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Review

Lipid nanoparticle-based delivery of small interfering RNAs: New possibilities in the treatment of diverse diseases

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ABSTRACT

RNA interference (RNAi) is a well-known post-transcriptional gene-silencing mechanism that has garnered significant attention as a potentially powerful therapeutic procedure for combating recalcitrant diseases. Small interfering RNA (siRNA) as an effective RNAi tool mediates gene silencing pathway by mRNA degradation in cells and presents a unique strategy for the treatment of rebellious diseases. However, the low stability and suboptimal pharmacokinetic behavior of naked siRNAs have made it necessary to employ a delivery vehicle to protect siRNA against degradation and allow for its intracellular delivery. Among a plethora of available delivery platforms, lipid nanoparticles (LNPs) have received significant research attention and are currently recognized as the most advanced delivery system for RNA-based therapeutic agents. This is exemplified by the approval of Onpattro® for treating amyloidosis in the US and the European Union in 2018, as well as the development of COVID-19 mRNA vaccines. This review aims to provide a comprehensive evaluation of the potential effectiveness of lipid-based nanoparticles as a delivery system for siRNA in treating a wide array of diseases.

1. Introduction

RNA-based gene therapy exploits therapeutic RNA to silence disease-related genes or produce specific proteins, paving the way toward precise and personalized treatment of various diseases including cancers, infectious, neurological, and immune diseases [1]. Small interfering RNAs (siRNAs) are short double-stranded RNA molecules that can be produced by cleavage of long double-strand RNA (dsRNA) by endoribonuclease Dicer in the cytoplasm [2]. As gene silencing via siRNA relies on the RNA-induced silencing complex (RISC) system, an inherent enzyme system, it possesses the capability to target virtually all genes. This implies that RNA interference (RNAi) mediated by siRNA or other relevant RNA molecules can effectively inhibit the expression of splicing variants and mutants, indicating its potential to target proteins that are currently considered undruggable (Fig. 1) [3–8].

siRNAs hold several advantages including exerting their function at the post-transitional stage of gene expression without DNA interference, higher gene silencing efficacy compared to other antisense strategies, and flexibility and adaptability in target selection due to their capability to target a wide range of mRNA sequence [8]. Although siRNAs offer therapeutic potential, there are strong physiological and intracellular barriers to their effective delivery and taken up into target sites, making its *in vivo* application unfounded and clinically dubious at best. Naked and unmodified siRNAs are negatively charged and hydrophilic macromolecules that are hindered from entering cells due to the electrostatic repulsion by the cellular membrane, leading to limited therapeutic efficacy [9].

The precision of RNAi appears to fall short of its initial expectations. It's widely acknowledged that many siRNAs have off-target effects, suggesting that most siRNA molecules are not as precise as previously

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believed. Introducing siRNA can lead to off-target effects such as suppressing genes other than the intended target, which can cause harmful alterations in gene expression and unforeseen outcomes. The off-target effect may also stem from the immune response. RNA can be detected by immune receptors like Toll-like receptors (TLRs), triggering cytokine release and alterations in gene expression. While the complete sequence dependence of this immune response remains unclear, certain immunostimulatory patterns have been recognized, highlighting the importance of avoiding them [10].

Furthermore, upon systemic delivery, rapid uptake by the reticuloendothelial system (RES), renal clearance, and nuclease degradation shorten the half-life of siRNA as short as 5 min and raise serious concerns about poor stability, bioavailability, and undesirable pharmacokinetic behavior of siRNA [11,12]. Therefore, it is necessary to establish a safe and suitable delivering system to protect siRNA in the harsh *in vivo* environment, ensure successful cellular uptake of siRNA, induce endosomal escape and reduce off-target side effects.

The introduction of nanotechnology has led to the development of various nanocarrier systems, revolutionizing the field of drug delivery [13,14,18–23]. There have been numerous examples of using nanomaterials for therapeutic purposes [21–28]. Lipid-based nanoparticles, particularly a promising class of nanocarriers, are considered to be nontoxic, biocompatible, easy to produce, and primarily prepared using physiological lipids [26]. These novel systems have proven to be effective in improving the delivery of drugs, proteins, genes, oligonucleotides and overcoming the challenges faced by traditional delivery methods [27].

2. Lipid-based nanoparticles

Lipid-based nanoparticles (LNPs) hold immense potential to develop safe carrier systems aiming at intracellular delivery of siRNA [28,29]. Since lipids constitute the primary component of cellular membranes, the incorporation of siRNA into lipid vesicles could be advantageous for facilitating passage through the cellular membrane and subsequent release of siRNA into the cytosol [30]. The self-assembly characteristic of lipids around the nucleic acid payload, along with cylindrical, conical, and inverted conical conformation of lipids in solution, has led to the selection of various structurally adequate lipids, either individually or in combination, for designing lipid-based gene delivery systems [31].

Up to now, liposomes, an early version of LNPs, and the next generation of LNPs including nanostructured lipid carriers (NLC), solid lipid nanoparticles (SLN), lipid-calcium-phosphate (LCP), lipid-polymer hybrid nanoparticles (LPH), cationic liposomal-protamine sulfate-DNA (LPD), stable nucleic acid lipid particles (SNALP), multifunctional envelope-type nanodevice (MEND) system and lipidoid also have been under investigation for the in vivo delivery of siRNA for management of several diseases.

2.1. Components of lipid-based delivery systems

It is generally accepted that LNPs have four main components: cationic lipid, cholesterol, a helper lipid, and polyethylene glycol (PEG)-lipid. However, the portion of these components, along with the ratio of the cationic lipid to RNA, needs to be considered to achieve promising efficacy both in vitro and in vivo. [32]. Cationic lipids (CLs) and ionizable lipids (ILs) are two principal lipids classes employed to form stable complexes with negatively charged siRNA via electrostatic interaction. The positive charge of cationic lipids made it possible for LNPs to exert fusogenic functionality, mediating high transfection and gene silencing [33]. The initial study by Felgner in 1987 for the first time confirmed the potential of DOTMA (N-[1-(2,3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride), a quaternary ammonium salt and unsaturated phosphatidylethanolamine (DOPE) for successful transfection of DNA in mammalian cells [34]. In the following, alterations in the linker part, the quaternary ammonium group in the headgroup region or the lipid chain of DOTMA seem to reduce toxicity and expand the range of related lipids. These lipid included DOTAP (1,2-dioleoyloxy-3-(trimethylammoniopropane chloride), DMRIE (1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide), GAP-DLRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide), DODAB (dimethyldioctadecylammonium bromide), DOSPA (2,3-dioleyloxy-N-[2-(sperminecarboxamido) ethyl]-N,N-dimethyl-1-propanaminium chloride) [34]. The structure of these lipids are shown in Fig. 2.

Compared to DOTMA, replacing degradable ester bonds as a linker in the structure of DOTAP reduced toxicity and increased transfection

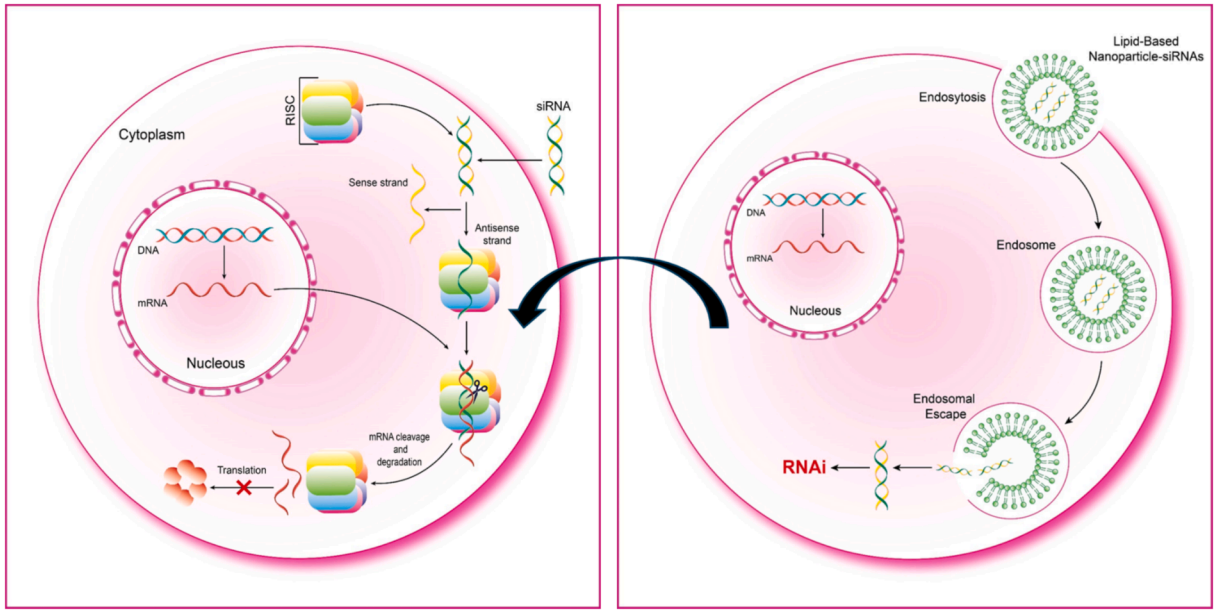

Fig. 1. Schematic illustration of utility of lipid-based nanoparticles as a delivery vehicle for siRNAs and siRNA-mediated gene silencing mechanism. After endosomal escape of siRNA, it enters the cytoplasm of cell where siRNAs are incorporate into a multiprotein RNA-induced silencing complex (RISC) assembly, where the sense (passenger) strand leaves the antisense (guide) siRNA strand. Then the antisense siRNA strand binds to the target mRNA, ultimately resulting in cleavage and degradation.

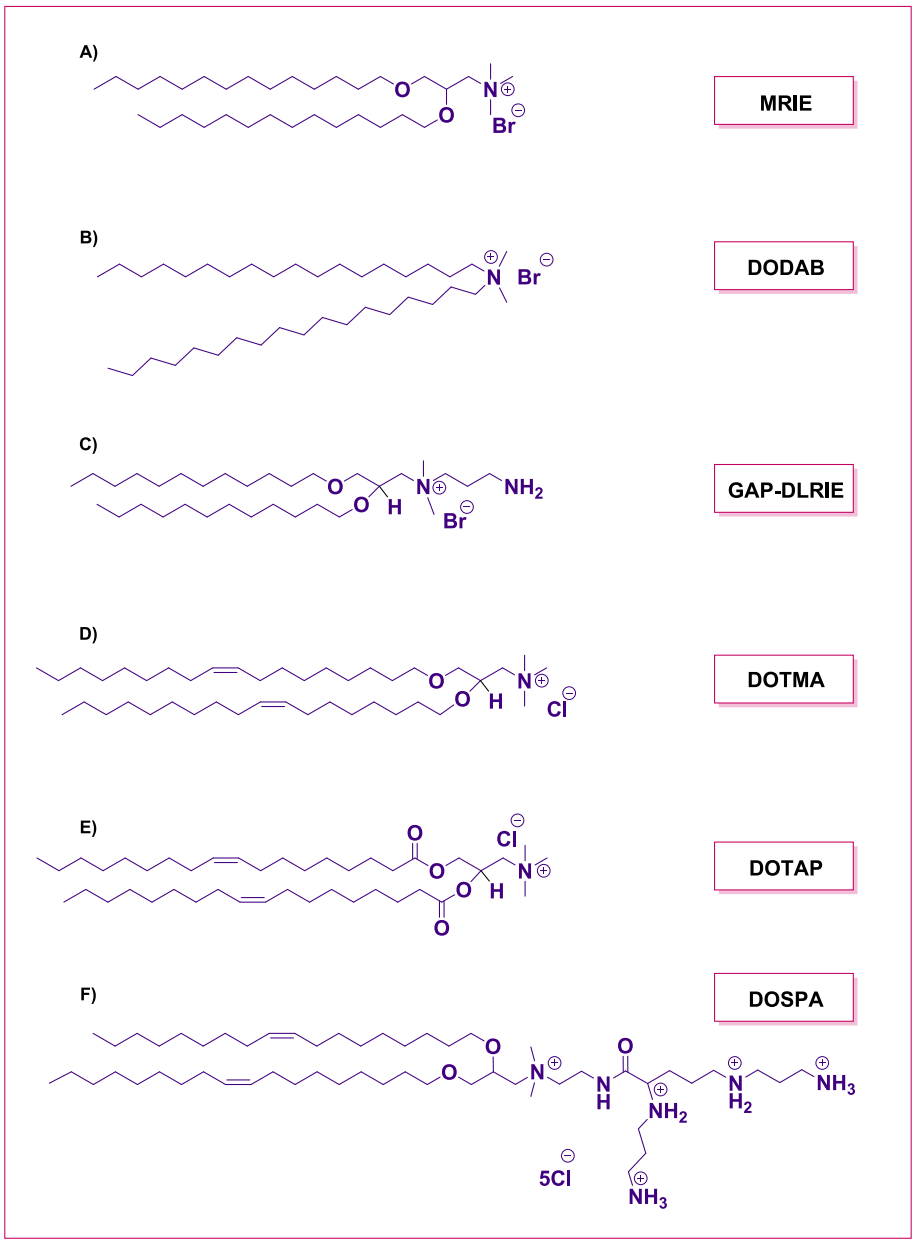

Fig. 2. Chemical formulation of different commercial cationic lipids. A) MRIE, B) DODAB, C) GAP-DLRIE, D) DOTMA, E) DOTAP and, F) DOSPA.

efficacy in a formulation with DOPE (dioleoylphosphatidylethanolamine) at a 1:1 ratio [35].

Commonly used CLs for siRNA delivery come with several shortcomings associated with toxicity issues, limiting electrostatic interaction with siRNA, and thus lack in vivo efficacy [30].

Commonly used CLs for siRNA delivery are a double-edged sword. They have several shortcomings associated with toxicity issues, limiting their in vivo application. A permanent positive charge of these lipids disrupts the integrity of the cellular membrane, releases lysosomal degradative enzymes, changes the biosynthesis and metabolism pattern of steroids, lipids, and cholesterol, causes oxidative stress, DNA damage, immunogenicity, mitochondrial dysfunction, and fast plasma elimination due to opsonization [36–45]. However, lipoplexes comprising CLs are still widely employed to transfer nucleic acids [40]. To tackle these issues, permanently cationic lipids are replaced with pH-sensitive ionizable lipids (ILs) that selectively become protonated at the lower pH of the endosome. These lipids are neutral at physiologic pH and become protonated at the lower pH of endosomes, thus facilitating endosomal escape, cytosolic delivery of their cargo, and reducing hazardous effects on cells [41]. Fig. 3 illustrates the structure of various ILs. 1, 2-dilinoleyloxy- N, N- dimethyl-3- amino propane (DLin- DMA) is the first ionizable lipid that was applied for siRNA delivery in 2010. Afterward, further investigation was accomplished by modifying the amine head group to generate the heptatriaconta-6,9,28,31- tetraen-19- yl 4-(dimethylamino) butanoate (DLin- MC3- DMA; MC3) [42].

These lipids usually make up 30–50 % of the total lipids in a formulation [30]. The structure of CLs and Ils has three main parts: 1) the headgroup, (2) the linker, and (3) the tails (Fig. 4).

The hydrophilic headgroup of a lipid plays a critical role in determining the performance of lipid-based delivery systems. Headgroup impacts the encapsulation percent, the toxicity issue, and the transfection efficiency. The positively charged moiety in the head group facilitates binding to negatively charged nucleic acids and enhances electrostatic interactions with the cell membrane to release encapsulated payloads. Multivalent polar headgroups provide a greater surface charge density, which exhibited a significant improvement in nucleic

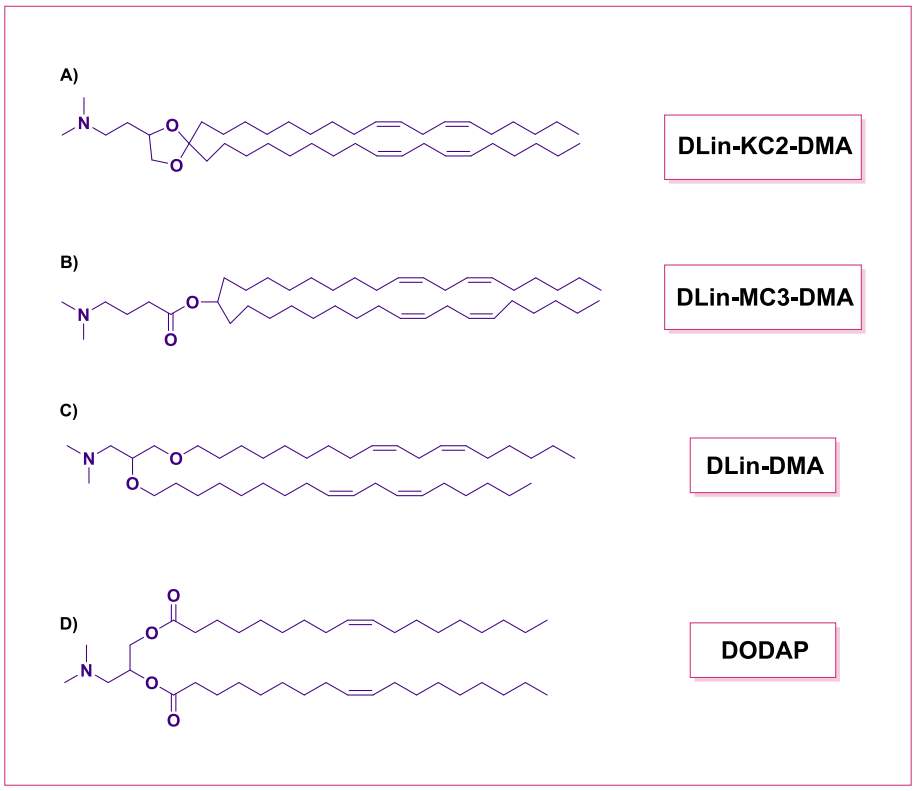

Fig. 3. Chemical formulation of ionizable lipids utilized for preparation of lipid-based nanoparticles. A) DLin-KC2-DMA, B) DLin-MC3-DMA and, C) DLin-DMA, D) DODAP.

acid delivery compared to monovalent headgroups [43]. Although Permanently charged headgroups have brought the merits of higher encapsulation rate and transfection efficacy rather than ionizable headgroups, they come with enhanced cytotoxicity [44].

Ionizable lipids offer the main advantage that they have ionizable head group, such as simple tertiary amines, branched polyamines, or cyclic structures including a piperazine, diketopiperazine, or benzene core, etc., which can be protonated in an acid environment [45]. It should be noted that the small head group is critical for forming inverted and non-bilayer structures, which destabilize the endosomal membrane and facilitate siRNA release into the cytoplasm of target cells [29].

Studies have suggested that the ionizable lipids' structure and dissociation constant (pKa) determine the efficient delivery of the burden to the target site [46]. The pKa value of ionizable lipids is a critical parameter for assessing transfection efficiency and the optimal pKa of the nanoparticles was found to be between 6.2 and 6.5 [47].

Structure modification of the hydrophilic head group region modulates the pKa of the ionizable amino lipids. For instance, Dlin-MC3-DMA, the ionizable lipid in Onpattro®, with a pKa of 6.44, demonstrated a 10-fold higher potency than (2,2-dilinoleyl-4-(2- dimethylaminoethyl)-[1]-dioxolane, DLin-KC2-DMA), which has a pKa of 6.7 [48,49]. The presence of hydroxyl groups in the head groups of ALC-0315 and SM-102 minimizes the hydration of the head region and promotes hydrogen-bounding interactions with the nucleic acid, improving transfection efficacy [30].

The nature and length of the linker directly affect the stability and biodegradability of lipid carriers. An ideal linker group should be biodegradable and possess good circulation stability to perform well in a biological environment. Ethers and esters, carbamate,amide urethanes, oxime ethers, phosphate or phosphonate linkers, glycerol-type moieties, peptides, acid-labile ketal bonds and redox-sensitive disulfide bonds[44] are frequently used linker groups. Biodegradable linkers are highly advantageous due to their rapid clearance in vivo, which allows for multiple dosing schedules and significantly reduces the probability of side effects [50,51]. Linker groups can be modified, ensuring stable enough for body circulation and storage, while they break down quickly at the target locations to let the RNA payload be released [44].

The hydrophobic tails of lipids can affect their pKa, lipophilicity, transition temperature, cytotoxicity, stability, and effectiveness in RNA delivery [52,53]. A cholesterol derivative, a hydrocarbon chain, or tocopherol derivative can serve as a hydrophobic tail of lipids. The hydrocarbon tails generally range from 8 to 18 carbon atoms in length and can exhibit varying degrees of unsaturation, such as in oleoyl and linoleoyl groups, without requiring symmetry [54]. Using unsaturated fatty acids as lipid tails has improved delivery efficiency in certain formulations, likely due to their low transition temperature and ability to enhance membrane fluidity [55]. Increasing the number of cis double bonds in the tail from 0 to 2 contributes to a higher tendency for bilayer lipids to form a nonbilayer phase. This change leads to increased membrane disruption and enhanced payload release [56]. Analysis of C18:0 (stearic) and C18:1 (oleyl) analogs indicates that oleyl (C18:1) lipids are more effective for delivering plasmid DNA [57]. Additionally, the lipid tails of most ionizable lipids are unsaturated, which may aid in the mRNA release [45]. A Research showed that a linoleic acid-derived ionizable lipid (OF-02) had the highest efficiency in hepatic mRNA delivery and protein expression compared to its analogs [58]. Multi-tail ionizable lipids possessing three or more tails can form a more cone-shaped structure, enhancing their ability to disrupt endosomes due to the increased cross-section of the tail region and releasing the loaded nucleic acid into the cytosol [56]. Tail branching is another factor that, parallel to long and saturation, can remarkably impact ionizable lipid function. Isodecyl acrylate (Oi10), which has a 1C branch at the end, significantly increased hepatic mRNA expression by up to 10-fold compared to its isomers with linear tails. This result can be related to improved endosomal escape due to the enhanced protonation of spaced ionizable lipids at endosomal pH. Moreover, the larger cross-section of the lipid tails facilitates the formation of a more cone-shaped structure [50,59].

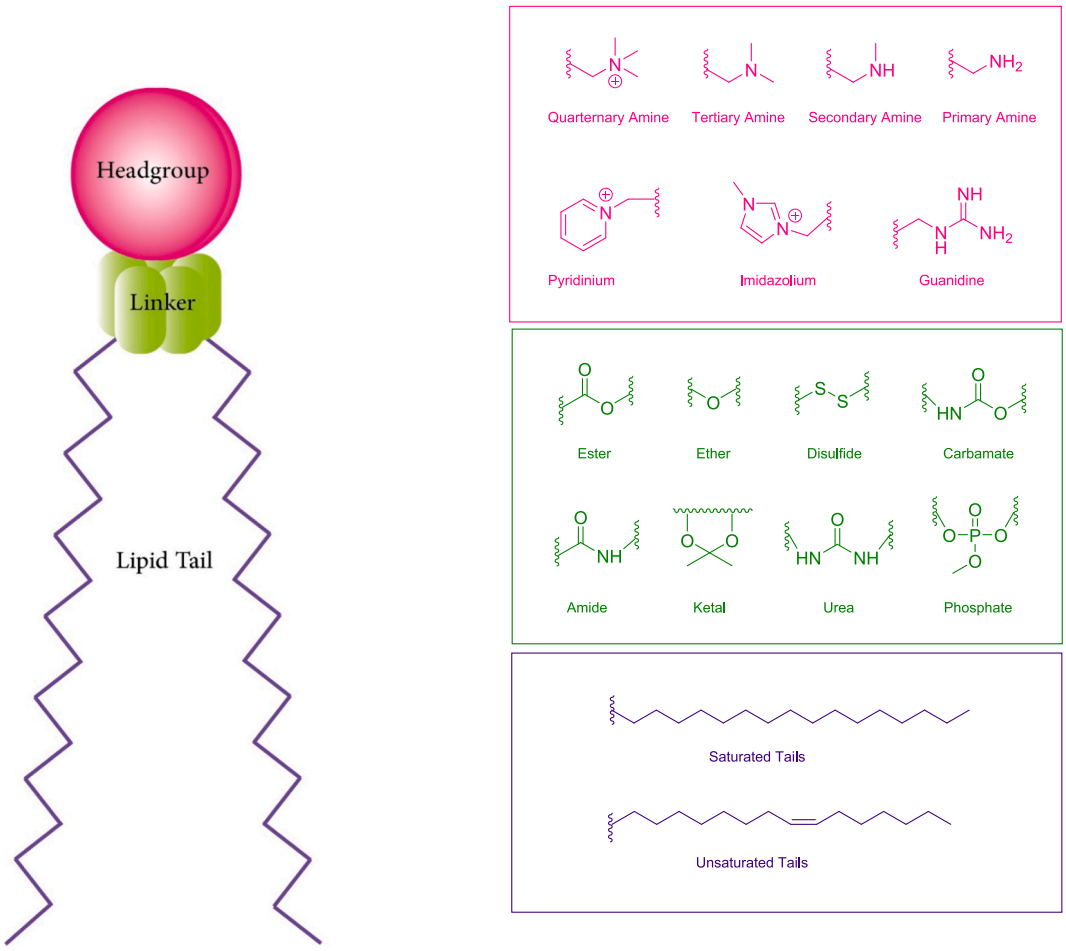

Fig. 4. Schematic illustration of cationic lipids and ionizable lipids and their different components (headgroup, linker, and tails).

