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Recent advances in siRNA delivery mediated by lipid-based nanoparticles

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ABSTRACT

Small interfering RNA (siRNA) has been expected to be a unique pharmaceutic for the treatment of broad-spectrum intractable diseases. However, its unfavorable properties such as easy degradation in the blood and negative-charge density are still a formidable barrier for clinical use. For disruption of this barrier, siRNA delivery technology has been significantly advanced in the past two decades. The approval of Patisiran (ONPATTRO™) for the treatment of transthyretin-mediated amyloidosis, the first approved siRNA drug, is a most important milestone. Since lipid-based nanoparticles (LNPs) are used in Patisiran, LNP-based siRNA delivery is now of significant interest for the development of the next siRNA formulation. In this review, we describe the design of LNPs for the improvement of siRNA properties, bioavailability, and pharmacokinetics. Recently, a number of siRNA-encapsulated LNPs were reported for the treatment of intractable diseases such as cancer, viral infection, inflammatory neurological disorder, and genetic diseases. We believe that these contributions address and will promote the development of an effective LNP-based siRNA delivery system and siRNA formulation.

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1. Introduction

Oligonucleotides such as small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs) are ideal drug candidates that are able to be chemically synthesized and directly act on a target gene in a sequence-dependent manner [1,2]. They are expected as a promising drug modality to fulfill unmet medical needs that have not been satisfied with the use of small molecule- or antibody-based drugs. Because gene silencing with siRNA is triggered by a RNA-induced silencing complex (RISC) system [3], an endogenous enzyme system, siRNA is capable of targeting all genes in principle. The expression of splicing variants and mutants can be also inhibited by RNA interference (RNAi), suggesting that siRNA is applicable for targeting undruggable proteins. Thus, the therapeutic application of siRNA is extremely promising for a variety of diseases.

Only a few years after the discovery of siRNA [4], the world's first clinical trial of intraocularly injected siRNA against vascular endothelial growth factor (VEGF) was conducted [5]. Since then, clinical application of siRNA has received great attention and widely studied for the development of siRNA-based medicines. Although early clinical trials of siRNA were conducted for the treatment of diseases such as age-related macular degeneration [6] and lung-infected respiratory syncytial virus (RSV) [7], these trials failed to show any clinical benefits of siRNA drugs. At that time, one of the concerns for the development of siRNA drugs was innate immunity stimulated by siRNA. Judge et al. reported that such innate immune responses can be abrogated by the use of 2'-*O*-methyl (2'OMe)-modified siRNA [8–10]. Another concern is the development of a drug delivery system (DDS) for siRNA drugs. Since siRNA is a polyvalent anionic and highly hydrophilic mid-sized molecule, it is hardly taken up into cells. In addition, siRNA is easily degraded by nucleases in the blood, resulting in poor accumulation of siRNA in a target tissue. Therefore, it is essential to establish a proper DDS for the development of siRNA drugs.

Lipid-based nanoparticles such as liposomes (Fig. 1a) are a suitable carrier for drug and nucleic acid delivery because of their excellent biocompatibility, biodegradability, low toxicity and immunity, structural flexibility, and ease of large-scale preparation. Many lipid-based nanoformulations have been approved and are being used around the world for the treatment of various diseases [11]. Liposomes containing a cationic or pH-sensitive lipid have been investigated for the delivery of nucleic acids since the 1980s [12,13]. Because positively charged liposomes can electrostatically interact with nucleic acids and form complexes called lipoplexes (Fig. 1b), they have been used for the transfection of cells with plasmid DNA, ASOs [14], and siRNA [15,16]. Various types of lipid-based nanoparticles have been commercially available as a standard transfection reagent. On the other hand, cationic liposomes have been developed for the treatment of diseases and have shown promising pharmacological effects in animal studies; but their instability in blood and their toxicities [17,18] are often major concerns for clinical application. It seems favorable that lipid-based nanoparticles should not have a positive charge in the physiological condition to avoid adverse events.

In 2006, Zimmermann et al. succeeded in long-term silencing of a target gene in cynomolgus monkeys by systemic administration of siRNA encapsulated in lipid nanoparticles (LNPs) containing an ionizable lipid [19]. LNPs have received considerable attention as a promising carrier for siRNA delivery [20]. Twenty years after the discovery of RNAi, Patisiran (ONPATTRO™), an LNP formulation of siRNA targeting transthyretin (TTR), was approved as the first siRNA drug by the Food and Drug Administration (FDA) in 2018 for the treatment of TTR-type familial amyloid polyneuropathy. Systemic administration of LNPs loaded with siRNA targeting TTR suppresses the deposition of amyloid fibrils of misfolded TTR in the peripheral nerves and heart [21,22]. The LNP is the most successful platform for siRNA delivery in the clinical setting and is expected to be applied for the treatment of various diseases.

2. Lipid-based nanoparticles for siRNA delivery

2.1. Stable nucleic acid-lipid particles (SNALPs)

RNAi-mediated gene silencing in cynomolgus monkeys was shown by intravenous injection of a LNP formulation (Fig. 2), called SNALPs, containing an ionizable lipid, 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLin-DMA, Fig. 3a) [19], which is an ether analog of 1,2-dioleoyl-3-(*N*,*N*-dimethylamino)propane (DODAP, Fig. 3b) [23]. DODAP is a pH-responsive ionizable lipid containing oleic acid chains, but a subsequent study revealed that ionizable lipids with linoleic acid chains are superior to those with oleic acid chains for the induction of LNP-mediated RNAi [24]. A further study systematically investigated the importance of the linker moiety and the head of the ionized lipid and found excellent *in vivo* effects of LNPs composed of 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA, Fig. 3c) at a siRNA dose of 0.1 mg/kg in mice [25]. This study also demonstrated that the incorporation of an adequate amount of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) into the LNPs improves their stability during formulation and in the blood circulation. In 2012, the gold standard ionizable lipid, heptatriaconta-6,9,28,31-tetraen-19-*yl* 4-(dimethylamino)butanoate (DLin-MC3-DMA, Fig. 3d) was generated for hepatic gene silencing *in vivo* by optimizing the structure of the head group of DLin-KC2-DMA [26]. LNPs containing DLin-MC3-DMA

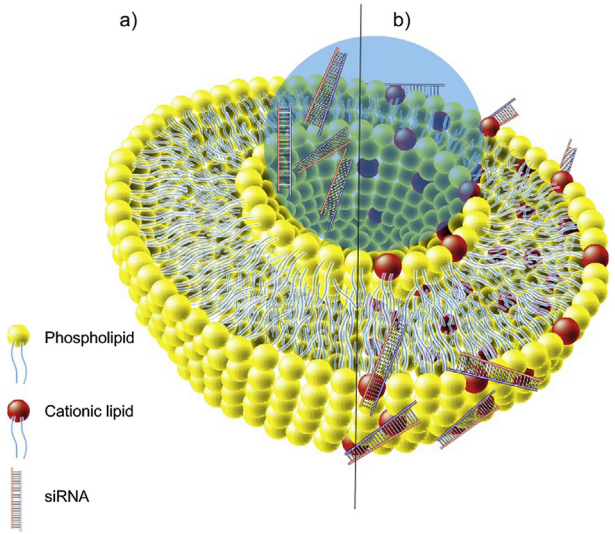

Fig. 1. Schematic illustration of liposome-mediated delivery of siRNA. a) siRNA is encapsulated in the inner water phase of liposomes. b) siRNA is complexed with liposomes containing cationic lipids.

