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## More than a delivery system: the evolving role of lipid-based nanoparticles

Senjuti Karmaker,<sup>†</sup> Plinio D. Rosales,<sup>†</sup> Barath Tirumuruhan, <sup>†</sup> Amartya Viravalli<sup>†</sup> and Natalie Boehnke \*

Lipid-based nanoparticles, including liposomes and lipid nanoparticles (LNPs), make up an important class of drug delivery systems. Their modularity enables encapsulation of a wide range of therapeutic cargoes, their ease of functionalization allows for incorporation of targeting motifs and anti-fouling coatings, and their scalability facilitates rapid translation to the clinic. While the discovery and early understanding of lipid-based nanoparticles is heavily rooted in biology, formulation development has largely focused on materials properties, such as how liposome and lipid nanoparticle composition can be altered to maximize drug loading, stability and circulation. To achieve targeted delivery and enable improved accumulation of therapeutics at target tissues or disease sites, emphasis is typically placed on the use of external modifications, such as peptide, protein, and polymer motifs. However, these approaches can increase the complexity of the nanocarrier and complicate scale up. In this review, we focus on how our understanding of lipid structure and function in biological contexts can be used to design intrinsically functional and targeted nanocarriers. We highlight formulation-based strategies, such as the incorporation of bioactive lipids, that have been used to modulate liposome and lipid nanoparticle properties and improve their functionality while retaining simple nanocarrier designs. We also highlight classes of naturally occurring lipids, their functions, and how they have been incorporated into lipid-based nanoparticles. We will additionally position these approaches into the historical context of both liposome and LNP development.

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Department of Chemical Engineering and Materials Science, University of Minnesota, Twin Cities Minneapolis, MN 55455, USA. E-mail: nboehnke@umn.edu

<sup>†</sup> Denotes equal contributions.



Natalie Boehnke

her multidisciplinary background, focusing on next-generation tools to advance nanomedicine. Specifically, her group takes a data driven approach to nanocarrier design, developing barcoded and pooled screening approaches and leveraging bioactive building blocks to develop intrinsically therapeutic drug delivery systems.

## Introduction

Lipid-based nanoparticles have emerged as a groundbreaking platform for drug delivery over the past 60 years.<sup>1</sup> As the name suggests, lipid-based nanoparticles include nanoparticle constructs that are composed of lipids, including liposomes, lipid nanoparticles (LNPs), solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs).<sup>2,3</sup> In this review, we will limit our focus to liposomes and LNPs. The evolution of lipid-based nanoparticles began with the discovery of the liposome structure in the 1960s<sup>4</sup> and has progressed to their development into sophisticated therapeutic nanocarriers. The modularity, biodegradability and biocompatibility of lipid-based nanoparticles have made them the gold standard for drug delivery. Lipid-based nanocarriers currently make the largest proportion of nanocarriers in clinical trials.<sup>5</sup>

Researchers in the field have primarily focused on the use of external modifications, such as peptides, proteins, and polymers, to impart lipid-based nanoparticles with additional functionality, including the use of targeting and immunomodulatory motifs.<sup>6-8</sup> This additive approach to designing functional nanocarriers can increase nanocarrier complexity and result in scale up and manufacturing challenges, hindering translational efforts.<sup>9,10</sup> Moreover, from an 'atom economy'

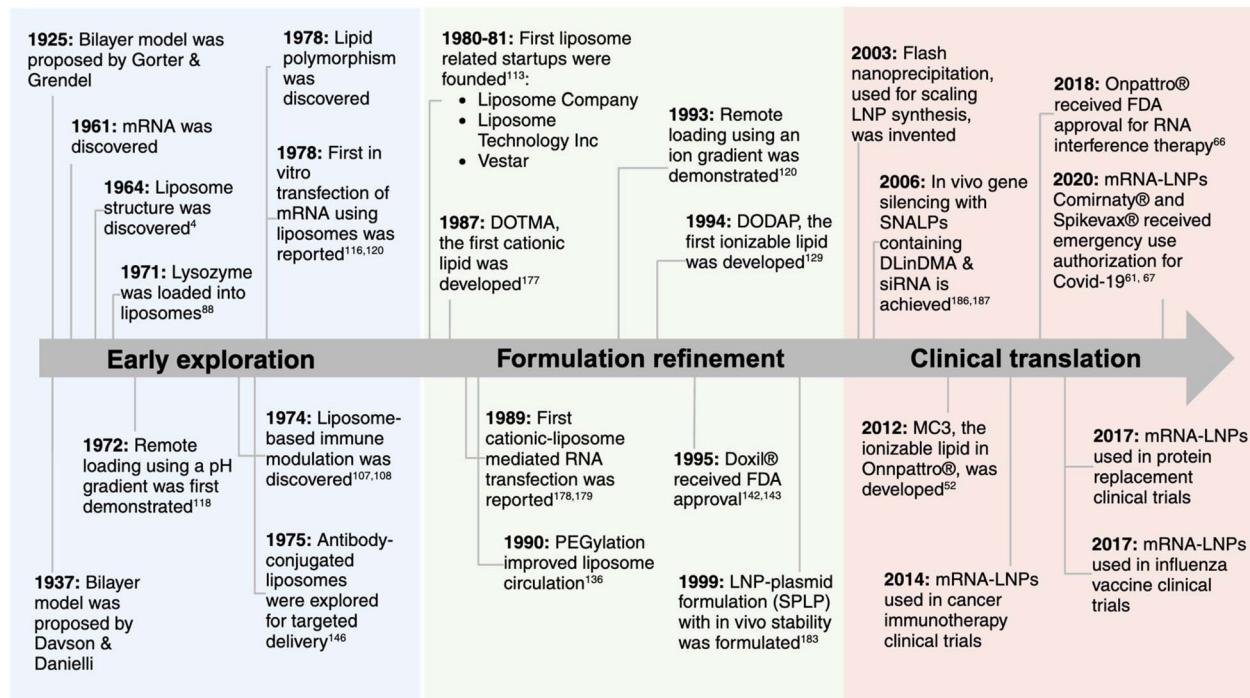


Fig. 1 Timeline of key discoveries and advancements in lipid-based nanoparticle development.

perspective, the majority of the nanocarrier is composed of lipids that serve only a carrier function, while the therapeutic itself constitutes an additional, and often smaller, portion.<sup>11</sup> This prompted the question whether an alternative approach to synthesizing functional lipid-based nanoparticles that leverages the inherent functionality of the lipid building blocks could address the limitations of additive nanocarrier design.

In this review, we focus on recent advances in lipid-based nanoparticle design which leverage existing understanding of lipid structure and function to engineer structurally simple yet intrinsically multifunctional carriers. The review begins by providing brief overviews of liposomes and LNPs in addition to a historical context of the development of these nanocarriers (Fig. 1). We then categorize recent formulation strategies into two key approaches – (1) incorporation of bioactive lipids and (2) modifying nanoparticle physical properties. These strategies enable functionalities beyond delivery such as targeting, immunomodulation, and antimicrobial activity. Specifically, we further classify bioactive lipid incorporation into two sub-categories, naturally occurring lipids and synthetically modified lipids. We highlight the vastness of naturally occurring lipids that are underexplored and can be leveraged to design functional nanoparticles. Ultimately, we hope this review inspires a multi-disciplinary effort in paving the way for the next generation of lipid-based nanoparticles.

## General overview of liposomes

Liposomes consist of amphiphilic phospholipid molecules that organize into bilayers in aqueous environments.<sup>12,13</sup> Due

to their amphiphilic nature, liposomes serve as versatile nanocarriers capable of transporting both hydrophobic and hydrophilic molecules, including small molecules, nucleic acids, proteins, and imaging agents, with hydrophobic drugs integrating into the lipid bilayer and hydrophilic drugs encapsulated within the aqueous core.<sup>14,15</sup>

### Liposome composition and structure

**Lipids.** The primary components of liposomes are phospholipids, which are biodegradable, biocompatible, and closely resemble the lipids present in the cell membranes.<sup>10</sup> Phospholipids such as phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylglycerols (PG), and phosphatidic acid (PA) are commonly used for the preparation of liposomes.<sup>14</sup> Phospholipids are predominantly selected for drug delivery applications based on their impact on stability, drug encapsulation and release, pharmacokinetics, and pharmacodynamics.<sup>16-19</sup>

**Cholesterol.** Cholesterol is incorporated to improve phospholipid packing, modulate membrane fluidity, and enhance bilayer stability. Below the transition temperature ( $T_m$ ) of the phospholipid, cholesterol decreases membrane fluidity,<sup>20,21</sup> reduces membrane stiffness, and promotes liposome deformability.<sup>22-24</sup> Additionally, cholesterol aids in drug encapsulation and retention<sup>19,25-27</sup> in the presence of serum by limiting phospholipid removal from bilayers by high density lipoproteins, thereby maintaining structural integrity.<sup>28</sup> Some studies have also shown that cholesterol can increase the circulation time of liposomes by decreasing the serum opsonin interactions.<sup>26</sup>

**Structure.** The structure of liposomes depends on the method of synthesis.<sup>12,29</sup> They can be classified into unilamellar vesicles (ULVs) with a single bilayer, oligolamellar vesicles (OLVs) containing two to five bilayers, multilamellar vesicles (MLVs) with multiple concentric bilayers and multivesicular vesicles (MVs). ULVs are further divided into small unilamellar vesicles (SUVs, 20–100 nm), large unilamellar vesicles (LUVs, 100–1000 nm), and giant unilamellar vesicles (GUVs, >1000 nm).<sup>14,17,30</sup> Liposomes in the 50–200 nm range, such as SUVs (e.g., Doxil),<sup>31</sup> smaller OLVs (e.g., Vyxeos with a bilamellar structure)<sup>32</sup> and smaller LUVs and MLVs have seen greater clinical success and are preferred due to their uniform drug encapsulation, release kinetics, and prolonged circulation times by escaping the phagocytic system.<sup>12,33,34</sup> While these structures are primarily used for therapeutic applications, GUVs serve as models for cell studies.<sup>35</sup> MLVs and MVs offer increased lamellarity and lipid content, enhancing their capacity for therapeutic loading (e.g. DepoCyt and DepoDur).<sup>30,36</sup> For instance, MVs are well-suited for encapsulating combination therapeutics and macromolecules, offering superior mechanical stability that enhances stability in physiological conditions while enabling a slower, controlled release of encapsulated cargo from the outer layers compared to unilamellar counterparts.<sup>37</sup>

### Anti-fouling coatings

Despite their advantages, liposomes face challenges like short circulation times and limited stability due to rapid clearance by the mononuclear phagocyte system (MPS). To overcome this, anti-fouling polymers, like polyethylene glycol (PEG), can be added to create “stealth” liposomes, providing steric protection that prolongs circulation by evading opsonization.<sup>12,38–40</sup> However, in recent years, PEG and PEG-lipid conjugates have been associated with drawbacks, such as hypersensitivity reactions with repeated administration and complement system activation, which can amplify the clearance of these nanocarriers.<sup>41–44</sup> To mitigate these issues, alternative materials, such as polysialic acids, glycoproteins, zwitterionic polymers, and polysaccharides, are being explored to enhance the circulation time of liposomes.<sup>45–47</sup>

### Targeting motifs

Liposomes can be functionalized with small molecule ligands, carbohydrates, proteins and nucleic acid aptamers to enhance the targeting of liposomes, thereby improving drug efficacy and reducing side effects.<sup>14,48</sup> Current efforts are focused on decorating the surface of liposomes with ligands that can target and bind to receptors overexpressed on the surfaces of cancer cells. Common targets include transferrin receptor (TfR), folate receptor (FR), and the epidermal growth factor receptor (EGFR).<sup>14</sup> Additionally, stimuli-responsive liposomes have been designed to release their cargo in response to specific environmental triggers such as pH, temperature, enzymes, or redox conditions within the tumor microenvironment, reducing premature degradation of the cargo and enhancing treatment efficacy.<sup>49,50</sup>

Despite extensive efforts to enhance liposome functionality through external modifications, challenges remain in evading premature clearance, addressing receptor expression heterogeneity, and minimizing ‘on-target, off-site’ effects, where liposomes bind to the intended receptor but accumulate in tissues other than the intended target.

As an alternative approach to targeted and controlled delivery, this review will focus on how lipid-based nanoparticle formulation components can be strategically selected, designed, and incorporated to modulate nanocarrier properties. This, in turn, can improve targeting efficacy, immune response, stability in blood circulation, antimicrobial activity and additional functions such as antitumor effects, transfection efficiency and stimuli-responsiveness.

## General overview of LNPs

In contrast to liposomes, LNPs are considered to have a less ordered structure. The primary focus of LNP development has been on nucleic acid encapsulation and delivery, with current efforts largely focused on synthesizing LNP formulations for delivery beyond the liver.

