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Microfluidic technologies and devices for lipid nanoparticle-based RNA delivery

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\section*{ABSTRACT}

In 2021, mRNA vaccines against COVID-19 were approved by the Food and Drug Administration. mRNA vaccines are important for preventing severe COVID-19 and returning to normal life. The development of RNA-delivery technology, including mRNA vaccines, has been investigated worldwide for \textasciitilde30 years. Lipid nanoparticles (LNPs) are a breakthrough technology that stably delivers RNA to target organs, and RNA-loaded LNP-based nanomedicines have been studied for the development of vaccines and nanomedicines for RNA-, gene-, and cell-based therapies. Recently, microfluidic devices and technologies have attracted attention for the production of LNPs, particularly RNA-loaded LNPs. Microfluidics provides many advantages for RNA-loaded LNP production, including precise LNP size controllability, high reproducibility, high-throughput optimization of LNP formulation, and continuous LNP-production processes. In this review, we summarize microfluidic-based RNA-loaded LNP production and its applications in RNA-based therapy and genome editing.

\section*{1. Introduction}

In recent years, RNA-delivery technologies have been investigated for use in vaccines and nucleic acid therapies \cite{1-4}. In 2020, mRNA vaccines for COVID-19 were authorized by the Food and Drug Administration (FDA) under an emergency use authorization. The mRNA vaccines BNT162 and mRNA-1273 produced by Pfizer–BioNTech and Moderna can prevent severe cases of COVID-19 worldwide \cite{5-9}. The mRNA vaccine was officially approved by the FDA in 2021, and other mRNA vaccines against malaria, human immunodeficiency virus, influenza, and cancer are also being developed.

RNA-delivery technology using lipid nanoparticles (LNPs) plays an essential role in the practical application of mRNA vaccines and RNA-based therapies. The encapsulation of RNA into the LNP prevents RNA cleavage by RNases in the blood. Additionally, LNP-based drug-delivery technologies enable the delivery of RNA to target organs. LNP characteristics, including lipid composition, the molar ratio of nitrogen/phosphate (N/P), LNP size, LNP size distribution, Z-potential, and RNA-encapsulation efficiency, affect biodistribution and therapeutic effects, suggesting that these conditions should be optimized to maximize the therapeutic effect. Therefore, the development of RNA-loaded LNP-production technologies with high reproducibility is strongly desired.

Microfluidic devices provide many advantages, such as high-throughput screening, reduced use of expensive samples, precise control of reaction times, wearable sensing, and on-site analysis in the biochemical, biomedical, diagnostics, drug-design, and pharmaceutical fields \cite{10-20}. Microfluidic devices and technologies promise precise size controllability with high reproducibility for the production of microdroplets, emulsions, microcapsules, microparticles, and NPs \cite{21-26}. Additionally, microfluidic devices have been employed for liposome and LNP production \cite{27-51}. Microfluidic device features, including continuous flow, precise control of reaction time, high-temperature controllability, and shorter diffusion distance in a microchannel, are advantageous for the production of microparticles and NPs. Other aspects of a microfluidic device suitable for LNP production include rapid optimization of LNP-production conditions and ease of scaling up. These microfluidics features provide outstanding contributions to LNP production and LNP-based RNA-delivery technology and enable transition from laboratory-scale use to practical applications. Recently, various microfluidic devices have been developed to produce LNPs and applied to RNA, DNA, ribonucleoprotein (RNP), drugs, and other NP-delivery platforms, as well as the development of Onpattro, the first approved RNA-interference therapeutic drug, employing a microfluidic device \cite{52}. Furthermore, several microfluidic devices, including

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NanoAssemblr and iLiNP are commercially available and used to study RNA delivery. In the future, microfluidic devices and technologies will be the gold standard for LNP production. This review focuses on RNA-delivery technologies using LNPs produced by microfluidic devices. We briefly introduce the characteristics of microfluidic devices used for LNP production and the mechanism of LNP formation using microfluidic devices. Additionally, we provide an overview of LNPs used for small-interfering (si)RNA, mRNA, and RNP delivery and produced by microfluidic devices.

