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Complexity, unpredictability and safety challenges of lipid nanoparticles - A multidisciplinary narrative review

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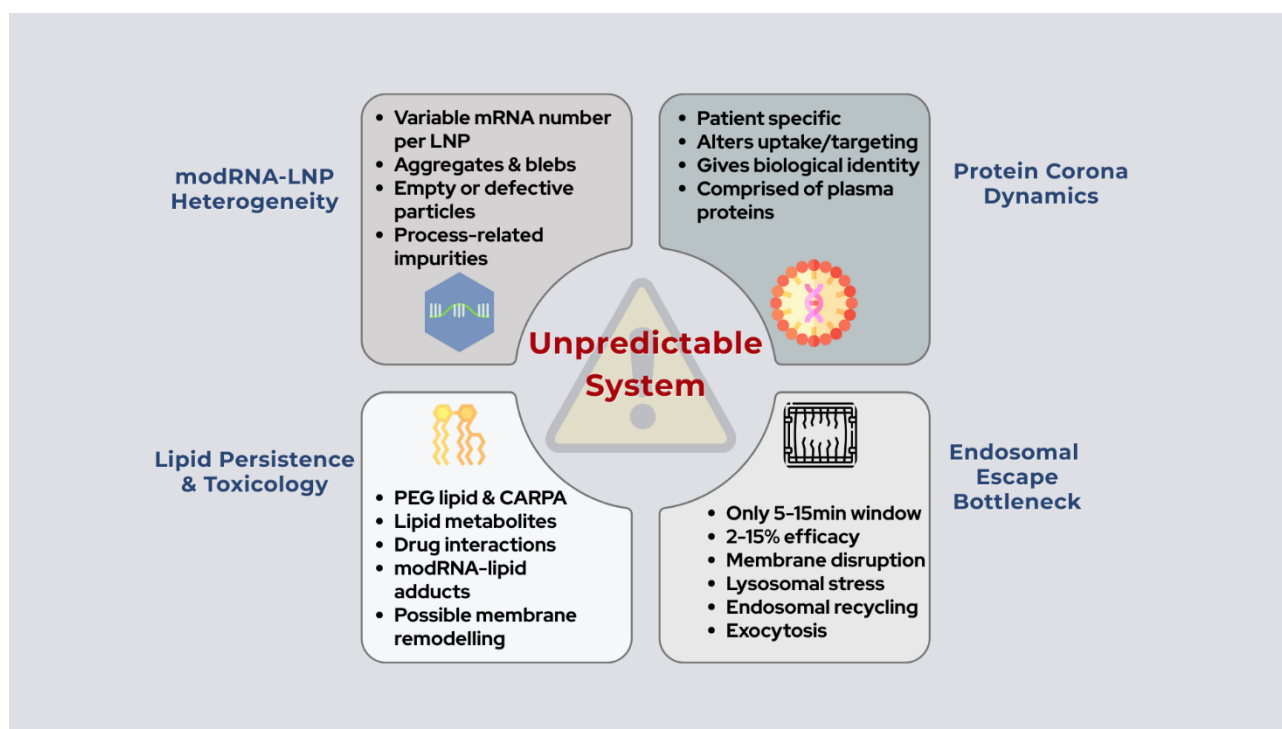
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Abstract

The lipid nanoparticle (LNP) platform for delivering modified messenger RNA (modRNA) represents a transformative yet inherently complex and unpredictable technology. This narrative review synthesizes multidisciplinary evidence to explore the physicochemical basis, biological interactions, pharmacodynamic uncertainties, and safety challenges associated with LNPs and LNP-modRNA interactions. We describe how LNP self-assembly gives rise to variable structures with inconsistent modRNA payloads, as well as dynamic protein corona formation and aggregation phenomena that complicate the reliable characterization of these systems. After injection, LNPs undergo rapid biotransformation, including PEG-lipid shedding, biodistribution, and cellular uptake, which current analytical techniques cannot fully capture.

Importantly, endosomal escape, which leads to the disruption of the endosome and the release of the payload, occurs within a narrow time window, is often inefficient, and results in inconsistent delivery. In addition, lipid metabolites, cell membrane modulation, and adduct formation pose poorly characterized risks.

Keywords: lipid nanoparticles, mRNA vaccines, protein corona, endosomal escape, unpredictability, drug interactions, safety



29
30 **Figure 1**

31 Conceptual overview of the unpredictable LNP platform. Four key challenges are highlighted. 1. LNP
32 heterogeneity (variable modRNA content, aggregates, impurities) 2. Protein corona dynamics (patient-
33 specific, uptake, biological identity) 3. Lipid persistence and toxicology (PEG lipid immunogenicity,
34 modRNA-lipid adducts) and 4. Endosomal escape bottleneck (5-15min window, low efficacy,
35 membrane disruption) Original work using Canva by S. Natsheh. Icons made by [Pixel](#)
36 [perfect](#) from www.flaticon.com

37

38 **1 Physicochemical Foundations of the LNPs**

39

40 **1.1 Introduction**

41

42 The physicochemical properties of lipid nanoparticles (LNPs), including their size, shape, surface reac-
43 tivity, and lipid composition, are crucial for their role in delivering modRNA to cells. These in vitro
44 properties govern LNP stability, encapsulation efficiency, and the ability to penetrate the cell membrane
45 and transport the modRNA into the cytosol. The physicochemical properties of LNPs profoundly af-
46 fect the lipid chemistry of the cell membrane, which varies between different cells and cell types. This is
47 important since the membrane is inherently connected to the intracellular signal transduction

48

49 network, which is initiated and regulated by endocytic processes and receptor conformational changes,
50 many of which depend on the physicochemical properties of the LNPs. This section thoroughly inves-
51 tigate the LNP composition, structure, and nanoparticle characteristics, establishing a foundation for
52 understanding their behavior in vivo.

53

54 LNPs are by no means new.([Cullis & Felgner, 2024](#); [Tenchov et al., 2021](#)) Research into lipid carrier
55 systems with a wide variety of formulations has been ongoing for over 60 years. Liposomes are an ear-
56 lier type of LNPs, consisting of one or multiple lipid bilayers with an aqueous core. They are commonly
57 used in drug delivery because hydrophilic drugs can be enclosed within the aqueous interior, while hy-
58 drophobic drugs are trapped within the hydrocarbon chains of the lipid bilayer. Liposomes cannot effi-
59 ciently carry nucleic acids, such as mRNA, due to the size, polyanionic nature, and hydrophilicity of the
60 mRNA, which motivated the development of ionizable lipid-based LNPs. Additionally, nucleic acids
61 are quickly degraded by endogenous nucleases in bodily fluids.([Kloczewiak et al., 2022](#)) To address
62 these issues, LNPs incorporating ionizable lipids have been developed as delivery vehicles for small in-
63 terfering RNA (siRNA) and mRNA, thereby protecting fragile cargo from degradation in vivo and fa-
64 cilitating cellular delivery.

65

66 Despite their widespread clinical application in SARS-CoV-2 vaccination, the complex multicomponent
67 nature of LNP systems leads to heterogeneity and unpredictability at multiple levels of biological inter-
68 action. Regulatory assessments have traditionally categorized LNPs as inert excipients, but accumulat-
69 ing evidence points to adjuvant-like properties, complement activation, immunomodulation, and poten-
70 tial drug–vaccine interactions caused by cytokine-mediated suppression of cytochrome P450 enzymes.
71 Taken together, these findings suggest that LNPs should be regarded as active pharmacological entities
72 rather than passive carriers, whose systemic and long-term effects remain incompletely understood.

73

74 While prior reviews have explored the properties of LNPs([Tenchov et al., 2021](#)) or safety as-
75 pects,([Bitounis et al., 2024](#)) the present work represents a first attempt to integrate the unpredictable
76 and partially stochastic nature of modRNA–LNP systems across their pharmacological dimensions.

77

We argue that this non-linear behavior introduces uncertainty into therapeutic application and challenges precision and predictability. Accordingly, we emphasize the need for enhanced regulatory oversight, thorough mechanistic studies, clinical pharmacology assessments, and the application of advanced analytical techniques to better characterize and evaluate this novel platform.

1.2 Composition

The currently approved LNP formulations for the COVID-19 vaccines contain four lipids: (1) an ionizable cationic lipid, (2) a helper lipid DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), (3) cholesterol, and (4) a polyethylene glycol (PEG)-lipid conjugate.[\(Chaudhary et al., 2024\)](#) Each lipid component of the nanoparticle and its molar ratio are critical to the activity and disposition of the modRNA. Similarly, the first approved LNP-RNA product, patisiran (Onpattro), contains short interfering RNA (siRNA) in an LNP formulation designed to deliver siRNA to the liver and silence the expression of transthyretin, a protein that causes transthyretin amyloidosis (ATTR).

Developing and scaling up Onpattro® paved the way for LNP-modRNA vaccines, which are the fastest vaccines ever produced.[\(Hald Albertsen et al., 2022\)](#)

The ionizable lipid is crucial for delivering nucleic acids across cell membranes. Composed of a tertiary amine head, a linker, and a hydrophobic tail, it undergoes protonation under acidic conditions. This allows it to bind to negatively charged modRNAs, specifically via the tertiary amine head, owing to the unique properties and pH-dependent surface charge of ionizable lipids [\(Han et al., 2021\)](#). The design of the ionizable lipid, such as tail length[\(Hashiba, Taguchi, Sakamoto, Otsu, Maeda, Suzuki, et al., 2024\)](#), saturation, and branched tails, [\(Petersen et al., 2024\)](#) influences the efficacy and toxicity of the LNPs. The helper phospholipid (DSPC) enhances LNP bilayer stability, thereby preventing leakage of nucleic acid cargo. It provides the structural foundation for membrane fusion, which is necessary for cellular uptake. Cholesterol is crucial for maintaining the overall shape, fluidity, and permeability of the bilayer membrane, as well as supporting other phospholipids for effective encapsulation and protection of the

107 modRNA cargo,[\(Wang et al., 2024\)](#). Cholesterol accounts for about 45% of the LNP content and can
108 exist in a crystalline-like state within the LNP.[\(Anindita et al., 2024\)](#)

109

110 The PEG lipid conjugate serves primarily to decrease LNP size, shield the LNP from rapid clearance
111 by the reticuloendothelial system (RES), stabilize LNPs via steric repulsion, and prevent protein
112 adsorption due to the hydrophilic chains extending from the surface.[\(Hald Albertsen et al., 2022\)](#) It
113 typically only comprises about 1.5% of the LNP content. The immunogenicity of PEG has drawn
114 attention due to the development of anti-PEG antibodies after repeated exposure.[\(Song et al., 2025\)](#)

115

116 1.3 Structure of the LNPs

117

118 For COVID-19 vaccines, the exact structures of modRNA-LNPs remain unknown due to their self-
119 assembly nature and the properties of the lipids used. These Janus particles, which exhibit two or more
120 distinct physical properties, remain poorly understood. Small-angle neutron scattering (SANS) reveals
121 that blebs (separate aqueous-filled compartment within a lipid nanoparticle, distinct from the main lipid
122 structure) are common, but they do not always indicate the presence of modRNA within them.[\(Chen et al., 2025\)](#) In fact, identifying modRNA-free LNPs has proved particularly challenging. Studies estimate
124 that 12-80% of LNPs (most recently 30-35%) may lack any modRNA, depending on the manufacturing
125 process, the ionizable lipid used, and the analytical method employed.

126 [\(Li et al., 2022; Münter et al., 2024; Pavlin et al., 2025; Schober et al., 2024\)](#) The modRNA payload is
127 especially important, particularly regarding the number of strands and the structure of the modRNA, as
128 the random packaging of modRNA constructs influences LNP behaviour and potency.[\(Liao et al., 2025\)](#) [\(Renzi et al., 2024\)](#) [\(Di et al., 2022\)](#) Therefore, the relationship between the declared dose (μg of
130 RNA) and the number of RNA-containing particles is not straightforward, and this correlation has yet
131 to be fully described.