### LNP composition and structure

**Ionizable cationic lipids.** Ionizable lipids play a key role in mediating encapsulation of nucleic acid cargo through the formation of electrostatic interactions with negatively charged nucleic acids, which enable the efficient complexation and encapsulation of the nucleic acid cargo within the LNP structure.<sup>51</sup> The apparent  $pK_a$  of ionizable lipids is tailored so that the lipid remains neutral at physiological pH (~7.4) but becomes protonated (positively charged) at acidic pH levels, such as those found in the endosomes (pH ~5.5–6.5), to facilitate successful cargo release and transfection.<sup>52–54</sup>

**Cholesterol.** Similar to liposomes, cholesterol is an essential component of LNP formulations because of its role in the formation of RNA-lipid complexes at neutral pH. Cholesterol, along with the helper lipid DSPC, forms lipid bilayers in close proximity which sequester RNA in the formation of LNPs.<sup>55</sup> This impacts encapsulation of nucleic acid, particle size and structural stability.<sup>55,56</sup> Cholesterol analogs can be incorporated in LNPs to modulate particle properties such as stability and encapsulation of cargo as well as subsequent transfection efficiency.<sup>57–59</sup>

**Helper lipids.** Helper phospholipids increase LNP structural stability and prevent premature release of nucleic acid cargo. They also aid in membrane fusion processes.<sup>60</sup> The most common phospholipids include 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), which is used in current FDA-approved LNP formulations,<sup>61</sup> and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE).<sup>62,63</sup>

**PEG-lipids.** As in liposomes, PEG-lipids are used to improve LNP stability and extend circulation times.<sup>55,64,65</sup> Examples of PEG-lipids in LNP systems include DMG-PEG as formulated in

Onpattro and Spikevax<sup>66,67</sup> and ALC-0159 as formulated in Comirnaty.<sup>61</sup>

**Structure.** The morphology and structure of LNPs has not yet been definitively established, though LNPs are usually represented as ionizable lipids arranged in an inverted micellar structure around nucleic acid cargo.<sup>68</sup> The structure of LNPs has been shown to be highly dependent on lipid components, lipid composition, type of cargo and synthesis method<sup>68</sup> and is being investigated *via* various methodologies including cryo transmission electron microscopy (CryoEM),<sup>69,70</sup> small X-ray scattering (SAXS),<sup>71</sup> and molecular dynamics (MD) simulations.<sup>72</sup>

### Targeting strategies

Similar to liposomes, LNPs can be functionalized externally using ligands like antibodies, proteins, and aptamers to enhance LNP targeting.<sup>73–76</sup> For instance, Palanki *et al.* were able to actively target the CD45 receptor for in-utero delivery of targeted ionizable lipid nanoparticles.<sup>77</sup> Aptamers have gained significant interest due to their size (6–30 kDa), which enables them to bind to smaller targets or hidden binding domains which are inaccessible for antibodies.<sup>78,79</sup>

Despite various efforts to incorporate external modifications using ligands, receptors, or aptamers to lipid-based nanoparticles to improve functionality, challenges remain in targeted delivery, extrahepatic delivery in the context of LNPs, and immunomodulation and evasion. This review will emphasize how lipid-based nanoparticle formulations can be leveraged to modulate biological responses and functions using both natural and synthetic lipids as building blocks. This, in turn, can improve targeting efficacy, immune response, stability in blood circulation, antimicrobial activity and additional functions such as transfection efficiency and stimuli-responsiveness.

## Timeline of lipid-based nanoparticle development

### Early exploration

The early development of lipid-based nanoparticles finds its roots in the study of biological membranes and the fundamental inquiry of “what holds biological membranes together”. In 1964, Alec Bangham published the first electron microscopy image of vesicles formed by ovolecithin, a natural phospholipid extracted from egg, following an accidental discovery made while studying the role of phospholipids in blood clotting.<sup>4</sup> Initially referred to as “multi-lamellar smectic mesophases” or “banghosomes”, these vesicles were later termed “liposomes” by Gerald Weissmann.<sup>15</sup> Bangham’s pioneering work on ion sequestration and transport in these vesicles sparked interest in using phospholipid liposome models for biological membranes and as potential drug delivery vehicles.<sup>80,81,82–87</sup>

The progression from liposome discovery to their application in drug delivery unfolded at a remarkable pace. In 1970, Gerald Weissmann provided the first experimental evidence of therapeutic loading into nanoparticles by encapsulating lysozyme into liposomes, positing their use for enzyme replace-

ment therapy.<sup>88</sup> Subsequently, Brenda Ryman and Gregory Gregoriadis’ pioneering work reported the delivery of *Aspergillus niger amyloglucosidase* and radiolabeled albumin encapsulated in liposomes to liver and spleen—the main organs of the reticuloendothelial system (RES), marking the first nanoparticle biodistribution study.<sup>89</sup> Notably, the observed challenges associated with RES clearance of nanoparticles still impede clinical translation to this day. Subsequent studies rapidly explored the encapsulation of a range of therapeutics, including proteins, enzymes, hormones, and small molecules, targeting diseases like cancer, microbial infections, and metabolic conditions.<sup>90,91</sup>

In parallel, several early investigations also aimed at interpreting liposome interactions with the biological environment. *In vitro* studies investigated the effect of physicochemical properties such as composition, fluidity, charge, and size on liposome uptake mechanisms.<sup>92–95</sup> Building on these insights, *in vivo* studies revealed how these properties modulate pharmacokinetics and biodistribution of encapsulated therapeutic cargo.<sup>96–98</sup> This prompted the development of several liposome synthesis techniques to enhance these physicochemical properties including ethanol dilution, thin lipid membrane hydration, and high-pressure extrusion.<sup>99–106</sup> Around the same time, the concept of using liposomes for immune modulation was introduced by Gregoriadis and Allison, who found that an inactivated form of diphtheria toxoid loaded into negatively charged liposomes elicited higher antibody responses compared to free antigens or antigen loaded into positively charged liposomes.<sup>107,108</sup>

### Formulation refinement

In the 1980s and 1990s, efforts to resolve key challenges in liposome technology, including drug leakage<sup>97,109–111</sup> and immune detection, to enhance intracellular delivery,<sup>112</sup> and to develop scalable synthesis protocols paved the way for the founding of companies like The Liposome Company, Liposome Technology Inc., Vical, and Acuitas, and the first clinical translation of lipid-based nanotherapeutics.<sup>112–114</sup>

Research efforts focused on optimizing formulations to enhance drug loading and retention, including the incorporation of cholesterol to modulate the nanoparticle’s membrane fluidity and bilayer thickness.<sup>115,116</sup> Another change was the shift from using lipids that were solids in physiological temperatures instead of fluids, which reduced drug leakage and liposome degradation.<sup>117</sup> Remote loading was also introduced as an approach that uses transmembrane pH or ion gradients to achieve high drug-to-lipid ratio loading and up to 100% encapsulation efficiency.<sup>118–125</sup>

There was also a focus to address the premature clearance of administered liposomes, including pre-dosing with ‘empty’ liposomes to block the RES system<sup>126–128</sup> and size modifications.<sup>129,130</sup> Interestingly, the former concept was revisited in Chan *et al.*’s recent work identifying a nanoparticle dose threshold for blocking liver’s Kupffer cells.<sup>131</sup> Concurrently, in late-1970s, it was hypothesized that liposome opsonization by serum proteins causes accumulation in the

RES organs.<sup>132–134</sup> Early reports of increased liposome circulation half-life involved surface modifications with monoasialo-glycoprotein GM1 and substituting egg PC with sphingomyelin.<sup>135</sup> These ‘stealth’ liposomes avoided RES clearance. In 1990, Klibanov *et al.* reported in their seminal work that grafting polyethylene glycol (PEG) chains onto the liposome surface enhanced circulation,<sup>136</sup> using hydrophilic interactions to repel opsonins.<sup>137–141</sup>

The cumulative outcome of addressing drug leakage *via* remote loading and clearance *via* PEGylation led to the development of Doxil, doxorubicin-loaded PEGylated liposomes, the first FDA-approved nanoparticle-based therapeutic in the US in 1995 after successful trials demonstrating prolonged circulation and improved safety.<sup>142,143</sup> The rapid translation of liposomes from discovery to FDA approval in just thirty years underscores the potential of nanoparticles as drug delivery vehicles.

### Intracellular and targeted delivery

The use of nanocarriers to serve as ‘magic bullets’<sup>144,145</sup> and improve accumulation of therapeutic cargo at target disease sites has been a long-standing challenge in the field. Targeted nanocarriers have traditionally been designed with external targeting motifs. This concept originated from attempts to improve intracellular delivery of liposomes, which were substantially larger and more complex compared to biomolecules typically endocytosed by cells. Initial attempts focused on the use of antibodies to enhance the homing of nanoparticles and utilize receptor-mediated endocytosis for uptake.<sup>146–150</sup> This concept, which is referred to as active or ligand-based targeting, was expanded to include targeting of cell surface receptors *via* the addition of ligands to nanoparticle surfaces to facilitate localization to target tissues, disease sites, and cell populations. Many classes of molecules capable of targeting cell surface receptors, including vitamins, peptides, and carbohydrates, have been since explored. Lipid-based nanoparticles modified with tissue and cell-penetrating peptides, polysaccharides, and proteins are a few of the notable strategies that have been explored for clinical use.<sup>151–167</sup> However, clinical translation remains elusive because of factors including toxicity, difficulty navigating biological barriers, serum protein interaction, and disease specific heterogeneities.<sup>168–170</sup>

### Lipid-based nanoparticles for nucleic acid delivery

In addition to small molecule delivery and targeting efforts, the field quickly recognized the potential of lipid-based nanocarriers to deliver a different class of therapeutics: nucleic acids. Early efforts to encapsulate nucleic acids in nanoparticles showed promise, but challenges including low encapsulation efficiency and charge-based repulsion between anionic nucleic acids and liposomes presented early barriers to translation.<sup>171–176</sup> In 1987, Phil Felgner created lipoplexes that achieved 100% DNA encapsulation by complexing DNA with the permanent cationic lipid DOTMA.<sup>177</sup> This process was refined to create Lipofectin, a commercial carrier with excellent *in vitro* DNA and RNA transfection.<sup>178,179</sup> However, the

*in vivo* success of cationic liposomes was hindered due to immunogenicity imparted by the overall positive charge of the nanocarrier.<sup>180–182</sup>

Stable plasmid lipid particles (SPLPs) represent an early attempt at mitigating immunogenicity. SPLPs are formed by detergent dialysis to incorporate small amounts of a permanent cationic lipid, DODAC, along with PC or PE, cholesterol, and PEG-lipids. SPLPs formed particles with a plasmid-encapsulated lipid complex enclosed by a bilayer.<sup>183</sup> SPLPs achieved 70% plasmid trapping efficiency while maintaining charge neutrality and demonstrated effective transfection at tumor sites in several murine models. However, the detergent dialysis process presented challenges for scale up, preventing its clinical translation. Despite this, the promising encapsulation efficiency and transfection outcomes of SPLPs helped evolve LNPs as specialized nucleic acid carriers, making SPLPs precursors to modern LNPs.

An alternative to permanent cationic lipids was the incorporation of ionizable lipids into LNPs. These lipids have apparent  $pK_a$  values slightly below physiological pH. This enables efficient nucleic acid complexation and retention of neutral nanoparticle surface charge while facilitating endosomal escape. The first ionizable lipid, 1,2-dioleoyl-3-dimethylammonium propane (DODAP),<sup>184</sup> was incorporated into LNPs for the delivery of antisense oligonucleotides, which achieved approximately 90% nucleotide encapsulation and a circulation half-life of around six hours in ICR mice.<sup>185</sup> These LNPs were termed stabilized antisense lipid particles (SALPs). The pH-dependent modulation of lipid charge continues to be leveraged to induce endosomal escape and enhance intracellular nucleic acid delivery, resulting in particles referred to as stable nucleic acid-lipid particles (SNALP),<sup>186,187</sup> optimized for encapsulating and delivering siRNA. DODAP used in SALPs evolved into the “DLinDMA” lipid family, from which 4-(*N,N*-dimethylamino)butyric acid (dilinoleyl) methyl ester, DLinMC3DMA (MC3), emerged as the present “gold standard” for siRNA delivery.<sup>52</sup>

In addition to nucleic acid delivery, current efforts are on the use of LNPs for the encapsulation and delivery of small molecules and protein therapeutics, including for CRISPR/Cas9 delivery.<sup>188</sup> Challenges in CRISPR/Cas9 delivery include the development of optimized delivery vectors, ensuring biocompatibility, and achieving efficient and targeted delivery to target cells.<sup>189,190</sup> LNPs have been successfully used to incorporate and deliver CRISPR/Cas9 technology,<sup>188,191–193</sup> including through the encapsulation of plasmid DNA (pDNA) encoding both Cas9 protein and gRNA or pDNA encoding Cas9 protein in combination with gRNA oligos, or Cas9 mRNA and gRNA.