2. Microfluidic devices for RNA-loaded LNP production

Generally, the ethanol-dilution method is used for LNP production using a microfluidic device. The lipid solution is dissolved in ethanol, and RNA is dissolved in appropriate buffer solutions, such as acetate, citrate, or malic acid buffer. To encapsulate RNAs into LNPs, a cationic lipid or pH-sensitive cationic lipid is employed for the lipid components. The lipid solution and RNA/buffer solutions are then introduced into the microfluidic device, where positively-charged lipids and negatively-charged RNAs form complexes via electrostatic interactions. The

![Fig. 1. Conceptual illustration of the relationship between LNP-formation behavior and fluid dynamics.](image1)

![Fig. 2. Schematic illustration of LNP formation process in a microfluidic device at (a) slower and (b) faster mixing. Reprinted from [30] with the permission of the Public Library of Science.](image2)

![Fig. 3. (a) Time evolution of microfluidic devices for LNP production. (b) A sheath-flow (3 inlets)-type microfluidic device. Reprinted from [34] with the permission of the American Chemical Society. (c) A chaotic mixer device. Reprinted from [28] with the permission of the American Chemical Society. (d) A planar asymmetric split-and-recombine micromixer. Reprinted from [58] with the permission of the American Chemical Society. (e) The iLiNP device. Reprinted from [28] with the permission of the American Chemical Society.](image3)
3. Application of microfluidic devices for RNA delivery

3.1. Microfluidic preparation of RNA-loaded LNPs

Fig. 4 shows a schematic illustration of the preparation method for RNA-loaded LNPs using a microfluidic device. A microfluidic device for RNA-loaded LNPs was designed with two inlets for a lipid/ethanol solution and an RNA/buffer solution. The lipid solution contains a cationic lipid or a pH-sensitive lipid as the main component, and other lipids, such as phospholipids, cholesterol, and polyethylene glycol (PEG)ylated lipids, are mixed into the solutions. The pHkAs of typical pH-sensitive lipids are lower than physiological pH in order to prevent electrostatic interactions between the LNP and serum proteins in blood. When pH-sensitive cationic lipid-based LNPs are taken up by cells via endocytosis, the LNPs show a positive charge at the late endosome, which enables fusion of positively-charged LNPs with the endosomal membrane and subsequent RNA release into the cytosol. The lipid solution is diluted with RNA/buffer solution to allow the formation of RNA-loaded LNPs, and acidic buffer solutions are used to encapsulate RNAs into pH-sensitive cationic lipid-based LNPs. RNA-loaded LNPs are collected at the outlet of the microfluidic device, after which the collected LNP suspension is dialyzed against appropriate buffer solutions to remove ethanol.