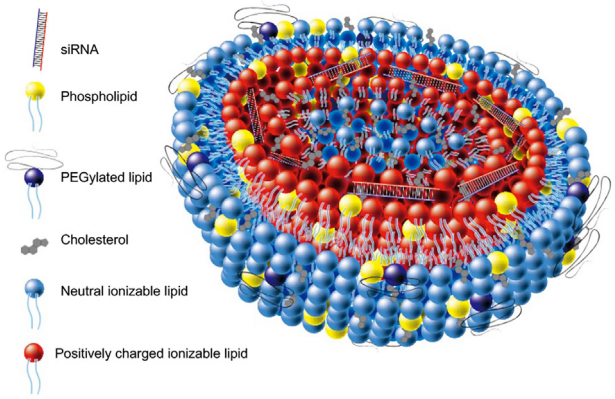

Fig. 2. Schematic illustration of LNPs. LNPs can be prepared by mixing a lipid mixture dissolved in an organic solvent and siRNA in aqueous solution with a microfluidic device followed by dialysis in water to remove the organic solvent. siRNA is encapsulated in the core of LNPs after dialysis.

achieved hepatic gene silencing in mice when used at a siRNA dose of around 0.005 mg/kg (ED_{50}). In addition, this study showed that the gene-silencing efficiency of LNPs well correlated with the p*K*a of the ionizable lipid and that the optimal p*K*a was in the range of 6.2–6.5. The final composition of DLin-MC3-DMA LNPs is DLin-MC3-DMA, DSPC, cholesterol, and 3-*N*-[(ω-methoxypoly(ethylene glycol)2000) carbamoyl]-1,2-dimyristyloxy-propylamine (DMG-mPEG2000) at a molar ratio of 50:10:38.5:1.5. These helper lipids (DSPC, cholesterol, and DMG-mPEG2000) generally contribute to the stability of lipid-based nanoparticles [27]. In the case of hepatic delivery, it is considered that DMG-mPEG2000 is gradually removed from the LNPs in the blood at the same time as apolipoprotein E (ApoE) gradually coats the surface of LNPs, which coating triggers the transport of LNPs into hepatocytes via low-density lipoprotein (LDL) receptor-mediated endocytosis [28].

The preparation methods of lipid-based nanoparticles for nucleic acid delivery have been significantly advanced [29,30]. One of the methods for LNP preparation involves microfluidic mixing of a lipid mixture in ethanol and siRNA in citrate buffer (pH 4.0) followed by dialysis in phosphate buffer to remove the ethanol [31]. The microfluidic mixing method enables the control of particle size and affords high siRNA encapsulation efficiency (~ 100%) and mass production. The structure of LNPs prepared by the microfluidic mixing method has been determined by molecular modeling [32]. The results showed that LNPs have a predominantly hydrophobic core consisting of reverse

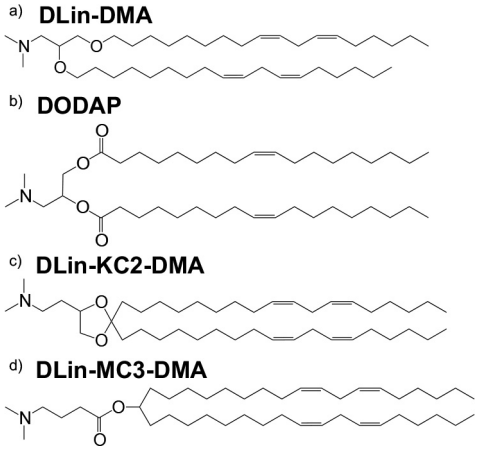

Fig. 3. Structural formulae of ionizable lipids used for preparation of SNALP. a) DLin-DMA, b) DODAP, c) DLin-KC2-DMA, d) DLin-MC3-DMA.

micelles of DLin-KC2-DMA that have interacted with siRNA, phospholipid, and cholesterol, with the surface of the lipid bilayer coated with PEG. Recent structural studies on LNPs performed by the use of cryo-transmission electron microscopy (TEM) and small-angle X-ray approaches showed that the siRNA/DLin-KC2-DMA complex is present in the form of a sandwich of siRNA in the lipid bilayer at pH 4.0 but that the ionizable lipids in the bilayer structure form an amorphous oil phase in the center of the LNP as the pH is brought closer to neutral [33,34].

2.2. Multifunctional envelope-type nano device (MEND) system

In order to develop siRNA-encapsulated nanoparticles with several functions to overcome multi-step barriers in the body, it is essential to mount functional molecules in one nano-particle for carrying out each function. For this purpose, Harashima et al. proposed the concept of a MEND for nucleic acid delivery [35]. As shown in Fig. 4a, this particle has a structure in which siRNA is encapsulated in the inner phase of a lipid bilayer modified with functional molecules such as peptides. For example, octaarginine (R8)-modified MEND has been reported to be taken up into cells by macropinocytosis followed by the efficient release of the nucleic acids into the cytoplasm [36]. Intracellular dynamics can be controlled by introducing fusible or pH-responsive lipids into the lipid membranes. Harashima's group has been working to develop their own pH-responsive ionizable lipids, resulting in the first-generation YSK05 [37], second-generation YSK13 [38], and third-generation CL4H6 (Fig. 4b) [39]. YSK05, an ionizable lipid designed based on the structure of DODAP, has a tertiary amine and unsaturated fatty acid chains and a p*K*a of 6.4, which lipid enhances the transfection efficiency by promoting endosomal escape. It has been shown that YSK05-MEND exhibits high membrane fusion and gene knockdown activity [37]. Next, YSK13 was developed to enhance the gene-silencing efficiency of MEND [38]. It was revealed that the ED_{50} (0.015 mg/kg) of blood-clotting factor VII (FVII) knockdown in mice intravenously injected with YSK13-MEND with a p*K*a of 6.45 was more than 4 times less than that of YSK05-MEND [38]. CL4H6 with a p*K*a of 6.25 was developed by a systematic study on the structure of the ionizable lipid [39]. It was shown that the structure of the head group of ionizable lipids is a primary key to determine the p*K*a and is important for liver distribution and endosomal escape of LNPs. It was also reported that the structure of the hydrophobic tail does not affect the apparent p*K*a. This systematic study revealed how the structure of the head group of ionizable lipids affects the carrier's characteristics. In addition, the ED_{50} of Factor VII knockdown after the intravenous injection of CL4H6 LNPs into mice was shown to be 0.0025 mg/kg [39], indicating that gene-silencing efficiency of LNPs is considerably improved by the systematic study on the structure of the ionizable lipid.

2.3. SS-cleavable and pH-activated lipid-like material (ssPalm)

Akita et al. developed ssPalmas a component of LNPs encapsulating nucleic acids [40–42]. LNPs containing ssPalm (ssPalm-LNPs) are neutral in charge at physiological pH. It has been shown that after internalization of ssPalm-LNPs into cells via endocytosis, ssPalm-LNPs destabilize the endosomal membrane under the acidic condition of the endosome and disintegrate under the reducing environment in the cytoplasm. ssPalm is a lipid derivative that has two tertiary amines, two hydrophobic chains, and a disulfide bond. The tertiary amines of ssPalm are charged positively in response to low pH, which charge contributes to the destabilization of the endosomal membrane. ssPalm-LNPs disintegrate by cleavage of the disulfide bond under the reducing environment, resulting in the release of the encapsulated nucleic acids. These mechanisms were demonstrated for the first-generation ssPalmM (Fig. 5a). ssPalmM-LNPs showed efficient endosomal escape and biodegradability, indicating them to be useful for delivering nucleic acids and controlling particle fate [40]. ssPalmA (Fig. 5b) and ssPalmE

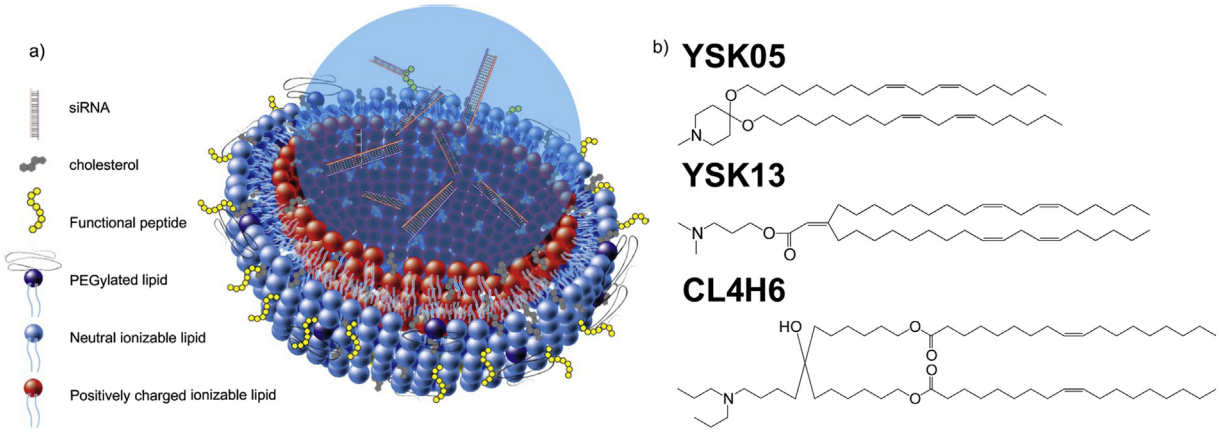

Fig. 4. Schematic illustration of MEND. a) siRNA is enclosed in the internal aqueous phase. The surface of MEND is made up of various functional lipid derivatives to give it multi-functionality to control internal and intracellular kinetics. b) Chemical structures of pH-responsive ionizable lipids YSK05, YSK13 and CL4H6.