### Clinical translation

Several liposomes were successfully translated into the clinic following Doxil® (1995) for a broad range of applications, including VYXEOS for acute myeloid leukemia, and Onivyde for metastatic pancreatic cancer.<sup>5,194,195</sup> In recent years, there has been growing interest in using liposomes in combination

therapy,<sup>196</sup> such as liposomal irinotecan and nituzumab for nasopharyngeal carcinoma, currently in phase 2 trials (NCT06414577), and liposomal nivolumab combined with pembrolizumab for platinum resistant ovarian cancer, currently in phase 3 (ARTISTRY-7, NCT05092360) trials.

On the LNP front, research showed that DLinMC3DMA-based LNPs preferentially accumulated in the liver due to Apolipoprotein B adsorption, which facilitates receptor-mediated endocytosis by hepatocytes.<sup>197</sup> Additionally, these LNPs were two orders of magnitude more potent than DLinDMA-containing LNPs in silencing transthyretin (TTR) protein in the liver.<sup>52</sup> Together, these factors resulted in the FDA approval of the first RNAi therapeutic, Onpattro®, in 2018.<sup>66</sup>

The COVID-19 pandemic spurred the unparalleled development and subsequent FDA approval of mRNA vaccines, Pfizer/BioNTech's Comirnaty®<sup>61</sup> and Moderna's Spikevax®,<sup>67</sup> using ionizable lipids ALC-0315 and SM-102, respectively.<sup>198–203</sup> Post-pandemic, LNP research has expanded to include cholesterol variants, biodegradable ionizable lipids, SORT lipids, and CRISPR/Cas9. These advances will be discussed in detail in the upcoming sections. Several LNP formulations that are currently in clinical trials have been reviewed previously.<sup>14,30,204–206</sup> Notable LNP formulations in clinical trials include Pfizer-BioNTech's BNT122-01 for colorectal cancer, Moderna's mRNA-4157 for melanoma, CureVac's CV7202 for rabies, and Moderna's mRNA-1345 for respiratory syncytial virus.

## Evolving role of lipid-based nanoparticle systems

It is interesting to note that, while the discovery and use of lipid-based nanoparticles originated from biological studies where researchers observed that lipid membranes enable cells to segregate their internal components from the external environment,<sup>207</sup> efforts to optimize lipid-based nanoparticles quickly shifted toward optimization of non-biological parameters, such as drug loading, stability and release, and the use of external modifications to add stealth and targeting capabilities. Lipids, as essential components of biological systems, serve a variety of functions, from modulating cell membrane fluidity to acting as signaling molecules that control cellular processes. The versatility and modularity of both lipid structure and function has inspired the creation of intrinsically functional nanocarriers, which we define as lipid-based nanoparticles in which a formulation component has been leveraged to serve a purpose beyond cargo encapsulation and stabilization. One main approach is through the use of bioactive lipids, which we define as both natural and synthetic lipids that serve a biological role or function. These formulations consist of relatively simple designs, helping to circumvent challenges associated with the complexity of multicomponent nanoparticles that rely on exogenous stealth and targeting modalities.

In this section we will explore how bioactive lipids have been incorporated into lipid-based nanoparticles as well as their subsequent uses. We will additionally provide examples on how formulation components can be used to modulate mechanical properties of lipid-based nanoparticles for added functionality, including tumor penetration, enhanced blood circulation, and immune cell targeting (Table 1).

### Use of natural bioactive lipids

The inherent biocompatibility and cellular recognition of naturally occurring lipids make them appealing building blocks for the design of intrinsically therapeutic nanocarriers (Fig. 2). In this section, we highlight how naturally occurring bioactive lipids are being used to create lipid-based nanoparticles for a range of biomedical applications. Beyond their role in maintaining structural integrity of cell membranes,<sup>207–210</sup> lipids are involved in energy metabolism,<sup>211–214</sup> signaling pathways,<sup>46,215,216</sup> protein-lipid interactions<sup>217–221</sup> and immune modulation.<sup>222,223</sup> Although not a direct function, the biosynthesis of lipids is a vital metabolic process essential for survival<sup>224–228</sup> (Table 2). Here we highlight key examples of how these natural lipids have been used to impart their functionality into nanocarriers.

**Targeting & delivery.** Natural interactions of lipids with cellular components can be leveraged to direct nanoparticles to specific tissues. For example, polyunsaturated fatty acids (PUFAs), which play a crucial role in disease progression<sup>229</sup> are readily taken up by tumor cells as biochemical precursors and energy sources. Incorporating PUFAs into nanoparticle formulations can leverage this property to enhance nanoparticle accumulation in tumors. Therapeutic nanoformulations containing PUFAs, including nano-emulsions, solid-lipid nanoparticles, and fatty acid-drug conjugates, are a growing area of study for applications against cancer and other diseases.<sup>230–232</sup> For instance, Ringhieri and coworkers demonstrated that liposomes made from cholesterol and egg PC, rich in unsaturated fatty acids, improved cisplatin accumulation in ovarian cancer (SKOV-3) and breast cancer (MCF7, MDA-MB-231) cell lines. These liposomes overcame drug resistance and were three times more effective than free cisplatin in killing cells.<sup>233</sup> Also, Bradley and colleagues improved the targeting potential of paclitaxel, a chemotherapeutic agent, by conjugating it to docosahexaenoic acid, which is readily taken up by tumor cells. This conjugation resulted in an eightfold increase in the area under the concentration-time curve and enhanced antitumor activity in the M109 lung tumor model compared to free paclitaxel at equimolar doses.<sup>234</sup>

Apart from PUFAs, glycolipids can be used to achieve tissue specific targeting by leveraging the specific interactions between the carbohydrate moieties of glycolipids and lectins or other carbohydrate-binding proteins on the surface of target cells.<sup>235,236</sup> For instance, Bae *et al.* incorporated a trehalose glycolipid into an LNP formulation by substituting half of the ionizable lipid and observed improved accumulation in spleen and lymph. The platform also demonstrated lower immunogenicity and biotoxicity compared to traditional LNP formu-

**Table 1** Examples of various natural and synthetic lipids used to create intrinsically functional nanocarriers

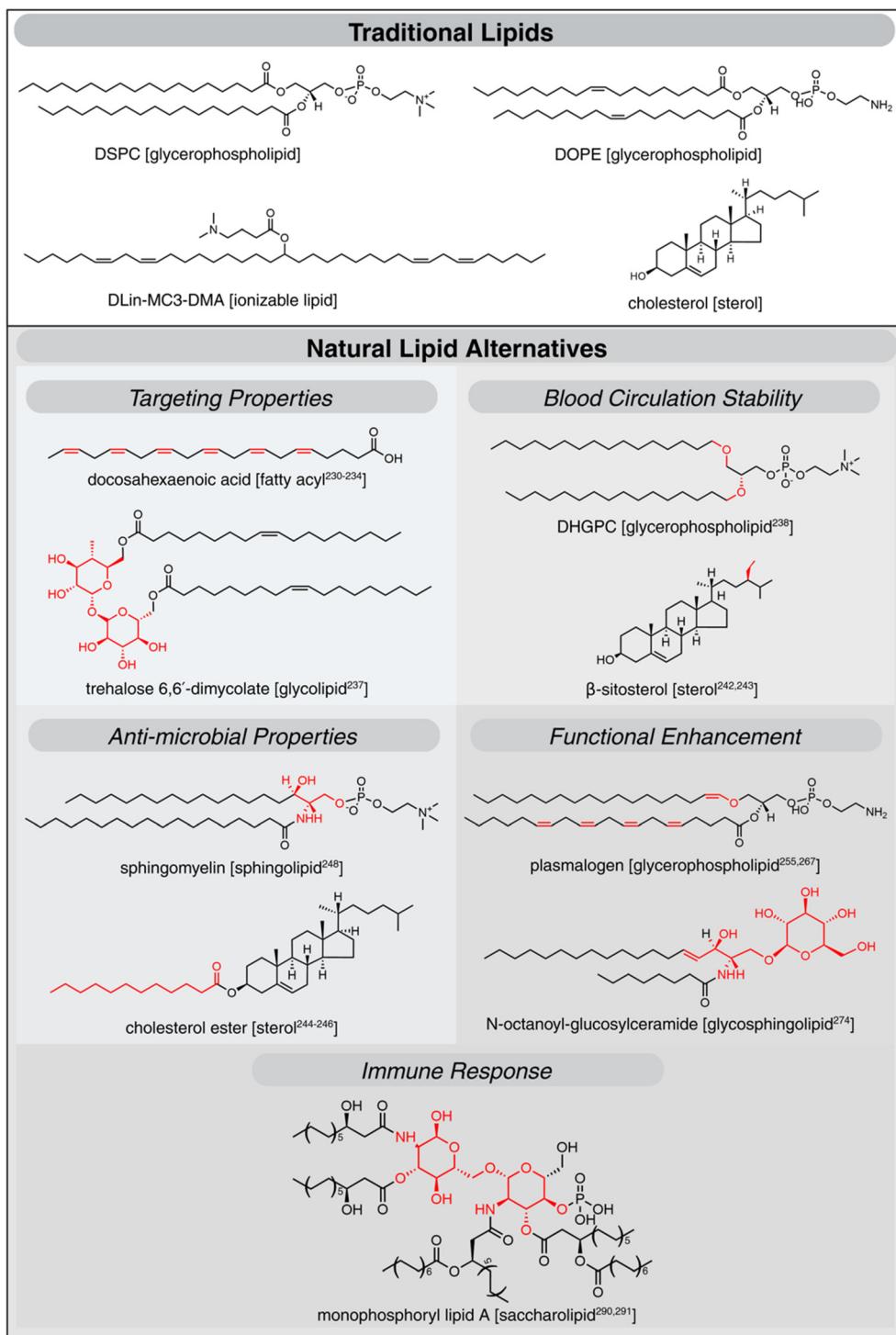
Type of lipid	Lipid replaced or formulation modified	NP form	Function(s) altered	Ref.
Natural	<i>N</i> -Octanoylglucosylceramide	Lipid nanovesicle	Biodistribution	274
	Monophosphoryl lipid A	Liposome	Immune response	290
	$\alpha$ GalCer-containing oligomannose-ooated liposomes (Mannotriose-DPPEconj)	Liposome	Immune response	294
	Saponin monophosphoryl lipid A (SMNP)	SMNP	Immune response	296 and 297
		LNP	Nucleic acid delivery, transfection	406
	Cholesteryl oleate, 7 $\alpha$ -hydroxycholesterol, 7 $\beta$ -hydroxycholesterol, and 4 $\beta$ -hydroxycholesterol	LNP	Nucleic acid delivery, transfection	57
	7 $\alpha$ -hydroxycholesterol	Liposome	Sequester bacterial endotoxins	248
	Sphingomyelin	Liposome	Therapeutic efficacy	233
	Egg PC	Liposome	Therapeutic efficacy	260
	Ceramide	Liposome	Therapeutic efficacy	274
Synthetic	<i>N</i> -Octanoylglucosylceramide	Liposome	Therapeutic efficacy, tumor penetration	22
	Cholesterol	Liposome	Therapeutic efficacy, tumor accumulation	277
	Archaeal ether lipids	LNP	Transfection, tumor accumulation	21
	Cholesterol	Liposome	Tumor accumulation	329
	Polyglycerol	Liposome	Circulation time	331
	Polyglycerol fatty acid esters	Liposome	Circulation time	332
	Folic-acid conjugated to DSPE-PEG	Liposome	Circulation time	334
	Polycarboxybetaine (PCB) variants	Liposome	Circulation time	335
	Polysialic acid-octadecyl dimethyl betaine (PSA-BS18)	Liposome	Circulation time	338
	CD47 mimicry self-peptide	Liposome	Immune response	400
Natural & synthetic	Dimethyldioctadecylammonium (DDA)	Liposome	Targeting	323
	Cetyltrimethylammonium bromide (CTAB) and DDAB	LNP	Transfection, nucleic acid delivery, immune response, immune evasion	301
	pSar BA12-50	LNP	Transfection, uptake, biodistribution, tumor accumulation	315
	DDAB	LNP	Transfection, nucleic acid delivery, immune response, immune evasion	300
	DOPE, DOPS, DOTAP	LNP	Transfection, therapeutic efficacy, biodistribution	362
	DODAP, DOTAP, 18PA	LNP		

lations in BALB/c mice. This was attributed to the reduction in ionizable lipid content, while the trehalose glycolipid stabilized the mRNA through hydrogen bonding, maintaining delivery efficacy.<sup>237</sup>

**Circulation.** Lipids, including ether phospholipids, sphingolipids and cholesterol analogs, can be used to improve nanoparticle stability, leading to improved circulation and biodistribution, which is essential for achieving prolonged therapeutic effects and reducing off-target toxicity. Ether phospholipids have an ether linkage at the sn-1 position instead of a traditional ester bond. This linkage confers hydrolytic stability and enhances resistance to metabolic conversion of liposomal formulations incorporating ether phospholipids, making them particularly useful for developing slow-release drug delivery systems. An example includes a study conducted by Derkx *et al.*, where the authors showed that multilamellar vesicles (MLVs) containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) roughly degraded 1.5 times quicker in the liver and spleen of male sprague-Dawley rats compared to MLVs containing its ether-linked 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHGPC) analog.<sup>238</sup> The use of sphingolipids in LNPs for improved circulation is explored in a study by Chander *et al.*, in which the authors showed that LNPs containing 40 mol% of the helper lipid egg sphingomyelin

extended the circulation time shown in a half-life of 3.7 hours relative to the 15 minutes half-life of the Onpattro LNP mRNA formulation in C57BL6 male mice. Increased accumulation in extrahepatic tissues, including spleen and bone marrow was also observed.<sup>239</sup> This was attributed to the unique morphology of ESM-containing LNPs, characterized by an outer monolayer enclosing an inner solid core. Additionally, these LNPs exhibited increased accumulation in extrahepatic tissues, such as the spleen and bone marrow.<sup>239</sup> More about extrahepatic delivery strategies are discussed in the **Use of Synthetic Bioactive Lipids** section.

