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Lipid nanoparticles for RNA delivery: Self-assembling *vs* driven-assembling strategies

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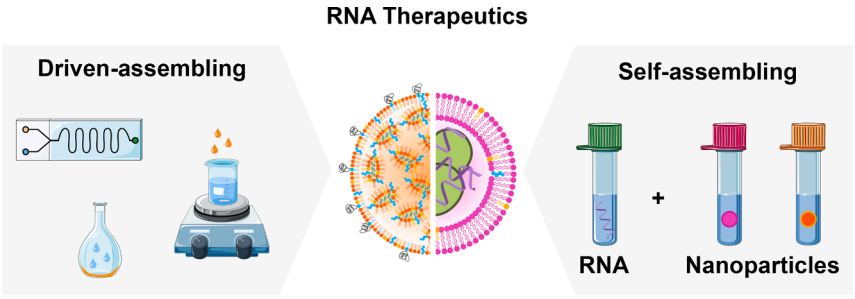
GRAPHICAL ABSTRACT



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ABSTRACT

Among non-viral vectors, lipid nanovectors are considered the gold standard for the delivery of RNA therapeutics. The success of lipid nanoparticles for RNA delivery, with three products approved for human use, has stimulated further investigation into RNA therapeutics for different pathologies. This requires decoding the pathological intracellular processes and tailoring the delivery system to the target tissue and cells. The complexity of the lipid nanovectors morphology originates from the assembling of the lipidic components, which can be elicited by various methods able to drive the formation of nanoparticles with the desired organization. In other cases, pre-formed nanoparticles can be mixed with RNA to induce self-assembly and structural reorganization into RNA-loaded nanoparticles. In this review, the most relevant lipid nanovectors and their potentialities for RNA delivery are described on the basis of the assembling mechanism and of the particle architecture.

1. Introduction

The therapeutic potential of RNA is widely recognized and can be ascribed to its ability to harness the fundamental principles of molecular biology to modulate protein expression and achieve precise gene editing [1]. The main types of therapeutic RNA with a brief description of their properties and mechanism of action are reported in Table 1. While research into RNA-based drugs has been ongoing for over 40 years, only recently RNA therapeutics have been brought to the public attention due to the approval of the two mRNA-based vaccines for COVID-19 [2]. Despite the great therapeutic potential of RNA, biopharmaceutical issues have hampered the clinical use of RNA-based medicines, with some delivery challenges to be addressed. Therapeutic RNA is a high molecular weight, anionic molecule and is susceptible to nuclease

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degradation; these features prevent RNA from entering the cells following *in vivo* administration and make the use of strategies for RNA delivery necessary [2,3].

Vectors for RNA delivery can be categorized into two broad classes: viral and non-viral vectors. Viral vectors have been approved for the delivery of plasmid DNA and have also been proposed for the delivery of RNA [10,11]. Nevertheless, there are some concerns on the immunogenicity and safety of viral-based systems [12], which has prompted the development of non-viral vectors. Among these, lipid-based nanovectors (LNVs) are the forefront technology for RNA delivery. A timeline of key milestones in the development of RNA-loaded LNV formulations is shown in Fig. 1. Liposomes were initially proposed for *in vitro* RNA delivery in 1978 [13,14]; subsequent work led to the introduction of cationic liposomes for DNA [15] and RNA [16] transfection in 1987 and 1989, respectively. The first clinical trial involving cationic liposomes dates back to 1993 and assessed the safety and feasibility of DNA delivery for the treatment of melanoma [17]. The introduction of stabilized antisense-lipid particles (SALP) (subsequently termed as Stable Nucleic Acid Lipid Particles or SNALPs and finally as Lipid Nanoparticles or LNPs) for the delivery of antisense oligodeoxynucleotides in 2001 [18] has revolutionized the RNA delivery field, with the first clinical trials on mRNA-loaded lipid nanoparticles (LNPs) in 2014–2017 and the approval of Onpattro® (LNPs for siRNA delivery) in 2018 [19]. The approval of mRNA-based vaccines against COVID-19 (Cominarty® and Spikevax®) has dramatically accelerated the development of LNP-based therapeutics, with several ongoing clinical trials (Tables 2 and 3).

LNVs offer various advantages: they can effectively encapsulate RNA, protect it from enzymatic degradation, and achieve high RNA intracellular delivery. Moreover, LNVs can be designed to reach target tissues or cells. With the approval of LNV-based formulations for RNA delivery, a growing attention has been paid on LNV preparation methods to identify procedures which are easy to perform, time-saving, and suitable to scale-up. With the increasing knowledge on prognostic markers and improved diagnostic tools, the need for precision and personalized therapies is rapidly emerging. In this context, LNVs that can be assembled by loading the RNA payload directly at the point-of-care may represent a promising approach to personalize RNA-based therapies tailored to the biomarker profile of the patients.

1.1. General concepts on lipid self-assembly

Lipid nanomedicines are based on LNVs formed by the assembly of lipid molecules and the subsequent formation of colloidal particles with different morphologies. Lipids are amphiphilic molecules characterized by the coexistence of a polar hydrophilic moiety and a non-polar hydrophobic region [20]. This feature enables their self-assembly in a plethora of structures once a threshold concentration (critical micellar concentration) is reached in aqueous environments [20]. Two competing factors drive the spontaneous lipid assembly: the hydrophobic effect (*i.e.*, the tendency of non-polar molecules to interact with each other to reduce water contact) and the attractive forces between the polar region of amphiphiles and water [21]. The final self-assembled structure will be the one ensuring the lowest assembly free energy [22]. Given the key role of molecule geometry in amphiphilic self-assembly, an a-dimensional packing parameter (*i.e.*, critical packing parameter, CPP) which accounts for the relative proportion of the hydrophobic and the hydrophilic domains is used to predict the final self-assembled entity [23].

With regards to lipid-based nanoparticles, a lamellar organization is obtained when CPP = 1; hexagonal liquid crystalline phases are formed when the lipids have a CPP > 1, while CPP values < 1 result in micellar structures [24]. Depending on the selected lipidic composition, various assembled/self-assembled structures can be formed [25]. Several phospholipids possess a cylinder-like geometry and spontaneously assemble to form particles with a lamellar bilayer surrounding an aqueous core (*i.e.*, liposomes), while lipids with a cone shape or inverted cone shape assemble into micellar structures [26]. The chosen lipid composition affects both the structure and the main properties of the LNVs in terms of drug retention upon storage, colloidal stability in blood, and interaction with the cell membrane and the sub-cellular compartments.

The final particle architecture can also be affected by the preparation method: for liposomes, the choice of the formulation method and the use of size reduction techniques affects the morphology and the size of the nanoparticles [27,28]. This is true also for other non-liposomal nanovectors, for which in defined experimental conditions lipids assemble to create nanovectors with a certain morphology. In the case of LNPs, for example, the change of the pH during the preparation induces a transition from liposomal vesicles to nanoparticles with hydrophobic core comprising inverted micelles [29]. From a general point of view, all the LNVs are formed by lipid self-assembling which yields colloids with a well-defined structure. A careful selection of the manufacturing process is required in some cases to drive lipid assembly into nanovectors; in other cases, specific components spontaneously and rapidly interact through simple mixing, leading to the formation of nanoparticles with the desired physical characteristics.

Within this framework, a classification of RNA-loaded LNVs which accounts for the specific mechanism underlying lipid assembly can be proposed. In particular, two main categories have been identified:

- LNVs obtained by process-driven assembling of individual lipid molecules in the presence of RNA, here termed as driven-assembling LNVs;
- LNVs that spontaneously assemble upon mixing RNA and pre-formed nanoparticles as a result of structural re-organization at a supramolecular scale, here termed as self-assembling LNVs.

Table 1
Main properties of therapeutic RNA.

RNA Type	Abbreviation	Type	Length	Description
Small interfering RNA	siRNA	Double-stranded	18 – 22 nucleotides	Double-stranded non-coding RNAs that promote degradation of complementary messenger RNA, effectively silencing specific target genes [4].
MicroRNA	miRNA	Single-stranded	18 – 22 nucleotides	Short, single-stranded non-coding RNAs involved in post-transcriptional gene regulation by binding specific sequences within the target RNA, which either degrades mRNA or inhibits its translation [5].
Antisense oligonucleotides	ASOs	Single-stranded	18 – 22 nucleotides	Short, synthetic nucleic acids that bind target RNAs through complementary base pairing, altering their biological functions [5].
Small activating RNA	saRNA	Double-stranded	18—22 nucleotides	Double stranded RNAs that enhance the expression of desired genes, although the mechanism of action is still unclear [6].
Ribozymes	Not used	Double-stranded	10–5000 nucleotides	Catalytic RNA molecules that can precisely edit RNAs by cleaving and splicing, with the potential to correct disease-causing mutations [7].
Messenger RNA	mRNA	Single-stranded	commonly ≥ 100 nucleotides	Long RNA sequence capable of inducing the intra-cellular expression of any encoded protein without the risk of genome integration [8].
Circular RNA	circRNA	Single-stranded	≥ 100 nucleotides	CircRNA arise from back-splicing of and are able to regulate gene expression through various mechanisms [9].

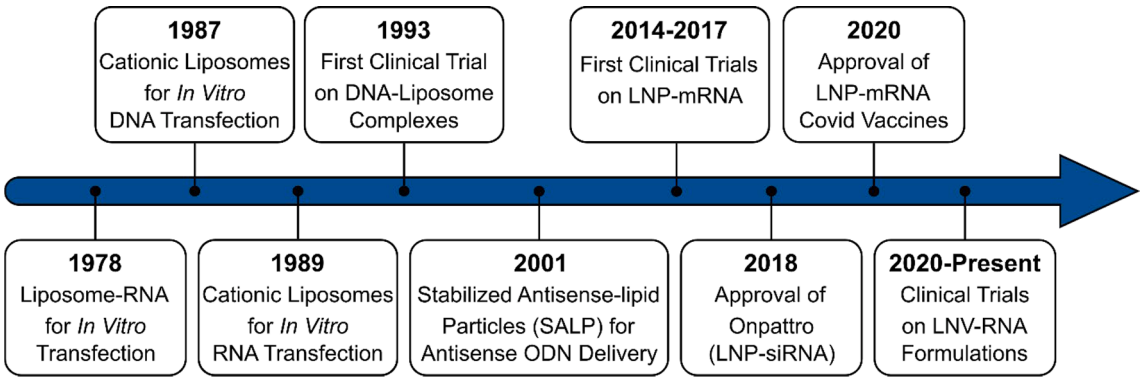

Fig. 1. Timeline of key developments in LNV-RNA formulations. Schematic of the most relevant milestones in LNV-mediated RNA delivery, from the discovery of liposome-RNA complexes for *in vitro* transfection to the approval of LNP-mRNA formulations as Covid vaccines.

