

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

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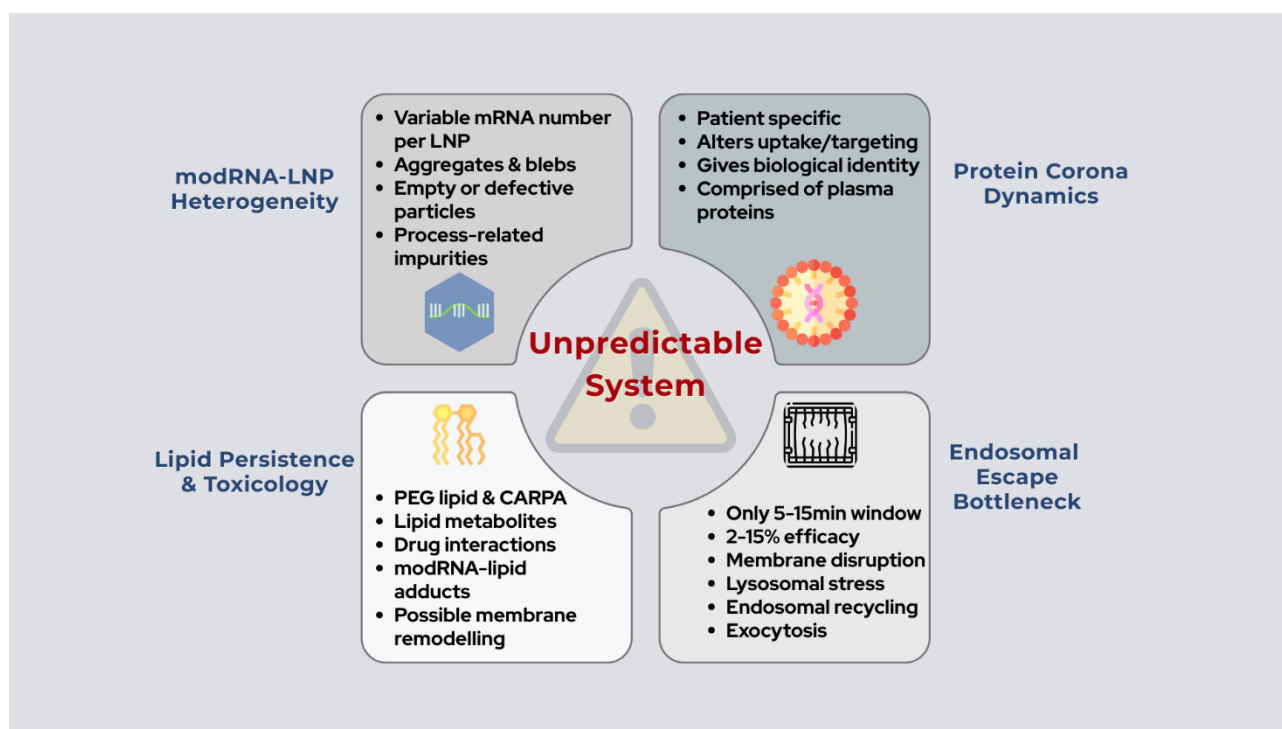
## Abstract

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 raise poorly characterized safety questions. This review summarizes published evidence to inform future safety evaluations. It does not provide medical or clinical recommendations.

**V2:** Legend formatting changes, clarification on endosomal bilayer and luciferase half-life, additional data on lipid adducts, in vitro/in vivo correlation, Methods section added, some wording changed

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



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

## Section 1: Physicochemical Foundations of the LNPs

### 1.1 Introduction

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

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

56

57 LNPs are by no means new.<sup>3,4</sup> Research into lipid carrier systems with a wide variety of formulations  
58 has been ongoing for over 60 years. Liposomes are an earlier type of LNPs, consisting of one or multi-  
59 ple lipid bilayers with an aqueous core. They are commonly used in drug delivery because hydrophilic  
60 drugs can be enclosed within the aqueous interior, while hydrophobic drugs are trapped within the hy-  
61 drocarbon chains of the lipid bilayer. Liposomes cannot efficiently carry nucleic acids, such as mRNA,  
62 due to the size, polyanionic nature, and hydrophilicity of the mRNA, which motivated the development  
63 of ionizable lipid-based LNPs. Additionally, nucleic acids are quickly degraded by endogenous nucle-  
64 ases in bodily fluids.<sup>5</sup> To address these issues, LNPs incorporating ionizable lipids have been devel-  
65 oped as delivery vehicles for small interfering RNA (siRNA) and mRNA, thereby protecting fragile  
66 cargo from degradation in vivo and facilitating cellular delivery.

67

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

76

77 While prior reviews have explored the properties of LNPs<sup>3</sup> or safety aspects,<sup>6</sup> the present work repre-  
78 sents a first attempt to integrate the unpredictable and partially stochastic nature of modRNA–LNP  
79 systems across their pharmacological dimensions.

80

81 We argue that this non-linear behavior introduces uncertainty into therapeutic application and chal-  
82 lenges precision and predictability. Accordingly, we emphasize the need for enhanced regulatory over-  
83 sight, thorough mechanistic studies, clinical pharmacology assessments, and the application of ad-  
84 vanced analytical techniques to better characterize and evaluate this novel platform.

## 85 86 **1.2 Composition**

88 The currently approved LNP formulations for the COVID-19 vaccines contain four lipids: (1) an  
89 ionizable cationic lipid, (2) a helper lipid DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), (3)  
90 cholesterol, and (4) a polyethylene glycol (PEG)-lipid conjugate.<sup>7</sup> Each lipid component of the  
91 nanoparticle and its molar ratio are critical to the activity and disposition of the modRNA. Similarly, the  
92 first approved LNP-RNA product, patisiran (Onpattro), contains short interfering RNA (siRNA) in an  
93 LNP formulation designed to deliver siRNA to the liver and silence the expression of transthyretin, a  
94 protein that causes transthyretin amyloidosis (ATTR).

96 Developing and scaling up Onpattro® paved the way for LNP-modRNA vaccines, which are the  
97 fastest vaccines ever produced.<sup>8</sup>

99 The ionizable lipid is crucial for delivering nucleic acids across cell membranes. Composed of a tertiary  
100 amine head, a linker, and a hydrophobic tail, it undergoes protonation under acidic conditions. This  
101 allows it to bind to negatively charged modRNAs, specifically via the tertiary amine head, owing to the  
102 unique properties and pH-dependent surface charge of ionizable lipids<sup>9</sup>. The design of the ionizable  
103 lipid, such as tail length<sup>10</sup>, saturation, and branched tails,<sup>11</sup> influences the efficacy and toxicity of the  
104 LNPs. The helper phospholipid (DSPC) enhances the LNP bilayer stability,<sup>12</sup> thereby preventing  
105 leakage of nucleic acid cargo. It provides the structural foundation for membrane fusion, which is  
106 necessary for cellular uptake. Cholesterol is crucial for maintaining the overall shape, fluidity, and  
107 permeability of the (bilayer) membrane, as well as supporting other phospholipids for effective

108

109 encapsulation and protection of the modRNA cargo, <sup>13</sup>. Cholesterol accounts for about 45% of the  
110 LNP content and can exist in a crystalline-like state within the LNP.<sup>14</sup>

111 The PEG lipid conjugate serves primarily to decrease LNP size, shield the LNP from rapid clearance  
112 by the reticuloendothelial system (RES), stabilize LNPs via steric repulsion, and prevent protein  
113 adsorption due to the hydrophilic chains extending from the surface.<sup>8</sup> It typically only comprises about  
114 1.5% of the LNP content. The immunogenicity of PEG has drawn attention due to the development  
115 of anti-PEG antibodies after repeated exposure. <sup>15</sup>

116

### 117 1.3 Structure of the LNPs

118

119 For COVID-19 vaccines, the exact structures of modRNA-LNPs remain unknown due to their self-  
120 assembly nature and the properties of the lipids used. These Janus particles, which exhibit two or more  
121 distinct physical properties, remain poorly understood. Small-angle neutron scattering (SANS) reveals  
122 that blebs (separate aqueous-filled compartment within a lipid nanoparticle, distinct from the main lipid  
123 structure) are common, but they do not always indicate the presence of modRNA within them. <sup>16</sup> In  
124 fact, identifying modRNA-free LNPs has proved particularly challenging. Studies estimate that 12-80%  
125 of LNPs (most recently 30-35%) may lack any modRNA, depending on the manufacturing process, the  
126 ionizable lipid used, and the analytical method employed. <sup>17-20</sup> The modRNA payload is especially  
127 important, particularly regarding the number of strands and the structure of the modRNA, as the  
128 random packaging of modRNA constructs influences LNP behaviour and potency. <sup>21 22 23</sup> Therefore,  
129 the relationship between the declared dose (µg of RNA) and the number of RNA-containing particles is  
130 not straightforward, and this correlation has yet to be fully described.

131

132 Currently, there is no reliable analytical method to accurately characterize either the content (i.e., the  
133 modRNA, <sup>24</sup>) or the structure of LNPs, <sup>25</sup> so orthogonal techniques are necessary.<sup>20,26</sup> Moreover,  
134 LNPs with blebs may also exhibit different immunogenicity, biodistribution, or *in vivo* properties that  
135 have not been adequately studied. <sup>27</sup> Mixing and filling parameters during manufacturing and sample  
136 handling of filled vials by clinicians also impact modRNA payload. <sup>28</sup> Furthermore, empty LNPs may  
137 reduce the effective dose, increase variability in therapeutic effectiveness since these are the ones most  
138 likely to transfect cells, <sup>21</sup> and accumulate in tissues possibly acting as adjuvants, <sup>29</sup> an understudied risk.  
139 These recent findings have raised questions about the formulation and composition of safe and  
140 effective LNPs for modRNA therapeutics and makes it difficult to comply with recommendations for  
141 LNP characterization by regulatory authorities. <sup>30</sup> Lyophilization (freeze drying) could reduce empty  
142 LNPs and improve stability at room temperature <sup>31</sup> and improve mixing, but remains investigational.

143

#### 144 1.4 The Nanoparticle Nature of LNPs

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146 Due to their small size, nanoparticles have an extremely high surface area relative to their volume,  
147 resulting in unique chemical, physical, and biological properties not found in bulk materials. These  
148 properties enhance the LNPs' reactive interactions with the cell membrane, such as immune responses  
149 and cellular uptake. <sup>32</sup>

150

151 Importantly, the biological behaviour of the LNP formulation cannot be inferred merely from the  
152 isolated properties of individual lipids. This is because the physicochemical characteristics of the entire  
153 LNP present in the final formulation, such as size distribution, shape, surface charge or zeta potential,  
154 agglomeration state, and lipid packing, <sup>33</sup> arise from interactions among all the components. For  
155 example, the degree of lipid unsaturation and branching affects not only membrane fusion capabilities  
156 but also biodegradability and systemic persistence. <sup>34</sup> Secondly, variations in size, modRNA payload,

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158 encapsulation rate, stability, lipid impurities, and other physicochemical factors may affect the safety  
159 and efficacy of these products, as demonstrated in preclinical and clinical studies. <sup>32</sup> (see **Table 1**).