132

133

134 Currently, there is no reliable analytical method to accurately characterize either the content (i.e., the
135 modRNA,[\(Webb et al., 2025\)](#)) or the structure of LNPs([Sanyal et al., 2021](#)), so orthogonal techniques
136 are necessary.[\(Parot et al., 2024; Pavlin et al., 2025\)](#) Moreover, LNPs with blebs may also exhibit
137 different immunogenicity, biodistribution, or *in vivo* properties that have not been adequately
138 studied.[\(Simonsen, 2024\)](#) Mixing and filling parameters during manufacturing and sample handling of
139 filled vials by clinicians also impact modRNA payload.[\(Matthessen et al., 2024\)](#) Furthermore, empty
140 LNPs may reduce the effective dose, increase variability in therapeutic effectiveness since these are the
141 ones most likely to transfect cells,[\(Liao et al., 2025\)](#) and accumulate in tissues possibly acting as
142 adjuvants,[\(Lee et al., 2023\)](#) an understudied risk. These recent findings have raised questions about the
143 formulation and composition of safe and effective LNPs for modRNA therapeutics and makes it
144 difficult to comply with recommendations for LNP characterization by regulatory authorities.
145 [\(EuropeanMedicinesAgency, 2025\)](#) Lyophilization (freeze drying) could reduce empty LNPs and
146 improve stability at room temperature [\(De & Ko, 2023\)](#) and improve mixing, but remains
147 investigational.

148

149 **1.4 The Nanoparticle Nature of LNPs**

150

151 Due to their small size, nanoparticles have an extremely high surface area relative to their volume,
152 resulting in unique chemical, physical, and biological properties not found in bulk materials. These
153 properties enhance the LNPs' reactive interactions with the cell membrane, such as immune responses
154 and cellular uptake.[\(Yuan et al., 2024\)](#)

155

156 Importantly, the biological behaviour of the LNP formulation cannot be inferred merely from the
157 isolated properties of individual lipids. This is because the physicochemical characteristics of the entire
158 LNP present in the final formulation, such as size distribution, shape, surface charge or zeta potential,
159 agglomeration state, and lipid packing,[\(Abbasi et al., 2023\)](#) arise from interactions among all the
160 components. For example, the degree of lipid unsaturation and branching affects not only membrane
161 fusion capabilities but also biodegradability and systemic persistence.[\(Yang et al., 2022\)](#) Secondly,
162 variations in size, modRNA payload, encapsulation rate, stability, lipid impurities, and other
163 physicochemical factors may affect the safety and efficacy of these products, as demonstrated in
164 preclinical and clinical studies.[\(Yuan et al., 2024\)](#) (see Table 1).

165

166 **The LNPs are dynamic and unstable**

167

168 Most recently, evidence suggests that the physiological stability of RNA-LNPs significantly impacts
169 their therapeutic efficacy, pharmacokinetics (PK), tissue-targeting ability, and toxicity. ([Zhang & Barz,](#)
170 [2025](#)) Instability in blood or plasma can lead to premature degradation of LNPs and the release of
171 modRNA, potentially altering biodistribution and immune effects or affecting potential inflammation,
172 depending on the specific formulation. ([Eygeris et al., 2022](#)) The Moderna and Pfizer/BioNTech
173 vaccines differ in LNP behaviour. The modRNA of the Moderna COVID-19 vaccine persisted longer
174 in plasma than the ionizable lipid SM-102 itself, suggesting lipid transfer to lipoproteins or extracellular
175 vesicles (EVs). ([Kent et al., 2024](#); [Y. Ren et al., 2025](#)). The implications for cellular function remain
176 uncertain. Conversely, the ionizable lipid of Pfizer/BioNTech's vaccine, ALC-0315, showed prolonged
177 lipid exposure but lower levels of modRNA in plasma. ([Y. Ren et al., 2025](#)) This could indicate
178 instability of the intact LNP in plasma, possibly caused by trace impurities of the ionizable lipid ([Liau et](#)
179 [al., 2024](#)) or complete disintegration in plasma ([Bitounis et al., 2024](#)) These differences between the
180 approved vaccines suggest that the specific formulation and manufacturing of the modRNA and lipid
181 components (**Figure 2**) are distinct both in composition and biological effects, which may influence
182 vaccine efficacy and outcomes. A comparison of the publicly available compositions, physicochemical
183 properties, and key formulation parameters of the currently approved LNP-RNA products is shown in
184 **Table 1.**

185

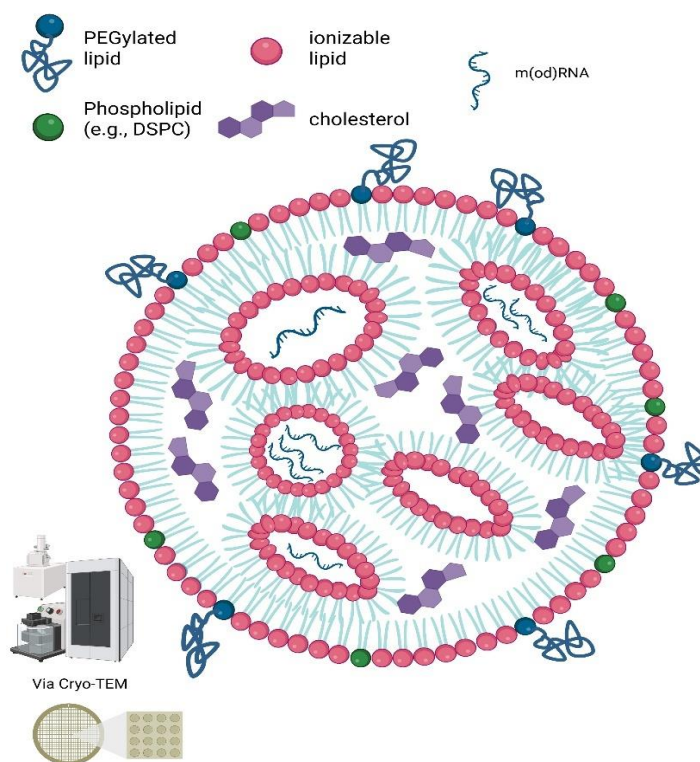


Figure 2 Schematic Structure of mRNA-Lipid Nanoparticle

Lipid nanoparticles are mainly composed of ionizable lipids, cholesterol, phospholipids, and polyethylene glycol (PEG)-lipid.

The ionizable lipids are cationic (positively charged) at a low pH (enabling negatively charged RNA complexation) and neutral at physiological pH (reducing potential toxic effects), allowing a better delivery of mRNA into the cells via endocytosis.

Phospholipids play a structural role, and cholesterol serves as a stabilizing element in lipid nanoparticles. Lipid-anchored PEGs dominantly deposit on the lipid nanoparticle surface as a barrier to sterically stabilize them and reduce nonspecific binding to proteins. **Created in BioRender. Seger, F. (2025)**

Category	Pfizer/BioNTech (modRNA)	Moderna (modRNA)
Name	BNT162b2, Comirnaty	mRNA-1273, SpikeVax
Dose, Route	30 µg/0.3 ml, IM	50 µg/0.5 mL, IM
Lipid Components	ALC-0315 (ionizable lipid, Acuitas) ALC-0159 (pegylated lipid) DSPC (neutral lipid) Cholesterol	SM-102 (ionizable lipid) PEG-DMG (pegylated lipid) DSPC (neutral lipid) Cholesterol
Molar Ratios (%) <i>(ionizable cationic lipid: neutral lipid: cholesterol: PEGylated lipid)</i>	46.3:9.4:42.7:1.6	50:10:38.5:1.5
Molar N/P ratios	6	6
Ionizable Lipid Properties	Apparent pKa=6.09 least stable 2 branched chains; moderate biodegradability 2 chiral centres, 3 stereoisomers (De et al., 2025)	Apparent pKa=6.68 more stable 1 branched chain; improved biodegradability No chiral centres
LNP Particle Size and Distribution (Hermosilla et al., 2023)	Widest distribution (60-5000nm)	Wider distribution (30-1000nm)
modRNA payload (number of intact modRNA constructs per LNP)	Variable (exact payload unclear)	Variable (exact payload unclear)
Encapsulation Efficiency (%EE)*	~50% (Schober et al., 2024)	Not reported but likely similar
Stability	Moderate (Y. Ren et al., 2025)	High (Y. Ren et al., 2025)
Buffer	Potassium dihydrogen phosphate; Disodium hydrogen phosphate dihydrate pH 7–8; Tris (tromethamine) in October 2021(USFDA, 2021)	Tris (tromethamine) pH 7–8

190 **TABLE 1:**Composition and Physicochemical Properties of LNPs in Approved modRNA Vaccines
191 (Abstracted from Schoenmaker ([Schoenmaker et al., 2021](#)), Zhang, Akinc([Akinc et al., 2019](#); [Zhang et](#)
192 [al., 2023](#)); EMA([EMA/707383/](#), 2020 Corr.1*¹),([EuopeanMedicinesAgency, 2021](#)))

193 ***United States Pharmacopeia** uses $EE(\%)$, defined as the percentage of RNA or therapeutic cargo that is suc-
194 cessfully enclosed within the LNPs relative to the total amount of RNA present in the final sample. Schober et al([Scho-](#)
195 [ber et al., 2024](#)) used encapsulation efficiency as the percentage of input RNA encapsulated in the final LNP product
196 ($EE_{input}(\%)$) and found encapsulation rates $<50\%$

197

198 1.5 Analytical Challenges and Knowledge Gaps

199

200 The physicochemical properties often differ from theoretical predictions based on behaviors observed
201 in non-biological systems. Despite significant progress, reliable techniques to determine physicochemi-
202 cal attributes are not yet fully standardized.([UnitedStatesPharmacopeia, 2024](#)) For instance, particle size
203 varies.([Hermosilla et al., 2023](#)) Using both expired and unexpired batches of BNT162b2 (Comirnaty®)
204 and m-1273 (Spikevax®), the authors identified three different populations of LNPs for Comirnaty®:
205 60–65 nm (90% of the total), 600–700 nm (5–10%), and, in two vials examined, 5000 nm (1.2% and
206 2.8% by volume). Similar results were observed for SpikeVax®, ranging from 30 nm to 1000 nm. These
207 large particles likely represent agglomerated LNPs, which are visible particles that may have specific
208 physical, microbiological, and chemical adverse effects.([Liu & Hutchinson, 2024](#)) Aggregates are higher
209 in thawed vials and may have *in vivo* risks (e.g., embolism or inflammation)

210

211 These issues complicate accurate assessment of their *in vivo* behaviors, as *in vitro* characterization re-
212 mains unpredictable and variable.([C. Chen et al., 2023](#)) The need for precise characterization of LNPs,
213 including size, blebs, empty structures, and other parameters, has driven the development of techniques
214 to identify, observe, and measure significant differences between formulations and batch-to-batch vari-
215 ability of the same LNP-RNA system.([Parot et al., 2024](#)) For instance, Pavlin *et al.* (2025) recently intro-
216 duced a two-dimensional chromatography method that simultaneously assesses encapsulation efficiency
217 ($\sim 65\text{--}70\%$), nucleic acid integrity, LNP size and impurities

218

219 enabling detection of empty particles and aggregates in heterogeneous samples simultaneously but re-
220 quires standardization.([Pavlin et al., 2025](#)) The physicochemical and structural complexities, as well as
221 the lack of a reference standard (a certified material for calibration) for LNP formulations([Simon et al.,](#)
222 [2023](#)) raise critical questions about their *in vivo* behavior. **Section 2** will expand on this foundation to
223 examine how these properties affect biodistribution, uptake, endosomal escape, therapeutic effective-
224 ness, and potential toxicities.

225

226 Section 2 - Biological Interaction and Pharmacodynamic Uncertainties of LNPs

227

Key Terms in modRNA-LNP Vaccines: Biodistribution, Transfection, and Gene Expression

1. **Biodistribution:** physical location of a drug, tracer, or intact LNP within a biological system.
 - a. depends on circulation, the protein corona, vascular permeability, and reticular endothelial system (RES) uptake
 - b. does not indicate cell entry
2. **Transfection:** process of delivering nucleic acids, such as modRNA, into eukaryotic cells using nonviral methods.
 - a. Requires cellular uptake and endosomal release
3. **Gene expression or Protein Production**
 - a. Translation of mRNA into target protein (ie spike protein)
 - b. Depends on intact mRNA, active ribosomes, and protection from degradation

Critical Note.

- Biodistribution, transfection and gene expression are time-dependent and distinct processes
- Many studies conflate LNP biodistribution with transfection or gene expression leading to inaccurate assumptions
- Preclinical trials or regulatory submissions often lack transfection and gene expression data, limiting understanding of efficacy and adverse events

228

229 2.1 Overview

230

231 The *in vivo* journey of modRNA-LNPs from injection to protein translation depends on a variety of in-
232 terdependent processes. The physicochemical properties, influenced by LNP manufacturing and chem-
233 istry, impact the *in vivo* response. This begins with the formation of a protein corona when the LNP in-
234 teracts with biological fluids. Cell uptake, target cell specificity, reliance on the protein corona, routes of
235 administration, and other factors are not fully captured by current biodistribution analysis methods. Ul-
236 timately, endosomal escape releases the modRNA for translation, and the lipids, modRNA, and newly
237 formed protein are cleared and degraded through various pathways. LNP-mediated delivery requires
238 entry into the target cell, traversal of biological barriers and release of modRNA into the cytosol (**Fig.**
239 **2**).