Table 2
Ongoing clinical trials on RNA-loaded LNPs.

Clinical trial number	RNA type	Administration route	Application
NCT05945485	mRNA	Intramuscular injection	Influenza vaccine
NCT05755620	mRNA	Intramuscular injection	Influenza vaccine
NCT05639894	mRNA	Intramuscular injection	Respiratory syncytial virus vaccine
NCT05581641	mRNA	Intramuscular injection	Malaria vaccine
NCT05712538	mRNA	Inhalation	Cystic fibrosis
NCT05295433	mRNA	Intravenous injection	Methylmalonic acidemia
NCT05095727	mRNA	Intravenous injection	Glycogen storage disease type Ia
NCT04416126	mRNA	Intravenous injection	Ornithine transcarbamylase deficiency
NCT04159103	mRNA	Intravenous injection	Propionic acidemia
NCT04990388	mRNA	Intravenous injection	Glycogen storage disease type III
NCT04710043	mRNA	Intravenous injection	Metastatic or unresectable solid tumors
NCT04455620	mRNA	Intravenous injection	Metastatic or unresectable solid tumors
NCT04683939	mRNA	Intravenous injection	Metastatic or unresectable gastric, pancreatic, ovarian and biliary tract tumors
NCT05262530	mRNA	Intravenous injection	CLDN6-positive solid tumors
NCT04601051	mRNA	Intravenous injection	Transthyretin amyloidosis
NCT05120830	mRNA	Intravenous injection	Hereditary angioedema
NCT05398029	mRNA	Intravenous injection	Heterozygous familial hypercholesterolaemia

Table 3
Ongoing clinical trials on RNA-loaded lipoplexes.

Clinical trial number	RNA type	Administration route	Application
NCT02410733	mRNA	Intravenous injection	Advanced melanoma
NCT04503278	mRNA	Intravenous injection	CLDN6-positive advanced solid tumours
NCT04163094	mRNA	Intravenous injection	Ovarian cancer
NCT04534205	mRNA	Intravenous injection	Unresectable Recurrent, or Metastatic Head and Neck Squamous Cell Carcinoma
NCT03418480	mRNA	Intradermal injection	HPV16 + head and neck squamous cell carcinoma
NCT05142189	mRNA	Intravenous injection	Advanced non-small cell lung cancer
NCT03289962	mRNA	Intravenous infusion	Locally advanced or metastatic tumors
NCT03815058	mRNA	Intravenous infusion	Melanoma
NCT04486378	mRNA	Intravenous injection	Surgically resected stage II/III rectal cancer or stage II (high risk)/Stage III colon cancer.
NCT04161755	mRNA	N/A	Surgically resected pancreatic cancer

In the following sections, a comprehensive overview of the various types of LNVs for RNA delivery is provided according to the proposed classification framework.

2. Lipid nanovectors obtained by the driven-assembling of lipids

Lipid-based nanoparticles can be obtained *via* bottom-up approaches, which drive lipid assembly and entail the formation of nanostructures with various morphologies starting from dissolved lipid molecules (Fig. 2) [30]. Bottom-up methods for lipid-based nanoparticle formulation include nanoprecipitation, thin film hydration, emulsification, microfluidic mixing, and impingement jet mixing [31]. The choice of the process parameters (*e.g.*, formulation buffers and solvents, pH, ionic strength, mixing speed, and lipid concentration) affects the physico-chemical properties of the obtained lipid-based nanoparticles (*e.g.*, size distribution, encapsulation efficiency of the payload, and morphology). In the following sections, the most common methods for the formulation of lipid-based nanoparticles and the main types of LNVs for RNA delivery will be described.

2.1. Formulation methods for driven-assembling lipid nanovectors

One of the early bottom-up approaches to produce lipid-based nanoparticles is the thin film hydration method, in which a dried lipid film is hydrated with an aqueous buffer; the resulting multilamellar vesicle suspension is then subjected to sonication or extrusion through membranes with a defined pore size to obtain a lipid nanoparticle suspension with a uniform size distribution [28]. The extrusion technique is generally preferred to sonication since it ensures a more uniform vesicle size distribution and improved reproducibility [28]; process parameters such as temperature, applied pressure for extrusion, membrane pore size, and the number of extrusion steps affect the colloidal properties of the obtained nanoparticles [31]. The thin film hydration method is used to produce liposomes, lipid-based nanoparticles comprising one or more lipid bilayers surrounding an aqueous core which have been extensively investigated for the delivery of various biomolecules, such as proteins, peptides, and anti-cancer drugs [32].

Various emulsion-based methods have also been used to produce

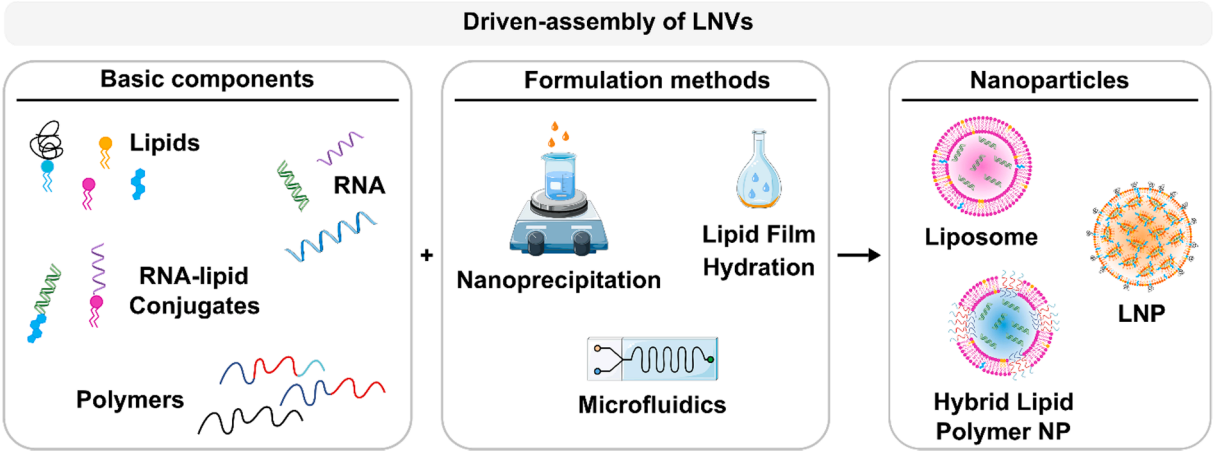

Fig. 2. Driven-assembly strategies for RNA-loaded LNVs. Representative formulation methods and nanoparticle types obtained by driven-assembling approaches. The basic components comprise lipids, RNA, RNA-lipid conjugates or polymers and can be assembled into nanoparticles with various morphologies *via* several formulation methods. The schematic was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

lipid-based nanoparticles comprising a solid lipid core loaded with the drug and stabilized by a surfactant shell. In the solvent emulsification approach lipids and payloads with poor water solubility are dissolved in an organic solvent and subsequently added to an aqueous solution to form an oil-in-water emulsion. Removal of the organic solvent *via* evaporation or diffusion results in the formation of lipid-based nanoparticles [33]. For hydrophilic payloads such as nucleic acids, a double emulsification technique is preferred; in this case a primary water-in-oil (w/o) emulsion is formed and dispersed in an aqueous phase to form a water-in-oil-in-water (w/o/w) emulsion by homogenization or sonication [34]. Solvent-free methods which use a lipid melt as the oil phase as opposed to a lipid solution in an organic solvent can also be leveraged to obtain lipid-based nanoparticles with good encapsulation efficiencies for both hydrophobic and hydrophilic model payloads [35]. While these methods do not require the use of organic solvents, their cumbersome and complex manufacturing steps often result in batch-to-batch variability [33]. Process parameters affecting the colloidal properties of the nanoparticles and the payload encapsulation efficiency comprise homogenization/sonication time and the relative ratios between lipids, drug, and surfactant [36].

Various payloads can be easily encapsulated by nanoprecipitation methods, in which an aqueous phase is mixed with a water-miscible organic solvent containing the lipids to guide lipid assembly in nanoparticles [37]. The organic solvent can be removed *via* dialysis or ultracentrifugation; however, both dialysis and ultracentrifugation are sub-optimal purification techniques for formulation volumes > 10 mL since they are time-consuming (dialysis) and can lead to potential sample loss and nanoparticle aggregation (ultracentrifugation) [38]. This has prompted the development of tangential flow filtration for lipid nanoparticle purification given its ability to process large formulation volumes with minimal sample losses [38]. The physico-chemical properties and the cargo encapsulation efficiency of lipid-based nanoparticles prepared by nanoprecipitation approaches depend on a multitude of process parameters including the mixing rate, the volumetric ratio between the two phases, and the relative concentrations of the lipids and the payload [37]. Incomplete mixing of the aqueous and the organic phases may lead to batch-to-batch variability and yield lipid nanoparticles with a non-uniform size distribution [31].

