* transcription (IVT), from a DNA template, and they are designed to be structurally like natural mRNAs [33]. Eukaryotic mRNA consists of a single-stranded RNA molecule built from nucleotides, which are linked together by phosphodiester bonds to form a backbone with a repeating pattern of sugar-phosphate groups—each nucleotide containing a ribose sugar, a phosphate group, and a nitrogenous base: adenine (A), guanine (G), cytosine (c), or uracil (U) (figure 2a) [34]. Mature mRNA has a tripartite structure consisting of an open reading frame (protein-coding sequence that gets translated into the desired protein) flanked by two untranslated regions (UTRs) at both the 5’ and 3’ ends (Figure 2b) [35]. The 5’ end is capped with a modified 7-methyl-guanosine (m7G) residue, and the 3’ end of mRNA is terminated with a poly(A) tail [34]. However, the development of mRNA-based drugs has not been straightforward. Unmodified mRNA is highly unstable under physiological conditions, and it is quickly degraded by extracellular and intracellular ribonucleases, which in turn leads to poor pharmacokinetics and biodistribution [15,21]. Furthermore, the large, negatively charged size of mRNA molecules makes cellular internalization very inefficient [5,19]. Lastly, exogenously delivered, unmodified IVT mRNAs stimulate the activation of various Toll-like receptors and/or cytoplasmic receptors e.g., RIG-I, PKR, and MDA5, which results in the activation of innate immune pathways that stall the cellular translational machinery, accelerate mRNA degradation and prevent effective protein production [36,37].