Cholesterol is a commonly used component of lipid nanoparticles (LNPs), comprising approximately 20–50 % of the total lipids in an LNP formulation [30]. Cholesterol affects the packing of phospholipid molecules, increases aggregation resistance, and decreases the lipid bilayer's permeability to electrolytes and non-electrolyte solutes [60]. The presence of cholesterol imparts more stability to LNPs in the presence of serum proteins and mediates membrane fusion [61]. The binding of cholesterol to Apo E and LDL receptors promoted the uptake of LNPs, specifically to the liver [62].

Cholesterol analogs, such as β-sitosterol and C-24 alkyl derivatives, modulate nanoparticle packing for improved intracellular delivery and increased transfection efficacy [63]. It was reported that increasing the cholesterol concentration increased cationic lipid activity, consequently promoting endosomal membrane fusion [64].

Helper lipids, including 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), are essential components of LNP formulations. They facilitate fusion with cell and endosomal membranes, which enhances cellular uptake and endosomal release [64]. DOPE is commonly used as a helper lipid to adjust the positive surface charges of LNPs, enhancing their transfection efficiency and reducing cytotoxicity [65].

The unique structure of DOPE, with its unsaturated chains and smaller amine group, forms a cone shape that significantly enhances the delivery of nucleic acid into cells, facilitating their escape from endosomes and improving overall effectiveness [66]. Phospholipids comprise about 10–20 % of total lipids in a formulation [30].

Incorporating PEG-lipids into LNP formulations is a proven strategy to optimize the pharmacokinetics of therapeutic LNPs. This modification effectively prevents interactions with serum proteins, reduces opsonization, and limits recognition by cells of the reticuloendothelial system (RES). As a result, this leads to significantly longer circulation times, enhancing the therapeutic efficacy of these innovative treatments [64]. It should be noted that the overuse of PEG molecules stimulates the production of anti-PEG antibodies, which specifically recognize and bind to PEG. The presence of these anti-PEG antibodies has been linked to reduced therapeutic efficacy and an increase in adverse effects [67].

2.2. *The advancement of LNP for gene delivery*

2.2.1. *Liposome*

Liposomes, the earliest version of LNPs, represent an excellent choice for gene delivery due to their safety, biocompatibility, biodegradability, and versatile structure [68]. In the beginning, liposomes consisting of neutral or zwitterionic lipids were tested for transporting DNA and siRNA. Despite their benefits, such as low macrophage uptake and extended circulation time in the blood stream, their deficient nucleic acid encapsulation efficiencies impeded the continued advancement of such liposomes [42]. Cationic lipids were later developed for more efficient DNA encapsulation in liposomes, resulting in improved transfection efficiency. Cationic liposomes incorporating cationic lipids in their structure have shown to be effective carriers. They can condense with negatively charged nucleic acids such as plasmid DNA, miRNA, and siRNA, thereby increasing gene transfection efficacy [69,70]. Type and molar ratio of cationic lipid, the use of helper lipid, the ratio of cationic lipid to siRNA, preparation method, size and surface charge are key factor affecting formulation of cationic liposomes [71]. Lipoplexes, cationic liposome-siRNA complexes, have been investigated for over a decade as a vector for siRNA delivery to specific organs through

systematic administration [8,78–81]. Also, it was reported that polycationic liposomes composed of cetylpolyethylenimine, dicetyl phosphate (DCP)-spermidine and DCP-spermine [76], dicetyl phosphate-tetraethylenepentamine conjugate (DCP-TEPA) [77], and dioleylphosphate–diethylenetriamine conjugate (DOP-DETA)[78] were found to be effective for siRNA delivery. Lipoplexes impose some limitations, including the formation of the protein corona, rapid clearance from blood, toxicity at high doses, and insufficient transfection capacity relative to viral vectors [68]. A significant advancement in developing pharmacologically acceptable lipid formulations was the introduction of ionizable cationic lipids. Using pH-sensitive lipids is an innovative strategy to overcome these limitations and efficiently deliver siRNA to target regions [79,80]. Pre-condensing the nucleic acids with polycations, such as poly-L-lysine and protamine, to form LPD NPs offers valuable potential for gene-silencing applications [81].

Additionally, other shortcomings of liposomes include poor stability, complex production methods that use organic solvents, and difficulties in scaling up production. This highlights the need to develop solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) [82].

2.2.2. Solid lipid nanoparticles and nanostructured lipid carriers

Solid lipid nanoparticles (SLNs) are lipid structures that feature a solid lipid core surrounded by an amphiphilic exterior shell. siRNA can be integrated into the core of cationic SLNs using the hydrophobic ion-pairing (HIP) technique [83,84]. This method relies on the formation of ionic complexes between cationic lipids and siRNA, resulting in the incorporation of the hydrophobic siRNA/lipid complex into the electrically neutral hydrophobic core of an SLN. The lipid core is immersed in a solution containing 67 % methanol along with PEGylated lipid and phosphatidylcholine, following which the organic solvent was evaporated to yield SLNs encapsulating siRNA. SLNs produced through this approach have demonstrated the ability to sustain the release of siRNA for over 10 days in mice model [85]. The composition of the lipids used in SLNs significantly impacts the physicochemical properties such as morphology, size, surface chemistry, and aggregation of the particles. For instance, triglyceride-based SLNs tend to form a platelet shape, while monostearates are known to produce spherical particles [31]. Also, the lipid composition in SLNs affects electrostatic interactions with nucleic acids, which in turn influence transfection efficacy and the interaction between nanoparticles and biological systems regarding activity and, toxicity [86]. Sustained release of siRNA is preferred to reduce the need for frequent injections and to minimize toxicity associated with high intracellular siRNA levels [85]. An intradermal injection of Tristearin solid lipid nanoparticles containing siRNA (4.4–5.5 wt % loading ratio) in mouse footpads sustainably released siRNA for 10–13 days [85]. Despite the assessment of SLN in the research, their application is limited due to the evident cytotoxicity of the cationic lipids [87]. NLC is composed of a combination of solid and liquid lipids, which minimizes the lipid matrix during NP preparation. This suppression prevents the explosion of payloads from the matrix, thus improving the drug-loading capacities of the carrier [13]. One significant challenge associated with NLCs is the necessity for an ideal ratio of solid and liquid lipids, which is crucial for creating stable NPs [88].

SLN and NLC demonstrate improved physical stability, overcoming a significant limitation of liposome-based formulations. They also present higher loading capacities and better cargo bioavailability. Additionally, they can be produced rapidly on a large scale without any need for organic solvents, and they are more stable during sterilization compared to other lipid nanoparticles (LNPs) [89].

2.2.3. Lipid-calcium-phosphate (LCP) NPs

LCP nanoparticles offer a unique class of LNPs, possessing the potential to be a promising and effective siRNA delivery system. LCP NP was prepared by replacing the protamine/DNA core in the LPD formulation with a pH-sensitive calcium phosphate (CaP) core [90]. The Ca/P molar ratio has been taught as a key factor in determining the optimized features of particle size, zeta potential, siRNA loading and protection in synthesized LCP NPs. The interaction between the phosphate group in nucleic acids and calcium ions facilitates efficient and complete encapsulation of nucleic acids [91]. CaP is an acid-sensitive material that quickly dissociates in an acidic endosomal or lysosomal condition, releasing its payload into the cytoplasm [90,92]. The LCP nanoparticle delivery system for siRNA operates on the principle that this vesicle can quickly respond to the acidic pH found in endosomes by rapidly dissolving in the low pH environment created by calcium phosphate (CaP) [90,93]. LCP nanoparticle offer advantages in siRNA delivery, including small size (around 50 nm), good stability, high endosomal disruption and siRNA release efficiency, as well as effective targeting capabilities [67]. The coating of CaP with lipid or polymer addresses the problems associated with the uncontrollable rapid growth of CaP precipitation, leading to colloidal stability and achieving nanosize formulation [91]. Also, the outer lipid layer allows further modification and functionalization. Khatri et al. developed a calcium phosphate-based neutral liposome for siRNA delivery by ethanol. In this formulation, calcium phosphate precipitation was encapsulated within a liposome composed of a neutral lipid (DPPC), a fusogenic lipid (DOPE), a PEGylated lipid (DSPE-mPEG2000), and cholesterol [94]. Adding ethanol increases the permeability of siRNA to liposomal bilayer and complexation with CaP inside the liposome, improving siRNA up to 80 %.

2.2.4. Stable nucleic acid lipid particles (SNALPs)

The SNALP bilayer consists of a combination of cationic and fusogenic lipids, which facilitate the cellular uptake and endosomal release of the particle's contents. Additionally, SNALP contains a diffusible poly (ethylene glycol)-lipid conjugate (PEG-lipid) that provides a neutral, hydrophilic coating to the exterior of the particle [95].

The development of the stable nucleic acid lipid particles (SNALPs) in 2001 revolutionized the field of LNP production. Before producing SNALPs through spontaneous vesicle formation via ethanol dilution, nucleic acid was passively encapsulated into lipid formulations by hydrating a thin film of lipid with a buffer that contains the nucleic acid [96]. Ionic strength and pH of the aqueous medium, as well as the lipidic composition, are critical parameters in the ethanol dilution procedure [97]. This technique is utilized for producing Onpattro and has been applied in several other clinical-stage LNP formulations [98]. SNALPs can encapsulate nucleic acids and efficiently transfect cells both in vitro and in vivo. Compared to commercial transfection reagents like Lipofectamine, they exhibit low or no toxicity, which is dose-dependent [99]. SNALP were specifically developed for encapsulating siRNA into liposomes with the following lipid composition: 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol, 3-N-[(ω-methoxypoly (ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA), and 1,2-dilinoleyloxy-3-(N,N-dimethyl)aminopropane (DLinDMA) in a ratio of 20:48:2:30 mol% [100]. It was shown that the additional double bond in the lipid tails of DlinDMA, a linoleic analogue of DODMA, could increase the tendency to form a non-bilayer phase, thereby enhancing the transfection efficiency of the LNP [101]. An examination of four lipids with the same alkyl chain length (C18), a protonatable tertiary amine head group, and ether linkages—specifically, DSDMA, DODMA, DLinDMA, and DLenDMA—in the SNALP formulation revealed that none exhibited any indication of adopting the fusogenic HII phase, even at relatively high temperatures. However, lipids containing one double bond per alkyl chain (DODMA) within the SNALP structure led to a significantly lower phase transition temperature for the HII phase transition. The presence of two double bonds (DLinDMA) further decreased the phase transition temperature, while adding a third double bond (DLenDMA) had little additional effect [95].

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

A multifunctional envelope-type nano device (MEND) system based on programmed Packaging has been proposed. This system integrates multiple devices into a nano gene delivery platform, allowing each

device to perform its specific function at the appropriate time and location according to a predetermined program [102]. The MEND system is composed of a nucleic acid core that is condensed or complexed with a polycation, and a lipid envelope structure that functionalized with various moieties such as PEG, specific target ligands, and cell-penetrating peptides (CPPs) [103]. It is beneficial to compact the nucleic acids into a dense core before including them in the lipid envelope. The complex core reduces the size of nucleic acids, enhancing packaging efficiency. The resulting core/shell structure safeguards the nucleic acids from enzyme degradation. MEND can enter cells through macropinocytosis and mediated transfection as effectively as an adenovirus [103] MEND is developed to manage the intracellular transporting of nanocarriers and control tissue distribution [104]. It was demonstrated that MEND modified with octaarginine (R8) significantly improved cellular uptake through macropinocytosis and ensured the effective release of nucleic acid into the cytoplasm [105].

YSK05 is an ionizable lipid which is employed to modify conventional MENDs. YSK05-MEND exhibited greater gene silencing activity than other MENDs containing conventional cationic lipids, DOTAP and DODAP, which are a pH-insensitive and a pH-sensitive cationic lipid, respectively [106]. This may be attributed to YSK05′s apparent pKa of approximately 6.6. It can rapidly switch to a cationic form in response to the acidification of the endosome, evading lysosomal degradation. In contrast, the DODAP-MEND, with an apparent pKa of less than 6.0, cannot achieve this, at least not as rapidly. Also, YSK05-MEND exhibits significantly higher hemolytic activity compared to DODAP-MEND, suggesting that YSK05-MEND can escape from endosomes more effectively through membrane fusion and consequently release siRNA to the cytoplasm [106]. The YSK13 is second-generation with two long unsaturated carbon tails, enhancing membrane disruption activity. YSK13-MENDs showed enhanced gene silencing efficacy. It was found that the ED50 (0.015 mg/kg) of blood-clotting factor VII (FVII) knockdown in mice, which were intravenously injected with YSK13-MEND possessing a pKa of 6.45, was more than four times lower than that of YSK05-MEND [107].

2.2.6. *Lipidoid-based NPs*

Lipidoid-based NPs are a newer class of LNPs for delivering siRNA appearing to be preferred over traditional LNPs due to enhanced stability and minimized toxicity [108]. The structure of some lipidoids are shown in Fig. 5.

The new synthetic lipid like materials called lipidoids improved delivery systems' efficacy. Lipidoids are created by adding amines to acrylates, acrylamides, or epoxides. Unlike frequently used cationic lipids, lipidoids contain multiple secondary and tertiary amines, which makes them more effective at interacting with siRNA without significantly increasing the delivery system's net charge [109]. The benefits of lipidoid nanoparticles are their malleable structures, which can be

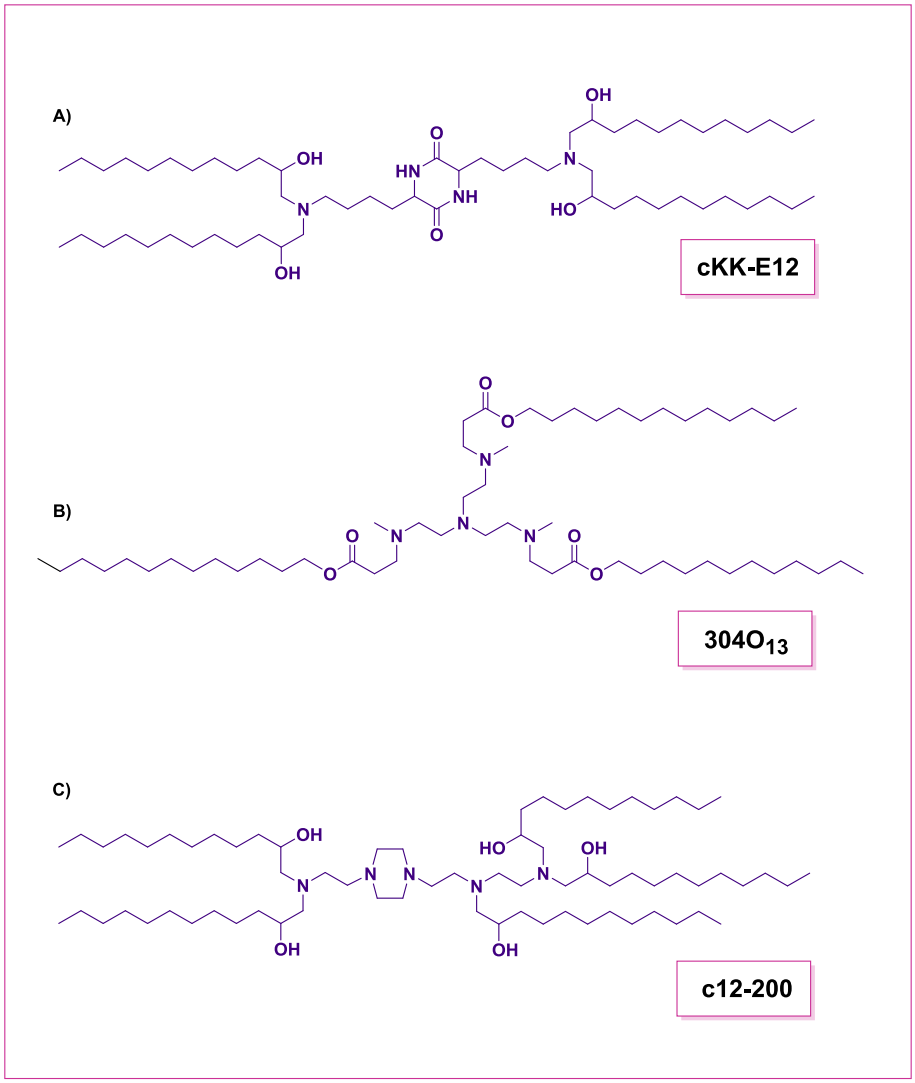

Fig. 5. Chemical formulation of lipidoids and lipid-like substances. A) cKKE12, B) 304O13 and, C) C12–200.

modified to optimize in vivo kinetics and enhance efficacy and safety, while also minimizing side effects [81].

Anderson and colleagues have set a combinatorial strategy aiming to prepare a library of lipidoids for siRNA delivery [110–118].

A study explored the effect of lipidoid tail structure on siRNA delivery potency [113]. Alkyl-acrylate-conjugated lipidoids induce superior gene silencing compared to alkyl-methacrylate-conjugated lipidoids. This difference is attributed to a detrimental alteration in the orientation of lipid tails and nanoparticle packing caused by the presence of methyl groups in the methacrylates. Also, the incorporation of the ether group lowered the knockdown efficiency of lipidoids compared to the counterpart alkyl-structured tails [113].

Fig. 6 depicts composition and siRNA loading of different LNPs used for siRNA delivery.

2.2.7. *Polymer and Lipid-Polymer nanoparticles*

Polymers are an excellent candidate for siRNA delivery due to their numerous advantages. Polymers offer advantages in producing ultrasmall sizes in a controllable manner, achieving a maximizing surface area-to-mass ratio, allowing for functionalizable structures, versatility, and straightforward manipulation. Cationic polymers and cationic block polymers are the most commonly utilized types of polymer for siRNA delivery [114–122]. Certain polymers enhance the transfection efficacy of RNA and reduce immune system activation, making them reliable strategies for non-vaccine-related applications [116–124]. Fig. 7 depicts the mechanism of polymer-based nanoparticles for siRNA delivery and compares it to lipid nanoparticles..

The positive charge is a crucial consideration for polymer design to achieve stable complexation with nucleic acids and ensure successful siRNA protection from nuclease degradation. The cationic charge confers buffer capacity to polymer-based nano-carriers, mediates endosomal release by proton sponges, and promotes the endosomal escape [119]. Cationic polymers and cationic block copolymers possess amine groups that can be protonated, enabling interaction with nucleic acids and the formation of polyplexes [120]. Natural polymers such as dextran, chitosan, cyclodextrin, and atelocollagen, as well as synthetic polymers including polyethyleneimine (PEI), poly (dimethyl aminoethyl methacrylate) (pDMAEMA), poly(L-lysine) (PLL), poly(beta-aminoesters) PBAEs, and poly (dimethyl aminoethyl methacrylate) (pDMAEMA), were employed for RNA delivery [42]. PEI is a well-known cationic gene delivery system that was developed in the early stages of gene therapy. It is recognized for its high transfection efficiency; however, its clinical applications are significantly limited due to excessive cellular accumulation and physiological metabolic toxicity. These issues primarily arise from its high molecular weight and lack of degradability [121,122].

The nitrogen to phosphorus (N/P) ratio is determinant factor for lipoplex formation, influencing the size, stability, and net charge of the polymer-based delivery systems. Higher N/P ratios result in smaller micelles and enhance gene expression in vivo [123,124]. Moreover, consideration of polymer length and molecular weight is crucial in polymer design. Low-molecular-weight polymers degrade more easily than high-molecular-weight ones, which subsequently affects the rate of siRNA release from the polymer matrix [125]. The toxicity concern of

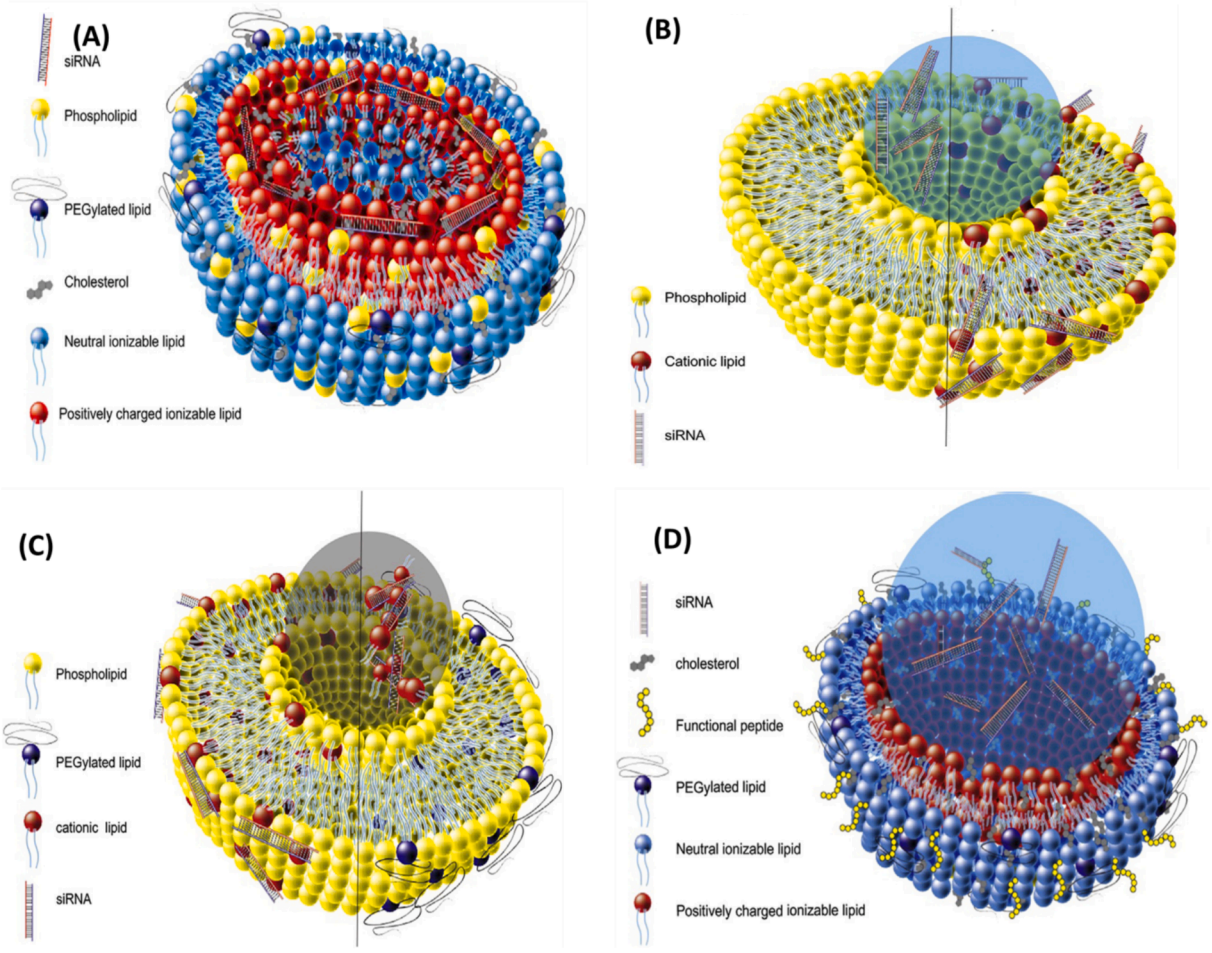

Fig. 6. Schematic illustration of composition and siRNA loading of (A) lipid nanoparticles (B) Liposome (C) Solid lipid nanoparticles (D) Multifunctional envelope-type nanodevice.
Reproduced with permission from [390].