To improve the reproducibility of lipid-based nanoparticle formulations, a plethora of approaches enabling the controlled mixing between the aqueous and the organic phase has been developed [39]. T-junction mixers were designed to induce the rapid mixing of two input streams into a turbulent output flow, which requires the use of high flow rates (>10 mL min^{-1}) to ensure a uniform nanoparticle size distribution and small nanoparticle sizes [40]. The need for high flow rates makes T-junction mixers ill-suited for nanoparticle formulation at laboratory scales [41] and has prompted the development of microfluidic mixers. One such example is microfluidic hydrodynamic focusing, in which the organic phase is focused by two parallel streams of aqueous phase; in this case T- or Y-shaped devices can be used to drive lipid nanoparticle formation at the liquid–liquid interface due to the slow ethanol diffusion into the aqueous phase [42].

More complex microfluidic systems equipped with asymmetric protrusions (*i.e.*, staggered herringbone mixers) able to induce chaotic mixing of the two phases ensure improved control over the lipid nanoparticle size at low flow rates compared to T- or Y-shaped microfluidic chips [43]. Other microfluidic devices include bifurcating or toroidal mixers, which have a larger flow rate range compared to staggered herringbone mixers and allow process scalability under good manufacturing practices conditions [44]. Key process parameters for lipid-based nanoparticle formulation *via* microfluidic mixing include the total flow rate, the relative ratio between the two phases (*i.e.*, flow rate ratio, FRR), and the lipid concentration [45]. Compared to bulk nanoprecipitation, microfluidics allows rapid mixing at the microscale and results in the formation of nanoparticles with uniform size distribution and high cargo encapsulation efficiencies [39]. For a comprehensive overview of microfluidic-based strategies for lipid nanoparticle formulation, the reader is referred to specialized reviews [41,46].

2.2. *Lipid-based nanocarriers for RNA delivery obtained by driven-assembly*

In this section, various types of lipid-based nanocarriers encapsulating RNA which are prepared by driven-assembling methods (section 2.1) starting from individual lipid molecules will be discussed. Examples include lipid nanoparticles (LNPs), lyotropic liquid crystalline nanoparticles, solid lipid nanoparticles, nanostructured lipid carriers, hybrid lipid-polymer nanoparticles, and micelles.

2.2.1. *Lipid nanoparticles*

Lipid nanoparticles (LNPs) are now the gold standard platform for RNA delivery, with three RNA-LNP formulations already on the market and several undergoing clinical trials [47,48]. LNPs typically comprise four lipid components in various ratios: an ionizable cationic lipid, a sterol, a phospholipid, and a PEG-functionalized lipid [49,50]. Ionizable cationic lipids possess a positively charged headgroup region at acidic pH which enables complexation with negatively charged RNA molecules [49]. Substantial synthetic efforts have been carried out to unravel the

role of the headgroup region, the linker moiety, and the hydrophobic tails of ionizable lipids on *in vitro* and *in vivo* RNA delivery [51–55]. The headgroup region affects the apparent acid dissociation constant (pKa) of the ionizable lipid and an optimal pKa of 6.44 has been identified for *in vivo* siRNA delivery to the liver; the lead lipid candidate discovered in this study is now used for Onpattro®, the first clinically approved LNP-RNA formulation [19,51].

The chemical or enzymatic stability against degradation of the linker moiety can also play a crucial role in the overall efficacy of LNPs: ionizable lipids with an alkoxy linker group outperformed ionizable lipids containing either a diester, carbamate or thioether linkages for *in vivo* hepatic gene silencing [52]. The authors speculated that this may be due to the different hydrolyzation rates of such moieties; the use of a ketal ring linker resulted in a further improvement in the *in vivo* gene silencing activity of LNPs [52]. Ionizable lipids with unsaturated hydrophobic tails can adopt inverted hexagonal phases that destabilize the cellular endosomes and favor RNA cytosolic release; two degrees of unsaturation ensures optimal gene silencing activity [53]. The ionizable lipids used for clinically approved LNP-mRNA formulations (*i.e.*, SM-102 and ALC-0315) contain branched hydrophobic tails, biodegradable ester linkages, and a hydroxyl-terminated tertiary amine head group. These features confer superior *in vivo* mRNA delivery efficacy and tolerability compared to non-degradable ionizable lipids [50,54]. A further improvement in mRNA transfection was obtained when the hydroxyl group was replaced with thiourea bioisosteres [55].

As mentioned above, LNPs comprise cholesterol and its derivatives and phospholipids which confer structural stability to the nanoparticles and affect their RNA delivery efficacy. Chemical modifications of cholesterol yielded cell-specific delivery of siRNA [56] and mRNA [57], up to a 200-fold enhancement in mRNA transfection *in vivo* [58], and improved LNPs fusogenicity with endosomal compartments [59]. Due to their bilayer-forming properties, phospholipids such as 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) have been included in the LNP composition of marketed products to facilitate nucleic acid packaging and ensure the LNP structural stability [60]. Less than 5 mol% of PEG-functionalized lipids are typically incorporated in the LNP composition to control the nanoparticle size and to impart colloidal stability against aggregation upon storage and *in vivo* administration [60]. By tuning the length of the lipidic backbone of PEGylated lipids it is possible to regulate their desorption kinetics from the LNP surface and the nanoparticle biodistribution [49,61]. All the clinically approved LNP formulations contain rapidly dissociating PEGylated lipids with 14 carbons alkyl chains (*i.e.*, DMG-PEG2000 and ALC-0159) [49].

In the preparation of LNPs, a driven assembling of the lipids is required. LNPs were originally formulated *via* a nanoprecipitation approach involving the injection of a lipid mixture dissolved in ethanol into an aqueous solution containing the nucleic acid at pH < 4.5, which ensured protonation of the ionizable lipid components and its complexation with the nucleic acid. The rapid increase in solvent polarity upon mixing induced the formation of oligonucleotide-loaded vesicles with encapsulation efficiencies > 70 % [18]. The remarkable improvements in the mixing process achieved with microfluidic techniques (described in section 2.1) have led to the adoption of these approaches for the manufacturing of RNA-loaded LNPs [39]. Furthermore, the parallelization of multiple microfluidic devices that operate simultaneously enables the production of RNA-loaded LNPs at industrially-relevant scales while meeting the nanoparticle quality requirements in terms of physico-chemical properties and RNA encapsulation efficiency [46,62].

Key process parameters for the manufacturing of RNA-loaded LNPs include the total flow rate (TFR) and flow rate ratio (FRR), the lipid composition, the molarity and pH of the formulation buffer, the type of RNA, and the ratio of ionizable lipid to nucleic acid (N/P) [39,63,64]. Fine control of the size of siRNA-loaded LNPs could be achieved by using the staggered herringbone microfluidic mixer, which yielded siRNA encapsulation efficiencies > 95 %, 55 nm-sized LNPs with low (<0.1) polydispersity index at total flow rates > 0.2 mL min^{-1} [65]. An increase in the polydispersity index was observed when the total flow rate was reduced [65]. Recently, Jürgens *et al.* have investigated the role of the microfluidic chip geometry and the type of RNA on the overall process efficiency when total flow rates < 5 mL h^{-1} were employed [66]. While for mRNA process efficiencies > 95 % and encapsulation efficiencies of 80 % could be obtained regardless of the microfluidic chip geometry, for siRNA-loaded LNPs the highest process efficiency (~ 60 %) could be achieved only with the T-junction mixer [66]. However, T-junction mixing yielded lower encapsulation efficiencies compared to the other microfluidic mixers [66].

LNP-mediated RNA delivery and in vivo fate. The adsorption of serum protein following the *in vivo* administration of LNVs leads to the formation of the so-called protein corona, which is known to affect the LNV biodistribution, colloidal stability in serum, and half-life in circulation [67]. For example, the adsorption of opsonins can trigger nanoparticle uptake by macrophages and leads to rapid blood clearance [68]. In other instances, the protein corona can endow LNPs with endogenous targeting properties and enhance the uptake of LNPs by specific cells [19,69–71]. It is now well-established that the remarkable clinical success of Onpattro® is due to the adsorption of apolipoprotein E (ApoE) on the surface of LNPs, which favors their uptake by hepatocytes through the low-density lipoprotein receptor (LDLR) and enables siRNA delivery [19]. ApoE-mediated LNPs uptake and enhanced intra-cellular siRNA delivery was also observed in neurons [71].

Modulation of the protein corona can be achieved by the addition of a charged lipid in the LNP composition, as shown by Dilliard and co-workers [72]. In their work, the inclusion of a cationic lipid in LNPs led to the preferential adsorption of vitronectin, which correlated to nanoparticle accumulation in the lungs [72]. An alternative strategy for extra-hepatic organ targeting entails the addition of charged lipids or cholesterol substitutes (*e.g.*, bile acids) able to promote nanoparticle accumulation into specific organs following *in vivo* administration [73–75]. Active targeting strategies which entail the functionalization of the LNP surface with ligands that interact with specific receptors on the surface of cells have also been explored. By decorating the LNP surface with vitamin A or platelet-derived growth factor receptor β-targeted peptide cell-specific siRNA delivery within the liver microenvironment for the treatment of hepatic fibrosis was achieved [76,77]. Targeting ligands have also been proposed to aid extra-hepatic *in vivo* RNA delivery from LNPs [78–81]. Examples include the use of antibodies to achieve selective siRNA delivery to leukocyte [78,79] or breast cancer cells [80] and the functionalization of the LNP surface with peptides derived by the rabies virus glycoprotein for siRNA delivery to the brain [81].

The versatility of LNPs makes them ideal candidates for the delivery of miRNA and mRNA for various therapeutic applications. LNPs loaded with an oncosuppressor miRNA could successfully stop tumor growth in a triple negative breast cancer model, overcome the blood–brain-barrier, and reduce breast cancer-derived brain metastases [82]. In another example, a combination of oncosuppressor miRNAs was encapsulated within LNPs, which were shown to potentiate target therapy against metastatic melanoma both *in vitro* [83] and *in vivo* [84]. A reduction in tumor growth could also be observed upon delivery of LNPs encapsulating miR-634 in a pancreatic cancer model [85] and prolonged mice survival could be achieved with LNPs loaded with miR-34a and functionalized with transferrin in a model of multiple myeloma [86].