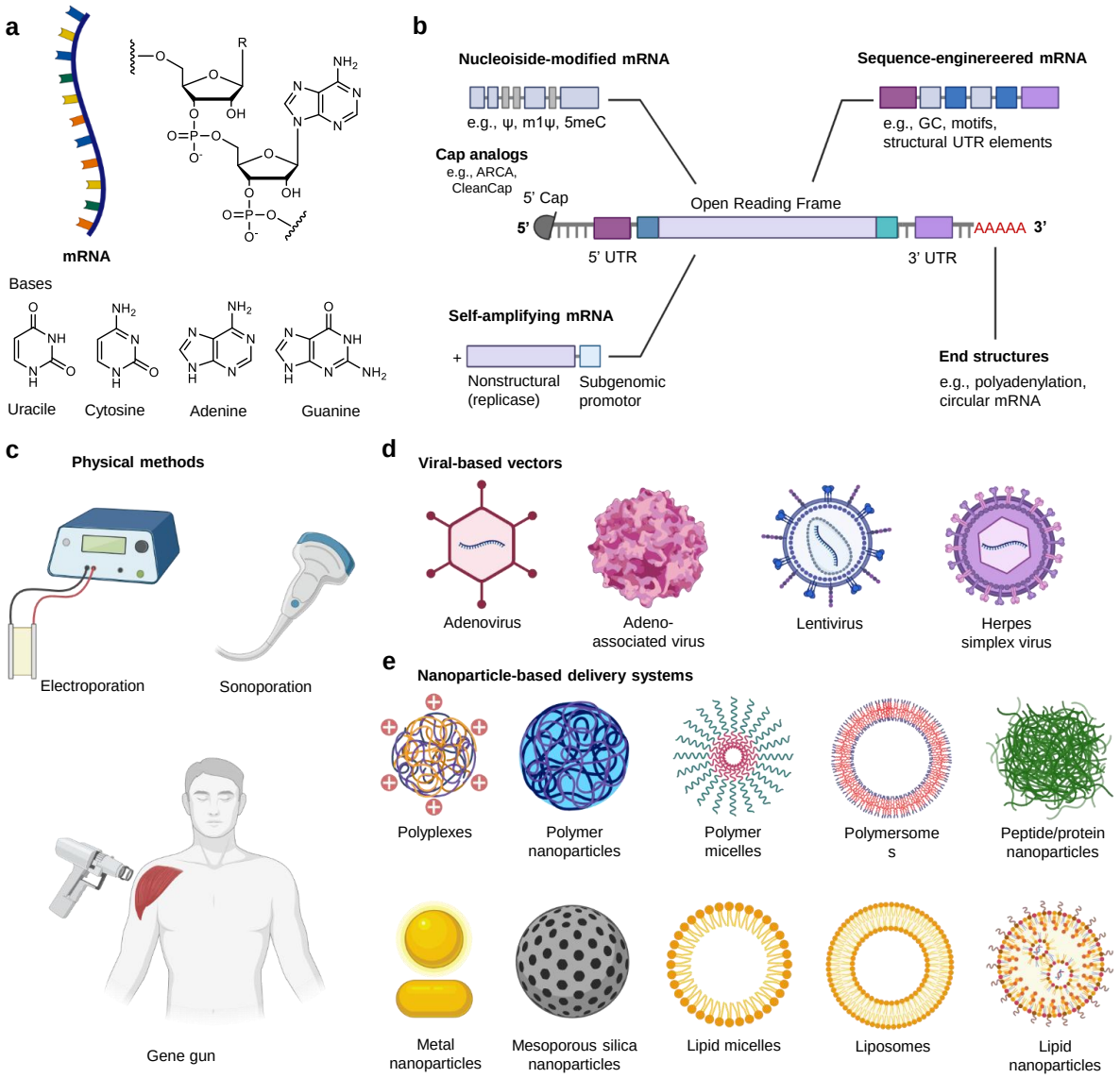

Figure 2. Optimization of mRNA structure and delivery strategies. **a** Chemical composition of mRNA, consisting of a ribose-phosphate backbone and the four nucleobases adenine (A), cytosine (C), guanine (G), and uracil (U), linked through phosphodiester bonds, with the ribose 2'-hydroxyl group distinguishing RNA from DNA [17,35]. **b** General architecture of therapeutic mRNA, including the 5' cap, untranslated regions (UTRs), open reading frame (ORF), and poly(A) tail; chemical modifications, such as incorporation of N1-methylpseudouridine (m1ψ), are introduced to enhance stability, reduce immunogenicity, and improve translational efficiency [33,38,39]. **c** Physical delivery approaches, including electroporation, sonoporation, and gene gun technology, enable direct introduction of mRNA but are largely restricted to localized or ex vivo applications [40]. **d** Viral vector-based systems allow efficient gene transfer but remain limited by immunogenicity, restricted payload capacity, and manufacturing complexity. **e** Nanoparticle-based platforms, most notably lipid nanoparticles (LNPs), are the predominant strategy for systemic delivery, offering protection of mRNA cargo, biocompatibility, and tunable targeting properties [41,42].

2.1. mRNA modifications for optimal therapeutic performance

Chemical modifications of the phosphate backbone, ribose sugar, and nucleobase in the mRNA components, the 5' cap, 5' and 3' UTRs, open reading frame and poly(A) tail, have helped prevent exonuclease degradation and enhance ribosomal catalysis, while reducing recognition by immune sensors, leading to an enhanced mRNA stability and translation efficiency [43]. These modifications are carried out during various stages of the IVT reaction [44]. In this regard, Cap analogs—modified nucleotides used to mimic the natural 5' cap structure—not only protect mRNA from 5' to 3' exoribonucleases and increase protein synthesis rate but also increases capping efficiency that improves the mRNA production yield [17]. Capping technologies such as anti-reverse cap analog (ARCA) and CleanCap® enable synthesis of mRNAs with 5'-cap1 or 5'-cap2 structures, known to increase the half-life of mRNA-based drugs [38,45,46]. The 3′ and 5′ UTRs can also be engineered for the target cell of interest, thereby improving translation efficiency and tissue-specificity [43,47]. On the other hand, the poly(A) tail at the 3' end of mRNA is engineered to act as a protective buffer against degradation by 3'-5' exonucleases in a length-dependent manner; a poly(A) tail length of 100-300 nucleotides has been shown to be optimal for balancing mRNA stability and translation efficiency [12,17]. Nucleotide modifications of mRNA constructs can also reduce immunogenicity and increase translation. For example, substituting uridine with pseudouridine (ψ) or its methylated derivative N1-methyl-pseudouridine (m1ψ) enhances base stacking and raises the melting point, thereby improving mRNA stability, as well as it induces changes in the secondary structure of mRNA which correlated with high translation yields, while minimizing cellular recognition by the innate immune system [39,48].

2.2. mRNA delivery systems

To achieve the desired biological effect, mRNAs must be delivered to specific target cells and translated efficiently to produce therapeutic levels of the protein of interest, and chemical modifications to mRNA are not yet sufficiently developed to overcome these obstacles [49,50]. To this end, several approaches are being considered for mRNA delivery, in particular, electroporation methods, viral vectors, and nano-sized delivery systems have proven to be the most promising mRNA delivery strategies (Figure 2c-e) [7,26,51]. Electroporation involves the application of voltage pulses to generate an electrical field that creates temporary pores in the cell membrane, which allows the mRNA to enter [52,53]. However, irreversible disruption of cell membrane and the loss of intracellular components compromise cell viability and

subsequent translation activity [26,53]. Viral vectors, particularly adeno-associated viruses (AAVs), can efficiently deliver mRNA into cells; nevertheless, issues such as immunogenicity, potential toxicity, insertional mutagenesis, limited loading capacity, and complex manufacturing processes have so far limited their clinical approval [40,54]. On the other hand, with the application of nanotechnology in medicine (i.e. nanomedicine), a variety of materials have been developed for mRNA delivery, including lipids, polymers, carbohydrates, peptides and proteins, and inorganic compounds (Figure 2e) [41,42,55–57]. Nanoparticle-based delivery systems have the potential to pass through biological barriers, improve mRNA stability *in vivo*, prolong mRNA circulation time, deliver mRNA to its target site and release it into the cytosol to be translated, and minimize side-effects [7,42]. Nanoparticle-mediated mRNA delivery to target tissues is significantly influenced by the physicochemical properties of nanocarriers, such as composition, size, shape, surface charge, surface functionalization, hydrophobicity/hydrophilicity, as well as their interaction with serum proteins and cell surface receptors [56,57]. However, targeted delivery and efficient endosomal escape remain key bottlenecks in the field of mRNA therapeutics, reinforcing the need for more effective mRNA delivery platforms [49,58,59].

