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Review of structural design guiding the development of lipid nanoparticles for nucleic acid delivery

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Abstract

Lipid nanoparticles (LNPs) are the most versatile and successful gene delivery systems, notably highlighted by their use in vaccines against COVID-19. LNPs have a well-defined core–shell structure, each region with its own distinctive compositions, suited for a wide range of *in vivo* delivery applications. Here, we discuss how a detailed knowledge of LNP structure can guide LNP formulation to improve the efficiency of delivery of their nucleic acid payload. Perspectives are detailed on how LNP structural design can guide more efficient nucleic acid transfection. Views on key physical characterization techniques needed for such developments are outlined including opinions on biophysical approaches both correlating structure with functionality in biological fluids and improving their ability to escape the endosome and deliver they payload.

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Keywords

Lipid nanoparticles, Nucleic acid delicery, Structure-function, Cationic ionizable lipids.

Introduction

The idea of gene transfer and gene therapy is not new, having first been proposed and explored during the second half of the last century [1]. The use of nucleic acids (NAs) as a therapeutic agent is based on their potential to treat and prevent a range of diseases including multifactorial genetic diseases, cancer mutations, and viral infections. Specifically, NAs can be engineered to cause either the knock-down or the induction of the expression of any gene, including those that encode for proteins that are considered to be 'undruggable' by conventional small therapeutic molecules [2]. However, despite their long history, gene therapies are still in the development phase, with two major delivery challenges to be overcome, namely: (1) protection of the NA payload from nucleases, which widely circulate in the extracellular environment and which degrade the NA before it reaches the target cell and (2) poor transport of the NA across the target cell membrane and the difficulty of its subsequent localization in the desired subcellular compartment. The poor transport of NA across cell membranes results in its confinement in intracellular organelles, such as the endosomes, from which the NA needs to rapidly escape into the cytosol to avoid its degradation and to be able to exert its therapeutic effect [3]. To achieve NA delivery, a range of different nanoparticle (NP)-based delivery systems have been developed, which when administered protect their NA cargo until they enter the target cells. This process occurs generally *via* a cellular mechanism known as endocytosis, whereupon they release their cargo in the cell. Viral vectors were first explored thanks to the birth of recombinant DNA methods and the understanding of how retroviruses work [1]. Later, non-viral vectors were envisioned for targeted organ gene-delivery and with decreased immunogenetic response, overcoming limitations existing in viral vectors. Among the early non-viral vectors developed are a range of polymer and lipid-based complexes. The latter, so-called lipoplexes, are complexes formed between a

Abbreviations

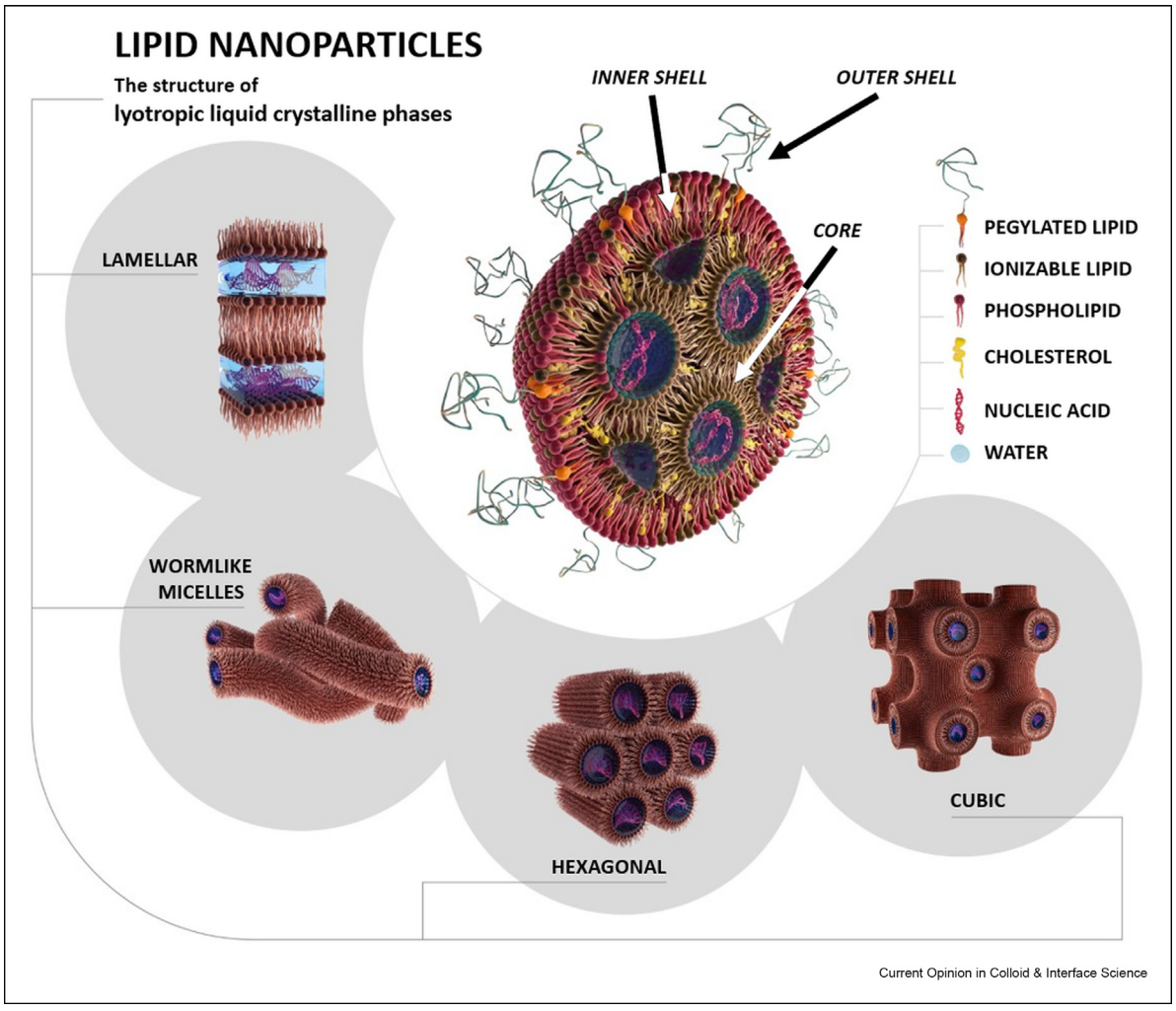
APCs Antigen presenting cells
ApoE Apolipoprotein E
BAM Brewster angle microscopy
CILs Cationic ionizable lipids
CPP Critical packing parameter
cryo-EM Cryogenic electron microscopy
cryo-TEM Cryogenic electron transmission microscopy
DLin-KC2-DMA or KC2 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane
DPH Diphenylhexatriene
DSPC Disteroyilphosphatidylcholine
DLS Dynamic light scattering
EE Early endosome
FCS Fluorescence correlation spectroscopy
GMO Glycerol monooleate
DLin-MC3-DMA or MC3 Heptatriaconta-6,9,28,31-tetraen-19-y14-(dimethylamino) butanoate
LE Late endosome
LNPs Lipid nanoparticles
MS Mass spectrometry
mRNA Messenger RNA
MALS Multi-angle light scattering
NP Nanoparticle
NA Nucleic acid
pSar Polysarcosinylated
TNS 6-(p-toluidino)-2-napthalenesulfonyl chloride
QCM-D Quartz crystal microbalance with dissipation monitoring
N/P Ratio of amino lipid nitrogen to NA phosphate
SLD Scattering length density
SORT Selective organ targeting
siRNA Short interfering RNA
SEC Size-exclusion chromatography
SAXS or SANS Small-angle X-ray or neutron scattering

cationic lipid (typically a permanent cation), frequently in combination with a helper lipid (added to modulate the liquid crystalline phase to be formed), and a NA. The resulting complex or lipoplex can have a range of architectures. A subsequent development was the lipopolyplex, a complex formed by reaction of the NA with a cationic polymer and cationic lipid. The lipopolyplex has been reported to comprise a core of a polyplex (i.e., complex of polymer and NA) surrounded by a bilayer of lipid. It is possible to attach targeting ligands as well as incorporate poly (ethylene) glycol containing (PEGylated) lipids on the exterior surface of these complexes. Unfortunately, some of these early vectors did not exhibit sufficient efficacy and exhibited safety concerns at high doses, as discussed in two notable comprehensive review articles [4,5].