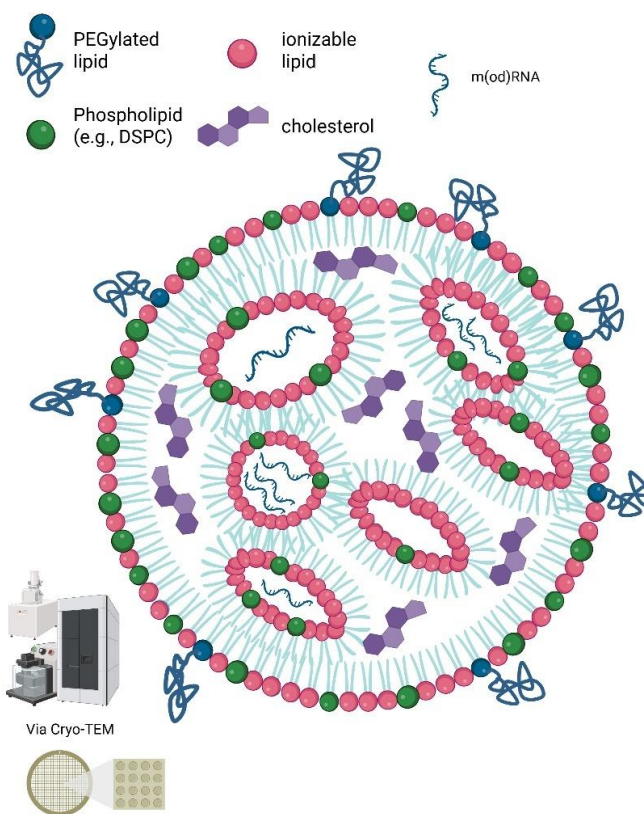
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## 161 **The LNPs are dynamic and unstable**

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163 Most recently, evidence suggests that the physiological stability of RNA-LNPs significantly impacts  
164 their therapeutic efficacy, pharmacokinetics (PK), tissue-targeting ability, and toxicity. <sup>35</sup> Instability in  
165 blood or plasma can lead to premature degradation of LNPs and the release of modRNA, potentially  
166 altering biodistribution and immune effects or affecting potential inflammation, depending on the  
167 specific formulation. <sup>36</sup> The Moderna and Pfizer/BioNTech vaccines differ in LNP behaviour. The  
168 modRNA of the Moderna COVID-19 vaccine persisted longer in plasma than the ionizable lipid SM-  
169 102 itself, suggesting lipid transfer to lipoproteins or extracellular vesicles (EVs). <sup>37,38</sup> The implications  
170 for cellular function remain uncertain. Conversely, the ionizable lipid of Pfizer/BioNTech's vaccine,  
171 ALC-0315, showed prolonged lipid exposure but lower levels of modRNA in plasma. <sup>37</sup> This could  
172 indicate instability of the intact LNP in plasma, possibly caused by trace impurities of the ionizable lipid  
173 <sup>39</sup> or complete disintegration in plasma. <sup>6</sup> These differences between the approved vaccines suggest that  
174 the specific formulation and manufacturing of the modRNA and lipid components (**Figure 2**) are  
175 distinct both in composition and biological effects, which may influence vaccine efficacy and outcomes.  
176 A comparison of the publicly available compositions, physicochemical properties, and key formulation  
177 parameters of the currently approved LNP-RNA products is shown in **Table 1**.

178



**Figure 2: Schematic Structure of an mRNA-Lipid Nanoparticle**

Lipid nanoparticles mainly consist of ionizable lipids, cholesterol, phospholipids, and polyethylene glycol (PEG)-lipid. The ionizable lipids are positively charged at a low pH (which allows negatively charged RNA to bind) and neutral at physiological pH (reducing potential toxic effects), enabling better delivery of mRNA into cells via endocytosis. Phospholipids provide structural support, while cholesterol acts as a stabilizing component in lipid nanoparticles. Lipid-anchored PEGs predominantly coat the surface of the lipid nanoparticle, forming a barrier that sterically stabilizes the particle and decreases nonspecific protein binding. Created in BioRender. Seger, F. (2025)

<https://BioRender.com/ysmgilk>

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**Table 1: Composition and Physicochemical Properties of LNPs in Approved modRNA Vaccines** [Abstracted from *Schoenmaker*<sup>40</sup>, *Zhang, Akin*<sup>41,42</sup>, *EMA*<sup>43, 44</sup>]

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:</i> <i>neutral lipid: cholesterol:</i> <i>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 <sup>45</sup>	Apparent pKa=6.68 more stable 1 branched chain; improved biodegradability No chiral centres
LNP Particle Size and Distribution <sup>46</sup>	Wider distribution (60-5000nm)	Wide distribution (30-1000nm)
modRNA payload (number of intact modRNA constructs per LNP)	Variable (exact payload unclear)	Variable (exact payload unclear)
Encapsulation Efficiency (%EE)*	≥80% (proposed specification) <sup>47</sup> ~50% <sup>19</sup>	≥80% (proposed specification) Not reported but likely similar
Stability in Plasma	Moderate <sup>37</sup>	High <sup>37</sup>
Buffer	Potassium dihydrogen phosphate; Disodium hydrogen phosphate dihydrate pH 7–8; Tris (tromethamine) in October 2021 <sup>48</sup>	Tris (tromethamine) pH 7–8

183 \*United States Pharmacopeia uses EE(%), defined as the percentage of RNA or therapeutic cargo that is successfully enclosed within  
184 the LNPs relative to the total amount of RNA present in the final sample. Schober et al.<sup>19</sup> used encapsulation efficiency as the percent-  
185 age of input RNA encapsulated in the final LNP product (EE<sub>input</sub>%) and found encapsulation rates <50%

186

## 187 1.5 Analytical Challenges and Knowledge Gaps

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189 The physicochemical properties often differ from theoretical predictions based on behaviors observed  
190 in non-biological systems. Despite significant progress, reliable techniques to determine physicochemi-  
191 cal attributes are not yet fully standardized. <sup>49</sup> For instance, particle size varies. <sup>46</sup> Using both expired  
192 and unexpired batches of BNT162b2 (Comirnaty®) and m-1273 (Spikevax®), the authors identified  
193 three different populations of LNPs for Comirnaty®: 60–65 nm (90% of the total), 600–700 nm (5–  
194 10%), and, in two vials examined, 5000 nm (1.2% and 2.8% by volume). Similar results were observed  
195 for SpikeVax®, ranging from 30 nm to 1000 nm. These large particles likely represent agglomerated  
196 LNPs, which are visible particles that may have specific physical, microbiological, and chemical adverse  
197 effects. <sup>50</sup> Aggregates are higher in thawed vials and may have *in vivo* risks (e.g., embolism or inflamma-  
198 tion)

199

200 These issues complicate accurate assessment of their *in vivo* behaviors, as *in vitro* characterization re-  
201 mains unpredictable and variable. <sup>51</sup> The need for precise characterization of LNPs, including size,  
202 blebs, empty structures, and other parameters, has driven the development of techniques to identify,  
203 observe, and measure significant differences between formulations and batch-to-batch variability of the  
204 same LNP-RNA system. <sup>26</sup> For instance, Pavlin *et al.* (2025) recently introduced a two-dimensional  
205 chromatography method that simultaneously assesses encapsulation efficiency (~65–70%), nucleic acid  
206 integrity, LNP size and impurities enabling detection of empty particles and aggregates in heterogene-  
207 ous samples simultaneously but requires standardization. <sup>20</sup> The physicochemical and structural com-  
208 plexities, as well as the lack of a reference standard (a certified material for calibration) for LNP formu-  
209 lations <sup>52</sup> raise critical questions about their *in vivo* behavior. **Section 2** will expand on this foundation  
210 to examine how these properties affect biodistribution, uptake, endosomal escape, therapeutic effec-  
211 tiveness, and potential toxicities.

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

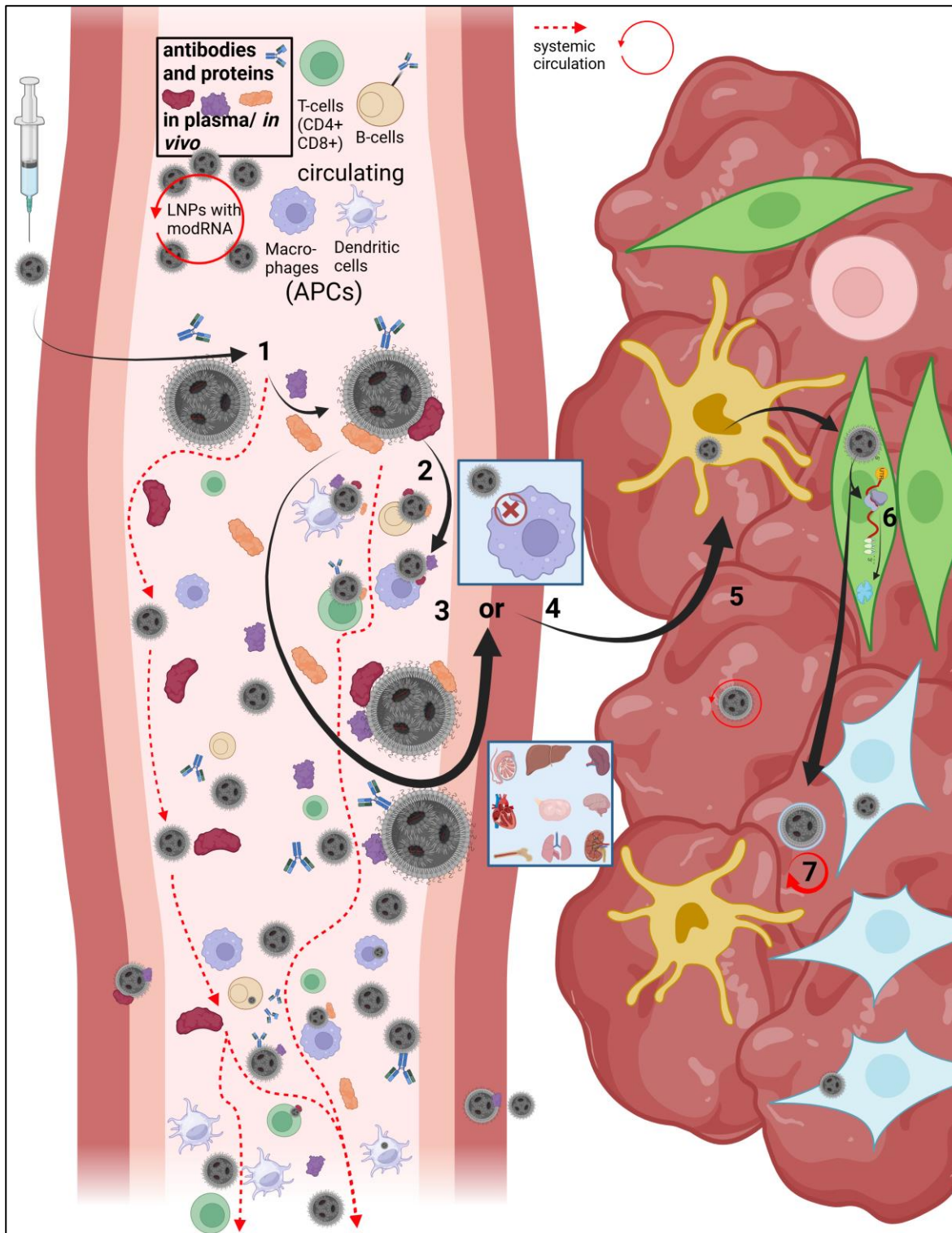
1. **Biodistribution:** physical location of a drug, tracer, or intact LNP within a biological system.
  - a. depends on circulation, the protein corona, vascular permeability, and reticular endothelial system (RES) uptake
  - b. does not indicate cell entry
2. **Transfection:** process of delivering 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