240

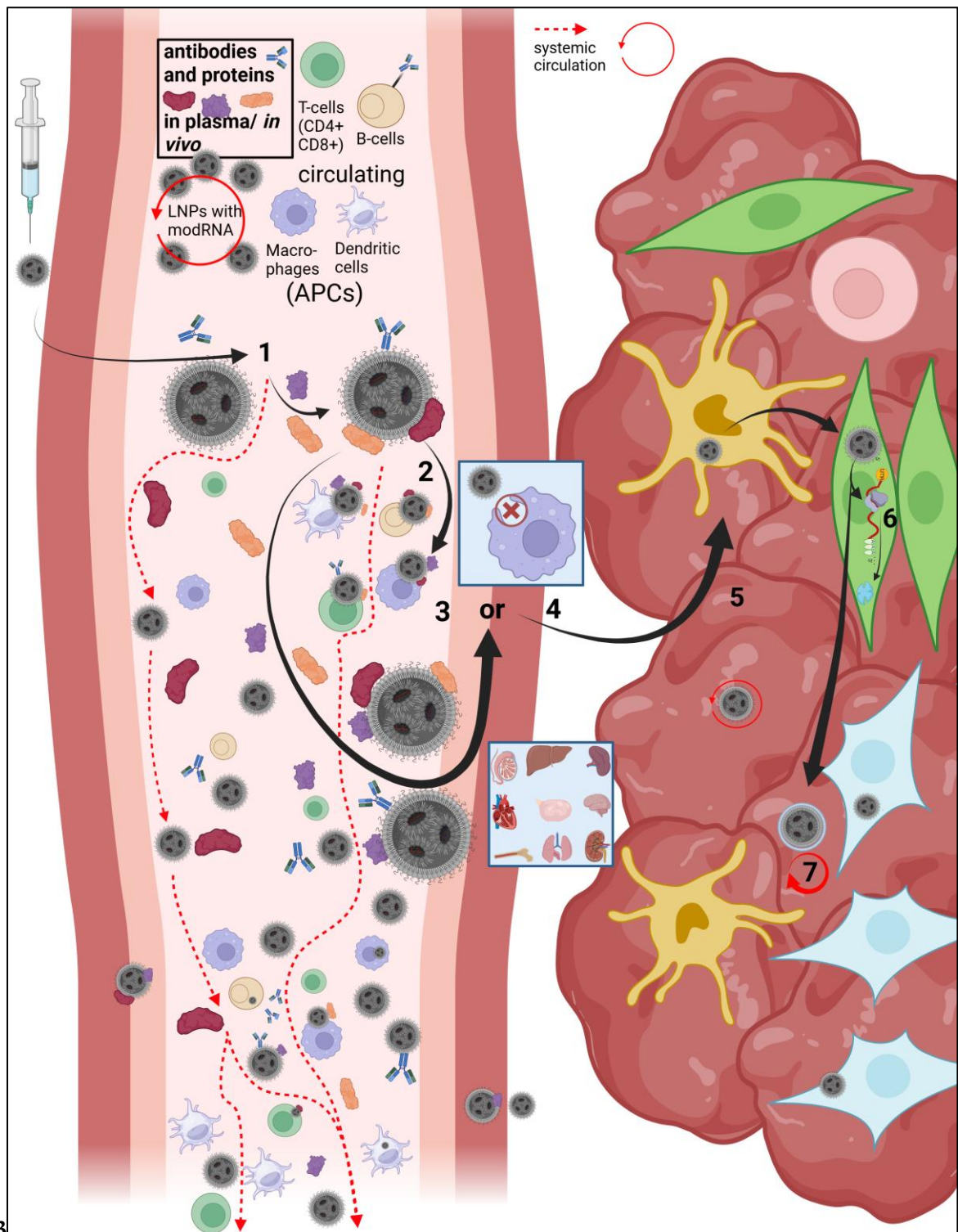


Figure 3
LNP *in vivo* Journey from Injection to Site of Action LNPs injected into muscle rapidly drain into lymph nodes and subsequently circulate in lymph and plasma (the LNP must remain stable in circulation); **2** Acquires an individualized protein corona; **3** Transfects circulating immune cells; **4** Avoids phagocytosis **5** Leaves circulation via fenestrated epithelium or transcytosis **6**. Random transfection of individual cells and release of modRNA into cytosol **7** Exocytosis via extracellular vesicles (EVs)/exosomes. **Created in BioRender. by Seger, F. (2025)**
<https://BioRender.com/byxe75h>

250 2.1 Biodistribution of the LNP-modRNA vaccines

251

252 Accurately determining the biodistribution of LNPs, the modRNA, and the expressed protein remains
253 a challenge. This issue affects many studies on modRNA-LNP technologies. Fluorescence-based re-
254 porter assays are primarily used to track protein production or gene expression, but they do not directly
255 indicate LNP localization or transfection ability. Regulatory guidelines also recommend using quantita-
256 tive whole-body autoradiography (QWBA) to visualize and quantify intact LNP concentrations across
257 almost all tissues and organs simultaneously, enabling systematic comparisons among tissues. ([Vervaeke](#)
258 [et al., 2022](#)) Hybridization techniques, such as fluorescence in situ hybridization (FISH) for localization
259 and branched DNA (bDNA) amplification for quantification, are used to study mRNA distribution
260 throughout the body. Luciferase mRNA is a useful reporter for examining biodistribution and protein
261 expression. However, as regulatory authorities note, its short half-life and lack of modifications mean it
262 may not accurately reflect the longer and more sustained protein production typical of modified
263 mRNAs. ([EMA/H/C/005735/RR, 2020](#)) Therefore, conclusions based solely on luciferase mRNA-
264 LNPs may underestimate the actual performance of the modRNA product. These points highlight the
265 complexity of evaluating the biodistribution of modRNA-LNP therapies and emphasize the im-
266 portance of a layered, comprehensive approach. ([Vervaeke et al., 2022](#))

267

268 No biodistribution studies using the actual modRNA from the Pfizer/BioNTech or Moderna vaccine
269 were included in the regulatory documents. As a result, there was no assessment of transfection effi-
270 ciency or gene expression levels. Further clarification from regulatory authorities and manufacturers is
271 needed to determine the necessary chemical, pharmacological, and toxicological studies for these lipids
272 to obtain approval. ([Hemmrich & McNeil, 2023](#))

273

274 *Ci et al.* ([Ci et al., 2023](#)) performed one of the few LNP-modRNA biodistribution studies, where the
275 methodology showed strong differentiation of the sequential process of LNP activity based on current
276 technical capabilities. Quantification of the ionizable lipid and its metabolites was accomplished using

277

278 LC-MS/MS. ModRNA quantification employed bDNA, and detection of the non-translating Factor IX
279 (NTFIX), a model protein, was analyzed using LC-MS/MS. This multi-faceted analytical approach, per-
280 formed in mice, allowed for a clear distinction between LNP distribution, modRNA delivery, and
281 downstream protein production. The authors demonstrated both LNP distribution and subsequent
282 protein expression across a wide range of tissues. Protein production was quickly detected in the liver,
283 ovary, and thymus, followed by the uterus and kidneys. As expected, the liver produced the most pro-
284 tein overall, followed by the ovaries, kidneys, and lungs. Protein production persisted at low levels up to
285 168 hours in the lungs, heart, liver, gastrointestinal tract, kidneys, and uterus, but not in the ovaries;
286 however, no further measurements were obtained. Notably, protein expression was observed in the
287 heart despite little to no corresponding mRNA at later time points, emphasizing the importance of an-
288 alyzing both mRNA and protein production separately over time to understand the therapeutic effects.
289 These results may indicate that macrophages or dendritic cells traffic to the heart; however, generaliza-
290 bility to humans is unknown. The ionizable lipid and its metabolites were concentrated in the urinary
291 and digestive tracts, suggestive of hepatobiliary and urinary clearance. The ethanolamine portion of the
292 ionizable lipid, radiolabeled with ^{14}C , showed no metabolism in vivo, ([Burdette et al., 2023](#)) indicating
293 tissue persistence.

294

295 Luo *et al.* ([Luo et al., 2025](#)) recently introduced Single Cell Precision Nanocarrier Identification (SCP-
296 Nano), a novel imaging and deep learning pipeline for single-cell resolution mapping fluorescence-la-
297 belled carriers such as LNPs across whole mouse bodies at doses as low as 0.0005 mg/kg, which are
298 typical for modRNA vaccines and are 100-1000 times lower than conventional imaging methods, such
299 as QWBA. Using reporter mRNA (e.g. EGFR), the study demonstrated heterogeneous nanocarrier up-
300 take and protein expression both within and across organs, with hotspots in the liver and spleen. This
301 punctuated pattern indicated that some cells successfully translated the mRNA, while neighboring cells
302 exhibited uptake without expression. Intramuscular injection of LNPs with SARS-CoV-2 spike mo-
303 dRNA revealed low-level heart endothelial delivery, confirming possible molecular and proteomic
304 changes beyond primary targets. These results in rodent models may not directly apply to humans. Still,
305 this may have important implications for potential off-target effects that standard diagnostic methods,
306 such as ultrasound or CT scans, might miss. Cellular or molecular changes could contribute to symp-
307 toms or disease risk, but may not be visible until they become extensive enough to be detected by con-
308 ventional tools.

309

310 The route of administration also influences the biodistribution of LNP-modRNA therapy. For intra-
311 muscular administration, such as that for the modRNA COVID-19 vaccines, syringe pressure, perfu-
312 sion rate, proximity to blood vessels and lymphatic vessels, local pH, and temperature, among others,
313 are important considerations. ([Naasani, 2022](#)) In contrast, other LNP-nucleic acid therapies, such as the
314 siRNA product patisiran (Onpattro®), are administered through intravenous infusion, which achieves
315 liver targeting almost exclusively through ApoE binding and LDL receptor uptake. Subcutaneous and
316 intranasal routes favour lymph nodes and lungs, respectively. ([Mendonça et al., 2023](#)) These differences
317 demonstrate that the biodistribution of LNPs differs significantly based on the route of administration,
318 making them distinct from traditional small-molecule therapeutics.

319

320 2.3 Formation and Biological Role of the Protein Corona

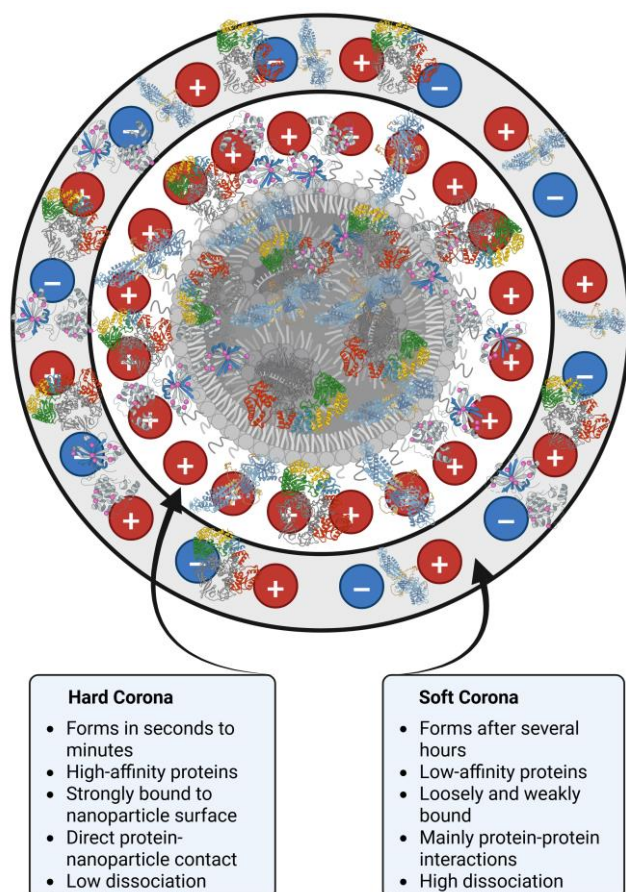
321

322 When LNPs encounter biological fluids, they are immediately transformed by their environment. They
323 acquire a dynamic and heterogeneous coating of biomolecules known as the protein corona. This layer
324 fundamentally changes how the body perceives and processes the LNPs, influencing biodistribution,
325 immune recognition, cellular uptake, and ultimately the efficiency of modRNA translation. Therefore,
326 the protein corona gives the LNPs a “biological identity”. ([Akhter et al., 2021](#))