Since mRNA could potentially induce the expression of any protein upon intra-cellular delivery, mRNA-loaded LNPs are now attractive platforms for anti-cancer vaccines and protein replacement therapy [87]. LNPs were recently proposed to deliver mRNA encoding tumor antigens, exhibit lymphoid tissue tropism, and induce a robust cytotoxic lymphocyte response in a melanoma mouse model [88]. A more pronounced immune response could be obtained by co-encapsulating mRNAs encoding tumor antigens and adjuvants able to activate type I interferon signaling into LNPs; this effect led to a reduction in tumor

growth and prolonged survival of vaccinated mice [89]. In other examples, the intratumoral injection of LNPs loaded with cytokine-encoding mRNA induced the *in vivo* production of such cytokines, which were able to recruit immune cells to the tumor site and to modulate the tumor microenvironment [90,91]. By changing the sequence of the encapsulated mRNA, researchers have successfully used LNPs to induce the production of therapeutic proteins for the treatment of liver and lung fibrosis [92], methylmalonic acidemia/aciduria [93], hemophilia B [94], or ornithine transcarbamylase deficiency [95] in various preclinical models. More recently, *in vivo* T cell reprogramming was achieved by injecting LNPs loaded with mRNA encoding a transient antifibrotic chimeric antigen receptor (CAR) for the treatment of cardiac failure; this approach is particularly relevant since it overcomes several limitations associated with conventional CAR T therapy [96].

LNPs in clinical studies. As mentioned above, several LNP-RNA formulations are being evaluated in clinical trials for vaccine and protein replacement therapy *via* various administration routes (Table 2). Examples include LNP formulations loaded with mRNA as vaccines against influenza (NCT05945485 and NCT05755620), respiratory syncytial virus (NCT05639894), and malaria (NCT05581641). While NCT05945485, NCT05755620, and NCT05581641 are Phase 1 trials, NCT05639894 has progressed to Phase 2 with ~ 800 patients currently enrolled. In the context of protein replacement therapy, an ongoing clinical trial on LNP-mRNA (NCT05712538) administered *via* inhalation may represent a substantial advancement in the treatment of cystic fibrosis; this formulation has recently received the orphan drug designation by the Food and Drug Administration (FDA).

A large proportion of LNP-RNA formulations undergoing clinical trials is designed for intravenous administration and leverages the versatility of mRNA for protein replacement therapy. LNPs loaded with mRNA encoding enzymes involved in key pathological processes are currently under investigation for the treatment of methylmalonic acidemia (NCT05295433), glycogen storage disease type Ia (NCT05095727), ornithine transcarbamylase deficiency (NCT04416126), propionic acidemia (NCT04159103), and glycogen storage disease type III (NCT04990388). Other ongoing clinical trials are assessing LNPs loaded with mRNA encoding interleukins (NCT04710043 and NCT04455620) or antibodies (NCT04683939 and NCT05262530) for the treatment of solid tumors and LNPs loaded with mRNA for gene editing in transthyretin amyloidosis (NCT04601051), hereditary angioedema (NCT05120830), and heterozygous familial hypercholesterolaemia (NCT05398029). The number of approved LNP-RNA therapies is expected to grow in the near future although some issues regarding the long-term stability of LNP formulations remain to be addressed to fully leverage their potential and allow their use in underdeveloped countries.

2.2.2. Lyotropic liquid crystalline nanoparticles: Cubosomes and hexosomes

Lyotropic liquid crystalline nanoparticles are an interesting class of lipid-based nanoparticles and are characterized by a complex, non-lamellar architecture of which cubic and inverse hexagonal mesophases are the most relevant examples [97]. Lyotropic liquid crystalline nanoparticles with cubic and inverse hexagonal mesophases are named cubosomes and hexosomes, respectively [97]. The internal structure of lyotropic liquid crystalline nanoparticles features a high surface area for the encapsulation of hydrophilic and lipophilic compounds and tunable membrane curvature [97,98], which has been shown to enhance the enzyme-responsiveness of cubosomes compared to liposomes [99]. Key components of lyotropic liquid crystalline nanoparticles comprise monoolein and phytantriol, which form non-lamellar mesophases such as the inverse hexagonal mesophase (H_{II}) and the inverse bicontinuous cubic mesophase (Q_{II}). In the H_{II} mesophase cylindrical micelles are packed in an inverted hexagonal structure while the Q_{II} presents curved bicontinuous lipid bilayers with a thickness of ~ 3.5 nm and water channel with diameters between 3 and 25 nm [97,98].

Top-down formulation approaches are typically used for the formulation of cubosomes and hexosomes [97,98]. A bulk lipid mesophase is formed by hydrating a lipid film with an aqueous buffer and is the dispersed in the presence of stabilizers such as non-ionic tri-block copolymers (*e.g.,* Pluronics) or lipopolymers (*e.g.*, PEGylated lipids) to obtain nanoparticles with diameters < 200 nm [98,100]. SiRNA-loaded cubosomes have been obtained by hydrating a lipid film with an aqueous siRNA solution and by dispersing the bulk lipid mesophase with tip sonication; in this case, a cationic lipid was included in the lipid composition to favor siRNA complexation [101]. *In vitro*, cubosomes exhibited higher gene silencing ability compared to siRNA-loaded lipoplexes, even at low charge ratios (*i.e.*, ratio between the positive charges of the cationic lipid and the siRNA negative charges) [101]. The authors ascribed this behavior to the membrane curvature of cubosomes, whose features enable the efficient endosomal escape and intracellular delivery of encapsulated siRNA [101].

Probe tip sonication is a standard dispersion method for the formulation of cubosomes and hexosomes. While ensuring good reproducibility, tip sonication suffers from limited process scalability, which has prompted the development of approaches based on microfluidic mixing for the formulation of hexosomes and cubosomes [102]. Examples include hydrodynamic flow focusing for the production of hexosomes with a good size distribution [103] and the use of a herringbone mixer microfluidic chip to obtain cubosomes *via* the chaotic mixing of an ethanolic lipid solution with an aqueous buffer [104]. In this example, the authors also investigated the possibility to add siRNA to the aqueous phase to obtain siRNA-loaded cubosomes; however, the addition of siRNA during lipid assembly resulted in the formation of lipoplexes and siRNA loading could be achieved with the subsequent mixing of pre-formed cubosomes with siRNA [104]. This approach has been leveraged for the formulation of RNA-loaded cubosomes [105–108] and will be discussed in more detail in section 3.

2.2.3. Solid lipid nanoparticles and nanostructured lipid carriers

Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) are another class of lipid-based nanoparticles and exhibit a solid hydrophobic core, as opposed to the aqueous core of liposomes [109]. This feature confers improved drug loading capacity and bioavailability, as well as higher colloidal stability following sterilizing filtration and superior control of drug release compared to other lipid-based nanoparticles [109]. The lipid core of SLNs includes solid lipids such as stearic acid, glyceryl behenate, tripalmitin, cetyl palmitate, glyceryl monostearate or tristearin, while for NLCs liquid lipids such as oleic acid and caprylic/capric triglycerides are incorporated alongside solid lipids [110]. As a result, NLCs have a reduced degree of crystallinity compared to SLNs, which leads to improved colloidal stability upon storage [109]. Non-ionic, charged, or amphoteric surfactants are also included as stabilizers [110]. The addition of a cationic lipid in the composition of SLNs and NLCs has unlocked RNA encapsulation and delivery for several applications [111–115]. Since the RNA was mixed with pre-formed SLNs and NLCs, these examples have been discussed in section 3.

2.2.4. Hybrid lipid-polymer nanoparticles

Lipid- and polymer-based nanoparticles are leading platforms for RNA delivery due to their favorable properties. High RNA encapsulation efficiency and biocompatibility are some of the advantages of lipid-based nanoparticles while polymer-based nanoparticles offer high chemical versatility and can be designed to achieve controlled drug release [116]. In order to integrate the advantages of both systems, hybrid lipid-polymer nanoparticles (LPNPs) have been developed [117]. Depending on the localization of the lipid and polymer components within the nanoparticle, hybrid LPNPs can be classified into core–shell and monolithic nanoparticles. Core-shell nanoparticles exhibit a lipid- (or a polymer-) based core enclosed by a polymer (or a lipid) shell; layer-by-layer structures or biomimetic core–shell nanoparticles can also be obtained. Monolithic hybrid lipid-polymer nanoparticles typically

feature a polymer matrix in which lipid molecules are dispersed [117]. RNA molecules are either in the aqueous core or are intercalated within cationic lipid layers. Common methods for the production of hybrid LPNPs comprise nanoprecipitation and solvent emulsification [117].

Clinically-established polymers such as polyethylenimine (PEI) [118], poly D,L-lactic-co-glycolic acid (PLGA), and poly(D,L-lactide) (PLA) [119] have been exploited for the formulation of LPNPs. PLGA was used as the hydrophobic layer in a hollow core–shell hybrid LPNP to enable the sustained release of siRNA for at least one month; the LPNPs were prepared *via* a double-emulsion solvent evaporation method [120,121]. The incorporation of a PEG-based shell to LPNPs extended the circulation half-life in blood to ~ 8 h [122], while the inclusion of a poly(disulfide amide) polymer imparted redox-triggered siRNA release in the cell cytoplasm [123]. A double emulsion-solvent evaporation technique was developed to obtain hybrid LPNPs comprising an amphiphilic block copolymer of PEG and PLA and a cationic cholesterol derivative able to encapsulate siRNA within their core at encapsulation efficiencies > 90 % and to achieve cytosolic siRNA delivery [124].

A similar emulsification method was employed to obtain hybrid LPNPs based on PLGA and a cationic lipid for siRNA delivery; the nanoparticle structure could be tuned by varying the ratios between the components and affected the siRNA release profile [125]. In this approach, siRNA-cationic lipid complexes were either entrapped into the PLGA core or sandwiched between the lamellar lipid layers surrounding the PLGA core [125]. Subsequent studies involving the use of a lipidoid as the cationic lipid showed that the interaction between the lipidoid and siRNA was influenced by pH and the inclusion of siRNA affected the thermotropic phase behavior of the lipidoid [126]. A systematic formulation screening informed by a quality-by-design approach led to the identification of an LPNP composition based on PLGA as the core material and an ionizable lipidoid which was able to achieve a 50-fold improved *in vitro* gene silencing compared to LPNPs based on cationic lipids [127]. A two-step microfluidic method has been recently proposed for the formulation of hybrid LPNPs loaded with mRNA, which was first complexed with a cationic polymer via microfluidics; the mRNA-polymer complexes were then mixed in a second step with a lipid mixture to obtain the hybrid LPNPs [128].