As mentioned previously, cholesterol is included in many clinically approved nanoparticle formulations due to its ability to enhance membrane stability.<sup>2,240</sup> Aside from cholesterol, other cholesterol analogues like stigmasterol and  $\beta$ -sitosterol (plant sterols) can be used to modulate properties such as membrane fluidity and nanocarrier stability over a wider temperature range. For instance, stigmasterol is bulkier than cholesterol and has been found to reduce overall lipid packing, whereas incorporation of  $\beta$ -sitosterol has led to formation of liposomes with improved long-term stability compared to cholesterol-containing formulations.<sup>241</sup> Incorporation of ergosterol, a major sterol found in fungi, was found to broaden the temperature range over which the membranes undergo phase



**Fig. 2** Representative structures of natural lipids used for a range of drug delivery applications, with specific references to studies where they have been utilized. Structural variations compared to traditional components are highlighted in red.

transition, which enables the membranes to maintain fluidity and stability over wider temperature ranges.<sup>242,243</sup> More about how incorporation of cholesterol analogues into LNP formulations affects the transfection efficiency is included in the *Enhanced Functionality* subsection.

**Antimicrobial activity.** Lipids, such as free fatty acids and cholestrylin esters, including cholestrylin linoleate, are predominantly present in human nasal fluid and have been identified as substrates with inherent antimicrobial activity,<sup>244</sup> this property can be leveraged to develop nanoparticle platforms to

**Table 2** Lipid classifications based on their functions with examples

Function	Description	Examples	Ref.
Structural role	Lipids maintain structural integrity, fluidity, and functionality of cellular membranes providing a dynamic platform for cellular processes	Glycerophospholipids (PC, PE, PS, PI, PA), sphingolipids (sphingomyelin, glycosphingolipids,) sterols (cholesterol)	207–210
Energy source	Lipid molecules act as energy substrates, stored by the body and systematically broken down to release energy in the form of ATP as needed	Triglycerides, fatty acids	211–214
Signaling molecules	Lipids mediate signal transduction, regulate immune responses, and influence cell survival and proliferation	Phosphatidylinositol (PIs), sphingolipids (ceramides, S1P), eicosanoids (prostaglandins, leukotrienes), steroid hormones (cortisol, testosterone, estrogens)	46, 215 and 216
Protein-lipid interactions	Lipids enable peripheral, integral, and lipid-anchored protein interactions, facilitating cellular signaling, transport, and structural integrity	GPI-anchored proteins, actin-binding proteins, integral membrane proteins (e.g., EGFR)	217–221
Immune regulation	Lipids act as antigens, modulating immune responses and inflammation	Eicosanoids (prostaglandins, leukotrienes), glycosphingolipids ( $\beta$ -GlcCer, $\beta$ -GalCer), phospholipids (LPC, eLPA), polyunsaturated fatty acids (Omega-3, Omega-6)	222–223
Lipid biosynthesis	Dysregulation of biosynthesis pathways can lead to diseases like cancer, cardiovascular diseases, and neurodegenerative conditions	Ether phospholipids (plasmalogens), cholesterol, fatty acid derivatives	224–228

tackle bacterial infections.<sup>245,246</sup> In a study by Lam *et al.*, the authors screened liposomes composed of six different fatty acids—palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and arachidonic acid (C20:4)—along with four cholesteryl esters—cholesteryl palmitate, cholesteryl oleate, cholesteryl linoleate, and cholesteryl arachidonate—against both Gram-negative and Gram-positive bacteria. Their findings revealed that palmitic acid, stearic acid, and cholesteryl linoleate exhibited the highest antibacterial activity against multidrug-resistant bacteria. Additionally, the study demonstrated a synergistic effect between these liposomes and antibiotics like vancomycin.<sup>247</sup>

Another strategy to develop antimicrobial liposomes includes the incorporation of lipids such as cholesterol and sphingomyelin, which are specific to animal cell membranes; these lipids are primary targets for bacterial toxins and have the ability to neutralize bacterial toxins and protect host cells from infection. Drug delivery systems composed of these lipids have been shown to sequester toxins, preventing mammalian cell lysis. For instance, Henry *et al.* demonstrated that liposomes composed of sphingomyelin and cholesterol (66 mol%), combined with antibiotics like vancomycin, effectively competed with host cells for toxin binding during active infections. The authors observed that the administered liposomes effectively rescued pneumonic mice (caused by *S. aureus* and *S. pneumoniae*) within 10 hours.<sup>248</sup>

**Enhanced functionality.** Apart from incorporating naturally occurring lipids into lipid-based nanoparticle formulations for the applications discussed above, other avenues that have been explored for enhancing the functionality of lipid-based nanoparticle platforms include imparting antitumor activity, promoting neuroprotection, enabling stimuli-responsive cargo release, and improving efficacy.

**Antitumor activity.** PUFAs and PUFA-containing phospholipids (PUFA-PLs) are highly susceptible to peroxidation<sup>249</sup> and play key roles in free radical reactions and lipid peroxidation

processes. They also contribute to ferroptosis, an iron-dependent, non-apoptotic form of cell death characterized by the accumulation of lipid peroxides.<sup>250–252</sup> For instance, Huang *et al.* demonstrated that liposomes enriched with linoleic acid, oleic acid, or  $\alpha$ -linolenic acid (C18:2) elevated cellular reactive oxygen species (ROS), inducing lipid peroxidation and triggering ferroptosis in B16 tumor cells *in vitro* and in B16 tumor-bearing models *in vivo*.<sup>253</sup> Induction of ferroptosis is a promising strategy that can be used to tackle cancers and a significant body of research has been conducted to highlight the pivotal role of PUFA-PLs in increasing the susceptibility of cells to ferroptosis. For instance, Zou *et al.*, through genome-wide CRISPR/Cas9 screens identified the role of peroxisome synthesized PUFA-containing ether phospholipids (PUFA-ePLs), predominantly plasmalogens, in ferroptosis induction. The authors showed exogenous treatment with PUFA-ePLs liposomes resulted in increased susceptibility to ferroptosis in high grade human serous ovarian cancer cells, with an additional potential for modulating cell membranes.<sup>250,254,255</sup> Qiu *et al.*, through systematic studies across 20 different cell lines, identified a specific class of phospholipids containing PUFA tails at both the sn-1 and sn-2 positions as critical drivers of ferroptosis. These phospholipids were shown to initiate ROS production in mitochondria and trigger lipid peroxidation within the endoplasmic reticulum, guiding the ferroptosis process.<sup>256</sup> Building on these studies, researchers have begun incorporating PUFA-PLs into liposomal formulations to enable ferroptosis, often encapsulating ferroptosis inducing compounds to effectively kill cancer cells.<sup>253</sup> For instance, Gao *et al.* synthesized surface-engineered liposomes with fucose ligands and incorporated phospholipids with two C20:4 tails, encapsulating immunostimulatory CpG oligodeoxynucleotides to enhance the synergy between ferroptosis and immunotherapy. These liposomes exhibited improved antitumor activity in 4T1 tumor-bearing mice, where ROS triggered the peroxidation of the incorporated phospholipid, which led

to the release of immunostimulatory cargo, boosting immune response and promoting ferroptosis.<sup>257</sup>

In addition, other lipids like ceramides, which are central intermediates of sphingolipid metabolism, exhibit tumor suppressive properties and are key mediators of cellular death programs like apoptosis and autophagy.<sup>258,259</sup> Ceramides have been incorporated into nanoparticle formulations to achieve anti-tumor effects. For example, Zhai *et al.*, synthesized ceramide (C6) containing liposomes to increase the apoptotic sensitivity induced by methotrexate and doxorubicin by twofold in U2OS human osteosarcoma xenograft model.<sup>260</sup>

**Treatment against neurodegenerative conditions.** The involvement of plasmalogens in various neurodegenerative conditions makes them an appealing therapeutic strategy.<sup>254,261–263</sup> Plasmalogens are essential for proper neuronal function and therefore are enriched in synaptic vesicles, where plasmalogens support release of neurotransmitters and vesicular fusion.<sup>264</sup> Their unique properties make them promising candidates for the treatment of disorders associated with neuroinflammation, oxidative stress, mitochondrial dysfunction, and perturbations in lipid metabolism. In a recent study, Wu *et al.* synthesized plasmalogen-containing nanoparticles for treating neurodegenerative diseases such as Parkinson's disease (PD), which is linked to reduced plasmalogen levels.<sup>265,266</sup> In this study, the authors ameliorated the behavioral PD symptoms in transgenic PD mouse models by treatment with nanoparticles containing scallop-derived plasmalogens intranasally.<sup>267</sup>

**Stimuli-responsive cargo release.** Unsaturated phospholipids have been explored for light-triggered cargo release in liposomal formulations.<sup>268</sup> For instance, a study by Miranda and Lovell demonstrated that increasing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) content in doxorubicin-loaded liposomes enhanced drug release rates up to tenfold under 665 nm NIR light due to membrane destabilization and permeabilization.<sup>269</sup> Apart from this, plasmalogens have also been incorporated into liposomes to enable stimuli-responsive drug release through the photoinduced cleavage of the labile *sn*-1 vinyl ether linkage.<sup>270,271</sup> Synthetic approaches to develop stimuli responsive platforms are discussed in the **Use of Synthetic Bioactive Lipids** section.

**Improved efficacy.** The trans-membrane diffusion of amphiphilic drugs can be improved by treating cells with liposomes enriched with short-chain sphingolipids, which cause lipid packing imperfections in cell membranes.<sup>272,273</sup> In a study by Lummel *et al.*, the authors observed almost twofold reduction in tumor growth rate when dosed with *N*-octanoyl-glucosylceramide-enriched doxorubicin-containing nanoparticles compared to only doxorubicin-containing nanoparticles in an A431 xenograft model, with no reported systemic toxicity.<sup>274</sup>

Ether lipids from archaea, owing to their fusogenic and lyotropic properties that enhance endosomal escape, have been incorporated into LNP systems, enhancing transfection efficiency for pDNA and mRNA.<sup>275,276</sup> For example, Sedlmayr *et al.* demonstrated that incorporating archaeal ether lipids into mRNA LNPs improved internalization and increased mRNA expression up to tenfold in primary (HSMM) and estab-

lished (Caco-2, C2C12) cell lines.<sup>277</sup> Additionally, incorporation of cholesterol analogues instead of cholesterol into LNP systems can impact particle surface morphology, which indirectly affects gene transfection. For example, in a study by Eygeris *et al.*, the authors compared the transfection efficiencies of LNPs containing either cholesterol or its analogs in HeLa cells. The authors observed that LNPs containing  $\beta$ -sitosterol had the highest transfection efficiency and the authors attribute this to reduced membrane ordering, resulting in polymorphic or faceted morphologies, which contribute to improved fusogenic properties.<sup>278,279</sup>