3. Lipid nanoparticles for mRNA delivery

Lipid nanoparticles (LNPs) are the most successful nanocarriers for the intracellular delivery of exogenous RNAs used for gene silencing (siRNA) [60,61] expression (mRNA) [62,63] and editing (CRISPR/Cas9) [64,65]. Three RNA-LNP products have already been approved for clinical use, including Onpattro®—the first siRNA-LNP drug for the treatment of polyneuropathies resulting from the hereditary transthyretin amyloidosis—and the two mRNA-based vaccines against SARS-CoV-2 [60,66,67].

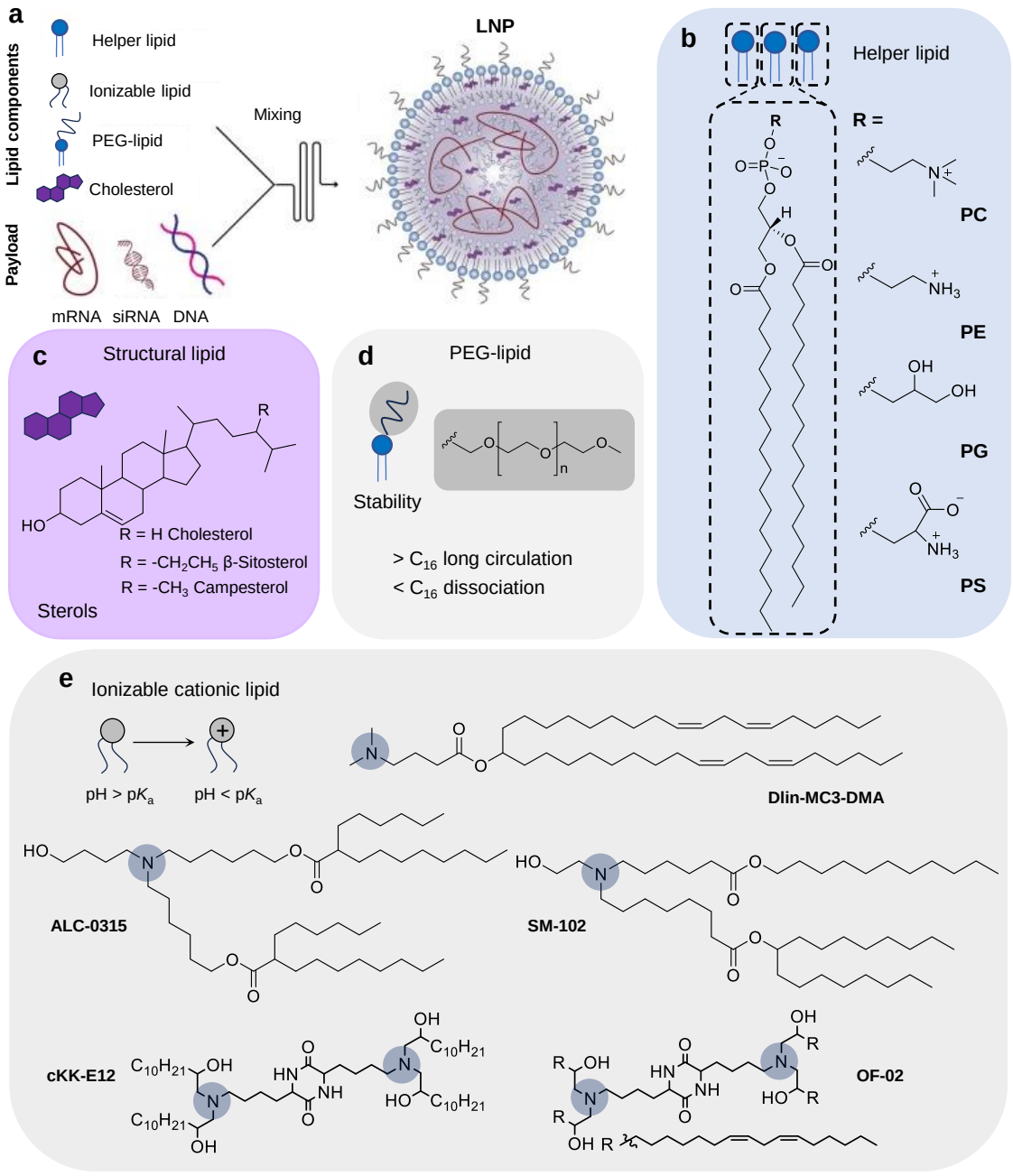

Figure 3. Molecular properties of nucleic acid-based LNPs. **a** Individual lipid components and nucleic acid payloads used in LNPs and formation of the nanoparticle after microfluidic mixing. **b** Molecular structures of commonly used helper lipids. Phospholipids can vary on head group or chain length and saturation. **c** Structural lipids are usually sterols. Clinically-approved LNPs incorporate cholesterol; however, other sterol analogs have been observed to influence LNP morphology and mRNA transfection. **d** PEG-lipids providing structural and long-term stability on the LNP are also used to prolong circulation lifetimes or can be designed to dissociate for LNP interaction when in contact with biological fluids. **e** ICLs contain tertiary amine-based head groups that, when the pH is lower than their p*K*a, they become protonated and thus positively charged. Abbreviations: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PG = phosphatidylglycerol.