Lipid nanoparticles (LNPs) are currently state-of-the-art with respect to lipid-based non-viral vectors. LNPs are NPs formed by a mixture of NA, cationic ionizable lipids (CILs) together with the helper lipids, cholesterol, phospholipids, and a PEGylated lipid. Figure 1 indicates LNP structure in terms of a core, an inner shell and an outer shell, the description of which is elaborated in a later section on LNP structure and composition. The most frequently incorporated NA is RNA, as opposed to DNA, since the former only needs to reach the cell cytosol compared to the nucleus for the latter, which is significantly more difficult to achieve. The encapsulation of RNA is obtained by mixing an ethanolic solution of lipids and sterols with an acidic aqueous RNA solution. This solution is then dialysed against a neutral buffer solution for ethanol removal, neutralization of the CIL and physiological compatibility. A key parameter in the formulation of LNPs for RNA delivery is the N/P value, which is the ratio of amino lipid nitrogen present in the CIL to NA phosphate. This value largely determines the level of encapsulated RNA. Another very important parameter is the critical packing parameter (CPP) of the CILs and helper lipids, which we will discuss in more detail in the next section. There are many other parameters important for LNP formation, structure, colloidal stability and ability to escape the endosome, including the surface concentrations of helper and PEGylated lipids.

The efficiency of NA delivery using NPs, including LNPs, is influenced by a number of factors, such as their ability to protect their NA cargo from biodegradation by nucleases until they reach the interior of the target cells, their ability to enter the target cells, generally through endocytosis, and their ability to ensure that their NA cargo safely reaches the cytosol either in its free form or encapsulated in the NPs to avoid the NA being degraded in the endosome and/or recycled out of the cell [8]. Subsequently, the NA must be trafficked through the cell to reach its area of function. Further, once the cellular delivery of NA is accomplished, the components of the NPs must be able to degrade safely, or if not, there is the risk of safety concerns such as apoptosis or necrosis caused by the biomaterial carrier [9] and mutagenesis caused from NA cargo [10].

Figure 1



Lipid nanoparticles have a core–shell structure. The outer shell is composed of a hydrated PEGylated layer while the inner shell contains cholesterol, phospholipids and cationic ionizable lipids [6,7]. The core is proposed to be a distorted hexagonal phase or worm-like phase of cationic ionizable lipids, cholesterol and RNA [6]. Other liquid crystalline phases found in different types of lipoplexes include lamellar phase and the cubic phases. PEG, poly(ethylene) glycol.

RNA-containing LNPs (RNA-LNPs) have attracted wide attention in the last few years especially due to the recent worldwide COVID-19 pandemic. In addition to the large number of biological and animal studies on RNA-LNP efficacy, knowledge of the relationship between their structure and functionality is an active area of research, mainly due to diverse range of lipid chemistry available and the complex mixture of lipids comprising the LNPs. Understanding the structure and composition of RNA-LNPs is of key interest for formulating a stable and efficacious particle [6,11].

In this review, we will start by introducing the physicochemical principles behind LNPs which determine LNP structure, we will present a few key characterization techniques used to characterize LNP structure and function, we will discuss how the structure dictates the route for endosomal escape, and we will put in perspective what is needed to go beyond current state-of-the-art by considering factors such as efficacy, safety, stability and manufacturability.

From lyotropic liquid crystals to LNPs

When designing non-viral gene delivery vehicles, the first challenge to overcome is to ensure efficient packing of the genetic encoding molecules into units that can be delivered intracellularly. This problem can be understood in terms of physicochemical concepts, particularly the associative phase separation between multivalent, negatively charged NA macromolecules and oppositely charged 'compacting' molecules. A pre-requisite for a NA-compacting moeity is that it must carry a

polycationic charge. This could be achieved either *via* assemblies of lipids or surfactants (lipoplexes [12]) or the use of polymers (polyplexes [13]). In this way, the large negative charge on the NA can be masked, allowing transport across the cellular membrane to take place.

For lipid-based systems and lipoplexes, which are the focus of this review, the structure of the compacted phase (or mesophase) very much depends on the type of cationic molecule and lipid helper used for NA compaction, and especially the CPP of the lipids or surfactants used (Figure 1). The CPP is defined as the molecular volume of the amphiphile's hydrophobic part divided by the product of the length of such hydrophobic part and the effective headgroup area. For amphiphiles with a CPP = 1, columnar lamellar liquid crystalline phases are typically found in which the NA molecules can be intercalated between two bilayers [14]. For amphiphiles with a CPP >1, inverse hexagonal liquid crystalline phases are produced in which cationic rod-like micelles can pack around columnar NA molecules [15]. Other liquid crystalline phases can be obtained depending on the acyl chain length of the amphiphile used [16]. A particular 'effective' CPP value (and thus mesophase) can be obtained by either using individual amphiphiles or a mixture of amphiphiles. Neutral helper lipids, as already mentioned, can be introduced to modulate the charge and mesoscopic liquid crystalline phase of the lipoplex. For example, by including lipids that self-assemble into bicontinuous gyroid liquid crystalline phases such as glycerol monooleate, cuboplexes containing messenger RNA (mRNA) can be formed [17].

Bulk thermodynamically stable liquid crystals cannot, however, be injected into the blood stream. Instead, these large and dense structures must be broken down into smaller and metastable particles with good colloidal stability (and shelf-life). Indeed, a certain size range, typically around 100 nm, has been reported as optimal for cellular uptake considering physiological barriers, such as liver fenestration, that must be overcome to reach the required organs [3]. The use of hydrophilic polymer-based lipids as PEGylated lipids was introduced to break the bulk liquid crystalline phases into small, well-defined NPs and tune their size, a point which has been discussed in a recent comprehensive review article [18]. The overall particle charge can be tuned by varying the composition of various lipid components.

Another important challenge is how to dissociate or decompact the NA once the gene delivery vehicle in which it is contained reaches the inside of the target cell. At the same time, it is important to decrease the toxicity of the permanently charged cationic molecules used as compacting agents. Several new lipids and surfactants have been designed including disulfide cationic lipids [19] (the disulfide bond breaks at low pH cleaving the cationic group from the lipid molecule, which leads to the collapse of the lipoplexes or lipid-based systems) and CILs [20] (lipids in which the cationic charge occurs at low pH, which means that modulation of the pH enables the collapse of the lipoplexes/lipid-based systems).