**Immunomodulation.** Traditionally, extrinsic antigens and adjuvants, including small molecules, proteins, polysaccharides, lipids, and nucleic acids, are used to induce immunogenicity of lipid-based nanoparticles.<sup>280,281</sup> Increasing effort is being focused on leveraging the intrinsic immunogenicity of lipid-based nanoparticles for immunotherapy and vaccine applications.<sup>282–284</sup> Physicochemical parameters including size, surface charge, and membrane fluidity of lipid-based nanoparticles are known to regulate immune response.<sup>285–287</sup> However, the bulk of lipid-based nanocarrier components – such as PC, PE, and cholesterol – are weakly immunogenic. Conversely, several naturally occurring exogenous lipids have been shown to be potent adjuvants and have been incorporated into lipid-based nanoparticles.<sup>223</sup> For example, lipid-based nanoparticles containing monophosphoryl lipid A (MPLA), a detoxified form of lipopolysaccharide derived from *Salmonella minnesota*<sup>288</sup> have been extensively studied<sup>289</sup> and had advanced up to phase III clinical trials as cancer vaccines for breast<sup>290</sup> (NCT00925548) and non-small-cell lung cancer<sup>291</sup> (NCT00409188, NCT01015443). Furthermore, Mosquirix® (FDA 2015) and Shingrix® (FDA 2017) are FDA-approved liposomal vaccines incorporating MPLA for preventing malaria and shingles, respectively.<sup>292</sup> Other natural lipids and their analogs that are adjuvant candidates in nanoparticle formulations include squalene (a natural precursor for cholesterol biosynthesis) and macrophage activating lipopeptide-2 (derived from cell walls of mycoplasma and *Escherichia coli*).<sup>280,293</sup> Additionally, lipids can also be employed as antigens inside nanoparticles. For example, Ishi and Kojima explored the selective delivery of  $\alpha$ -galactosylceramides ( $\alpha$ -GalCer), glycosphingolipids derived from the sea sponge *Agelas mauritianus*, to dendritic cells for invariant natural killer T cells (iNKT cells) activation using oligomannose-coated liposomes ( $\alpha$ GC-OMLs). *In vitro*,  $\alpha$ GC-OMLs induced a stronger Th1-biased immune response indicated by higher IFN- $\gamma$ /IL-4 production in splenocytes and bone marrow-derived dendritic cells compared to free  $\alpha$ GC. This effect was confirmed *in vivo*, where intraperitoneal injection of  $\alpha$ GC-OMLs into C57BL/6 mice effectively activated iNKT cells, highlighting their potential as a targeted delivery system.<sup>294</sup>

Researchers have also designed lipid-based nanoparticles composed of multi-functional lipids that are immunogenic in addition to serving as critical structural components in nanoparticle assemblies.<sup>282,295</sup> For instance, Irvine and coworkers reported the self-assembly of saponin and monophosphoryl lipid A (MPLA), resulting in caged nanoparticles, referred to as

saponin/MPLA nanoparticles (SMNPs), which have substantial adjuvanticity in non-human primate models. This formulation has been fast-tracked to phase I human-clinical trials for use as an HIV vaccine (NCT06033209).<sup>296,297</sup>

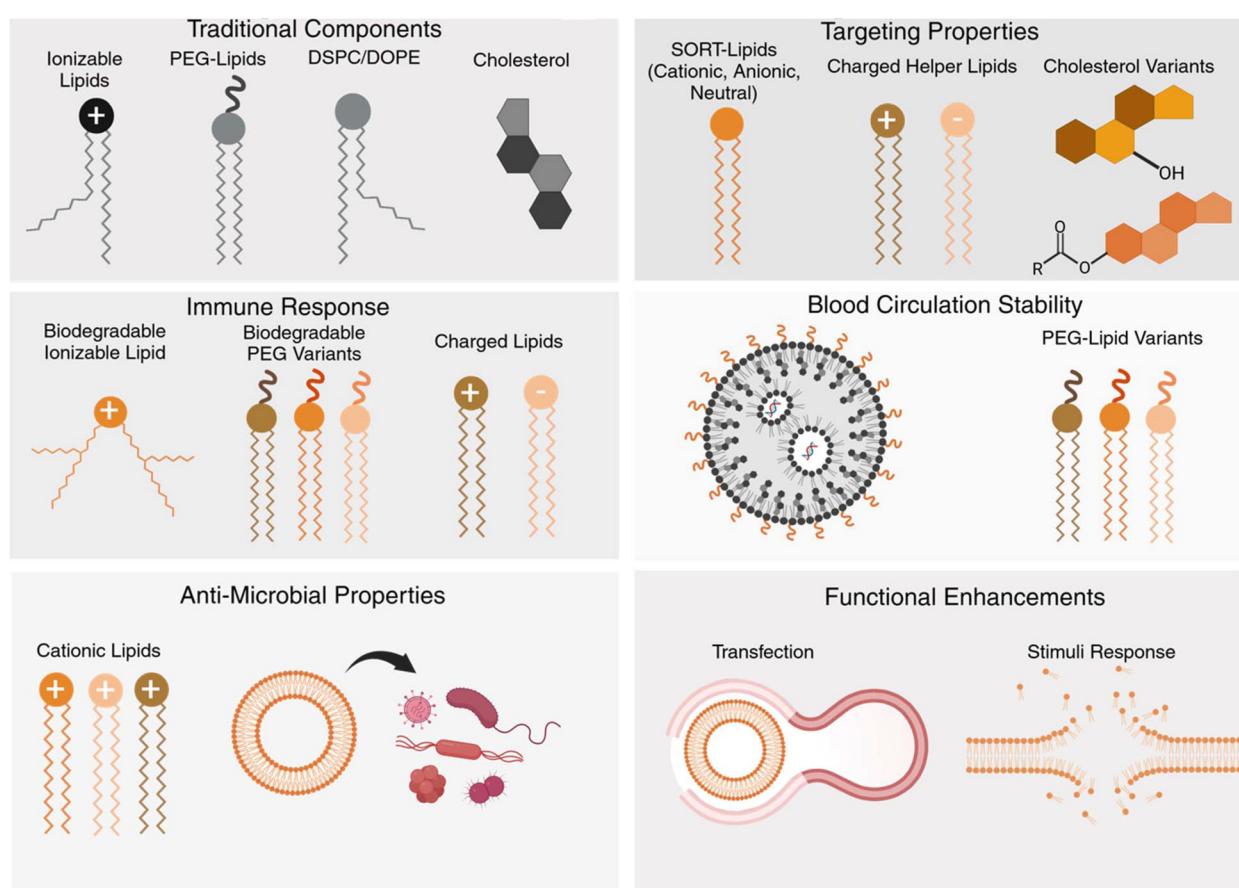
As research advances, new lipid species and their functions will continue to be discovered, and the potential for developing functional lipid-based nanoparticle systems will continue to expand. As discussed in this section, naturally occurring lipids can be incorporated into lipid-based nanoparticles for a wide range of biomedical applications. By taking inspiration from the diverse array of lipids present in the body and their functions, it is possible to strategically select and incorporate bioactive lipids into nanoparticle systems tailored to meet specific functional requirements.

### Use of synthetic bioactive lipids

While naturally-occurring lipids provide the field with a comprehensive toolkit of building blocks for designing functional nanocarriers, synthetic approaches have also been taken to design lipids and nanocarriers with specific and systematically tailored properties (Fig. 3). As LNPs continue to garner interest in the field, a large part of our discussion in this section will center around LNP development in the context of synthetic bioactive lipids.

**Targeting & delivery.** Like many other types of nanoparticles, traditional lipid-based nanoparticles show significant liver accumulation.<sup>52,197,298–300</sup> In this section, modification to lipid-based nanoparticle formulations including modifications to traditional formulations such as varying cholesterol and PEG variants, adding additional lipid components, and replacing LNP helper lipids will be discussed.

**Modifications to traditional formulation components: cholesterol and PEG-lipids.** Systematic alterations of the structure of cholesterol can be used to modulate lipid-based particle functionality. Various studies have synthesized cholesterol variants to improve delivery. For example, Dahlman and coworkers investigated the impact of esterified cholesterol variants, including cholesteryl stearate and cholesteryl oleate, and oxidized cholesterol variants, like  $7\alpha$ -hydroxycholesterol,  $7\beta$ -hydroxycholesterol, and  $4\beta$ -hydroxycholesterol, on LNP function. They concluded that LNPs formulated with esterified cholesterol delivered DNA barcodes to hepatic endothelial cells in mice with deficiency in the low-density lipoprotein receptor ( $LDLR^{-/-}$ ) and mice with deficiency in the very low-density lipoprotein receptor ( $VLDLR^{-/-}$ ) more efficiently than LNPs formulated with regular or oxidized cholesterol, likely due to differential interactions with serum proteins and the protein corona.<sup>57</sup> Patel *et al.* used a hydroxycholesterol substitution in



**Fig. 3** Synthetic bioactive lipids and their respective functions. In grey, the traditional lipid-based nanoparticles are highlighted. In shades of orange, the synthetic bioactive lipids used to enhance carrier functions are indicated.

LNPs for mRNA delivery to T-cells and found that the substituting cholesterol with  $7\alpha$ -hydroxycholesterol LNP-mediated mRNA delivery and targeting to primary human T cells *ex vivo* by 1.8-fold and 2.0-fold in protein expression.<sup>58</sup> In a recent study, Radmand *et al.* explored the tropism of charge-dependent lipid-based nanoparticles by investigating the substitution of cholesterol with positively charged cholesterol variants including DC cholesterol, a cholesterol derivative with an ionizable tertiary amine and GL67, a spermine-based cholesterol variant. They observed a 25–75% increase in tdTomato + expression across all liver cell lines in Ai14 and BL/6 mice—including endothelial cells, dendritic cells, hepatocytes, and T-cells—when using cationic cholesterol compared to LNPs with cationic helper lipids and neutral cholesterol.<sup>59</sup> Overall, the addition of modified cholesterol variants has been shown to serve as a useful tool for modulating lipid-based nanoparticle delivery and targeting.

While PEG-lipids have been shown to control the size and stability of both liposomes and LNPs,<sup>55,64,65</sup> chemical modifications to the PEG end groups can also be used to modulate LNP targeting. In a recent study by Sahay and coworkers, lipid-based particles with three different surface modifications were designed to deliver mRNA into the retina using 661w cell line model. The LNP surfaces were modified by amine-, carboxy-, and carboxyl ester-modified PEG lipids. They showed that incorporating carboxy and carboxyl ester-modified PEG lipids in LNPs increased mRNA delivery by 20 and 35% relative to LNPs containing unmodified PEG lipids.<sup>301</sup>

**SORT lipids.** Selective organ targeting (SORT) LNPs were developed by Siegwart and coworkers as a means to create LNPs capable of targeting organs other than the liver.<sup>302–305</sup> Although not the first example of LNPs for extrahepatic delivery,<sup>306–308</sup> SORT technology shows an alternative to circumvent liver tropism. SORT-LNP formulations consist of traditional components (ionizable lipid, helper phospholipid, cholesterol, and PEG lipid) with the addition of a SORT lipid, which can be a cationic, ionizable or negatively charged lipid. A recent study showed that the addition of unsaturated lipids to SORT LNPs can promote transfection efficiency relative to other traditional saturated LNPs *in vivo*.<sup>309</sup> SORT lipids have been shown to alter the apparent  $pK_a$  of LNPs, leading to changes in transfection efficacy and tropism.<sup>300</sup> In addition, it was shown that the interaction of quaternary ammonium-containing lipids, used as SORT lipids, with serum proteins can influence protein corona formation and impact targeting. Dilliard *et al.* characterized a panel of quaternary ammonium lipids to assess how chemical structure affects the organ-targeting of SORT LNPs and found that chemical structure of both the lipid alkyl tail and headgroup impact the potency and specificity of mRNA delivery to the lungs due to alterations in protein corona identity.<sup>310</sup> This work and other studies<sup>300,304,311</sup> have shown that the chemical structure of the SORT lipid impacts plasma protein interactions, leading to organ-specific delivery. In a more recent study, SORT technology with crosslinked lipids has been leveraged for LNP delivery for bone marrow gene editing.<sup>312</sup> Bone targeting is a

growing area of interest to treat osteoporosis, bone cancer, and osteoarthritis. As bone marrow is the primary site of blood cell production, including red blood cells, white blood cells, and platelets, it is an important target for leukemia and other genetic disorders.<sup>313,314</sup> Another study by Alvarez-Benedicto *et al.* has demonstrated that splenic T cells and macrophages can be separately transfected by LNPs containing an anionic SORT lipid.<sup>311</sup> In addition to these advantages, the change in apparent  $pK_a$  as a result of incorporating SORT lipids have been shown to lead to changes in transfection efficacy.<sup>370</sup>

Overall, SORT technology has been shown to efficiently deliver LNPs to the lungs, spleen, kidney, heart and bone marrow. Some generalizable rules that have been discovered in these studies include the use of permanently cationic SORT lipids (e.g. DOTAP) to increase lung tropism and permanently anionic SORT lipids (e.g. 18PA) to increase spleen tropism. These modifications to LNPs change the apparent  $pK_a$  of LNPs to be greater than 11 for lung tropism and less than 6 to increase spleen tropism, respectively.<sup>304,305,310</sup>