### 2.1 Overview

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



**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>

## 237 2.1 Biodistribution of the LNP-modRNA vaccines

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239 Accurately determining the biodistribution of LNPs, the modRNA, and the expressed protein remains  
240 a challenge. This issue affects many studies on modRNA-LNP technologies. Fluorescence-based re-  
241 porter assays are primarily used to track protein production or gene expression, but they do not directly  
242 indicate LNP localization or transfection ability. Regulatory guidelines also recommend using quantita-  
243 tive whole-body autoradiography (QWBA) to visualize and quantify intact LNP concentrations across  
244 almost all tissues and organs simultaneously, enabling systematic comparisons among tissues. [53](#) Hybrid-  
245 ization techniques, such as fluorescence in situ hybridization (FISH) for localization and branched  
246 DNA (bDNA) amplification for quantification, are used to study mRNA distribution throughout the  
247 body. Luciferase mRNA is a useful reporter for examining biodistribution and protein expression.  
248 However, as stated in the EMA Rapporteur's Rolling Review Overview, the luciferase mRNA construct  
249 lacks nuclear modifications. It is short-lived, [54](#) and may not accurately reflect the longer, more sus-  
250 tained protein production typical of modified mRNAs. [55,56](#) Therefore, conclusions based solely on lu-  
251 ciferase mRNA-LNPs may underestimate the actual performance of the modRNA product. These  
252 points highlight the complexity of evaluating the biodistribution of modRNA-LNP therapies and em-  
253 phasize the importance of a layered, comprehensive approach. [53](#)

254

255 No biodistribution studies using the actual modRNA from the Pfizer/BioNTech or Moderna vaccines  
256 were included in the regulatory documents, which described only luciferase and non-modified mRNA  
257 biodistribution studies. [43,44](#) As a result, no assessment of transfection efficiency or gene expression lev-  
258 els was performed. Further clarification from regulatory authorities and manufacturers is recommended  
259 to determine the necessary chemical, pharmacological, and toxicological studies required to obtain ap-  
260 proval for these lipids. [57](#)

261

262 Ci *et al.* [58](#) performed one of the few LNP-modRNA biodistribution studies, where the methodology  
263 showed strong differentiation of the sequential process of LNP activity based on current technical ca-  
264 pabilities. Quantification of the ionizable lipid and its metabolites was accomplished using

265

266 LC-MS/MS. ModRNA quantification employed bDNA, and detection of the non-translating Factor IX  
267 (NTFIX), a model protein, was analyzed using LC-MS/MS. This multi-faceted analytical approach, per-  
268 formed in mice, allowed for a clear distinction between LNP distribution, modRNA delivery, and  
269 downstream protein production. The authors demonstrated both LNP distribution and subsequent  
270 protein expression across a wide range of tissues. Protein production was quickly detected in the liver,  
271 ovary, and thymus, followed by the uterus and kidneys. As expected, the liver produced the most pro-  
272 tein overall, followed by the ovaries, kidneys, and lungs. Protein production persisted at low levels up to  
273 168 hours in the lungs, heart, liver, gastrointestinal tract, kidneys, and uterus, but not in the ovaries;  
274 however, no further measurements were obtained. Notably, protein expression was observed in the  
275 heart despite little to no corresponding mRNA at later time points, underscoring the importance of an-  
276 alyzing mRNA and protein levels separately over time to understand therapeutic effects. These results  
277 may indicate that macrophages or dendritic cells traffic to the heart; however, generalizability to hu-  
278 mans is unknown. The ionizable lipid and its metabolites were concentrated in the urinary and digestive  
279 tracts, suggestive of hepatobiliary and urinary clearance. The ethanolamine portion of the ionizable li-  
280 pid, radiolabeled with  $^{14}\text{C}$ , showed no metabolism in vivo, <sup>59</sup> indicating tissue persistence. (see **Section**  
281 **2.7**)

282

283 Luo *et al.* <sup>60</sup> recently introduced Single Cell Precision Nanocarrier Identification (SCP-Nano), a novel  
284 imaging and deep learning pipeline for single-cell resolution mapping fluorescence-labelled carriers  
285 such as LNPs across whole mouse bodies at doses as low as 0.0005 mg/kg, which are typical for mo-  
286 dRNA vaccines and are 100-1000 times lower than conventional imaging methods, such as QWBA. Us-  
287 ing reporter mRNA (e.g. EGFR), the study demonstrated heterogeneous nanocarrier uptake and pro-  
288 tein expression both within and across organs, with hotspots in the liver and spleen. This punctuated  
289 pattern indicated that some cells successfully translated the mRNA, while neighboring cells exhibited  
290 uptake without expression. Intramuscular injection of LNPs with SARS-CoV-2 spike modRNA re-  
291 vealed low-level heart endothelial delivery, confirming possible molecular and proteomic changes be-  
292 yond primary targets. These results in rodent models may not directly apply to humans. Still, this may  
293 have important implications for potential off-target effects that standard diagnostic methods, such as  
294 ultrasound or CT scans, might miss. Cellular or molecular changes could contribute to symptoms or  
295 disease risk but may not be visible until they become extensive enough to be detected by conventional  
296 tools.

297



298 The route of administration also influences the biodistribution of LNP-modRNA therapy. For intra-  
299 muscular administration, such as that for the modRNA COVID-19 vaccines, syringe pressure, perfu-  
300 sion rate, proximity to blood vessels and lymphatic vessels, local pH, and temperature, among others,  
301 are important considerations. <sup>61</sup> In contrast, other LNP-nucleic acid therapies, such as the siRNA prod-  
302 uct patisiran (Onpattro®), are administered through intravenous infusion, which achieves liver targeting  
303 almost exclusively through ApoE binding and LDL receptor uptake. The subcutaneous and intranasal  
304 routes favour the lymph nodes and the lungs, respectively. <sup>62</sup> These differences demonstrate that the  
305 biodistribution of LNPs differs significantly based on the route of administration, making them distinct  
306 from traditional small-molecule therapeutics.

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### 308 2.3 Formation and Biological Role of the Protein Corona

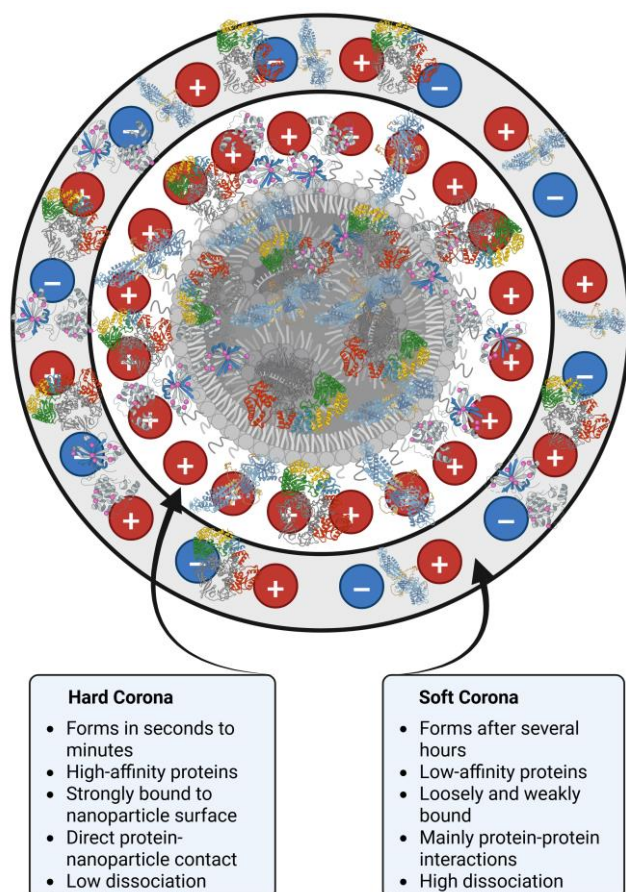
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310 When LNPs encounter biological fluids, they are immediately transformed by their environment. They  
311 acquire a dynamic and heterogeneous coating of biomolecules known as the protein corona. This layer  
312 fundamentally changes how the body perceives and processes the LNPs, influencing biodistribution,  
313 immune recognition, cellular uptake, and ultimately the efficiency of modRNA translation. Therefore,  
314 the protein corona gives the LNPs a “biological identity”.<sup>63</sup>

315

316 The protein corona formation occurs within minutes through van der Waals forces, hydrophobic inter-  
317 actions, electrostatic interactions, and other biochemical and biophysical interactions, resulting in an  
318 individual, heterogeneous *in vivo* LNP pool. <sup>64</sup> For the modRNA-LNPs, this process is accelerated be-  
319 cause the PEG-lipids on the surface of the modRNA-LNPs dissociate and exchange with plasma pro-  
320 teins, a mechanism known as PEG-lipid shedding. <sup>65</sup> It is a dynamic, complex, and unpredictable pro-  
321 cess that is crucial for biodistribution, transfection, and cellular responses, since this is what the cell it-  
322 self “sees.” <sup>66</sup>

323



325 **Figure 4: The Biocorona**  
 326 The illustration displays the characteristics of the hard and  
 327 soft biocorona. Created with BioRender. Seger, F. (2025)  
 328 <https://BioRender.com/edw2fru>  
 329

## 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



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

331

332 The protein corona can potentially mask targeting ligands or alter interactions with cell membranes.

333 This can reduce the efficacy of targeted delivery by shielding functional moieties or, in some cases, en-

334 hance functionality by presenting a new protein-based signal. [67](#)

335

336 One of the most critical aspects of the protein corona was demonstrated by Sebastiani *et al.* [68](#) When

337 ApoE binds to the protein corona of LNPs, the entire biodistribution pattern of the original

338 formulation is altered by internal structural changes, potentially affecting modRNA encapsulation,

339 agglomeration and premature RNA release. Accordingly, the entire surface structure changes,

340 facilitating the opsonization of phagocytes, such as macrophages and dendritic cells. Further work also

341 emphasized the importance of the protein corona for not only biodistribution but also transfection

342 efficiency and translation yield. [69-72](#)