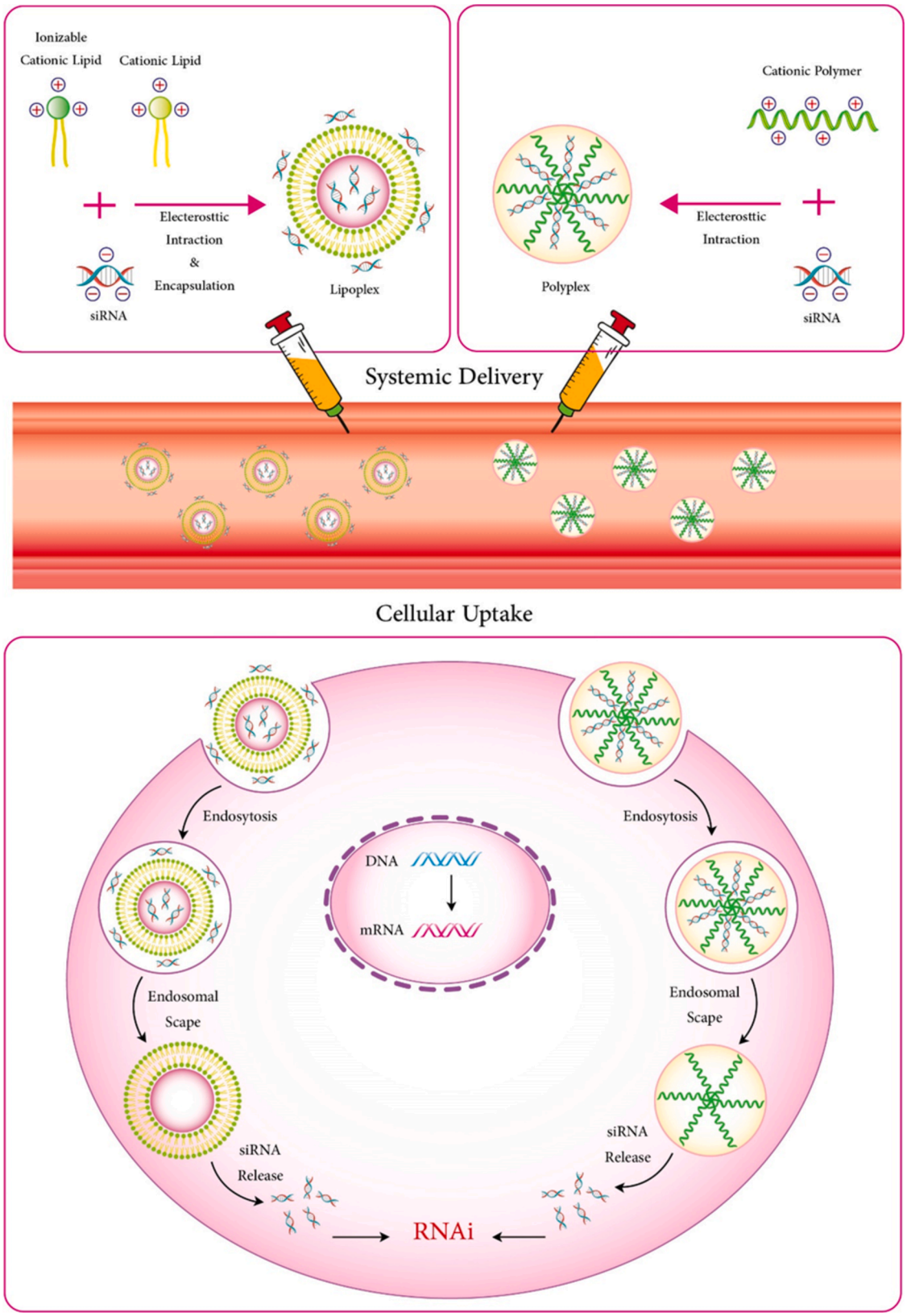

Fig. 7. Schematic illustration of lipoplex and polyplex gene delivery systems, including their formation, systemic delivery, and mechanisms of action in cells.

cationic polymers has prompted the suggestion of using non-cationic polymers for nucleic acid delivery, as they are more biocompatible than cationic polymers [42]. Hyaluronic acid, an anionic polymer, stabilizes siRNA through hydrogen bonding and Van der Waals interactions. This stabilization leads to gene silencing of approximately 60 %, which is influenced by the size and concentration of hyaluronic acid, in both a CD44-positive human osteosarcoma cell line (MG-63) and human mesenchymal stromal cells (hMSCs) [126].

To give an example of utilizing polymersomes in siRNA delivery, polymersomes consist of poly(N-vinylpyrrolidone)14-block-poly (dimethylsiloxane)47-block-poly(N-vinylpyrrolidone)14 triblock copolymer (PVPON14–PDMS47–PVPON14) were tested for delivering siRNA to silence PARP1 in breast cancer cells [127]. Lipid-based systems offer advantages such as better encapsulation efficiency, scalability, and low-cost raw materials; however, they face limitations, including instability, rapid drug release, and restricted surface modification. On the other hand,

Polymeric nanoparticles (NPs) have potential benefits for encapsulating siRNA, including fine particle sizes, low polydispersity indices (PDI), convenient surface functionalization, controlled release, stability, and reproducible synthesis process. However, they also face challenges such as low drug loading capabilities and toxicity, which can arise from traces of organic solvents or the degradation of the polymer. Hybrid nanocarriers that combine lipids and polymers can address some

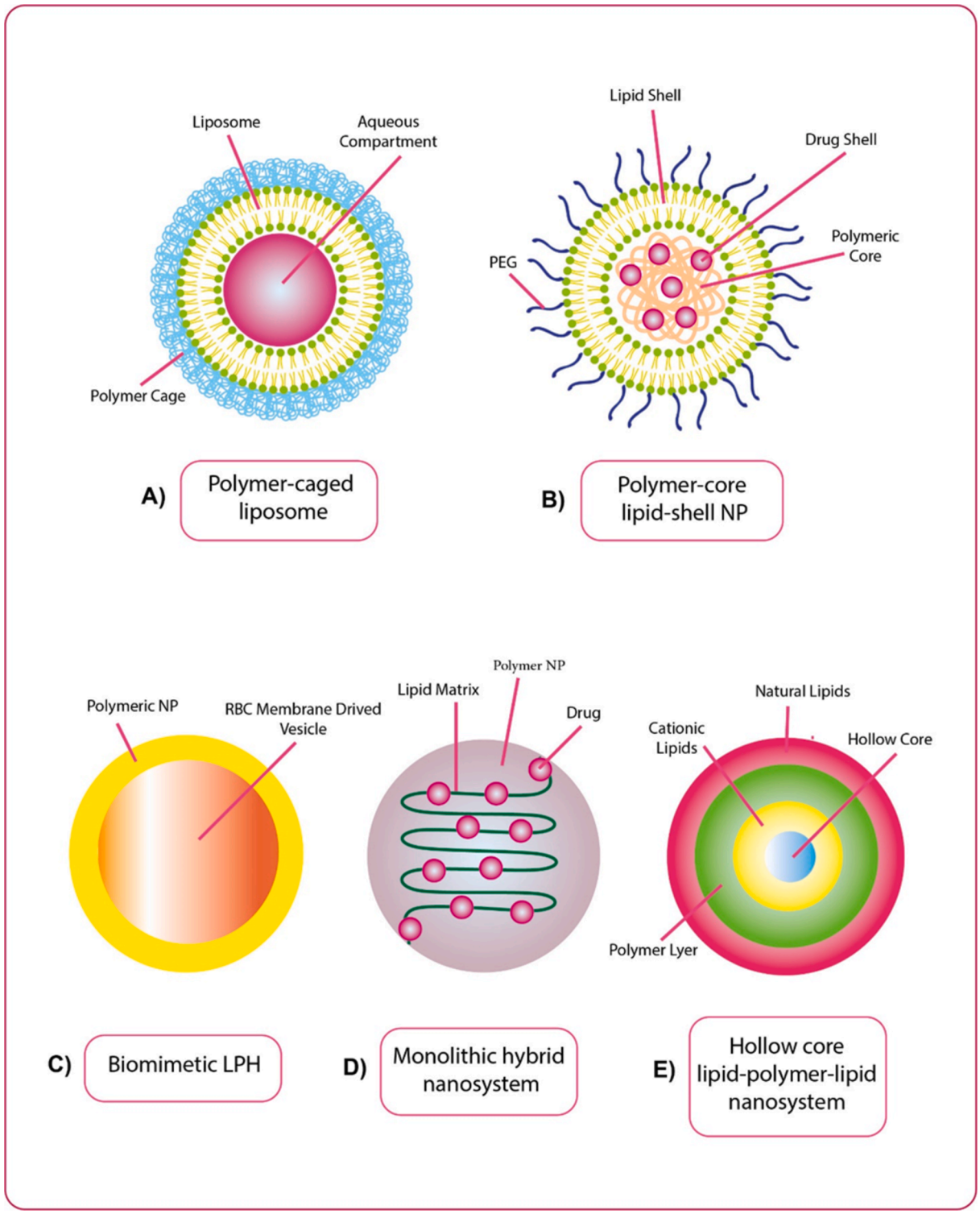

Fig. 8. Schematic illustration of the different LPHNPs. A) Polymer-caged liposome, B) Polymer core-lipid shell hybrid nanosystem, C) Biomimetic lipid-polymer hybrid nanosystem, D) Monolithic hybrid nanosystem and, E) Hallow core–shell vector.

limitations while leveraging the advantages of both systems [128].

The incredible versatility of lipid- and polymer-based NPs opens up exciting possibilities to integrating various materials into a single carrier such as lipids, polymers, and targeting agents. The integration of diverse materials aids in addressing deficiencies and boosts efficacy, as well as ameliorating physicochemical properties such as size, loading capacity, surface modification, stability, and scalability of the delivery system [42].

LPH NPs are gaining prominence in drug delivery due to their key advantages over traditional nanocarriers. Their structural reliability, storage stability, and precise drug release are enhanced by the polymer core's high biocompatibility, thanks to PEG-lipid [129]. LPH NPs typically consist of three distinct functional components: a hydrophobic polymer core, a lipid layer covering the core, and a hydrophilic polymer stealth layer surrounding the lipid shell [130].

LPH nanoparticles can be categorized into five distinct groups based on the different arrangements of lipids and polymers: (1) monolithic hybrid nanosystems, (2) core–shell nanosystems, (3) hollow core–shell nanoparticles, (4) biomimetic lipid-polymer hybrid nanosystems, and (5) polymer-caged liposomes [131]. (Fig. 8).

Lipid-Polymer Hybrid (LPH) nanoparticles are a type of delivery system used for siRNA delivery. The main components of LPH nanoparticles are a lipid bilayer and a polymeric core, which enhance siRNA encapsulation and stability [132]. Fabricated HNPs composed of PLAPEG-PLA copolymer and DDAB cationic lipid encapsulating IGF-1R targeting siRNA exhibited down-regulation of IGF-1R gene expression in MCF7 cells [133].

2.2.8. Considerations for the rational design of LNPs for siRNA delivery

While LNPs have been authorized for clinical use with siRNA and mRNA, they encounter several barriers; overcoming these barriers is essential to achieving the optimum therapeutic effect of nucleic acids. as the first barrier, upon systemic administration of LNPs into the bloodstream, they face significant hurdles due to the formation of the protein corona (PC) and the mononuclear phagocyte system (MPS) [134]. The formation of PC impacts the physicochemical characteristics of LNPs, such as size, morphology, rigidity, diversity, and integrity. The PC formation leads to the dissociation of nucleic acid, disruption of the integrity of LNPs, and rapid elimination of LNPs from blood circulation, ultimately influencing transfection efficacy [135,136]. The most common strategy for solving this problem is PEGylating, which forms a hydrophilic layer protecting nanoparticles from the MPS after systemic administration and extending their circulation lifetime. However, PEGylation causes steric hindrance and may strict their interaction and uptake into the tumor cells (known as the PEG dilemma). Therefore, novel strategies such as cleavable PEG coatings and tumor microenvironment-responsive appears to be possible to overcome this issue [137]. Using the MMP-cleavable PEG-PEP-DOPE modified MEND which selectively dissociated PEG from the MEND in tumor cells resulted in approximately 70 % silencing activity in tumors, compared to non-treatment [138].

Some approaches, such as reducing the size of LNPs, preferring negative surface charge over positive and neutral LNPs, and pKa adjustments, are used to avoid MPS recognition [134]. As the pKa increases, the preferred cells for LNPs shift from hepatocytes (pKa 6.2–6.5) to LSECs (pKa ~7.15) and finally to Kupffer cells (pKa > 7.4) [134]. Particle size, surface characteristics, and surface charge are crucial parameters that influence the behavior of LNPs and their effectiveness in vivo after systemic administration. Delivery systems ranging in size from 50 to 200 nm are highly effective for the intracellular delivery of therapeutic siRNA to the target cells [139]. Particles ranging from 150 to 300 nm in diameter efficiently distribute within the liver and spleen, while larger molecules tend to be readily captured by the MPS [140].

Upon reaching the target cells, LNPs should be taken up and internalized into the cytoplasm. Modifying surfaces with CPPs or ligands that bind to cell surface receptors is a method to improve cellular uptake. Asai et al. developed a new protamine-derived cell-penetrating peptide and attached it to lipid nanoparticles for the delivery of siRNA; the cellular uptake was found to be greater than that achieved with Lipofectamine 2000-mediated transfection [134]. Most nanoparticles enter cells through the endocytosis pathway and are trapped within endosomal vesicles. Inspired by the hypothesis that endosomal escape is the limiting step for siRNA delivery, many researchers have set out to facilitate endosomal escape. It has been reported that only 2 % of RNA delivery systems can successfully escape the endosomes [141]. Cationic liposomes increase electrostatic interactions with anionic endosomal membrane components, which consequently mediates fusion and cargo release into the cytoplasm [142]. Also, the use of ILs aiming the protonation at low pH of endosomes and becoming cationic results in endosomal membrane rupture and promotes endosomal escape [13]. Headgroups containing tertiary amines able to protonation, branched tail lipids, Optimizing the pKa values of the ILs as well as Lipid type and ratio in LNP formulation are crucial parameters that affect the endosomal escape [13]. It was demonstrated that LNPs with a pKa lower than 5.5 are ineffective in achieving gene knockout in both in vitro and in vivo systems [143].

Heyes et al. demonstrated that the presence of unsaturated hydrocarbon chains in pH-sensitive cationic lipids plays a crucial role in enhancing their ability to trigger membrane fusion and enable endosomal escape. They also identified DLinDMA, a pH-sensitive cationic lipid, which marked the first instance of successfully inducing RNAi formation in non-human primates through systemic injection [144]. One method to enhance endosomal escape in lipid nanoparticle (LNP) structures is the incorporation of a fusogenic moiety. DOPE, a fusogenic lipid, has been widely used in lipid-based siRNA delivery systems. At low pH levels, DOPE undergoes a conformational change from a lamellar phase to an inverted hexagonal phase, which destabilizes the endosomal membrane [145]. Another challenge to resolve is the selective targeting of LNPs to specific cell types or organs, aside from the natural accumulation in the liver and lymph nodes [42]. While active targeting strategies can redirect nucleic acid expression from the liver to other organs, the accumulation of LNPs in the liver persists overall [134]. The key physical and chemical properties of LNPs, including size, surface charge, lipid composition and compositional ratio, nitrogen/phosphate portion, as well as RNA-loading efficiency, all influence their biodistribution and therapeutic effects, necessitating their optimization for maximum therapeutic impact [146].

Although LNP-based siRNA therapy has achieved an excellent position in experimental studies, attempting to seek promising ways around the existing barrier could be hopeful for designing effective delivery systems and translating into clinical.

Forthcoming section thoroughly discussed the therapeutic potential of siRNA-loaded LNPs and extensively reviewed various LNPs that have been used to deliver different therapeutic siRNAs for treatment of several ailments. The results of *in vitro* and *in vivo* experiments are mentioned in Table 1 and Table 2.

3. Therapeutic applications of lipid nanoparticles against various diseases

3.1. Cancer

Cancer progression is a multifactorial and complicated phenomenon involving various genes like oncogenes or mutated genes, specific knocking down of targeted genes by siRNA seems to be a promising and potent approach in cancer treatment [259]. Vigorous efforts have been devoted to applying lipid based nanoparticles for the delivery of specific cancer-targeting siRNAs to achieve the best therapeutic effect.

Hirai et al. developed anti polo-like kinase 1 siRNA containing charge-reversible DOP-DEDA conjugated LNPs [79]. These LNPs were found to be negatively charged at basic pH, positively charged at slightly weak acidic pH, whereas no charge was observed at neutral pH. The

Table 1
Pharmacological effects of nanocarriers *in vitro*.

Formulation	Surface modification	siRNA type	Cell line	Effect	Ref
Cancer					
PCLs	–	siAgo2 siRNA	HUVECs,Human umbilical vein endothelial cells / HT1080,human fibrosarcoma cells.	siAgo2 lowered Ago2 mRNAs expression in HUVECs.	[76]
Cationic liposome	–	SiGLO siRNA	A-549 human lung carcinoma cells	Cationic liposomes were observed in the cellular cytoplasm and nucleus. Despite the large size of liposomes, they were able to penetrate to the cytoplasm.	[147]
LPD	Anti-EGFR antibody	Luciferase and RhoA siRNA	SMMC-7721, LM3 and Hep3B human hepatocellular carcinoma cells	Transfection efficacy, specific interaction with EGFR, prevention of luciferase expression, RhoA expression, and cell invasion significantly occurred by targeted LPD which was better than non-targeted LPD.	[148]
ALP	Anti-EGFR antibody or polyarginine peptide (R12)	FITC-siRNA	A549, NCI-H322 and NCI-H460, nonsmallcell lung carcinoma (NSCLC) cells and NIH-3 T3 fibroblast cells	R12 and Anti-EGFR antibody conjugation enhanced cellular uptake of ALPs. Anti-EGFR-conjugated ALPs increased EGFR-mediated uptake into NSCLC cells which was higher relative to NIH-3 T3 cells without the receptors.	[149]
LPD	Anti-EGFR antibody	Luciferase siRNA	MDA-MB-231human breast cancer cells	Compared with non-targeted liposomes, anti-EGFR-Fab conjugated LPD substantially showed a high affinity to EGFR receptors and potent luciferase gene silencing.	[150]
Liposome	Anti ICAM antibody	Lcn2 siRNA	MDA-MB-231 human breast cancer cells, MCF10A non-neoplastic human cells	Stronger interaction of ICAM-Lcn2-LP with MDA-MB-231 cells than non-neoplastic human cell lines was observed. ICAM-Lcn2-LP caused potent knocking down of Lcn2, which consequently reduced significantly VEGF production in MDA-MB-231 cells.	[151]
Liposome	Anti HER1 antibody	RhoA or HER1 siRNA	SK-BR3 human breast cancer cells	Targeted liposomes silenced HER1 gene expression and selectively bound to SK-BR3 cells. Also, this formulation knocked down RhoA expression and prevented cell invasion in SK-BR3 cells.	[152]
liposome	Anti TfRscFv antibody	6-FAM-labeled siRNA	PANC-1 human pancreatic cancer cells	Intracellular localization study confirmed the higher transfection efficacy of TfRscFv-liposomes compared to free siRNA.	[153]
liposome/HoKC	Anti TfRscFv antibody	HER-2 siRNA	PANC-1 human pancreatic cancer cells, MDA-MB-435 metastatic human breast cancer cells	Due to small size and ligand targeting, enhanced cellular uptake and cytoplasmic delivery of TfRscFv-liposome/HoKC was observed in PANC-1 cells which was higher than free siRNA. Also, TfRscFv-liposome/HoKC induced sensitization of both cancer cell lines to gemcitabine and docetaxel.	[154]
liposome	Anti- EpCAM antibody	EpCAM siRNA	MCF-7 human breast cancer cells	modification with EpCAM elevated cellular uptake and internalization, which consequently silenced the EpCAM gene in cells compared with non-targeted liposomes and free siRNA.	[155]
hybrid liposome	Anti- EpCAM antibody	EpCAM siRNA	MCF-7 human breast cancer cells	Enhanced cellular uptake of targeted liposomes.	[156]
Liposomes complexed with polyethylenimine (PEI)	Anti-CD33scFv antibody	AML1/MTG8 leukemia fusion protein siRNA	SKNO-1, Kasumi-1 myeloid leukemia cells	Immunolipoplexes demonstrated specific interaction and uptake by CD33-expressing cell lines. Also, a reduction in AML1/MTG8 mRNA and protein amount, and leukemic clonogenicity was observed.	[157]
Cationic liposome	Anti-EphA10 antibody	MDR1-siRNA	MCF-7/ADR multidrug-resistant breast cancer cells	Immunoliposomes increased siRNA transfection and significantly diminished the MDR1 protein level in MCF-7/ADR cells. At 4 h post-transfection, liposomes showed *endo*-lysosomal escape and siRNA release into the cytoplasm.	[158]
Cationic liposome	Octaarginine (R8) peptide	HDM2 si RNA	SK-MES-1 human lung cancer cells	High transfection efficacy, low off-target toxicity, significant reduction in tumor cell proliferation	[159]
Cationic liposome	Y (K16GACYGLPHKFCG) peptide	endogenous luciferase and GAPDH si RNA	1HAEo human airway epithelial/ B104 rat neuroblastoma/ Neuro2A-Luc mouse neuroblastoma cells	Successful gene silencing in cell lines with high efficiency which was comparable with lipofectamine 2000	[160]

(*continued on next page*)

Table 1 (*continued*)

Formulation	Surface modification	siRNA type	Cell line	Effect	Ref
PCL	RGD peptide	Luciferase si RNA	B16F10/ murine melanoma cell line	Targeted liposomes demonstrated higher knockdown efficiency compared with non-targeted liposomes.	[161]
LPD	RGD peptide	VEGFR-2 siRNA	H5V murine endothelial/ HUVECs Human Umbilical Vein Endothelial cell lines	RGD-PEG-LPD showed enhanced uptake and VEGFR-2 expression silencing in comparison with non-targeted LPD nanoparticles.	[162]
LPC	T7 peptide	EGFR siRNA	MCF-7 human breast cancer cells	EGFR siRNA was delivered into cells through receptor-mediated endocytosis and the EGFR expression was effectively suppressed.	[163]
Cationic Liposomes	AS1411aptamer	BRAF siRNA	A375 human melanoma cell line	Real-time PCR and Western blot analysis demonstrated that prepared liposomes strongly inhibited the activity of the BRAF gene.	[164]
lipid polymer hybrid liposomes	A6 Aptamer	p-gp siRNA	breast cancer cell MDA MB-231, MCF-7, SKBR-3 human breast cancer cells, and 4 T1-R and 4 T1-S mouse breast cancer cells	Her-2 (+) SKBR-3 and 4 T1-R cells showed increased cell transfection in comparison with Her-2 low-expressed MDA MB-231 and MCF-7 cells. Aptamer conjugation significantly enhanced the silencing of the p-gp gene.	[165]
LCP	Anisamide	Luciferase si RNA	H-460 human lung cancer cell	Targeted LCP showed an improved silencing effect compared to untargeted LCP.	[90]
LCP	Anisamide	HDM2, c-myc and VEGF siRNA	A549 and H460 human lung cancer cell	Pooled siRNA co-loaded targeted-LCP efficiently and simultaneously silenced HDM2, c-myc and VEGF expressions and substantially prevented tumor growth.	[166]
LCP	FA and ABX-EGFscFv	CD siRNA	MDA-MB-468 triple negative breast cancer cell	Co-conjugation of LCPs with a sub-optimal number of two ligands (50 FA and 75 ABX-EGFscFv) enhanced the cellular uptake in comparison with mono-targeted LCP NPs.	[167]
LCP or LDH	–	PD-1 siRNA	EL4 murine lymphoblast cell	LCP NPs showed higher cellular uptake and knocking down of PD-1 gene expression in comparison with LDH NPs.	[168]
LCCP	–	PD-1	B16F10 murine melanoma cell line	LCCP entered into cell in a dose and time-dependent manner. Expectedly, LCCP-loaded PD-1 siRNA released siRNA in the endosome.	[169]
SNALP	–	PDL1 siRNA and mRNA expressing OX40L	B16F10 murine melanoma cells and macrophage J774 cells	Transfection of B16F10 cells and macrophages by SNALP led to 75 % silencing PDL1 expression and simultaneously express OX40L with low toxicity.	[170]
LDCP	SP94 tumor targeting peptide	PDL1 siRNA and IL-2 pDNA	He3B human and HCA-1 murine hepatocellular carcinoma cells	Higher cellular uptake and enhanced transfection efficacy of targeted LDCP than non-targeted NPs or LDCP NPs without dendrimer.	[171]
NC100 lipidoid	–	PARP1 siRNA and Myc siRNA	BR5FVB1 a genetically defined Brca1-deficient murine ovarian epithelial cells	Lipidoid-delivered siRNA showed ~65 % silencing of PARP1 expression and inhibited cell growth by apoptosis induction.	[172]
306O13 lipidoid	–	GAPDH siRNA	Caco-2 human colorectal adenocarcinoma cells	siRNA-loaded lipidoid NPs demonstrated strong, stable, and dose-dependent gene silencing in Caco-2 with no sign of cytotoxicity.	[173]
LNP	–	RUNX1 siRNA	T-cell leukemia cells/ patient-derived primary human AML cells	Suppression of RUNX1, apoptosis and cell cycle arrest, growth inhibition effect in cell lines	[174]
LNP	–	BCR-ABL siRNA	K562, Human leukemia BCR-ABL positive cells	LNP-siRNA formulations dose-dependently knocked down the BCR-ABL gene in cells. Also, this formulation targets CD34 + primary human CML cells without any toxicity effect on healthy CD34 + bone marrow cells.	[175]
lipidoid 306O13	–	Cyclin D1, Bcl-2, Mcl-2 siRNAs	JeKo-1 and MAVER-1 mantle cell lymphoma cells	SiRNA at concentration as low as 10 nM suppressed related genes and induced lymphoma cell apoptosis.	[176]
Neurological disorders					
LNP		PTEN siRNA	Hippocampal cultures	LNPs were accumulated in neurons in an apolipoprotein E–dependent manner, demonstrating an increase of 100 % in cellular uptake.	[177]
Peptide-lipid nanocomplex	Peptide Y (K16GACYGLPHKFCG) and	BACE1 siRNA	Neuro-2A mouse neuroblasts cell	Targeted nanoparticles silenced gene expression with minimal cytotoxicity.	[178]