327

328 The protein corona formation occurs within minutes through van der Waals forces, hydrophobic inter-
329 actions, electrostatic interactions, and other biochemical and biophysical interactions, resulting in an
330 individual, heterogeneous *in vivo* LNP pool. ([Cedervall et al., 2007](#)) For the modRNA-LNPs, this pro-
331 cess is accelerated because the PEG-lipids on the surface of the modRNA-LNPs dissociate and ex-
332 change with plasma proteins, a mechanism known as PEG-lipid shedding. ([Escalona-Rayó et al., 2024](#))
333 It is a dynamic, complex, and unpredictable process that is crucial for biodistribution, transfection, and
334 cellular responses, since this is what the cell itself “sees.” ([Walczyk et al., 2010](#))

335



Composition and Determinants of the Biocorona

Composition

- Lipoproteins, immunoglobulins, albumin, complement, etc
- Species-specific, impact on animal models for toxicity studies

Influencing Factors

- Physicochemical properties (size, shape PEG-lipid density, shedding rate)
- Environmental factors; temperature, pH, incubation time, biological fluid (plasma, lymph), age, gender, comorbidities

Human biocorona characterization

- Not fully characterized
- LNP size and density resemble natural serum lipoproteins
- Voke *et al* found consistent proteins, i.e. ApoE, C-reactive protein, alpha-2 macroglobulin, vitronectin

Dynamic Remodelling

- Biocorona changes as the LNP moves through biological fluids, hard and soft corona
- Complicates LNP behaviour prediction

338 **The biocorona can alter the internal structure of the LNPs.**

339

340 The protein corona can potentially mask targeting ligands or alter interactions with cell membranes.
341 This can reduce the efficacy of targeted delivery by shielding functional moieties or, in some cases, en-
342 hance functionality by presenting a new protein-based signal. ([Voke et al., 2025](#))

343

344 One of the most critical aspects of the protein corona was demonstrated by Sebastiani *et al.* ([Sebastiani](#)
345 [et al., 2021](#)) When ApoE binds to the protein corona of LNPs, the entire biodistribution pattern of the
346 original formulation is altered by internal structural changes, potentially affecting modRNA
347 encapsulation, agglomeration and premature RNA release. Accordingly, the entire surface structure
348 changes, facilitating the opsonization of phagocytes, such as macrophages and dendritic cells. Further
349 work also emphasized the importance of the protein corona for not only biodistribution but also
350 transfection efficiency and translation yield. ([da Costa Marques et al., 2023](#); [Huang et al., 2023](#); [K. Liu et](#)
351 [al., 2023](#); [Sengottayan et al., 2023](#))

352

353 **The immunological effects of the biocorona in plasma**

354

355 The accelerated blood clearance (ABC) phenomenon, often triggered by repeated administration of
356 PEGylated LNPs, results from the production of anti-PEG antibodies. These antibodies can quickly
357 clear subsequent doses of PEGylated LNPs from the bloodstream through accelerated blood clearance,
358 reducing therapeutic effectiveness but also potentially increasing the risk of adverse reactions due to the
359 rapid and unpredictable distribution of the nanoparticles. ([Wang et al., 2024](#)) PEGylated nanoparticles
360 are known to interact with circulating complement proteins, activating the complement cascade and
361 producing opsonins and anaphylatoxins, which are associated with acute infusion reactions in patients,
362 known as complement activation-related pseudoallergy (CARPA). ([Szebeni et al., 2018](#)) Anaphylactic
363 and allergic reactions observed after modRNA COVID-19 vaccination may partly reflect this phenom-
364 enon. ([Bakos et al., 2024](#))

365

366 **Implications and Challenges**

367

368 Since processing and administration into a living organism involve many interfering factors, it seems
369 plausible that the biocorona causes a nonlinear distribution route depending on the formulation of the
370 LNPs, the biological environment, and the route of administration. Overall, these data challenge the
371 idea of a uniform LNP formulation and predictable biodistribution. One might assume that if these
372 factors heavily influence biodistribution, administering the same dose to two subjects is unlikely to
373 result in similar responses. Recent approaches to addressing the inherent issues with the biocorona of
374 LNPs have utilized liposomal LNP-modRNA nanoparticles, which exhibit extra-hepatic targeting and
375 longer circulation lifetimes, likely due to the formation of fewer proteins in the protein corona. ([M. H.
376 Y. Cheng et al., 2025](#)) This represents a return to the original nanosized lipid particles, liposomes.

377

378 **2.4 Target Sites and Tissues**

379

380 What are the main sites and tissues targeted by the LNPs? The primary target sites for the LNPs
381 include the liver, spleen, and draining lymph nodes, as these organs comprise a significant portion of
382 the Reticuloendothelial System (RES), a component of the immune system that involves phagocytes,
383 such as macrophages and monocytes. These cells are primarily located on the vascular wall of the liver
384 (Kupffer cells), spleen (splenic macrophages), kidneys (mesangial cells), and lungs (lung
385 macrophages). ([Ngo et al., 2022](#)) Given the dynamic nature of the biocorona and the common presence
386 of ApoE in it, it is not surprising that hepatocytes in the liver are the primary target for the
387 LNPs. ([Hosseini-Kharat M, 2025](#)) Additionally, because the liver functions as a biological filter system,
388 LNPs that are up to 200 nm in size tend to undergo fenestration unless specifically engineered
389 otherwise, which helps their uptake into liver sinusoidal endothelial cells (LSECs). ([He et al., 2024](#)) Since
390 LNPs first enter through the sinusoidal lumen, Kupffer cells are also the initial targets for
391 transfection. ([Hosseini-Kharat M, 2025](#))

392

393 Similarly, LNPs tend to distribute to the spleen due to its sinusoidal endothelium, which facilitates LNP
394 uptake. Depending on the LNP formulation and composition, macrophages and dendritic cells can be
395 targeted, which is essential for the efficacy of modRNA-LNP vaccines.([Haghighi et al., 2024](#)) Based on
396 the proportions, shape, charge, and other factors of the LNP lipid, gene expression or protein
397 production can sometimes exceed levels in the liver.([Hald Albertsen et al., 2022](#))

398

399 Draining lymph nodes are a common target for LNPs. The size of their fenestrations (<200nm)
400 allows the LNPs to migrate through lymphatic channels and be taken up by antigen-presenting cells
401 (APCs).(Hassett et al., 2024) Various modifications, especially surface engineering of the LNPs and
402 other adjustments, improve targeting and retention. Depending on their size and other factors, LNPs
403 can also drain directly into lymph nodes. Additionally, larger particles are transported by APCs (mainly
404 dendritic cells) to different locations, such as the heart, which has been suggested to explain immune
405 reactivity and responses.(Milano et al., 2021)

406

407 In addition, other organs may exhibit detectable LNP presence in preclinical studies, sometimes
408 referred to as “off-target effects,” which are caused by the physicochemical properties of the LNPs and
409 the resulting protein corona. The heart, lungs, adrenal glands, and ovaries are frequently reported in
410 studies involving rodents and non-human primates (NHP).(TherapeuticGoodsAdministration, 2021)

411

412 Transcytosis or direct penetration can occur, allowing LNPs to bypass blood-organ barriers. This is
413 important because LNPs can leave the vasculature and cross the blood-brain barrier ([Khare et al., 2023](#))
414 or the intestinal barrier.([Neves et al., 2016](#)) Zhang *et al.* ([Zhang et al., 2024](#)) in a comprehensive review
415 list various target cells, such as epithelial, basal, and endothelial cells, and explain how these are
416 particularly likely to be targeted. Other notable examples include cardiac and skeletal muscle, bone
417 marrow-derived dendritic cells and macrophages, as well as various cell types and tissues. ([J. Chen et al.,](#)
418 [2023](#); [Dey et al., 2021](#); [Han et al., 2021](#); [Khare et al., 2023](#); [Swingle et al., 2023](#); [Younis et al., 2023](#); [Zak](#)
419 [et al., 2023](#))

420

421 Regulatory Gap

422

423 In official FDA and EMA documents, the “target cells” believed to be transfected by the LNPs are not
424 specified. Notably, the US FDA mentions transfection([USFDA, 2020](#)) Conversely, the EMA states that
425 the viral protein antigen is expressed in the desired conformation([EuopeanMedicinesAgency, 2021](#)) It is
426 unclear whether both agencies refer to the same process or if the EMA distinguishes between
427 transfection and protein expression, as previously discussed. This lack of clear communication and
428 precise data presentation regarding the modRNA-LNP target cells and delivery, combined with support
429 from public health agencies, ([CentresforDiseaseControlandPrevention, 2022](#)) has contributed to the
430 common belief that the vaccine is limited to the deltoid muscle. These misconceptions, have led to a
431 limited understanding of the vaccine’s potential for broader use and distribution, leaving safety profiling
432 incomplete.

433

434 2. 5 Cellular Uptake Mechanism

435

436 As we have seen, the adsorption of biomolecules onto the LNP surface establishes a dynamic
437 biocorona overriding the synthetic nanoparticle design. This identity governs cellular interactions by
438 dictating which membrane receptors are engaged, leading not only to biodistribution patterns but also
439 to endocytic pathways and, consequently, the intracellular fate of the encapsulated modRNA. This
440 membrane uptake into cells is termed endocytosis. The efficiency of uptake is profoundly affected by
441 the biocorona, particle size, shape, and net surface charge. ([Hald Albertsen et al., 2022](#))

442

443 Transfection occurs when the LNPs are endocytosed and the modRNA subsequently escapes the
444 endosome into the cytosol (**Figure 5**).

445

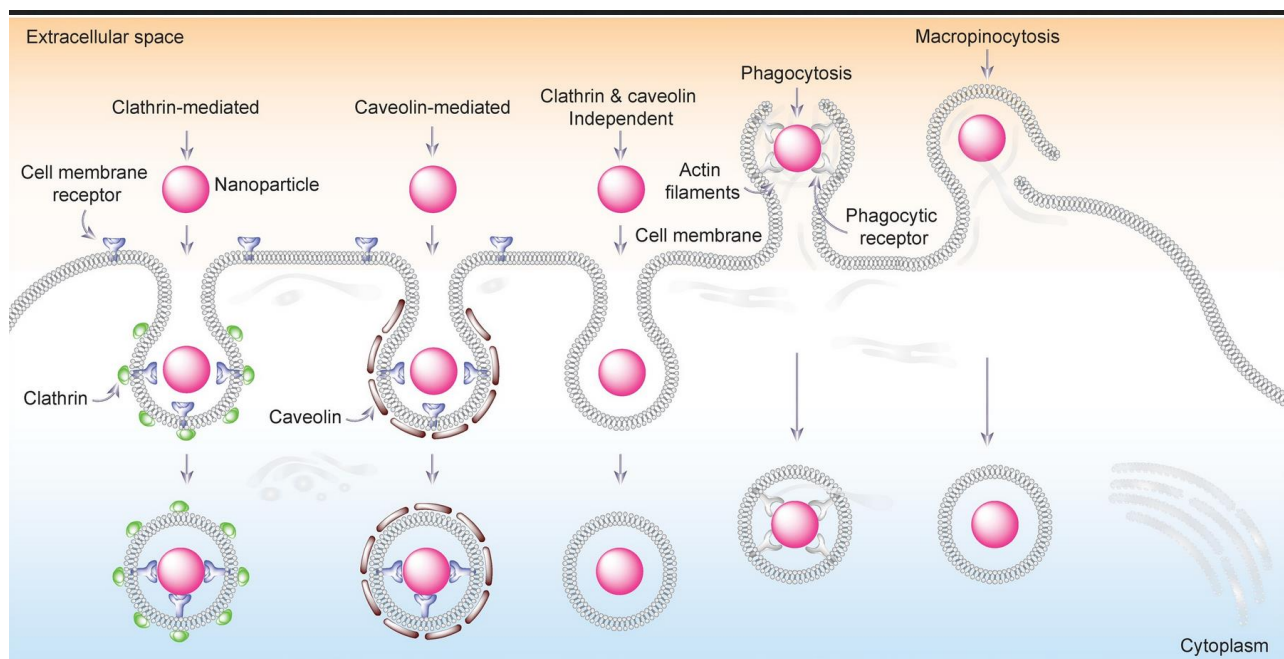


Figure 5. Schematic representation of nanoparticle cellular internalization pathways, including clathrin-mediated, caveolin-mediated, clathrin- and caveolin-independent, phagocytosis, and macropinocytosis. Adapted from Augustine R, Hasan A, Primavera R, et al. *Materials Today Communications* (2020) 25:101692. <https://doi.org/10.1016/j.mtcomm.2020.101692>. Licensed under CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