3.1. LNP self-assembly

LNPs are kinetically stable spherical particles exhibiting a diameter of ~100 nm and near-neutral surface charge at physiological pH [66–68]. The "optimal" combination of the LNP components allows for efficient RNA entrapment and cytosolic delivery. Moreover, lipid components can individually dictate circulation lifetimes, biodistribution, cell selectivity, or transfection potency and therefore rational design of LNPs are of great importance for the outcome of each individual therapeutic. LNPs have a sophisticated nanostructure based on the synergistic effect of individual lipid components comprising the assembly (Figure 3) [69]. Components include "helper" lipids that provide structural integrity (i.e., phospholipids and PEG-lipids), hydrophobic "structural" lipids which reside in the LNP core (i.e., cholesterol) and ionizable cationic lipids (ICLs), that complex RNA and facilitate transfection. ICLs with an apparent pK_a between 6-7, allow electrostatic interaction driven complexation with nucleic acid molecules in acidic pH, followed by LNP assembly [70].

3.2. Nano-bio interactions

LNPs—like other nanocarriers—encounter several biological barriers, which could potentially reduce their performance (Figure 4). Upon intravenous administration, LNPs must avoid immune detection, prevent non-specific interactions with proteins and non-target tissues, reach and internalize into the target cells, and finally facilitate endosomal escape for payload release in the cytosol [39,49,71]. Notably, it has been estimated that only a small percentage of the injected LNP dose reaches its target cells, and that less than 2% of the LNP-siRNA reaches the cytosol after endocytosis [72]. On this journey, LNPs interact with several biomolecules (serum proteins) and biological structures (cell membranes), which influence the ability of LNPs to deliver their payload into the target site. Such nano-bio interactions, which in turn determine biodistribution and LNP fate, are mainly determined by the physicochemical properties of LNPs, including lipid composition, particle size, surface charge, apparent pK_a and morphology.