(Fig. 5c), which contain vitamin A and E as hydrophobic chains of ssPalm, respectively, have been developed for nucleic acid delivery as the second generation of ssPalm. Since vitamin A in ssPalmA makes it possible to utilize the nuclear transport system operated by cellular retinoic acid-binding protein, the gene-transfer efficiency of ssPalmA-LNPs was dramatically increased compared with that for ssPalm-LNPs [43]. ssPalmE-LNPs are a potential gene carrier with anticancer activity derived from α-tocopherol [44]. In siRNA delivery, intravenously administered ssPalmE-LNP, which uses vitamin E as a hydrophobic scaffold, knocked down FVII more efficiently than ssPalmM or ssPalmA [41]. Furthermore, an improved ssPalmE has been developed in which the tertiary amine of ssPalmE is fixed to the piperidine structure to increase the distance between the surface of the particle and the tertiary amine. The FVII knockdown effect of the improved ssPalmE-LNP achieved an ED_{50} of 0.035 mg/kg.

On the other hand, activated hepatic stellate cells, which have the ability to produce extracellular matrix, are thought to promote hepatic fibrosis and liver cirrhosis [45]. These cells are known to store vitamin A intracellularly in order to maintain homeostasis [46]. Comparing the type I collagen a-1 knockdown effect of 3 kinds of ssPalm-LNPs, ssPalmA-LNP showed the highest inhibitory effect on hepatic fibrosis, with an ED_{50} of 0.25 mg/kg [47].

2.4. Lipidoid nanoparticles

Nanoparticles (NPs) composed of lipid-like materials, termed "lipidoids," have also been studied for siRNA delivery [48]. The structures of some such lipidoids are shown in Fig. 6. The advantages of lipidoids are that their structure can be freely arranged to improve *in vivo* kinetics, efficacy, and safety of lipidoid NPs. The effect of differences in the partial structure of lipidoids on the properties of lipidoid NPs can be analyzed in the process of screening lipidoids, which enable to predict their potential from the structure and reduce the number of experiments.

There have been three screening research reports on lipidoid NPs from Anderson's group over the last decade [49–51]. The ED_{50} of FVII knockdown obtained by intravenous injection of lipidoid NPs into

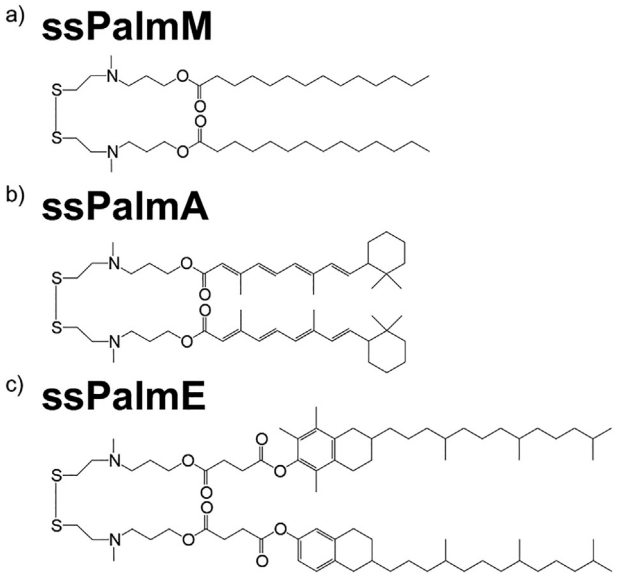

Fig. 5. Structural formulae of ssPalms. a) ssPalmM is characterized by having miristic acid, 2 tertiary amines, and a disulfide bond, b) ssPalmA contains Vitamin A, c) ssPalmE contains Vitamin E. The tertiary amine of ssPalm is positively charged in response to the acidic pH in the endosome. The disulfide bond is cleaved in response to the reducing environment in the cytoplasm.

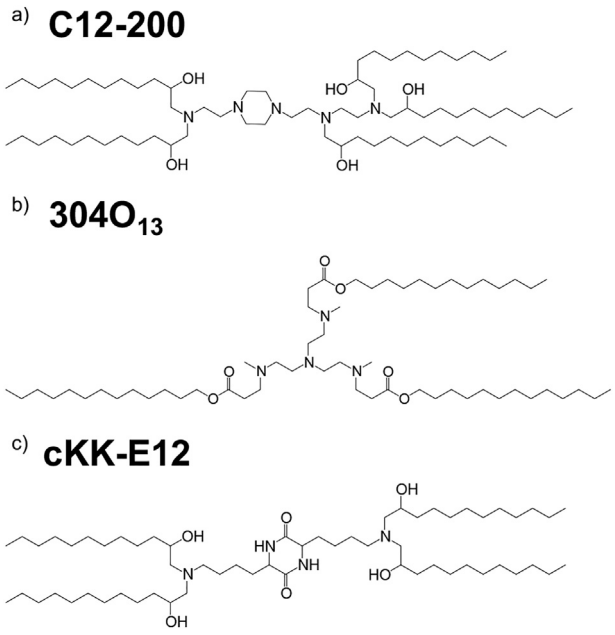

Fig. 6. Structural formulae of lipidoids, lipid-like materials. a) C12–200. b) $304O_{13}$. c) cKK-E12.

mice was determined after each screening. The ED_{50} of C12–200 (Fig. 6a) lipidoid NPs developed by Love et al. was 0.01 mg/kg of siRNA [49]. Whitehead et al. performed screening of lipidoids for improving the biocompatibility of lipidoid NPs [50]. Their results showed that $304O_{13}$ (Fig. 6b) NPs induced gene silencing with an ED_{50} of 0.01 mg/kg and that even with high-dose siRNA (1 mg/kg) no severe cytokine induction or inflammation was induced. Dong et al. identified cKK-E12 (Fig. 6c) by screening with a peptide-based lipidoid library that mimics lipoproteins [51]. The ED_{50} of cKK-E12 NPs for FVII knockdown was 0.002 mg/kg, being lower than that of DLin-MC3-DMA-LNP.

Lipidoid NPs used in these screenings are composed of lipidoid, DSPC, cholesterol, and DMG-mPEG2000 at a molar ratio of 50:10:38.5:1.5. The average particle size of lipidoid NPs is less than 90 nm. Similar to LNPs, lipidoid NPs are considered to be coated with ApoE in the blood and taken up by hepatocytes via hepatic LDL receptors. The particle size of lipidoid NPs also seems to contribute to liver accumulation, because these NPs can pass through the fenestrae of hepatic vessels, which have a diameter of about 100–150 nm [52,53]. However, the precise delivery mechanism has not been fully elucidated. In recent years, siRNA delivery with lipidoid NPs has been reported for the treatment of inflammation [54] and intestinal disease by oral administration [55]. These studies will provide new insights into siRNA delivery to various target tissues other than the liver.

2.5. Solid lipid nanoparticles (SLNs)

SLNs composed of non-toxic lipids show high biocompatibility [56,57] and have been investigated for drug delivery of therapeutics [58–60] and cosmetics [61]. The structure of SLN is characterized by a lipid core coated with a lipid membrane (Fig. 7). Lipophilic drugs can be incorporated in the core formed by lipids with a high melting point, which feature contributes to sustained drug release from SLNs.