Hybrid LPNPs have also been proposed for the co-delivery of RNA and other therapeutic agents to enhance their efficacy or impair the development of drug resistance. For example, siRNA and gemcitabine were co-delivered *via* hybrid LPNPs with a PEGylated lipid shell enclosing a polymeric core comprising PLGA and polylysine [129]. The polymeric nanoparticle core was prepared by emulsion solvent evaporation; the resulting emulsion was used to hydrate a lipid film and form a core–shell nanoparticle. Gemcitabine was encapsulated within the nanoparticle core while negatively charged siRNA was adsorbed on the nanoparticle surface. The PEGylated lipid coating protected siRNA from nucleases, minimized gemcitabine leakage, prevented the desorption of siRNA from the nanoparticle surface in circulation, and reduced nanoparticle aggregation. *In vivo*, the LPNP exhibited prolonged circulation and synergistic antitumor effects when co-delivering siRNA and gemcitabine [129].

In another example, a cisplatin prodrug and siRNA were loaded into hybrid LPNPs with a lipid-polymer shell and an aqueous siRNA core which were able to potentiate the anti-tumor efficacy of cisplatin [130]. An anti-inflammatory drug, capsaicin, has also been co-delivered with siRNA *via* hybrid LPNPs, which were prepared by nanoprecipitation. Capsaicin was entrapped in the PLGA core while the siRNA molecules were located in the lipid shell comprising a phosphatidylcholine, a cationic lipid, and a PEGylated lipid. Results showed that the nanoparticles were able to deliver capsaicin into the dermis up to a depth of ~ 360 μm and that a synergistic effect in reducing skin inflammation could be obtained upon the topical administration of the LPNPs [131].

2.2.5. Nanostructures obtained from RNA-lipid conjugates

While for the majority of LNVs physical entrapment or electrostatic interaction have been employed to encapsulate RNA, alternative approaches such as RNA-lipid chemical conjugation have been investigated to load the nucleic acid into LNVs characterized by the absence of an aqueous phase or positive charges. Conjugation of hydrophobic moieties to the 5′ or 3′ end of the nucleic acid sequence yields RNA-based amphiphiles that assemble into micelles in aqueous media [132,133] or that can be included into micelles [134,135]. Driven-assembling process, such as the hydration of a lipid film containing a PEGylated lipid, can be used to prepare the formulation. The use of cleavable disulfide bonds enables siRNA release once inside the target cells [134].

RNA-based micelles exhibit a lipophilic core and an RNA outer layer, consistent shapes and sizes, and high thermodynamic stability. One of the advantages of RNA-based micelles lies in the absence of cationic lipids to facilitate delivery, which bypasses the immunotoxicity issues associated to these lipids [136]. RNA-based micelles have been proposed for various applications including the delivery of therapeutic RNA and poorly water-soluble drugs, targeted delivery, or bioanalysis. The chemical structure of RNA-based micelles controls drug loading, release and tissue accumulation of RNA; for example, the lipid and oligonucleotide sequences affect the ability of RNA-based micelles to encapsulate hydrophobic drugs within their core [137]. Similarly, Biscans et al. found that the structure and valency of the fatty acid backbone alters the physicochemical properties of conjugated siRNA, which in turns affect its pharmacokinetic profile [138].

RNA-based micelles have been recently proposed as carriers for paclitaxel, a common anti-cancer drug [139]. By conjugating cholesterol to one end of a helical RNA structure it was possible to obtain RNA amphiphiles which could form micelles in aqueous solution. The encapsulation of paclitaxel within these RNA-based micelles greatly improved its water solubility, cellular uptake, and ability to induce cancer cell death. *In vivo* animal studies further demonstrated that the paclitaxel-loaded RNA micelles could selectively target tumors without building up or accumulating in healthy tissues and organs [139]. In another example, folate-decorated RNA -based micelles were used for anti-miR21 delivery; the amphiphiles were prepared by 3-way junction including RNA conjugated cholesterol, anti-miR21, and folate- conjugated RNA. Micelles were prepared by mixing in phosphate buffer and were shown to deliver anti-miR21 to cancer cells, which effectively inhibited cancer growth [140].

Alternatively, siRNA molecules have been covalently conjugated to cationic squalene derivates *via* different synthetic approaches [141–144]; the resulting siRNA-squalene bioconjugates assembled as supramolecular entities connected one to another to form spherical nanoparticles with diameters of ~ 130 nm. SiRNA-squalene nanoparticles were effective in reducing tumor growth in two cancer models with fusion oncogenes [141–143]. SiRNA-squalene nanoparticles were also used to target PMP22, an overexpressed gene causing hereditary neuropathy. The intravenous administration of siRNA restored both motor and neuromuscular activities in transgenic mouse models [145]. Interestingly, the nanoparticles successfully entered the targeted tissue and cells (*i.e.*, the sciatic nerve and Schwann cells). The siRNA-squalene nanoparticles interacted with serum components such as bovine serum albumin and low-density lipoproteins via hydrophobic interactions through the squalene moiety. This interaction has been suggested as an endogenous nanoparticle functionalization approach to target pathological tissue, such as tumor tissues that use LDL as carrier for cholesterol transport [145].

While most research has employed DNA as a building block for self-assembled structures, the use of RNA molecules as building blocks has recently emerged; given the high potential and advantages of RNA-based nanoassemblies, it is anticipated that more studies will be carried out in this area.

3. Supramolecular self-assembly of lipid-based nanoparticles

An alternative approach for the formulation of RNA-loaded LNVs

entails mixing pre-formed nanovectors with an aqueous RNA solution to induce their structural re-arrangement in RNA-containing supramolecular assemblies of various morphologies (Fig. 3). This structural re-arrangement occurs spontaneously upon mixing and yields a plethora of LNV formulations including lipoplexes, non– lamellar lipid-RNA complexes, RNA-loaded SLN and NLC, and hybrid self-assembled lipid nanoparticles with inorganic or polymeric cores. In the following sections, a detailed description of these LNV formulations is provided alongside their applications for RNA delivery.

3.1. Lipoplexes

Liposomes are the most largely investigated lipid nanovectors for RNA delivery. One of the first mRNA-liposome formulations was developed in 1978, highlighting that lipid vesicles can facilitate the cellular incorporation of administered RNA [13,14]. Since then, several liposomal formulations have been proposed, bringing to light the crucial role of cationic lipid components to maximize the intracellular delivery of nucleic acids. Cationic lipids have a head group with a permanent positive charge able to interact with the negative charges of nucleic acids. This interaction leads to the formation of a complex through electrostatic interactions between the opposite charges of RNA sequences and cationic lipids [146]. Due to their long history as transfection agents, cationic liposomes for RNA delivery are the system with the highest number of published studies. As such, the physico-chemical characteristics of cationic liposomes/RNA complexes (*i.e.*, lipoplexes) as well as their biological behaviour are well-known.

The structure resulting from cationic liposomes/RNA interaction has been largely investigated since the efficient intracellular delivery of RNA is clearly linked to the formation of their architecture. Lipoplex structures form by a spontaneous self-assembly induced by electrostatic interaction forces between the positively charged exposed on cationic liposomes and the negatively charges of RNA, that in turn trigger lipid structure rearrangements and condensation events [146,147]. Compared to process-driven assembly, the self-assembly *via* electrostatic interactions is spontaneous and rapid and allows the formation of stable complexes due to the occurrence of several and simultaneous binding events between opposite charges with consequent RNA condensation. This spontaneous phenomenon is generally irreversible, even if local electrostatic bindings may be reversible and change and thermal fluctuations may occur resulting in "small" rearrangements of lipoplexes structure [147].

The RNA lipoplex geometry is mainly dependent on the lipid components, having the RNA a minor effect on the lamellar structure. However, the physico-chemical properties of lipoplexes are strongly influenced by the formulation, such as components and their ratio, and experimental conditions, such as incubation medium, order and volumes of components mixing, incubation time and temperature, and phase mixing rate [148–152]. In the same experimental conditions, changes in the cationic lipid required a different charge ratio to achieve lipoplexes with optimal colloidal size and siRNA transfection efficiency [153]. This has been attributed to a different arrangement structure of stable lamellar domains with a spacing of 67 Å [153]. The introduction of neutral lipids did not influence the morphology of lipoplexes although an increase of the interlamellar spacing was observed [154]. Lipoplexes in which increasing amounts of dioleoyl-L-α-phosphatidylethanolamine (DOPE) was associated to the cationic lipid led to a structural transition from a lamellar (Lα) phase to an inverted hexagonal (H_{II}) phase with a region of coexistence in between [155].

The lipoplex structure is also influenced by the preparation methods, as reported by the preparation of lipoplexes using "2X mixing" or the "jump method", which led to lipoplexes with different size, colloidal stability, and biological effects [156]. Finally, the ionic strength of the incubation medium can have a dramatic effect on lipoplex formation. The use of high concentration of buffer solution could hamper complex formation by affecting the interaction between the bilayer surface and the nucleic acids and favouring aggregation; this was especially evident when more heterogeneous buffers such as cell culture media were used [151,156,157]. The efficient complexation of RNA by cationic liposomes results in enhanced stability of nucleic acids against enzymatic degradation and improved transfection efficiency [154].

As mentioned above, lipoplexes are obtained by mixing cationic liposomes and RNA immediately before administration, with no standardised mixing procedures. This can lead to operator-dependent variability in the physico-chemical properties of the formulation, which could in turn affect its efficacy. To improve the reproducibility of lipoplex-RNA formulations, controlled mixing strategies based on microfluidics or pre-filled, double-channel syringes may be explored. The performance of the available mixing strategies can be assessed by analytical methods able to determine the colloidal properties of the lipoplexes in terms of size, surface charge, and RNA loading. It is worth mentioning that while the use of microfluidic mixing for lipoplex formation is still at the pre-clinical development stage [158], pre-filled, double-channel syringes have already been employed in a phase II clinical trial involving plasmid DNA-loaded lipoplexes [159].