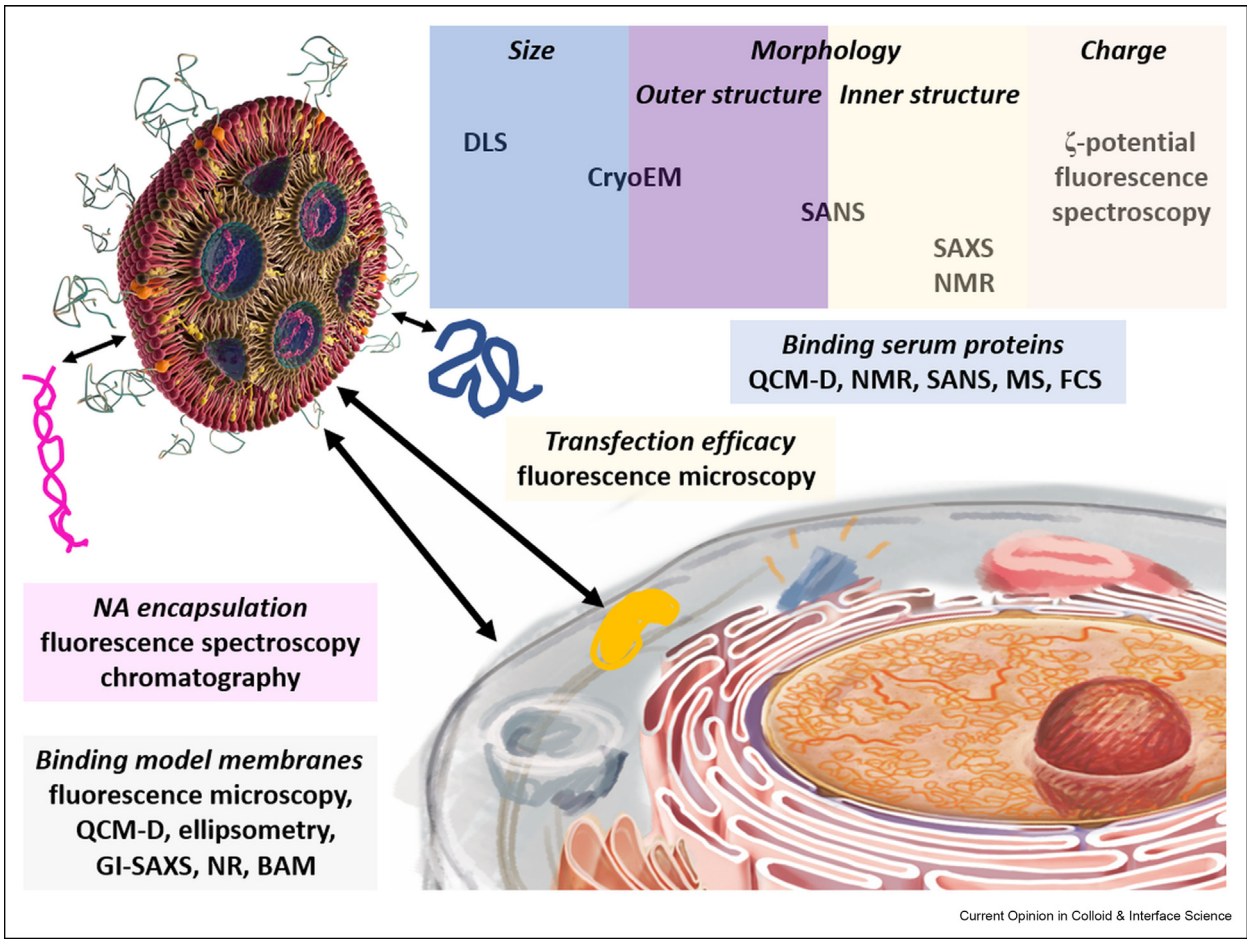
The presence of non-lamellar forming phospholipids such as phosphatidylethanolamine and cholesterol are known to promote inverse structures, which are thought to facilitate membrane fusion and endosomal escape [21]. This property is very important since the genetic carrying molecules would otherwise be degraded inside the acidic lysozyme due to the presence of enzymes. In LNPs, CILs are key to formulating NPs that have colloidal stability and can be efficiently delivered into the cell with low toxicity. A very popular CIL, used to encapsulate short interfering (si)RNA, is heptatriaconta-6,9,28,31-tetraen-19-y14-(dimethylamino) butanoate [22] also known as DLin-MC3-DMA or simply MC3. Indeed, MC3 is the CIL component of Patisiran (Onpattro©) LNPs, which became the first siRNA product to reach the market to treat patients with polyneuropathy caused by hereditary transthyretin-mediated amyloidosis [23]. Since then, the design of CILs has evolved [18] both to improve NA delivery and to support a largely competitive IP landscape.

Since LNPs are metastable complexes, their structure depends to some extent on the method of preparation. LNPs for RNA delivery can be formulated in a variety of methods from basic (ethanol injection of lipid components into the RNA rich aqueous phase) to more complex ones (i.e., rapid mixing using microfluidics). Rapid mixing is the most suitable method to make LNPs of reproducible size and polydispersity. The choice of the preparation method will influence the size distribution of the LNPs (which in some cases limit the production of colloidally stable, low polydisperse LNPs without damaging the RNA), but it does not influence the core structure (which is set by the bulk liquid crystalline phase dictated by the core composition). Finally, we stress that the identity of the NA-encoding molecule is largely irrelevant from the physicochemical point-of-view, as the same liquid crystalline phase of LNP should broadly be obtained when using two distinctive NAs that have the same length for a given lipid composition of the formulation.

Methods for LNP structural characterization

The use of advanced characterization techniques to resolve the physical properties of LNPs with or without their encapsulation of genetic cargo is a growing area in this field. Different physical aspects including LNP size, polydispersity, shape, surface charge, composition, internal structure and stability are now routinely

Figure 2



LNPs interact with serum proteins and with the cell membrane in order to cross it and deliver the NA. Several analytical and physical characterization techniques currently used to study LNP size, morphology and charge are summarized together with those used to measure NA encapsulation, transfection efficacy and their ability to bind with serum proteins and model membranes. LNP, lipid nanoparticle; NA, nucleic acid.

resolved in such analysis. Additionally, their mode of interactions with model cellular membranes can be explored through the use of a number of complementary techniques. In the following, some of the key bulk characterization techniques are discussed before a note on the potential for exploitation of interfacial characterization techniques in this field in the future. The techniques currently used for LNP characterization as well as notes on additional ones for future research are summarized schematically (Figure 2).

Bulk characterization techniques

Dynamic light scattering (DLS) is a key technique to characterize both the size and polydispersity of LNPs suspended in solution [24,25]. The technique measures the diffusion of the particles, which can be converted to the hydrodynamic diameter through the Stokes–Einstein equation. In terms of the detection angle, backscattering can be particularly useful for concentrated or turbid samples, as can be the case for LNPs. Jia et al. [26] proposed the use of size-exclusion chromatography in combination with multi-angle light scattering detector for characterizing LNPs based on size and RNA content.

Cryogenic electron microscopy, including cryogenic transmission EM (cryo-TEM), is a technique used to image frozen LNP samples, revealing their size, size distribution and shape [24,25]. The technique works by plunging aqueous LNP dispersions into liquid ethane or ethane/propane mixtures so as to avoid the formation of crystalline ice. Cryo-TEM has the advantage that the LNP samples do not need to be stained or labelled like with fluorescence-based techniques (see below). Technically, the dispersion is spread on an electron microscopy grid prior to freezing and various image analysis approaches including Bayesian and maximum likelihood estimation can be used. Kulkarni et al. [11] used the

technique, for example, to show that LNPs containing ionizable lipids lack a high-ordered inner structure. This type of microscopy can be used for giving a size population distribution, although it is very important to count enough particles to have statistical significance.

Fluorescence techniques, such as microscopy and fluorescence correlation spectroscopy, as well as mass spectrometry, are used to quantify NA encapsulation [7], using specific RNA and DNA binding dyes as Ribogreen and Picogreeen, respectively. These are also used to locate tagged components in LNPs including co-localization of different lipids and/or NA [24,25]. Fluorescence techniques work through illumination of the sample with a specific wavelength of light that is absorbed by a fluorophore tag. Different colours produced by different fluorophores can also be used to infer co-localization or interactions between different components in cell-based assays. A drawback of using flurophore tags is that they are often bulky organic molecules that may alter the functionality of the lipids and molecules [27]. Therefore, the type, position and effect of labelling must be carefully considered when planning a set of experiments. Majzoub et al. [28] used the technique to show the proportion of tagged LNPs interacting with the early endosome (EE). They used the data to reason that endosomal escape of the LNPs occurs from the late endosome (LE) rather than EE. Improved *in vitro* assays are key for understanding the differences between NP uptake, endosomal escape and effective delivery [29], complemented to pre-clinical *in vivo* studies, that lead to the rational design of lipids and LNPs.

An important property of NPs is their surface charge since this will impact their biological activity and safety. ζ-potential measurements are typically used to determine the surface charge of colloids and NPs, including LNPs [24,25]. The magnitude of the surface charge indicates the degree of electrostatic repulsion between the LNPs in dispersion. A low surface charge results in lack of colloidal stability of the LNPs, while a high surface charge can enhance non-specific interactions with proteins. The PEGylated coating of LNPs has many roles including enhancement of their colloidal stability through steric repulsion, which is dependent on the molecular weight of the polymeric head group. While the ζ-potential measurement of LNPs exhibits quite a low surface charge at neutral pH, which makes measurement typically unreliable, the LNPs are still colloidally stable due to the presence of their hydrated PEG coating. To determine LNP charge, especially as a function of pH, several dyes that respond to changes in the hydrophobic environment have been evaluated such as 6-(p-toluidino)-2-napthalene-sulfonyl chloride (TNS), diphenylhexatriene and laurdan. The exact location of the different dyes within the LNPs is still a matter of intense investigations since they indicate different transitions in the LNPs as a response to pH changes. From those dyes, TNS is the most commonly used to measure the reported 'apparent pK_a' of the LNPs, which indicates the degree of ionization of the CIL in the LNP [30].