SLNs can be applied to siRNA delivery by adding cationic lipids to SLNs for electrostatic complex formation (Fig. 7a). Similar to other lipid-based NPs, SLNs complexed with siRNA have been investigated for the treatment of cancer [62] and liver diseases [63]. On the other hand, as shown in Fig. 7b, siRNA can be incorporated into the core of SLN by the hydrophobic ion-pairing (HIP) approach [64,65]. This method is based on ionic complex formation between siRNA and cationic lipids by which the hydrophobic siRNA/lipid complex is incorporated into the electrically neutral hydrophobic core of an SLN. For instance, siRNA/DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) can be encapsulated in the triolein core [65]. Then, the lipid core is put into 67% methanol containing phosphatidylcholine and PEGylated lipid, after which the organic solvent is evaporated to obtain siRNA-encapsulated SLNs. SLNs produced by this method were shown to be capable of sustained release of siRNA in mice over a 10-day period [66]. In addition, an *in vitro* experiment revealed that siRNA gradually released from SLNs for 7 days showed a gene-silencing effect. It has recently been reported that betamethasone, an anti-inflammatory corticosteroid, co-encapsulated with siRNA in the core of SLNs suppressed the induction of proinflammatory cytokines, such as IL-6 and monocyte chemotactic protein-1 (MCP-1), in the blood in mice [67]. The number of outer membrane layers can be controlled by changing the ratio of core lipids to surface ones [68], which can be applied to the control of sustained siRNA release.

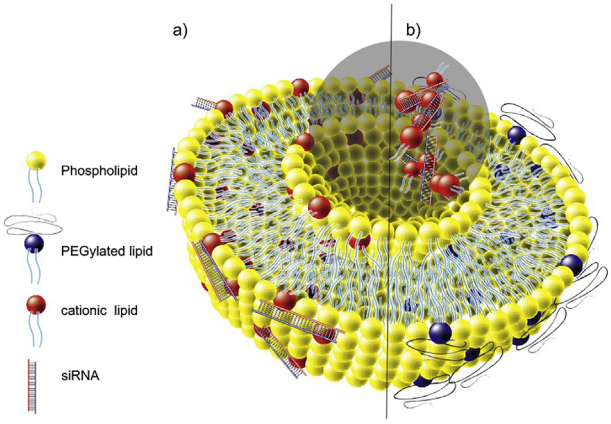

Fig. 7. Schematic illustration of SLN. a) SLN has a crystalline triolein core surrounded by phospholipids, cationic lipids, and PEGylated lipids. siRNA interacts with cationic lipids. b) HIP consists of siRNA and cationic lipids is incorporated in the crystalline core of SLN.

2.6. Exosomes

Exosomes, endogenous vesicles carrying nucleic acids and proteins, have been received a lot of attention in the field of siRNA delivery research, and are expected to be a safe and efficient DDS carrier [69,70]. It has been clarified that tissues in which exosomes accumulate differ depending on the cells that produce exosomes [71]. It has also been reported that the surface molecules expressed on exosomes are different depending on the type and state of the exosome-producing cells [72,73]. When exosomes are used as an siRNA carrier, the exosome-producing cells are preferably cells derived from self-tissues in consideration of safety and immunogenicity. Although the use of exosomes as an siRNA carrier is still highly experimental, it has been reported that exosomes collected from human serum can be encapsulated with siRNA by electroporation and introduced into human monocytes and lymphocytes [74,75]. Exosomes produced by the recipients' own cells will be a potential carrier for siRNA delivery.

The surface of exosomes can be modified with ligand molecules to increase selectivity for target cells. Alvarez-Erviti et al. established dendritic cells that produce exosomes constitutively expressing rabies viral glycoprotein (RVG) peptide for selectively targeting neuronal cells [76]. In order to display the RVG peptide on the exosome surface, the dendritic cells were transfected with a chimeric gene encoding the RVG peptide and Lamp2b, an exosome membrane protein. siRNA encapsulated in the RVG peptide-expressing exosomes induced gene silencing of β-site amyloid precursor protein cleaving enzyme 1 (BACE1), a therapeutic target in Alzheimer's disease, in the brain when administered to mice. These results indicate that the RVG peptide-expressing exosomes can cross the blood-brain barrier (BBB) and deliver siRNA into neuronal cells.

ExoCarta (www.exocarta.org), an exosome database, contains information on constituent molecules of exosomes, including proteins, mRNAs, miRNAs, and lipids [77]. From the findings of exosome studies, it is expected to construct artificial exosomes or exosome mimetics useful for tissue-specific delivery of siRNA.

3. Strategy to achieve efficient siRNA delivery

3.1. Improvement of siRNA properties by chemical modification

Chemical modification of siRNA can suppress off-target effects and improve gene silencing efficiency, although too much modification may interfere with RISC formation [78]. The position and frequency of chemical modification should be appropriate to use siRNA for therapeutic applications without off-target effects [79]. Appropriate triazole modification of the guide strand of siRNA has been reported to suppress off-target effects induced by the guide strand, increase the stability of siRNA, and attenuate unfavorable immune stimulation [79–82]. The effect of nucleotide analogues at the 3'-overhang on gene silencing has been clarified to design the sequence of siRNA [83]. In addition, innate immune responses can be prevented by use of 2'OMe-modified siRNA which is used in Patisiran. Thus, chemical modification of siRNA is important for the development of siRNA-encapsulated LNP formulations.

Another interesting siRNA chemical modification is the preparation of lipid-siRNA conjugates such as those with cholesterol [84]. It has been reported that such modification not only results in exonuclease resistance but also promotes cellular uptake and increases the blood retention time by binding to LDL and high-density lipoproteins (HDL). Conjugates of *N*-acetyl-*D*-galactosamine (GalNAc) and siRNA have been used for *in vivo* targeting to hepatocytes [85]. GalNAc conjugated with siRNA binds to the asialoglycoprotein receptor on hepatocytes and can reduce the distribution of siRNA to peripheral tissues. The conjugation of GalNAc to siRNA allows high and specific gene silencing in the liver and reduces the risk of gene silencing in different organs [85]. Givosiran, a GalNAc-modified siRNA targeting aminolevulinate synthase 1 (ALAS1) mRNA, was approved in 2019 as the second siRNA drug for the treatment of acute hepatic porphyria [86].

3.2. Enhancement of cellular uptake of LNP

The cell membrane is a barrier to be overcome for efficient delivery of siRNA. Cellular uptake and endocytosis pathway of LNP are determinants for delivery of siRNA into cytoplasm. Conjugation of siRNA or LNP with cell-penetrating peptide (CPP), antibody or other ligands improves the cellular uptake of siRNA [87–90]. Enhancement of membrane fusion with LNP by use of 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or fusogenic peptides also improves the cellular uptake of siRNA [37,91].

MEND modified with two kinds of CPP peptides, R8 and GALA, has been shown to be a potential carrier for siRNA delivery into dendritic cells [92]. It was shown that MEND modified with R8 is effectively internalized into cells via macropinocytosis [93]. Modification of MEND with GALA which adopts a random coil structure in physiological pH and an α-helix structure in acidic pH [94] was shown to facilitate endosomal escape [37,95]. Therefore, MEND modified with R8 and GALA can overcome multiple barriers, resulting in efficient delivery of siRNA into dendritic cells.

Ligand-mediated targeting of LNP to surface receptors on cancer cells has been achieved for siRNA delivery by use of transferrin [96,97], folic acid [98] or antibodies [99,100]. Internalizing receptors suitable for cellular uptake enhancement of LNP have been identified in various tissues [101–103]. We previously developed LNP decorated with an Fab' antibody against heparin-binding epidermal growth factor-like growth factor (HB-EGF) for siRNA delivery to triple-negative breast cancer cells overexpressing HB-EGF on their cell surface [99,104]. Decoration with anti-HB-EGF Fab' antibody significantly increased the association and internalization of LNP encapsulating siRNA. Intravenous injection of siRNA targeting polo-like kinase 1 (PLK1) with this formulation significantly suppressed the growth of MDA-MB-231 human triple-negative tumors in mice. These findings support that enhancement of cellular uptake of LNP by ligand-mediated targeting is highly effective for delivery of siRNA.