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Table 1 (*continued*)

Formulation	Surface modification	siRNA type	Cell line	Effect	Ref
Chitosan-SLN	Peptide ME27 (K16RVRRGACRGDCLG) rabies virus glycoprotein (RVG-9r) peptide	BACE1 siRNA	Caco-2 cells (as a model of epithelial-like phenotypes)	Chitosan-coated formulation notably enhanced the permeation of siRNA through epithelial cells primarily at 60 min confirming its ability for nose-to–brain delivery.	[179]
SNALPs	rabies virus glycoprotein (RVG-9r) peptide	MutAtax3 siRNA	Neuro2a mouse neuroblasts cells, HT22 mouse hippocampal cells.	Targeted LNPs with RVG-9r-peptide showed enhanced internalization by neuronal cells via receptor-mediated endocytosis and improved mutant ataxin-3 knockdown.	[180]
Cationic SLN	–	c-Met siRNA	U87MG human glioblastoma cells	Down-regulation of c-Met expression and inhibition of cell proliferation	[181]
Ionizable LNP		SAT1 siRNA	U251, LN229, 42MGBA Human Glioblastoma cells, hCMEC/D3 human brain microvascular endothelial cell, Primary human astrocytes, ANA-1 murine macrophage cell	LNPs effectively suppressed SAT1 expression in the cells at the mRNA and protein levels. Also, SAT1 knocking down in glioblastoma cell lines sensitized them towards radiation and chemotherapy treatments.	[182]
Ionizable cationic LNP	–	PD-L1 and CD-47 siRNA	bEnd.3 mouse brain endothelial cells/ GL261 murine GBM cells murine GBM cells	Enhanced cellular uptake, BBB penetration and endosomal escape of siRNA-LNP in cells	[183]
DoGo/LNP	Mannose	TLR4 siRNA	BV2 murine microglia cells	NPs were specifically internalized into cells through the mannose receptor-mediated manner, leading to silencing TLR4 expression and stimulating downregulated M1 polarization and upregulated M2 polarization markers.	[184]
DoGo/LNP		TLR4 siRNA	BV2 murine microglia cells	SiRNA/ DoGo310/LNPs were entered by the oxygen–glucose deprived (OGD) mouse primary microglia, silenced TLR4, and switched the cell phenotype to the anti-inflammatory mode.	[185]
modified –PCL LNP	Angiopep	Double-stranded luciferase GL3 siRNA	bEnd.3 Murine brain endothelial cells and U87MG human glioblastoma cells	The combination of Angiopep with a mechanism for removal of PEG shielding by MMP enzyme resulted in 10-fold higher mediated-receptor cellular uptake and gene silencing relative to non-targeted LNPs.	[186]
SLN	TfR	FITC siRNA	hCMEC/D3 human Cerebral Microvascular Endothelial cells	TfR-functionalized SLN were safe and non-toxic and their affinity to brain endothelial cells was raised to 3-fold compared to unmodified SLN.	[187]
Liver diseases					
SNALP	pPB peptide	gp46 siRNA, the rat homologue of human HSP47	LX-2 human hepatic stellate cells and mouse primary HSC cells	Due to the active targeting role of the pPB peptide, cellular uptake of SNALP was increased in cells.	[188]
liposome	vitamin A	MMP-2 siRNA	HSC–T6 rat hepatic stellate cells	Significant reduction in mRNA expression and MMP-2 activity, and protein expression levels of type I collagen and α–smooth muscle actin were observeded.	[189]
SNALP	pPB peptide	HMGB1 siRNA	mouse primary HSC cells	pPB modification of SNALPs led to higher internalization of NPs and gene silencing than unmodified NPs. Also, these NPs promote cell growth inhibition and apoptosis in HSC cells.	[190]
SNALP	Mannose	HMGB1 siRNA	Raw264.7 murine macrophages cells	Mannose modification confers specific targeting ability to NPs, which strengthens the cellular uptake of nanoparticles and gene silencing in macrophages.	[191]
Viral diseases					
YSK13-MEND		HBV siRNA	PXB, Primary human hepatocytes	MEND/ HBV siRNA effectively reduced the amount of HBsAg and HBeAg and the amount of HBV DNA	[192]
LNP		PKL1siRNA	PHH, Primary human hepatocytes	100 ng/mL of PKL1siRNA/LNP reduced the amount of secreted HBV DNA which was as effective as LNP siHBV or tenofovir (TFV) treatment. Also, the secretion of viral RNA and HBs /HBe Ag were considerably inhibited.	[193]
SNALP		HBV263M or HBV1583M siRNAs	HBV-replicating HepG2 cells	The siRNAs-SNALP lessened HBsAg in HepG2 cells in a dose-dependent manner	[194]

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Table 1 (*continued*)

Formulation	Surface modification	siRNA type	Cell line	Effect	Ref
				compared to untreated cells and control siRNA.	
Epoxide-based lipidoid		PRK2 siRNA	Huh-7 hepatocellular carcinoma cells	The results suggest that lipidoid/siRNA nanoparticle complexes entered into cells by micropinocytosis, leading to enhanced significantly silencing PRK2 expression (~80 %) and HCV replication suppression.	[195]
cationic liposomes	vitamin E	HCV 2a replicon (NS3) si RNA and core gene siRNA	Huh7.5.1 human hepatocellular carcinoma cells	The gene and protein expression was markedly suppressed in vitamin E-liposomes/siRNAs treated cells compared to naked siRNA or cationic liposome/siRNA.	[196]
chitosan-lipid nanocomplexes (CNs)		plasmid DNA encoding an siRNA cocktail (siCCR5,siCXCR4, siTat,siGag, si5′LTR, andsiRev)	HEK-293, Human Embryonic Kidney cells, and 3-dimensional human vaginal ectocervical tissue (3D-VEC) model	The results showed that siRNA cocktail –CNs HIV considerably reduced HIV titers as compared with the control groups.	[197]
Lipid-based-Neutraplex nanosystems		CXCR4 co-receptor siRNA	THP-1 macrophage cells, and TZM-bl human epithelial cancer cervical cells	These nanoparticles showed low toxicity with no sign of pro-inflammatory effect. Nanoparticles efficiently delivered siRNA into cytoplasm due to escape the late endosome/lysosome compartment and HIV replication was inhibited.	[198]
Inflammatory diseases					
Cationic liposome complexed with carrier DNA		TNF-α siRNA	B16 mouse melanoma cells	The TNF-α expression was reduced in both mRNA and protein level.	[199]
CCP-LPD		RRM2 siRNA	MH7A human RA–FLS cells	CCP-LPD/siRRM2 substantially silenced RRM2 gene expression by 80 %. As a result of targeting RA–FLS by CCP-LPD/siRRM2, a marked reduction in the proliferation, enhancement in apoptosis level, and considerable reduction in TNF-α and IL-6 levels was observed.	[189]

PCL, Polycation liposomes; Ago2, Argonaute2; LPD; Liposome-polycation-DNA complex;, ALPs; asymmetric liposome particles, ICAM-1; intercellular adhesion molecule-1, Lcn2; Lipocalin 2, VEGF; vascular endothelial growth factor, TfRscFv; transferrin receptor single-chain antibody fragment, HoKC; pH-sensitive histidine-lysine peptide, EpCAM; epithelial cell adhesion molecule, Eph; anti-EphA10 antibody, R8; Octaarginine, HDM2; human double minute gene 2, LPC; liposome-protamine- chondroitin sulfate, nanoparticles; Apt1; anti-CD44 aptamer, LCP; lipid coated calcium phosphate, FA; folic acid, scFv; EGFR-specific single chain fragment antibody, LDH; layered double, hydroxide, CD-siRNA; cell death siRNA, PD-1; programmed cell death protein 1, LCCP; lipid coated calcium phosphate/carbonate hybrid NPs, SNALP; stable nucleic acid lipid particles, PLK1; polo-like kinase 1, KSP; kinesin spindle protein, LDCP; lipid-dendrimer-calcium-phosphate, PARP1; DNA repair enzyme poly(ADP-ribose) polymerase1, GAPDH; glyceraldehyde 3-phosphate dehydrogenase, PCL; PEGylated cleavable lipopeptide, MMP; matrix metalloproteinase, BACE1; Beta-site amyloid precursor protein cleaving enzyme 1, MutAtax3; mutant ataxin-3, RVG; virus glycoprotein, SAT1; Spermidine/spermine N1-acetyltransferase 1, DoGo; biocompatible short peptidomimetics, pPB; cyclic oligopeptide with amino acid sequence C*SRNLIDC*, HSP47, heat shock protein 47, HMGB1; High mobility group box-1, MEND; multifunctional envelope-type nanodevice, HBV; Hepatitis B virus, PKL1; Serine/threonine polo-like-kinase 1, PRK2; protein kinase C-related kinase 2, pyropheophorbide phosphatydic acids.

hemolysis assay revealed that DOP-DEDA@LNPs induced disruption of red blood cells solely under low-pH conditions, indicating a pH-dependent interaction between biological membranes and DOP-DEDA@LNPs. Also the prepared LNPs were taken up by cells via caveola and clathrin- mediated endocytosis pathways and were successfully able to suppress PLK1 mRNA and its protein when checked in MDA-MB-231 cells, *in vitro* [260].

The usage of LNPs as a vehicle for transcription factor RUNX1 [174] and LINC01257 [261] siRNAs presented a promising approach for management of Acute myeloid leukemia (AML), a rapidly progressing malignancy of the white blood cells [91]. In a recent research, Jyotsana et al formulated LNP for siRNA delivery targeting breakpoint cluster region-abelson (BCR-ABL) oncogene to bone marrow as treatment of chronic myelogenous leukemia (CML). It was shown that synthesized LNP effectively transfers siRNA both *in vitro* and *in vivo*, demonstrating a reduction of leukemia burden in a mouse model [175].

It was shown that an optimized LCP NP with a hollow and spherical structure can maintain its payload and colloidal stability in a culture medium, leading to efficient cellular accumulation of siRNA and cell growth inhibition in MDA-MB-468 human breast cancer cells [262]. After uptake by the cells, CaP is dissolved in the acidic pH of endosome environment which consequently increases the osmotic pressure, creates endosome swelling and rupturing for the release of encapsulated siRNA into the cytoplasm [201]. It should be noted that uncoated LCP NPs displayed limited transfection efficacy and reproducibility due to their instability, which is rarely utilized for *in vivo* nucleic acid delivery [166].

Anisamide-functionalized LCP NPs with different lipid coating layers are established as efficient delivery agents for siRNA into the cytoplasm of cells along with improved antitumor activity in lung, breast, and metastatic cancers [90,93,166,201].

Tang and team developed LCP for delivering cell death siRNA to breast cancer tumor cells and analyze their stability in blood serum. Results showed that naked siRNA was extensively degraded after 1 h of incubation with serum, with complete degradation observed after 2 h. Furthermore, siRNA encapsulated in LCP NPs prepared with a Ca/P ratio of 400 was rapidly degraded, and after 4 h no siRNA was left. Conversely, when LCP NPs were prepared with Ca/P ratios of 50, 100, and 200, and incubated with serum, a significant portion of siRNA was protected by an hour, and this protection persisted for 4 h. The highest level of siRNA protection was observed with LCP NPs prepared at a Ca/P ratio of 100. This protection of siRNA from RNase degradation in serum is primarily is attributed to CaP cores of LCP NPs [263].

In a comparative study, LCP and layered double hydroxide (LDH) NPs were used to deliver programmed cell death protein 1 siRNA (PD-1siRNA) to T cells. LCP NPs more efficiently suppressed the PD-1 expression in human ex vivo tumor-infiltrating lymphocytes (TILs),

Table 2
Pharmacological effects of nanocarriers in vivo.

Formulation	Animal model	Dose	Administration route	diseases	Effect	Ref
Cationic liposome	Athymic nu/nu mice	100 μL	intratracheal/ intravenous	Lung cancer	Local intratracheal administration of cationic liposomes increased the concentration of siRNA in the lung for an extended period with a significant reduction in siRNA concentration in non-targeted tissues in comparison with intravenous administration.	[147]
Anti-EFGR Fab-LPD	Balb/c nude mice	1.2 mg siRNA/kg	intravenous	Hepatocellular carcinoma (HCC)	*In vivo* experiments represent that targeted LPD is greatly distributed in tumors than non-targeted LPD in the orthotopic HCC mice model. Targeted LPD was successful in knocking down luciferase expression.	[148]
Anti-EFGR Fab-LPD	Balb/c nude mice	1.2 mg siRNA/kg	intravenous	Breast cancer	After 24 h, Anti-EFGR Fab-LPD was significantly accumulated in tumor compared to non-targeted liposomes in mice bearing MDA-MB-231 tumor. Upon three successive *i.v.* injection, a ~ 20 % luciferase silencing expression was observed by targeted LPD, while non-targeted liposomes had little effect.	[150]
TfRscFv-liposome	athymic nude mice	9 mg/kg	intravenous	Pancreatic and prostate cancer	TfRscFv-liposomes selectively targeted and transfected tumor cells and deeply penetrated tumors.	[153]
TfRscFv-liposome/ HoKC	female athymic nude mice	SiRNA: (3 mg/kg (days 0–15), 2 mg/kg (days 15–21), and 1.5 mg/kg (days 21–33)/ gemcitabine: twice weekly at 60 mg/kg	intravenous	primary/metastatic tumors	The results have included: Tumor-targeting ability, effective delivery of HER-2 siRNA to tumor, gene silencing and modulating its downstream pathway components, and inhibited tumor growth in animals administrated with combination treatment of TfRscFv-liposome/HoKC and gemcitabine.	[154]
EpCAM antibody-liposome	Female BALB/c SCID mice	0.15 mg/kg twice a week for 4 weeks	intratumoral	Breast cancer	A 35 % reduction was observed in tumor volume in mice treated with EpCAM-targeted liposomes in comparison with positive control group.	[155]
EpCAM antibody-hybrid liposome	Female BALB/c SCID mice	0.15 mg/kg twice a week for 4 weeks	intratumoral	Breast cancer	After 28 days, a 45 % reduction was observed in tumor volume in mice treated with EpCAM antibody-hybrid liposome in comparison with the positive control group. Also, hybrid immunoliposomes reduced the expression level of EpCAM compared to free siRNA and positive control	[156]
anti-EphA10 antibody-cationic liposome	Female BALB/c nude mice	–	intravenous	Breast cancer	Immunoliposomes effectively accumulate in the tumor tissue.	[158]
RGD-PEG-PCL	C57BL/6 male mice	40 μg/mouse as siRNA-C	intravenous	B16F10 lung metastatic model	High knockdown efficiency against metastatic B16F10 tumors	[161]
cRGD-pH sensitive liposome	ICR mice and BALB/ cAJcl-nu/nu	4.0 mg/kg	intravenous	Renal carcinoma	A gene reduction in tumor endothelial cells compared to other endothelial cells of other organs.	[200]
T7-LPC	Female Balb/c nude mice	1 mg/kg siRNA every other day	intravenous	Breast cancer	Effective EGFR suppression and tumor growth inhibition compared to the control group and non-targeted liposomes.	[163]
AS1411aptamer-Cationic Liposomes	Balb/c nude mice	1.2 mg/kg once a day for three consecutive days	intravenous	Melanoma	Tumor targeting ability of targeted liposomes led to a higher accumulation of siRNA in tumors in comparison with normal cells. Also, prepared liposomes considerably knocked down the activity of the BRAF gene and inhibited the melanoma growth in mice bearing A375 tumor.	[164]
Anisamide-LCP	female nude mice	24 μg anti-luciferasesiRNA	intravenous	Lung cancer	Targeted LCP down-regulated about 50 % of luciferase activity in xenografted tumor cells.	[90]

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Table 2 (*continued*)

Formulation	Animal model	Dose	Administration route	diseases	Effect	Ref
Anisamide-LCP	Female C57BL/6 mice	0.36 mg/kg	intravenous	Lung metastasis	Targeted LCPs co-formulating MDM2, c-myc, and VEGF siRNA simultaneously suppressed related oncogenes in metastatic tumors. siRNA-loaded Targeted LCP successfully reduced lung metastasis (70–80 %) and extended the survival time of the treated mice (28,7%) in comparison with the control group.	[201]
Anisamide-LCP	female nude mice	0.36 mg/kg	intravenous	Non-small cell lung cancer	Upon i.v. administration of mice bearing A549 xenografted tumor, siRNA was successfully accumulated in the tumor. Also, pooled siRNA formulated in the targeted LCP delayed tumor progress for both A549 and H460 tumors.	[166]
FA/ ABX-EGFscFv-LCP	athymic 190 nude (nu/nu) mice	3 μM Cy5-dsDNA per mouse	intravenous	Triple negative breast cancer	Dual-targeted LCP NPs significantly penetrated tumors and improved tumor cell uptake.	[167]
SNALP	Female SCID/beige mice	2 mg/kg	intravenous	Cancer	Cleavage of targeted mRNA, silencing of PLK1 and KSP genes expression in tumor cells	[202]
SNALP	naive female C57BL/6 mice	13 μg of RNA per dose per mouse	Intratumoral	Cancer immunotherapy	Reduced tumor growth, a greater density of CD4 + and CD8 + infiltrates in the tumor, and immune activation within tumor-draining lymph nodes.	[170]
SP94-LDCP	C3H/HeNCrNarl male mice	1.2 mg/kg per dose, three doses per week	intravenous	Hepatocellular carcinoma	Increased uptake and accumulation of targeted LDCP NPs, and greater siRNA/pDNA delivery in tumor tissue than non-targeted LDCP NPs. Also, enhanced tumoral infiltration, $CD8^+$ T cell activation, improved efficacy cancer vaccine immunotherapy, and HCC prevention were observed.	[171]
98ND12-5 lipidoid	female athymic nude-*Foxn1nu/ Foxn1 mice*	–	Intratumoral	Ovarian cancer	Suppression of tumor growth, reduction of CLDN3 protein production and cell proliferation, increased apoptosis induction in tumor cells in comparison with tumors injected with control siRNA.	[203]
98ND12-5 lipidoid	Male MISIIR/TAg mice/female BALB/c nude mice	–	Intraperitoneal	Ovarian cancer	Reduction in ascites development due to metastasis suppression in mice treated with lipidoid-CLDN3 siRNA formulation.	[203]
NC100 lipidoid	female FVB/NJ mice	0.5 mg/kg	Intraperitoneal	Ovarian cancer	Local delivery of lipidoid-siRNA NPs extended survival time and increased apoptosis in tumor cells.	[172]
LNP	female NOD/SCID mice	3 injections of 5 mg/kg	Intravenous	Chronic myeloid leukemia	Intravenous injection of ionizable cationic LNPs loaded with BCR-ABL siRNA reduced leukemia burden and suppressed BCR-ABL expression in leukemia cells isolated from myelosarcoma tissue	[175]
Neurological disorders						
LNP	Sprague–Dawley rats	5 mg/ml siRNA	intracortical or intracerebroventricular	Neurological disorders	PTEN gene was knocked down in distinct areas around the injection site or in more broad regions after intracerebroventricular injection with no toxicity and immune reaction.	[177]
Peptide-lipid nanocomplex	Rat	5 μl (5.6 μg of siRNA or 0.02 mg/kg)	corpus callosum or striatum by CED	Neurological disorders	Direct delivery of NPs resulted in BACE-1 gene silencing	[178]
RVG-9r-targeted SNALPs	C57 BL/6 ataxin-3 [Q69] transgenic mice	3 consecutive days with 2.5 mg/kg of siRNA	intravenous	Machado-Joseph disease	Injected NPs were accumulated in the brain and suppressed mutant ataxin-3 in the cerebellum of mice.	[180]
Cationic SLN	Balb/c-nu mice	2 mg/kg, three times once a week	intravenous	glioblastomas	Significant silencing of c-Met expression at the tumor tissue, suppressed tumor growth with no evidence of systemic toxicity in mice, and accumulation of SLNs in tumor of the brain	[181]
Ionizable cationic LNP	Female C57BL/6 mice	100 nmol/kg of siRNA	intravenous	glioblastomas	Cationic LNPs efficiently delivered PL-D1 and CD-47 siRNAs across the	[183]

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Table 2 (*continued*)

Formulation	Animal model	Dose	Administration route	diseases	Effect	Ref
					BBB into cranial GBM in mice, and down-regulated related gene expression in the tumor, leading to synergistically induction of a T cell-dependent immune response.	
DoGo310/LNP	Balb/c mice	15 mg/kg body	intravenous	Cerebral Ischemia/ Reperfusion Injury	TLR4 siRNA was accumulated in the microglia marker Iba1 positive cells in the *peri*-infarct brain tissue, leading to significant suppression of TLR4 expression, switching the pattern of cytokines expression, and amending neurological operation in the mice model.	[185]
Liver diseases						
pPB peptide-modified SNALP	Male Kunming mice	dose of 0.023 mg/kg of siRNA every other day for 2 weeks	intravenous	Hepatic fibrosis	The presence of pPB peptide would be of great help in enhanced accumulation of SNALP in the liver and in PDGFR-β receptor targeting ability to HSCs. 48 h post-injection, NPs were passively taken up by macrophages and spleen. In Thioacetamide (TAA)-induced mice, the serum level of AST and ALT enzymes was significantly reduced with modified SNALP compared to non-modified ones.	[188]
pPB peptide-modified SNALP	Male Kunming mice	0.1 and 0.75 mg/kg every other day for 3 weeks	intravenous	Hepatic cirrhosis	Active targeting of HSC cells by pPB-modified LNPs, effective HMGB1 gene silencing, prevention of the activation and proliferation of HSCs, reduction of the HMGB1 protein release, inhibition of fibrosis formation and collagen deposition in the liver, and substantial extension of survival time in TAA −Induced Cirrhosis mice model.	[190]
Mannose modified SNALP	C57BL/6 mice	0.75 mg/ml for 8 times	intravenous	non-alcoholic steatohepatitis (NASH)	Mannose-SNALP/si HMGB1 could considerably reduce inflammatory factors production in the liver, silence HMGB1 gene, shift liver macrophage to anti-inflammatory M2 phenotype, return liver function to normal condition in NASH model mice.	[191]
Viral diseases						
YSK13-MEND	uPA+/+/SCID mice	5 mg/kg	intravenous	Hepatitis B	A Single dose of YSK13-MEND/HBV siRNA more effectively suppressed the HBV antigens levels and HBV DNA in comparison with the entecavir (ETV) treatment.	[192]
GalNAc-modified PEG-coated LNP	chimeric mice infected with HBV	5 mg siRNA/kg	intravenous	Hepatitis B	Significant reduction in the HBV-genomic DNA and their antigens	[204]
Ionizable liposome	C57BL/6	1 mg/kg	intravenous	Hepatic disorders	Mice treated with siApoB/liposomes considerably and dose-dependently minimized apoB mRNA expression and levels of triglyceride and cholesterol in circulation. Also, siApoB/liposomes significantly accumulated in the liver.	[205]
Ionizable liposome	C57BL/6J-TgN (AlblHBV)/ C57BL/ 6J-M−Tg (HBV C1.0)	1 mg/kg	intravenous	Hepatitis B	siHBV/liposomes significantly suppressed the expression of viral RNAs, viral DNAs, and antigens (HBsAg and HBeAg), dose-dependently and time-dependently in transient and transgenic mouse models, in both liver tissue and circulation.	[205]
SNALP	NOD.CB17-Prkdcscid/J mice	3 mg/kg/day for 3 consecutive days	intravenous	Hepatitis B	SiRNAs-SNALP administration dose-dependently decreased serum HBV DNA > 1.0 $\log_{10}$ and continued for 7 days after dosing.	[194]
apo A-I-Cationic liposome	Female C57BL/6 mice	2 mg/kg	intravenous	Hepatitis C	The use of apo A-I can promote cationic liposomes not only to target liver, but also release therapeutic siRNA cargo into HCV-protein expressing hepatic cells. Also, viral	[206]