For RNA delivery, LNP characteristics, including size, size distribution, the polydispersity index (PDI), Z-potential, and RNA-encapsulation efficiency, are controlled by lipid composition and concentration, preparation methods, and the N/P ratio (the ratio between the positively-charged amino in lipid to the negatively-charged phosphate in RNA) [60,61]. Recently, the influences of these critical formulation parameters on the LNP size and mRNA vaccine immunogenecity have been reported using microfluidic devices. A variety of nucleic acids, such as plasmid DNA, oligonucleotides, siRNA, mRNA, and RNP, can be encapsulated into LNPs using microfluidic devices. In this review, we provide an overview of siRNA, mRNA, and RNP delivery by LNPs prepared using microfluidic devices.
| Lipid | Device | Size [nm] | PDI [-] | Z potential [mV] | RNA EE [%] | In vitro target | In vivo target | Ref. |
|-------|--------|-----------|---------|-----------------|-----------|----------------|---------------|-----|
| Cationic lipid/DSPC/cholesterol/mPEG2000-DMG = 2:0.28:0.52:0.13 mg/mL | CM | 60-90 | – | – | 80 | Firefly luciferase in dual-glow HeLa cells | VII factor in mouse liver | [39] |
| DLinKC2-DMA/DSPC/PEG-c-DMA = 40:11.5:47.5:1 mol% | CM | 28-54 | > 0.1 | – | > 95 | – | VII factor in mouse liver | [37] |
| YSK05/Chol/DMG-Peg = 50:50:1 | iLiNP | 40 | – | – | > 90 | – | VII factor in mouse liver | mRNA in liver and spleen | [29] |
| ClinDMA/Cholesterol/PEG-DMA = 50:44:6 mol% | T-junction | 140± | – | – | 82% | – | – | – | [62] |
| KC2/DSPC/Cholesterol/PEG = 50:10:38.5:1.5 mol% | CM | 60 | – | – | – | – | – | – | [63] |
| DLin-KC2-DMA/DSPC/Cholesterol/PEG = 20:31.5:47.5:1 mol/mol | T-junction | 20-60 | – | – | > 0.1 | – | – | – | [38] |
| DMAP-BLP/DSPC/Cholesterol/PEG = 50:10:39.5-39.75 mol/mol | CM | 27-117 | – | – | > 95 | – | VII factor in mouse | – | [64] |
| Dlin-MC3-DMA/DSPC/Cholesterol = 51:10:9 mol% DMG-PEG (a) 1.4-1.5% (b) 4-5% (c) 6-8% | T-junction | 110-130 | – | – | 82% | – | VII factor in mouse | – | [65] |
| DMAP-BS/DlinMC3 PEG-DMG/DSPC/Cholesterol = 50:10:39.5:39.75:0.25-5 mol/mol | CM | 84.5 ± 32.5 | 0.18 ± 0.12 | 0.04 ± 0.08 | 82 ± 1 | – | (Cytokin in human whole blood) | (Cytokin in human whole blood) | [66] |
| DMAP-BS/DSPC/Cholesterol/PEG-DSG = 40:17.5:40:2.5 mol% | CM | 84.5 ± 32.5 | 0.18 ± 0.12 | 0.04 ± 0.08 | 82 ± 1 | – | (Cytokin in human whole blood) | – | [67] |
| DOPE/DSPE-PEG/Cholesterol = 13:1:1 | CM (Original) | 120.2 ± 1.4 | 0.18 ± 0.12 | 0.04 ± 0.08 | 82 ± 1 | – | (Cytokin in human whole blood) | – | [68] |
| C12-200/DSPC/Cholesterol/PEG2000-PE = 50:10:38.5:1.5 mol% | CM (Original) | 84.5 ± 32.5 | 0.18 ± 0.12 | 0.04 ± 0.08 | 82 ± 1 | – | (Cytokin in human whole blood) | – | [69] |
| YSK05/DOPE/Cholesterol/PEG-S94/PEG = 5:3:1:0.3 | iLiNP | 60.47 ± 6.9 | 0.101 ± 0.011 | – | > 95 | – | Midkine gene in HepG2 cells | HCC in mouse | [70] |
| DODMA/DOTMA/egg PC/Chol/mPEG-Chol = 40:5:18:35:2 | CM (Original) | 132.6 ± 1.6 | 0.19 ± 0.02 | 7.3 ± 0.7 | – | – | TI receptor in HepG-2 | Survivin mRNA in mouse | [71] |
| DNN-MC3-DMA/Chol/DSPC/PEG-DMG = 50:38:10.5:1.5 | NanoAssembler | 73 ± 6.0 | 0.13 ± 0.01 | 0.03 ± 0.01 | 95 ± 9 | CD44 in CCL cells | – | [72] |
| MC3/DSPC/Chol/DMG/PEG/DSPE-PEG = 50:10:38:1:0.5 | NanoAssembler | 129 ± 5 | 0.12 ± 0.02 | 9.3 ± 0.4 | 95 ± 9 | CD44 in CCL cells | – | [73] |
| Dendrimer/DSPC/Cholesterol/PEG-GnCm = 50:10:38:2 | CM | 50-100 | – | – | > 90 | – | Luciferase in HeLa cells | VII factor in mouse | SOST-mRNA in MEF | [75] |
| DOPE/DSPE-PEG/Cholesterol = 13:1:1 | CM (Original) | 132.6 ± 1.6 | 0.19 ± 0.02 | 7.3 ± 0.7 | – | Luciferase in HeLa cells | VII factor in mouse | SOST gene in mouse | [76] |
| DMAP-BLP/DSPC/Cholesterol/PEG = 50:10:39.5-39.75:0.25-5 mol/mol | CM | 30-115 | – | – | – | VII factor in mouse | – | – | [77] |
| DODAPE/DSPE-PEG/Cholesterol = 13:1:1 | CM (Original) | 350-1400 | 0.1-0.4 | < 40 | – | Luciferase in HeLa cells | – | – | [78] |
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were controlled according to PEG in hepatocytes using various sizes of siRNA-loaded LNPs, sizes of which the molar ratio of PEGylated lipid allowed control of the size of siRNA-associated characteristics [75], and developing new approaches for LNP production as compared with the bulk-mixing method, the microfluidics method produced small-sized LNPs with a narrow size distribution. Further, the Tf-LNPs produced by the microfluidic device inhibited the expression of survivin mRNA [72]. Moreover, peptide- and antibody-conjugated LNPs were also produced using a microfluidic device for targeting hepatic fibrosis [66] and T lymphocytes [74].