343

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

345

346 The accelerated blood clearance (ABC) phenomenon, often triggered by repeated administration of

347 PEGylated LNPs, results from the production of anti-PEG antibodies. These antibodies can quickly

348 clear subsequent doses of PEGylated LNPs from the bloodstream through accelerated blood clearance,

349 reducing therapeutic effectiveness but also potentially increasing the risk of adverse reactions due to the

350 rapid and unpredictable distribution of the nanoparticles. [13](#) PEGylated nanoparticles are known to in-

351 teract with circulating complement proteins, activating the complement cascade and producing opso-

352 nins and anaphylatoxins, which are associated with acute infusion reactions in patients, known as com-

353 plement activation-related pseudoallergy (CARPA). [73](#) Anaphylactic and allergic reactions observed after

354 modRNA COVID-19 vaccination may partly reflect this phenomenon. [74](#)

355

## 356    **Implications and Challenges**

357

358    Since processing and administration into a living organism involve many interfering factors, it seems  
359    plausible that the biocorona causes a nonlinear distribution route depending on the formulation of the  
360    LNPs, the biological environment, and the route of administration. Overall, these data challenge the  
361    idea of a uniform LNP formulation and predictable biodistribution. One might assume that if these  
362    factors heavily influence biodistribution, administering the same dose to two subjects is unlikely to  
363    result in similar responses. Recent approaches to addressing the inherent issues with the biocorona of  
364    LNPs have utilized liposomal LNP-modRNA nanoparticles, which exhibit extra-hepatic targeting and  
365    longer circulation lifetimes, likely due to the formation of fewer proteins in the protein corona. <sup>75</sup> This  
366    represents a return to the original nanosized lipid particles, liposomes.

367

## 368    **2.4 Target Sites and Tissues**

369

370    What are the main sites and tissues targeted by the LNPs? The primary target sites for the LNPs  
371    include the liver, spleen, and draining lymph nodes, as these organs comprise a significant portion of  
372    the Reticuloendothelial System (RES), a component of the immune system that involves phagocytes,  
373    such as macrophages and monocytes. These cells are primarily located on the vascular wall of the liver  
374    (Kupffer cells), spleen (splenic macrophages), kidneys (mesangial cells), and lungs (lung  
375    macrophages).<sup>76</sup> Given the dynamic nature of the biocorona and the common presence of ApoE in it,  
376    it is not surprising that hepatocytes in the liver are the primary target for the LNPs. <sup>77</sup> Additionally,  
377    because the liver functions as a biological filter system, LNPs that are up to 200 nm in size tend to  
378    undergo fenestration unless specifically engineered otherwise, which helps their uptake into liver  
379    sinusoidal endothelial cells (LSECs). <sup>78</sup> Since LNPs first enter through the sinusoidal lumen, Kupffer  
380    cells are also the initial targets for transfection. <sup>77</sup>

381

382 Similarly, LNPs tend to distribute to the spleen due to its sinusoidal endothelium, which facilitates LNP  
383 uptake. Depending on the LNP formulation and composition, macrophages and dendritic cells can be  
384 targeted, which is essential for the efficacy of modRNA-LNP vaccines. <sup>79</sup> Based on the proportions,  
385 shape, charge, and other factors of the LNP lipid, gene expression or protein production can  
386 sometimes exceed levels in the liver. <sup>8</sup>

387

388 Draining lymph nodes are a common target for LNPs. The size of their fenestrations (<200nm) allows  
389 the LNPs to migrate through lymphatic channels and be taken up by antigen-presenting cells (APCs). <sup>80</sup>  
390 Various modifications, especially surface engineering of the LNPs and other adjustments, improve  
391 targeting and retention. Depending on their size and other factors, LNPs can also drain directly into  
392 lymph nodes. Additionally, larger particles are transported by APCs (mainly dendritic cells) to different  
393 locations, such as the heart, which has been suggested to explain immune reactivity and responses, <sup>81</sup>  
394 and may account for the results of Ci *et al.* <sup>58</sup>(see **Section 2.1**)

395

396 In addition, other organs may exhibit detectable LNP presence in preclinical studies, sometimes  
397 referred to as “off-target effects,” which are caused by the physicochemical properties of the LNPs and  
398 the resulting protein corona. The heart, lungs, adrenal glands, and ovaries are frequently reported in  
399 studies involving rodents and non-human primates (NHP). <sup>82</sup>

400

401 Transcytosis or direct penetration can occur, allowing LNPs to bypass blood-organ barriers. This is  
402 important because LNPs can leave the vasculature and cross the blood-brain barrier <sup>83</sup> or the intestinal  
403 barrier. <sup>84</sup> Zhang *et al.* <sup>85</sup> in a comprehensive review list various target cells, such as epithelial, basal, and  
404 endothelial cells, and explain how these are particularly likely to be targeted. Other notable examples  
405 include cardiac and skeletal muscle, bone marrow-derived dendritic cells and macrophages, and various  
406 cell types and tissues. <sup>9, 83, 86-90</sup>

407

## 408 **Regulatory Gap**

409

410 In official FDA and EMA documents, the “target cells” believed to be transfected by the LNPs are not  
411 specified. Notably, the US FDA mentions transfection <sup>91</sup> Conversely, the EMA states that the viral  
412 protein antigen is expressed in the desired conformation <sup>44</sup> It is unclear whether both agencies refer to  
413 the same process or if the EMA distinguishes between transfection and protein expression, as  
414 previously discussed. This lack of clear communication and precise data presentation regarding the  
415 modRNA-LNP target cells and delivery, combined with support from public health agencies, <sup>92</sup> has  
416 contributed to the common belief that the vaccine is limited to the deltoid muscle. These factors have  
417 contributed to an incomplete understanding of the vaccine’s biodistribution and safety profile.

418

## 419 **2. 5 Cellular Uptake Mechanism**

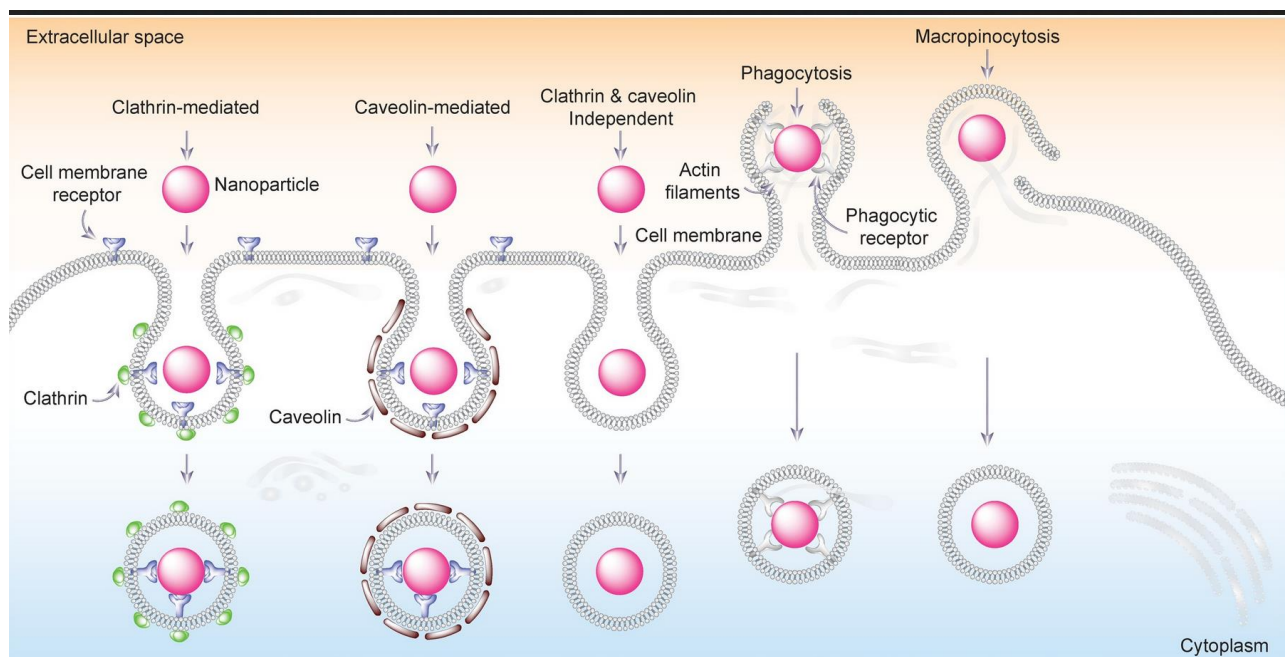
420

421 As we have seen, the adsorption of biomolecules onto the LNP surface establishes a dynamic  
422 biocorona overriding the synthetic nanoparticle design. This identity governs cellular interactions by  
423 dictating which membrane receptors are engaged, leading not only to biodistribution patterns but also  
424 to endocytic pathways and, consequently, the intracellular fate of the encapsulated modRNA. This  
425 membrane uptake into cells is termed endocytosis. The efficiency of uptake is profoundly affected by  
426 the biocorona, particle size, shape, and net surface charge. <sup>8</sup>

427

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

430



**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.*<sup>93</sup> 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<sup>94</sup> 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<sup>95</sup> (pH, bradykinin, prostaglandins, etc). Paunovska *et al.*<sup>96</sup> reported that LNPs can bind to apolipoprotein E and low-density lipoprotein receptors (LDL-R), whereas Chaudhary *et al.*<sup>7</sup> reported that Toll-like receptor (TLR)4 and CD1d can be internalized with the endosome.

451 Both receptor-mediated and receptor-independent cellular uptake [63](#) likely occur simultaneously within  
452 the same cell. Uptake may also occur under specific conditions without direct binding to membrane  
453 components; instead, nonspecific hydrophobic or electrostatic interactions ultimately initiate the  
454 process. (see **Table 2**)

455

#### 456 **Measuring how cell receptors bind is challenging.**

457

458 The current methods used to study the mechanisms by which LNPs interact with the cell membrane  
459 often disrupt the natural protein corona composition, making it challenging to identify which cell  
460 receptors recognize and bind to LNPs accurately. Identification of the corona proteins is not sufficient  
461 because not every protein in the corona can interact with cell receptors, as they may require correct  
462 orientation on the nanoparticle surface. Therefore, identifying which epitopes on the biomolecular  
463 corona are accessible to cell receptors is essential for determining potential interactions. Likewise, not  
464 all exposed proteins can necessarily bind to receptors, especially if there is competition with other  
465 proteins with higher affinity for the same receptors. It is, therefore, important to identify which  
466 proteins genuinely participate in these interactions. [97](#)

467

468 Lipid–membrane interactions can also influence cell membrane receptor activity and thereby contribute  
469 to the uptake of lipid nanoparticles (LNPs). As summarized by Lavington & Watts, [98](#) nanodisc and  
470 SMA lipid nanoparticle (SMALP) studies demonstrated that specific lipid components (such as helper  
471 lipids) modulate the surrounding membrane environment without directly binding to G-protein cou-  
472 pled receptors (GPCR). Such lipid-induced alterations affect GPCR conformation, ligand binding, and  
473 signal transduction, supporting functional receptor interactions. The elements of the protein corona,  
474 uptake pathways and primary tissues affected are reviewed in **Table 2**.