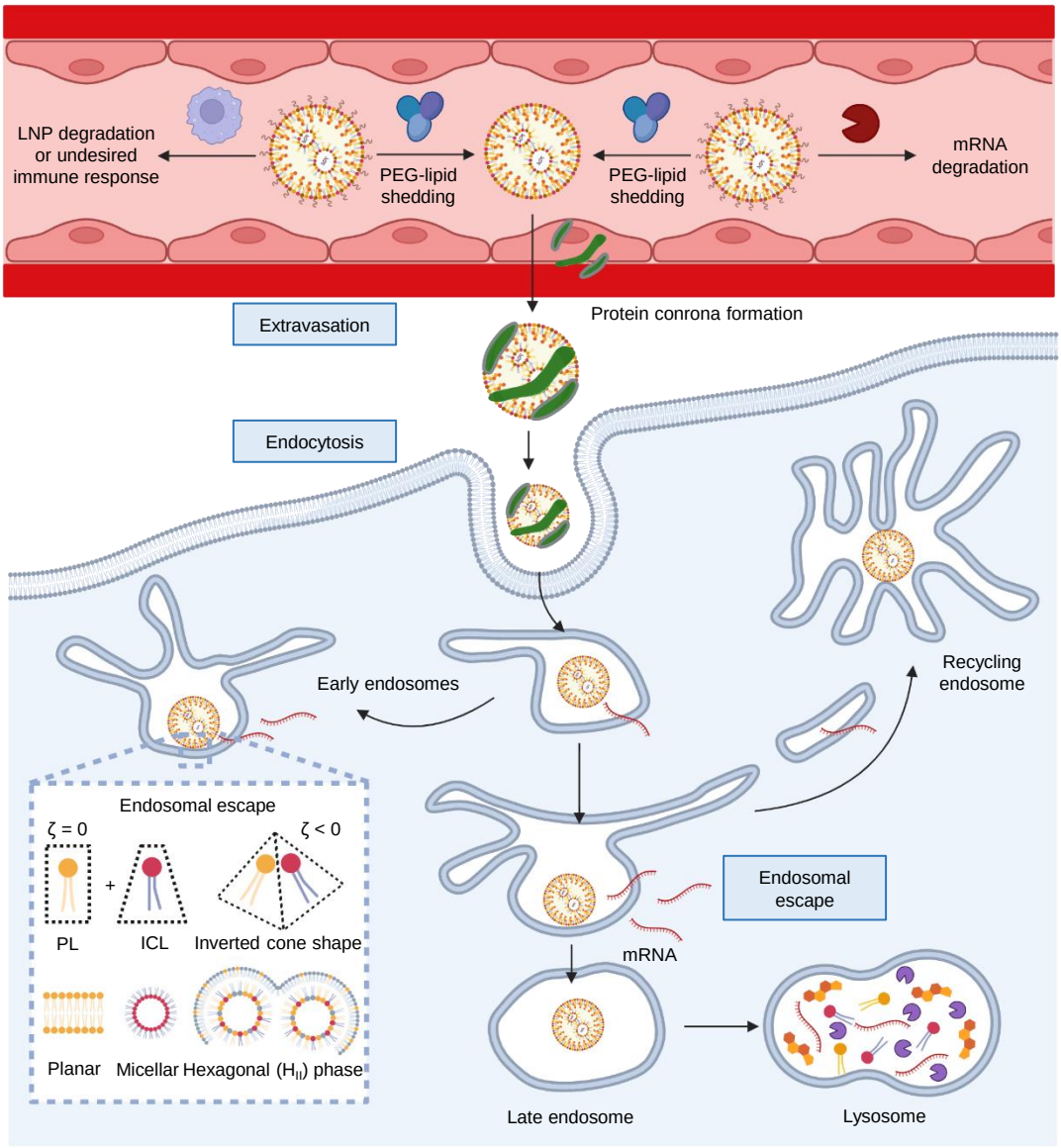

Figure 4. *In vivo* fate of mRNA-LNPs following systemic administration. After mRNA-LNPs reach the bloodstream, PEG-shedding can take place, which facilitates adsorption of serum proteins onto the LNP surface, which in turn could influence nanoparticle clearance and phagocytosis, but also enable receptor-mediated endocytosis in target cells. Once endocytosed, mRNA-LNPs are entrapped in early, recycling, and late endosomes where pH drops to acidic values (pH ~6.0–6.5). At this stage, the ICLs will be positively charged in the acidic lumen of the endosomal compartments, and therefore electrostatically interact with anionic endosomal phospholipids. Abundant membrane phospholipids prefer planar membrane geometries. However, electrostatic interaction with the ICLs leads to the formation of conical-shaped lipid pairs inducing liquid-crystalline phases in the endosomal membrane (hexagonal phase H_{II}). Such non-bilayer phases disrupt the endosome, resulting in subsequent cytosolic escape of the RNA payload. If would not escape the endosomes, mRNA-LNPs will either recycle back to the bloodstream via the recycling endosomes or will be degraded when endosomes fuse with lysosomes. Abbreviations: PL = phospholipid, ζ = membrane curvature, inv. hexagonal = inverse hexagonal.

3.2.1. LNP-serum protein interactions

After administration, LNPs instantly interact with biological components, particularly, plasma proteins and mononuclear phagocytic cells (monocytes, macrophages and dendritic cells) responsible for clearance. PEGylation has demonstrated to prolong the blood circulation half-life of LNPs by offering a "stealth effect" that reduces interactions with immune system-triggering proteins e.g., opsonins, which recognize and bind to macrophage and phagocyte receptors [73,74]. However, PEGylation, especially in high mol% content, also decreases particle-cell membrane interactions and hence reduces transfection potency [73,75]. Considering this, PEG-lipids can be designed to dissociate from the LNP surface upon contact with biological fluids, and exchange with plasma lipoproteins, a process termed PEG-lipid shedding [76]. The PEG-lipid dissociation rate from LNPs in blood circulation is highly determined by the PEG-lipid anchor length. C14 anchor PEG-lipids dissociate faster (>45%/h) than C18 anchor PEG-lipids (0.2%/h) [77]. After PEG-lipid shedding, serum proteins are rapidly adsorbed on the LNP surface leading to the formation of a protein layer called protein corona, which modifies the physicochemical features of RNA-LNPs and determines their biodistribution and cellular uptake [78,79]. The protein corona of several LNP formulations has been recently found to be rich in apolipoproteins (Apo), which are known to facilitate cellular uptake via receptor-mediated endocytosis [80,81]. For instance, the selective recognition and hepatic uptake of Onpattro® relies on the adsorption of ApoE, which binds to low-density lipoprotein receptor (LDLR), abundantly expressed on hepatocytes [60]. The strong interaction between the LNPs and N-terminal lipid-binding region of ApoE via tryptophan residues causes conformational changes in ApoE, which results in a high affinity to LDLRs [78].

3.2.2. Interactions of LNPs with tissues and cells

Considering that nanoparticle physicochemical properties will profoundly influence (desired and undesired) nano-bio interactions, LNPs can be engineered for cell and tissue-selective targeting and payload delivery (Table 1).

Table 1. A selection of reviewed LNPs which demonstrated enhanced tissue/cell targeting.