**Replacement of helper lipids with charged lipids.** As charged lipids can be used to alter the homing capabilities of lipid-based nanoparticles, replacing the helper lipid with charged lipids can be used to modulate targeting and trafficking. A study from Whitehead and coworkers demonstrated that replacing the helper phospholipid with a cationic or anionic lipid enhanced extrahepatic targeting properties. They achieved both lung and spleen homing based on only four lipid components by changing the apparent  $pK_a$  of the particle replacing the neutral helper phospholipid with a permanent cationic or anionic lipid, respectively.<sup>315</sup> Consistent with other studies and SORT technology, adding an anionic helper lipid containing PS achieved spleen tropism, while adding a cationic helper lipid like DOTAP significantly improved lung tropism. More specifically, substituting the neutral phospholipid with PS altered the ratio of liver to spleen protein expression from 8:1 to 1:3, while substituting the neutral phospholipid with DOTAP changed the protein the ratio of liver to lung protein expression from 36:1 to 1:56. In a more recent study, Kuzminich *et al.* used a similar strategy using the cationic lipid dimethyldioctadecylammonium bromide (DDAB) to replace the helper phospholipid in an ionizable LNP formulation to target the lungs *in vivo* via intravenous administration. This study shows that extrahepatic lung tropism of these cationic lipid-based nanoparticles was enhanced even compared to SORT formulation and also showed central nervous system (CNS) tropism in these particles in Ai9 and Ai14 mice models, in addition to showing a 30% increase in protein expression in endothelial cells relative to LNP formulation without the cationic lipid.<sup>316</sup> In addition, Melamed *et al.* have shown that LNPs containing cationic lipids may benefit pancreatic mRNA delivery in C57BL/6 mice in combination with intraperitoneal delivery. A 40% luminescence increase was observed in the pancreas in formulations containing DOTAP and the ionizable lipid 306O<sub>110</sub> relative to formulations containing DOPE and PS, indicating that the presence of the cationic lipid can improve specificity for the pancreas.<sup>317</sup> A

limitation of this approach that needs to be highlighted lies in previously reported toxicity concerns associated with positively charged lipids.<sup>318</sup> Nonetheless, these studies show decreased immune response either due to LNPs being significantly smaller than liposomes<sup>315</sup> or due to intraperitoneal delivery which causes peritoneal macrophage extracellular vesicle (EV) transfer, which refers to the process by which macrophages in the peritoneal cavity release extracellular vesicles that can transfer various bioactive molecules to other cells, thereby reducing overall immunogenicity.<sup>317</sup> Overall, incorporation of cationic helper lipids has been shown to increase extrahepatic delivery, while maintaining lower immunogenicity relative to cationic lipoplexes.

The incorporation of charged lipids can also enhance targeting and delivery in liposomal formulations. Perrie and co-workers demonstrated that upon intramuscular injection anionic formulations, including dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG) and PS, facilitated lymphatic targeting.<sup>319</sup> Among these, PS-containing liposomes exhibited greater accumulation in draining lymph nodes, likely due to preferential uptake by macrophages, highlighting their potential for lymphatic-targeted therapy. The same study also reported that cationic DOTAP-containing liposomes showed retention at the injection site. Sun *et al.* synthesized a cationic lipid, HC-Y2, and incorporated it into sialic acid (SA)-modified cationic liposomes loaded with Paclitaxel to assess their antitumor efficacy.<sup>320</sup> Their study showed that increasing the proportion of cationic lipids enhanced uptake in tumor-associated immune cells (RAW264.7) and tumor cells (4T1). *In vivo*, these cationic liposomes reduced lung metastases in a 4T1 metastatic tumor-bearing BALB/c mouse model of breast cancer, likely due to their ability to target tumor-associated macrophages. Another study reported that cationic liposomes containing stearyl amine loaded with resveratrol, an anti-cancer drug, enhanced targeting of hepatocellular carcinoma.<sup>321</sup> This was attributed to electrostatic interactions with anionic PS on liver cancer cell surfaces. In addition, Mooney and coworkers revealed that encapsulating 2'3' cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) in cationic liposome containing DOTAP lipid resulted in targeted delivery to metastatic melanoma tumors in the lung, leading to antitumor activity.<sup>322</sup> Lin *et al.* demonstrated the potential of cationic liposomes made of cetyltrimethylammonium bromide (CTAB) and DDAB encapsulating doxorubicin for targeted glioma treatment with focused ultrasound in C6 glioma model.<sup>323</sup> These studies highlight that lipids can enhance the targeting of liposomes to desired tissues, offering a promising strategy for improving the specificity and efficacy of treatments.

Although many advancements have been made in the design and use of lipid-based nanoparticles for targeted delivery, immunogenicity remains a significant challenge.<sup>283</sup> We will next discuss the immune response of lipid-based nanoparticles, different strategies to circumvent these, as well as how immunogenicity has been leveraged for immune-based therapies.

**Immune evasion and enhanced circulation.** PEGylation is widely used to circumvent premature nanocarrier clearance due to opsonization.<sup>324</sup> In this subsection we will discuss the use of synthetic bioactive lipids to enhance circulation in lipid-based nanocarriers. PEGylated lipid-based nanoparticles suffer from accelerated blood clearance (ABC) as an immunogenic response elicited after repeated exposure to PEG-based therapeutics.<sup>42,325</sup> To avoid ABC, researchers have come up with various synthetic strategies to replace or modify PEG.

A main approach is surface modification of lipid-based nanoparticles with hydrophilic polymers, including pSar,<sup>326–328</sup> polyglycerol,<sup>329–331</sup> folic acid,<sup>332</sup> zwitter-ionic polymers,<sup>333,334</sup> and polysaccharides.<sup>332,335</sup> For example, Ito and coworkers investigated the effect of replacing PEG with pSar using identical HSPC-based liposomes and found that that pSar-liposomes elicited significantly lower antipolymer antibodies' levels compared to PEG-liposomes.<sup>327</sup> In a separate study, replacing PEG with polyglycerol in liposomes made of hydrogenated egg PC and cholesterol, loaded with doxorubicin, resulted in reduced ABC and antibody responses. This formulation also enhanced anti-tumor activity in a mouse model of Lewis lung carcinoma.<sup>329</sup>

Instead of replacing PEG, chemical structure modification of PEG-lipids with moieties like CD47-derived self-peptides can also enhance stealth behavior.<sup>336–339</sup> These self-peptides transmit a “don't eat me” signal to macrophages constituting the MPS and thus mask the liposomes against ABC during circulation. One study demonstrated enhanced antitumor efficacy of self-peptide incorporated liposomal doxorubicin in subcutaneous tumor of B cell lymphoma-A20 in Balb/c mice.<sup>338</sup>

Another challenge for PEG lipids is their detachment from the nanoparticles during circulation, which affects its stability. One approach for tackling this was demonstrated by Pasut *et al.*, who used  $\beta$ -glutamic acid as an anchor to conjugate multiple DSPE molecules to PEG to form dendrite-like structures.<sup>340</sup> This enabled more stable interactions of the PEG lipids during circulation, resulting in a 6.2 fold increase in distribution half-life of doxorubicin-loaded liposomes when compared to conventional PEGylation.

**Antimicrobial activity.** Similar to naturally occurring lipids, synthetic lipids can also be leveraged to enhance antimicrobial properties in lipid-based nanoparticles. It has been previously reported that cationic liposomal formulations generally exhibit higher antibacterial activity than anionic or neutral formulations.<sup>341</sup> An example can be seen in how cationic liposomes bind electrostatically at the Gram-negative bacteria outer membrane, which is not observed in neutrally charged liposomes.<sup>341,342</sup> Liposomes containing the synthetic cationic lipid dimethyldioctadecylammonium bromide (DDAB) were shown to elicit an antimicrobial response in HT29-MTX-E12 mucus-secreting epithelial cells *in vitro* by binding to the negatively charged mucins that *Helicobacter pylori* binds to, in addition to delivering amoxicillin to an *in vitro* model cultivating *H. pylori* J99.<sup>343</sup> In a recent study, Amer *et al.* showed that cationic liposomes containing DOTAP display the strongest interaction with a *in vitro* inoculum of *Staphylococcus*

*aureus* biofilm matrix compared to neutral and anionic liposomes.<sup>344</sup> Similarly, Porolnik *et al.* synthesized cationic liposomes using DOTAP and BODIPY dimers as photosensitizers to synergistically promote antimicrobial properties by promoting photodynamic inactivation on both Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* *in vitro*. A 3.3 and 3.5 fold reduction in bacterial cells were observed when using cationic liposomes relative to negatively charged POPG-containing liposomes in *S. aureus* and *E. coli*, respectively.<sup>345</sup> A more extensive overview of cationic liposomal formulations and their antimicrobial properties can be found in the following reviews.<sup>346–348</sup>

**Enhanced functionality.** Apart from incorporating synthetic lipids into lipid-based nanoparticle formulations for the applications discussed above, we can also enhance carrier functionality such as enabling stimuli-responsive cargo release and improving transfection efficiency by leveraging the bioactivity provided by synthetic lipids.

**Stimuli response.** To establish stimuli-responsive liposomes, researchers have developed synthetic lipids which undergo conformational changes in the presence of specific stimuli. When these lipids are incorporated into liposomes, their structural alterations can disrupt lipid packing within the membrane, triggering the release of encapsulated contents.<sup>349</sup> For instance, in a study by Lou and Best, the authors developed a ROS-responsive liposome platform by incorporating a synthetic lipid bearing a DOPE lipid scaffold with a quinone-methide linker and boronate ester headgroup, which oxidatively breaks down, resulting in membrane disruption and release of both hydrophobic and hydrophilic cargo.<sup>350</sup> Another example includes a study by Chander *et al.*, where the authors developed light triggerable liposomal release systems by incorporating small amounts of (~10 mol%) photoswitchable DSPC analogs which have azobenzenes incorporated into their lipid tails. The authors demonstrated that upon irradiation with UV-A light source (365 nm) the azobenzene group in the lipid tails undergoes photoisomerization (from the trans to the cis form) inducing cargo release up to 80% in 24 hours.<sup>351</sup> Apart from using ROS as stimuli, researchers have developed liposomal platforms which use metal cations, ATP, pH, electric fields, magnetic fields, ultrasound waves or enzymes as triggers for cargo release.<sup>352–357</sup>

**Transfection.** Synthetic lipids have also been used to improve transfection of lipid-based nanoparticles. A platform for synthesizing a class of branched ionizable lipids called Branched Endosomal Disruptors (BEND) lipids that improve endosomal escape was reported. BEND lipids incorporate terminally branched groups that increase hepatic mRNA delivery and gene editing efficiency, *in vitro* in HeLa cells and *in vivo* in C57BL/6J female mice, as well as T cell transfection compared to non-branched lipids *via* Apo-E mediated mechanism.<sup>358</sup> In the context of liposomes, Best and coworkers recently developed ROS-triggered liposomes by incorporating both anionic and cationic synthetic lipids. Initially, the cationic DOTAP positively charge is balanced through the inclusion of anionic lipid containing a boronate headgroup to

produce approximately neutral liposomes. These anionic boronate lipids interact with ROS, which leads to cleavage of the group unveiling the neutral lipid DOPE. They found that ROS-driven cleavage of anionic lipids was effective at unveiling the positively charged liposomes, which increased the transfection efficiency relative to PC liposomes.<sup>359</sup>

As mentioned above, pSar has shown promising results as an excipient, providing stealth properties, together with enhanced nanoparticle stability and reduced immunogenicity relative to PEG-containing formulations.<sup>360–362</sup> pSar has also been used to increase transfection in lipid-based nanoparticles. Recently, Langguth and coworkers developed a pSar containing LNP platform tailored to avoid the immune response elicited by PEG lipids and found that pSar-containing particles generally have higher transfection efficiency in human peripheral blood mononuclear cells *in vitro* relative to traditional formulations containing PEG lipids.<sup>362</sup>

**Immunomodulation.** Immunogenicity of lipid-based nanoparticles remains a significant challenge due to a number of reasons. Research has shown that RNA, a common cargo in LNPs, stimulates Toll-Like Receptors (TLRs) to activate the innate immune system, leading to production of proinflammatory cytokines, interferons, and chemokines.<sup>363</sup> In addition, a study by Ritsma *et al.* examined both Moderna and Pfizer's SARS-CoV-2 vaccines for anaphylaxis and identified pre-existing antibody recognition of PEG as a possible contributor to this reaction.<sup>364</sup> This is supported by other studies that also pointed to the PEG-lipid as one of the main causes of anaphylaxis.<sup>364–366</sup> Another challenge arises from the innate immunogenicity of permanently cationic lipids. Cationic molecules have been shown to promote immune response *via* oxidative stress, plasma membrane disruption, and opsonization.<sup>367</sup>

Various strategies have been employed to combat these responses. As mentioned previously, the development of DODAP, and, by extension, the DLinDMA family and other engineered ionizable lipids were engineered to have a neutral charge in blood (avoiding immune response) but a positive charge in endosome (facilitating endosomal escape).<sup>66,368,369</sup>