475

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

Biocorona Components	Main Receptors Engaged	Dominant Uptake Pathways	Cell Types Most Affected	Comments/Impact on transfection
<b>Albumin</b>	gp60 (albondin), SPARC, FcRn <sup>92</sup>	Caveolae-Mediated Endocytosis <sup>94, 100</sup> Trancytosis	Hepatocytes Endothelial cells Epithelial cells Tumor cells	Can bypass lysosomes, improved cytosol delivery, recycling endosomes Preferred mechanism for modRNA-LNPs
<b>Apolipoproteins (ApoE, ApoB, ApoA-1)</b>	LDL-R LRP-1 SR-B1	Clathrin-Mediated <sup>68, 85, Borah, 2025 #1697</sup> or Caveolae Mediated Endocytosis	Hepatocytes, Spleen, macrophages  Tissues with LDL-R include adrenals, ovaries, testes Neurons <sup>101</sup>	Classic receptor-mediated LNP uptake route with ApoE  May lead to lysosomal degradation if clathrin-mediated
<b>Vitronectin/ Fibronectin</b>	Integrins ( $\alpha v \beta 3$ , $\alpha 5 \beta 1$ )	Clathrin or Caveolae-mediated (lipid rafts) <sup>102, 98</sup>	Endothelial cells Fibroblasts Epithelial cells Tumor and parenchymal cells Heart in murine models <sup>60</sup>	Off-target effects Affected by nanoparticle shape; size, etc.
<b>Alpha-2 macroglobulin</b>	LRP1 <sup>103</sup>	Primarily clathrin-mediated	Hepatocytes, endothelial cells	Traps LNPs for lysosomal degradation <sup>104</sup> Reduces efficacy
<b>*C-Reactive Protein</b>	FcγR, C1q	Phagocytosis, complement activation	Macrophages, neutrophils	Complement activation, CARPA Reduces transfection efficiency
<b>*Immunoglobulins (IgG, IgM), Anti-PEG antibodies</b>	FcγR, FcαR CSF2RB (new finding) <sup>105</sup>	Phagocytosis <sup>102, 105</sup> 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
<b>*Complement proteins (C3b, C4b etc)</b>	Complement receptors	Phagocytosis Macropinocytosis <sup>100, 106, 107</sup>	Macrophages Neutrophils Dendritic cells	Strongly degradative; opsonization

<b>Direct</b>	TLR4/CD14	TLR4 is internalized along with the forming endosome (promotes lipid-raft formation), <sup>7, 108, 96</sup>	Dendritic cells, macrophages	Initiates cell signalling and immune activation Leads to lysosomal degradation Receptor recycling
<b>Direct</b>	None	Direct Membrane Penetration GPCR interactions (lipid rafts) <sup>109, 98</sup>	Driven by pH and lipid destabilization (small size, specific surface chemistry or external physical forces (e.g., electroporation))	Bypasses endosomal uptake

477  $\alpha v\beta 3$ =integrin alpha-v beta-3;  $\alpha 5\beta 1$ =alpha-5 beta-1; ABC = accelerated blood clearance; APC = antigen-presenting cells; ApoE =  
 478 apolipoprotein E. C1q=complement C1q component; CSF2RB=colony stimulating factor 2 receptor beta; CR3b=complement receptor  
 479 3; Fc $\gamma$ R= Fc gamma receptor; Fc $\alpha$ R=Fc alpha receptor; GPCR=G-protein-coupled receptor; LDL-R=low-density lipoprotein receptor;  
 480 LRP-1=low-density lipoprotein receptor protein-1; PEG=polyethylene glycol; SR-B1=scavenger receptor class B Type 1; TLR4=toll-like  
 481 receptor 4.

482 **\*Opsonins** (e.g. CRP, IgGs, complement) act in the vasculature, whereas integrins and others, mediate uptake at the cell membrane  
 483

484 The main challenge isn't whether transfection occurred, but how much happens and how conditions in  
 485 systems biology influence this process. According to current knowledge, organ fenestrations and the  
 486 pKa value mainly determine biodistribution and cellular uptake. The  $\zeta$ -potential primarily affects  
 487 protein corona formation and the likelihood of its formation. <sup>110, 64</sup> Given the numerous mechanisms,  
 488 cell receptors, and a wide range of cell types, along with cells at different stages of maturation and  
 489 division within the same lineage, it is not surprising that efforts to systematically target receptor-driven  
 490 signalling pathways within a highly complex biological system are inherently problematic.

491



492 Interestingly, Zelkoski *et al.* [111](#) demonstrated in THP-1 cells that ionizable LNPs can activate both  
493 TLR4 signalling pathways, the TIRAP/MyD88-NF- $\kappa$ B pathway and the TRAM/TRIF-IRF  
494 pathway, albeit with differences in magnitude and kinetics: NF- $\kappa$ B signalling was rapid and robust,  
495 while IRF activation was weaker and delayed. This observation supports the concept that ionizable  
496 LNPs, by altering lipid raft dynamics, can induce overlapping but temporally shifted TLR4 signaling  
497 responses, diverging from the canonical temporal segregation of these pathways. [112](#)(Table 2).

498

## 499 2.6 Endosomal Escape as Key Bottleneck

500

501 Transfection, as previously discussed, occurs in a receptor-dependent and/or receptor-independent  
502 manner, indicating a bioactive behaviour that extends beyond the traditional pharmacokinetic  
503 approach. Transfection is completed when the modRNA escapes the endosome. Assessing how LNPs  
504 are metabolized from a traditional pharmacokinetic perspective is challenging because they are not  
505 degraded through organ uptake during cell transfection. Instead, endosomal escape and degradation  
506 define the entire spectrum of pharmacodynamics. [113, 114](#) The classic absorption, distribution,  
507 metabolism, and excretion (ADME) pharmacokinetic model does not apply to liposomal or  
508 nanoparticle delivery systems.

509

## 510 The Endosomal Escape Mechanism is Based on Biophysical and Chemical Processes

511

512 Endosomes consist of a lipid monolayer similar to the cell membrane, which prevents nucleic acid  
513 escape as an evolutionary defense against foreign viral RNA entering the cell. For LNPs carrying  
514 modRNA, successful endosomal escape is essential for therapeutic action. After endocytosis, the  
515 endosomes increase the acid gradient, which protonates the ionizable lipids within the LNPs. For  
516 example, ALC-0315 has an apparent pKa value of  $\sim 6.09$ , and SM-102 has a value of  $\sim 6.6$ . This  
517 protonation event triggers the rearrangement of lipid molecules into a lamellar phase within the  
518 endosomes, promoting membrane destabilization and releasing the payload into the cytosol, a process  
519 known as the proton-driven osmotic swelling or the proton sponge effect. [115 116](#)

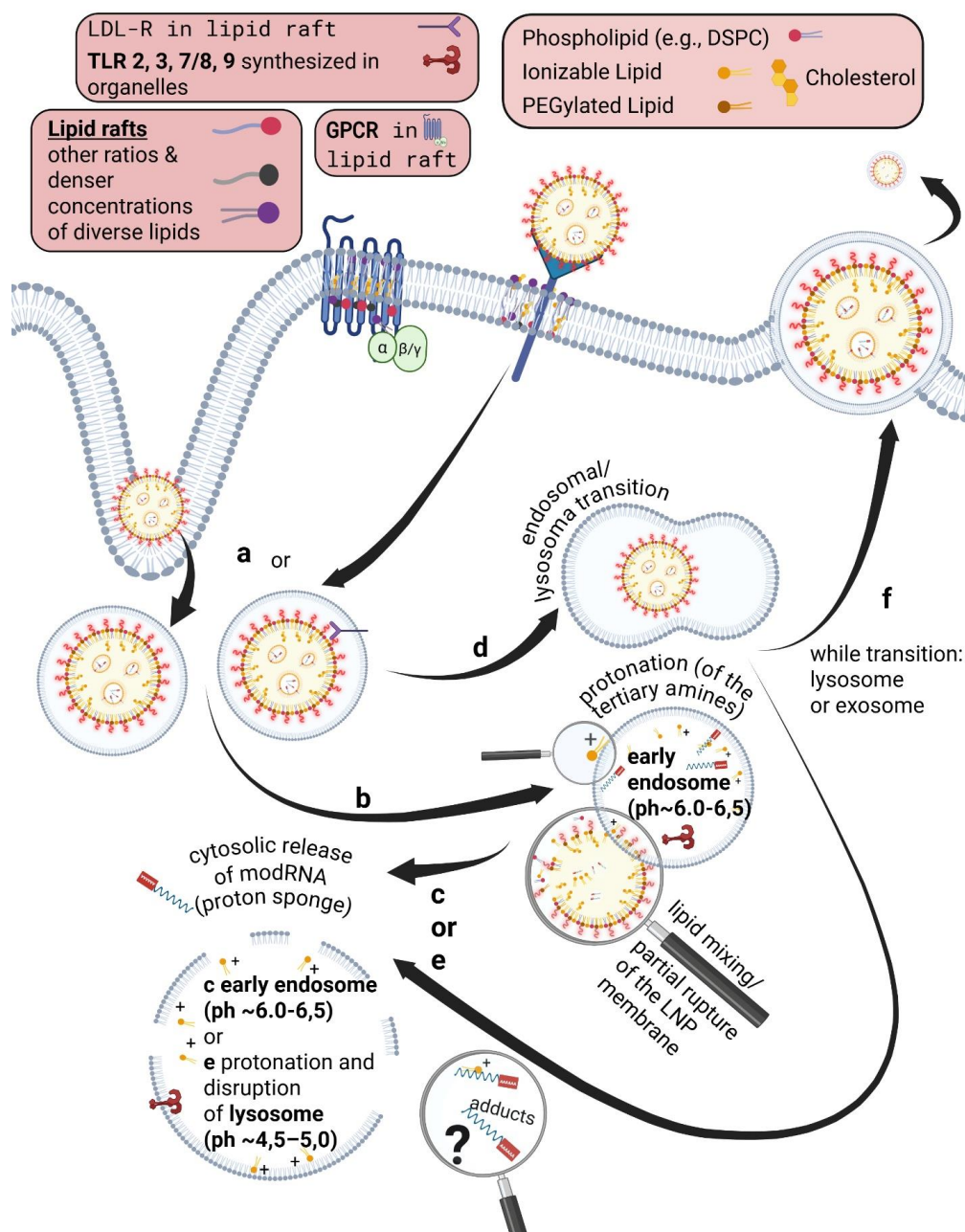
520

521 As the pressure rises, the membrane destabilizes and may rupture, releasing its contents into the  
522 cytosol. Endosomal damage, as indicated by galectin recruitment, can occur solely from the presence of  
523 ionizable lipids and does not require cytosolic delivery of the RNA molecule. <sup>117</sup> Lipid geometry  
524 facilitates this process. The conical shape of the branched, unsaturated fatty acid chains promotes  
525 negative curvature stress within the membrane, increasing destabilization. <sup>11</sup> Computational free energy  
526 calculations have shown that both ALC-0315 and SM-102 insert into the cell membrane favourably, <sup>118</sup>  
527 suggesting that ionizable lipids in the current LNP-modRNA vaccines embed into the lipid bilayer.