Microfluidic devices have been applied for other applications, including the production of functional NPs [28,38,68,70,73,76], investigating the mechanisms of siRNA-loaded LNP formation [63] and their associated characteristics [75], and developing new approaches for LNP production [69,71,80]. Wei et al. [68] produced hybrid NPs using siRNA, policaprolactone–polyethyleneimine (PCL–PEI), cholesterol, DOPE, and DSPE–PEG. In the first step, siRNA and PCL–PEI complexes were prepared by microfluidic mixing, and the complex was coated with lipids in the microchannel (Fig. 7), resulting in NPs of ~120 nm with a PDI of 0.18, the properties of which were well controlled by microfluidic production as compared with bulk mixing. Their results showed that siRNA-loaded hybrid NPs targeting epidermal growth factor receptor (EGFR) mRNA resulted in reduced mRNA levels and a 57% decrease in tumor-growth rate. Additionally, we previously reported one-step production of siRNA-loaded exosome-like NPs using the ILiNP device [28]. Because encapsulation of negatively-charged siRNA using the non-cationic lipid system is a major challenge due to the lack of a driving force for RNA encapsulation (i.e., electrostatic interactions), the ILiNP-based approach enabled production of size-controlled siRNA-loaded exosome-like NPs, which demonstrated luciferase knockdown in HeLa cells that was proportional to NP size.

3.3. Microfluidics for mRNA-delivery applications using LNPs

mRNA delivery using LNPs is a popular research topic worldwide, and mRNA-loaded LNPs are prepared similar to siRNA-loaded LNPs.
Fig. 6. (a) Intrahepatic distribution of siRNA. Blood vessels and siRNA are colored green and red, respectively. Scale bar represents 50 μm. (b) FVII-silencing activity of the 1% and 3% PEG-LNPs. Reprinted from [56] with the permission of Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Table 2 summarizes the mRNA-loaded LNPs produced by microfluidic devices \[3,35,81–99\]. Kauffman et al. \[81\] reported an optimization strategy for lipid formulation using a microfluidic device. They optimized the types of lipids, molar ratio of the lipids, and N/P ratio for mRNA delivery to the liver in vivo, which resulted in a 3-fold improvement in the delivery and expression of luciferase mRNA (1653 nucleotides) relative to the original formulation (Fig. 8). For a shorter mRNA (583 nucleotides) encoding erythropoietin (EPO), the optimized LNP formulation showed 7-fold higher EPO expression in the liver relative to that of the original LNP formulation. Interestingly, the FVII-silencing activities of the original and optimized formulations for siRNA-loaded LNPs were the same. The results demonstrated the combined use of microfluidics and design-of-experiment (DoE) approaches for optimizing the LNP formulation. Additionally, Sato et al. \[35\] reported a two-step DoE approach for preparing liver-targeted mRNA-loaded LNPs using original pH-sensitive cationic lipids (CL4H6 and CL15H6) and the iLiNP device. They optimized several formulation parameters, including the mRNA/lipid ratio, cationic lipid concentration, phospholipid concentration, and molar ratios of lipids, to design mRNA-loaded LNP libraries based on previously reported siRNA-loaded LNPs and DoEs. The results identified LNP size and the PEG–DMG/phospholipid ratio as critical factors, which was similar to other lipid formulations, and that the optimized mRNA-loaded LNP promoted liver-specific gene expression. Moreover, mRNA-loaded CL4H6-based LNPs showed higher protein expression in hepatocytes than the mRNA-loaded MC3-LNPs \[35\].

Specific organ targeting and protein expression are essential advantages of LNP-based RNA-delivery technology; therefore, the development of mRNA-delivery technology for targeted organs is strongly desired for protein-replacement therapy. B lymphocytes play an important role in antibody production, and dysregulation of B-cells induces autoimmune disorders. B-cells are attractive targets for gene delivery in order to modulate their functions; however, in vivo mRNA-delivery systems targeting B-cells to allow sufficient protein expression have not been demonstrated. Fenton et al. \[85\] synthesized a novel lipid (OF-Deg-Lin) for spleen-selective mRNA delivery and confirmed that OF-Deg-Lin-based LNPs could specifically produce proteins in B-cells. OF-Deg-Lin is an ionizable lipid that contains degradable linkers. The authors prepared Cy5-labeled mRNA-loaded LNPs and evaluated their biodistribution and luciferase expression, revealing that OF-Deg-Lin-based LNPs were mainly delivered to the liver; however, luciferase-expression efficiency was lower than that of the spleen, which demonstrated >85% of detected luciferase expression (Fig. 9). They hypothesized that the organ-dependent degradation of OF-Deg-Lin ester linkers affected spleen-specific luciferase expression and tested this hypothesis by preparing non-degradable OF-02-based mRNA-loaded LNPs. They found that OF-02-based mRNA-loaded LNPs were primarily distributed in the liver along with observed luciferase expression. Their findings suggested the importance of designing lipid molecules for selective targeting and protein expression \[85\]. Additionally, Cheng et al. \[84\] investigated selective organ-targeting (SORT) NPs for mRNA and CRISPR-Cas9 delivery, demonstrating SORT of lung, spleen, and liver by LNPs as a result of controlling internal and/or external charge.
### Table 2
mRNA-loaded LNPs produced by microfluidic devices.