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Table 2 (*continued*)

Formulation	Animal model	Dose	Administration route	diseases	Effect	Ref
					protein expression was significantly suppressed.	
vitamin E-coupled cationic liposomes	Female BALB/c mice	siRNA dose of 0.8 mg/kg	intravenous	Hepatitis C	Nanoparticles were successful at transporting siRNA into hepatocytes. Also, E-cationic liposomes/siRNA significantly accumulated in liver. Core protein production and luciferase activity was knocked down with no innate immune response and toxicity	[196]
Lipidoid ND98	NOD-SCID male mice	once every three days for a total of three injections at a dose of 3 mg kg^{-1}	intravenous	Hepatitis C	The administration of PRK2/lipidoid to subcutaneous and orthotopic xenograft mouse models led to a reduction in in serum HCV RNA level.	[207]
lipidoid-based liposome	BALB/c mice	siRNA dose of 1 mg $kg^{-1}1$	intravenous	Hepatitis C	Systemic administration of NPs significantly reduced PKR2 in the mouse liver by up to 60 % and suppressed HCV replication in an HCV-xenograft mice model. Also, galactosylated lipidoids induced more suppression compared to unmodified lipidoids.	[195]
YSK05-MEND	Male ICR mice	1.0 mg siRNA per kg	intravenous	Hepatitis C	The results demonstrated that HCV-targeting dh-siRNAs-loaded MEND can suppress HCV replication.	[208]
LFA-1 mAbs-liposome	NOD/SCID/IL2rγ^{null} mice	50 μg siRNA/injection	intravenous	HIV infection	Systemic administration of LFA-1 integrin-Immunoliposomes containing CCR5 siRNA led to specific uptake of siRNAs by macrophages and T cells, leukocyte-specific gene silencing for 10 days, and a decrease in plasma viral burden and disease-associated CD4 T-cell loss.	[209]
chitosan-lipid nanocomplexes (CNs)	rhesus monkeys	Triple dose of 25 μg per plasmid /animal	intravaginal	SHIV infection	It was observed that prophylactic treatment of siRNAs-CNs with cream formulation in monkeys substantially reduced the level of SHIV viral load.	[197]
SNALP	guinea pigs	0.75 mg/kg	Intraperitoneal	Ebola virus infection	SNALPs containing pool of siRNAs completely protected guinea pigs against death and viremia and were more efficient compared to PEI Polyplexes delivering the same siRNAs.	[210]
SNALP	rhesus macaque	2 mg/kg per dose	intravenous	Ebola virus infection	Rhesus macaque treated with pooled anti-ZEBOV siRNAs in seven dose (days 1, 2, 3, 4, 5, and 6 after challenge with ZEBOV) were completely protected against lethal ZEBOV infection. This treatment regimen was more effective than four postexposure treatments.	[211]
LNP	rhesus macaques	total of seven daily doses of 0.5 mg kg^{-1} (siRNA dose)	intravenous	Ebola virus infection	VP35 siRNA-LNPs treatment prolonged survival and controlled viral replication, resulting in up to 4 log10 reductions after dosing. Also, Animals treated with VP35 siRNA-LNPs only experienced minor clinical symptoms when the treatment was started 4 days after sudan ebolavirus challenge.	[212]
LNP	rhesus macaques	0.5 mg/kg	intravenous	Marburg virus (MARV) and Ravn virus (RAVV) infection	Acceptable survival rate was observed in animals treated with NP siRNA-LNPs.	[213]
Inflammatory diseases						
Acid-sensitive-SLN	male BALB/c mice	On days 1, 3, 5, and 7, TNF-α siRNA, 2 mg/kg	intravenous	Rheumatoid arthritis (RA)	Intravenous administration of acid-sensitive-TNF-α siRNA loaded SLN in a collagen antibody-induced arthritis mouse model considerably minimized bone loss, paw thickness, and histopathological scores.	[214]
Cationic liposome complexed with	DBA/1 mice	every other day with 10 μg of TNF-α siRNA	intravenous	Rheumatoid arthritis (RA)	Upon weekly administration of TNF-α siRNA/liposome-carrier DNA	[199]

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Table 2 (*continued*)

Formulation	Animal model	Dose	Administration route	diseases	Effect	Ref
carrier DNA (lipoplex)					complex, collagen-induced arthritis was completely improved. The presence of liposome-carrier DNA complex enhanced liver and spleen uptake. A reduction in interleukin-6 and monocyte chemotactic protein 1 led to the prevention (50–70 %) of articular and systemic TNF-α production.	
Cationic liposome complexed with carrier DNA (lipoplex)	DBA/1 mice	0.5 mg/kg	intravenous	Rheumatoid arthritis (RA)	This formulation containing IL-1, IL-6, and IL-8 siRNAs improves all symptoms of RA, such as joint damage, inflammation, and Th1 response as compared with TNF-α siRNA-lipoplex-based treatment.	[215]
lipidoid-polymer hybrid nanoparticle (FS14-NP)	male DBA/1JRj mice	equivalent dose of 1 mg/kg on days 2,4,5, and 7	intravenous	Rheumatoid arthritis (RA)	Intravenous administration of the lipid nanoparticle formulation FS14-NP/ IL-1ßsiRNA resulted in a prompt accumulation of the siRNA within the arthritic joints, specifically within macrophages.	[216]
Lipidoid	C57BL/6 mice/ Sprague–Dawley rats/monkey	5 mg/kg (mice and rat) 5 mg/kg, 30 min i.v. infusion (monkey)	intravenous	hypercholesterolemia	Liver-specific silencing of PCSK9 minimized PCSK9 mRNA level (50–70 %), leading to a 60 % reduction in cholesterol reduction in plasma. in monkeys, a single dose administration of PCSK9 siRNA caused a prompt, prolonged, and reversible lowering of plasma PCSK9, LDLc, and apolipoprotein B, without a noticeable impact on HDLc and TGs.	[217]

LPD; Liposome-polycation-DNA complex; TfRscFv; transferrin receptor single-chain antibody fragment, EpCAM; epithelial cell adhesion molecule, Eph; anti-EphA10 antibody, LPC; liposome-protamine- chondroitin sulfate, nanoparticles, SNALP; hydroxide, CD-siRNA; cell death siRNA, PD-1; programmed cell death protein 1, LCCP; lipid coated calcium phosphate/carbonate hybrid NPs, SNALP; stable nucleic acid lipid particles, PLK1; polo-like kinase 1, KSP; kinesin spindle protein, LDCP; lipid-dendrimer-calcium-phosphate, PARP1; DNA repair enzyme poly(ADP-ribose) polymerase1, GAPDH; glyceraldehyde 3-phosphate dehydrogenase, PCL; PEGylated cleavable lipopeptide, MMP; matrix metalloproteinase, BACE1; Beta-site amyloid precursor protein cleaving enzyme 1, MutAtax3; mutant ataxin-3, RVG; virus glycoprotein, SAT1; Spermidine/spermine N1-acetyltransferase 1, DoGo; biocompatible short peptidomimetics, pPB; cyclic oligopeptide with amino acid sequence C*SRNLIDC*, HSP47, heat shock protein 47, HMGB1; High mobility group box-1, MEND; multifunctional envelope-type nanodevice, HBV; Hepatitis B virus, PKL1; Serine/threonine polo-like-kinase 1, PRK2; protein kinase C-related kinase 2, pyropheophorbide phosphatydic acids,

holding a promising approach for TIL-related cancer immunotherapy [168].

To release siRNA in the endosome, lipid-coated calcium phosphate/carbonate (LCCP) hybrid NPs were developed by partially replacing phosphate in LCP NPs with carbonate [169]. The substitution of carbonate in the composition of NPs enable them to release siRNA in milder acidic condition (pH 6.0–5.5) than LCP (pH 4–5). LCCP-loaded with PD-L1 siRNA down-regulated the expression of the PD-L1 faster than that of LCP NPs in the B16F10 cells, confirming the release of siRNA in the endosome.

Also, Huang et al developed SP94 peptide-targeted lipid- thymine-functionalized dendrimers containing −calcium phosphate (TT-LDCP) NPs. Thymine-functionalized dendrimers activate activating the stimulator of interferon genes (STING)–cGAS pathway, thus possessing enhanced gene delivery capabilities and immune adjuvant properties. TT-LDCP NPs were employed to deliver siRNA targeting the immunostimulatory IL-2–encoding plasmid DNA and immune checkpoint ligand PD-L1 to hepatocellular carcinoma cell (HCC), both *in vitro* and *in vivo*. This approach resulted in increased infiltration and activation of CD8 + T cells within the tumor, thereby enhancing the efficacy of cancer vaccine immunotherapy and suppressing HCC progression [171].

It has been shown that cholesterol increases transfection efficacy by facilitating the fusion of nanoparticles with the cell membrane [264]. Therefore, the incorporation of the intermediate concentration of cholesteryl oleate in the cationic SLN formulation presented a stable, non-toxic nanocarrier with high transient transfection efficacy that intracellularly release siRNA in the HEK293T and HeLa cells cytosol [265]. In a study, a reducible PEG-siRNA conjugate was electrostatically adsorbed on the surface of cationic SLNs [266].

Systemic administration of siRNA-loaded stable nucleic acid lipid particles (SNALP) into hepatic and xenografted tumor model induced RNAi mechanism for specific mRNA cleavage in tumors, sufficient knocking down of polo-like kinase 1 (PLK1) and kinesin spindle protein (KSP) genes. This process caused the mitotic cycle disruption and apoptosis in tumor cells [202].

Simultaneously combined use of both inhibiting and stimulating targets offers a cocktail strategy for the next generation of cancer immunotherapy [267]. The immunosuppressive tumor microenvironment (TME) seems to be a major cause of cancer immunotherapy failure [268]. The combination of siRNA and mRNA to simultaneously knock down the expression of immune checkpoint targets and induce immunostimulating factors could modulate and reprogram TME from immunosuppressive to immunostimulatory phenotype. In this context, a group of researchers investigated intratumoral administration of siPDL1-mOX40L SNALP for cancer immunotherapy. When checked in B16F10 cancer cell line, in vitro, SNALPs demonstrated the ability to transfect macrophages and cancer cells, leading to a 75 % reduction in PDL1 expression while simultaneously expressing high levels of stimulatory checkpoint OX40L. Also, SNALPs promotes selective tumor targeting together with a reduction in tumor growth, infiltration of the high density of CD4 + and CD8 + in the tumor, and immune stimulation in tumor-draining lymph nodes in mice bearing B16F10 tumor cells (Fig. 9) [170].

It was reported that lipidoid-formulated siRNA may present potential efficacy to combat ovarian [172,203], prostate [269], and gastrointestinal cancers [173]. Huang et al. showed that intratumoral and

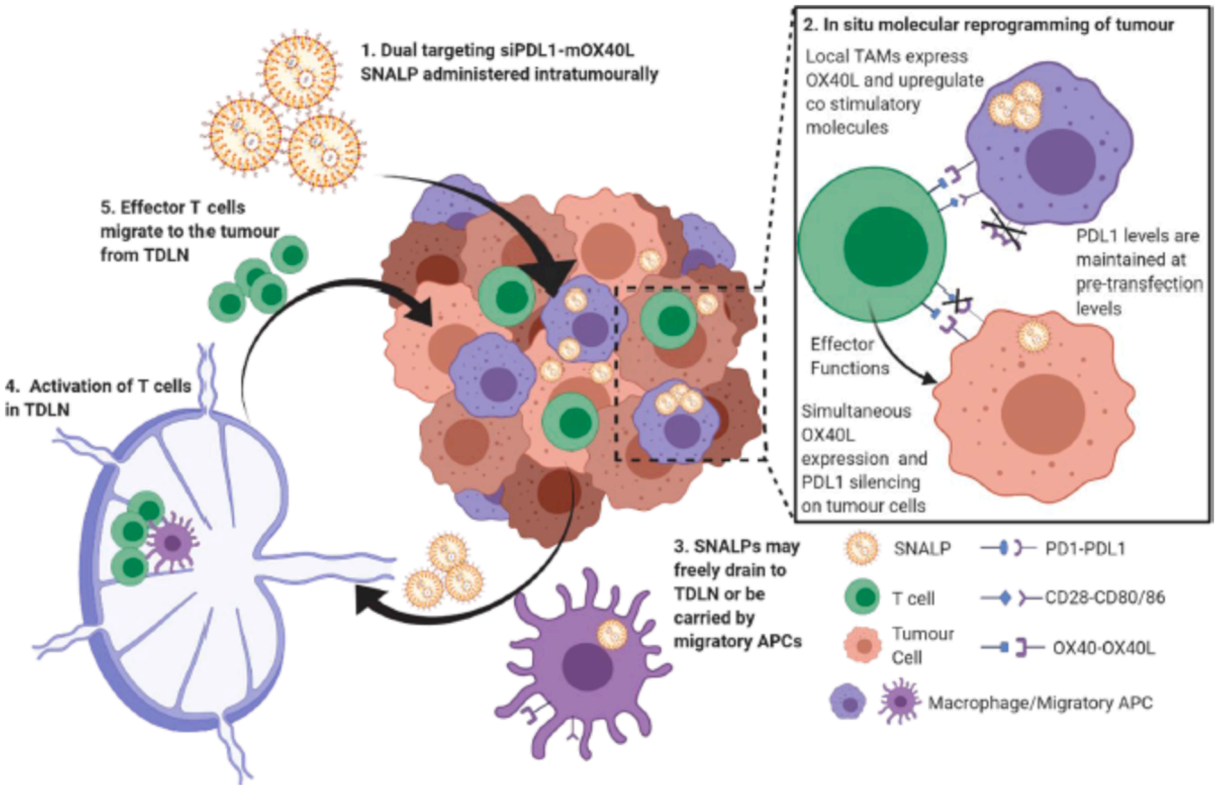

Fig. 9. Graphical representation of siPDL1-mOX40L SNALP for cancer immunotherapy. The immunosuppression mediated by PDL1 alleviated by siRNA, while the tumor prompted to express the positive checkpoint molecule OX40L through mRNA. SNALPs will activate antigen-presenting cells (APC), leading to the expression of a co-stimulatory molecule alongside OX40L. This process will reprogram the tumor microenvironment to favor an immunostimulatory state. Reproduced with permission from [170].

intraperitoneal injection of lipidoid-formulated Claudin-3 (CLDN3) siRNA in three different mouse models i.e., OVCAR-3 xenografts nude mice, MISIIR/TAg mice, and nude mice bearing mouse ovarian surface epithelial cells (MOSEC) tumor overexpressing firefly luciferase (ID8-Fluc cells) successfully reduced ovarian tumor growth, metastasis, and CLDN3 expression in tumors [203]. Also in another study, lipidoid-DNA repair enzyme poly (ADP-ribose) polymerase1 (Parp1) siRNA formulation was found to be effective against ovarian cancer with BRCA mutations through extending survival time in treated mice and induction of apoptosis in tumor cells [172].

Mantle cell lymphoma (MCL) is a malignancy that originates from mature B cells and contributes to approximately 5 % of all cases of non-Hodgkin's lymphoma (NHL) [270]. Considering the overexpression of a protein Cyclin D1 (CCND1) as a cell cycle regulator and up-regulation of anti-apoptotic proteins Bcl-2 and Mcl-2 in Mantle cell lymphoma (MCL), the Knapp group designed a lipidoid 306O13 –based NPs to target these proteins at mRNA level in MCL [176]. The results showed that lipidoid nanoparticles encapsulating siRNA cocktails against Cyclin D1, Bcl-2, and. Mcl-2 considerably prevented JeKo-1 and MAVER-1 mantle cell lymphoma cell proliferation.

In another study, intravenous administration of rabies virus glycoprotein (RVG-9r) targeted −SNALP successfully silenced mutant ataxin-3, alleviating motor impairments and neuropathology in Machado-Joseph disease mouse model [180],cationic SLN encapsulating c-Met siRNA [181] and LNP-siSAT1 containing ionizable DODAP lipid [182] were proposed as a promising therapeutic way for glioblastoma treatment. The *in vitro* and *in vivo* results provide important insight into the clinical application of these LNPs of therapeutic siRNA for the systemic treatment of glioblastoma. In another study, ionizable cationic LNPs were found to be effective in transporting PD-L1 and CD-47 siRNAs across the blood brain barrier (BBB) into cranial GBM in mice and suppressing gene expression in the tumor [183].

Rinaldi and team utilized cationic lipid 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) to prepared two polymer-lipid hybrid nanomedicine (HNMed) with poly(lactic-co-glycolic acid) as polymer. Following the characterization of the HNMed, a model siRNA was surface enginnered onto their exterior of NPs to create siRNA/HNMed complexes (Fig. 10). *In vitro* studies using the mouse fibroblast cell line, NIH3T3 revealed that compared to HNMed based on DC-Chol, DOTAP-based HNMed exhibited greater cytocompatibility. The bio-efficacy and cellular uptake and of the formulations were further evaluated *in vitro* using the U87MG human glioblastoma cell line expressing the luciferase gene. The complexes successfully delivered anti-luciferase siRNA, leading to a considerably significant suppression of gene expression. Importantly, in comparison to the DC-Chol-based HNMed, the effect of the DOTAP-based formulation approximately three times higher [271].

A LPH NPs composed of a cationic lipid, N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxycarbonyl aminoethyl) ammonium bromide (BHEM-Chol), and PLGA polymers was developed to carry polo-like kinase 1 (Plk1)-specific siRNA (siPlk1) [272]. Obtained LPH NPs. The hybrid nanoparticles delivered siRNA into BT474 cells and facilitated the escape of the loaded siRNA from the endosome to the cytoplasm. Systemic administration of LPH NPs specifically reduced expression of the oncogene Plk1 and induced cancer cell apoptosis both in vitro and in vivo and remarkably suppressed tumor progression.

Indeed, nonspecific targeting poses a significant challenge in both traditional cancer treatment approaches and newly developed nanoparticles. Surface modification of nanoparticles to enable active targeting specifically to tumor cells represents a promising solution. This approach allows for minimal invasion of healthy cells, enhancing the precision and efficacy of cancer therapy while minimizing side effects. In the context of targeted LNPs, diverse moieties including monoclonal

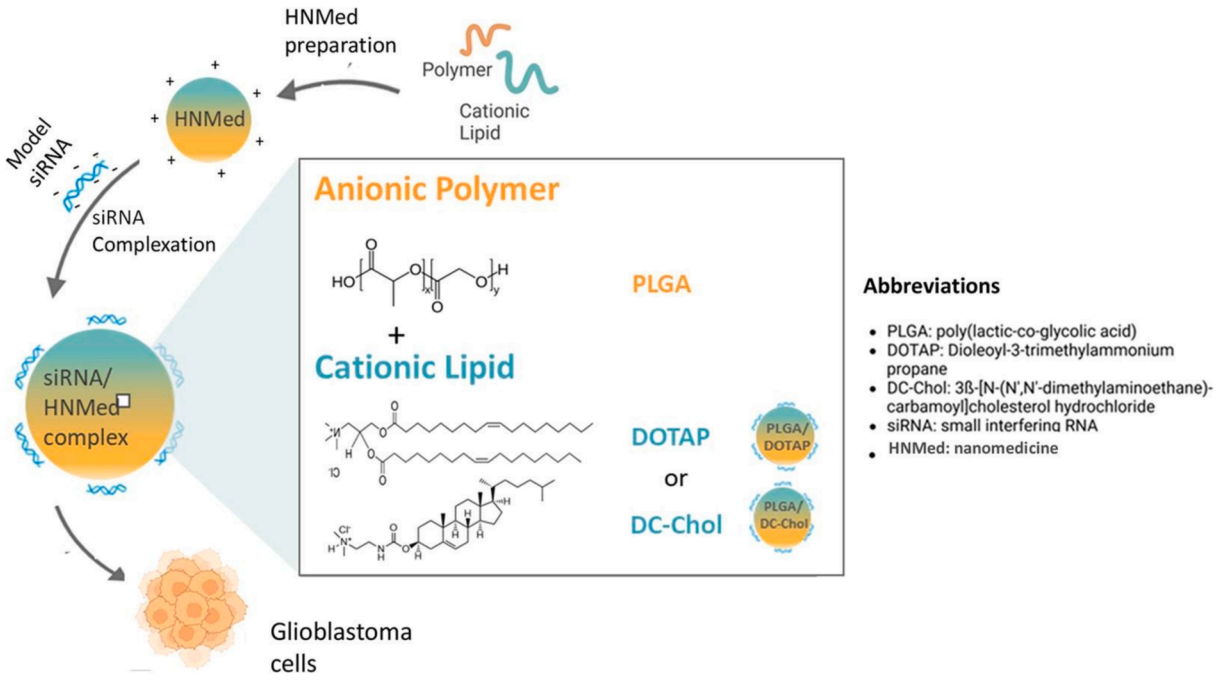

Fig. 10. Formulation of polymer-lipid hybrid nanomedicine using two cationic lipids, 3ß-[N(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and polymer poly(lactic-co-glycolic acid) as polymer to deliver anti-luciferase siRNA to glioblastoma cells. Reproduced with permission from [271].

antibodies, aptamers, peptides, folate receptors, transferrin and oligosaccharides served as ideal targeting ligands for siRNA delivery. Immunoliposomes, antibody-conjugated liposomes, are accepted by a majority of researchers for active delivery of siRNA and improvement of therapeutic outcome. Some liposomes are functionalized with anti-EGFR antibodies [148–156,273], anti-intercellular adhesion molecule-1 (ICAM-1) antibody [151], anti-HER2 antibody [152], anti-transferrin receptor (TfR) antibody [153,154], Epithelial cell adhesion molecule (EpCAM) antibody [155,156], anti-CD33 antibody [157], anti-EphA10 antibody [158], anti-CD20 antibody [274], and Th17 antibody [275].

Guo et al developed Lipocalin 2-siRNA containing liposomes and surface modified it with ICAM-1 to target triple negative breast cancer (TNBC).

When compared to non-neoplastic MCF-10A cells, the produced liposomes bound human TNBC MDA-MB-231 cells far more strongly. MDA-MB-231 TNBC cells produce much less vascular endothelial growth factor (VEGF) when Lipocalin 2 is effectively knocked down by siRNA. As a result, there was less angiogenesis in vivo and in vitro. Since angiogenesis is necessary for the growth and progression of solid tumors, blocking it is an important treatment approach for malignancies in humans [151].

Peptides are another class of modification moieties with the advantages of easy production and having tunable sequence to adjust properties of charge, hydrophobicity, solubility and stability [276]. Two subclasses of peptides, Cell Penetrating Peptides (CPPs) and Tumor Targeting Peptides (TTPs), have been used for facilitating liposome delivery to cell cytoplasm [277]. Octaarginine (R8) CPP [159], targeting peptides of Y (K16GACYGLPHKFCG) [160], RGD peptide [161,162,200,278], and T7 were brought into apply [163].

Recent studies have indicated that all-*cis*-configured anandamide (AEA), also known as arachidonoylethanolamin, has the capability to direct siRNA towards cannabinoid receptors. This phenomenon facilitates the effective receptor-mediated internalization of siRNA–AEA complexes. Given that cannabinoid receptors are present on immune and neural cells, this method facilitates the successful delivery of siRNAs into these critical cell types.