Lipoplex-mediated RNA delivery. A formulation based on the synthetic cationic lipid 1,2-di-O-octadecenyl-3-trimethylammonium-propane (DOTMA), a quaternary ammonium lipid, together with DOPE was the

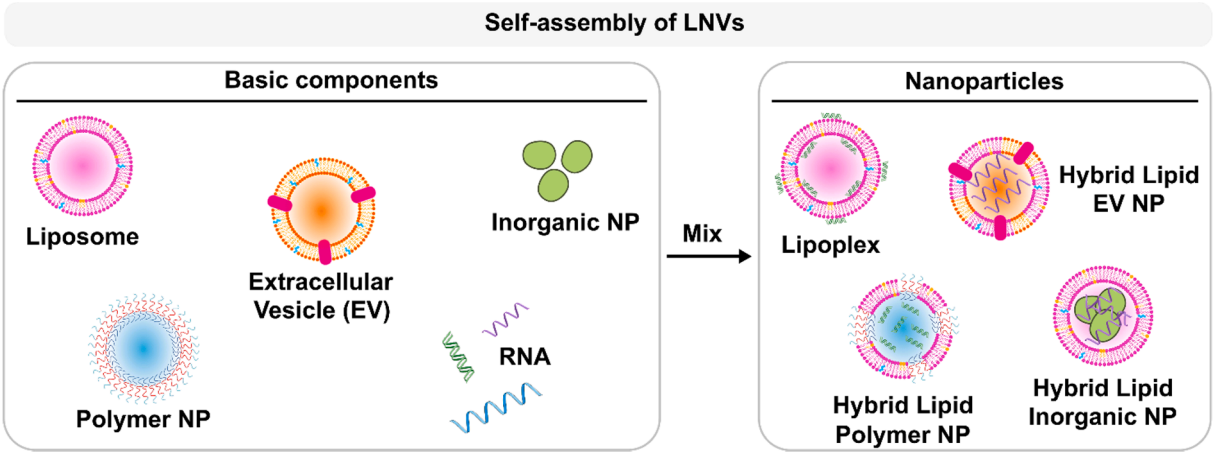

Fig. 3. Self-assembly strategies for RNA-loaded LNVs. Representative formulation methods and nanoparticle types obtained by self-assembling approaches. The basic components comprise liposomes, extracellular vesicles, inorganic nanoparticles, polymeric nanoparticles, and RNA which can be mixed to obtain RNA-loaded LNVs with varying morphologies. The RNA schematics were generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

first marketed lipid-based transfection agent (Lipofectin™) in 1990 [16]. Since then, many cationic lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were synthesized and used to prepare cationic liposomes. The presence of a hydrolysable chemical ester bond in the DOTAP structure reduced the cytotoxicity of the systems due to the biodegradability of the lipidic chain [160]. Lipoplex formulations based on DOTAP and DOPE as the helper lipid have been largely investigated for mRNA delivery [161–164]. The addition of cholesterol can improve the colloidal stability, the membrane fusion ability, the encapsulation efficiency, and the endosomal escape of lipoplexes. In alternative to DOTMA and DOTAP, a cationic cholesterol derivative (3β [N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol, DC-Chol) has been proposed for the formulation of lipoplexes to increase the biocompatibility and the stability of the lipid membrane [165]. Multivalent cationic lipids with multiple cationic groups such as 2,3-dioleyloxy-N- [2(sperminecarboxamido)ethyl]-N,N-dimethyl-l-propanaminium trifluoroacetate (DOSPA) or dioctadecylamidoglycylspermine (DOGS) have been shown to allow more efficient ionic interactions with the negative charges of RNA and can be used for lipoplex formulations [166].

Despite their widespread use for RNA delivery, cationic liposomes suffer from some limitations: they are rapidly eliminated following the formation of a protein corona upon intravenous administration [146,167,168]. Among the components of cationic liposomes, lipids conjugated to a polyethylene glycol moiety can be included to minimized vesicles aggregation and opsonization *in vivo* [169]. The cytotoxicity of cationic liposomes is strongly related to the exhibition of the net positive charge of the polar head group, but it is also strongly linked to the formulation composition and to the structure of the cationic lipid [170,171]. It has been demonstrated that the lipid DOTMA with a hydrophobic double-tail chain is less toxic compared to cetyltrimethylammonium bromide; the counterpart with a single-tail aliphatic chain as well as a heterocyclic ring to replace a linear positive head group reduce the cellular toxicity [172]. As mentioned above, the presence of biodegradable ester groups in DOTAP led to a reduced toxicity compared to DOTMA [173]. Negatively charged lipids such as dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) or PEGylated lipids can be used to "shield" the surface charge of vesicles and reduce the toxicity [170,171].

In vivo RNA delivery *via* lipoplexes is affected by the net positive charge that limits their systemic administration. As such, local administration routes have been explored for RNA-loaded lipoplexes for various applications. In one example, lipoplexes were used for the local delivery of siRNA to the basal epidermal layer for the treatment of melanoma [173]. *In vivo* studies carried out on lipoplexes administered *via* vaginal instillation for the treatment of lethal herpes simplex virus (HSV-2) showed that the formulations were able to deliver siRNA to the epithelial cells and target HSV-2 in treated mice [174]. Direct instillation or nebulization has been proposed to deliver mRNA to the lungs bypassing systemic circulation [175]; RNA delivery to the lungs can also be achieved by intravenous administration [176].

An optimized self-assembled lipid nanocarrier, obtained by simply adjusting the composition of the formulation, was used to target specific cells. For instance, lipoplexes loaded with RNA encoding the reporter gene firefly luciferase were optimized to specifically target dendritic cells for cancer immunotherapy [177]. RNA lipoplexes based on cationic lipids and cholesterol were developed and optimized in term of size and charge ratio. *In vivo* experimental results showed that a gradual decrease of the cationic lipid content shifted luciferase expression from the lungs towards the spleen. Thus, the optimized RNA-based vaccine formulation was systemically administered in mouse tumour models of lung metastasis or lung cancer. These systems showed a high RNA delivery to antigen presenting cells in spleen lymph nodes and bone marrow and elicited the immunostimulatory response mediated by interferon-α (IFNα), highlighting the potential antitumor activity of these formulations [177].

Helper lipids can modify the biodistribution of lipoplexes, reducing the side effects related to their accumulation in non-targeted organs [172,178–181], while cholesterol enhances their interaction with the cell surface and facilitates their accumulation in the lung [181]. The introduction of a PEG moiety on the surface of cationic liposomes results in a prolonged lipoplex circulation time in the bloodstream upon systemic administration [182–184], with a different biodistribution and a higher probability to target solid tumors [185]. To improve the selectivity of lipoplexes towards tumour cells, different ligands for specific receptors overexpressed on cancer cells have been used [179,182–186]. Further functionalization of lipoplexes has been proposed for a more specific and efficient delivery. Namely, *in vivo* studies demonstrated that lipoplexes functionalized with hyaluronic acid or aptamers strongly improve siRNA lipoplexes antitumoral efficacy both in lung and breast cancer [179,187].

Clinical studies. Several clinical studies based on cationic liposomes for RNA delivery have been carried out, although there are currently no approved products on the market for use in humans. Early clinical studies on lipoplexes were aimed at gene delivery with a plasmid DNA [159,188]. Lipoplexes composed of DC-chol were evaluated in a phase I clinical trial on patients with HER-2/neu-overexpressing and low HER-2/neu-expressing breast and ovarian cancers evidencing the antitumor efficacy of the treatment [188]. In another example, a phase II clinical trial (NCT01621867) was aimed at assessing the safety, tolerability, and gene expression after the administration of lipoplexes to treat cystic fibrosis patients [159]. To limit the variability of extemporaneous preparations, the assembling of the formulation was carried out by a simple automated mixing device that allows a more accurate control of the mixing components with a simultaneous extrusion of two mixing phases. Therefore, a coextrusion of plasmid pGM169 and GL67A lipid mixture was carried out to obtain pGM169/GL67A lipoplexes. This formulation was delivered to patients by a pneumatic nebulizer within 6 h from mixing. Results showed that monthly application of the formulation was associated with significant benefits in terms of forced expiratory volume in 1 s at 1 year, indicating a stabilization of lung infection [159].

Lipoplexes have been also investigated for RNA based vaccination. A tetravalent RNA-lipoplex vaccine (FixVac-BNT111) developed by BioNTech and encoding four different tumour antigens was assessed for the treatment of advanced malignant melanoma upon intravenous administration and entered clinical evaluation (NCT02410733; LipoMerit trial). Results demonstrated a strong CD4 + and CD8 + T cell immunity against the vaccine-encoded antigens suggesting that FixVac-BNT111 vaccination is a potent immunotherapy in melanoma patients and that non-mutant shared tumour antigens are a potent target for cancer vaccination [189]. In 2021 BioNTech announced a phase II cancer vaccine trial in which the vaccine is used in combination with Libtayo® (cemiplimab) in patients with anti-PD1-refractory/relapsed unresectable Stage III or IV melanoma. Lipoplexes encapsulating CLDN6-encoding mRNA (CarVac) for intravenous administration are currently undergoing phase 1–2 trial (NCT04503278) for the treatment of CLDN6-positive advanced solid tumours. Results showed that the combination of CarVac and autologous CLDN6 targeting CAR T-cell therapy showed no dose limiting and important side effect with a partial response in 57 % of patients [190]. Other clinical trials are assessing different FixVac mRNA lipoplex vaccines for various cancers (NCT04163094, NCT04534205, NCT03418480, and NCT05142189) or mRNA-lipoplex formulations for individualised neoantigen-specific immunotherapy (BNT122) and are based on mRNA that encodes individual tumor mutations (NCT03289962, NCT03815058, NCT04486378, and NCT04161755).