Mechanisms of Uptake

There is little focus on how LNPs penetrate the cell membrane, or which receptors and ligands are most likely to interact with LNPs for uptake into cells or endocytosis. In 2008, Loney et al ([Loney et al., 2008](#)) stated that it was unclear whether receptor-dependent or receptor-independent “endocytosis-like” uptake of liposomes into cells was involved. A reassessment by the same authors in 2012 ([Loney et al., 2012](#)) noted that the exact nature of the endocytic vesicles involved in endocytosis or “endocytosis-like” uptake of LNPs was still “a matter of debate.” Whether a receptor- or receptor-independent “endocytosis-like” process occurs strongly depends on the protein corona and the state of the cell encountered by the LNP and the local microenvironment ([Behzadi et al., 2017](#)) (pH, bradykinin, prostaglandins, etc). Paunovska et al. ([Paunovska et al., 2022](#)) reported that LNPs can bind to apolipoprotein E and low-density lipoprotein receptors (LDL-R), whereas Chaudhary et al ([Chaudhary et al., 2024](#))

467 reported that Toll-like receptor (TLR)4 and CD1d can be internalized with the endosome. Both
468 receptor-mediated and receptor-independent cellular uptake([Akhter et al., 2021](#)) likely occur
469 simultaneously within the same cell. Uptake may also occur under specific conditions without direct
470 binding to membrane components; instead, nonspecific hydrophobic or electrostatic interactions
471 ultimately initiate the process. (see **Table 2**)

472

473 **Measuring how cell receptors bind is challenging.**

474

475 The current methods used to study the mechanisms by which LNPs interact with the cell membrane
476 often disrupt the natural protein corona composition, making it challenging to identify which cell
477 receptors recognize and bind to LNPs accurately. Identification of the corona proteins is not sufficient
478 because not every protein in the corona can interact with cell receptors, as they may require correct
479 orientation on the nanoparticle surface. Therefore, identifying which epitopes on the biomolecular
480 corona are accessible to cell receptors is essential for determining potential interactions. Likewise, not
481 all exposed proteins can necessarily bind to receptors, especially if there is competition with other
482 proteins with higher affinity for the same receptors. It is, therefore, important to identify which
483 proteins genuinely participate in these interactions. ([Aliyandi et al., 2020](#))

484

485 Lipid–membrane interactions can also influence cell membrane receptor activity and thereby contribute
486 to the uptake of lipid nanoparticles (LNPs). As summarized by Lavington & Watts, ([Lavington & Watts,
487 2020](#)) nanodisc and SMA lipid nanoparticle (SMALP) studies demonstrated that specific lipid compo-
488 nents (such as helper lipids) modulate the surrounding membrane environment without directly binding
489 to G-protein coupled receptors (GPCR). Such lipid-induced alterations affect GPCR conformation, lig-
490 and binding, and signal transduction, supporting functional receptor interactions. The elements of the
491 protein corona, uptake pathways and primary tissues affected are reviewed in **Table 2**.

492

Biocorona Components	Main Receptors Engaged	Dominant Uptake Pathways	Cell Types Most Affected	Comments/Impact on transfection
Albumin	gp60 (albondin), SPARC, FcRn(Ji et al., 2024)	Caveolae-Mediated Endocytosis(Lonez et al., 2012 ; L. Ren et al., 2025) Trancytosis	Hepatocytes Endothelial cells Epithelial cells Tumor cells	Can bypass lysosomes, improved cytosol delivery, recycling endosomes Preferred mechanism for modRNA-LNPs
Apolipoproteins (ApoE, ApoB, ApoA-1)	LDL-R LRP-1 SR-B1	Clathrin-Mediated (Sebastiani et al., 2021 ; Zhang et al., 2024 , Borah, 2025 #1697) or Caveolae Mediated Endocytosis	Hepatocytes, Spleen, macrophages Tissues with LDL-R include adrenals, ovaries, testes Neurons(Martins et al., 2024)	Classic receptor-mediated LNP uptake route with ApoE May lead to lysosomal degradation if clathrin-mediated
Vitronectin/Fibronectin	Integrins ($\alpha v\beta 3$, $\alpha 5\beta 1$)	Clathrin or Caveolae-mediated (lipid rafts) (Sousa de Almeida et al., 2021),(Lavington & Watts, 2020)	Endothelial cells Fibroblasts Epithelial cells Tumor and parenchymal cells Heart in murine models (Luo et al., 2025)	Off-target effects Affected by nanoparticle shape; size, etc.
Alpha-2 macroglobulin	LRP1(Yamamoto et al., 2024)	Primarily clathrin-mediated	Hepatocytes, endothelial cells	Traps LNPs for lysosomal degradation(Tomihari et al., 2023) Reduces efficacy
*C-Reactive Protein	FcγR, C1q	Phagocytosis, complement activation	Macrophages, neutrophils	Complement activation, CARPA Reduces transfection efficiency

*Immuno-globulins(IgG, IgM), Anti-PEG antibodies	FcγR, FcαR CSF2RB (new finding)(Baimanov et al., 2025)	Phagocytosis (Sousa de Almeida et al., 2021); (Baimanov et al., 2025) Also clathrin-mediated	Uptake by APC when LNPs are opsonized Spleen, macrophages	Leads to lysosomal degradation Triggers immune response (ABC) CSF2RB potential role for CARPA
*Complement proteins (C3b, C4b etc)	Complement receptors	Phagocytosis Macropinocytosis (Borah et al., 2025 ; Miao et al., 2020 ; L. Ren et al., 2025)	Macrophages Neutrophils Dendritic cells	Strongly degradative; opsonization
Direct	TLR4/CD14	TLR4 is internalized along with the forming endosome (promotes lipid-raft formation), (Chaudhary et al., 2024); (Korzun et al., 2023); (Paunovska et al., 2022)	Dendritic cells, macrophages	Initiates cell signaling and immune activation Leads to lysosomal degradation Receptor recycling
Direct	None	Direct Membrane Penetration GPCR interactions (lipid rafts) (Sakurai et al., 2022); (Lavington & Watts, 2020)	Driven by pH and lipid destabilization (small size, specific surface chemistry or external physical forces (e.g., electroporation))	Bypasses endosomal uptake

493 **Table 2 Biocorona, Receptors and Mechanisms of Uptake**

494 αvβ3=integrin alpha-v beta-3; α5β1=alpha-5 beta-1; ABC = accelerated blood clearance; APC = anti-
495 gen-presenting cells; ApoE = apolipoprotein E. C1q=complement C1q component; CSF2RB=colony
496 stimulating factor 2 receptor beta; CR3b=complement receptor 3; FcγR= Fc gamma receptor;
497 FcαR=Fc alpha receptor; GPCR=G-protein-coupled receptor; LDL-R=low-density lipoprotein recep-
498 tor; LRP-1=low-density lipoprotein receptor protein-1; PEG=polyethylene glycol; SR-B1=scavenger
499 receptor class B Type 1; TLR4=toll-like receptor 4.

500 *Opsonins (e.g. CRP, IgGs, complement) act in the vasculature, whereas integrins and others, mediate
501 uptake at the cell membrane.

502

503 The main challenge isn't whether transfection occurred, but how much happens and how conditions in
504 systems biology influence this process. According to current knowledge, organ fenestrations and the
505 pKa value mainly determine biodistribution and cellular uptake. The ζ-potential primarily affects

506 protein corona formation and the likelihood of its formation. ([Patel et al., 2021](#)),([Cedervall et al., 2007](#))
507 Given the numerous mechanisms, cell receptors, and a wide range of cell types, along with cells at
508 different stages of maturation and division within the same lineage, it is not surprising that efforts to
509 systematically target receptor-driven signalling pathways within a highly complex biological system are
510 inherently problematic.

511

512 Interestingly, Zelkoski *et al*([Zelkoski et al., 2025](#)) demonstrated in THP-1 cells that ionizable LNPs can
513 activate both TLR4 signalling pathways, the TIRAP/MyD88-NF κ B pathway and the
514 T2025RAM/TRIF-IRF pathway, albeit with differences in magnitude and kinetics: NF- κ B signalling
515 was rapid and robust, while IRF activation was weaker and delayed. This observation supports the
516 concept that ionizable LNPs, by altering lipid raft dynamics, can induce overlapping but temporally
517 shifted TLR4 signaling responses, diverging from the canonical temporal segregation of these
518 pathways([Kim et al., 2023](#))(**Table 2**).

519

520 **2.6 Endosomal Escape as Key Bottleneck**

521

522 Transfection, as previously discussed, occurs in a receptor-dependent and/or receptor-independent

523

manner, indicating a bioactive behaviour that extends beyond the traditional pharmacokinetic approach. Transfection is completed when the modRNA escapes the endosome. Assessing how LNPs are metabolized from a traditional pharmacokinetic perspective is challenging because they are not degraded through organ uptake during cell transfection. Instead, endosomal escape and degradation define the entire spectrum of pharmacodynamics. ([Ait-Oudhia et al., 2014](#); [He et al., 2019](#)) The classic absorption, distribution, metabolism, and excretion (ADME) pharmacokinetic model does not apply to liposomal or nanoparticle delivery systems.

531

532 **The Endosomal Escape Mechanism is Based on Biophysical and Chemical Processes**

533

Endosomes consist of a lipid bilayer similar to the cell membrane, which prevents nucleic acid escape as an evolutionary defense against foreign viral RNA entering the cell. For LNPs carrying modRNA, successful endosomal escape is essential for therapeutic action. After endocytosis, the endosomes increase the acid gradient, which protonates the ionizable lipids within the LNPs. For example, ALC-0315 has an apparent pKa value of ~6.09, and SM-102 has a value of ~6.6. This protonation event triggers the rearrangement of lipid molecules into a lamellar phase within the endosomes, promoting membrane destabilization and releasing the payload into the cytosol, a process known as the proton-driven osmotic swelling or the proton sponge effect. ([Fell et al., 2025](#)) ([Chatterjee et al., 2024](#)) As the pressure rises, the membrane destabilizes and may rupture, releasing its contents into the cytosol. Endosomal damage, as indicated by galectin recruitment, can occur solely from the presence of ionizable lipids and does not require cytosolic delivery of the RNA molecule. ([Johansson et al., 2025](#)) Lipid geometry facilitates this process. The conical shape of the branched, unsaturated fatty acid chains promotes negative curvature stress within the membrane, increasing destabilization ([Petersen et al., 2024](#)) Computational free energy calculations have shown that both ALC-0315 and SM-102 insert into the cell membrane favourably, ([Ermilova & Swenson, 2023](#)) suggesting that ionizable lipids in the current LNP-modRNA vaccines embed into the lipid bilayer. Even transient tearing may contribute to escape. Such tearing has been demonstrated with other nanoparticles. ([Er-Rafik et al., 2022](#)). Most recently, LNPs were found tethered to the endosomal membrane and associated with membrane destabilization. ([Johansson et al., 2025](#)) Finally, Pilkington et al ([Pilkington et al., 2021](#)) suggest that LNPs may perturb lipid raft organization, implying that endosomal escape involves not only endocytosis but also broader effects on membrane dynamics. **Figure 5** shows the typical intracellular journey of a modRNA-LNP.