Brunner and colleagues devised a dendrimer-based siRNA, with an anandamide receptor ligand targeting the vital nucleoprotein (N protein) and phosphoprotein (P protein) of the rabies virus (RABV), achieving suppression of the virus titer in neurons to levels undetectable by conventional methods. These cell-penetrating siRNA dendrimers, synthesized through click chemistry, present promising avenues for discovering novel compounds capable of combating this lethal ailment [262].

Similarly in another study, the anandamide-modified RNA exhibits remarkably effective transfection capabilities, facilitating the delivery of siRNA even into difficult-to-transfect RBL-2H3 cells, which mimic neuronal uptake. Also, when this system was checked in human immune cells (BJAB), it demonstrates silencing effects comparable to those achieved with a cationic, well-established transfection agent. Moreover, the anandamide conjugates were observed to be non-toxic. The chemistry outlined and the properties demonstrated by the anandamide siRNA broaden the potential applications of siRNA-mediated gene silencing in both neuronal and immune cells [279].

Aptamers are defined as short (20–60 nucleotides) and single-strand DNA or RNA molecules with high affinity to specifically bind various types of ligands including small molecules, proteins, drugs, peptides, cells and tissues [280]. The utility of some aptamers as targeting ligand present some insight for designing functionalized liposomes for siRNA delivery with improved transfection efficacy [164,165].

In a study by Alshaer et al, liposomal-siRNA delivery system comprising a core consisting of siRNA:protamine complex and a shell tailored with anti-CD44 aptamer for active targeting of CD44-expressing cells. Among its various functions, CD44 stands out as the most common cancer stem cell surface biomarker and is frequently overexpressed in many tumors, making it an appealing receptor for therapeutic targeting. This innovative non-cationic system was assessed for its ability to silence the reporter gene of luciferase (luc2) in TNBC model both in vitro and in vivo [281].

In another study, aptamer-siRNA chimeric RNAs were created with the ability to bind specifically to certain cell types and deliver functional siRNAs into cancer cells. The aptamer component of these chimeras facilitates binding to PSMA, a cell-surface receptor that is highly

expressed in prostate cancer cells and tumor vascular endothelium. Meanwhile, the siRNA portion of the chimeras targets the expression of survival genes [282].

Also, some reports have shown that the application of folate receptors [283–291] and transferrin [286–294] as targeting ligands could effectively derive the process of selective traversing of siRNA into cells, both *in vitro* and *in vivo*. In a nanoparticle designing study done by Tang and team, co-conjugation of LCP NPs with folic acid and ABX-EGFscFv successfully improved cellular uptake and increased tumor penetration in MDA-MB-468-bearing nude mice. This drug/gene delivering, dual-targeting system utilizing LCP NPs to greatly enhance siRNA uptake by cancer cells and its transfection ability. The siRNA loaded LCP NPs, were conjugated with a controlled number of EGFR-specific single chain fragment antibody and/or folic acid (scFv ABX-EGF). The uptake of folic acid-modified LCP NPs (FA-LCP) and scFv ABX-EGF-modified (scFv-LCP) and by MDA-MB-468 breast cancer cells was significantly increased with an optimal ligand density on each NP surface [167].

Similarly, an LNP formulation was synthesized which could benefit from the over expression of transferrin receptor on the cell membrane, and the elevated matrix metalloproteinases (MMPs) in tumor sites and inflammatory BBB condition [186]. The aforementioned nanocarrier was composed of a PEGylated cleavable lipopeptide (PCL) sensitive to MMPs and angiopep as a targeting ligand aiming to dual targeting both glioma cells and brain endothelial. The removal of the protective PEG layer in the presence of MMPs at the tumor site together with angiopep ligand make it a favorable siRNA delivery system with high cellular uptake and gene silencing capability. Also, transferrin-modified SLN exhibited considerably higher cellular interaction with brain endothelial cells (hCMEC/D3) compared to unmodified nanoparticles [187]. Despite *in vitro* promising results, further *in vivo* studies are required to confirm mentioned findings.

Anisamide, a different targeting ligand, showed a moderate affinity for sigma receptors, which improved intracellular transport into lung and prostate cancer cells. A team of scientists created a self-assembling LNP that can effectively administer siRNA intravenously (IV) to tumors. Carrier DNA, siRNA, protamine, and lipids were combined to create this LNP. Polyethylene glycol and the ligand anisamide were then added as a post-modification step. Four hours after the formulation was IV injected into a xenograft model for in vivo testing, about 70–80 % of the injected siRNA/g accumulated in the tumor, 20 % was recovered in the lung, and 10 % was found in the liver. Confocal imaging demonstrated that the targeted NPs successfully delivered fluorescent-labeled siRNA into the cytoplasm of the sigma receptor-expressing NCI-H460 xenograft tumor, while free siRNA and non-targeted NPs exhibited minimal uptake [289].

NLC was created by Taratula et al. and tested for effective inhalation delivery of siRNA and an anticancer medication. A modified synthetic analog of luteinizing hormone-releasing hormone (LHRH) was added to NLC, and siRNA targeting MRP1 mRNA to suppress pump drug resistance and siRNA targeting BCL2 mRNA to suppress non-pump cellular resistance served as a targeting moiety specific to receptors overexpressed on the plasma membrane of lung cancer cells. Human lung cancer cells were used for in vitro testing, while a mouse orthotopic model of human lung cancer was used for in vivo research. Compared to IV administration, the NLCs considerably reduced exposure to healthy organs and efficiently delivered its payload into lung cancer cells after inhalation [250].

Similarly, among other targets, the gonadotropin-releasing hormone receptor type I (GnRHR I), a member of the G-protein-coupled receptor family, is expressed on the surface of both healthy and cancerous cells. Its presence in cancer cells has made it a primary focus for the development of innovative anti-cancer agents. Consequently, Biniari and their team rationally designed and synthesized a series of anthraquinone/mitoxantrone–GnRH conjugates (con1–con8). These conjugates exhibited in vitro binding affinities ranging from 0.06 to 3.42 nM, with six out of eight (con2–con7) showing higher affinities for GnRH than the established drug leuprolide [290].

3.1.1. Combination therapy

The complexity of hard-to-treat diseases, such as cancer, makes it difficult for available treatment options to achieve optimal therapeutic benefit. In recent years, both clinical practice and research studies have demonstrated the limitations of singular treatment approaches in effectively managing more complex diseases, thereby necessitating a shift towards multi-modal and combination therapies [291].

The combination of chemotherapy and gene therapy has generated a significant level of interest in cancer treatment, due to their powerful anti-tumor activity, reduced side effects, and potential opportunity to circumvent drug resistance [233]. The successful implementation of combined treatment greatly relies on the identification of ideal drugs, appropriate formulations that carry both chemotherapies and gene drugs and the specialized targeting of tumors to ensure optimal efficacy and minimize adverse effects. Ideally, both agents should show either synergistic or additive effects, without any opposing or antagonistic effects [233]. Numerous research efforts have aimed to utilize LNPs for the co-delivery of chemotherapeutic agent doxorubicin and siRNA molecules. Various reported delivery systems include: NGR-modified PEGylated LPD for co-delivery c-myc siRNA [218], TPGS- modified cationic liposomes for deliver Bcl-2 siRNA [219], galactosylated-cationic liposomes co-delivering Vimentin [220], cationic liposomes folate receptor targeted liposomes co-delivering Bmi1 siRNA [292] and SNALP co-encapsulating CD47 siRNA [221], liposomes functionalized with anti-CD44 and anti-PD-L1 DNA aptamers containing IDO1 siRNA [293] and doxorubicin for cancer treatment. In addition to the co-delivery approach described above, in which siRNA and chemotherapeutic agents are loaded in one delivery system, sequential administration of RGD-functionalized cationic liposomes entrapping MDR1 siRNA or doxorubicin [222], polo-like kinase 1 (PLK1) siRNA-MEND and liposomal Dox formulations [252], and F3 targeted liposomes encapsulating PLK1 siRNA in combination with free paclitaxel [223] have been another methods for combination therapy. Also, liposomal co-delivery of STAT3 siRNA and curcumin [224,230], pH sensitive carboxymethyl chitosan modified-liposome co-delivering siRNA and sorafenib [231], cationic liposomes co-delivering paclitaxel, crizotinib and Bcl-2 siRNA [294], co-encapsulation of bortezomib and SHARP1 siRNA into RGD-liposome [295] aptamer functionalized liposomes co-encapsulating paclitaxel and PLK1 siRNA [296], co-delivery of IGF-1R siRNA and lycopene encapsulated hybrid lipid NPs [297], and co-delivery of GPR78 siRNA and docetaxel [235], VEGF siRNA and gemcitabine monophosphate [253], and Beclin 1 siRNA and FTY720 [236] in LCP NPs have been evaluated for cancer therapy. These studies have presented convincing evidence of the potential synergistic effect of combining siRNA and chemotherapeutic agents. This approach promoted sensitivity to anti-cancer drugs by reducing cancer-related genes expression and drug resistance, while also improving the anti-tumor effect and influencing the pharmacological behavior of the co-delivery systems both *in vitro* and *in vivo*.

In addition to chemotherapy drugs, phototherapy can be combined with gene therapy to enhance the overall potency of the nanoscale delivery platform. Phototherapy, as a non-invasive and modern method, is further segmented into two main subtypes: photothermal therapy (PTT) and photodynamic therapy (PDT). In PTT, a photosensitizer absorbs the near-infrared (NIR) light with an appropriate wavelength and produces heat energy to locally induce apoptosis in cancer cells [298]. Therefore, combination treatment using anti-EGFR bispecific antibody (BsAb) conjugated-LCP NPs co-delivering Cell Death (CD) siRNA and indocyanine green (ICG) as a photosensitizer plus laser irradiation was found to be effective in suppressing tumor growth in MDA-MB-468 breast tumors mice model [237]. Alternatively, Photodynamic therapy (PDT) is based on the selective absorption of light at a specific wavelength by a photosensitizer molecule, which triggers a series of events like generating free radicals or singlet oxygen, leading to the targeted destruction of inappropriate cells [299].

It has been offered that more effective cancer therapies can be

achieved by targeting multiple cellular pathways, rather than solely focusing on individual proteins [300]. In this regard, it was reported that a mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor (PD0325901)-loaded N',N''-dioleylglutamide-based cationic liposomes complexed with Mcl1 siRNA prohibited tumor growth by 79 % and considerably induced apoptosis of cancer cells [241]. Fraxinellone-loaded CGKRK-modified poly (ethylene glycol) (PEG)-poly (lactic acid) (PLA) nanoparticles modulated the suppressive TME by regulating TGF-ß signaling and reduced stroma barrier density. Subsequently, siKRAS-LCPA-poE3 displayed efficient internalization by pancreatic cancer cells, resulting in specific silencing of the KRAS mutation and extending the survival time of pancreatic tumor-bearing mice.

The design and development of nanocarriers that take advantage of immunotherapeutic strategy and specifically normalization of TME that is highly immunosuppressive has enabled the conquering of obstacles that immunotherapy on their own would struggle to overcome, such as high level of immune-suppressive cytokines within the TEM. To meet this demand, xu et al, augmented the efficacy of a mannose-modified LCP vaccine co-delivering tumor antigen (Trp 2 peptide) and an adjuvant (CPG oligonucleotide) on an advanced melanoma tumor by suppressing TGF-ß in tumor cells [256].

In another combined strategy, a cyclic peptide iRGD (CCRGDKGPDC)-conjugated SLN co-delivering EGFR and PL-D1 in combination with radiation therapy was evaluated against glioblastoma [243]. Application of short bursts of radiation therapy altered tumors to enhance uptake of this targeted SLN in glioblastoma, resulting in activated immune response, tumor inhibition, and improved survival in mice models.

Theranostic-based strategies that combine gene therapy and diagnosed/imaging modalities have attracted much attention in cancer therapy [301]. Core-shell quantum dots have garnered significant interest due to their diminutive size, adjustable fluorescent emission properties, and relatively high photostability [302].

Inspired by the fluorescence property of CdSe/ZnS quantum dot (QD) nanocrystals, theragnostic anti EGFR-Apt liposomes containing Bcl-2 siRNA [244], and SLN/ Bcl-2 siRNA encapsulating paclitaxel and CdSe/ZnS QD [245] have been developed for in situ tracking of their delivery to cancer cells by fluorescent imaging techniques. Other imaging modalities, non-invasive imaging technologies based on nuclear medicine, like single photon emission computer tomography (SPECT) in combination with computed tomography (SPECT/CT) have been developed in clinical practices [303]. *In vivo* imaging of lymph node metastasis using SPECT/CT confirmed preferentially accumulation of ^{111}In LCP NPs in the lymph nodes via lymphatic drainage [257].

Ultrasound (US)-enhanced delivery is one promising theranostic method for delivering drugs or genes, which utilizes energy-induced gas cavities (cavitation bubbles) generated by US pressure oscillation [304]. The combination of ultrasound (US) and microbubbles has been shown to produce temporary changes in cell membrane permeability, allowing for targeted delivery of a variety of therapeutic compounds, both in vivo and in vitro, including dextran, plasmids (pDNA), siRNA, and peptides [305]. It was reported that PEG-liposomes containing a US imaging gas (bubble liposomes) combined with US may be used for siRNA delivery *in vitro* and *in vivo* [246,247,306].

In another study, magnetic fluid Fe_3O_4, thermosensitive lipid, and CPP were combined into a platform to develop magnetic and thermal dual-sensitive liposome (TML) for siRNA delivery [248]. C-myc siRNA-CPP/TML under alternating current (AC) magnetic fields showed enhanced *in vivo* targeted delivery efficacy and antitumor efficacy in a murine model of MCF-7 tumor.

Furthermore, a few other combined treatment approaches have been implemented for various diseases treatments, including the delivery of Bcl-2 siRNA and paclitaxel in SLN for cervical cancer [249], co-delivery of atorvastatin and LOX-1 siRNA by core–shell nanoparticle for atherosclerosis [307], pulmonary co-delivery of prostaglandin E (PGE2) and MMP3, CCL12, and HIF1Alpha siRNAs [258], doxorubicin or paclitaxel and MRP1 and Bcl-2 siRNA [250], and co-delivering tacrolimus and TNF-α for psoriasis treatment [251] using NLC (Table 3 and 4).

3.2. Neurological disorders

Blood-brain barrier (BBB) is a selective/semipermeable barrier composed of continuous endothelial membrane and microvessels with tight junctions and is shielded by mural vascular cells, astrocytes and pericytes [308]. The existence of protective arrangements including, tight junctions between brain capillary endothelial cells (BCECs), multidrug resistance protein-1 (MRP-1), P-glycoprotein (P-GP), breast cancer resistance protein (BCRP), and degrading enzyme transforms the BBB into a significant obstacle that impedes the accumulation of drug molecules in the brain [309]. Therefore, addressing these challenges has heightened the need to develop efficient, safe and stable brain delivery systems.

Apolipoprotein E (ApoE), a predominant lipoprotein in the brain, is produced by astrocytes and plays a critical role in cholesterol/lipid transporter in the central nervous system (CNS) through interaction with members of the receptor (LDLR) family followed by endocytosis [310].

In this regard, it was shown that PTEN siRNA-LNPs containing ionizable lipids distributed broadly in the brain and manipulated the expression of the PTEN gene, upon intracortical or intracerebroventricular (ICV) injection [177]. The natural affinity of LNPs to the endogenous ApoE can describe the mechanism underlying LNP delivery into neurons *in vivo* [177].

The efficacy of β-site amyloid precursor protein cleaving enzyme 1 (BACE1) siRNA, potentially beneficial to treat Alzheimer's disease, was evaluated by a team of scientists, using anionic siRNA nanocomplexes consisting of anionic PEGylated liposomes and cationic targeting peptide. Since BACE1 enzyme is involved in the formation of amyloid plaques, the prepared NPs successfully exert considerable gene silencing, however their non-PEGylated counterparts were less effective. For treating neuronal diseases, siRNA to BACE1was directly administered into rat brains. *In vivo* silencing of BACE1 was accomplished following a single injection of anionic NPs using convection-enhanced delivery. The specificity of RNA interference was confirmed through Western blot analysis of protein and 5′ RACE-PCR [311]. BACE1 serves as the rate-limiting enzyme in the production of Aβ peptides, which are key markers in the pathophysiology of AD. As the expression of BACE1 is elevated in the brains of AD patients so is their enzymatic activity [312]. The prepared anionic PEGylated nanoparticles led to approximately a 60 % decrease in BACE1 mRNA levels compared to the untreated group, along with a 30 % reduction in protein expression. Therefore, the reduction in BACE1 expression observed through protein quantification, mRNA quantification and mRNA cleavage analysis, supports an RNA-mediated mechanism of gene silencing [178].

Traumatic brain injury (TBI) triggers the pro-inflammatory polarization of astrocytes and leads to secondary disruption of BBB and subsequent brain damage. Xiao and team proposed astrocyte-targeted delivery of siRNA using ligand-functionalized, adenosine-conjugated lipids dependent LNPs, denoted as Ad4 LNPs. Following systemic administration of siRNA-Ad4 LNPs against Toll-like receptor 4 (TLR4) to the TBI xenograft model, specific internalization of the LNPs by astrocytes was observed in the vicinity of damaged brain tissue. This resulted in a substantial knockdown of TLR4 at both protein and mRNA levels in the brain. Consequently, there was a significant reduction in key pro-inflammatory cytokines and consecutively increase in key anti-inflammatory cytokines in the serum [313].

Rapid microglia activation is a typical response of the neuro-inflammation process to brain damage, especially occurring following ischemic stroke [314,315]. Microglia polarization is referred to as a process of inhibiting the proinflammatory (M1) phenotype while inducing the anti-inflammatory (M2) phenotypes, assisting in brain

Table 3
Combination therapy of siRNA-LNPs with *in vitro* application.

Formulation	Combined strategy	SiRNA target	Cell line	Effect	Ref
NGR-PEG-LPD	Chemotherapy/ Doxorubicin	c-myc siRNA	HT-1080 human fibrosarcoma cells	NGR ligand improved delivery of Dox and siRNA to cells. C-myc siRNA- NGR-PEG-PLD treatment considerably inhibited the c-myc mRNA and protein expression in cell line.	[218]
TPGS-cationic liposome	Chemotherapy/ Doxorubicin	Bcl-2 siRNA	H22, Murine hepatic carcinoma cell	SiBcl-2/Dox-TPGS cationic liposomes increased the cellular uptake of Dox and toxicity against 3D H22 tumor spheroids via tumor priming	[219]
Galactosylated cationic liposomes	Chemotherapy/ Doxorubicin	Vimentin siRNA	Huh7,human hepatocarcinoma cells	Dox and siRNA delivered by Galactosylated cationic liposomes significantly caused cell growth inhibition or cell death.	[220]
SNALP	Chemotherapy/ Doxorubicin	CD47 siRNA	CT26 murine colon carcinoma cells	SNALP improved Dox and siRNA uptake in cells with increased cytotoxicity. Also, siCD47 SNALPs efficiently reduce the expression of CD47 without any hindrance from Dox. This combination therapy elicited a synergistic effect on macrophage-mediated phagocytosis of the treated cells.	[221]
RGD- liposome	Chemotherapy/ Doxorubicin	MDR1 siRNA	MCF7/A, Dox resistant human breast cancer cells	RGD-modified liposomes demonstrated increased siRNA transfection than non-modified liposomes. Combination use of siRNA formulation via interfere with drug resistance related P-glycoprotein could considerably enhance Dox concentration and cytotoxicity in Dox-resistant cells compared to liposomal Dox alone.	[222]
F3-pH sensitive liposome	Chemotherapy/ Paclitaxel	PLK1 siRNA	PC3 human prostate cancer cell line, HMEC-1 human microvascular endothelial cells	Treatment of cells with F3-liposome containing PLK1 siRNA considerably lessened cell viability with a significant PLK1 knocking down at mRNA and protein levels. Also, pretreatment of pc3 cells with F3-liposome containing PLK1 siRNA led to the sensitization of cells to paclitaxel up to 3-fold.	[223]
Cationic liposome	Chemotherapy/ Curcumin	STAT3 siRNA	A431 human epidermoid carcinoma cells	curcumin-loaded liposome–siRNA complex was rapidly entered to cells via clathrin-mediated endocytosis pathway. Co-delivery of curcumin and siRNA significantly increased cell growth inhibition and apoptosis compared with free curcumin and STAT3 siRNA. topical iontophoresis modality enhanced penetration of NPs to the epidermis.	[224]
Cationic liposome	Chemotherapy/ Curcumin	STAT3 siRNA	B16F10 mouse melanoma cells	Co-delivery of curcumin – an antitumor phytochemical [225–235], and STAT3 siRNA reduced cell viability compared with STAT3 siRNA alone or liposomal curcumin.	[230]
CMCS-modified-pH sensitive cationic liposome	Chemotherapy/ Sorafenib	nonspecific siRNA	HepG2 human hepatocellular carcinoma cell	The release of Sorafenib from NPs a showed pH-dependent pattern. Cellular uptake of Sorafenib/siRNA-CMCS liposome was higher at pH 6.5 than at pH 7.4.	[231]
Cationic liposome	Chemotherapy/ docetaxel	Bcl-2 siRNA	A549 and H226 human lung carcinoma cell	The co-encapsulation of docetaxel and siRNA prohibited cell proliferation in a time-dependent manner and showed notable apoptosis of cancer cells with elevated levels of apoptosis markers such as caspase 3/7 activity. Also, the sub-G0/G1 phase was increased, confirming the occurrence of apoptosis and hypodiploidy.	[232]
anti-EGFR Fab'LPD	Chemotherapy/ Adriamycin	RRM2 siRNA	SMMC-7721, HepG2, Huh-7 human hepatocellular carcinoma cells	Adriamycin/ RRM2 siRNA-TLPD exhibited the most inhibitory effect, apoptosis inducing, and potent PRM2 suppressing expression than non-targeted LPD in EGFR overexpressing cells not EGTR-lowly expressing cells.	[233]
pH sensitive-MEND PEG-DSPESS	Chemotherapy/ Epirubicin	Bcl-2 siRNA	HepG2 human hepatocellular carcinoma cells	Epirubicin/siBcl-2 dual-loaded LNPs exhibited improved inhibition efficacy compared to individual epirubicin-loaded liposomes in cells. As well as this combination therapy reduced P-gp expression in contrast to free epirubicin and epirubicin-loaded liposomes.	[234]
RGD-PEG-LCP	Chemotherapy/ Docetaxel	GRP78 siRNA	PC-3 human prostate cancer cell	Co-delivery of docetaxel and siRNA using NPs showed synergistic effects on cell viability reduction, G2/M cell cycle arrest, cell apoptosis and autophagy, which were more potent than the simultaneous administration of free docetaxel and siRNA.	[235]
LCP	Chemotherapy/ FTY720	Beclin 1 siRNA	SMMC-7721 cells human hepatocellular carcinoma cells	The results showed that NPs were internalized into cells and silenced gene expression. Also, the co-delivery of si Beclin 1 and FTY720 caused the highest level of cytotoxicity.	[236]
ICG-BsAb-LCP	PTT	Cell death (CD) siRNA	MDA-MB-468 human breast cancer cell	ICG-BsAb/CD siRNA-LCP NPs were efficiently taken up by cells, subsequently cell apoptosis was significantly induced and synergically prohibited cell growth upon 808 nm near-infrared laser irridation.	[237]
AA-LCP	PDT	HIF1α siRNA	SCC4 and SAS human squamous cell carcinoma cells	LCP NPs encapsulating HIF1α siRNA potently knocked down HIF1α expression, enhanced cell death, and considerably prohibited cell proliferation after photosan-mediated photodynamic therapy.	[238]

(*continued on next page*)

Table 3 (*continued*)