| Lipid | Lipid: mRNA | Device | Size [nm] | PDI [-] | Z potential [mV] | RNA EE [%] | pKa | In vitro | In vivo | Ref. |
|-------|-------------|--------|-----------|---------|------------------|-----------|-----|----------|--------|------|
| CL4H6/ESM/chol/PEG-DMG (60/5/35/1.5 mol%) | mRNA/lipid = 18.3 (g/mol) | iLiNP | 66.9 | 0.129 | 6.2 | 95.1 | – | – | ICR mouse(4 weeks year), BALB/c mouse (4–5 weeks year), CKOR/Stm Slc-Apoe (4 – 5 weeks year) | [35] |
| C12–200:DOPE/Chol/C14-PEG2k (35/16/46.5/2.5 mol%) | C12–200:mRNA = 10/1 (w/w) | CM | 102 | 0.158 | –5 | 43 | 6.96 | – | C57BL/6 mouse (18–22 g) | [81] |
| X/DSPC/Chol/DMG-PEG2k (50/10/38.5/1.5 mol%) | X: selected from library | CM | 86.2 ± 1.7 | 0.04 ± 0.06 | – | 97.5 ± 0.2 | 6.56 ± 0.13 | – | CD-1 mouse (18–22 g), Sprague-Dawley rat (225–250 g), Naive cynomolgus monkeys (2–4 years old, 2-6 kg) | [82] |
| MC3/DSPC/Chol/DMG-PEG2k (50/10/38.5/1.5 mol%) | – | CM | 80–100 | – | – | ≥ 90 | – | HeLa | BALB/c mouse (5–8 weeks old), Ferret (13-15 weeks old), Naive cynomolgus monkeys (2–4 years old, 2-6 kg) | [3] |
| X/DSPC/Y/DMG-PEG2k (50/10/38.5/1.5 mol%) | X: selected from library | CM | – | – | – | – | – | – | HeLa | – | [83] |
| N/P = 5.67 | Total lipid/mRNA = 40/1 (w/w) | CM | – | – | – | – | – | – | – | C57BL/6 mouse (18–20 g) | [84] |
| OF-Deg-Lin/DOPE/Chol/C14-PEG2k (35/16/46.5/2.5 mol%) | – | CM | 75 ± 10 | 0.197 | – | – | 5.7 | – | C57BL/6 mouse (18–22 g) | [85] |
| APE/DOPE/Chol/C14-PEG2k (50/25/23.5/1.5 mol%) | APE: selected from several types | CM | 65–100 | 0.136–0.151 | –2.1–10 | 93–98 | – | – | C57BL/6 mouse (18–22 g) | [86] |
| CL2–200:DOPE/Chol/C14-PEG2k (35/16/46.5/2.5 mol%) | – | CM | 80± | – | – | 55–65 | – | HeLa | C57BL/6 mouse (16–20 g) | [87] |
| C14-4:DOPE/Chol/C14-PEG (35/16/46.5/2.5 mol%) | – | CM | 65.19–70.17 | 0.176–0.189 | – | 86.3–92.53 | 6.143–6.505 | Jurkat cell, Nalm-6 cell | – | [88] |
| X/DSPC/Chol/DMG-PEG2k (50/10/38.5/1.5 mol%) | X: selected from library | CM | 75–95 | – | – | 69–100 | 6.6–6.9 | – | – | BALB/c mouse (5–8 weeks old), CD-1 mouse, Sprague-Dawley rat, Naive cynomolgus monkeys (2–5 years old, 2-3 kg) | [89] |
| X/DSPC/Chol/C14-PEG2k (50/10/38.5/1.5 mol%) | X: N/A | CM | 78 ± 3.5 | 0.15 ± 0.03 | – | 95.8 ± 0.5 | – | Human fibroblasts | HeLa | C57BL/6 mouse (16–20 g) | [90] |
| X/DSPC/Chol/DMG-PEG2k (50/10/38.5/1.5 mol%) | X: N/A | CM | 80–100 | – | – | ≥ 90 | – | – | HeLa | mouse, rabbit, non human primates | [91] |
| X/DSPC/Chol/DMG-PEG2k (50/10/38.5/1.5 mol%) | N/P = 5.67 | CM | 80–100 | – | – | ≥ 90 | – | – | K562 cell, Vero cell | AG129 mouse, C57BL/6 mouse, BALB/c mouse (8 weeks old) | [92] |
| ATX/DSPC/Chol/DMG-PEG2k (50/7/40/3 mol%) | – | CM | N/A | – | – | N/A | – | – | CD-1 mouse, C57Bl6 mouse (6–8 weeks old), BALB/c mouse (7 weeks old) | [93] |
| X/DOPE/Chol/C14-PEG2k (35/16/46.5/2.5 mol%) | X: selected from library | CM | 82.5–135.2 | – | – | 74.0–98.0 | 5.05–7.14 | Human dendritic cells | BALB/c mouse, C57BL/6 mouse | [94] |
| TT3/DOPE/Chol/C14-PEG2k (20/30/40/0.75 mol%) | N/P = 4 | CM | 99–178 | <0.2 | ≥ 0 | 15–82 | – | HeP3B cell | C57BL/6 mouse (6–8 weeks old) | [95] |
| Ionizable lipid/DSPC/Chol/DMG-PEG2k or pSar (40/10/50-x/x mol %) | – | CM | PEG: 90 pSar: 150 | <0.25 | – | – | – | HeP3B | BALB/C mouse | [96] |
| TT3/DOPE/Gd-DTPA-BSA/Chol/DMG-PEG2k (20/12/18/4.0/0.75 mol%) | – | CM | Gd18: 110± | – | 6± | 91 | – | HeP3B | C57BL/6 mouse (6–8 weeks old) | [97] |
| MC3/DSPC/Chol/DMPE-PEG2k (50/10/38.5/1.5 mol%) | N/P = 3 | CM | 81–87 | 0.020–0.13 | – | 95–98 | – | – | – | [98] |
| Ionizable lipid/DSPC/Chol/DMPE-PEG2k (40/10.5/47.5/2 mol%) | N/P = 3 | CM | Lipid14: 50 | 0.2 ± | – | – | – | VeroE6 cell | BALB/c mouse (6–8 weeks old) | [99] |
The LNPs were formulated with ionizable cationic lipids (5A2-Sc8, DLin-MC3-DMA, or C12–200), phospholipids, cholesterol, and PEGylated lipids, with the SORT LNPs harboring supplemental components relative to basic LNP formulations (Fig. 10). These included positively-charged lipids, such as DOTAP, DDAB, and EPC, that play an important role in lung targeting, whereas negatively-charged lipids, including 14PA, 18PA, and 18BMP, promote specific delivery to the spleen and show a high level of luciferase expression. Additionally, liver-specific SORT LNPs can be produced by adding other ionizable lipids. The authors confirmed that the primary ionizable lipid did not affect SORT according to supplemental lipid component [84].