556

Figure 6: Endosomal Escape

a The modRNA is introduced into the early endosome after being taken up via clathrin-mediated endocytosis or LDLR as example internalization, which is governed by the biocorona and lipid raft interactions. b The early endosome and protonation of the ionisable lipids. c The disruption of the early endosome and the release of modRNA, impurities, and modRNA-lipid adducts. d Meanwhile, a portion of engulfed LNPs are recycled back into the extracellular space as EVs or exosomes. e Another fraction progresses into late endosomes and eventually into lysosomes, where they are degraded. f Endosomal maturation from early to late stages determines the fate of the cargo: either delivery to the lysosome (e) or secretion via exosomes, unless the endosome is disrupted (f). **Created in BioRender. Seger, F. (2025)**

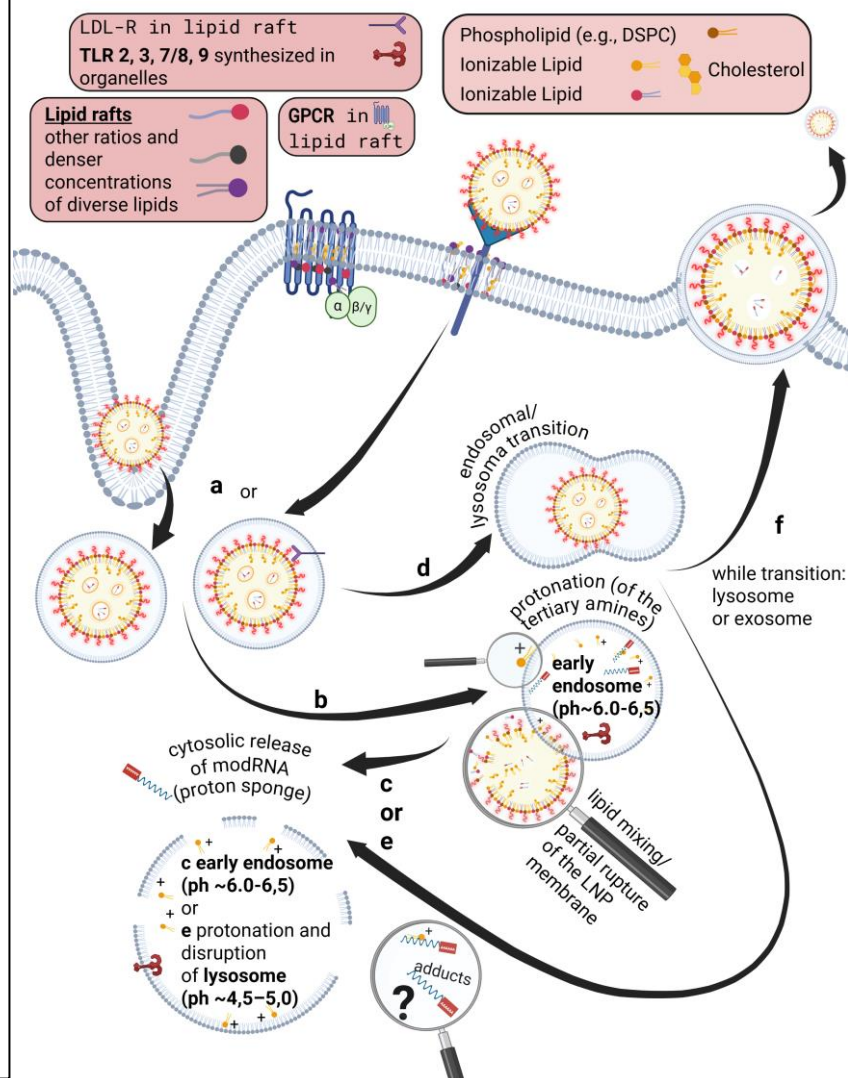


Figure 6 <https://BioRender.com/0921vhh>

560 Endosomal Escape is Inefficient

561

562 Only within a narrow window of opportunity do conditions allow LNPs to escape through endosomal
563 fusion during the endosomal maturation process.([Chatterjee et al., 2024](#)) ([Müller et al., 2024](#)) ([Hald](#)
564 [Albertsen et al., 2022](#))

565

566 This window is brief, lasting about 5-15 minutes([Schlich et al., 2021](#)) when conditions in the endosome
567 enable the LNPs to fuse with the endosomal membrane and deliver their cargo into the cytosol.
568 Beyond this period, escape efficiency drops significantly.

569

570 This process is highly inefficient, with only about 1-15% of all internalized LNPs resulting in the
571 production of the target protein.([Sabnis et al., 2018](#)) ([Aliakbarinodehi et al., 2024](#); [Chatterjee et al.,](#)
572 [2024](#))([Müller et al., 2024](#)) LNPs that do not escape the endosome at this stage are degraded or
573 exocytosed.([Maugeri et al., 2019](#)) Degradation through lysosomal fusion enriches the endosome with
574 degradative contents and enzymes, moving endosomes toward the plasma membrane and enabling
575 fusion for exocytosis. Most LNPs follow these pathways and fail to deliver mRNA to the cytosol, since
576 endosomal escape is the main “bottleneck” of mRNA therapeutics.([Chatterjee et al., 2024](#)). Over the
577 past four decades, numerous methods have been attempted to improve delivery. However, significant
578 improvements in endosomal escape often come at the cost of increased cytotoxicity, such as
579 endosomal bursting and release of entire contents into the cytosol.([Dowdy et al., 2022](#))

580

581 Failure to Escape the Endosomes Results in Cellular Stress

582

583 After endocytosis, if the modRNA is not released into the cytoplasm, the endosomes mature into late
584 endosomes and then fuse with lysosomes.([Chatterjee et al., 2024](#)) Lysosomes contain various enzymes
585 such as lipases, proteases, nucleases, and glycosidases that dismantle both the modRNA and lipids. An

586

587 accumulation of undegraded materials from the LNPs can trigger cellular stress, oxidative stress, and
588 potential inflammatory signalling. This accumulation has been compared to aspects of lysosomal
589 storage disorders, ([Paramasivam et al., 2021](#)) though a direct link to human disease has not been
590 established.

591

592 Lysosomal retention blocks expected degradation and recycling processes in the cell, including
593 receptor recycling such as LDL-R. This can create a cellular “traffic jam” that impairs the uptake of
594 new ligands and receptors. ([Y. Cheng et al., 2025](#)) Although the lipids comprising the LNPs are
595 considered biodegradable, high local concentrations can impair lysosomal function, slow degradation,
596 and prolong the retention of the disassembled lipids. ([Sahay et al., 2013](#)) Consequently, a blockade or
597 arrest of normal endosomal maturation and acidification not only reduces therapeutic efficacy but can
598 also lead to toxicological effects. ([Paramasivam et al., 2021](#))

599

600 **LNPs May be Expelled Intact or Partially Degraded in Exosomes**

601

602 Not all LNPs successfully escape the endosomes or are degraded in lysosomes. A significant portion is
603 recycled back into the extracellular space, repackaged in extracellular vesicles (EVs) or exosomes. This
604 pathway enables cells to eliminate undigested LNPs or those that fail to escape the
605 endosomal/lysosomal pathway. Maugeri ([Maugeri et al., 2019](#)) showed that LNPs in recycling
606 endosomes are expelled either intact or partially degraded, which affects transfection efficiency.
607 Exocytosis serves as both a clearance route and a secondary distribution mechanism; vesicle-mediated
608 transport may transfer the modRNA or lipid fragments to the surrounding microenvironment in a
609 paracrine manner. ([Sahin et al., 2014](#))

610

611 These EVs can also transfect cells, influencing pharmacodynamic outcomes and contributing to
612 variability and off-target effects. In fact, natural exosomes are being engineered for RNA delivery
613 ([Iqbal et al., 2024](#)) ([Bost et al., 2021](#)) because they can cross physiological barriers effectively, have
614 improved biocompatibility, low toxicity, cell-specific tropism, and can evade the mononuclear
615 phagocytic system. ([Wu et al., 2021](#)) This recycling of endosomes, as well as empty LNPs or those with
616 blebs, may cause cellular stress, oxidative damage, and chronic inflammation, ([Y. Cheng et al., 2025](#))
617 which could be linked to adverse effects such as injection-site reactions or immune activation. These
618 factors are not considered in biodistribution studies and may contribute to cumulative toxicity,
619 especially with repeated doses. Long-term studies are needed to determine if these adverse events are
620 causally related, as current regulatory focus is on immediate effects and may overlook these delayed
621 responses. Endosomal escape of siRNA-loaded LNPs, such as those for Onpattro, is minimal, typically
622 around 1%, ([Akinc et al., 2019](#); [Dowdy, 2023](#)) which restricts cytosolic delivery and helps minimize
623 cytotoxicity. This low efficiency means that only a small subset of internalized siRNA particles reaches
624 the cytosol. The escape events themselves tend to produce small, transient membrane disruptions that
625 are readily repaired by the cell. ([Bates et al., 2025](#); [Johansson et al., 2025](#)) As a result, siRNA-mediated
626 delivery elicits slower and weaker cytotoxic effects compared to delivery systems that induce more
627 extensive endosomal damage.

628

Endosomal Escape Key Barriers and Open Questions

Endosomal escape is the critical bottleneck for modRNA-LNP therapeutics. Only 1-15% of internalized particles successfully release mRNA into the cytosol

Main Barriers

- **pH gradient.** Protonation of ionizable lipids destabilized the endosomal membrane, but the window is narrow (5-15 min)
- **Lipid geometry.** Branched or conical tails of the ionizable lipid promote curvature stress, but also raises toxicity
- **Particle size and number per cell;** too few results in low transfection, too many may lead to lysosomal stress and degradation
- **Cell type:** Hepatocytes and dendritic cells favour endosomal escape, quiescent or specialized cells like neurons or fibroblasts are less permissive

Unresolved Questions

- Is protein production driven by a few highly productive escape events, or many inefficient
- How do free ionizable lipids behave once released? (pKa shifts, ROS generation, immune activation, reactive aldehydes)?
- What happens to the modRNA immediately after escape (the ‘dark hour of transfection’)? before translation begins?
- Do failed events contribute to chronic inflammation or lipid accumulation with repeated dosing
- How much variability is stochastic (intrinsic) vs cell type dependent and thus controllable?

Implication: Escape is both inefficient, unpredictable and context-dependent, leading to high variability in transfection and protein expression. Strategies to promote endosomal escape often increase cytotoxicity, resulting in the need for a better mechanistic understanding and safer lipid design

631 **Single cell Analysis: a pharmacokinetic perspective**

632

633 The pharmacokinetics of LNP delivery and protein expression are a complex, multi-step stochastic pro-
634 cess involving uptake, endosomal processing, and mRNA escape. Using single-cell analysis, Müller *et al.*
635 ([Müller et al., 2024](#)) found that cellular uptake was variable and ranged from minutes to hours depend-
636 ing on LNP shape, composition, and cell type. Endosomal escape varied among individual cells and
637 was inversely related to protein production; faster release and translation of RNA led to increased pro-
638 tein output. A theoretical “area under the curve” (AUC), used to describe overall pharmaceutical pro-
639 tein availability, was found to depend equally on four factors: the number of mRNA molecules deliv-
640 ered, the translation rate, the mRNA lifetime, and the protein lifetime. Moreover, Müller *et al.* noted
641 that little is known about the fate of nucleic acids after they escape from the endosome. Before any
642 measurable action, such as protein expression occurs, there is what Müller calls “the dark hour of trans-
643 fection,” the intracellular biochemical and physical processes that occurs following endosomal escape
644 but before protein synthesis. What happens during this period remains unclear, which limits a full un-
645 derstanding. Additionally, the amount of modRNA released into the cytosol does not reliably predict
646 the level of protein expression, previously noted by Liu *et al.* ([Liu et al., 2024](#))

647

648 **2.7 Lipid Degradation and Metabolite Persistence**

649

650 Once the modRNA is released, the fate of the lipid components determines the final pharmacodynamic
651 stage of LNP activity. This aspect, concerning the fate of the individual lipids after they deliver their
652 payload, is rarely discussed or addressed. The LNPs do not simply vanish; instead, they are disassem-
653 bled *in vitro*, metabolized, and cleared at different rates depending on the lipid chemistry. For example,
654 cholesterol may form oxysterols with immune effects, while DSPC can accumulate in organs, poten-
655 tially altering membrane fluidity.

656

657 Both cholesterol and DSPC are natural lipids, but they are manufactured synthetically. Clearance path-
658 ways remain poorly characterized, necessitating further study. These are further delineated in **Table 4**.