3.3. Facilitation of endosomal escape of LNP

Intracellular dynamics of LNPs is one of great concern to improve the efficiency of siRNA delivery. When LNPs that encapsulate siRNA are taken up into cells by endocytosis, they are sent to late endosomes followed by lysosomes and then degraded [105]. For this reason, LNPs should have a function for endosomal escape and for release of the siRNA into the cytoplasm [24]. The p*K*a of the ionizable lipid is an important parameter that strongly affects the efficiency of siRNA delivery [26]. The optimal p*K*a range for gene silencing in hepatocytes has been shown to be 6.2–6.5, suggesting that cationization of ionizable lipids in the weakly acidic environment of the endosome is critical for efficient siRNA delivery. The neutral surface charge of LNPs reduces nonspecific adsorption of serum proteins in the blood to the LNP surface compared with a cationic surface charge [25,106,107]. Such adsorption causes phagocytosis of LNPs in the blood and the liver [108], resulting in a reduced efficiency of delivery to target tissues. After LNPs are taken up into cells by endocytosis, their surface charge changes from neutral to positive at the low endosomal pH, which change enhances the interaction of the positively charged LNPs with the negatively charged endosomal inner membrane. This electrostatic interaction induces membrane fusion via the hexagonal II structure, allowing the release of the encapsulated siRNA into the cytoplasm [15,25]. In addition to the p*K*a of the ionizable lipid, the lipid composition of LNPs and the molar ratio of siRNA and lipids in LNPs affects the efficiency of siRNA delivery [25,26,31]. The design of an appropriate formulation of LNPs must be required to capture siRNA in LNPs and induce efficient endosomal escape of LNPs [109].

3.4. Enhancement of intracellular siRNA bioavailability

The control of siRNA kinetics after internalization of LNPs into cells is critical for siRNA delivery. It has been reported that only a small amount of the siRNA internalized into cells contributes to gene silencing, as most of the siRNA internalized is released to the outside of cells by endocytic recycling [105].

The Niemann-Pick type C-1 protein (NPC-1), a lysosomal membrane protein that mediates intracellular cholesterol transport, is considered to play an important role in the regulation of major recycling pathways of siRNA. In NPC-1 knockout cells, the extracellular recycling pathway of siRNA transfection with LNPs is inhibited, resulting in an increased intracellular concentration of siRNA and enhanced gene-silencing efficiency [110]. These findings suggest that inhibition of NPC1 may improve the effect of siRNA transfection via LNPs. Actually, Cullis et al. investigated the effect of incubating various cell lines with LNP encapsulating siRNA in the presence of NP3.47, a NPC1 inhibitor obtained from screening for drugs that prevent Ebola virus infection [111]. The presence of NP3.47 increased LNP accumulation in late endosomes/lysosomes by more than 3-fold and enhanced the knockdown effect by up to 4-fold suggesting that pharmacological inhibition of NPC1 might be an attractive strategy to enhance the therapeutic effect of LNP-siRNA (Fig. 8).

3.5. Improvement of siRNA pharmacokinetics

The physicochemical properties of LNPs such as particle size and surface charge are critical for siRNA delivery via systemic administration. When their properties are not satisfactory for systemic drug delivery, almost all LNPs injected are captured by the mononuclear phagocyte system (MPS) and rapidly eliminated from the blood, resulting in poor

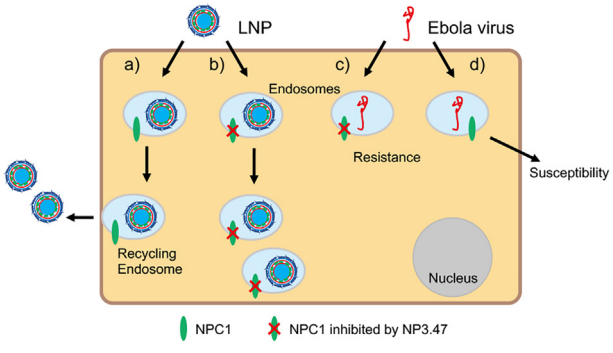

Fig. 8. Mechanisms of enhanced cellular retention of LNPs in cells treated with NP3.47, a NPC1 inhibitor. a) LNPs are often exocytosed to the outside of the cells. b) Endosomal recycling mechanism is impaired by NP3.47 treatment, resulting in enhanced retention of LNPs. c) In cells treated with NP3.47, infection of the Ebola virus is suppressed by inhibiting endosomal recycling mechanism. d) The Ebola virus is exocytosed to the outside of the cells, resulting in infection spread. By NP3.47 treatment, LNP increases the chance of exerting its function by endosomal escape, but Ebola virus reduces the chance of infection because it cannot endosomal escape.

accumulation in a target tissue [108]. To avoid such unfavorable biorecognition, the particle size of LNPs is commonly in the range of 30 to 150 nm; and the surface of LNPs is often covered with PEG. Modification of LNPs with PEG is well known to enhance their dispersibility in aqueous solution and to improve their stability in the blood. On the other hand, the presence of PEG on the surface of LNPs reduces the interaction between LNPs and target cells, which reduction diminishes their internalization into the cells. Therefore, the formulation of PEGylated LNPs should be optimized for the purpose of PEGylation. The amount of a PEG-lipid conjugate for modifying the surface of LNPs strongly influences the physicochemical properties and gene-silencing efficiency of the LNPs [112,113]. It has been reported that the size of LNPs decreases as the proportion of the PEG-lipid conjugate in LNPs increases [31]. Increased PEG density on the surface of LNPs has been shown to reduce the immune response [114]. The structural differences in the length of PEG, fatty acids, and linker of the PEG-lipid conjugate used for modification of LNPs also strongly affect the potential of LNPs. LNPs modified with (*R*)-3-[(ω-methoxy-PEG-carbamoyl)]-1,2-di-O-octadecyl-sn-glyceride (C18) showed high blood retention but dramatically reduced gene-silencing effects in the liver [112,113]. This is probably because the interaction between LNPs and cells is reduced or the ability of LNP to adsorb ApoE is reduced, resulting in difficulty in uptake into hepatocytes. The length of fatty acid chains of the PEG lipid is responsible for the time of dissociation from the LNPs. Since PEGylated lipids with myristoyl (C14) chains are rapidly dissociated from the LNPs in the blood circulation, they do not interfere the process of FVII knockdown. We previously showed in mice that long circulation of siRNA-loaded pH-sensitive liposomes modified with PEG was observed only when DSPE-PEG but not distearoylglycerol (DSG)-PEG was used. Our results suggest that the electrostatic interaction between lipid molecules on the surface of the liposomes is a critical determinant for the *in vivo* effect of PEGylation [115].

PEGylation of LNPs and chemical modification of siRNA reduce their immunogenicity, but it is difficult to completely eliminate the possibility of immune reactions, especially in susceptible individuals [116]. Recently, Chen et al. reported that incorporation of a small amount (4 mol% of total lipid content) of dexamethasone into LNPs can suppress the immune response [117]. Such an approach provides a positive perspective for future clinical trials and commercialization.

4. Development of LNPs for siRNA therapy

4.1. Cancer

Since siRNA specifically inhibits the target protein functions via the specific cleaving of the target mRNA, it has been receiving significant attention as an anti-cancer agent. To date, there have been several successful and unique reports for treating tumors using LNPs. Before the development of LNPs (siRNA-encapsulated lipid nanoparticles), siRNA was delivered to the target organs via systemic administration by attaching siRNA onto the particle (liposome) surface (lipoplex) [118,119]. Since the accumulation of lipoplex in a tumor relies on the enhanced permeability and retention (EPR) effect [120,121], the surface of the lipoplex can be modified with a hydrophilic PEG polymer to increase its circulation time after intravenous injection; otherwise, the lipoplex is rapidly captured by the reticuloendothelial system. On the other hand, it was reported that LNPs without a large amount of PEG modification are highly effective for liver targeting [28,122,123]. LNPs prepared with ionizable lipids form a protein corona in the bloodstream [124]. ApoE in the protein corona is recognized by receptors such as LDLR, which are overexpressed by hepatocytes [125]. Then, the complexes are internalized into hepatocytes via the ApoE-LDLR pathway [28,122,123]. Since the formation of the protein corona in the bloodstream is important for the liver targeting of LNPs, only a small amount of PEG modification (~1% as a molar ratio) of the LNP is standard protocol in the case of liver targeting. This means that the PEG modification for liver targeting is not for obtaining long circulation of LNPs after systemic administration but for increasing their stability in the bloodstream, such as by inhibition of aggregation.