**Biodegradable Ionizable Lipids.** Biodegradable ionizable lipids, such as ester and disulfide-containing lipids, are thought to be one solution to mitigating the immune response to lipid-based nanoparticles.<sup>53,370–372</sup> For example, introducing ester bonds into MC3 resulted in a biodegradable variant, L319, which has been shown to increase delivery efficacy and elimination from the liver in 6 to 8 weeks old male C57BL/6 mice.<sup>370</sup> Another example is the lipid 304O<sub>13</sub>, which showed increased potency relative to its non-biodegradable variant c12-200 *in vitro* in HeLa cells and *in vivo* in Female C57BL/6 mice.<sup>53</sup>

To increase potency of biodegradable ionizable lipids, branched-tail ionizable lipids can be employed. In a recent study by Whitehead and coworkers, a library of 580 ionizable lipids, including branched-tail ionizable lipids for extrahepatic targeting, was developed to screen delivery to extrahepatic targets. This study identified a new ionizable branched-tail

lipid with a unique ability to selectively transfect lung natural killer and dendritic cells when incorporated to LNP formulations, without the need for cationic helper lipids *in vitro* in HeLa cells and *in vivo* in 6–8 weeks old C57BL/6 female mice.<sup>373</sup> Separately, Chaudhary *et al.* identified potent branched-tail ionizable lipids for LNPs that deliver mRNA selectively to both the placenta and non-reproductive maternal organs without fetal delivery due to biodegradable ionizable lipids maintaining immune homeostasis in order to treat pregnancy disorders in Pr CD-1 mice and Pr A19 mice.<sup>374</sup>

**Charged lipids & immune stimulation.** Synthetic bioactive lipids can also be leveraged to elicit intrinsic immunomodulatory effects. For instance, a study evaluated the intrinsic immunostimulatory effect of liposome synthesized from two types of cationic lipids, Arg-C3-Clu2C14 (R3C14) or Arg-C5-Clu2C14 (R5C14), revealing the liposomes composed of R5C14 resulted in activation and differentiation of T cells.<sup>375</sup>

A recent study evaluated liposomes containing various cationic lipids and their effects on immune responses, and it was found that liposomes containing 1,2-Dioleoyl-*sn*-glycero-3-ethylphosphocholine (EPC), cholesterol, DOPC promoted greater immunomodulation than formulations with other lipid compositions, such as DOTAP, DODMA, and DDA, as indicated by increased expression of cell surface activation markers, enhanced cellular uptake, antigen-specific T-cell activation, cytokine production, and improved cellular viability in an *in vitro* model using human monocyte-derived dendritic cells (MDDCs).<sup>376</sup> Kranz *et al.* synthesized lipoplexes composed of RNA encoding the firefly luciferase gene (luc-RNA) with lipids such as DOTMA, DOTAP, and DOPE and found that controlling the lipid composition influenced targeting, with negatively charged liposomes accumulating in the spleen and lymph nodes.<sup>377</sup> This resulted in dendritic cell targeting *in vivo*, resulting in immunostimulatory and antitumor effects. Münter *et al.* developed a cationic liposomal platform by incorporating lipopeptide (cholesterol-anchored tri-arginine peptide) into liposomes<sup>378</sup> and demonstrated that monocyte targeting was dependent on lipopeptide content.

Efforts to design synthetic lipids and nanocarriers with intrinsic adjuvanticity are gradually increasing. For instance, Xia and coworkers screened cationic lipid-like materials generated *via* epoxide ring-opening by poly(-amidoamine) (PAMAM) dendrimers for developing mRNA cancer nanovaccines. They identified C1, a 12-carbon tail molecule, incorporated into lipid-based nanoparticles, was capable of activating TLR-4 signaling in mouse bone marrow dendritic cells and effectively deliver antigen encoding mRNA for OVA expressing tumor cell lines, resulting in significant tumor growth inhibition.<sup>379</sup>

Specifically, designing amino ionizable lipids for LNP formulations with self-adjuvanticity has garnered considerable interest.<sup>380–383</sup> For instance, Anderson and co-workers studied tested 1000 lipids for melanoma and human papillomavirus mRNA vaccines and found that LNPs made with lipid A18, containing a heterocyclic piperidinyl-based head group, triggered 10-fold greater IFN- $\gamma$  secretion than LNPs with MC3 (used in Onpattro). Other LNP components included cholesterol,

DOPE, and C14-PEG 2000. Further, A18-LNP selectively activated adaptive immunity the STING pathway over TLR pathway to enhance antitumor efficacy.<sup>384</sup> Similarly, Zhang and co-workers developed STING agonist-derived amino lipids (SALs) SARS-CoV-2 vaccine carriers. LNPs made of SAL, DOPE, cholesterol, and DMG-PEG demonstrated significantly higher IFN levels bone marrow dendritic cells *in vitro* and STING related cytokine levels in mouse plasma *in vivo*.<sup>383</sup>

### An alternate design strategy: use of nanoparticle mechanical properties to modulate function

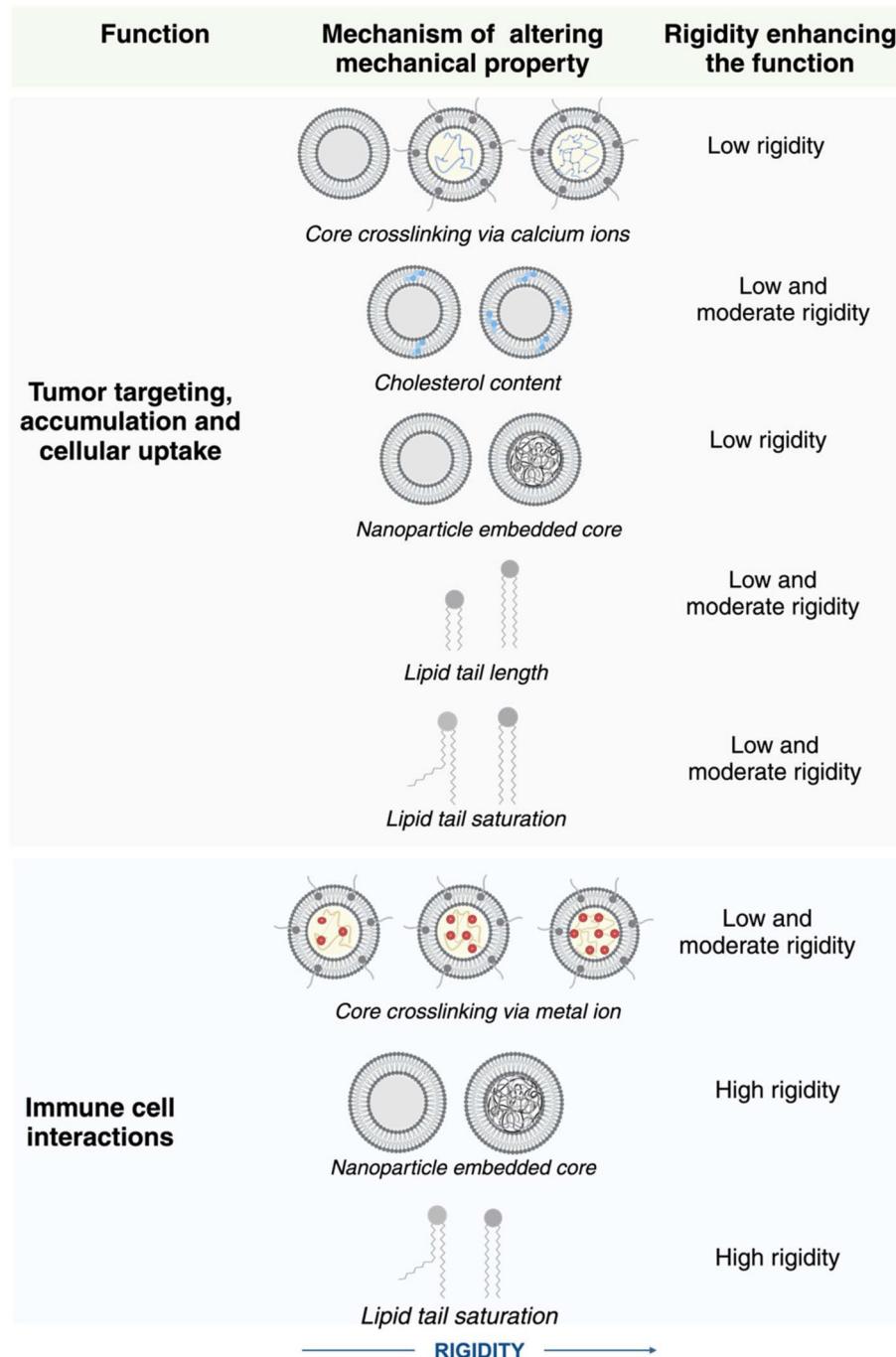
In this section, we will discuss an additional design parameter, the mechanical properties of lipid-based nanocarriers. There are directly linked to the types and combinations of lipids used. Here, we will explore how mechanical properties influence nanocarrier function and biological responses (Fig. 4).

The effects of different physicochemical properties of nanoparticles, such as shape, size, and surface properties, in regulating biological performances have been extensively investigated.<sup>6,7,385</sup> However, there have been limited studies on how mechanical properties, such as stiffness and elasticity, govern biological interactions, such as blood circulation, immune cell interactions, tumor accumulation, and cellular uptake and trafficking of lipid-based nanocarriers.<sup>386</sup>

**Mechanical properties of lipid-based nanoparticles.** Mechanical properties primarily refer to a material's behavior under load.<sup>386</sup> The field often uses the terms elasticity and stiffness interchangeably when discussing the mechanical properties of nanoparticles; however, they have different meanings. Elasticity is an intrinsic property of a material, defined as the ability of an object to resist deformation and return to its original state when stress is removed.<sup>386,387</sup> The elasticity is usually presented as the elastic modulus (N m<sup>-2</sup> or Pa). In contrast, stiffness is an extrinsic property affected by Young's modulus and the material's geometry, including its shape and size, which defines its ability to resist deformation under applied forces.<sup>386–388</sup> In this article, the terminology used by each work will be used to describe the rigidity of those nanoparticles.

Atomic force microscopy is commonly used to characterize the mechanical properties of nanoparticles.<sup>389</sup> As discussed in other review articles, various strategies, including physical, chemical, and biological methods, can be employed to tailor the mechanical properties of nanoparticles.<sup>386,388</sup> The influence of mechanical properties of various nanoparticles such as hydrogel particles, layer-by-layer templated capsules, hybrid polymer-lipid nanoparticles, and silica nanoparticles on their biological processes have been covered.<sup>386</sup> However, this review will specifically highlight the role the mechanical properties of lipid-based nanoparticles play in key biological processes such as tumor penetration and accumulation, immune cell interactions, and cellular uptake.

The influence of LNP mechanical properties on biological responses is an emerging field, with limited studies effectively characterizing these properties due to challenges in measuring mechanical compliance, which stem from their small size,



**Fig. 4** Nanoparticle mechanical properties can influence their biological functions, including tumor targeting, accumulation, cellular uptake and immune cell interactions.

structural complexity, and soft, deformable nature.<sup>390</sup> However, a study found that soft LNPs, with elasticity modulated by the calcium ion density in alginate gel, exhibited enhanced permeation through endothelial layers.<sup>390</sup> Additionally, several studies have investigated the impact of LNP rigidity on endosomal escape, including the role of lipid rafts,<sup>391</sup> the fusogenicity of lipids,<sup>392</sup> and the packing and shape of lipids in facilitating the escape of mRNA-loaded LNPs

from endosomes.<sup>393</sup> Given the emerging nature of this field, our review primarily focuses on liposomal mechanical properties.

**Tumor penetration and accumulation.** The mechanical properties of nanoparticles impact cellular uptake and tumor accumulation. Additionally, they help overcome biological barriers like epithelial and mucosal layers, improving the delivery of therapeutic agents to tumor sites.<sup>394,395</sup> There are discrepan-

cies between studies regarding whether less rigid or more rigid particles accumulate more effectively in tumors, which can be attributed to differences in tumor models and the use of different nanoparticle systems across studies. However, elasticity-dependent uptake is well-documented in the literature.<sup>395–397</sup>

Elasticity-mediated tumor accumulation of a nanolipogel (NLG) system has been reported.<sup>398</sup> Hybrid NLGs with an alginate hydrogel core and lipid bilayer shell were synthesized to evaluate the effect of nanoparticle elasticity on tumor accumulation. NLG elasticity was modulated by varying the degree of cross-linking of the alginate core. Additionally, nanoliposomes (NLPs) without a hydrogel core were also synthesized, exhibiting Young's modulus of  $45 \pm 9$  kPa. The Young's Modulus of the NLGs ranged from  $1.6 \pm 0.6$  MPa to  $19 \pm 5$  MPa. *In vitro* studies in MDA-MB-231 and MCF7 breast cancer cell lines revealed that the softer NLPs exhibited greater (5-fold increase) cellular internalization in breast cancer cell lines compared to the NLGs, which was hypothesized to be due to different uptake mechanisms for the soft and stiff nanoparticles. The enhanced internalization of soft NLPs was attributed to internalization through both energy-independent fusion and endocytosis processes compared to the stiffer nanoparticles, which were shown to depend only on energy-dependent clathrin-mediated endocytosis. Furthermore, the soft NLPs demonstrated about 2.6-fold increase in tumor accumulation in an orthotopic mouse 4T1 breast cancer model, which can be attributed to their lower elastic modulus, leading to increased tumor penetration *in vivo*.