To improve mRNA delivery and protein expression, the development of new ionizable lipids is a major research topic in the field of drug delivery; however, the development and role of helper lipids, such as phospholipids and cholesterol, have not been well investigated. Patel et al. [83] studied the structural effects of cholesterol derivatives on mRNA delivery in combination with different LNP compositions. The authors used DLin-MC3-DMA, lipid 9, and DODMA as ionizable lipids along with...
a fixed LNP formulation at molar ratios of 50/38.5/10/1.5 mol% [ionizable lipid/cholesterol-derivative/distearylophosphatidylcholine (DSPC)/PEG–DMG].

Antibody-dependent enhancement (ADE) is a significant problem in the development of vaccines against infections, because ADE exacerbates symptoms caused by the production of non-neutralizing antibodies. ADE has been reported for dengue vaccines [100]. Richner et al. [92] demonstrated the induction of neutralizing antibodies and reduction of non-neutralizing antibodies by mRNA-loaded LNPs for ZIKV infection. The authors used DAMP–BLP/DSPC/cholesterol/PEG–DMG at a molar ratio of 50/10/38.5/1.5 and evaluated the effects of dose and boosting on survival rate, antibody titer, and weight change. They found that two doses of mRNA-loaded LNPs induced high neutralizing-antibody titers that protected against ZIKV infection and conferred sterilizing immunity [100].