Small-angle X-ray and neutron scattering (SAXS and SANS) are used to resolve the internal composition and structure of LNPs [24,25]. Their principles are based on the deflection at small angles (typically of $<10°$) of a collimated beam of radiation as a result of interactions with particles that are much larger than the wavelength(s) of the radiation. As with some of the techniques described above, scattering from very small particles can be used to resolve LNP shape and polydispersity, although in addition small-angle scattering holds additional advantages. Specifically, Bragg diffraction peaks can reveal the internal order, and therefore, the phase of the lipid and specific molecular deuteration in SANS can reveal the locations of different components in the core or shell of the LNPs. The latter is possible thanks to the significant difference in the scattering length density (which can be though of as the neutron refractive index) between hydrogen and deuterium, which manifests as a large difference in the scattering length density of molecules or regions of molecules containing deuterium compared to those containing hydrogen. The high sensitivity of SANS to resolve light atoms, in comparison with SAXS, positions it as a unique tool for the characterization of structure and composition of soft matter systems. For example, the relationship of LNP structure to encapsulation ability of different genetic payload was demonstrated by two independent studies that showed a positive correlation between the intensity of the Bragg peak in the SANS data (corresponding to the inner core structure) and *in vitro* performance [30,31].

There are, of course, other bulk characterization techniques that have been applied and hold promise in their application to resolve the physicochemical properties of LNPs [24,25], such as nuclear magnetic resonance [32]. A single method cannot resolve all aspects of the complexity of LNP physicochemical properties. We believe that a combination of structural techniques such as SAXS and SANS as well as cryo-TEM are ideal to address the full structure of LNPs. The overall morphology and structure suggested by the latter are corroborated and enhanced by detailed structural information on the inner and outer parts of the LNP, down to the individual lipid composition. However, a distinct approach using surface-sensitive techniques that enable the study of membrane curvature [33] and/or coarse-grained molecular dynamics simulations may provide a more complete mechanistic picture of the LNP structure in the future.

Although mRNA vaccines are currently paving the regulatory way for these drug products, most of the United States Pharmacopeia (USP) analytical guidelines available are focused on the identity and purity of the RNA [34]. However, the LNP physicochemical properties such as size, polydispersity, surface charge and encapsulation are also part of the drug product critical quality attributes (CQAs). These CQAs are standard for the characterization during the preparation of an LNP formulation and can be measured using some of the described methods as DLS, zeta potential, fluorescence and chromatography that are available in many research labs [35].

Interfacial characterization techniques

The use of interfacial characterization techniques, in particular various implementations of reflectometry, also offers potential for the study of LNP interactions at model cellular membranes [36], although there have been relatively few studies to date. In addition to solid supported membranes that can be prepared on substrates such as silicon or quartz, the use of a Langmuir trough allows the surface pressure of the membrane to be controlled and monitored during the interaction. The lipid composition of the model membrane can be tuned to mimic that of different cell types.

Various interfacial characterization techniques hold potential to offer key insight into interfacial interaction mechanisms [36], including infrared reflection absorption spectroscopy offering the possibility to detect NA binding to a model membrane through resolution of resonances by specific functional groups. Surface-sensitive fluorescence spectroscopy allows resolution interaction kinetics with sensitivity to single binding events. Neutron reflectometry can resolve the composition and structure normal to an interface and has the potential to quantify lipid exchange between LNPs and model membranes. Ellipsometry can resolve changes in the interfacial thickness. Lastly, Brewster angle microscopy (BAM) offers insight into the in-plane lipid morphology. Additional techniques of potential use include quartz crystal microbalance with dissipation monitoring (QCM-D), atomic force microscopy and grazing incidence X-ray diffraction amongst others.

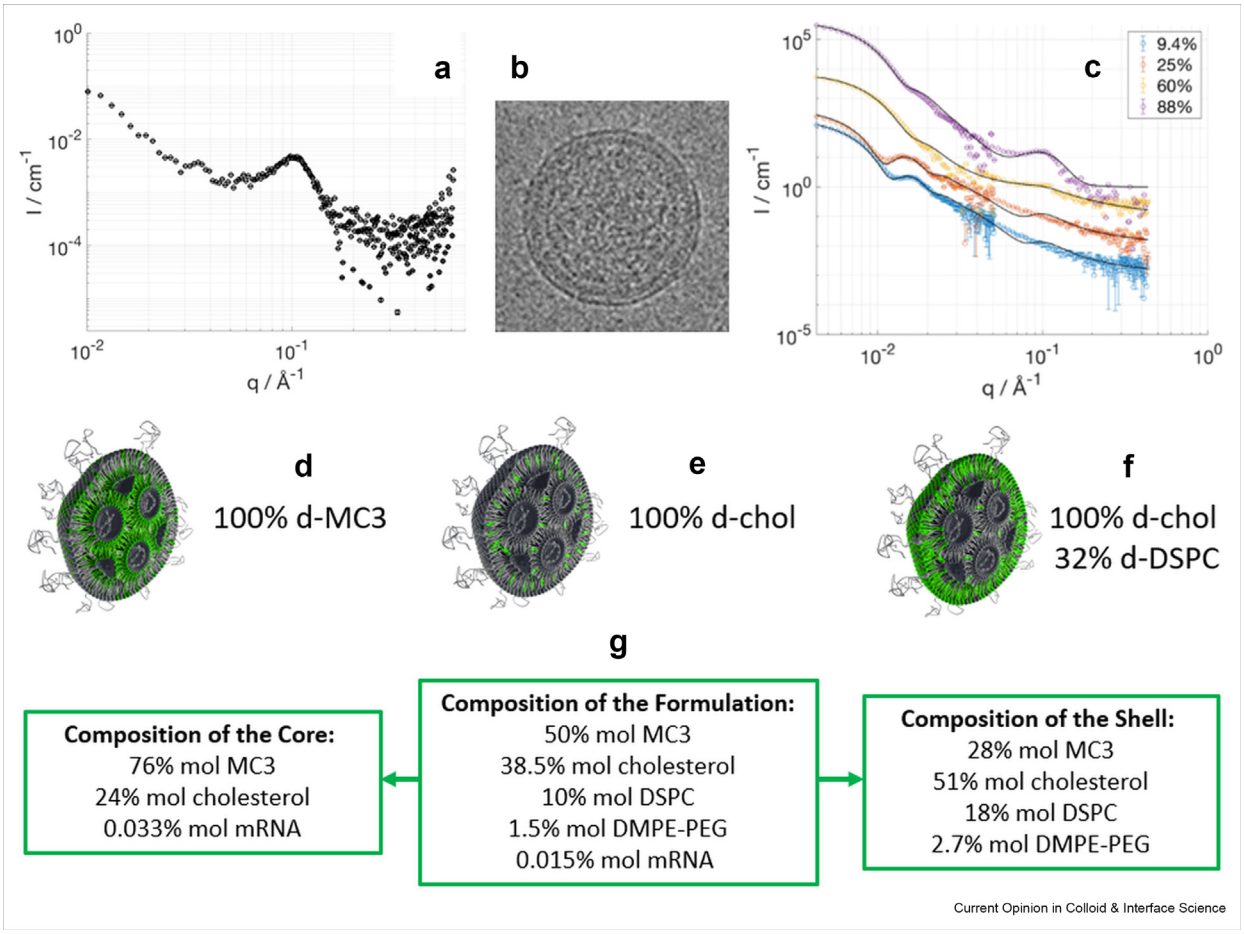
Spadea et al. [37] have recently used ellipsometry and BAM in combination with a low-volume stainless steel Langmuir trough to resolve differences in MC3-based LNPs interacting with model early- and late-endosomal membranes. They showed that insertion of lipid was greatest as pH 5.5 and 6.5 whereas intact LNPs bound to the model membranes at pH 7. Additionally, delivery was shown to be more efficient for mRNA than poly (adenylic acid), where the latter NA is typically used as a mRNA surrogate model, suggesting that it is possible to tune the extent of interaction by the type of cargo. Most surprisingly perhaps, it was shown that the effects of changing lipid composition mimicking the EE and LE were minimal. Aliakbarinodehi et al. [38] went on to show that the binding to a solid-supported endosomal membrane was greater at pH 5 than pH 6 and that the onset of LNP binding was shifted to lower pH when the LNPs had been incubated with a protein corona. Complementary to these studies on the binding of LNPs to model membranes, serum protein binding to LNPs have been assessed using QCM-D to probe protein binding affinity on LNP-coated surfaces and to correlate this binding with LNP protein expression [39]. It will be intriguing to follow the ways in which such works extend in the future, such as the use of additional techniques to quantify aspects already discussed such as lipid exchange and transport across the membrane, among others. In this way, the ideas behind the molecular mechanisms for endosomal escape will be advanced, which will certainly represent a key step forward in the development of LNP-based therapies.