Formulation	Combined strategy	SiRNA target	Cell line	Effect	Ref
AEAA-LCP Pyro PA + AEAA EGFR siRNA-LCP	PDT	EGFR siRNA	SCC4 and SAS human squamous cell carcinoma cells	The results demonstrated that EGFR siRNA was substantially suppressed after PDT therapy with significant prevention of cancer growth.	[239]
pcCPP/NGR-Cationic liposome	PDT	c-Myc siRNA	HT-1080 cells human fibrosarcoma cell line	NIR-pretreated pcCPP/NGR cationic liposomes showed improved cellular uptake, endosomal escape, significant c-myc gene silencing, and cell apoptosis induction, indicating pcCPP activation by NIR light	[240]
N',N''-dioleylglutamide-based cationic liposomes	Signaling pathway inhibition	Mcl1 siRNA	KB human epithelial carcinoma cells	Co-delivery of Mcl1 siRNA and PD0325901 (MEK inhibitor) to cells lessened Mcl1 expression and PD0325901 amount and more potently decreased tumor cell survival compared to other treatments. s	[241]
CGKRK modified-Frax-loaded PLA NPs + siKRAS-LCP- ApoE3	Signaling pathway inhibition	KRAS siRNA	Panc-1 human pancreatic cancer cells / NIH3T3 mouse fibroblast cells	The evidence indicated that siKRAS-LCP- ApoE3 entered into cells by macropinocytosis pathway and potently suppressed KRAS mutation. As well as, CGKRK modified-Frax-loaded PLA NPs downregulated the TGF-ß signaling pathway, reversed the activated CAFs, and reduced M2 macrophages and the stroma barrier density.	[242]
iRGD-SLN	Radiation therapy	EGFR and PD-L1 siRNA	U87, human glioma cell, GL261 mouse glioma cell	Dose-dependently suppressing related genes, higher cellular uptake and affinity than non-targeted NPs were observed.	[243]
Anti-EGFR aptamer-liposome-Quantum dot (Apt-QLs)	Theranostic strategies	Bcl-2 siRNA	MDA-MB-231 Human breast adenocarcinoma cell	MDA-MB-231 cells treated with Apt-QLs showed the highest transfection of Q-dots and siRNA co-localizing in the cytoplasm, potent endosomal escape of siRNA. Also, the presence of anti-EGFR ligands on the surface of liposomes facilitated siRNA delivery into cells overexpressing RGFR-receptor.	[244]
Quantum dots-loaded −LDL-mimetic SLN/ siRNA complex	Theranostic strategies	Bcl-2 siRNA	A549, Human lung carcinoma cell line	The combination therapy synergistically induced apoptosis in cancer cells by activation of the caspase mechanism. In addition, the robust fluorescence generated from quantum dots within the SLN provides an in-situ visualization of the internalization of the nanoparticles into the cancer cells.	[245]
PEG- bubble cationic liposome (BL)	Ultrasound	Luciferase siRNA	COS-7 monkey kidney fibroblast cells	SiRNA-entrapped BLs were stable in serum. Transfection of siRNA with BLs showed specific gene-silencing.	[246]
Chol-siRNA/ PEG-bubble liposome (BL)	Ultrasound	Luciferase siRNA	Colon26 cells	The presence of cholesterol in Chol-siRNA conjugates stabilized siRNA against RNase. The combination of ultrasound and Chol-siRNA BL led to specific gene silencing.	[247]
SiRNA-CPPs-magnetic and thermo sensitive liposome (TML)	Magnetic field	c-Myc siRNA	MCF7 human breast cancer cells	The results showed that siRNA-CPP/TML benefited from the effective internalization of CPPs, enhanced cellular uptake, distribution and endosome escape. Also, upon magnetic hyperthermia condition, transportation of c-Myc siRNA and gene silencing were increased in MCF7 cells.	[248]
SLN	Chemotherapy/ Paclitaxel	Bcl-2 siRNA	HeLa, human cervical cancer cell line	*In vitro* results revealed that simultaneous release of siRNA and paclitaxel is achievable using SLN NPs after local vaginal administration.	[249]
LHRH-cationic NLC	Chemotherapy/ doxorubicin	MRP1 and Bcl-2 siRNA	A549 human lung adenocarcinoma cells	The experiments using LHRH-Dox-NLC-siRNA and LHRH-TAX-Dox-NLC-siRNA revealed that chemotherapeutic agent and siRNA were efficiently delivered into cells and the expression of both BCL2 and MRP1 was significantly suppressed.	[250]
NLC	Chemotherapy/ tacrolimus	TNF-α siRNA	T3/NIH Murine fibroblast 3	Controlled release of tacrolimus and the permeation and penetration pattern in the skin tissue were appropriate for tropical application	[251]

NGR; aspargine–glycine–arginine, LPD; Liposome-polycation-DNA complex; TPGS; D-α-tocopherol polyethylene glycol 1000 succinate, SNALP; stable nucleic acid lipid particles, PLK1; Polo-like Kinase 1, CMCS, carboxymethyl chitosan, HCC; hepatocellular carcinoma, Pyro PA; pyropheophorbide phosphatydic acids, AEAA, aminoethylanisamide, pcCPP; photolabile-caged CPP, TGF-β; transforming growth factor β, LPH; Liposome-protamine-hyaluronic acid, Frax; fraxinellone, PLA; poly (ethylene glycol) (PEG)-poly(lactic acid), PGE2; prostaglandin E, PTT; photothermal therapy, PDT; photodynamic therapy, SLN; solid lipid nanoparticles, CAF; cancer-associated fibroblasts.

recovery after ischemic stroke [316]. Taking into consideration the role of toll-like receptor 4 (TLR4) in inducing M1 phenotype, biocompatible peptidomimetic (DoGo) LNPs containing TLR4 siRNA were designed [184,185]. The results of intravenous administration of these LNPs into transient middle cerebral artery occlusion (tMCAO) mice model demonstrated siRNA accumulation in the *peri*-infarct brain tissue, silencing of TLR4 expression, switching the mode of cytokines expression, and recovery of neurological function, showing their ability for CNS-targeted siRNA delivery.

3.3. Liver diseases

Chronic liver disease (CLD) is a major public health concern due to the lack of effective treatment and is caused by chronic liver abnormalities non-alcoholic steatohepatitis (NASH), alcoholic steatohepatitis (ASH), genetic and autoimmune diseases [317]. CLD is accompanied by durable inflammatory responses, chronic parenchymal injuries, and liver fibrosis formation [318]. Liver fibrosis is a dynamic pathological process that is described by the excessive deposition of extracellular matrix (ECM) proteins and followed by the activation of hepatic myofibroblasts (MFs), inflammatory/immune response and angiogenesis [318,319]. As the liver is the main organ in charge of lipid metabolism,

Table 4
Combination therapy of siRNA-LNPs with *in vivo* application.

Formulation	Animal model	Dose	Administration route	Diseases	Effect	Ref
NGR-PEG-LPD	Mice	siRNA (1.2 mg/kg) or Dox (0.3 mg/kg) once per day for 3 days	intravenous	Cancer	The results showed that targeted LPD can successfully deliver Dox and siRNA to the tumor cells and suppress c-myc expression. Co-delivery of Dox and c-myc to tumor cells can work synergistically to inhibit tumor growth and enhance the therapeutic effect.	[218]
TPGS-cationic liposome	BALB/c female mice	5 mg Dox equiv./kg or 1.2 mg siBcl-2 equiv./kg at five days	intravenous	Cancer	SiBcl-2/Dox- TPGS-cationic liposome treatment exhibited higher tumor growth inhibition (75 %) compared with monotherapy with Dox. Also, extended Dox circulation in the blood and improved tumor accumulation of Dox were observed.	[219]
Galactosylated cationic liposomes	male Balb/c athymic nude mice	single dose of 150 μg/kg vimentin siRNA and/or 5 mg/kg DOX	intravenous	Hepatocellular Carcinoma	Gal-Dox/siRNA cationic liposomes showed more tumor accumulation than free Dox and Dox/siRNA cationic liposomes. As well as co-delivery of Dox and siRNA promoted tumor inhibition and therapeutic effect improvement.	[220]
SNALP	BALB/c mice	5 mg/kg (two injections)	intravenous	cancer	50 % of the animals that received the combined therapy of siCD47 and Dox using SNALPs were successful in removing the tumor, and the remaining animals experienced significantly reduced tumor burden as compared to those treated with either drug alone.	[221]
RGD-liposomes containing MDR1 siRNA or Dox	Female Balb/c mice	Four doses of RGD-Liposome-siRNA (2 mg/kg), and Four doses of RGD-Dox-liposome (4 mg/kg)	intravenous	cancer	Sequential administration of RGD/siRNA liposomes and RGD/Dox liposome led to optimal tumor growth inhibition and tumor accumulation.	[222]
SiPLK1 MEND and Dox-liposome	BALB/cAJcl-nu/nu	Three dose of si-PLK1 MEND (3 mg/kg), Dox liposome (1.5 mg/kg)	intravenous	Renal carcinoma	As a result of a combination of Dox and siRNA, a mild reduction in tumor growth along with apoptotic cell induction with no sign of acute toxicity was observed.	[252]
Cationic liposome	C57BL/6 mouse	75 μl, four doses	Intratumoral or topical	Skin cancer	Combination treatment of curcumin and STAT3 siRNA using cationic liposomes substantially suppressed tumor development compared with liposomal curcumin or STAT3 siRNA alone in melanoma mice model. As well as, the iontophoretic administration of curcumin-loaded liposome-siRNA complex was as effective as an intratumoral injection for tumor regression and STAT3 protein repression.	[230]
CMCS-modified-pH sensitive cationic liposome	Female Kunming mice	1.0 mg/kg (siRNA)	intravenous	cancer	The results confirmed the *in vivo* stability and si RNA delivery to the tumor region.	[231]
Cationic liposome	BALB/c nude mice	DTX equivalent dose of 10 mg/kg	intravenous	Lung cancer	*In vivo* anti-tumor study demonstrated acceptable tumor inhibition response with a 100 % survival rate due to the synergistic effect of MDR reversing ability of BCL-2 siRNA and docetaxel potency in tumor tissue.	[232]
anti-EGFR Fab'-LPD	BALB/c nude mice	a single dose (1.2 mg siRNA/kg, 0.5 mg ADR/kg).	intravenous	Hepatocellular carcinoma	Treatment of mice bearing orthotopic HCC with Adriamycin/ RRM2 siRNA-TLPD resulted in enhanced accumulation in tumor and liver compared to non-targeted LPD and significantly suppressed tumor progression.	[233]
RGD-PEG-LCP	BALB/c mice	1.3 mg/kg GRP78 ssiRNA and 0.5 mg/kg docetaxel every 3 days	intravenous	Prostate cancer	The complete inhibition of tumor growth and longest survival time was observed in the group that received docetaxel/ GPR78 siRNA NPs. These results may be related to the suppression of the GPR78 gene in the tumor.	[235]

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Table 4 (*continued*)

Formulation	Animal model	Dose	Administration route	Diseases	Effect	Ref
LCP	Female nude mice	Four injection with a dose of 50.4 μmol/Kg GMP and/or 0.2 mg/Kg VEGF siRNA	intravenous	non-small–cell lung cancer	Combination therapy using LCP co-loaded with VEGF siRNA and gemcitabine monophosphate (GMP) as compared with VEGF siRNA or GMP therapy alone led to 30–40 % induction of tumor cell apoptosis, reduction of cell growth by 8-fold, substantial decrease in tumor microvessel density, and remarkable tumor growth inhibition with subtle *in vivo* toxicity.	[253]
LCP	Female nude mice	Four injections at a dose of 25 μmol/Kg GMP and/or 0.2 mg/Kg c-Myc siRNA.	intravenous	non-small–cell lung cancer	Co-formulating of c-Myc siRNA and GMP in LCP dramatically prevented tumor growth with low toxicity.	[254]
LCP	Male BALB/c nude mice	Daily injection of 5 mg/kg total FTY720, 2 mg/kg siBeclin 1 for 3 weeks	Intravenous	Hepatocellular carcinoma	The co-delivery of FTY720 and Beclin 1 siRNA inhibited more efficiently tumor growth than FTY720-loaded LCP NPs.	[236]
ICG-BsAb/CD siRNA LCP	Female Balb/c nude (nu/nu) mice	1.6 mg/kg ICG and 0.125 mg/kg CD siRNA at days 0 and 5	intravenous	Breast cancer	Targeted multifunctional LCP NPs more efficiently accumulated in the tumor region. Also, the combination of CD siRNA and photothermal therapy (ICG) using mentioned NPs roughly cleared small tumors (~100 mm^3) and large tumors (~ 500 mm^3) in mice bearing MDA-MB-468 breast tumors.	[237]
AA-LCP	female nude mice	2 mg/kg PS + 0.36 mg/kg HIF1α loaded −LCP NPS, a total of 3 times at 24 h intervals	intravenous	Head and neck cancer	Systemic administration of HIF1α siRNA-loaded LCP NPs into human SAS or SCC4 xenografted model led to siRNA accumulation and suppression of HIF1α expression in the tumor region. Combination therapy with PDT regressed tumor size after 10 days which was more effective than either PDT or HIF1α siRNA alone.	[238]
AA-LCP	male BALB/cAnN.Cg-Foxn1nu	2 mg/kg PS + irridation with 640 nm light + 0.36 mg/kg siVEGF-A-LCP NPS on days 1, 2,3,6,7, 8	intravenous	Head and neck cancer	The combined therapy of siVEGF-A-loaded LCP and PDT was found to be effective in tumor growth inhibition by suppressing in vivo VEGF-A after PDT, tumor volume decrease, reduction in VEGF-A protein, inducing apoptosis tumor cells in SCC4 and SAS xenografted mice as compared with PBS group.	[255]
AEAA-LCP Pyro PA + AEAA EGFR siRNA-LCP	male BALB/cAnN.Cg-Foxn1 nude mice	0.78 mg/kg LCP Pyro PA NPs + 0.36 mg/kg LCP siEGFR NPs + red light at 663 ± 9 nm wavelength	intravenous	Head and neck cancer	Combined therapy led to significant tumor volume reduction in the SAS and SCC4 xenografted model compared with groups treated with PBS, control siRNA, and PDT alone.	[239]
pcCPP/NGR-Cationic liposome	Female BALB/c nude mice	1.2 mg/kg siRNA for 10 days (once every other day)	intravenous	Cancer	upon illumination using NIR light at the tumor site, accumulation of pcCPP/NGR-cationic liposome in the tumor, enhanced c-myc silencing and delayed tumor development were observed in HT-1080 xenografted nude mice.	[240]
N',N''-dioleylglutamide-based cationic liposomes	Mice	0.7 mg/kg of siRNA in complexed with liposome containing 0.72 mg/kg PD0325901 every other day (five injections total).	intratumoral	cancer	Co-delivery of Mcl1 siRNA and PD0325901 (MEK inhibitor) prevented tumor growth by 79 % in KB tumor xenografted mice with significant tumor cell apoptosis.	[241]
CGKRK modified-Frax-loaded PLA NPs + siKRAS-LCP- ApoE3	nude mice	Preinjection with Frax-NP-CGKRK once a day for four times, and then administration with both formulations every other day for three times. (20 mg kg^{-1} fraxinollen, 0.36 mg kg^{-1} siKras)	Intravenous	Pancreatic cancer	As a result of sequential combination therapy, CGKRK-Frax-loaded PLA NPs modified TME by reducing fiber collagen, preventing the fibronectin expression, and normalizing the blood vessels, following siKras-LCP- ApoE3 suppressed the KARAS gene and destroyed tumor cells. Also, tumor volume and longest survival time were observed in mice treated with combination therapy than single formulation treated group.	[242]
Mannose modified-Trp 2 peptide and CPG ODN loaded LCP and TGF-β siRNA LPH	female C57BL/6 mice	LCP vaccination on day 4 and 13 + LPH NP containing siRNA (0.6 mg/kg) on days 13, 15, and 17	Intravenous	Cancer	SiRNA delivery using LPH NPs suppressed TGF-β expression by 50 % in the advance stage of tumor microenvironment. TGF-β suppression augmented the LCP vaccine efficacy	[256]

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Table 4 (*continued*)

Formulation	Animal model	Dose	Administration route	Diseases	Effect	Ref
					and prevented tumor proliferation by about 52 % compared with vaccine therapy alone.	
iRGD-SLN	C57BL/6 female mice	*retro*-orbitally injected with 75 μg (in 100 μL of PBS), 3 days after irridation	*retro*-orbitally injection	Glioblastoma	Mice treated with radiation therapy and iRGD-SLN loaded with EGFR/PDL1 siRNA showed the most therapeutic outcome with decreased tumor growth at day 21 and elongated mouse survival.	[243]
Anti-EGFR aptamer-liposome-Quantum dot (Apt-QLs)	female BALB/c nude mice	15 mg lipid/kg	intravenous	Cancer	*In vivo* imaging of MDA-MB-231-xenografted mice treated with Apt-QLs demonstrated a higher fluorescent signal in the tumors compared with the mice received non-targeted QLs.	[244]
^{111}In-LCP	Athymic nude (nu/nu) mice and wild type C57BL/6 mice	10 μL	Intravenous and intramuscular	–	Small size, PEGylated lipid surface, and negative charge of LCP NPs led to tissue penetration, lymphatic system entrance, and lymph node accumulation via lymphatic drainage.	[257]
SiRNA-CPPs-magnetic and thermo sensitive liposome (TML)	Female nude mice	1.2 mg/kg siRNA (every other day for 10 days)	intravenous	Brest cancer	c-mycSiRNA-CPPs/TML under alternating current (AC) magnetic field demonstrated improved *in vivo* targeted delivery efficacy, anti-tumor efficacy, and gene silencing efficiency in the MCF-7 tumor mice model.	[248]
NLC	SKH1-hr hairless mice	twice weekly for three weeks, starting after bleomycin administration at a dose of 1.5 U/kg	inhalation	idiopathic pulmonary fibrosis	PGE2/MMP3, CCL12, HIF1Alpha siRNAs/NLC remarkably reduced mouse body mass, confined hydroxyproline amount in the lung, suppressed the protein expression, and prohibited fatality and lung tissue damage after local inhalation delivery. These results were more efficient than using NLC with PEG2 or siRNA alone.	[258]
LHRH-cationic NLC	athymic nu/nu mice	2.5 mg/kg paclitaxel and 170 μg/kg siRNA for intravenous injection	Intravenous or inhalation		Upon inhalation of NLC formulations, NPs delivered their cargo into lung cancer cells with no hurting healthy tissues and increased antitumor activity compared with intravenous administration of paclitaxel.	[250]
NLC	HRS/J mice	100 μL	tropical	psoriasis	*In vivo* results showed a reduction in the TNF-α expression by 7-fold and a synergistic effect between TNF-α and tacrolimus.	[251]

LPD; Liposome-polycation-DNA complex; TfRscFv; transferrin receptor single-chain antibody fragment, EpCAM; epithelial cell adhesion molecule, Eph; anti-EphA10 antibody, LPC; liposome-protamine- chondroitin sulfate, nanoparticles, SNALP; hydroxide, CD-siRNA; cell death siRNA, PD-1; programmed cell death protein 1, LCCP; lipid coated calcium phosphate/carbonate hybrid NPs, SNALP; stable nucleic acid lipid particles, PLK1; polo-like kinase 1, KSP; kinesin spindle protein, LDCP; lipid-dendrimer-calcium-phosphate, PARP1; DNA repair enzyme poly(ADP-ribose) polymerase1, GAPDH; glyceraldehyde 3-phosphate dehydrogenase, PCL; PEGylated cleavable lipopeptide, MMP; matrix metalloproteinase, BACE1; Beta-site amyloid precursor protein cleaving enzyme 1, MutAtax3; mutant ataxin-3, RVG; virus glycoprotein, SAT1; Spermidine/spermine N1-acetyltransferase 1, DoGo; biocompatible short peptidomimetics, pPB; cyclic oligopeptide with amino acid sequence C*SRNLIDC*, HSP47, heat shock protein 47, HMGB1; High mobility group box-1, MEND; multifunctional envelope-type nanodevice, HBV; Hepatitis B virus, PKL1; Serine/threonine polo-like-kinase 1, PRK2; protein kinase C-related kinase 2, pyropheophorbide phosphatydic acids,

it would make sense to considered lipid-based delivery systems for means for siRNA delivery to liver [320,321]. Also, the discontinuous structure of the hepatic vasculature leads to the accumulation of LNPs in the liver, even without the use of targeting moieties [207].

A study conducted by Jia et al has shown the success of polypeptide pPB-modified SNALPs to specifically deliver siRNA against heat shock protein 47 to the liver for hepatic fibrosis treatment [188]. This success was obtained due to the valuable role of the pPB ligand to target PDGFR-β receptors on the hepatic stellate cells (HSC), promoting considerable liver distribution and improved hepatic fibrosis symptoms in mice models.

Also, vitamin E-coupled liposomes carrying matrix metalloproteinase–2 (MMP-2) suggests that suppressive action against MMP-2 expression in HSC-T6 cells could be crucial in preventing the progression of hepatic fibrosis [322].

Liver fibrosis and liver inflammation are two important factors in cirrhosis development. [323].

Given the role of (HMGB1) protein in the development of inflammation and liver fibrosis, inhibiting its activity represents a valuable mechanism of treatment for cirrhosis treatment [190].

To target liver macrophages through mannose receptor mediation, a mannose-modified HMGB1-siRNA-loaded stable nucleic acid lipid particle delivery system (mLNP-siHMGB1) was developed. This allowed for the silencing of HMGB1 protein expression and the treatment of NASH (Fig. 11). The researchers also explored the impact on NASH of co-administration of the unsaturated fatty acid docosahexaenoic acid (DHA). The outcomes demonstrated that mLNP-siHMGB1 could effectively silence the HMGB1 gene, decrease the amount of HMGB1 protein released in the liver, control the phenotype of liver macrophages to be an anti-inflammatory M2, decrease hepatic lobular inflammation and bullous steatosis in the liver, and return the liver function of NASH model mice to normal. Following eight weeks of combination mLNP-siHMGB1 and DHA treatment, NASH model mice's liver function improved [191].

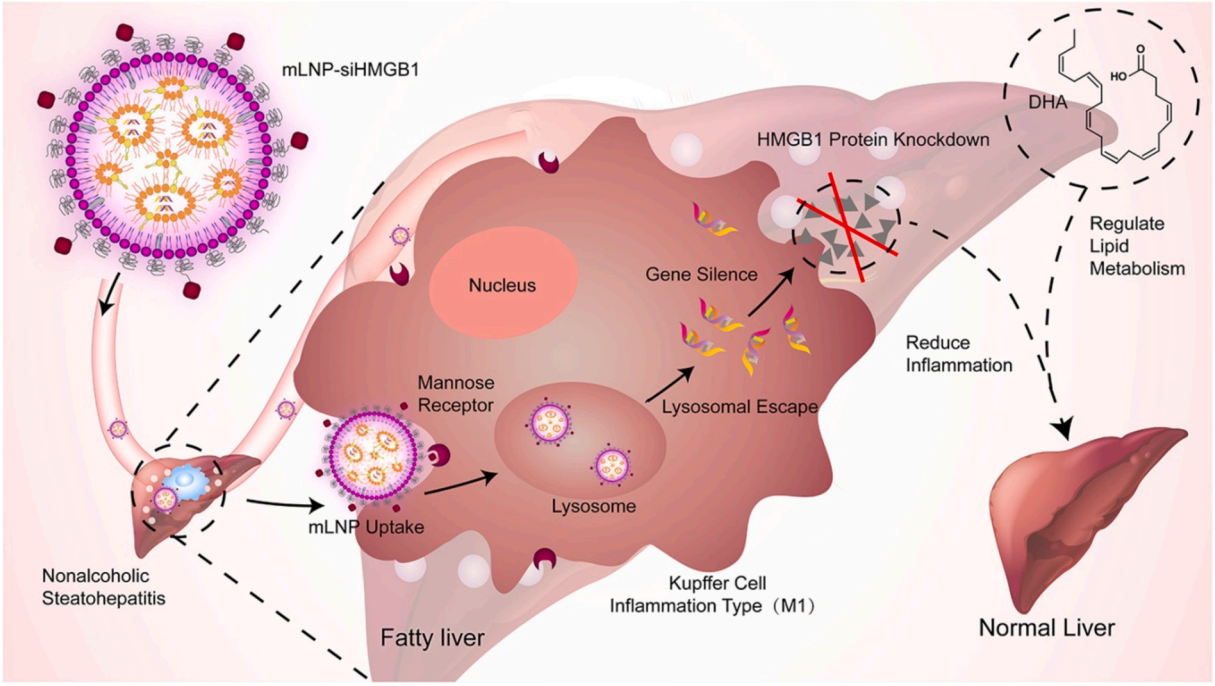

Fig. 11. Mannose-modified HMGB1-siRNA loaded stable nucleic acid lipid particle delivery system (mLNP-siHMGB1) targeting the liver macrophages with mannose receptor mediation, thereby silencing HMGB1 protein expression and treating NASH.
Reproduced with permission from [191].