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Fig. 10. (a) LNPs used for SORT. (b) Effect of cationic lipid (DOTAP) molar ratio on lung-targeted delivery using SA2-SCB SORT LNPs. (c, d) Importance of a cationic lipid (DOTAP) on lung-targeted delivery and luciferase expression. (e) Induction of mRNA delivery of an anion SORT molecule (18PA) to the spleen. (f) Luciferase expression in major organs administrated by D-Lin-MC3-DMA SORT LNPs and C12–200 SORT LNPs. (g) Detailed lipid formulations of SORT molecules. Reprinted from [81] with the permission of Springer Nature Publishing Group.

Antibody-dependent enhancement (ADE) is a significant problem in the development of vaccines against infections, because ADE exacerbates symptoms caused by the production of non-neutralizing antibodies. For example, ADE has been reported for dengue vaccines [100]. Richner et al. [92] demonstrated the induction of neutralizing antibodies and reduction of non-neutralizing antibodies by mRNA-loaded LNPs for ZIKV infection. The authors used DAMP–BLP/DSPC/cholesterol/PEG–DMG at a molar ratio of 50/10/38.5/1.5 and evaluated the effects of dose and boosting on survival rate, antibody titer, and weight change. They found that two doses of mRNA-loaded LNPs induced high neutralizing-antibody titers that protected against ZIKV infection and conferred sterilizing immunity [100]. mRNA delivery for human T cells is essential for producing chimeric antigen receptor (CAR) T cells to develop non-viral cell-based therapies. Conventional CAR T cell engineering uses viral vectors or electroporation, which can induce adverse effects and cytotoxicity. Billingsley et al. [88] described mRNA-loaded LNP-mediated mRNA delivery for CAR T cell engineering. They synthesized 24 ionizable lipid libraries for mRNA delivery to Jurkat cells, finding that C14–4 showed the best mRNA-delivery and luciferase-expression performance. Furthermore, T cells treated with C14–4-based LNPs killed tumor cells to a degree similar to those treated by electroporation or lentivirus and with low cytotoxicity [88].

Other applications of mRNA-loaded LNPs have been studied using microfluidics. Sebastiani et al. [98] investigated the structural and compositional effects of apolipoprotein (ApoE) on mRNA-loaded LNPs by evaluating mRNA-loaded LNPs using small-angle neutron scattering and small-angle X-ray scattering, with their findings revealing that ApoE binding to the LNP induced a redistribution of the lipid. Additionally, Luo et al. [97] demonstrated co-delivery of mRNA and magnetic resonance imaging contrast agents [gadolinium (Gd)-based contrast agents] for theranostics. The particle size of TT3-Gd lipid-like NPs was almost 100 nm and resulted in encapsulation efficiencies of mRNA and the Gd contrast agent of 91% and 74%, respectively. Moreover, the TT3-Gd lipid-like NPs showed good stability over the course of 1 month at 4 °C, and the authors confirmed the ability of the optimized lipid-like NPs to co-deliver mRNA and Gd in vitro and in vivo [97].
3.4. RNA delivery for genome editing

For genome editing-based therapy, the CRISPR/Cas9 RNP is a major target of LNP-based delivery systems. However, LNP-based RNA delivery has primarily focused on siRNA and mRNA. Therefore, there are few studies on the application of RNPs delivery using LNPs prepared by microfluidic devices as compared with siRNA and mRNA delivery. Table 3 summarizes the RNP-loaded LNPs prepared using microfluidic devices [34,84,101–110]. Miller et al. [101] demonstrated the first non-viral co-delivery of Cas9 mRNA and single-guide (sg)RNA using LNPs in vitro and in vivo. They designed a new lipid-like material (Z3-Ep10; a zwiterionic amino lipid) suitable for the delivery of long nucleic acids and prepared LNPs using NanoAssemblr. Treatment of HeLa-Luc-Cas9 cells with sgRNA-loaded LNPs and siRNA (siluc) resulted in similar luciferase-silencing activity by the sgRNA-loaded LNPs relative to that observed by the siluc-loaded LNPs. Additionally, co-delivery of Cas9 mRNA and sgRNA using LNPs was confirmed in vitro and in vivo. Fig. 11 shows CRISPR/Cas9 editing by co-delivery of Cas9 mRNA and sgRNA using LNPs in vivo. The authors showed that Cas9 mRNA and sgRNA-loaded LNPs were able to delete the target area in the genome and induce the expression of tdTomato [101]. Their findings demonstrated the ability to deliver multiple long RNAs using LNPs.