LNP structure and composition

An important characteristic differentiating LNPs from lipoplexes is that their structure cannot be predicted simply from the molar composition of the formulation. It is well established that LNP structure depends on N/P ratio as well as the choice of CIL and helper lipids including cholesterol. Indeed, the different physicochemical properties of the lipid components in LNPs imply that the lipid composition in the shell and the core may differ due to immiscibility of the components. For example, cholesterol has low solubility in CILs, and since a typical LNP formulation contains between 35% mol and 50% mol of cholesterol, it follows that the cholesterol distribution cannot be homogeneous across the LNP. The helper lipids also have limited solubility in CILs, despite the two lipids having distinctive CPPs (CPP = 1 for DSPC versus CPP >1 for CIL). Thus, due to geometrical consideration, it follows that curvature-induced phase separation could take place, leading to spatial separation of DSPC and CIL in different regions of the LNP.

The above considerations about the relation between LNP composition and structure have led to several experimental efforts seeking experimental evidence of the detailed core–shell structure, considerations of which are illustrated (Figure 3). Indeed, two independent studies showed the limited solubility of cholesterol in CILs, which was found to be 24% mol in MC3 [6] and just 8% mol in 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA or KC2) [11]. These works focused on resolution of LNP size and morphology investigated by SAXS and Cryo-TEM (Figures 3a and 3b). Upon dialysis for ethanol removal and pH neutralizations, the LNPs aggregated up to a limited dimension determined by the molar ratio of KC2 [11]. The analysis of the Cryo-TEM images led to the

Figure 3



The detailed structure of LNP core and shell is resolved using a combination of techniques. (**a**) SAXS data give the size of the pore of the liquid crystalline phase in the LNP core; (**b**) cryo-TEM images give the overall size and morphology including a visualization of the complex structure in the LNP core; (**c**) SANS data exploit isotopic contrast variation to resolve the locations of the LNP components between the core and shell (data shown involve 100% deuteration of the cholesterol and 32% of the DSPC); (**d–f**) schematics show in green the result of deuterating stated components where the prefix 'd-' signifies deuteration of the molecule and 'chol' refers to cholesterol; (**g**) summary of molar compositions of an LNP formulation as well as those resolved of the core and shell. Data in A and C are replotted from the study by Sebastiani et al. [7]; data in B are reproduced from the study by Kulkarni et al. [11]; panels D–G summarize results from the study by Kulkarni et al. [11]. cryo-TEM, cryogenic electron transmission electron microscopy; DSPC, distearoylphosphatidylcholine; LNP, lipid nanoparticle; SANS, small-angle neutron scattering; SAXS, small-angle X-ray scattering.

conclusion that KC2 was a main component of the core since the electron density of the LNP core increased with KC2 mol ratio. Moreover, cryo-TEM images for LNPs with varying N/P ratio (from 1 to 6) showed that the higher the N/P, the larger the electron dense core. Simultaneously, the lamellarity decreased with increasing N/P ratio, which suggested that siRNA drove the formation of lamellar structure surrounding the KC2-rich core. In the following study, the same research group performed a systematic investigation of the combined effect of DSPC and cholesterol on the structure and morphology of siRNA LNPs with Cryo-TEM [40]. The DSPC to cholesterol molar ratio was kept to 1:1, while the combined content was varied from 5 to 80% mol, showing that the entrapped siRNA increased with DSPC-cholesterol combined content up to 40% mol and then remained stable. This result suggested that helper lipids, such as DSPC and cholesterol, are essential in the formulation of LNPs and contribute to high encapsulation of the genetic material.

Even though cryo-TEM has provided detailed information on LNP morphology, all of its components have a very similar contrast which limits the ability of this technique to distinguish where a single component is located. SANS combined with isotopic substitution excels at highlighting a specific component in a multi-component system. Using this approach, Yanez Arteta et al. [6] demonstrated that LNPs consist of a core-double shell model in which PEGylated chains exist in a

mushroom conformation (outer shell), followed by a lipid mixture dense in DSPC (inner shell) and a dense core dominated by CIL with only ~ 24% vol water content (Figures 3c–g). Such a core-double shell model is illustrated schematically in Figure 1. A continuation study in 2021 fully determined the distribution of the other lipid components in mRNA-LNPs: deuterated versions of cholesterol, MC3 and DSPC were used to determine the core/shell size and their exact compositions in terms of lipids and sterols [7]. In the latter case, the particles were modelled with a simpler core–shell sphere since the scattering contribution of the PEG layer in the outer shell was negligible given the deuteration scheme used. The thickness of the inner shell was found to be larger than a DSPC monolayer and closer to that of a lipid bilayer, which could be the result of the highly unsaturated lipid MC3 presence in the shell (ca. 30% mol). Moreover, cholesterol was distributed across the inner shell and core, with the inner shell having roughly double the molar fraction of the core. In contrast, the MC3 fraction in the inner shell was less than half of that occupied in the core. Recently, a different research group used SANS to explore the effect of acidification in the LNP structure, revealing that ionization of the lipids can lead to redistribution of components across the LNPs [41].

In summary, these recent studies demonstrate that a systematic and detailed structural investigation of LNPs, for any given formulation, is needed. Such work serves as a start for our research community to construct a map of the resulting structures and in this way advance the knowledge needed, in time, to predict LNP structures based on the composition of their formulation.