Lipid Composition	Lipid ratio	Target tissue	Target cell	Payload	Function	Ref
Dlin-MC3-DMA, Chol, DSPC, DMG-PEG2k	50/38.5/10/1.5	Liver	Hepatocytes	siRNA	Transthyretin silencing (treatment for amyloidosis)	[60,82]
ALC-0315, Chol, DSPC, DMG-PEG2k	50/38.5/10/1.5	Liver	HSCs	siRNA	ADAMTS13 silencing (biodistribution studies)	[83]
Dlin-MC3-DMA, Chol, **DSPG**, DMG-PEG2k	50/38.5/10/1.5	Liver	LSECs	mRNA	eGFP/mCherry expression (biodistribution studies)	[84]
246C10, Chol, DOPE, **mannose-DSPE-PEG2k**	26.5/50.5/20/3	Liver	LSECs	mRNA	mFLuc expression (biodistribution studies)	[85]
306O10, Chol, **DOPE**, DMPE-PEG2k	22.5/40/35/2.5	Liver	-	mRNA	mFLuc expression (biodistribution studies)	[86]
306O10, Chol, **DOPS**, DMPE-PEG2k	22.5/40/35/2.5	Spleen	-	mRNA	mFLuc expression (biodistribution studies)	[86]
306O10, Chol, **DOTAP**, DMPE-PEG2k	22.5/40/35/2.5	Lung	-	mRNA	mFLuc expression (biodistribution studies)	[86]
5A2-SC8, Chol, DOPE, DMG-PEG2k, **18PA** (SORT)	16.7/33.3/16.7/3.3/30	Spleen	Macrophages	mRNA	mFLuc expression (biodistribution studies)	[87,88]
5A2-SC8, Chol, DOPE, DMG-PEG2K, **DODAP** (SORT)	19.05/38.1/19.05/3.8/20	Liver	Hepatocytes	mRNA	mFLuc expression (biodistribution studies)	[87,88]
5A2-SC8, DOPE, CHO, DMG-PEG2k, **DOTAP** (SORT)	11.9/23.8/11.9/2.4/50 11.9/11.9/23.8/2.4/50	Lung	Endothelial cells	mRNA	mFLuc expression (biodistribution studies)	[87,88]
PBA-Q76-O16B, **NT1-O14B** (NT-lipidoid)	5/5 and 3/7[a]	Brain	Neuronal cells	ASO	Tau silencing (biodistribution studies)	[89]
BP-lipid, Chol, DOPE, DMPE-PEG2k	35/46.5/16/2.5	Bone Surface and marrow	-	mRNA	mFLuc expression (biodistribution studies)	[90]

Abbreviations in the table that cannot be found in main text: ASO = antisense oligonucleotide. 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG); 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); 1,2-dioleoyl-3-dimethylammonium-propane (DODAP). aw/w ratio.

Systemic administration of Onpattro®, that exploits the ApoE-LDLR uptake pathway, resulted in liver accumulation and preferential uptake by hepatocytes [60]. In turn, by replacing the ICL Dlin-MC3-DMA for ALC-0315, a higher LNP accumulation in hepatic stellate cells (HSCs) was observed, likely due to differences in protein corona composition [83]. Moreover, hepatocyte targeting could be achieved by producing LNPs with sizes smaller than the fenestration diameter in the liver [91]. We also recently demonstrated that switching the LNP surface charge from neutral to anionic, leads to the preferential accumulation of Dlin-MC3-DMA-based LNPs in the hepatic reticuloendothelial system (RES), mediated by the scavenger receptor Stabilin-2 [84]. In a similar fashion, Kim et al., explored the ability of LNPs to target liver sinusoidal endothelial cells (LSECs) by functionalizing their surface with mannose-based targeting ligands, resulting in selective mRNA delivery to LSECs [85].

LNPs can be also engineered to target non-liver tissues. by modulating the lipid composition and therefore modifying properties such as surface charge and apparent pK_a of LNPs. For instance, the replacement of neutral phospholipids (DSPC or DOPE) with anionic (DSPG or 18:PA) or cationic (DOTAP or EPC) alternatives, leads to redirection of mRNA delivery from liver to spleen and lungs, respectively (Figure 2a) [86,92]. These differences in organ targeting can be only achieved when the phospholipid concentration in LNPs is 40 mol % [86]. On the other hand, the Siegwart group recently developed a new strategy called selective organ targeting (SORT) wherein a fifth component (SORT molecules) is added to LNP formulations to selectively target extrahepatic tissues through passive targeting (Figure 2b) [87,93]. In this context, systemically administered LNPs containing SORT molecules such as cationic (DOTAP), anionic (18PA), and ionizable (DODAP) lipids enabled selective mRNA delivery and CRISPR-Cas gene editing to lung, spleen, and liver, respectively [88]. Particularly, 50%-DOTAP-SORT-LNPs preferentially accumulated in lung endothelial cells, while 30%-18PA-SORT-LNPs did in splenic macrophages. Mechanistically, tissue-specific targeting by SORT LNPs occurs via PEG-lipid shedding, protein corona formation—of which composition is determined by the SORT molecule employed—and subsequent interactions with receptors abundantly expressed in the target tissue [94]. The addition of SORT molecules modifies the apparent pK_a of LNPs and further affects the LNP-serum protein interactions. Liver-targeting SORT LNPs exhibited an apparent pK_a in the range of 6-7 and acquired an ApoE-rich protein corona. SORT LNPs with higher apparent pK_a values (>9) had a protein corona rich in vitronectin, which mediated lung targeting due to the abundant expression of vitronectin receptors by lung endothelial cells. On the other hand, the protein corona displayed on spleen-