Rosencent et al. [105] reported genome editing for cancer therapy using Cas9 mRNA and sgRNA-loaded LNPs (sgPLK1-cLNPs). The optimized lipid system enabled up to 70% gene editing of polo-like kinase I (PLK1), inhibited tumor growth by 50%, and improved the survival rate of mice by 30%. Additionally, they developed EGFR-targeting sgPLK1-cLNPs, which improved overall survival by 80% [105]. Additionally, Qiu et al. [107] described co-delivery of Cas9 mRNA and sgRNA for liver-specific in vivo genome editing by designing tail-branched biodegradable lipidoids to explore suitable LNP formulations for liver targeting. They found that 306-O12B LNP showed higher knockdown performance than LNPs with MC-3, which is the gold standard lipid for liver-targeted delivery.

LNP-based CRISPR-Cas9 RNP delivery provides several advantages relative to Cas9 mRNA and sgRNA co-delivery using LNPs. RNP delivery does not require expression of Cas9 protein or complexation of CRISPR, Cas9, and sgRNA in the cell. Therefore, RNPs should enable higher genome-editing efficacy than co-delivery systems, such as Cas9 mRNA/pDNA and sgRNA. Additionally, an RNP-delivery system can minimize off-target effects, because Cas9 protein does not continue to be expressed in cells. However, critical problems include RNP denaturation during the production process and issues with the encapsulation efficiency of large-sized RNPs into LNPs. Specifically, the principle method for microfluidic-based LNP production involves ethanol dilution; however, critical problems include RNP denaturation upon contact with ethanol. Therefore, rapid dilution of ethanol is important to avoid RNP denaturation. Suzuki et al. [34] reported the delivery of an RNP/single-stranded oligonucleotide (ssON) complex using an iLiNP device. Fig. 12 shows a schematic illustration of the RNP-loaded LNP preparation using a three-inlet iLiNP device. Inclusion of ssON added an additional negative charge following complexation with RNP, thereby promoting LNP encapsulation via strong electrostatic interactions with cationic lipids. The three-inlet iLiNP device reduced RNP denaturation by introducing a buffer.
solution between the lipid flow stream and the RNP–ssON complex flow stream. Their results showed that the optimized RNP-loaded LNPs successfully convert the \textit{EGFP} gene sequence into one encoding blue fluorescent protein. Moreover, they demonstrated 95% \textit{EGFP} knockout using 5 nM of spCas9 RNP in HEK-GFP cells [34]. The optimized RNP-loaded LNPs were subsequently applied for hepatitis B virus (HBV) inhibition, showing effective suppression of both HBV DNA and covalently-closed circular DNA in HBV-infected human liver cells as compared with effects on adeno-associated virus type 2. Based on the advantages of RNP delivery using LNPs, the applications of microfluidic technologies for RNP-loaded LNP production should be expanded in the future.

4. Conclusion and outlook

In this review, we focused on microfluidic approaches for LNP-based RNA-delivery systems and their applications. Microfluidic devices and technologies allow the production of a variety of RNA-encapsulated LNPs, including those for siRNA, mRNA, and RNPs. Compared with conventional RNA-loaded LNP-production methods, microfluidic approaches can produce homogeneous-sized LNPs with high reproducibility. Additionally, optimized LNP-production conditions at a laboratory scale did not change when applied for mass production. These findings suggest that microfluidic devices have accelerated the development of LNP-based nanomedicines.

The LNP-production performance of microfluidics should be improved for vaccine production. Numbering-up and piling-up techniques can be expanded to increase LNP-production rates using microfluidic devices. A PDMS-based microfluidic device is usually used in laboratory studies, because it can be fabricated by rapid prototyping and under reasonable production costs. The pressure resistance and rigidity of the microfluidic device are required for LNP mass production. For this reason, microfluidic devices made from glass or metal are expected to be developed for LNP mass production. For the development of glass or metal-based microfluidic devices, a microchannel design is essential to allow fabrication of micrometer-scale structures via microfabrication processes, such as chemical etching and micromachining. We consider that the simple structure of the iLiNP device is suitable for fabricating glass or metal-based microfluidic devices.

In the future, RNA delivery using microfluidic devices will be
indispensable for personalized nanomedicine. In particular, a combination of digital twin and personalized nanomedicine can lead to the next generation of RNA, cell, and gene therapies. We believe that microfluidic technologies are a critical and core technology for next-generation therapies and can potentially promote a global paradigm shift.

Conflicts of interest

There are no conflicts to declare.

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