LNP formulated with analogues and derivatives: Effects on structure and function

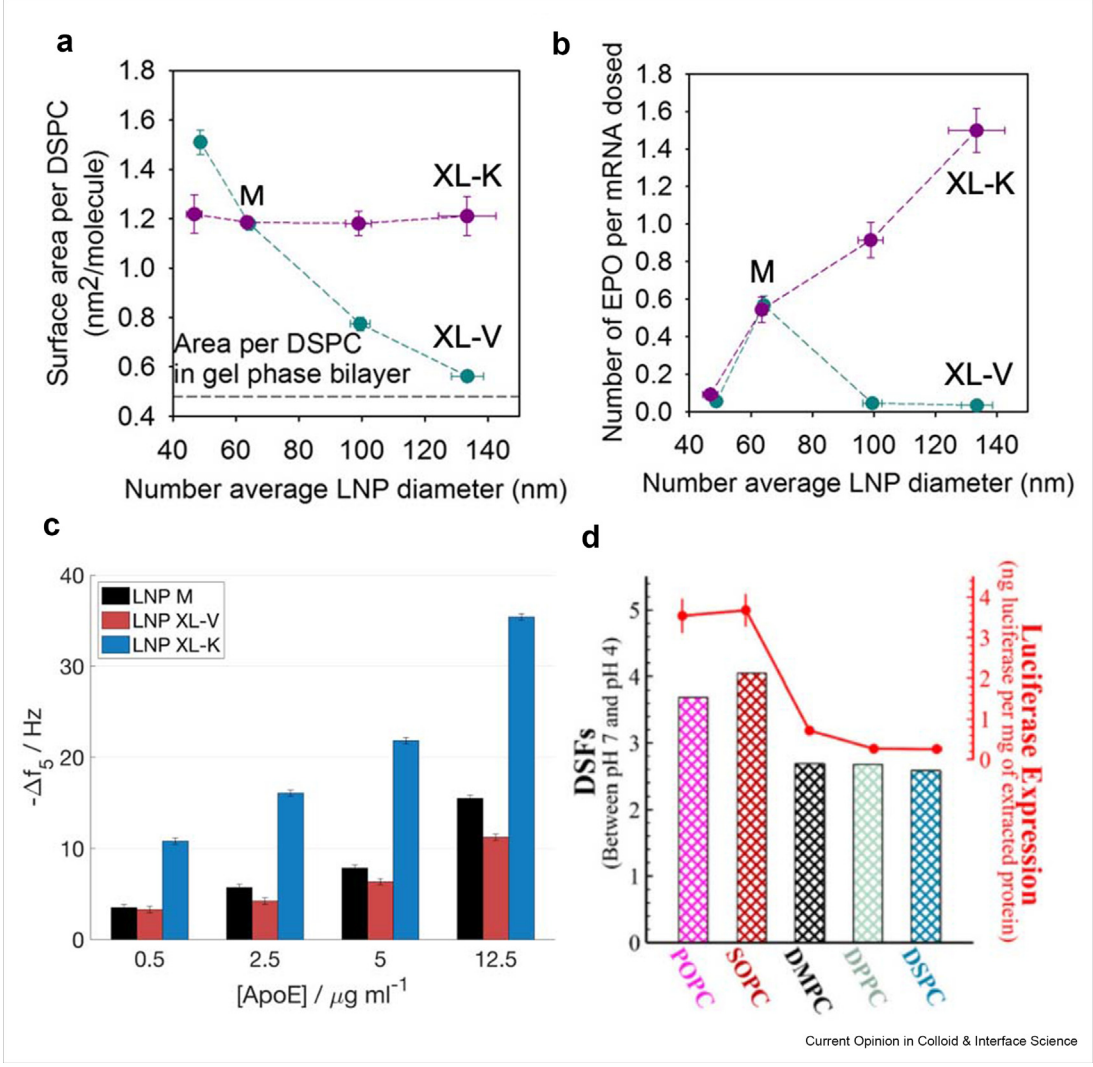
Knowledge of LNP structure and composition linked to efficacy of biological function is fundamental to improving the therapeutic capabilities of LNPs. Several studies have demonstrated the impact that finding the connection among composition–structure–function can have in the improvement of LNPs as NA delivery vehicles. Following on from the SANS studies described above [6,7], a set of LNP formulations was designed that resulted in similar sizes (albeit larger than the standard composition). One of the larger LNP formulations kept a constant DSPC area/molecule (1.2 nm^2) in the shell, while the other presented a variable DSPC surface density. These formulations were dosed to cells (adipocytes and hepatocytes) to compare their protein production ability. While the level of encapsulation of mRNA per LNP was similar in the two series of formulations, the protein expression was higher when surface area per DSPC was kept to 1.2 nm^2 in both cell lines (Figures 4a and b). Based on this result, the authors suggested that the surface composition of mRNA-LNPs is key for efficient protein production. Subsequently, using a surface-sensitive characterization technique (QCM-D), the extent of protein binding to LNPs was determined, and the binding affinities of various apolipoproteins to mRNA-LNP were ranked [39]. It was found that apolipoprotein E (ApoE) binding to mRNA LNP formulations correlated with protein expression *in vitro* upon mRNA-LNP dosing [6] (Figures 4b and c). The results confirmed the key role of ApoE in cellular uptake of mRNA-LNPs and the importance of the surface structure of mRNA-LNP in the binding to ApoE, as suggested earlier [6].

Other studies reported that the choice of helper lipid can affect both structure and function of the resulting LNP (Figure 4d). In particular, the use of unsaturated helper lipids instead of saturated ones in the LNP formulation resulted in higher luciferase expression *in vitro*, and this was linked to a larger structural remodelling upon pH decrease for the LNP containing unsaturated helper lipid than for the LNP containing DSPC [41]. The latter was quantified by the structural change in LNP taking place when changing the pH from 7.4 to 4 as measured by SANS; the so-called Euclidean distance in the structure feature space.

Another recent study shows that the type of sterol used also influences LNP structure and function [42]. In particular, the use of β-sitosterol induced a faceted LNPs surface and resulted in higher intracellular uptake, retention and transfection efficacy than the other analogues. The authors hypothesized that the presence of defects on the surface of β-sitosterol LNPs facilitated fusion with the endosomal membrane. Finally, a slight change in the inner structure of the core occurred as measured by SAXS, as expected, since the core contains sterols to some extent (see previous section and Figure 3g).

The use of PEGylated lipids in LNP formulations raises some concerns about activity and safety. For example, PEGylation can accelerate blood clearance through the formation of anti-PEG antibodies, which are increasingly found in healthy population due to the high probability of exposure to PEG-containing product [43,44]. To prevent the disadvantages of PEGylation, efforts are being made to replace this molecule while keeping its essential function. Among these, polysarcosinylated (pSar) lipid was reported to improve safety profile of LNPs and maintain the same *in vitro* and *in vivo* effects [45]. LNPs containing pSar lipids mirrored the properties of LNP containing PEGylated lipids: LNP particle size decreased with increasing pSar headgroup and pSar-lipid molar ratio, and longer pSar headgroup disrupted the internal order (at lower molar ratios than PEGylated lipids though). The *in vitro* and *in vivo* performance of the LNPs showed a similar protein expression for PEG-lipid and pSar-lipid, while the safety profile improved for

Figure 4



LNP structure correlation with function. (**a**) A standard MC3-based LNP formulation (M) was modified to increase its size having a constant (XL-K) or variable (XL-V) surface area occupied by DSPC. The horizontal line corresponds to the reported value of the area per DSPC in the gel phase. (**b**) Protein expression for LNP formulations in A in terms of the number of hEPO expressed per mRNA dosed after 48 h of dosing adipocytes showed increased function for the formulation with constant surface area of DPSC. A and B adapted with permission from the study by Yanez Arteta et al. [6] (Copyright 2018 the Authors). (**c**) Serum ApoE protein binding to LNP in terms of the net changes in Δfrequency (fifth overtone) as a function of ApoE3 concentration showed increased binding to LNP XL-K as measured by QCM-D. C adapted from the study by Sebastiani et al. [39]. (**d**) The compositions for LNP formulation M, XL-V and XL-K correspond to molar ratio for DSPC:Cholesterol:MC3:PEGylated lipid that is 10:38.5:50:1.5, 10:39.75:50:0.25 and 4.65:41.18:53.47:0.7, respectively. *In vitro* luciferase expression correlates with the extent of LNP acidification induced structural evolution, which depends on the phospholipid used, and the Euclidean distance in the structure feature space (DSF) value as the distance between the LNP's structural feature space (LSF) positions of each LNP at pH 7.4 and 4. The scatter plot (red) shows the optimal luciferase expressions (the right y-axis). D adapted from the study by Li et al. [41]. DSPC, disteroylphosphatidylcholine;LNP, lipid nanoparticle; PEG, poly(ethylene) glycol; QCM-D, quartz crystal microbalance with dissipation monitoring.

LNPs containing pSar-lipid. Another category of molecules to explore in the future is polyoxazolines, for example, which have been used for other type of NPs instead of PEGylated lipids.

Administration of LNPs

For the progression of LNP technology as gene delivery system, combining the knowledge on structure and function is essential and the selection of works reviewed in this work clearly demonstrates the advantages of such an approach. The control of LNP structure can lead to tuning of the function, either in a direct or indirect way. For example, LNPs can be engineered to target specific organs either including a targeting molecule (direct [46]) or having affinity for serum proteins that promote a certain uptake pathway or (indirect or endogenous [47]). While the first approach is the most common and exploited, it is highly valuable to investigate the indirect

targeting through control of the biomolecular corona, as suggested in a recent review by Francia et al. [47], and in this way overcome the liver accumulation which represents a significant barrier in the exploitation of LNPs for NA delivery.

Moreover, LNPs can be administered in a variety of ways depending upon the disease that they are intended to treat. For example, LNPs intended to treat lung disease can be administered into the lungs *via* instillation or inhalation, while others are injected intravenously such as Patisiran, an Food and Drug Administration approved LNP-based siRNA therapeutic, which is administered through the vein because its target is hepatocyte produced transthyretin amyloid [48]. In contrast, the COVID-19 RNA vaccines are injected intramuscularly where they result "*in transient local inflammation that drives recruitment of neutrophils and antigen presenting cells (APCs) to the site of delivery. Recruited APCs are capable of LNP uptake and protein expression and can subsequently migrate to the local draining lymph nodes where T cell priming occurs*" (EMA Assessment Report, 2000 [49]).