targeting SORT LNPs (apparent pK_a between 2 and 6) was found to be rich in β2-glycoprotein I, which bound to phosphatidyl serine-exposing blood cells, promoting LNP filtration from blood circulation to the spleen [88,94]. Instead of incorporating additional components into LNP formulations, synthesis of a new generation of ICLs containing targeting head groups has been also exploited to target different organs via active targeting. For instance, Xu and coworkers synthesized a series of neurotransmitter-based lipoids that can cross blood-brain barrier (BBB), leading to a higher accumulation of LNPs and subsequent payload release in various regions of the mouse brain, including cerebral cortex, hippocampus, and cerebellum [89]. A similar approach has been employed by the Mitchell group to deliver mRNA into the bone marrow microenvironment [90]. They designed ICLs containing bisphosphonates (BP) head groups that displayed a high affinity for hydroxyapatite and bone surfaces, which improved LNP accumulation and mRNA transfection in the bone microenvironment.

3.2.3. Cellular uptake

Receptor-mediated endocytosis is the main process by which RNA-LNPs are taken up by cells. Early studies showed that fluorescently-labeled siRNA-LNPs enter cells via clathrin-mediated endocytosis [72], which was further corroborated for mRNA-LNPs [78]. This endocytic pathway is responsible for the cellular uptake of lipid- and cholesterol-enriched LDLs by binding to ApoE that targets them to LDLRs [95]. As mentioned above, the LNP-protein corona complex is enriched with ApoE after PEG-lipid shedding, to target LDLRs [72,96]. It has recently been shown that ApoE adsorption causes rearrangement of LNP components, affecting the LNP nanostructure, which is expected to affect the intracellular RNA delivery mediated by LNPs [78]. LNPs can also be functionalized with antibodies for cytosolic mRNA delivery via caveolae-mediated endocytosis in lung endothelial cells expressing caveolae-associated proteins [97]. Importantly, studies using small molecules to block endocytic pathways revealed that macropinocytosis and phagocytosis also contribute to the cell internalization of some LNP formulations [72,98]. In fact, immunes cells commonly use phagocytosis to engulf and destroy exogenous particles, including LNPs [99,100]. Note that positively charged LNPs may be taken up by clathrin- or caveolae-mediated endocytosis after electrostatic interactions with anionic macromolecules, such as proteoglycans and glycosaminoglycans of cell membranes [99]. Finally, membrane fusion has been recently employed to deliver siRNA and mRNA in mouse and human cells via LNPs containing membrane-fusogenic cationic lipids or fusogenic coiled-coil peptides [100,101]. Direct fusion

with the cell membrane allows for payloads to be directly delivered to the cytosol, avoiding therefore endocytic routes from where LNPs need to escape [101].

3.2.4. Intracellular trafficking

Following endocytosis, LNPs are entrapped in endosomes that, eventually, fuse with lysosomes [70,96]. In early endosomes, physiological pH (~7.4) decreases to acidic values (pH~6.5), and after further endosome maturation (late endosome) pH decreases even further (pH~6.0) before final fusion with lysosomes (pH~5.0) [102]. For cytosolic mRNA delivery, LNPs must help mRNA escape from the endosomes before lysosome formation which eventually leads to LNP and mRNA degradation (Figure 4). The pH-responsive property of ICLs is pivotal for endosomal escape. In the acidic endosomes, ICLs are positively charged and electrostatically interact with negatively charged phospholipids of the endosomal membrane, creating non-bilayer liquid crystalline phases (i.e., hexagonal H_{II} phase), which facilitate endosomal disruption and escape of mRNA into the cytosol [68,70,103]. The optimal pK_a of ICLs has been intensively studied and LNPs with excellent transfection potency display apparent pK_a values between 6.0 and 6.7 [62,63,104]. However, not only the pK_a, but also other structural properties affect the ability of ICLs to induce transfection. ICLs with an accentuated coned-shape molecular structure, such as the clinically-approved Dlin-MC3-DMA, ALC-0315 and SM-102, have shown to destabilize the lipid bilayer of cell membrane [104–106]. Moreover, a recent study showed that LNPs with cubic and hexagonal nanostructures fuse more easily with endosomal membranes than lamellar LNPs, resulting in higher endosomal escape rates [103]. In a similar fashion, induction of phase-separated blebs in the LNP nanostructure led to enhanced transfection efficiency [107].