528

529 Even transient tearing may contribute to escape. Such tearing has been demonstrated with other  
530 nanoparticles. <sup>119</sup> Most recently, LNPs were found tethered to the endosomal membrane and  
531 associated with membrane destabilization. <sup>117</sup> Finally, Pilkington et al <sup>120</sup> suggest that LNPs may  
532 perturb lipid raft organization, implying that endosomal escape involves not only endocytosis but also  
533 broader effects on membrane dynamics. **Figure 6** shows the typical intracellular journey of a  
534 modRNA-LNP.

535



**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 is transported into late endosomes and eventually into lysosomes, where it is 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 **(f)**, unless the endosome is disrupted. Created in BioRender. Seger, F. (2025) <https://biorender.com/0921yhb>

## 539    **Endosomal Escape is Inefficient**

540

541    Only within a narrow window of opportunity do conditions allow LNPs to escape through endosomal  
542    fusion during the endosomal maturation process. [116](#) [121](#) [8](#)

543

544    This window is brief, lasting about 5-15 minutes [122](#) when conditions in the endosome enable the  
545    LNPs to fuse with the endosomal membrane and deliver their cargo into the cytosol. Beyond this  
546    period, escape efficiency drops significantly.

547

548    This process is highly inefficient, with only about 1-15% of all internalized LNPs resulting in the  
549    production of the target protein. [123](#) [116](#), [124](#), [121](#) LNPs that do not escape the endosome at this stage are  
550    degraded or exocytosed. [125](#) Degradation through lysosomal fusion enriches the endosome with  
551    degradative contents and enzymes, moving endosomes toward the plasma membrane and enabling  
552    fusion for exocytosis. Most LNPs follow these pathways and fail to deliver mRNA to the cytosol, since  
553    endosomal escape is the main “bottleneck” of mRNA therapeutics. [116](#). Over the past four decades,  
554    numerous methods have been attempted to improve delivery. However, significant improvements in  
555    endosomal escape often come at the cost of increased cytotoxicity, such as endosomal bursting and  
556    release of entire contents into the cytosol. [126](#)

557

## 558    **Failure to Escape the Endosomes Results in Cellular Stress**

559

560    After endocytosis, if the modRNA is not released into the cytoplasm, the endosomes mature into late  
561    endosomes and then fuse with lysosomes. [116](#) Lysosomes contain various enzymes such as lipases,  
562    proteases, nucleases, and glycosidases that dismantle both the modRNA and lipids.

563

564 An accumulation of undegraded materials from the LNPs can trigger cellular stress, oxidative stress,  
565 and potential inflammatory signalling. This accumulation has been compared to aspects of lysosomal  
566 storage disorders, [127](#) though a direct link to human disease has not been established.

567

568 Lysosomal retention blocks expected degradation and recycling processes in the cell, including  
569 receptor recycling such as LDL-R. This can create a cellular “traffic jam” that impairs the uptake of  
570 new ligands and receptors. [128](#) Although the lipids comprising the LNPs are considered biodegradable,  
571 high local concentrations can impair lysosomal function, slow degradation, and prolong the retention  
572 of the disassembled lipids. [129](#) Consequently, a blockade or arrest of normal endosomal maturation and  
573 acidification not only reduces therapeutic efficacy but can also lead to toxicological effects. [127](#)

574

#### 575 **LNPs May be Expelled Intact or Partially Degraded in Exosomes**

576

577 Not all LNPs successfully escape the endosomes or are degraded in lysosomes. A significant portion is  
578 recycled back into the extracellular space, repackaged in extracellular vesicles (EVs) or exosomes. This  
579 pathway enables cells to eliminate undigested LNPs or those that fail to escape the  
580 endosomal/lysosomal pathway. Maugeri [125](#) showed that LNPs in recycling endosomes are expelled  
581 either intact or partially degraded, which affects transfection efficiency. Exocytosis serves as both a  
582 clearance route and a secondary distribution mechanism; vesicle-mediated transport may transfer the  
583 modRNA or lipid fragments to the surrounding microenvironment in a paracrine manner. [130](#) These  
584 EVs can also transfect cells, influencing pharmacodynamic outcomes and contributing to variability  
585 and off-target effects. In fact, natural exosomes are being engineered for RNA delivery [131](#) [132](#) because  
586 they can cross physiological barriers effectively, have improved biocompatibility, low toxicity, cell-  
587 specific tropism, and can evade the mononuclear phagocytic system. [133](#) This recycling of endosomes,  
588 as well as empty LNPs or those with blebs, may cause cellular stress, oxidative damage, and chronic  
589 inflammation, [128](#) which could be linked to adverse effects such as injection-site reactions or immune

590

591 activation. These factors are not considered in biodistribution studies and may contribute to  
592 cumulative toxicity, especially with repeated doses. Long-term studies are needed to determine if these  
593 adverse events are causally related, as current regulatory focus is on immediate effects and may  
594 overlook these delayed responses. Endosomal escape of siRNA-loaded LNPs, such as those for  
595 Onpattro, is minimal, typically around 1%, [42, 134](#) which restricts cytosolic delivery and helps minimize  
596 cytotoxicity. This low efficiency means that only a small subset of internalized siRNA particles reaches  
597 the cytosol. The escape events themselves tend to produce small, transient membrane disruptions that  
598 are readily repaired by the cell. [117, 135](#) As a result, siRNA-mediated delivery elicits slower and weaker  
599 cytotoxic effects compared to delivery systems that induce more extensive endosomal damage.

600

### 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 ones?
- 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

601

## 602    **Single cell Analysis: a pharmacokinetic perspective**

603

604    The pharmacokinetics of LNP delivery and protein expression are a complex, multi-step stochastic pro-  
605    cess involving uptake, endosomal processing, and mRNA escape. Using single-cell analysis, Müller *et al.*  
606    [121](#) found that cellular uptake was variable and ranged from minutes to hours depending on LNP shape,  
607    composition, and cell type. Endosomal escape varied among individual cells and was inversely related  
608    to protein production; faster release and translation of RNA led to increased protein output. A theoreti-  
609    cal “area under the curve” (AUC), used to describe overall pharmaceutical protein availability, was  
610    found to depend equally on four factors: the number of mRNA molecules delivered, the translation  
611    rate, the mRNA lifetime, and the protein lifetime. Moreover, Müller *et al.* noted that little is known  
612    about the fate of nucleic acids after they escape from the endosome. Before any measurable action,  
613    such as protein expression occurs, there is what Müller calls “the dark hour of transfection,” the intra-  
614    cellular biochemical and physical processes that occurs following endosomal escape but before protein  
615    synthesis. What happens during this period remains unclear, which limits a full understanding. Addi-  
616    tionally, the amount of modRNA released into the cytosol does not reliably predict the level of protein  
617    expression, previously noted by Liu *et al.* [136](#)

618

## 619    **2.7 Lipid Degradation and Metabolite Persistence**

620

621    Once the modRNA is released, the fate of the lipid components determines the final pharmacodynamic  
622    stage of LNP activity. This aspect, concerning the fate of the individual lipids after they deliver their  
623    payload, is rarely discussed or addressed. The LNPs do not simply vanish; instead, they are disassem-  
624    bled *in vitro*, metabolized, and cleared at different rates depending on the lipid chemistry. For example,  
625    cholesterol may form oxysterols with immune effects, while DSPC can accumulate in organs, poten-  
626    tially altering membrane fluidity. Both cholesterol and DSPC are natural lipids, but they are manufac-  
627    tured synthetically. Clearance pathways remain poorly characterized, necessitating further study. These  
628    are further delineated in **Table 3**.

629



## 630 **PEGylated Lipids**

631

632 The pegylated lipid plays a key role in the lipid matrix of the LNP, despite its small molar ratio, because  
633 it extends outward on its surface, which is necessary for LNP stability during formulation and storage.  
634 [137](#) This also allows for increased *in vivo* circulation time since the PEG lipid impedes cellular uptake and  
635 endosomal escape, but this then creates the so-called “PEG dilemma.” As a result, PEG-lipids with  
636 shorter C-14 acyl chains were used in the LNPs, which gradually diffused out of the particles and pro-  
637 vided temporary stealth properties, achieving higher transfection efficiency than longer, more persistent  
638 PEG-lipids. [138](#) Once the PEG-lipid is sloughed off, it is metabolized by the liver and kidneys, where  
639 the lipid component undergoes enzymatic hydrolysis and  $\beta$ -oxidation which is standard processes for  
640 lipids. The pegylated part, being a polymer of ethylene glycol, is either excreted in urine or broken  
641 down into smaller oligomers. Although PEG-lipids are designed to quickly detach from the LNP sur-  
642 face once in circulation or shortly after uptake, they can remain associated. Then they can be internal-  
643 ized with the particle and undergo endosomal trafficking to lysosomes, where the lipid portion is de-  
644 graded and the PEG chains are either excreted or slowly metabolized. [139](#)

645

## 646 **Ionizable Lipid**

647

648 No clinical data exist for ALC-0315 and SM-102 regarding their retention and duration of activity in  
649 humans. Although they are labeled as "biodegradable" after their ester bonds are hydrolyzed within tis-  
650 sues and release their fatty acid tails, their overall ability to degrade doesn't truly improve, since com-  
651 mon degradation pathways like  $\beta$ -oxidation are not consistently used. [140](#)

652

653 Due to their sterically hindered ester structure, they are slowly hydrolyzed over several days. Jørgensen  
654 *et al.* highlight that these lipids usually have stable structures and multiple tertiary amines, which slow  
655 down their degradation and may cause toxicity. [140](#)

656



657 When ALC-0315 undergoes ester cleavage, it forms a doubly de-esterified metabolite that remains cationic and can reach metabolic sites such as mitochondrial membranes more quickly than longer lipids, 658 [36, 140](#) possibly leading to ROS production, cytokine release and membrane disruption. As a result, the 659 persistence of these shorter-chain lipids could lead to ongoing toxicity after exposure, [141, 142](#) but data in 660 humans is sparse. The ethanolamine head of Lipid 5, as studied by Burdette *et al.* [59](#) demonstrated in 661 vivo persistence, suggesting chemical stability. Such persistence could facilitate covalent or non-covalent 662 adduct formation with proteins or lipids, as tertiary amine head groups are capable of forming N-glucuronides 663 and quaternary ammonium metabolites [143](#) which are associated with enhanced tissue retention. Therefore, 664 there is an urgent need to develop new combinatorial reactions that can generate 665 degradable ionizable lipids for potent RNA delivery. <sup>9</sup> 666