In general, ionizable lipids in LNPs are positively charged at lower pH (pH ~6.0); therefore, highly hydrophobic LNPs tend to aggregate at physiological pH (pH 7.4). A small amount of PEG modification increases LNP stability in the bloodstream and may not inhibit ApoE binding in the bloodstream. For example, Huang et al. prepared polymer-lipid hybrid nanoparticles (P/LNPs) containing anti-VEGF siRNA [126]. Encapsulation of siRNA into these P/LNPs increased the amount of siRNA delivered into the cells. In addition, the circulation time of siRNA was significantly increased after systemic administration by encapsulation into the P/LNPs compared with that of free siRNA. The intravenous injection of P/LNPs into HepG2 hepatocellular carcinoma cell-implanted mice significantly inhibited the tumor growth and decreased the amount of VEGF mRNA in the tumor. In their report, Huang et al. indicated that the increase in the amount of LNPs (siRNA) delivered into the tumor and a good level of safety make P/LNPs a hopeful approach for cancer therapy. To increase the amount of LNPs delivered into a tumor, Li et al. modified LNPs with a transferrin receptor-targeting ligand in addition to PEG [127]. Also, a CPP was incorporated into the LNPs to enhance their cell-penetration efficacy. These LNPs containing anti-survivin siRNA significantly inhibited tumor growth after intravenous injection.

Now, a large amount of PEG modification and active targeting systems developed by modification with ligand molecules such as peptides and antibodies have been used for the delivery of LNPs to other organs. By intravenous injection, Yamamoto et al. delivered siRNA in LNPs to prostate tumors *in vivo* by increasing the amount of PEG modification from 1% to 2.5 or 5%, resulting in inhibition of the growth of the implanted tumors [128]. We modified the LNP surface with HB-EGF antibody for the targeting of breast tumors [99,104]. HB-EGF is a ligand that binds to the EGF receptor (EGFR) [129]. It was reported that triple-negative breast cancer (TNBC; no estrogen receptor, progesterone receptor or human epidermal growth factor receptor2 [HER2]) [130] overexpresses HB-EGF [129]. The use of anti-HB-EGF antibody-modified LNP increased the amount of siRNA delivered into the breast tumor after the intravenous injection. It is known that TNBC is refractory and has high malignancy and poor prognosis. Several nano-materials such as functionalized mesoporous silica [131], chitosan-layered gold particles [132], and cationic lipid-based LNPs [133] are used for siRNA delivery to tumors via passive-targeting. On the other hand, we delivered LNPs containing siRNA via active targeting. We succeeded in inhibiting TNBC growth *in vivo* by silencing PLK1 protein expression in the tumor after the intravenous injection of anti-HB-EGF antibody-modified LNPs containing siRNA against PLK1. This antibody modification strategy is a promising approach for the treatment of triple-negative breast cancer. Sakurai et al. modified the surface of MEND with epithelial cell adhesion molecule (EpCAM)-targeting peptide for targeting several types of cancers [134]. They found that 1.0% modification of the LNP surface with the peptide significantly enhanced the cellular uptake of LNP into several kinds of cancer cells (HT-1080, HEK293T, A549, and HeLa). These results indicate the peptide-based modification would be a useful strategy for delivering LNPs to various types of tumors.

In cancer treatment, targeting hematopoietic tissues is still a big challenge because of the lack of specificity. Jyotsana et al. delivered ionizable cationic LNPs containing anti-breakpoint cluster region-abelson (BCR-ABL) siRNA to bone marrow as a therapy for chronic myelogenous leukemia [135]. BCR-ABL, a chimeric fusion oncogene, is a leukemia-specific fusion transcript that occurs in acute lymphoblastic leukemia [136]. LNPs containing anti-BCR-ABL siRNA dose-dependently inhibited target mRNA expression in human leukemia K562 cells. In addition, significant knockdown was observed in CD34+ primary chronic myelogenous leukemia (CML) cells compared with the expression level in healthy control cells. They intravenously injected LNPs into a xenograft

leukemia mouse model and observed a 60% knockdown of BCR-ABL expression by LNP-anti-BCR-ABL siRNA in leukemia cells sorted from the myelosarcoma tissue, thus indicating that LNPs efficiently delivered siRNA to human leukemia cells *in vivo*. Knapp et al. tried to cure non-Hodgkin lymphoma by using cationic LNPs formulated with lipidoid [137]. The gold standard for therapy of non-Hodgkin lymphoma is now chemotherapy such as that with R-hyper-CVAD (rituximab, cyclophosphamide, vincristine, doxorubicin, dexamethasone), high-dose methotrexate and cytarabine, and R-CHOP (rituximab, cyclophosphamide, vincristine, prednisone, and doxorubicin) [138,139]. However, treatment options are limited. Knapp's group focused on several growth-related genes such as cyclin D1, Bcl-2, and Mcl-1 and found them to be overexpressed in mantle cell lymphoma cells; and they reported that LNPs encapsulating a cocktail of siRNAs against cyclin D1, Bcl-2 and Mcl-1 significantly inhibited mantle cell lymphoma cell growth [140–142], indicating RNAi therapy to have great potential to enhance currently available treatment. Recently, Huang et al. reported a unique tumor immunotherapy approach [143]. They hypothesized that the production of anti-PD-1 antibody should accelerate tumor immunotherapy. However, an immunosuppressive tumor microenvironment (TME) limits immunotherapeutic efficacy [144]. Since TME stimulates the release of immunosuppressive cytokines from both tumor and stromal cells, Huang's group encapsulated both siRNA against PD-L1, an immune checkpoint, and pDNA encoding IL-2, an immunostimulating cytokine, into tumor-targeting lipid-dendrimer-calcium-phosphate nanoparticles to promote antitumor immunity and increase the efficacy of cancer vaccines. The LNPs enhanced tumor CD8+ T cell infiltration and activation, leading to the suppression of hepatocellular carcinoma (HCC) progression *in vivo*. This unique cocktail strategy has potential as a novel immunotherapy.

Despite the many efforts to improve siRNA delivery for cancer treatment [145], an anticancer siRNA drug has not been launched yet. The anticancer effects of siRNA-based therapeutics may be still insufficient possibly due to delivery problems. Although LNP-mediated delivery of siRNA to liver tumors is promising because LNPs coated with ApoE in the blood are expected to be internalized into liver cancer cells via the ApoE-LDLR pathway, delivery systems of siRNA to other cancer tissues are still under exploration. Identification and use of the internalization pathway selective for target cells will open the way to construct innovative LNP systems for cancer therapy. Because siRNA has great potential for cancer treatment, we strongly believe that the efforts in overcoming delivery problems contribute to the development of siRNA-based cancer therapy.

Table 1 lists clinical trials in which lipid-based nanoparticles were used as a tool for siRNA delivery to cancers. EphA2-targeting DOPC-encapsulated siRNA (NCT01591356) is an siRNA-encapsulated liposome composed of DOPC. This candidate is expected to have a high therapeutic effect due to its excellent pharmacokinetics based on the high biocompatibility of neutral lipids [146]. The detailed lipid composition of the lipid-based nanoparticle called DCR-MYC (NCT02314052) has not been clarified, but the greatest feature is that the siRNA molecule encapsulated in DCR-MYC is designed to be efficiently cleaved by Dicer. Knockdown of the oncogene MYC was expected to be applicable for the treatment of various types of cancer, but its development is currently on hold as the results of a trial fell short of expectations [147]. TKM-080301 (NCT02191878) is a so-called SNALP containing DLin-DMA as a constituent lipid, and though well tolerated, its antitumor effect was limited [148]. It should be noted that trials using other ionizable lipids may be carried out in the future.

4.2. Viral diseases

The rapid development of drug resistance and the harmful side effects of long-term use are serious concerns for antivirus therapies. NPs-mediated delivery of antiviral drugs is a promising approach to improve antivirus therapies. The small size and adjustable surface-charge properties of NPs aid their delivery to a variety of target cells [149–152].

4.2.1. Human immunodeficiency virus (HIV)

A variety of antiretroviral (ARV) drugs are available to treat HIV/acquired immune deficiency syndrome (AIDS) [153–158]. Combinations of three or more drugs, known as highly active ARV therapies (HAART), have significantly improved the expectations and quality of life of HIV-infected individuals; but these therapies are not without side effects. A variety of reviews have been published specifically focusing on the development of HIV/AIDS vaccines [159,160] and siRNA drugs for HIV treatment [149,161–163].