659

660 **PEGylated Lipids**

661

662 The pegylated lipid plays a key role in the lipid matrix of the LNP, despite its small molar ratio, because
663 it extends outward on its surface, which is necessary for LNP stability during formulation and stor-
664 age.[\(Zhang et al., 2025\)](#) This also allows for increased *in vivo* circulation time since the PEG lipid im-
665 pedes cellular uptake and endosomal escape, but this then creates the so-called “PEG dilemma.” As a
666 result, PEG-lipids with shorter C-14 acyl chains were used in the LNPs, which gradually diffused out of
667 the particles and provided temporary stealth properties, achieving higher transfection efficiency than
668 longer, more persistent PEG-lipids.[\(Mukai et al., 2022\)](#) Once the PEG-lipid is sloughed off, it is metab-
669 olized by the liver and kidneys, where the lipid component undergoes enzymatic hydrolysis and β -oxi-
670 dation which is standard processes for lipids. The pegylated part, being a polymer of ethylene glycol, is
671 either excreted in urine or broken down into smaller oligomers. Although PEG-lipids are designed to
672 quickly detach from the LNP surface once in circulation or shortly after uptake, they can remain associ-
673 ated. Then they can be internalized with the particle and undergo endosomal trafficking to lysosomes,
674 where the lipid portion is degraded and the PEG chains are either excreted or slowly metabolized.[\(Mui](#)
675 [et al., 2013\)](#)

676

677 **Ionizable Lipid**

678

679 No clinical data exist for ALC-0315 and SM-102 regarding their retention and duration of activity in
680 humans. Although they are labeled as “biodegradable” after their ester bonds are hydrolyzed within tis-
681 sues and release their fatty acid tails, their overall ability to degrade doesn't truly improve, since com-
682 mon degradation pathways like β -oxidation are not consistently used. [\(Jørgensen et al., 2023\)](#)

683

684 Due to their sterically hindered ester structure, they are slowly hydrolyzed over several days. Jørgensen
685 *et al.* highlight that these lipids usually have stable structures and multiple tertiary amines, which slow
686 down their degradation and may cause toxicity. ([Jørgensen et al., 2023](#)) When ALC-0315 undergoes es-
687 ter cleavage, it forms a doubly de-esterified metabolite that remains cationic and can reach metabolic
688 sites such as mitochondrial membranes more quickly than longer lipids, ([Eygeris et al., 2022](#); [Jørgensen](#)
689 [et al., 2023](#)) possibly leading to ROS production, cytokine release and membrane disruption. As a result,
690 the persistence of these shorter-chain lipids could lead to ongoing toxicity after exposure, ([Hou et al.,](#)
691 [2021](#); [Inácio Â et al., 2011](#)) but data in humans is sparse. Therefore, there is an urgent need to develop
692 new combinatorial reactions that can generate degradable ionizable lipids for potent RNA delivery.
693 ([Han et al., 2021](#))

694

695 **Lipid Adducts**

696

697 An underrecognized risk for LNPs is the potential for lipid adduct formation, which occurs in storage.
698 The head groups of tertiary amine-based lipids can form N-oxides and, consequently, fatty aldehyde
699 impurities due to the thermodynamic instability of the LNPs and the oxidative impurities generated
700 during the complex processing of the ionizable lipid. ([Birdsall et al., 2024](#); [Zhichang Yang, 2023](#)) These
701 aldehydes can react with modRNA nucleobases, especially adenine and cytidine, inside the LNP to
702 form covalent bonds (**Figure 6e**). Adduct levels increase with storage time and temperature, making
703 the modRNA untranslatable once injected. Moderna scientists ([Packer et al., 2021](#)) first reported adduct
704 formation in 2021, highlighting the lack of validated assays for detecting these adducts during manufac-
705 turing. Moderna also noted that the Tris buffer used in their product acts as an aldehyde sink, ([Moderna,](#)
706 [2022](#)) enabling more extended storage at 2–8°C and reducing adduct formation with the modRNA.
707 Notably, Pfizer switched from PBS to Tris buffer in October 2021, raising questions about the amount
708 and reactivity of adducts in their early batches (**Table 1**).

709

710 The damaged adducted modRNA, once taken up by the cell, may be perceived as abnormal or viral-like
711 by cellular sensors, which may trigger inflammatory signals or interferon responses. ([Cordes et al., 2025](#);
712 [Maelfait et al., 2020](#)) Post-transcriptional interference, including adduct-induced damage, is
713 hypothesized to contribute to systemic immune dysregulation, ribosomal stalling and collision with
714 trailing ribosomes, and exaggerated inflammatory responses ([Cordes et al., 2025](#)), especially in
715 vulnerable individuals. ([Acevedo-Whitehouse & Bruno, 2023](#); [Rigby & Rehwinkel, 2015](#)) Research on
716 secondary amines and reactive aldehydes (e.g., 4-HNE from lipid peroxidation) indicates they are
717 cytotoxic and may affect protein folding or function, leading to the formation of neoantigens that can
718 provoke undesired immune responses or contribute to oxidative stress and lysosomal dysfunction.
719 ([Bitounis et al., 2024](#); [Dalleau et al., 2013](#); [Fritz & Petersen, 2013](#)) However, direct *in vivo* evidence of
720 adduct formation after LNP uptake has not been confirmed. Moderna is actively exploring strategies to
721 reduce covalent bonds and RNA-LNP adducts, acknowledging their potential toxicity. ([Meredith Packer
722 et al., 2022](#)) Similarly, DNA-LNP adducts could form with residual DNA in the vaccines, potentially
723 triggering interferon production. ([Atianand & Fitzgerald, 2013](#)) It is unclear whether BioNTech
724 considers these phenomena. Alternative ionizable lipids with piperidine heads have been developed to
725 mitigate this risk and enhance thermal stability. ([Hashiba, Taguchi, Sakamoto, Otsu, Maeda, Ebe, et al.,
726 2024](#)) However, the risks of adduct formation anticipated by developers have not yet been
727 systematically evaluated in vaccine studies.

728

729 The recent EMA draft guideline for modRNA vaccines ([EuropeanMedicinesAgency, 2025](#)) emphasizes
730 the control of adduct formation in manufacturing but does not delineate the possible adverse effects.
731 Continuous pharmacovigilance and advanced *in vivo* assays are essential to clarify these uncertainties *in*
732 *vivo*, particularly for vulnerable groups. The lipid components, metabolic pathway, and knowledge gaps
733 are summarized in **Table 4**.

734

Lipid Component	Metabolic Pathway	Clearance	Persistence/Risks	Knowledge Gaps
Cholesterol	Sterol metabolism to HDL/LDL; possible oxidation to oxysterols	Likely recycled endogenously, but this has not been studied	Oxysterols are immunologically active; (Back et al., 2024) cholesterol crystals form depending on saturation, may contribute to CARPA (Anindita et al., 2024)	No direct oxysterol data available after LNP uptake,
DSPC (helper lipid)	Phospholipase degradation is incorporated into membranes. Displays unusual rigidity. (Li et al., 2015), favours bleb formation. (Simonsen, 2024 ; Zhang & Barz, 2025)	Days-weeks can accumulate in the liver, spleen, heart, kidney, lung (Quick et al., 2022)	DSPC can produce phospholipid-derived products that may alter membrane structure and stability (Jeschek et al., 2016); (Rezaei et al., 2025). Can affect lipid raft integrity and functions like increased T-cell signaling (Zech et al., 2009) May also lower immune host surveillance. (Sfera et al., 2022)	The effect on lipid rafts across tissues is not fully understood nor thoroughly examined with repeated dosing.
PEGylated lipids (ALC-0159, PEG-DMG) See text	Lipid moiety hydrolyzed, PEG excreted renally	Renal and hepatobiliary	PEG accumulation with repeated dosing; CARPA risk Vacuolations due to incomplete metabolism in lysosomes have been seen in animal studies (class effect) (Therapeutic Goods Administration, 2021) but not in humans (Obeng et al., 2025)	Human persistence and dose thresholds unclear; PEG allergy may limit LNP use for other indications (Song et al., 2025)
Ionizable lipids (ALC-0315, SM-102) See text	Hydrolysis to amines/fatty acids; branched tails resist β -oxidation (ALC-0315 >> SM-102)	Slow hepatic clearance; ALC-0315 takes up to 3 weeks to fully metabolize ($t_{1/2}$ =139 hrs) (EMA/707383/, 2020 Corr.1*1) SM-102 half-life shorter at 7.3h (Y. Ren et al., 2025)	Tissue persistence of metabolites, including in mitochondria. In silico experiments demonstrate membrane embedding, which may enhance persistence (Aliakbarinodehi et al., 2024 ; Ermilova & Swenson, 2023) ROS production, cytokine release, membrane disruption or tearing	Identity of metabolites; long-term accumulation not well studied

Lipid Adducts See text	Reactive amines/aldehydes covalently bind proteins and nucleic acids(Packer et al., 2021)	Clearance uncertain	Persistent adducts, potential neoantigen formation, oxidative damage are possible	No standardized <i>in vivo</i> assays, frequency, and their impact <i>in vivo</i> are unknown.
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735 **Table 4: Lipid Components, Metabolic Pathways and Knowledge Gaps** HDL=high density
736 lipoprotein; LDL=low density lipoprotein; PEG=pegylated lipid CARPA=complement activation
737 reaction pseudoallergy; ROS=reactive oxygen species

738

739 2.8 Drug Interactions

740

741 Although regulatory agencies generally assume vaccines do not cause drug–drug interactions, early evi-
742 dence suggests this may not hold for modRNA–LNP vaccines. Case reports and cohort analyses docu-
743 ment clinically relevant changes in clozapine pharmacokinetics post-vaccination, in some cases leading
744 to neutropenia and hospitalization ([Bayraktar et al., 2021](#); [Imai et al., 2022](#); [Thompson et al., 2021](#)). The
745 mechanism is consistent with inflammation-mediated suppression of CYP450 enzymes, particularly
746 CYP1A2 and CYP3A4, central to clozapine metabolism ([Eiermann et al., 1997](#)).

747

748 While most effects appear mild or transient ([Demler & O'Donnell, 2023](#)), therapeutic drug monitoring
749 has been recommended for narrow-index drugs like clozapine ([Veerman et al., 2022](#)). Substantial
750 increases in escitalopram, fluoxetine, trazodone, and quetiapine levels have also been reported ([Kuzin](#)
751 [et al., 2023](#)), and a case of neuroleptic malignant syndrome with adrenal insufficiency occurred in a
752 patient on valproic acid ([Mizuno et al., 2022](#)).

753

754 This concern extends beyond psychotropic or antiepileptic medications. Inflammatory cytokines such
755 as IL-6, TNF- α , and interferon- γ , induced by both infection and vaccination, down-regulate multiple
756 hepatic CYP isoenzymes ([Lim et al., 2023](#)). Clinical studies in COVID-19 patients have shown that
757 elevated C-reactive protein levels are associated with reduced metabolism of midazolam and tacrolimus,
758 potentially leading to oversedation or immunosuppressant toxicity.

759

760 Because many common drugs, such as statins, benzodiazepines, antiepileptics, and
761 immunosuppressants, are CYP3A4([Villemure et al., 2023](#)) or CYP2C9 substrates([Lim et al., 2023](#)),

762 transient suppression of these pathways after vaccination could alter drug exposure in a clinically
763 significant way. Yet regulators do not currently require pharmacokinetic interaction studies for
764 vaccines, leaving these risks under-characterized, and clinicians may be unaware.

765

766 The possible pharmacodynamic interactions with lipid nanoparticles themselves may be overlooked.
767 Recent work has shown that small-molecule drugs can directly influence endosomal trafficking and
768 escape. Tricyclic cationic amphiphilic drugs (TCADs), such as tricyclic antidepressants, first-generation
769 antipsychotics, and certain antihistamines, share structural features with ionizable lipids and have been
770 repurposed in experimental systems to improve intracellular delivery of nucleic acids. ([Debisschop et al.,](#)
771 [2024](#)) In animal studies, nortriptyline-containing “CADosomes” demonstrated delivery efficiency
772 without the need for synthetic ionizable lipids, ([Bogaert et al., 2022](#)) suggesting a structural and
773 functional overlap between cationic amphiphilic drugs (CADs) and LNP excipients. While this may be
774 exploited experimentally to enhance delivery, it raises the question of whether patients already taking
775 CAD-class drugs (e.g., antipsychotics, some antidepressants, etc) may experience altered LNP
776 trafficking or immune responses following vaccination.

777

Vaccine-Drug Interactions May Be Underreconized

Pharmacokinetic and pharmacodynamic interactions are not regularly evaluated during vaccine development, as regulatory agencies generally assume there are no clinically significant drug–vaccine interactions. ([WorldHealthOrganization, 2005](#)) However, rare case reports with influenza ([Carnovale et al., 2018](#)) and COVID-19 modRNA–LNP vaccines challenges this assumption.

Case reports of clozapine toxicity, ([Thompson et al., 2021](#)), observations of altered serum levels of antiepileptics, ([Mizuno et al., 2022](#)) and observational studies linking inflammation-induced cytokines to CYP450 suppression ([F. Liu et al., 2023](#)) all suggest a potential for transient but clinically significant interactions.

Additionally, lipid nanoparticles exhibit pharmacodynamic interactions, such as altered endosomal trafficking, which can occur in patients taking psychotropic or other medications and remain largely unexplored.

Overall, these gaps suggest that vaccine–drug interactions are both possible and clinically relevant, but are currently underestimated due to existing regulatory frameworks.