Another study showed the effect of liposome stiffness on nanoparticle trafficking, pharmacokinetics and tumor accumulation.<sup>21</sup> This research reported that the stiffness of targeted layer-by-layer (LbL) modified liposomes can be modulated by varying the cholesterol content of the liposomal core. While the core composition was varied, the hyaluronic acid (HA) surface chemistry remained unchanged to ensure consistent targeting across formulations. The influence of their mechanical properties on *in vivo* trafficking in subcutaneous mouse model of OVCAR8 tumors expressing GFP/Luc2 was analyzed, revealing that soft liposomes resulted in longer elimination half-life (3.5 times higher than stiff counterparts), enhanced tumor accumulation, and exhibited higher penetration of particles in subcutaneous tumors *in vivo*. Wu *et al.* used a similar strategy of changing cholesterol content in liposomes to alter rigidity, determining that liposomes with moderate membrane (21.7 to 33.9 MPa) rigidity resulted in enhanced tumor penetration *in vitro* and *in vivo* compared to lower (11.5 MPa) or higher rigidities (53.3 MPa).<sup>22</sup> Montizaan *et al.* also reported that softer liposomes were taken up faster and to a greater extent in HeLa and A549 cells compared to harder liposome-coated silica nanoparticles.<sup>399</sup>

Altering the membrane fluidity and stiffness of liposomes can enhance their tumor penetration. Liposomes with increased fluidity can exhibit improved fusion capabilities, which can enhance the accumulation in tumor cells.<sup>400</sup> Bompard *et al.* engineered liposomes by modulating their

phospholipid composition, specifically by varying acyl chain lengths and degrees of saturation of lipid tails and assessed their uptake in various non-tumor and tumor cell lines *in vitro*.<sup>400</sup> The study found that less rigid liposomes, comprised of DMPC, DOPC and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids, had increased uptake in cancer cells and favored membrane fusion, while stiffer liposomes, comprised of DSPC, preferentially targeted healthy (WPMY-1) cells than cancer (LNCaP, C4-2B, and PC-3) cells. In contrast to these findings, when researchers compared hard polymeric PLGA core and lipid shell nanoparticles with soft liposomes in a separate study, the soft liposomes exhibited lower cellular internalization in HeLa cells,<sup>401</sup> highlighting the need for the field to gain a more comprehensive understanding of the influence of biological heterogeneity on nanocarrier uptake.

Research has focused on the effect on nanoparticle mechanical property on penetrating biological barriers to enhance tumor accumulation. Gao and coworkers highlighted the importance of nanoparticle rigidity in penetrating biological barriers and enhancing tumor accumulation by developing liposomes with varying stiffness.<sup>395</sup> In this work, core-shell nanoparticles with poly(lactic-*co*-glycolic acid) (PLGA) cores surrounded by lipid bilayer shells and hollow liposomes were separately engineered to evaluate how nanoparticle stiffness impacts diffusion through mucosal barrier. They observed that nanoparticles with intermediate rigidity (50 MPa) demonstrated enhanced diffusion through mucosal barriers and showed increased tumor penetration and accumulation compared to hard core-shell nanoparticles (110 MPa) and soft liposomes (5 MPa) *in vitro* in HT29-MTX-E12 cell line, human colon carcinoma cell line (Caco-2), *in situ* in pancreatic adenocarcinoma cell (BxPC-3), and human pancreatic star cells (HPSC).<sup>395</sup> To understand the difference in cellular interaction patterns of nanoparticles with varying rigidities, super-resolution microscopy and MD simulations were performed. The study showed that semi-elastic (50 MPa) nanoparticles can deform into ellipsoids, which allows rotation-facilitated diffusion, and enhanced penetration capabilities. It was reasoned that hard nanoparticles (110 MPa) cannot deform, and soft nanoparticles (5MPa) irregularly deform, causing them to exhibit the lowest permeation of the tested formulations.

In another study by Dai *et al.*, the influence of liposome rigidity on penetration through the tumor extracellular matrix (ECM) was investigated.<sup>402</sup> Liposome rigidity was modulated by changing the lipid tail saturation and chain length. The lipids used in this study, listed in order of increasing rigidity in liposomes, include DOPE (C18:1), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC, C12:0), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DEPC, C22:1), DMPC (C14:0) DPPC (C16:0), and DSPC (C18:0). Liposomes comprised of DPPC (C16:0) lipids with intermediate stiffness (19.9 MPa) exhibited superior diffusivity through the ECM, leading to enhanced internalization in pancreatic adenocarcinoma cells (BxPC-3) and HPSC cells, improved accumulation, and prolonged retention at tumor sites compared to liposomes with lower (below 10 MPa) or higher (42.6 MPa) stiffness used in the study. The research-

ers concluded that the deformation of the nanoparticles of moderate stiffness into ellipsoids could explain the enhanced diffusivity through the ECM.

Overall, these studies have shown that while the mechanical properties of nanoparticles impact penetration and tumor accumulation, there are discrepancies across nanoparticle formulations and biological systems. The differences in the physicochemical properties of nanoparticles, the variabilities in the magnitude of elastic moduli across studies, and the fabrication process of modulating liposomal stiffness may explain the differences between the studies.<sup>402</sup> Despite these differences, the findings highlight that tuning the mechanical properties of liposomes can be leveraged to enable penetration across biological barriers and enhance tumor accumulation.

**Immune cell interactions.** The mechanical properties of liposomes can also govern their interactions with immune cell populations. Yuan *et al.* modulated liposome elasticity by varying metal ion cross-linking to evaluate their fate in APCs including macrophages and dendritic cells, within the tumor microenvironment (TME) and transport to tumor-draining lymph nodes (tdLNs).<sup>403</sup> When evaluated *in vitro* in RAW 264.7 cells and B16F10 cells, soft liposomes (15 MPa) were more readily internalized by APCs and demonstrated enhanced lymph node transport *in vivo*, in a subcutaneous-B16F10 tumor bearing mouse model, compared to the stiff versions (149 MPa). Liposomes with medium elasticity (40 MPa) activated the STING pathway and enhanced transport through tdLNs, promoting greater tumor-infiltrating lymphocyte (TIL) infiltration and producing antitumor effects in mice. The study also explored the potential of these liposomes with medium elasticity as a synergistic therapy with immune checkpoint blockade (ICB) therapy, highlighting their promise in improving cancer immunotherapy outcomes. However, a different study demonstrated that stiff liposomes exhibited superior uptake in APCs.<sup>404</sup> The rigidity of the liposomes was modulated by the phase state of the lipid membrane as dictated by the lipid transition temperature. It was revealed that stiff (gel phase) liposomes loaded with a vaccine fusion protein (CTA1-3Ea-DD) showed an enhanced antigen-presentation ability compared to softer (fluid phase) counterparts. Additionally, they promoted superior upregulation of costimulatory molecules and increased release of cytokines, highlighting how liposome stiffness can enhance vaccine immunogenicity.

Vu *et al.* studied the interactions between liposomes and a core–shell polymeric nanoparticle, which had a polystyrene core and a poly(ethylene glycol) shell. The polymeric nanoparticle was synthesized using reversible addition–fragmentation chain transfer (RAFT) polymerization combined with polymerization-induced self-assembly (PISA).<sup>405</sup> They assessed how these nanoparticles interacted with immune cells (B cells, monocytes, and neutrophils) in the blood and found that polymeric nanoparticles had stronger interactions with these immune cells than liposomes, which could be attributed to the distinct mechanical properties of the nanoparticle platforms, with polymeric nanoparticles exhibiting greater stiffness. However, these systems differ from one another in

more ways than one, and other factors besides the mechanical properties could explain the results.

The importance of mechanical properties on vaccine-induced immune response has also been demonstrated.<sup>406</sup> Liposome rigidity was altered by modulating the degree of saturation of lipid tails. It was observed that rigid dimethyl-dioctadecylammonium (DDA)-based liposomes triggered a stronger immune response than softer dimethyldioleoyl-ammonium (DODA)-based liposomes through increased recruitment and activation of APCs. Moreover, liposome rigidity influences nanoparticle retention at the site of injection. Softer, DODA-based liposomes were cleared more rapidly from the injection site compared to their rigid counterparts, resulting in a lower immune response.

## Conclusions and future perspectives

The development of lipid-based nanoparticles has resulted in significant clinical successes, from the use of liposomes to change the safety and delivery profiles of key chemotherapy agents to LNP formulations that have enabled the first clinical uses of RNA-based therapeutics and vaccines. These developments have occurred within a remarkably short period of time, starting with the discovery of mRNA and liposome structures just sixty years ago. One of the main challenges in the field of nanomedicine remains the successful development and translation of targeted nanocarriers. In this review, we highlighted strategies and recent advances in the use of formulations to impart targeting and functional properties without adding complexity to the nanocarrier design in the form of exogenous antifouling and targeting motifs. This strategy of imparting intrinsic functionality into the nanocarrier has gained increasing popularity for LNP design, resulting in promising clinical candidates for a number of preventative and therapeutic applications. We also emphasized the biological origin of liposomes, and how our understanding of biological lipid structure and function can be used to design intrinsically functional nanocarriers. As the number of biological lipid classes and functions continues to expand, we look forward to seeing how use of bioactive and bio-inspired lipids will continue to yield new types of targeted and functional nanocarriers.

In addition to the naturally occurring bioactive lipids already incorporated into lipid-based nanoparticles, several other lipid classes have received less attention despite their potential. Among these are lipids belonging to the polyketide and prenol classes. For instance, polyketide-based small molecule drugs such as doxorubicin (anticancer), lovastatin (anti-cholesterol), and rapamycin (immunosuppressant), are clinically significant due to their bioactive functions.<sup>407–409</sup> Traditionally, these are administered either as free drugs or as nanoparticle formulations prepared *via* hydrophobic encapsulation. However, forming functional nanoparticles with these polyketides by themselves has not been widely explored. The vastness and diversity within this group of molecules adds to the difficulties. Recently, there has been interest in utilizing

machine learning algorithms to investigate clinically relevant drugs and excipients to discern their nanoparticle formation or co-aggregation capabilities to enhance their pharmacological properties.<sup>410</sup> Prenol lipids also serve diverse biological functions, including cellular signaling and acting as precursors to essential molecules like vitamins.<sup>411,412</sup> Due to their biocompatibility and biological activity, vitamin-lipid conjugates have been explored for incorporation into LNPs. For instance, Hou *et al.* developed various vitamin-lipid conjugates (Vitamin B3, C, D, E) with ionizable lipids containing tertiary amines and incorporated them into LNPs to treat multidrug-resistant bacteria-induced sepsis in C57BL/6 immunocompromised mice. They found that Vitamin C-lipid LNPs successfully delivered cathepsin-B mRNA to macrophage lysosomes, a key target for bactericidal activity.<sup>413</sup> Further physical and biological characterization of lipid-based nanoparticles incorporating these lipids will be essential to fully understand their properties and optimize their functionality for therapeutic use.

Besides the aforementioned evolving areas, non-lamellar lyotropic liquid crystalline nanoparticles (LLC NPs) have also emerged as promising drug delivery vehicles. While structurally similar to liposomes, they exhibit more complex architectures, such as inverse hexagonal and cubic mesophases, often referred to as cubosomes and hexosomes.<sup>414–421</sup> Studies have demonstrated their ability to modulate biological responses, including cellular interactions,<sup>422–424</sup> blood components interactions, which in turn can affect their circulation times and biodistribution fates,<sup>425–428</sup> and immune responses.<sup>424,429–431</sup> However, further research is needed to elucidate how formulation components, particle structure, and morphology impact cellular responses and therapeutic efficacy.

Additionally, there has been effort in the field to advance lipid-based nanoparticles design through computational means that was not covered in this review including artificial intelligence/machine learning<sup>432–435</sup> and molecular dynamic simulations.<sup>72,436,437</sup> These computational tools can aid the design optimization of lipid-based nanoparticles to effectively include bioactive lipids. A lot of advancements have also been made in unfolding the role of the protein corona in the context of lipid-based nanocarriers.<sup>438–440</sup> It will be of great importance to further explore the effect of adding bioactive lipids to lipid-based nanoparticles in protein corona formation and composition. Finally, in the era of personalized medicine, we also anticipate that patient and disease-specific lipid signatures will be identified and leveraged to design uniquely functional nanocarriers. The continued exploration of functional lipids and innovative design strategies to create simple, yet functional nanocarriers holds promise, and we look forward to seeing how these delivery systems will be translated to the clinic.

## Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

## Conflicts of interest

There are no conflicts of interest to declare.

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