3.2. Other lipid-based self-assembling nanoparticles

Squalene is a natural cholesterol precursor which is gaining a growing attention as a pharmaceutical excipient in drug delivery

systems, due to its high biocompatibility and self-assembling properties [161,191]. It can act as neutral helper lipid, mimicking cholesterol, or as complexation agent for RNA delivery [50,192]. Nanoparticles formed rapidly when squalene or its derivates were used in a nanoprecipitation-solvent evaporation method. This procedure allowed a spontaneous rearrangement of squalene derivates leading to the formation of nanoparticles of 100–200 nm without the need of surfactants. Cationic squalene derivates, such as trimethylammonium substituted or hydrazinoguanidinium substituted, have been proposed as siRNA delivery systems. Cationic squalene nanoparticles were obtained by the nanoprecipitation-solvent evaporation method and siRNA encapsulation was achieved upon mixing the squalene nanoparticles with a siRNA solution; siRNA-squalene nanoparticles formed due to simple electrostatic interactions between the negatively charged siRNA and the positively charged squalene derivates. The highest siRNA complexation was obtained for N/P = 3, alongside siRNA protection from nuclease degradation and efficient intracellular delivery [192].

As mentioned above, it is possible to form RNA-based lipid complexes with a non-lamellar structure by simple component mixing. The addition of siRNA to cationic lyotropic liquid crystalline nanoparticles resulted in the formation of complexes whose internal structure was dependent on the content of glycerol monooleate (GMO) [105]. At low GMO fractions lamellar and hexagonal phases were observed and the siRNA was either sandwiched between the lamellae or distributed within the water channels of the hexagonal phase. Higher GMO fractions yielded complexes with a double gyroid cubic phase (*i.e.*, cuboplexes) and siRNA within the water channels. SiRNA-loaded cuboplexes exhibited efficient gene silencing *in vitro* at low charge ratios with negligible toxicity [105]. The internal organization of cubosomes confers high RNA loading capacity without the need of adding large amounts of cationic lipids: by incorporating only 0.1 mol% cationic lipid, Sarkar and co-workers were able to form siRNA-loaded cuboplexes with 170 μg/mL encapsulated siRNA and encapsulation efficiencies > 70 % [106]. Alternatively, zinc-containing lipids can be used to achieve high siRNA loading by leveraging the specific interaction between zinc ions and the phosphate groups on the RNA backbone [107].

Recently, Zheng and co-workers have carried out a systematic investigation of the effect of the RNA length and lipid composition on the internal structure and fusogenicity of RNA-based lipid complexes [108]. At high GMO content, complexation of lipid-based nanoparticles with siRNA or mRNA yielded hybrid complexes in which the hexagonal and the cubic phase coexisted, as shown by small-angle X-ray scattering and cryogenic transmission electron microscopy [108]. Coexistence of a lamellar phase and a hexagonal phase were observed by small-angle X-ray scattering when the GMO was partly replaced by a phosphatidylcholine, irrespective of the RNA length [108]. SiRNA-loaded cuboplexes showed enhanced cellular uptake and endosomal escape ability compared to siRNA-loaded lipoplexes, which may be due to their intrinsic superior fusogenicity [108]. Interestingly, siRNA-loaded cuboplexes exhibited lower gene silencing efficiency compared to siRNA-loaded LNPs, which were used as the gold standard [108]. However, cuboplexes led to superior mRNA transfection compared to LNPs, while lipoplexes yielded the lowest intra-cellular delivery of both siRNA and mRNA [108].

SLNs and NLCs have been explored for RNA delivery by using simple component mixing approaches to achieve RNA loading. SLNs were used to deliver a fluorescently-labelled oligonucleotide [111] or a combination of siRNA and paclitaxel for combinatorial chemotherapy [112]; in these examples, the favourable properties of SLNs were leveraged to achieve high cargo loading and low toxicity. Combinatorial therapy with an antagomir to miR-155 and teriflunomide was also proposed for the treatment of multiple sclerosis; in this case, NLCs were used as nanocarriers and administered *via* the intranasal route in a mouse model. Successful RNA and teriflunomide delivery was achieved and resulted in reduced neuron demyelination [113]. More recently, the versatility and colloidal stability of NLCs has been leveraged for the development of RNA-based vaccines with enhanced long-term stability and efficacy [114,115]. NLCs were colloidally stable for at least one year upon storage at 4 °C and could be complexed with RNA at the point-of-care immediately before administration [114,115]. Alternatively, the RNA-NLC complexes could be lyophilized in the presence of a cryoprotectant and stored at 4 °C for at least 21 months without losing their RNA *in vivo* delivery efficacy upon reconstitution [114,115].

3.3. Hybrid self-assembling lipid nanoparticles

Self-assembling LNVs can also be designed by integrating non-lipid components such as inorganic, metal, and polymeric materials in the formulation. This approach ensures a finer tuning of the physicochemical and biological properties of the resulting nanoparticles. In order to trigger the self-assembling process, ionic or ionizable materials are generally needed and the resulting hybrid nanoparticles may exhibit a core–shell structure. The RNA is often first complexed with the non-lipid components and then mixed with the pre-formed, lipid-based nanoparticles. Some examples of hybrid self-assembling lipid nanoparticles which have been classified based on the type of non-lipid material are reported below.

3.3.1. Calcium-based self-assembling lipid nanoparticles

Inorganic, calcium-based materials are a well-established platform for nucleic acid delivery to mammalian cells [193]. The use of transfection agents based on the formation of complexes between DNA and calcium phosphate (CaP) dates back to the early 1970 s [194] and many products are now available on the market for *in vitro* DNA delivery. These systems rely on the interaction between the calcium ions present on the surface of CaP particles and the phosphate groups in the nucleic acid backbone [193]. Once internalized in the endosomal compartments (pH < 6.5), the CaP particles dissolve and induce endosomal rupture, potentially by osmotic swelling [195]; this cascade of events leads to the release of the encapsulated nucleic acid in the cell cytoplasm. Calcium-based materials exhibit excellent biocompatibility and biodegradability since they mimic the composition of natural biomaterials (*e.g.*, bone) and are metabolized into non-toxic ions [196,197]. However, calcium-based materials suffer from poor colloidal stability, which thwarts their *in vivo* applications and makes the use of stabilizing molecules such as lipids and biopolymers necessary [197]. In this Review, we will focus on self-assembled structures comprising lipids as stabilizing molecules.

A micro-emulsion approach was used to obtain CaP nanoparticles encapsulating siRNA, which were then mixed with cationic liposomes to form hybrid lipid-CaP [195]. These nanoparticles were able to induce gene silencing both *in vitro* and *in vivo* with minimal immunotoxicity [195]. The addition of an anionic lipid during the synthesis of the CaP nanoparticles yielded hybrid lipid-CaP nanoparticles with an asymmetric bilayer, whose composition could be tuned to achieve high siRNA intra-cellular delivery [198]. More recently, this approach was leveraged for the simultaneous delivery of mRNA encoding a melanoma-associated antigen and siRNA targeting programmed cell death protein ligand 1 in a melanoma mouse model. A mannose-functionalized PEGylated lipid was incorporated in the hybrid lipid-CaP nanoparticles to target dendritic cells [199]. In order to tune the pH-responsive properties of hybrid lipid-CaP nanoparticles, Wu *et al.* developed a modified micro-emulsion method to incorporate calcium carbonate, which dissolves at pH ~ 6.8, in the calcium phosphate nanoparticles for a faster nucleic acid release at pH 5.5–6 compared to CaP nanoparticles [200].

In the above-mentioned examples hybrid lipid-CaP nanoparticles encapsulating RNA were obtained with a complex assembly protocol involving the preparation of RNA-CaP nanoparticles *via* microemulsion, their subsequent mixing with lipids in an organic solvent, which is removed to obtain the hybrid lipid-CaP nanoparticles. Extrusion or ultrasonication were used to obtain nanoparticles with a narrow size distribution. An alternative approach based on simple component

mixing at room temperature was employed to produce hybrid lipid-CaP nanoparticles, named self-assembling nanoparticles (SANP). SANP could be produced by mixing CaP nanoparticles and the payload, followed by the addition of cationic liposomes without the need of further downstream processing. SANP were originally developed to deliver bisphosphonates for the treatment of prostate cancer [201,202] and glioblastoma [203–205]; these nanoparticles are characterized by an inorganic core surrounded by two lipid bilayers [206]. Given the ability of CaP nanoparticles to complex nucleic acids, they were also proposed for the delivery of miRNA [207] and siRNA to treat glioblastoma [208]. These formulations exhibited excellent biocompatibility, high RNA encapsulation efficiency and intracellular delivery. By tuning the composition of the cationic liposomes used to assemble SANP, it was possible to improve miRNA delivery to the brain [207] or to achieve selective brain targeting following transferrin functionalization on the nanoparticle surface [208].

It is worth mentioning that the components of RNA-loaded SANP formulations (*i.e.*, the CaP NPs precursor solutions, the liposomes, and the freeze-dried RNA) can be easily transported and stored at refrigerated temperatures and simply mixed in the appropriate ratios when needed. Targeting ligands such as transferrin can be included on the SANP surface by chemical conjugation to the PEGylated lipid [208] or added as fourth component to the "assembling kit" [205]. This feature makes these formulations attractive candidates for RNA delivery with applications spanning from prophylactic and anti-cancer vaccines to personalized nanomedicines. The possibility to prepare SANP from freeze-dried RNA could also overcome stability issues encountered with the LNP-based vaccines. Furthermore, it has been shown that the incorporation of cerium-based materials within the CaP nanoparticles endowed SANP with the ability to scavenge reactive oxygen species and to preserve the functionality of neurons subjected to oxidative stress [209].