Mechanism of cellular uptake and endosomal release of LNPs

Cellular uptake

As noted above, specific serum proteins (such as ApoE) adsorbed on the surface of LNPs after administration into the body can drive the cell internalization [50]. This phenomenon has been demonstrated for ApoE-coated LNPs, which are taken up by hepatocyte cells following the interaction between ApoE-adsorbed on the LNPs and low-density lipoprotein receptors on the cell membrane [51]. Alternately, by deliberately decorating the surface of LNPs with a targeting ligand such as an antibody, it is possible to exploit other receptors either uniquely or over-expressed on the surface of the target cells in the cellular uptake of LNPs [46].

Cellular uptake processes typically require the formation of the EE, a cellular vesicle containing fluid which can range in pH between 5.5 and 6.5 which can engulf NPs, including LNPs, in close proximity to the cell membrane. The EE then undergo a maturation process leading to the formation of the LE with its lower internal pH (5.0−5.5). Subsequently, the LE will fuse with the lysosome (Ly) which results in a further reduction of the vesicles' interior pH down to 4.5−5.5. Significantly, the Ly contain a range of enzymes including lipases, nucleases, glycosidase, proteases, phosphatases, sulfatases which together can destroy LNPs as well as degrade the NA cargo [52]. It is essential, therefore, that the LNPs and their NA cargo 'escapes' the endosomal compartment, being released into the cytosol, before the LE fuses with the Ly hence the degradation cascade can begin.

The cationic lipids used to prepare lipoplexes and lipopolyplexes and the ionizable lipids used to prepare LNPs, which are predominately cationic at the low pH of the endosome, are an essential component of the NPs/NAs escape. This escape is believed to be achieved by the binding of these exogenous cationic lipids to the negative lipids expressed on the interior side of the endosomal membrane which results in the formation of non-bilayer, hexagonal (HII) structures which perturb the organization of the endosomal membrane resulting in the consequent escape of the NPs/NA.

Endosomal release

Significantly, it is not the uptake of the NPs by the EE that is thought to limit delivery with NPs as many types of NPs have been shown to be successfully internalized by cells into EE. The real bottleneck is the escape of NPs and their intact payload from the endosome into the cytosol [53]. Indeed, it has been estimated that less than 2−3% of their NA payload escape the endosome to reach the cytosol [53,54], even though roughly 95% of LNPs (CM3:DSPC:cholesterol: DMPE-PEG molar ratio of 50:10:38.5:1.5) are endocytosed by cells within 6 h [54]. A more recent study investigating the fate of LNP-delivered mRNA and other components of LNPs after cellular uptake showed that less than 1% of administered mRNA was detected in the cytosol of the LNP-treated cells. The study also demonstrated that the nature of the cationic lipid used to prepare the LNPs influenced the extent of endosomal escape of its payload. It was shown that ionizable lipids could form complex salts, where the simple small counterion of the RNA is replaced by an ionizable lipid inside the endosomes acidic pH and this could affect its subsequent release into the cytoplasm [8]. Therefore, it is recognized that to fully harness the potential of LNPs, the amount of RNA escaping the endosomes into the cytosol must increase. Unfortunately, at the present time, the fate of *endocytosed* LNPs and how and why only a small amount of the LNPs payload reaches the cytoplasm is not completely understood.

There is consensus among the scientific community that changes in LNPs structure by tuning formulation are a promising approach to increase endosomal escape and hence transfection efficacy [6]. However, most of the structural studies of LNPs have been performed at physiological pH. Endosomal compartments present a mildly acidic pH, and hence, it is relevant to investigate LNPs in such conditions. As already mentioned [41], a correlation between structure (measured by SANS) and *in vitro* gene transfection was reported for plasmid DNA−LNPs in both physiological pH and acidic pH (Figure 4d).

Liu et al. [55] demonstrated that improved mRNA delivery could be obtained by tuning the chemical

structure of the ionizable lipid. The authors developed a phospholipid with one ionizable amide, one phosphate group and three hydrophobic alkyl tails (iPhos). This new lipid showed high mRNA delivery efficacy, which correlated with the strong ability to rupture endosomal model membrane. iPhos lipids showed a superior ability to fuse with endosomal model membrane compared to simple tertiary amine lipids. Furthermore, thanks to the iPhos lipid large tail body and inverse conical shape, disruption of the endosomal bilayer was promoted through phase transition to hexagonal phase.

Lastly, biophysical models for endosomal escape are most needed to be able to easily correlate those changes in composition and LNP structure with their ability to fuse and escape the endosome at acidic pH. As discussed earlier, initial studies using microscopy [38] and surface-sensitive techniques [37] were published very recently to follow LNP binding to model endosome membranes. However, more work is needed in this respect to unravel mechanistic details of not only LNP fusion but also translocation across the endosome membrane.

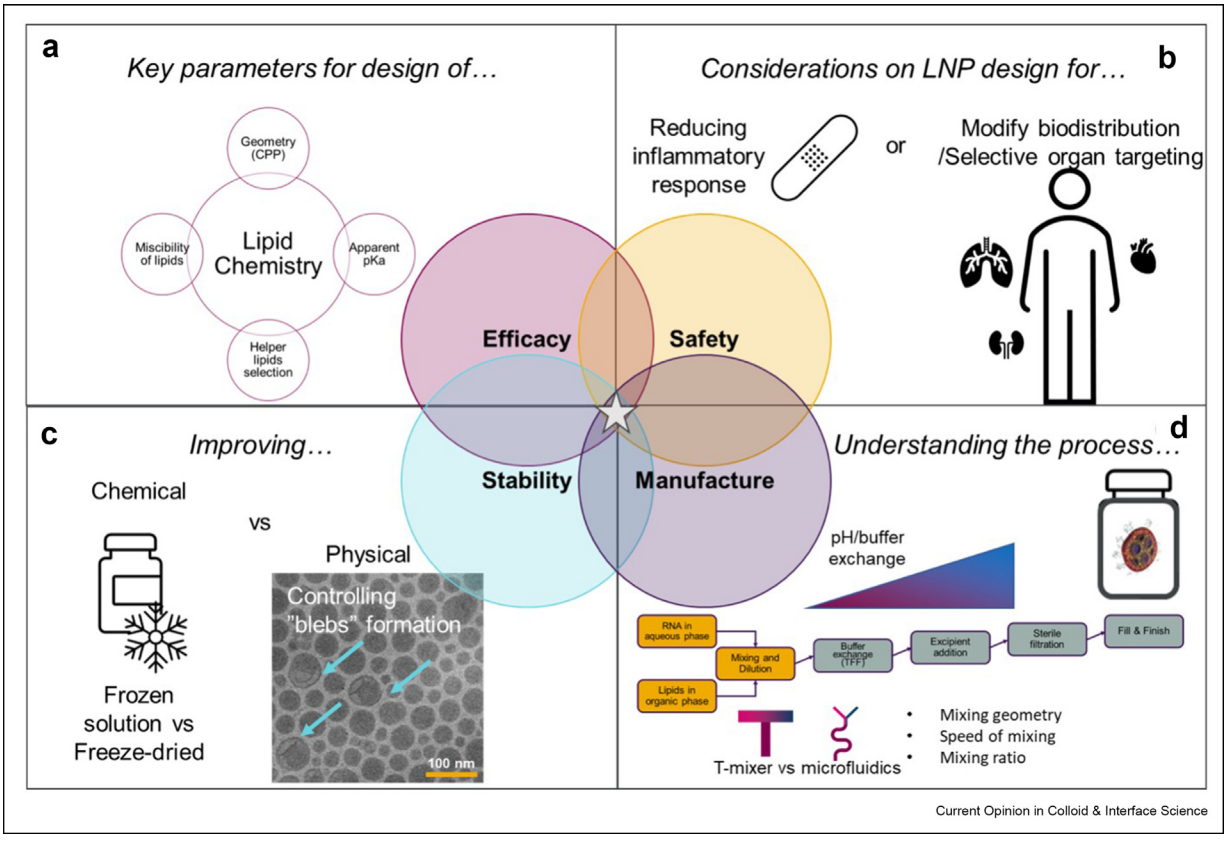
The future of LNP development: planning success for the next generation of NA therapeutics

The advancement of NA-based therapies relies on translating the early research efforts to patient-centric drug products and the development of the next generation of LNPs has many challenges yet to address. Several RNA-LNP products have been commercially approved or are being evaluated in clinical studies, especially for intravenous or intramuscular administration, while other routes are also being explored for similar systems, including intranasal [56]. It is clear, though, that one LNP will not achieve a 'one-size-fits-all' approach, and this platform will require tailoring to the intended therapy. In this section, we will focus on how understanding the molecular interactions in an RNA- LNP system will be fundamental for the future of their advancement with four key fundamental pillars: efficacy, safety, stability and manufacturability (Figure 5).