667

## 668 **Lipid Adducts**

669

670 An underrecognized risk for LNPs is the potential for lipid adduct formation, which occurs in storage. 671 The head groups of tertiary amine-based lipids can form N-oxides and, consequently, fatty aldehyde 672 impurities due to the thermodynamic instability of the LNPs and the oxidative impurities generated 673 during the complex synthesis of the ionizable lipid. [144, 145](#) These aldehydes can react with modRNA nucleobases, 674 especially adenine and cytidine, inside the LNP to form covalent bonds (**Figure 6e**). Adduct 675 levels increase with storage time and temperature, rendering the affected modRNA untranslatable once 676 injected. Moderna scientists<sup>[146](#)</sup> first reported adduct formation in 2021, highlighting the lack of validated 677 assays for detecting these adducts during manufacturing. Moderna also noted that the Tris buffer 678 used in their product acts as an aldehyde sink, [147](#) enabling more extended storage at 2–8°C and reducing 679 adduct formation with the modRNA. Notably, Pfizer switched from PBS to Tris buffer in October 680 2021, raising questions about the amount and reactivity of adducts in their early batches (**Table 1**). An 681 anionic supported lipid bilayer mimicking the endosomal membrane as used by Aliakbarinodehi *et al.* [124](#) 682 showed that a fraction of mRNA remained associated with the bilayer after LNP fusion. This persistence 683 may have arisen from hydrophobic or electrostatic

684

685 rebinding or possibly from a covalent lipid-mRNA adduct, although the mechanism was not experi-  
686 mentally characterized. Lipid adducts have also been observed in MC3-based siRNA LNPs, as used in  
687 Onpattro, where oxidation of the ionizable lipid Dlin-MC3-DMA led to covalent RNA-lipid adduct  
688 formation. [148](#)

689

690 The damaged adducted modRNA, once taken up by the cell, may be perceived as abnormal or viral-like  
691 by cellular sensors, which may trigger inflammatory signals or interferon responses. [149, 150](#)

692

693 Post-transcriptional interference, including adduct-induced damage, is hypothesized to contribute to  
694 ribosomal stalling and collision with trailing ribosomes, systemic immune dysregulation, and exagger-  
695 ated inflammatory responses [150](#), especially in vulnerable individuals. [151, 152](#)

696

697 Research on secondary amines and reactive aldehydes, such as 4-hydroxynonenal (4-HNE) from lipid  
698 peroxidation, indicates they are cytotoxic and can impair protein folding or function, leading to the  
699 neoantigen formation, oxidative stress and lysosomal dysfunction. [6, 153, 154](#) However, direct *in vivo*  
700 evidence of adduct formation after LNP uptake has not been confirmed.

701

702 Moderna is actively exploring strategies to reduce covalent bonds and RNA-LNP adducts,  
703 acknowledging their potential toxicity. [155](#) DNA-LNP adducts may similarly form with residual DNA in  
704 the vaccines, potentially triggering interferon production. [156](#) It is unclear whether BioNTech has  
705 evaluated these phenomena. Alternative ionizable lipids with piperidine heads have been developed to  
706 mitigate adduct formation and enhance thermal stability. [157](#) Despite these innovations, the biological  
707 risks associated with adduct formation have not yet been systematically evaluated in vaccine studies.

708

709 The recent EMA draft guideline for modRNA vaccines <sup>30</sup> emphasizes the control of adduct formation  
710 in manufacturing but does not delineate the possible adverse effects. Continuous pharmacovigilance  
711 and advanced *in vivo* assays are essential to clarify these uncertainties *in vivo*, particularly for vulnerable  
712 groups. The lipid components, metabolic pathway, and knowledge gaps are summarized in **Table 3**.

713  
714 **Table 3: Lipid Components, Metabolic Pathways and Knowledge Gaps**

Lipid Component	Metabolic Pathway	Clearance	Persistence/Risks	Knowledge Gaps
<b>Cholesterol</b>	Sterol metabolism to HDL/LDL; possible oxidation to oxysterols	Likely recycled endogenously, but this has not been studied	Oxysterols are immunologically active; <sup>158</sup> cholesterol crystals form depending on saturation, may contribute to CARPA <sup>14</sup>	No direct oxysterol data available after LNP uptake,
<b>DSPC (helper lipid)</b>	Phospholipase degradation is incorporated into membranes.  Displays unusual rigidity. <sup>159</sup> , favours bleb formation. <sup>27, 35</sup>	Days-weeks can accumulate in the liver, spleen, heart, kidney, lung <sup>160</sup>	DSPC can produce phospholipid-derived products that may alter membrane structure and stability <sup>161; 162</sup> .  Can affect lipid raft integrity and functions like increased T-cell signaling <sup>163</sup>  May also lower immune host surveillance. <sup>164</sup>	The effect on lipid rafts across tissues is not fully understood nor thoroughly examined with repeated dosing.
<b>PEGylated lipids (ALC-0159, PEG-DMG)</b> <i>See text</i>	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) <sup>82</sup> but not in humans <sup>165</sup>	Human persistence and dose thresholds unclear; PEG allergy may limit LNP use for other indications <sup>15</sup>
<b>Ionizable lipids (ALC-0315, SM-102)</b> <i>See text</i>	Hydrolysis to amines/fatty acids; branched tails resist $\beta$ -oxidation (ALC-0315>>SM-102)	Slow hepatic clearance; ALC-0315 takes up to 3 weeks to fully metabolize ( $t_{1/2}$ =139 hrs) <sup>43</sup>  SM-102 half-life shorter at 7.3h <sup>37</sup>	Tissue persistence of metabolites, including in mitochondria.  In silico experiments demonstrate membrane embedding, which may enhance persistence <sup>118, 124</sup>  ROS production, cytokine release, membrane disruption or tearing	Identity of metabolites; long-term accumulation not well studied
<b>Lipid Adducts</b> <i>See text</i>	Reactive amines/aldehydes covalently bind proteins and nucleic acids <sup>166</sup>	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.

715 \*HDL=high density lipoprotein; LDL=low density lipoprotein; PEG=pegylated lipid CARPA=complement activation reaction  
716 pseudoallergy; ROS=reactive oxygen species

## 718 2.8 Drug Interactions

719

720 Although regulatory agencies generally assume vaccines do not cause drug–drug interactions, early evi-  
721 dence suggests this may not hold for modRNA–LNP vaccines. Case reports and cohort analyses docu-  
722 ment clinically relevant changes in clozapine pharmacokinetics post-vaccination, in some cases leading  
723 to neutropenia and hospitalization [167-169](#). The mechanism is consistent with inflammation-mediated  
724 suppression of CYP450 enzymes, particularly CYP1A2 and CYP3A4, central to clozapine metabolism.  
725 [170](#)

726

727 While most effects appear mild or transient [171](#), therapeutic drug monitoring has been recommended  
728 for narrow-index drugs like clozapine [172](#). Substantial increases in escitalopram, fluoxetine, trazodone,  
729 and quetiapine levels have also been reported, [173](#) and a case of neuroleptic malignant syndrome with  
730 adrenal insufficiency occurred in a patient on valproic acid [174](#).

731

732 This concern extends beyond psychotropic or antiepileptic medications. Inflammatory cytokines such  
733 as IL-6, TNF- $\alpha$ , and interferon- $\gamma$ , induced by both infection and vaccination, down-regulate multiple  
734 hepatic CYP isoenzymes [175](#). Clinical studies in COVID-19 patients have shown that elevated C-  
735 reactive protein levels are associated with reduced metabolism of midazolam and tacrolimus, potentially  
736 leading to oversedation or immunosuppressant toxicity.

737

738

739 Because many common drugs, such as statins, benzodiazepines, antiepileptics, and  
740 immunosuppressants, are CYP3A4 [176](#) or CYP2C9 substrates [175](#), transient suppression of these  
741 pathways after vaccination could alter drug exposure in a clinically significant way. Yet regulators do  
742 not currently require pharmacokinetic interaction studies for vaccines, leaving these risks under-  
743 characterized, and clinicians may be unaware.

744

745 The possible pharmacodynamic interactions with lipid nanoparticles themselves may be overlooked.  
746 Recent work has shown that small-molecule drugs can directly influence endosomal trafficking and  
747 escape. Tricyclic cationic amphiphilic drugs (TCADs), such as tricyclic antidepressants, first-generation  
748 antipsychotics, and certain antihistamines, share structural features with ionizable lipids and have been  
749 repurposed in experimental systems to improve intracellular delivery of nucleic acids. [177](#) In animal  
750 studies, nortriptyline-containing “CADosomes” demonstrated delivery efficiency without the need for  
751 synthetic ionizable lipids [178](#), suggesting a structural and functional overlap between cationic  
752 amphiphilic drugs (CADs) and LNP excipients. While this could be exploited experimentally to  
753 improve delivery, it also raises the question of whether patients already taking CAD-class drugs (e.g.,  
754 antipsychotics, some antidepressants, etc.) may experience altered LNP trafficking or immune  
755 responses following vaccination or face toxic effects.

756

757 Other drug classes have also been implicated in modifying  
758 endosomal escape. Proton pump inhibitors, such as  
759 esomeprazole, have recently been investigated as adjuvants  
760 in preclinical LNP formulations, by raising endosomal pH,  
761 enhancing LNP delivery and immune responses via lysoso-  
762 mal destabilization in murine models. [179](#) PPI use has also  
763 been shown to increase risk of severe COVID-19 out-  
764 comes. [180](#) For chronic PPI users, altered transfection effi-  
765 ciency could amplify AEs, warranting caution and further  
766 research into vaccine safety profiles. These findings sug-  
767 gest that the LNP itself functions as a cationic amphiphilic  
768 drug (CAD), and its toxicological profile may overlap with  
769 that of CAD drugs [181](#). Endosomal escape enhancers,  
770 whether intentionally incorporated into formulations or  
771 present coincidentally in patient medications, can increase  
772 cytosolic release but also exacerbate lysosomal damage and  
773 galectin-mediated inflammation. [134, 182](#) This dual potential  
774 to both enhance efficacy and intensify toxicity underscores  
775 the need for pharmacovigilance analyses examining out-  
776 comes in patients on CADs, or other drugs at the time of  
777 vaccination. Together, these observations argue that vac-  
778 cine–drug interactions are not only possible but clinically  
779 relevant, and their continued neglect in regulatory assess-  
780 ment represents a substantial oversight.