Although the process of endosomal escape by LNP has not been completely resolved yet, pioneering studies reported that LNP-RNA escape occurs in the early and late endosomes [72,108,109]. Recruitment of cytosolic galectins by damaged endosomes in response to LNPs as well as colocalization of LNPs with endosomal trafficking proteins, such as EEA1, APPL1 and Rab11, corroborate endosomal escape [72,99,110]. More recently, Zerial and coworkers showed that mRNA-LNPs displaying high transfection efficiency accumulated in early- and recycling-endosomes, where escape mainly occurred, probably because of their positive and negative membrane curvatures that favor interactions with ICLs, leading to membrane leakages [99]. In this respect, it is interesting that Patel et al. showed that by substituting cholesterol to 25% and 50% 7a-hydroxycholesterol in mRNA-LNPs reduced the presence of recycling

endosomes and observed enhanced mRNA delivery to primary human T cells *ex vivo* by 1.8-fold and 2.0-fold, respectively [111]. Moreover, other sterol analogues have been also observed to induce morphological changes in LNPs and increase mRNA potency, likely due to different endosomal trafficking and enhanced endosomal escape [112,113].

4. Scopus and outline of the thesis

Despite the clinical success of mRNA-LNP vaccines, major challenges remain in achieving efficient, targeted, and safe delivery for diverse therapeutic applications. Key limitations include incomplete understanding of how LNP composition influences biodistribution and immune responses, the poor predictability of *in vitro* potency for *in vivo* efficacy, and insufficient tools to visualize and track LNPs at high spatial resolution within cells. Addressing these knowledge gaps is essential for improving both the performance and the rational design of next-generation mRNA-LNP systems.

This thesis explores the design, optimization, and mechanistic characterization of LNP systems for mRNA-based therapeutics and vaccines to address current limitations in delivery efficiency, targeting precision, and mechanistic understanding. It encompasses a critical overview of nucleic acid medicine and nanomedicine advances, with emphasis on LNP-mediated delivery, followed by systematic experimental investigations into how LNP composition, particularly ionizable lipids, influences physicochemical properties, cellular uptake, protein expression, and immune responses. This thesis further explores novel immunization strategies, biodistribution, macrophage interactions, and intracellular trafficking, complemented by advanced imaging approaches for super-resolved visualization of LNP localization. Together, these studies integrate materials science, immunology, and cell biology to address key barriers to the clinical translation of mRNA-LNP technology.

The main aim of this thesis is to advance the rational design and functional optimization of LNP-based mRNA delivery systems for therapeutic and vaccine applications. This involves systematically evaluating how variations in LNP composition, particularly ionizable lipids, affect delivery performance, immune activation, and biodistribution in both *in vitro* and *in vivo* contexts. Furthermore, it seeks to develop and validate innovative immunization strategies, such as heterologous prime–boost regimens with costimulatory mRNA-LNP boosters, to enhance antigen-specific cellular immunity. Another core objective is to establish state-of-the-art analytical and imaging tools, including bioorthogonal click chemistry combined with expansion microscopy, for the precise visualization of LNPs within cells. Through these combined approaches, the thesis aims to generate both fundamental insights and practical methodologies to improve the efficacy, specificity, and translational potential of LNP-mRNA therapeutics and vaccines.

Chapter 1 provides a focused exploration of nucleic acid-based medicine modalities and recent advances of nanomedicine in delivering them to their target sites, particularly, LNPs as delivery systems mRNA-based therapeutics and vaccines.

Chapter 2 explores the role of ionizable lipids in determining the effectiveness of LNPs for mRNA delivery and vaccination. Four ionizable lipids were tested for their impact on LNP properties, cellular uptake, protein expression, and immune response. The findings show that LNP composition significantly influences biological outcomes and that *in vitro* potency does not consistently predict *in vivo* efficacy.

Chapter 3 presents a novel heterologous prime-boost vaccination regimen designed to maximize antigen-specific cellular immunity. The strategy involves primary immunization with LNPs encapsulating mRNA encoding a model antigen, followed one week later by a costimulatory booster to enhance T cell priming. The study further examines the application of CD40L-mRNA-LNPs as costimulatory boosters, assessing their potential to enhance antigen-specific immune responses.

Chapter 4 provides a comprehensive examination of the biological interactions between LNPs and macrophages, focusing on biodistribution, cellular uptake, and intracellular trafficking. Utilizing advanced analytical techniques in combination with both cell culture and zebrafish models, the chapter elucidates the key biological barriers that hinder efficient mRNA delivery to target cells.

Chapter 5 describes an alternative approach for the intracellular visualization of LNPs using confocal microscopy. This strategy involves the chemical modification of an ionizable lipid to incorporate a clickable moiety, followed by its formulation into LNPs and subsequent delivery to cells. Once internalized, the clickable moiety is covalently conjugated with fluorescent dyes via bioorthogonal click chemistry, enabling its precise intracellular localization and tracking. When combined with expansion microscopy, this approach allows imaging of the labeled lipids at super-resolution, providing unprecedented spatial detail of their intracellular distribution.

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