T cells and macrophages are early targets of HIV, which cells are very important for controlling infections and preventing their expansion. The delivery of anti-HIV siRNA to virus-infected immune cells has been investigated as an RNAi-based HIV/AIDS treatment. Kim et al. reported that lymphocyte function-associated antigen-1 (LFA-1) integrin-targeted immunoliposomes encapsulating anti-CCR5 (chemokine receptor 5) siRNA reduced the viral load of plasma in an HIV-infected humanized mouse model over a 1-day period [164]. Berger et al. developed Neutraplex lipid-based NPs to deliver chemokine receptor type 4 (CXCR4)-siRNA and showed suppression of HIV virus replication in human macrophage cells by CXCR4 knockdown [165]. On the other hand, a complex of NPs composed of chitosan-lipid and a plasmid DNA encoding an siRNA cocktail (siCCR5, siCXCR4, siTat, siGag, si5'LTR, and siRev) was prepared and administered to chimeric simian human immunodeficiency virus (SHIV SF162)-infected rhesus monkeys intravaginally, leading to a significant drop in viral titer [166].

4.2.2. Hepatitis B virus (HBV)

HBV causes liver inflammation and is the cause of subsequent chronic infections. Chronic HBV infection progresses to hepatic cirrhosis and then to liver cancer. Current anti-HBV therapy includes interferon (IFN)-α, PEGylated IFN, nucleoside analogue reverse transcriptase Inhibitors (lamivudine, adefovir, entecavir, terbivudine, and tenofovir) [167]. Anti-HBV treatment is generally considered to cause drug resistance and has the risk of side effects such as liver failure [168]. Despite the development of many compatible drugs, it is difficult to completely eliminate HBV antigens such as Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) and covalently closed circular (ccc) DNA.

Inhibition of synthesis of viral antigens with siRNAs is an ideal strategy for HBV treatment. Yamamoto et al. showed that the expression of antigenic proteins (HBsAg and HBeAg) was successfully suppressed by siRNA-loaded YSK13-MEND in mice with persistently infected hepatitis B virus by silencing the sequences highly conserved among HBV strains [38]. In addition, GalNAc-modified PEG-coated LNP-siRNA reduced HBV replication in hepatocytes compared with that achieved with unmodified LNP [169].

ARB-001467, which is an HBsAg-siRNA formulated in LNPs, has been shown to inhibit HBV protein production and to further reduce the cccDNA content in preclinical studies; and its phase 2 trial has been completed (Table 2). A clinical trial of DCR-HBVS, a synthetic RNAi drug that consists of a double-stranded oligonucleotide conjugated to GalNAc ligands, is undergoing (phase1 recruiting, NCT03772249).

4.2.3. Hepatitis C virus (HCV)

HCV causes both acute and chronic hepatitis, killing approximately 400,000 people each year. Previously, "interferon treatment" using PEGylated IFN and ribavirin was used; but recently, "interferon-free treatment" has become mainstream. "Interferon-free treatment" is a treatment with direct-acting antiviral drugs including redipasvir, which can cure most HCV-infected individuals and shorten the treatment period. [170].

In addition to virus-targeted therapy, siRNA-mediated knockdown of the viral replication mechanism in the host has been proposed.

Table 1
Clinical trials of siRNA-encapsulated lipid-based nanoparticles against cancer.

Drug Name	Target	Vehicle	Disease	Status	ClinicalTrials.gov Identifier	Company
EphA2-targeting DOPC-encapsulated siRNA	EphA2	Liposome	Advanced Malignant Solid Neoplasm	Recruiting, Phase I	NCT01591356	M.D. Anderson Cancer Center
DCR-MYC	Oncogene MYC	LNP	Hepatocellular Carcinoma	Terminated, Phase I, II	NCT02314052	Dicerna Pharmaceuticals, Inc.
TKM-080301	PLK1 (polo-like kinase-1)	LNP	Advanced Hepatocellular Carcinoma	Completed, Phase I, II	NCT02191878	Arbutus Biopharma Corporation

Lipidoid NPs containing siRNA targeting protein kinase C-related kinase 2 (PRK2), effectively inhibited HCV replication in a xenograft model and is attracting attention as a new HCV drug therapy [171]. Vitamin E-labeled cationic liposomes loaded with siRNA targeting HCV non-structural protein 5A was developed for hepatic delivery and shown to suppress both HCV core antigen production and HCV replication [172].

4.2.4. Other viruses

Ebola virus disease (EVD) is an often deadly and contagious disease in primates, causing Ebola hemorrhagic fever [173,174]. siRNAs using lipid-based nanosystems (TKM-130803) have progressed to clinical trials, but unfortunately no significant protection has been achieved in infected patients [175]. However, it has been reported that the administration of LNPs containing siRNA to multiple targets (EK-1 mod, VP24, and VP35) to rhesus monkeys infected with lethal Zaire ebola virus (ZEBOV) improved the survival rate after infection [176]. To develop broad-spectrum therapies against members of the Marburg virus (MARV) genus, investigators have examined the therapeutic effect of single nucleoprotein–targeting siRNA-loaded LNPs composed of 3-(dilinoleylmethoxy)-*N*,*N*-dimethylpropan-1-amine on nonhuman primates at advanced stages of MARV or Ravn virus (RAVV) disease; and siRNA-LNP treatment conferred a high survival rate in both disease models, suggesting the possibility of broad-spectrum therapy against both MARV and RAVV [177]. A study in which an siRNA targeting Sudan Ebola virus VP35 was delivered by LNP also showed a prolonged survival of similarly infected non-human primates [178].

Currently, coronavirus disease 2019 (COVID-19) is in a rage all over the world. New therapeutic drug and vaccine candidates for COVID-19 are undergoing clinical trials. Remdesivir was recently approved by the Food and Drug Administration (FDA) for emergency use in patients with COVID-19. The viral genome that causes COVID-19 was reported to be closest to the severe acute respiratory syndrome (SARS) coronavirus group and was officially named SARS-CoV-2 [179]. It was recently revealed that SARS-CoV-2 utilizes angiotensin converting enzyme II (ACE2) as a receptor for intracellular entry [179]. With the progress of SARS-CoV-2 genome analysis, the sequences that can be targets of siRNA therapeutics have been identified [180–182]. Lipid-based nanoparticles are expected as a potential carrier useful for the development of siRNA therapeutics and mRNA vaccine for COVID-19.

4.3. Inflammatory diseases

LNPs targeting immune cells such as antigen-presenting cells are expected to be applicable for siRNA treatment of inflammatory and autoimmune diseases. The usefulness of LNPs containing various ionizable lipids (DLin derivatives) has been investigated for siRNA delivery to immune cells. LNP-mediated gene silencing of a target protein (GAPDH) on bone marrow-derived macrophages and dendritic cells was successful both *in vitro* and *in vivo*, and this system holds promise for the delivery of therapeutic siRNAs to treat various immune disorders [183]. Since delivery by the LNP system can deliver siRNA targeting CD45 to immune cells, it can be expected to be applied to neuroinflammation and autoimmune diseases related to Alzheimer's disease. Nakamura et al. showed that MEND composed of YSK12-C4, a second-generation ionizable lipid, induces remarkable gene silencing of target protein (GAPDH) on various human immune cell lines (Jurkat, THP-1, KG-1, and NK92) and dendritic cells *in vivo* [184,185]. In addition, they reported that some of the toxicological challenges of YSK12-C4 were overcome, with significantly reduced cytotoxic effects on natural killer cells (NK-92) and hemolytic activity [186]. Controlling the function of T cells by RNAi is expected to be useful for the treatment of various diseases such as cancer and viral infections, and is therefore of great interest. Ramishetti et al. reported that LNPs modified with anti-CD4 monoclonal antibodies efficiently delivered siRNA to CD4 + T lymphocytes *in vitro* and increased the accumulation of these cells in the spleen, lymph nodes, and bone marrow *in vivo* [103]. Furthermore, in recent years the expression of constitutive molecules (CD40, CD80, CD86) has been successfully knocked down by using LNP-siRNA to which an antibody against mouse DEC205+ dendritic cells had been bound [187]. Vitamin A-coupled lipid nanoparticles (Table 2, ND-L02-s0201), which inhibit the expression of collagen-specific chaperone HSP47, was clinically tested for the treatment of liver fibrosis; but it is currently used for the treatment of idiopathic pulmonary fibrosis (NCT03538301, Recruiting, Phase II).

Table 2
Clinical trials of siRNA-encapsulated lipid-based nanoparticles against various diseases.