781

## Vaccine-Drug Interactions May Be Underrecognized

- Pharmacokinetic and pharmacodynamic interactions are not regularly evaluated during vaccine development, as regulatory agencies generally assume there are no clinically significant vaccine-drug interactions.<sup>1</sup> However, rare case reports with influenza<sup>2</sup> and more commonly with COVID-19 mRNA-LNP vaccines challenge this assumption
- Case reports include
  - clozapine toxicity
  - altered serum levels of antiepileptics
  - inflammation-associated cytokine CYP450 suppression
- LNPs exhibit pharmacodynamic interactions
  - Altered endosomal trafficking especially in those taking antipsychotropic drugs
  - Other medications implicated (i.e. proton pump inhibitors)

### 3 Challenges, Gaps and Future Directions

783

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

788

**TABLE 4: Critical Uncertainties and Challenges of modRNA-LNP Technology**

Category	Documented Challenges	Broader Uncertainties	Implications
<b>Physico-chemistry</b>	Reliable characterization of particle size, encapsulation, payload, and stability remains challenging <sup>183</sup> No benchmark lipid formulation exists. <sup>52</sup> Standards and assays are continually evolving. <sup>20, 24, 49</sup>	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.
<b>Biodistribution and Transfection</b>	Conflation of biodistribution with gene expression, <sup>53, 58</sup> widespread off-target distribution. <sup>60, 184</sup>	Limited ability to achieve tissue-specific delivery beyond the liver. <sup>77</sup> Transfection is random and uneven; emerging tools like single-cell Nano mapping are still experimental. <sup>60</sup>	Therapeutic outcomes and adverse effects remain difficult to predict; single-cell methods are needed.
<b>Protein Corona</b>	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. <sup>67</sup> Measurement remains challenging. <sup>185</sup>	Patient variability (including age, sex, and comorbidities) <sup>186</sup> complicates predictability.	Results in nonlinear uptake, increased risk of immune activation, and reduced targeting accuracy.
<b>Endosomal Escape</b>	Low efficiency (1-15%); high stochastic cell-to-cell variability; <sup>117, 127</sup> “dark hour” between escape and gene expression is not well understood. <sup>121</sup> Attempts to improve endosomal escape raise toxicity <sup>134</sup> LNPs alter cell membranes <sup>65, 122</sup>	Escape remains nonlinear, context-dependent, with a bottleneck that limits potency <sup>116, 117, 127</sup>	“Bottleneck” increases unpredictability of therapeutic efficacy. <sup>116</sup> Non-linear and context-dependent; bell-shaped curve. <sup>135</sup>
<b>Persistence and Lipid Metabolism</b>	PEG-lipid immune effects, <sup>74</sup> possible lysosomal stress, <sup>6, 127</sup> unknown toxic ionizable lipid metabolites, <sup>140</sup> and cholesterol crystallization <sup>14</sup> , 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
<b>Manufacturing and stability</b>	Documented batch heterogeneity regarding variable LNP size and RNA integrity; <sup>43</sup> instability in plasma; <sup>35</sup> post-injection remodelling; <sup>37</sup> cold-chain and scale-up challenges. <sup>187</sup>	Effects of instability on potency and safety remain uncertain.	Variable potency, potential side effects, and administrative challenges can compromise efficacy

	Lipid adduct formation	and increase adverse event risk.  Lipid adduct formation could further influence therapeutic outcomes and AE profile
<b>Drug Interactions</b>	<p>Case reports of clozapine toxicity <sup>167</sup> and altered antiepileptic levels after vaccination; <sup>188</sup> CYP450 suppression during inflammation is well established. <sup>176</sup></p> <p>The degree to which mRNA–LNP vaccines transiently alter drug metabolism (CYP3A4, 2C9, 1A2) or interact with lysosomotropic drugs (e.g., psychotropics) or other drugs remains unknown</p>	Vaccine–drug interactions are not systematically assessed; potential underrecognized risk for patients on narrow therapeutic index drugs (clozapine, tacrolimus, midazolam).
<b>Regulatory and Data Gaps</b>	<p>LNPs have adjuvant-like activity, as acknowledged by the FDA, <sup>189</sup> but were classified as excipients in regulatory submissions.</p> <p>Pfizer/BioNTech’s Comirnaty lacked transfection and target-cell-specific data, and CARPA testing was not described. <sup>43</sup></p> <p>The FDA did not evaluate Moderna’s LNPs separately. <sup>57</sup></p> <p>Lipid adducts are assessed as process-related impurities, not as new toxicological impurities</p> <p>New EMA guidelines on the quality of mRNA vaccines reinforce the classification of excipients. <sup>30</sup></p> <p>Current regulatory framework does not capture transfection and nanoparticle-specific risks; transparency and public trust remain unresolved issues.</p> <p>Drug interactions were not assessed</p>	<p>Incomplete safety evaluation, risks confusion, and skepticism.</p> <p>Advanced methods, including proteomics <sup>30, 190</sup> and lipid profiling, <sup>191</sup> are needed to fully characterize LNP–mRNA formulations and their pharmacological and immunostimulatory properties.</p> <p>Secondary pharmacology, drug interactions, assessment of long term and lipid adduct risks recommended for regulatory assessment of LNPs</p>



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

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

798

799 These concerns are not isolated technical issues but interconnected challenges. The physicochemical  
800 heterogeneity and the dynamic structure of LNPs influence biodistribution, which in turn depends on  
801 the dynamic protein corona; meanwhile, inefficiencies in endosomal escape exacerbate variability in  
802 therapeutic outcomes. The toxicological dynamics of the extracellular LNPs are unstudied <sup>6</sup>, as is the  
803 possibility of lysosomal stress or dysfunction which is increasingly linked to numerous diseases, such as  
804 neurodegenerative disorders. <sup>192</sup> Likewise, patient heterogeneity amplifies these uncertainties, making it  
805 unreasonable to expect uniform efficacy or safety across populations and making it difficult to predict  
806 clinical response or an adverse event profile. Gaps in regulatory requirements, such as critical quality  
807 attributes, target-cell specificity, biodistribution,<sup>53</sup> immune effects, drug interactions, and long-term tox-  
808 icology, further undermine public confidence and complicate post-marketing safety and surveillance.

809

810 We assert that the interplay between protein corona composition, cellular uptake pathways, endosomal  
811 escape and lipid metabolism critically influences cell tropism, protein production, and the stability of

812

813 both the lipid and RNA components. These aspects should be carefully considered and require further  
814 investigation.

815

816 Given the dependencies shown, it is worth questioning whether parameters reliant on highly individual  
817 physiological factors, such as age-related metabolic changes, pre-existing conditions, medications, base-  
818 line protein levels, or temporal fluctuations in protein concentrations, can be effectively controlled or  
819 standardized.<sup>[193](#), [194](#)</sup>

820

821 Furthermore, these factors are inherently difficult to quantify and measure because they vary on an in-  
822 dividual basis and because in vitro measurements do not always reflect the in vivo behaviour of this  
823 technology and its immunogenic effects,<sup>[195](#)</sup> highlighting the complexity of translating LNP designs  
824 across biological contexts and the need for more predictive evaluation strategies. This presents funda-  
825 mental challenges for the translation of LNP-based therapeutics into clinical practice.

826

## 827 **Discussion**

828

829 Looking ahead, various strategies are being explored to address the unpredictability of current mo-  
830 dRNA–LNP systems. One approach involves developing liposomal LNP hybrids, which may lower bi-  
831 ocorona complexity and enable extra-hepatic targeting.<sup>[75](#)</sup> Exosome-inspired or engineered extracellular  
832 vesicles offer another promising avenue,<sup>[131](#)</sup> leveraging their natural ability to cross physiological barriers  
833 and evade immune clearance.<sup>[125](#)</sup>

834

835 On the chemistry front, new classes of ionizable lipids with improved degradation profiles are being  
836 developed to reduce persistence and toxicity.<sup>[9](#), [140](#), [182](#)</sup> Simultaneously, advances in single-cell mapping  
837 technologies aim to clarify stochastic uptake and expression at unprecedented resolution,<sup>[60](#), [117](#), [121](#), [135](#)</sup>

838

839 potentially making delivery more predictable. Improvements in assay methodology<sup>20,24</sup> and in formula-  
840 tions such as lyophilization<sup>31</sup> look promising. Together, these innovations and others suggest that alt-  
841 hough current formulations remain a biological “black box,” an expanding toolkit is being developed to  
842 potentially make modRNA delivery more controllable, targeted, and safer.

843

844 These uncertainties highlight the nonlinear and context-dependent nature of LNP-modRNA interac-  
845 tions, suggesting a pathogen-like effect on the cell beyond its inherent cytotoxicity. Insights from cationic  
846 amphiphiles such as antipsychotic drugs may enhance the understanding of these complex particles.  
847 <sup>164,181</sup> Progress will likely require integrating advanced *in vitro* and *in vivo* models, <sup>6</sup> single-cell resolution  
848 technologies, <sup>60</sup> and standardized analytical frameworks <sup>49,52</sup> to achieve this goal.

849

850 However, it must be considered that *in vitro* experiments with such a highly variable technology *in vivo*  
851 require a systems biology perspective. Neither membrane structural processes nor downstream signal  
852 transduction<sup>196,197</sup> follow linear dynamics. Additionally, incorporating longitudinal human data and  
853 comprehensive regulatory strategies will be crucial to ensure both efficacy and long-term safety. This  
854 will be a challenging task given the nonlinear dynamic nature of this technology. <sup>198</sup>

855

## 856 **Summary**

857

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

861

<b>Declaration of competing interest</b>  The authors declare that they have no competing interests.
<b>Author contribution</b>  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 and other AI-tools to improve the manuscript's readability. After using this tools, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.
<b>Materials</b>  All slides were created by F. Seger using BioRender (unless otherwise indicated) and are used under BioRender's <a href="#">Terms Of Service</a> and <a href="#">Academic License</a> .
<b>Acknowledgements</b>  Dr Susan Natsheh, MD for visuals and proofreading

862 **Methods**

863

864 This narrative review followed a structured literature search and critical synthesis approach.

865

866 **Search Strategy**

867

868 Databases searched included PubMed and Google Scholar (2018-2025) using the keywords “lipid nano-  
869 particles”, “ionizable lipids”, “mRNA vaccines”, “endosomal escape”, “protein corona”, “biodistribu-  
870 tion”, “drug interactions”, “lipid adducts”, “degradation”, “target cells”.

871

872 Regulatory documents from the EMA, FDA, and the Australian Therapeutic Goods Administration  
873 (TGA) were obtained from public or archived sources, including Freedom of Information requests.

874

## 875 **Selection Criteria**

876

877 Peer-reviewed studies, preprints from institutional servers, regulatory or pharmacopeial documents ad-  
878 dressing LNP composition, structure, analytical methods, critical quality attributes, biodistributions,  
879 pharmacodynamics, mechanistic and biophysical methods, or safety, including pharmacokinetic and  
880 pharmacodynamic drug-vaccine interactions, were included.

881

## 882 **Reference Chaining**

883

884 In addition to database searches, reference chaining was used to identify more relevant studies from the  
885 bibliographies of key publications. This approach helped capture regulatory documents, nanotechnol-  
886 ogy reports, and emerging pharmacology data not yet indexed in PubMed, allowing for a more compre-  
887 hensive synthesis across disciplines.

888

## 889 **Data Synthesis**

890

891 Findings were structured into four main domains: physicochemical, biological, pharmacological/phar-  
892 macodynamic, and safety/interactions. Cross-referencing was conducted to identify recurring mecha-  
893 nisms and unresolved knowledge gaps.

894

## 895    **Quality Control**

896

897    All quotations and regulatory attributions were independently verified for accuracy. No funding or ex-  
898    ternal editorial support was received.

899

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