Efficacy

During early drug discovery, the research is mostly focused on designing novel lipid chemistry and LNPs

Figure 5



Schematic description of the four pillars guiding the rational design of the next generation of LNPs for nucleic acid delivery. (**a**) Efficacy and the key parameters guiding lipid and LNP design, (**b**) safety and considerations for LNP design towards reducing inflammatory responses or decreasing dose by improved biodistribution, (**c**) Stability and challenges and opportunities around physical–chemical stability. Cryo-TEM micrograph adapted with permission from the study by Yanez Arteta et al. [6] (Copyright 2018 the Authors) and (**d**) manufacturability and schematic of scale up process and some of the critical process parameters. cryo-TEM, cryogenic electron transmission electron microscopy; LNP, lipid nanoparticle.

with improved efficacy that can overcome the commonly reported bottleneck of poor single digit percentage endosomal escape efficacy [54]. It is clear that CILs are fundamental for cell transfection, with CILs that have a cone-like geometry, i.e., CPP >1, been more efficient to disrupt the lamellar-shape endosomal membrane [18]. Additionally, the 'apparent pKa', which relates to the degree of ionization of the CIL headgroup in the LNP mixture environment, will determine the particle surface charge and eventual their ability to interact *via* coulombic attractions to the endosomal membrane [57].

The LNPs also contain helper lipids such as cholesterol, a phospholipid and a PEGylated lipid. Their role was largely assumed to support the structure and colloidal stability of the LNPs as well as to control size [18]. Understanding the different distribution of lipids across the LNP, including their potential to phase separate, needs to be incorporated in the overall selection of the lipid composition and molecular dynamic simulations could provide additional insights on these phenomena [58].

Safety

Another important factor on selecting the LNPs, besides their efficacy, is the right safety profile. This is largely determined on the disease, the target tissue and the route of administration. In the case of Patisiran, aimed at patients who suffer of a rare and fatal disease, the LNP formulation is administered with dexamethasone as glucocorticoid for reducing reactogenicity previously attributed to the lipid components, especially the CILs. An approach to improve the LNP safety profile is to include anti-inflammatory compounds, for example by incorporating aliphatic ester prodrugs of anti-inflammatory steroids to enable subcutaneous administration [59]. The location within the LNP of these pro-drugs is critical since they need to be available at the LNP surface to undergo enzymatic cleavage. This appears to be related to the length of the alkyl chain of the steroid lipid, with longer alkyl chains delaying their rate of hydrolysis.

Another limitation for the safe development of LNPs is their accumulation in the liver due to LDL receptor-mediated uptake, which limits their applications to other tissues. The discovery of selective organ targetingLNPs that include an additional lipid with diverse charge has been reported as a way to modulate delivery between the liver, lung and spleen [50]. Although the role of this additional lipid on the LNP structure is still subject of investigation, it is clear that the net charge of the LNP has a role on modulating their activity. Targeted approach opens the possibility to improve the development of safe RNA treatments by decreasing the dose required to achieve therapeutic effect.

Stability

From the stability perspective, it is known that the marketed mRNA LNP vaccines are frozen due to their lack of long-term stability in solution at 2−8 °C. The limited shelf-life at refrigerated conditions is mainly attributed to chemical degradation of the mRNA as well as reactogenicity of lipid impurities with nucleotides in solution [60,61]. To improve the supply chain of these products, strategies such as freeze-drying have been identified as a promising approach using sugars as lyoprotectants. Although this process is known to affect the size and encapsulation of the LNPs, it has been reported that function could still be preserved which points out that these attributes are not the sole determinant of the LNP performance [62]. The selection of the excipients in the solution, e.g., buffers and sugars will be critical for this approach, indicating that the solution media has a large impact on the formation and colloidal stability of the LNPs upon reconstitution.

The colloidal stability of the LNPs will be influenced by their lipid composition, with some of them showing features that been described as "blebs" where the RNA can potentially be separated in an aqueous compartment [63]. Although the composition of the 'blebs' has not been elucidated, the phase separation of phospholipids as DSPC towards the LNP surface might play a significant role [6]. Thus, the long-term physical stability of LNPs showing 'blebs' might be limited compared to those without these features, indicating that effective lipid mixing as well as interactions with the RNA cargo are necessary to achieve better products.

Manufacturability

From the manufacturability perspective, there are major differences between the process used to prepare LNPs during the research stage compared to those used for clinical and commercial manufacture, which ultimately can impact their performance. In general, LNPs are non-equilibrium (or metastable) structures, and their long-term colloidal stability will be impacted by their manufacture processes. There are many approaches to formulate LNPs, but most of them involve the preparation of the lipid mixture in an organic solvent due to their low polarity. The advances of microfluidics have facilitated the preparation of LNPs in early research using a bottom-up approach due to their high reproducibility and low volume required [64]. Besides microfluidics, other approaches, including the confined impinging jets developed by Prud'homme's lab [65] and simple t-mixers, are used for scaling-up clinical and commercial manufacture of RNA LNP drug products since they are easy and cheap to scale and have the potential to form NPs with similar CQAs based on the organic NP precipitation principle depending on the geometry and the speed of mixing [66]. Given the non-

equilibrium nature of LNPs, many factors will impact the outcome after mixing including.

- *pH and ionic strength of the NA buffer during mixing*
  - o The lipids in the organic solvent are mixed with the NA dissolved in an acidic buffer. The low pH is required for electrostatic complexation between the CIL and the NA and ions in solution can screen those interactions. Although Coulombic attractions have been attributed as the main reason for LNP formation, hydrophobic interactions based on the lipid chemistry and nucleotide modifications have also been identified as significant for the LNP formation, and therefore, the ideal buffer conditions might require optimization between different lipid mixture and for diverse cargos [67].
- *Ratio between the lipids and the NA and total concentration during mixing*
  - o LNPs are less stable and, thus, more difficult to manufacture close to the 1:1 CIL:nucleotide molar ratio, which correspond well with typical behaviour of strongly interacting oppositely charged amphiphiles/polyelectrolyte systems. Close to charge neutrality, these mixtures exhibit macroscopic phase separation and, therefore, higher N/P ratios are favourable for manufacturability. On the other hand, a high load of CIL might pose a risk for adverse reactions and thus, N/P ratio will need to be balanced through the manufacture process optimization.
- *Down-stream processing*
  - o During research, dialysis is employed to exchange the organic solvent/acidic solution mixture to a physiologically relevant buffer for storage. Dialysis is not a scalable process, and this is replaced by dilution post-mixing and ultrafiltration/diafiltration. However, dilution factors and holding times during down-stream processing affect the final LNP size and structure, leading to variable performance [68], which points again to the non-equilibrium nature of the system.

Thus, scientific understanding of the mechanisms of the formation of LNPs will be the key to achieve drug products of consistent quality, which is also required by the regulatory authorities.

Concluding remarks

Although Patisiran marked the first LNPs for siRNA delivery, and COVID-19 gave mRNA-LNP vaccines their big breakthrough, LNPs have the potential to enable future therapies such as gene repair with the aim of not only treating but curing diseases. Their application will also need to adapt to novel cargos, including even longer NAs (such as self-amplifying mRNA) or highly modified nucleotides. This will continue challenging the research and development community to generate further knowledge on the intermolecular interactions governing the success of the LNPs. For this, addressing the relation between LNP structure and functionality, specifically in relation to their abilities to bind serum proteins and escape the endosome, is key to moving the state-of-the-art forward. The recent methodological development to address key aspects that are believed to control LNP functionality such as apolipoprotein binding and LNP binding and fusion to model endosome membranes must be continued and strengthened since these will help test hypotheses in less time-consuming and expensive *in vitro* assays.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
Marianna Yanez-Arteta reports a relationship with AstraZeneca R&D Gothenburg that includes: employment.

Data availability

No data was used for the research described in the article.

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