778

779 Other drug classes have also been implicated in modifying endosomal escape. Proton pump inhibitors,
780 such as esomeprazole, have recently been investigated as adjuvants in preclinical LNP formulations, by
781 raising endosomal pH, enhancing LNP delivery and immune responses via lysosomal destabilization in
782 murine models. ([Kim et al., 2025](#)) PPI use has also been shown to increase risk of severe COVID-19
783 outcomes. ([G.-F. Li et al., 2021](#)) For chronic PPI users, altered transfection efficiency could amplify
784 AEs, warranting caution and further research into vaccine safety profiles. These findings suggest that
785 the LNP itself functions as a cationic amphiphilic drug (CAD), and its toxicological profile may overlap
786 with that of CAD drugs ([Gould & Templin, 2023](#)). Endosomal escape enhancers, whether intentionally
787 incorporated into formulations or present coincidentally in patient medications, can increase cytosolic
788 release but also exacerbate lysosomal damage and galectin-mediated inflammation. ([Dowdy, 2023](#);
789 [Omo-Lamai et al., 2025](#)) This dual potential to both enhance efficacy and intensify toxicity underscores
790 the need for pharmacovigilance analyses examining outcomes in patients on CADs, or other drugs at
791 the time of vaccination. Together, these observations argue that vaccine–drug interactions are not only
792 possible but clinically relevant, and their continued neglect in regulatory assessment represents a sub-
793 stantial oversight.

794

3 Challenges, Gaps and Future Directions

796

797 The modRNA-LNP platforms are transformative technologies with significant clinical potential. How-
 798 ever, several critical uncertainties remain. These challenges come from the complex physicochemical
 799 properties of the technology and from broader translational and regulatory issues. As a result, there is
 800 an ongoing need for sustained mechanistic research and transparent long-term studies.

Category	Documented Challenges	Broader Uncertainties	Implications
Physicochemistry	Reliable characterization of particle size, encapsulation, payload, and stability remains challenging(Nogueira et al., 2024) No benchmark lipid formulation exists.(Simon et al., 2023) Standards and assays are continually evolving.(Pavlin et al., 2025; UnitedStatesPharmacopeia, 2024; Webb et al., 2025)	Black box formulation, the dynamic nature of LNPs results in unpredictable <i>in vitro</i> and <i>in vivo</i> behaviour	Comprehensive analytical standards are required, including proteomic and lipidomic profiling.
Biodistribution and Transfection	Conflation of biodistribution with gene expression,(Ci et al., 2023; Vervaeke et al., 2022) widespread off-target distribution.(Luo et al., 2025; Pateev et al., 2023)	Limited ability to achieve tissue-specific delivery beyond the liver.(Hosseini-Kharat M., 2025) Transfection is random and uneven; emerging tools like single-cell Nano mapping are still experimental.(Luo et al., 2025)	Therapeutic outcomes and adverse effects remain difficult to predict; single-cell methods are needed.
Protein Corona	Formation is dynamic, species-specific, and patient-dependent, affecting biodistribution and immune recognition. Levels of cell uptake do not correlate with increased mRNA translation likely due to protein corona-induced lysosomal trafficking (Voke et al., 2025) Measurement remains challenging.(Francia et al., 2024)	Patient variability (including age, sex, and comorbidities)(Sun et al., 2024) complicates predictability.	Results in nonlinear uptake, increased risk of immune activation, and reduced targeting accuracy.

Endosomal Escape	Low efficiency (1-15%); high stochastic cell-to-cell variability;(Johansson et al., 2025; Paramasivam et al., 2021) “dark hour” between escape and gene expression is not well understood.(Müller et al., 2024) Attempts to improve endosomal escape raise toxicity(Dowdy, 2023) LNPs alter cell membranes (Escalona-Rayó et al., 2024; Schlich et al., 2021)	Escape remains nonlinear, context-dependent, with a bottleneck that limits potency(Chatterjee et al., 2024; Johansson et al., 2025; Paramasivam et al., 2021)	“Bottleneck” increases unpredictability of therapeutic efficacy.(Chatterjee et al., 2024) Non-linear and context-dependent; bell-shaped curve(Bates et al., 2025)
Persistence and Lipid Metabolism	PEG-lipid immune effects,(Bakos et al., 2024), possible lysosomal stress,(Bitounis et al., 2024; Paramasivam et al., 2021), unknown toxic ionizable lipid metabolites,(Jørgensen et al., 2023), and cholesterol crystallization (Anindita et al., 2024), DSPC membrane effects	Long-term safety of repeated dosing remains unclear.	Risks of chronic accumulation, inflammation, or metabolic disruption may be possible; requires further investigation and focused studies
Manufacturing and stability	Documented batch heterogeneity;(EMA/707383/, 2020 Corr.1*¹) instability in plasma; (Zhang & Barz, 2025) post-injection remodeling;(Y. Ren et al., 2025) cold-chain and scale-up challenges.(Oude Blenke et al., 2023) Lipid adducts an unrecognized concern	Effects of instability on potency and safety remain uncertain.	Variable potency, potential side effects, and administrative challenges can compromise efficacy and increase adverse event risk. Lipid adduct formation may affect therapeutic outcomes and AE profile
Drug Interactions	Case reports of clozapine toxicity(Thompson et al., 2021) and altered antiepileptic levels after vaccination;(Kow & Hasan, 2021) CYP450 suppression during inflammation is well established.(Villemure et al., 2023)	The degree to which modRNA–LNP vaccines transiently alter drug metabolism (CYP3A4, 2C9, 1A2) or interact with lysosomotropic drugs (e.g., psychotropics) or other drugs remains unknown	Vaccine–drug interactions are not systematically assessed; potential underrecognized risk for patients on narrow therapeutic index drugs (clozapine, tacrolimus, midazolam).

Regulatory and Data Gaps

LNPs have adjuvant-like activity, as acknowledged by the FDA, [\(Peden, 2022\)](#) but were classified as excipients in regulatory submissions.

Pfizer/BioNTech's Comirnaty lacked transfection and target-cell-specific data, and CARPA was not assessed. [\(EMA/707383/, 2020 Corr.1*¹\)](#)

The FDA did not evaluate Moderna's LNPs separately. [\(Hemmrich & McNeil, 2023\)](#)

New EMA guidelines on the quality of modRNA vaccines reinforce the classification of excipients. [\(EuropeanMedicinesAgency, 2025\)](#)

Current regulatory framework does not capture transfection and nanoparticle-specific risks; transparency and public trust remain unresolved issues.

Drug interactions were not assessed

Incomplete safety evaluation, risks confusion, and skepticism.

Advanced methods, including proteomics [\(Boros et al., 2024; EuropeanMedicinesAgency, 2025\)](#) and lipid profiling, [\(USFDA, 2022\)](#) are needed to fully characterize LNP-modRNA formulations and their pharmacological and immunostimulatory properties.

Secondary pharmacology, drug interactions, assessment of long term risks required for regulatory assessment of LNPs

802 **TABLE 5 Critical Uncertainties and Challenges of modRNA-LNP Technology**

803

804 Considering the factors discussed, processing and administering into a living organism involves numer-
805 ous disruptive factors. As a result, neither biodistribution nor transfection follows a linear pattern, and
806 unpredictable variations in the measured values occur depending on the *in vivo* model.

807 It also remains plausible that both a nonlinear distribution pathway and the transfection rate, dependent
808 on the formulation of the LNPs and the specific lipid components, may occur. From a pharmacoki-
809 netic perspective, the challenges associated with LNP technology, as identified in earlier research, have
810 not been fully addressed.

811

812 These concerns are not isolated technical issues but interconnected challenges. The physicochemical
813 heterogeneity and the dynamic structure of LNPs influence biodistribution, which in turn depends on
814 the dynamic protein corona; meanwhile, inefficiencies in endosomal escape exacerbate variability in
815 therapeutic outcomes. The toxicological dynamics of the extracellular LNPs are unstudied([Bitounis et](#)
816 [al., 2024](#)), as is the possibility of lysosomal stress or dysfunction which is increasingly linked to numer-
817 ous diseases, such as neurodegenerative disorders.([Feng et al., 2024](#)) Likewise, patient heterogeneity
818 amplifies these uncertainties, making it unreasonable to expect uniform efficacy or safety across popu-
819 lations and making it difficult to predict clinical response or an adverse event profile. Gaps in regulatory
820 requirements, such as critical quality attributes, target-cell specificity, biodistribution,([Vervaeke et al.,](#)
821 [2022](#)) immune effects, drug interactions, and long-term toxicology, further undermine public confi-
822 dence and complicate post-marketing safety and surveillance.

823

824 We assert that the interplay between protein corona composition, cellular uptake pathways, endosomal
825 escape and lipid metabolism critically influences cell tropism, protein production, and the stability of
826 both the lipid and RNA components. These aspects should be carefully considered and require further
827 investigation.

828

829 Given the dependencies shown, it is worth questioning whether parameters reliant on highly individual
830 physiological factors, such as age-related metabolic changes, pre-existing conditions, medications, base-
831 line protein levels, or temporal fluctuations in protein concentrations, can be effectively controlled or
832 standardized.([J. Li et al., 2021](#); [Wegler et al., 2019](#))

833

834 Furthermore, these factors are inherently difficult to quantify and measure because they vary on an in-
835 dividual basis, and because *in vitro* measurements do not always reflect the *in vivo* behaviour of this tech-
836 nology. This raises fundamental challenges for the translation of LNP-based therapeutics into clinical
837 practice.

838

839 Discussion

840

841 Looking ahead, various strategies are being explored to address the unpredictability of current mo-
842 dRNA–LNP systems. One approach involves developing liposomal LNP hybrids, which may lower bi-
843 ocorona complexity and enable extra-hepatic targeting.([M. H. Y. Cheng et al., 2025](#)) Exosome-inspired
844 or engineered extracellular vesicles offer another promising avenue,([Iqbal et al., 2024](#)) leveraging their
845 natural ability to cross physiological barriers and evade immune clearance.([Maugeri et al., 2019](#))

846

847 On the chemistry front, new classes of ionizable lipids with improved degradation profiles are being
848 developed to reduce persistence and toxicity.([Han et al., 2021](#); [Jørgensen et al., 2023](#); [Omo-Lamai et al.,](#)
849 [2025](#)) Simultaneously, advances in single-cell mapping technologies aim to clarify stochastic uptake and
850 expression at unprecedented resolution,([Bates et al., 2025](#); [Johansson et al., 2025](#); [Luo et al., 2025](#); [Mül-](#)
851 [ler et al., 2024](#)) potentially making delivery more predictable. Improvements in assay methodol-
852 ogy([Pavlin et al., 2025](#); [Webb et al., 2025](#))and in formulations such as lyophilization([De & Ko, 2023](#))
853 look promising. Together, these innovations and others suggest that although current formulations re-
854 main a biological “black box,” an expanding toolkit is being developed to potentially make modRNA
855 delivery more controllable, targeted, and safer.

856

857 These uncertainties highlight the nonlinear and context-dependent nature of LNP-modRNA interac-
858 tions, suggesting a pathogen-like effect on the cell beyond its inherent cytotoxicity. Insights from cati-
859 onic amphiphiles such as antipsychotic drugs may enhance the understanding of these complex parti-
860 cles.([Gould & Templin, 2023](#); [Sfera et al., 2022](#))

861

862 Progress will likely require integrating advanced *in vitro* and *in vivo* models,([Bitounis et al., 2024](#)) single-
863 cell resolution technologies,([Luo et al., 2025](#)) and standardized analytical frameworks([Simon et al.,](#)
864 [2023](#); [UnitedStatesPharmacopeia, 2024](#)) to achieve this goal.

865

866 However, it must be considered that *in vitro* experiments with such a highly variable technology *in vivo*
867 require a systems biology perspective. Neither membrane structural processes nor downstream signal
868 transduction([Thiemicke & Neuert, 2023](#); [Vijay & Gujral, 2020](#))follow linear dynamics.

869

870 Additionally, incorporating longitudinal human data and comprehensive regulatory strategies will be
871 crucial to ensure both efficacy and long-term safety. This will be a challenging task given the nonlinear
872 dynamic nature of this technology.([Fung et al., 2024](#))

873

874 **Summary**

875

876 To the best of our knowledge, this work is the first to systematically synthesize the current understand-
877 ing of LNP properties while highlighting unresolved challenges that have become increasingly evident
878 in recent years but remain insufficiently addressed in clinical applications.

879

Declaration of competing interest

The authors declare that they have no competing interests.

Author contribution

L.M. Gutschi and F. Seger wrote this manuscript equally and discussed every aspect. The manuscript was published with the consent of both authors. The authors used Grammarly to improve the manuscript's readability. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

Materials

All slides were created by F. Seger using BioRender (unless otherwise indicated) and are used under BioRender's non-commercial license.

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