Drug Name	Target	Vehicle	Disease	Status	ClinicalTrials.gov Identifier	Company
ND-L02-s0201	HSP47	LNP	Hepatic fibrosis	Completed, Phase I	NCT02227459	Bristol-Myers Sqyubb Pharmaceuticals
ARB-001467	HBsAg	LNP	Hepatitis B, Chronic	Completed, Phase II	NCT02631096	Arbutus Biopharma Corporation
ALN-PCS02	PCSK9	LNP	Elevated LDL-cholesterol	Completed, Phase I	NCT01437059	Alnylam Pharmaceuticals
PRO-040201	ApoB	LNP	Hypercholesterolemia	Terminated, Phase I (Potential for immune stimulation to interfere with further dose escalation.)	NCT00927459	Arbutus Biopharma Corporation

4.3.1. Arteriosclerosis

Long-term gene silencing effects over 10 days can be obtained with systemic administration of siRNA-encapsulated LNPs [19], suggesting that this system may be applicable for treating atherosclerosis and the chronic inflammation found in cardiovascular disease. Lauschner et al. reported that lipidoid C12–200-LNPs containing siRNA targeting chemokine receptor CCR2 was localized in the spleen and bone marrow after systemic administration [49] and suppressed atherosclerotic plaque formation associated with mouse macrophages [188,189]. Sager et al. prepared LNPs composed of a lipid derivative of polyethyleneimine and an encapsulated siRNA cocktail against 5 adhesion molecules (Icam1, Icam2, Vcam1, Sele and Selp) [190]. Gene silencing of these adhesion molecules by the systemic administration of the LNPs significantly reduced vascular neutrophil and monocyte recruitment induced by myocardial infarction. Bifunctional core-shell NPs modified with hyaluronic acid and apolipoprotein A-I for targeting endothelial cell and macrophage, respectively, have been developed for the delivery of siRNA targeting Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1) and atorvastatin [191]. These dual-targeting NPs where shown histochemically to suppress plaque formation, neutral lipid accumulation, and MCP-1 expression in aortic root lesions, indicating their anti-atherosclerotic activity in endothelial cells and macrophages.

In 2003, Abifadel et al. identified a proprotein convertase subtilisin/kexin type 9 (PCSK9) gene in an analysis of an autosomal dominant inheritance-hyper-LDL-C family and showed that the cause of autosomal dominant hypercholesterolemia (ADH) was a gain-of-function due to a missense mutation in the PCSK9 gene [192]. Standard treatment of ADH is statin treatment, but a significant proportion of patients do not reach their treatment goals even at the highest tolerated doses. In addition, because long-term administration of statins shows severe side effects, a new therapeutic approach has been awaited.

Lipidoid-NP loaded with PCSK9-siRNA (siPCSK9) was shown to reduce the LDL concentration in the blood for 3 weeks after intravenous administration in rodents and nonhuman primates [193]. Thereafter, a trial for LNPs encapsulating siPCSK9 for intravenous injection was clinically conducted (Table 2, NCT01437059) [194]. On the other hand, trials for the delivery of siPCSK9 conjugated with GalNAc for subcutaneous administration were conducted: a Phase I trial in 2014 (NCT02314442) and a Phase II one in 2016 (NCT02597127) and 2017 (NCT03060577). In the phase I trials, the knockdown effect remained for over 6 months, with safety [195].

The most important risk factor for atherosclerosis is hyperlipidemia [197]. The development of atherosclerosis correlates with high levels of LDL. A high correlation between blood levels of apolipoprotein B (ApoB) and the risk of arteriosclerosis is known, but it is difficult to target ApoB with small molecule drugs. As such, siRNAs have been viewed as attractive treatment option, and an LNP-siRNA system consisting of DLin-DMA was previously reported to reduce plasma lipid levels [197]. A clinical trial of PRO-040201 using the SNALP system was started in 2010 but was terminated due to side effects on the immune system in high-dose patients (Table 2, NCT00927459) [198].

4.3.2. Rheumatoid arthritis

Silencing of inflammatory cytokine genes including TNF-α, NF-κB, and IL-1β is a promising approach for the treatment of rheumatoid arthritis. The delivery of TNF-α-siRNA by acid-sensitive sheddable PEGylated SLNs reduced collagen-induced inflammation in arthritis model mice that did not respond to methotrexate treatment [199]. It was also reported that NF-κB-siRNA and methotrexate encapsulated in folate-conjugated liposomes reduced the progression of arthritis and reduced inflammatory cytokines in a collagen-induced arthritis mouse model [200]. Lipidoid-polymer hybrid nanoparticles efficiently delivered siRNA targeting IL-1β to macrophages, resulting in effective suppression of pathologies such as paw swelling and bone destruction in experimental arthritis [201].

4.4. Neurological disorders

BBB is a major obstacle for drug delivery to the brain. Unlike peripheral tissues, capillaries in the brain form a tight junction and have no fenestrae [202]. In addition, P-glycoprotein (P-gp), a kind of ATP-dependent drug transport protein, at the luminal side of endothelial cells selectively inhibits the accumulation of drug molecules in the brain [203]. The development of a brain delivery system is thus required to overcome these hurdles. Recent studies focused on brain delivery have offered a novel strategy for the treatment of neurological disorders. In addition to reducing drug size, delivering drugs to the brain via carrier-mediated transport involved in uptake such as that of glucose can be a very attractive strategy [204,205].

In 2013, it was reported that ionizable cationic lipid-LNP containing siRNA spread widely in the brain and showed significant knockdown of target mRNA (GRIN1, coding GluN1 subunit of NMDA [*N*-methyl-D-aspartate] receptor) in the brain after intracortical or intracerebroventricular injection without an immune response [206]. Conceicao, et al. treated polyglutamine neurological disease (Machado-Joseph disease; MID) via the systemic administration of LNPs [207]. They prepared DODAP-LNP incorporating a short peptide derived from RVG-9r and siRNA targeting mutant ataxin-3. Intravenous injection of the LNPs into MID model mice improved motor behavior deficits. These findings show the potential of LNPs as a noninvasive neuropathy therapy.

Drugs that target cholinergic and glutamatergic neurotransmission have been developed for the treatment of cognitive impairment seen in Alzheimer's disease [208]. For instance, acetylcholinesterase inhibitors improve the symptoms of patients, but further drug development is required to address the unmet needs of Alzheimer's disease. Chitosan-coated SLN modified with a CPP derived from RVG was shown to deliver siRNA into monolayer Caco-2 cells that mimics olfactory epithelium [209]. The results suggested that this SLN-siRNA may be a potential therapeutic candidate for Alzheimer's disease via the intranasal (nose-to-brain) delivery route. In another study to avoid the toxicity of cationic NPs, siRNA-anionic NP complexes were prepared by utilizing anionic PEG-liposomes and cationic targeting peptide [210]. Then, via intra brain injection of the complexes, the knockdown effect of siRNA against BACE1 was examined, with the result being a reduction in the number of amyloid plaques in the brain. Delivering siRNA with LNPs might be a useful approach for intractable neurodegenerative diseases by optimizing the formulation and injection route.

5. Conclusions

After the discovery of the RNAi phenomenon, numerous researchers thought that many intractable diseases might be cured by using synthetic siRNA; because siRNA is expected to be high specificity and low side effects. Although it is well known that the potential of siRNA is very promising for disease therapy, unfavorable siRNA properties such as easy degradation, low cellular uptake, and low endosomal escapability stand in the way of drug development. Up to now, a number of siRNA delivery carriers have been developed for the application of siRNA to intractable diseases. Finally, the first siRNA therapeutic was approved in 2018, i.e., Patisiran (ONPATTRO™). However, ONPATTRO™ is just the opening player of many future RNAi therapeutics. As highlighted in this review, many lipid-based nanoparticles have been developed for the improvement of siRNA properties, bioavailability, and pharmacokinetics. In addition, several siRNA-encapsulated LNPs show a therapeutic effect against cancer, inflammation, neurological disorder, and genetic disease. The challenges faced regarding the above-mentioned unfavorable properties of siRNAs will be surmounted by the designing of high-performance LNPs. On the other hand, it is true that current strategies still lack *in vivo* efficiency and targeting ability (delivery to organs other than the liver). For future RNAi therapeutics, the discovery and development of LNP with innovative targeting functions that deliver therapeutic siRNA specifically into target cells are

awaited. We strongly hope that attractive RNAi therapeutics for the treatment of intractable diseases will be developed shortly.

Contributions

SY, HK, and TA conceived and co-wrote the manuscript. The manuscript was approved by all authors.

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