The complexity of the preparation procedure of hybrid lipid-CaP nanoparticles *via* emulsion-based approaches makes this process difficult to scale-up. The evolution of preparation protocols from a complex lipid assembling process by evaporation towards a self-assembling approach starting from components in aqueous solution or in freeze-dried form could pave the way to the industrialization of these nanoparticles and their clinical use. The self-assembling approach could also be proposed for future personalized medicine in which the RNA sequence could be tailored to the patient based on diagnostic information (*e.g.*, miRNA profile). In this context, SANP could be prepared before use in the hospital pharmacy to obtain ready-to-administer personalized nanomedicines. On the other hand, the preparation protocol based on a self-assembling of three or four components could pose challenges associated to mixing errors by the operator. In order to address these concerns, it has been demonstrated that it is possible to freeze-dry hybrid lipid SANP, which retain the ability to effectively deliver their cargo for the treatment of brain tumors [205].

3.3.2. *Metal-based self-assembling lipid nanoparticles*

A wide range of metallic nanoparticles has been combined with synthetic or cell-derived lipids for nanotheranostic applications which combine drug delivery and imaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET) [210]. Among these, gold, iron oxide and mesoporous silica nanoparticles have been widely investigated, given their simple synthesis, ease of surface functionalization, and good biocompatibility [211]. Gold nanoparticles have been used as the core material for nanoparticles mimicking high-density lipoprotein assemblies [212,213]. When mixed with a cationic lipid and siRNA, these assemblies formed hierarchical, 100 nm-sized nanostructures which were successfully employed for *in vivo* gene silencing in a prostate cancer model [214].

The superparamagnetic properties of iron oxide nanoparticles have prompted their development as drug carriers able to induce localized hyperthermia upon exposure to magnetic fields for cancer therapy [215]. Surface engineering of iron oxide nanoparticles with cationic lipids allows complexation with negatively charged nucleic acids [211] and the subsequent magnetic field-guided transfection, an approach called "magnetofection" [216]. In this case, magnetic fields are used to facilitate nanoparticle accumulation in the target site and enhance the intra-cellular delivery of nucleic acids both *in vitro* and *in vivo* [217]. While some studies have relied on the use of solvent exchange methods to prepare lipid-coated iron oxide nanoparticles for nucleic acid delivery [218,219], current strategies aim at mixing RNA-loaded iron oxide nanoparticles with cell membrane-derived vesicles to obtain hybrid nanostructures whose size can be controlled by extrusion [220].

Mesoporous silica nanoparticles are characterized by tunable particle and pore size, versatile surface chemistry and good biocompatibility, which makes them promising drug nanocarriers [221]. Various research groups have focused on the development of lipid-coating strategies to confer mesoporous silica nanoparticles targeting ability, *in vivo* stability against aggregation, and hemocompatibility [222–230]. Such strategies comprise solvent exchange methods [222], the hydration of a lipid film with a mesoporous silica nanoparticle suspension followed by sonication [223–225], or mixing a liposome suspension with mesoporous silica nanoparticles [226–230]. By mixing cationic liposomes with negatively charged mesoporous silica nanoparticles in the presence of RNA, it is possible to obtain hybrid nanostructures with a mesoporous silica core coated by a lipid bilayer and encapsulating siRNA with loading efficiencies up to 80 % [221]. These nanoparticles had a higher siRNA loading capacity compared to lipid nanoparticles with a minimal increase in the hydrodynamic diameter due to their porous core and were able to induce gene silencing *in vitro* [227]. The same approach has been used to obtain lipid-coated mesoporous silica nanoparticles encapsulating miRNA for the treatment of hepatocellular carcinoma [228] and CRISPR-Cas9 payloads for gene editing applications [229,230].

Metal-organic frameworks (MOFs) are another interesting class of inorganic nanomaterials for drug delivery and biosensing applications [231]. MOFs are formed by multivalent metal ions, the most prevalent being zirconium(IV), iron(III), and zinc(II) for drug delivery, and organic ligands with multiple carboxyl or amine functional groups linked to an alkyl chain or a ring-based group like benzene or imidazole [232]. A crystal-like lattice with a well-defined porosity is obtained due to the coordination of the organic ligand with the ion [232]. Careful design of the MOF synthesis protocols ensures precise control on the final nanoparticle size, structure, and pore dimensions; other favorable properties include the ease of surface functionalization, high drug loading capacities and controlled release of therapeutics in biological environments [231,232]. Modifications of the MOF surface with a lipid shell improved the nanoparticle colloidal stability and ability to retain a model payload, as reported by Wuttke and co-workers [233]. They proposed a solvent exchange method to coat iron-based MOFs with a lipid bilayer; lipid-coated MOFs exhibited higher uptake by cancer cells compared to uncoated MOFs [233]. More recently, a straightforward approach entailing mixing preformed vesicles and MOFs and incubation for at least 2 h has been proposed to obtain liposome-[234] or exosome-[235] coated MOFs; in both cases, the authors speculated that the coating is driven by the fusion of the vesicles with the MOF surface.

Biomimetic MOF nanoparticles have been recently developed by coating the nanoparticle surface with cell-derived membranes which confer homotypic targeting properties and low intrinsic immunogenicity [236–238]. In one example, MOF nanoparticles encapsulating CRISPR-Cas9 were coated with lipid membranes derived from various cell sources and specific nanoparticle uptake by the parent cell was observed [236]. These findings were confirmed *in vivo*: MOF coated with breast cancer cell-derived membrane accumulated at the tumor site and successfully delivered CRISPR-Cas9 [236]. Compared to bare nanoparticles, coating zeolitic imidazolate framework-8 MOFs with platelet-derived membranes greatly enhanced the nanoparticle colloidal stability in serum, minimized their uptake by macrophages, and reduced their short-term immunogenicity [237]. The nanoparticles were able to

effectively deliver siRNA in an *in vivo* model of breast cancer with negligible systemic toxicity [237]. A similar approach was leveraged by Zhang and co-workers, who demonstrated that MOFs coated with cancer cell-derived membranes exhibited homotypic targeting *in vivo* and induced effective tumor suppression by delivering a siRNA targeting Polo-like kinase 1 [238]. In all these examples, the nucleic acid payload was incorporated into the MOF structure during synthesis following a biomineralization approach, while the cell membrane coating was achieved by mixing the MOF nanoparticles with cell-derived membrane followed by extrusion to control the nanoparticle size.

3.3.3. Polymer-based self-assembling lipid nanoparticles

Hybrid LPNPs have also been prepared by mixing RNA and pre-formed nanoparticles as a result of structural re-organization at a supramolecular scale. Ternary complexes composed of cationic liposomes, cationic polymers (polycations), and negatively charged nucleic acids, termed lipopolyplexes, have been largely described [239]. In the preparation of lipopolyplexes the components spontaneously self-assemble through electrostatic and hydrophobic interactions and encapsulate the nucleic acid. A typical lipopolyplex formulation can be obtained by mixing siRNA-PEI complexes with cationic liposomes [240]. PEGylated lipopolyplexes have also been developed to encapsulate a mixture of siRNA and DNA for anti-cancer applications [241]. An optimal transfection efficiency could be obtained by a careful selection of the polymer type in terms of its molecular weight and structure [242]. In an alternative strategy, hyaluronic acid has been employed to coat mRNA-loaded lipoplexes by the simple addition of an aqueous hyaluronic acid solution to the mRNA-loaded lipoplexes. In this example, the hyaluronic acid coating neutralized the positive surface charge of the nanoparticle and modified their stability without affecting the properties of core system [243].

Cholesterol-modified cationic polymers have also been explored for siRNA delivery [244]. Hybrid LPNPs were obtained by mixing cholesterol-modified nanoparticles complexed with siRNA with cationic liposomes; the presence of a bioreducible moiety within the polymer backbone enabled effective intra-cellular siRNA delivery [245]. This platform was further adapted for the co-delivery of siRNA and paclitaxel by modifying the nanoparticles with a targeting peptide ligand. Briefly, cationic liposomes containing paclitaxel and anionic nanoparticles containing siRNA, chondroitin and protamine were prepared separately and then mixed together to form the final hybrid LPNPs [246].

4. Conclusions and perspectives

The approval of the first medicinal products based on RNA and LNPs have paved the way for a widespread use of RNA therapeutics. LNPs represent the gold standard for RNA delivery and are the delivery system onto which research teams and companies are focusing their attention. However, LNPs have some limitations with regards to long-term stability and efficacy as well as immunogenicity and intrinsic toxicity, as recently highlighted [247–250]. The development of alternative RNA delivery systems should address these limitations and open new perspectives. Preparation methods with a more precise control of lipid assembling may enable the formulation of LNVs with the desired physico-chemical properties and the possibility to modulate their biodistribution or to optimize RNA delivery into the target cells. Alternatively, LNVs preparation methods based on self-assembling can offer further advantages compared to driven-assembling methods. As an example, the self-assembling of RNA-loaded LNVs upon component mixing could overcome the stability issues observed with the approved LNP formulations. For most of the self-assembling processes described in this review, the use of starting materials in freeze-dried form is possible, an aspect which would significantly increase the long-term stability of the formulations. It is also clear that self-assembling formulations can be easily updated by replacing the starting components, *e.g.*, by including "bioactive" excipients or further components for *e.g.*, active targeting applications.

Inspired by this approach, various self-assembling platforms have been proposed by including non-lipid materials into the LNV composition. While this strategy increases the complexity of the system, a more careful modulation of the LNV properties can be obtained. A higher degree of complexity could pose some challenges for self-assembling methods due to a higher risk of manufacturing errors. In order to minimize such risks, the self-assembling protocol should be robust and the number of components to mix should be minimal. Devices such multi-channel, pre-filled syringes may be designed to facilitate the assembly of the formulation at the point-of-care. Self-assembling processes able to support extemporaneous preparation of RNA-based nanomedicines represent a unique opportunity to move towards personalized therapies. In cancer, the intrinsic complexity of patients and the advances in patient profiling (*i.e.*, mutational oncology) require appropriate tools to address the variability of the therapies. Lipid self-assembling nanoparticles can be prepared on the basis of the patient profile to encapsulate the RNA sequence(s) required for the specific patient. In this context, it is of outmost importance that regulatory science takes into account such approaches for RNA-based medicines.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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