[191] Similar to this, pPB-modified and HMGB1 siRNA-loaded SNALPs were constructed by Zhang et al., based on the dual functionality of anti-fibrotic and anti-inflammation [190]. The aforementioned formulation showed more potent therapeutic efficacy in thioacetamide (TAA)-induced liver cirrhosis mice model than pPB modified-HSP47siRNA loaded SNALP with only antifibrotic effect, confirming the influential role of anti-inflammatory effect in cirrhosis treatment.

3.4. Viral diseases

Hepatitis B virus (HBV) is a hepatotropic and noncytopathic virus that belongs to the epadnaviridae family and causes both acute and chronic hepatitis in human [324]. This virus infects the liver and causes complex interactions with the host, leading to a range of clinical manifestations including an asymptomatic carrier state, hepatitis, and liver cancer with or without cirrhosis [325]. Clinically available antivirals used to treat hepatitis B, such as Interferon or PEGylated Interferon and nucleoside/nucleotide analogs (NAs) reverse transcriptase inhibitors (e.g., telbivudine, lamivudine, entecavir (ETV), adefovir, tenofovir alafenamide (TAF), tenofovir disoproxil (TDF) have been found to suppress HBV replication and reduce the progression of liver diseases, but they reportedly result in limited efficacy and viral resistance [326]. Therefore, focusing on suppression of expression of viral antigens and main factors involved in HBV by RNAi therapy appears to be a promising option for HBV treatment.

Fig. 12 illustrates the strategy of combining glycyrrhizic acid (GA) and polyene phosphatidylcholine (PPC) to create lipid nanoparticles known as GA/PPC-modified LNPs. These LNPs have the capacity to

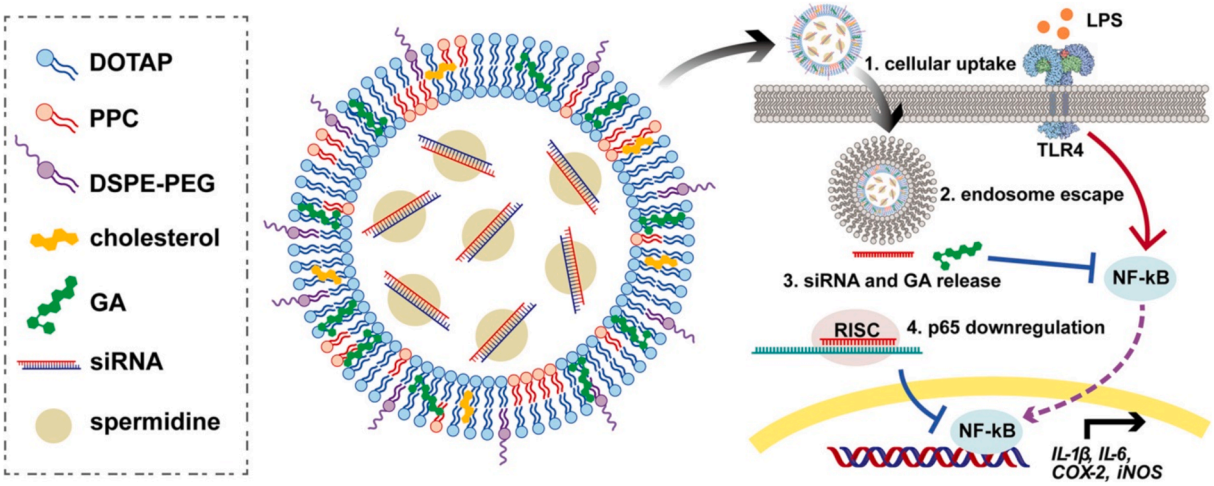

Fig. 12. Incorporating glycyrrhizic acid (GA) and polyene phosphatidylcholine (PPC) into lipid nanoparticles was developed, which has been demonstrated capable of promoting cellular uptake, enhancing gene-silencing, reducing cytotoxicity and improving siRNA stability.
Reproduced with permission from [391]

enhance gene-silencing, decrease cytotoxicity, and improve siRNA stability. Liver histology, hematological analysis, and pro-inflammatory cytokine analysis all demonstrate that acute liver injury is lessened by GA/PPC-modified LNP and siRNA lipoplex targeting NF-κB, a critical mediator of inflammation. Additionally, intracellular delivery of antisense oligonucleotides (ASOs) and mRNA that suppress viral infection is demonstrated by GA/PPC-modified LNPs. Finally, GA/PPC-modified LNPs represent a potentially effective delivery method for treatments based on nucleic acids [327].

Yamamoto et al reported that a single dose of HBV siRNA-loaded YSK13-C3-MEND continuously for 14 days suppressed the expression of HBsAg or HBeAg *in vitro* and chimeric mice with humanized liver infected with HBV [192]. Also, it was shown that a single injection of GalNAc-modified PEG-coated YSK13-C3-LNP-siRNA considerably repressed the HBV-genomic DNA and their antigens in chimeric mice infected with HBV which was more effective than unmodified LNPs [204].

In another study, a novel ionizable lipidoid (LC8) was employed to deliver siRNAs targeting HBV gene or apolipoprotein B (APOB) into the hepatocytes [205]. The results indicated that the aforementioned liposomes were able to effectively deliver siRNAs into hepatocytes in mice model and suppress targeted gene expression via RNA interference mechanism, highlighting its potential as a therapeutic agent for hepatitis B treatment.

Serine/threonine polo-like-kinase 1 (PKL1) is a critical factor in HBV infection and is expressed at higher levels in various human cancers. The importance of PKL1 in mediating HBV-induced HCC has been the focus of a study conducted by Foca et al, in which PKL1siRNA-loaded LNPs decreased the secreted HBV DNA (viral particles) in HBV-infected primary human hepatocytes [193].

A study reported that SNALP formulating HBV siRNAs efficiently delivered siRNAs to mouse liver and reduced HBV DNA level. The improved efficacy was attributed to the extension of half-life in plasma and liver and the reduction of immunostimulatory adverse effects and toxicity.

[194]. Hepatitis C virus (HCV), a positive-strand RNA virus belonging to the Flaviviridae family, primarily infects hepatic cells, leading to acute and chronic forms of liver disease [328]. Direct-acting antiviral agents (DAAs) and host-targeting antivirals (HTAs) are the two main classes of hepatitis C antiviral agents [329]. Currently, available DAAs targeting HCV nonstructural protein (NS)3 protease, NS5A, and NS5B RNA-dependent RNA polymerase (RdRp) have certain limitations such as additional side effects, viral resistance, and limitations to specific genotypes [330]. HTAs target the host factors required for viral replication and are regarded as a promising strategy for potentially addressing drawbacks of existing treatments and developing broad-spectrum antiviral therapies [331]. Protein kinase C-related kinase 2 (PRK2) as a proviral host factor necessary for HCV replication seems to be an ideal target for siRNA-based HCV therapy [332]. Correspondingly, two studies verified that systemic administration of lipidoid nanoparticles delivering PRK2 siRNA successfully inhibited HCV replication *in vivo* [195,207].

Also, Apolipoprotein A-I (apo A-I)-cationic liposomes [206] and vitamin E-coupled cationic liposomes [196,333], MEND [208] were known as a suitable nanocarriers for siRNA delivery to liver and hepatitis C treatment.

In the context of Human immunodeficiency virus (HIV) infection treatment, lymphocyte function-associated antigen-1 (LFA-1) modified immunoliposomes encapsulating anti-CCR5 (chemokine receptor 5) siRNA [209], a multifunctional chitosan-lipid nanocomplexes delivering plasmids encoding siRNAs [197], and neutraplex nanolipoplexes containing chemokine receptor type 4 (CXCR4) siRNA [198] were found to have the potential *in vivo* application against HIV infection

Ebola virus disease (EVD) is a serious and potentially fatal condition caused by the Ebola virus. EVD can be transmitted through direct contact with blood, bodily fluids, or objects that contaminated with the virus [334]. A phase II clinical trial evaluating an anti-Ebola virus siRNA-loaded LNPs (TKM-130803) reported safe, but unsuccessful results, probably due to the high viral dose and existing organ injury in the end-stage patients [335].

A combination of siRNAs targeting the polymerase (L) gene of the Zaire species of EBOV (ZEBOV) were either encapsulated in SNALP or complexed with polyethylenimine (PEI) [210].Guinea pigs treatment with two systems proved that a pool of the L gene-specific siRNAs delivered by SNALP was more efficient when administrated shortly after the ZEBOV infection. Subsequently, the evaluation of the combination of modified non-immunostimulatory siRNAs targeting the ZEBOV L polymerase (EK-1 mod), viral protein (VP) 24 (VP24-1160 mod), and VP35 (VP35-855 mod) formulating in SNALPs provide complete protection against ZEBOV-induced hemorrhagic fever in non-human primates [211]. Also, another study using Sudan Ebola virus VP35 siRNA-LNPs displayed significant survival benefits, along with rapid control of the viral replication in fighting lethal *Sudan ebolavirus* infection in non-human primates [212]. The use of a single nucleoprotein–targeting (NP-targeting) siRNA-loaded LNPs in non-human primates has shown a high survival rate at advanced stages of Marburg virus (MARV) and Ravn virus (RAVV) diseases, indicating the potential for broad-spectrum therapy against both MARV and RAVV [213].

Severe Acute Respiratory Syndrome (SARS) coronavirus 2 (SARS-CoV-2), A new coronavirus strain, has caused the coronavirus diseases 2019 (COVID-19) pandemic, leading to a global health emergency with a far-reaching impact worldwide [336]. SiRNA has demonstrated significant potential as a safe and specific option against COVID-19 by interfering with viral mRNA and inhibiting viral replication [337].

The encapsulation of siRNA targeting ultra- conserved regions of the SARS-CoV-2 virus into a novel LNP displayed potent suppression of the virus in the lungs and improved mouse survival rates following IV administration [338].

3.5. *Inflammatory diseases*

The progression and management of inflammatory diseases including cardiovascular diseases, autoimmune and neuropsychiatric disorders, allergic, and cancer are closely affected by the dysregulation of the immune system [339]. RNAi therapy that is based on siRNA is a potentially effective approach for reducing the expression of specific genes involved in inflammatory processes [340,341]. Concerning the treatment of inflammatory diseases, LNPs can transfer siRNA to splenocytes and attenuate inflammatory responses. For example, liposome coated with a specific antibody targeting integrin β7, and loaded with anti-cyclin D1 siRNA was assessed in a model of dextran sulfate sodium (DSS)-induced colitis [342].

Bangen and colleagues have applied a lipoplex system to target inflamed liver cells, observing an increase in leukocytes through using an anti-cyclin E1 siRNA for specific targeting [343]. Macrophages and dendritic cells (DCs) have been linked to inflammatory and autoimmune diseases. Activated macrophages are capable of producing several types of inflammatory cytokines and DCs are professional antigen-presenting cells that play a significant role in initiating autoimmune and malignant diseases by provoking chronic inflammation in the body [344,345]. Several reports have found that siRNA-incorporated LNPs effectively caused a knockdown effect on macrophages and DCs [346–360].

In a study, Dan Peers and colleagues demonstrated successful targeting of Ly6C + myeloid leukocytes, including all myeloid cells except neutrophils, via siRNA against Interferon regulatory factor 8 (IRF8) mRNA, as a strategy for reducing inflammation [355]. Prior attempts to transfect lymphocytes via the use of cationic liposomes have failed during both pre-clinical and clinical trials, as a result of the unique composition of the plasma membrane of these cells, which differs from that of other cell types [356]. This may be attributed to lack or low levels of negatively charged molecules, such as heparin sulfate proteoglycans, leading to an abundance of positively charged entities on their surface

[356]. It was reported that c-Rel targeting siRNA-encapsulated in distearoyl-phosphatidylserine (DSPS)-containing nanoparticles were shown to be effective in transfecting macrophages, neutrophils, and lymphocytes with low toxicity to cells [357].

Rheumatoid arthritis (RA) is a chronic, inflammatory, and potentially debilitating auto-immune illness associated with systemic complications and an increased risk of early mortality [358]. RA is characterized by bone and cartilage destruction, synovial inflammation and hyperplasia, autoantibody production, and systemic complications including psychological, pulmonary, skin, cardiovascular and skeletal diseases [359]. During conditions of RA inflammation, multiple molecules, including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interleukin-17 (IL-17), and hypoxia-inducible factor-1 alpha (HIF-1α), are synthesized and released, potentially leading to bone loss [360]. In this context, acid-sensitive SLN/TNF-α siRNA [214], lipoplexes containing TNF-α siRNA [199] and IL-1, IL-6. IL-8 siRNAs [215], Lipidoid-Polymer Hybrid NPs with IL-1ß [216] or TNF-α siRNA [361], wrapsome consist of a core cationic lipid bilayer and TNF-α siRNA complex enveloped in a neutral lipid bilayer [362] were proposed. The findings from studies have provided evidence that the mentioned formulations can play a therapeutic role in RA through the effective knockdown of inflammatory molecules.

Fibroblast-like synoviocytes (FLSs) are a critical component of the hyperplastic synovial pannus in RA, which is responsible for cartilage and bone damage. FLSs exhibit abnormal behaviors, such as excessive proliferation, migration, invasion, and the secretion of inflammatory cytokines, like IL-1β, IL-6, IL-8, and TNF- α [363]. Therefore, Wang et al, developed a CPP-conjugated LPD loaded with ribonucleotide reductase M2 (RRM2), aiming to enhance the apoptosis level and suppress FLS proliferation [364].

Atherosclerosis is a chronic inflammatory cardiovascular disease characterized by abnormal accumulation of lipids, immune cells, and fibrous tissue within artery walls, leading to the development of atherogenic plaques that obstruct blood flow and increase the risk of cardiovascular diseases [307]. Sageret et al. prepared lipid nanoparticles (LNPs) made of a polyethyleneimine lipid derivative and encapsulated a mixture of siRNAs targeting 5 adhesion molecules (ICAM1, ICAM2, VCAM1, SELE, and SELP) to combat immune-mediated inflammatory responses associated with atherosclerosis [365]. The silencing of specific genes through the systemic administration of mentioned LNPs can lead to a substantial decrease in the recruitment of neutrophils and monocytes to the endothelial wall of the blood vessels in the context of myocardial infarction [366]. Also, Lipid-based nanoparticles containing CCR2 siRNA prevented the recruitment of monocytes and reduced inflammation in mice model [367]. A genome-wide analysis performed in 2003 by Abifadel et al. found that a loss-of-function mutation in the PCSK9 gene was responsible for autosomal-dominant hyper-LDL cholesterol. This finding emphasized the potential of PCSK9 as a promising target for the development of medication to manage lipid levels [368]. Lipidoid- loaded with PCSK9 siRNA efficiently reduced plasma LDL cholesterol (LDLc) after 3 weeks post-injection in rodent and monkey [217].

Table 5
Ongoing clinical trials of siRNA-LNP therapies.

Name	Carrier	Target	Administration Route	Disease	Status	Company	NTC number
CALAA-01	Cyclodextrin nanoparticles	RRM2	intravenous	Advanced solid tumors	Phase I/ Terminated	Calando Pharmaceuticals	NCT00689065
siG12D LODER	Polymeric NPs (LODER)	KRAS G12D	EUS biopsy injection	Pancreatic Ductal Adenocarcinoma, Pancreatic Cancer	Phase I/ Completed PhaseII/ Ongoing	Silenceed Ltd	NCT01188785 NCT01676259
ALN-VSP02	SNALPs	KSP and VEGF	intravenous	Solid tumors	Phase I/ Completed Phase I/ Completed	AlnylamPharmaceuticals	NCT 00,882,180 NCT01158079
TKM- PLK1 (TKM-080301)	SNALPs	PLK1	intravenous	Advanced Hepatocellular Carcinoma	Phase I/II Completed	Arbutus Biopharma Corporation	NCT02191878
Atu027	Cationic LNPs	PKN3	intravenous	Advanced or MetastaticPancreatic Cancer(II) , SolidTumors (I)	Phase I Phase II/ Completed	Silence Therapeutics GmbH	NCT00938574 NCT01808638
DCR-PHXC-101	LNPs	Oncogene MYC	intravenous	Solid Tumors, Hepatocellular Carcinoma, Multiple Myeloma, NonHodgkins Lymphoma, Pancreatic Neuroendocrine Tumors	Phase I Phase Ib/ 2/ Terminated	Dicerna Pharmaceuticals, Inc.	NCT02110563 NCT02314052
EphA2-siRNA-DOPC	Liposome	EphA2	intravenous	Advanced Malignant Solid Neoplasm	Phase I/Not completed yet	M.D. Anderson Cancer Center	NCT01591356
NBF-006	LNPs	GSTP	intravenous	Non-Small-Cell Lung, Colorectal, and Pancreatic Cancer	Phase I/ Recruiting	Nitto BioPharma, Inc.	NCT03819387
ALN-PCS02	LNPs	PCSK9	intravenous	Elevated LDL-cholesterol	Phase I/ Completed	Alnylam Pharmaceuticals	NCT01437059
ND-L02-s0201	Liposome	HSP47	intravenous	Hepatic fibrosis	Phase I /Completed	Bristol-Myers Sqyubb Pharmaceuticals	NCT02227459
ARB-001467	LNPs	HBsAg	intravenous	Hepatitis B, Chronic	Phase I /Completed	Arbutus Biopharma Corporation	NCT02631096
PRO-040201	SNALPs	ApoB	intravenous	Hypercholesterolemia		Arbutus Biopharma Corporation	NCT00927459

RRM2; ribonucleotide reductase protein, EUS; endoscopic ultrasound, KSP; spindle protein, VEGF; vascular endothelial growth factor, PLK1; polo-like kinase 1, PKN3; protein kinase N3, GSTP; glutathione S-transferase P, PCSK9; Proprotein convertase subtilisin/kexin type 9, HSP47;heat shock protein 47.

3.5.1. siRNA-based lipid nanoparticles in clinical and ongoing clinical trials

siRNA-based lipid nanoparticles as an innovative modality have opened the way to clinical trials and broadened the new horizon to modern remedies. As illustrated in Table 5, numerous clinical t rials for treating various diseases are progressing through various phases.

Patisiran (ONPATTRO®) is the earliest FDA approved LNP-based nucleic acid drug, in which siRNA is incorporated into LNP to silence the expression of the protein transthyretin in the liver and treat the hereditary transthyretin-mediated amyloidosis [82]. ILs of dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA) in the formulation of Onpattro™, or the lipid H (SM-102) or ALC-0315 in the Moderna and BioNTech/Pfizer COVID-19 vaccines, respectively, were used to improve their pH-dependent endosomal escape [369]. Also, targeted lipid nanoparticles have been engineered to deliver their payloads specifically to specific cell populations and to increase retention in particular cell types, thus eliminating off-target effects via specific biochemical interactions with appropriate receptors and ligands [276]. Fitusiran is another example of how conjugation chemistry advances the clinical progression of targeted siRNA delivery and merits inclusion is the attachment of siRNA to ligands that specifically bind to receptors overexpressed on cancer cells. A synthetically devised siRNA-based therapeutic molecule fitusiran is a vital prophylactic treatment for bleeding episodes in individuals with hemophilia A and B. When intricately conjugated with a targeting moiety, such as a triantennary N-acetylgalactosamine, it allows the attached genetic moiety to specifically bind to anti-thrombin mRNA, thereby reducing antithrombin production. Studies conducted on mice have shown that when fitusiran is administered through subcutaneous injection, it leads to a significant and long-lasting reduction in antithrombin levels in both normal mice and those with haemophilia. The corresponding reduction in antithrombin levels has been attributed to an elevation in thrombin production and enhanced hemostasis. The devised siRNA-based therapeutic molecule is currently being tested in Phase 3 clinical studies to determine its efficacy and safety in patients with haemophilia A or B who are devoid of inhibitory antibodies to Factor VIII or IX [370].

In 2008, CALAA-01 was introduced as the pioneering drug to enter clinical trials for cancer treatment, exploiting the innovative modality of siRNA-mediated nanoparticles [371]. Calando Pharmaceuticals developed a Tf-modified cyclodextrin-based polymer NP containing a siRNA targeting the M2 subunit of the ribonucleotide reductase protein (RRM2). The trial was stopped in 2012.

Biodegradable LOcal Drug EluteR copolymer (LODER) embedded by anti-KRAS siRNA (siG12D) was evaluated by Silenseed Ltd with the aim of slow and prolonged-release local delivery system [372]. The data from the phase 1 trial showed that a single dose of siG12D, administered directly into the pancreas via endoscopic ultrasound (EUS) biopsy injection, along with standard chemotherapy agents, showed favorable safety and efficacy. The trial used dosages of 0.025 mg, 0.75 mg, and 3 mg in patients with non-metastatic advanced pancreatic ductal adenocarcinoma (PDA). notably, there were no symptom of dose-limiting toxicities, even at the highest dose of 3 mg/kg. Then, the phase II trial was commenced in 2017 in patients with unresectable local PDA treated with repeated doses of 3 mg siG12D LODER with gemcitabine and nab-paclitaxel. This phase is still ongoing (NCT01676259) [373].

Alnylam Pharmaceuticals, which collaborated with Tekmira in 2009, designed a SNALP formulation (ALN-VSP02) containing a combination of two siRNAs targeting KSP (spindle protein) and VEGF (vascular endothelial growth factor) genes in Patients with advanced solid tumors with liver involvement [374]. In the Phase I clinical trials, the drug was successfully tolerated at the highest dose of 0.1 to 1.5 mg/kg, administered by I.V. infusion (NCT 00882180). Then, an expansion study was started and completed in patients who had responded to ALN-VSP02 treatment (NCT01158079).

Tekmira Pharmaceuticals fabricated SNALP nanocarriers encapsulated PLK1 (polo-like kinase 1) aimning to treatment of patients with solid tumors involved in primary or secondary liver cancer (TKM-080301) [375]. I.V. administration of TKM-080301 showed an accepted safety profile in Phase I/II clinical trials for advanced hepatocellular carcinoma, while no sign of improved overall survival was observed (NCT02191878). Also, a Phase I clinical trial investigated by the intratumor administration of TKM-080301 for primary or secondary liver cancer, but the data were not reported (NCT01437007) [62].

In 2014, a cationic lipoplex nanocarrier called Atu027 was produced by Silence Therapeutics GmbH to silence the expression of protein kinase N3 (PKN3) for patients with advanced solid tumors [376]. I.V. administration of Atu027 at doses of up to 0.18 mg/kg in 24 patients in a dose-escalation phase I clinical trial resulted in disease stabilization or pulmonary metastasis regression without any dose-limiting toxicities. Additionally, a phase Ib/IIa trial was initiated in 2013 to test the efficacy of Atu027 in combination with gemcitabine for treating advanced pancreatic adenocarcinoma in 29 patientes [377]. (NCT01808638).

DCR-PHXC-101 was fabricated by Dicerna Pharmaceuticals, where DCR-MYC (also known as DCR-M1711) as a Dicer substrate siRNA (DsiRNA) targeting Myc was encapsulated into an EnCoreTM lipid nanoparticle (NCT02110563) [378]. In 2014, the phase I trial was conducted to treat solid tumors, multiple myeloma, non-Hodgkin's Lymphoma, and pancreatic neuroendocrine tumors. The trial showed desirable safety profiles and satisfying clinical outcomes. The company found the evaluation of DCR-PHXC-101 for hepatocellular carcinoma in a Phase Ib/II clinical trial unacceptable in 2015, prompting the termination of the DCR-MYC trials (NCT02314052) [379].

EphA2-siRNA encapsulated into DOPC-based liposomal formulation is the most recent anticancer siRNA-mediated nanoparticle under investigation in phase 1 for patients with advanced and recurring solid tumors.

A DOPC-based liposomal formulation targeting the tyrosine kinase EphA2, known as EPHARNA (EphA2-siRNA-DOPC), is the latest anticancer siRNA-mediated nanoparticle currently under investigation in a phase 1 trial for patients with advanced and recurrent solid tumors (NCT01591356). EPHARNA started its phase 1 trial in 2015, and it is still ongoing [380].

Another lipid nanoparticle (LNP) formulation containing siRNA that targets glutathione S-transferase P (GSTP) is referred to as NBF-006. The preclinical study proved the efficacy and well-tolerability of NBF-006 in the KRAS mutant non-small-cell lung cancer (NSCLC) xenograft model in animal models [381]. Therefore, this formulation was tested in 2019 in Phase I/Ib clinical trials on patients with non-small cell lung cancer and pancreatic and colorectal cancer (NCT03819387) [380].

Alnylam Pharmaceuticals formulated a LNP formulation encapsulated with proprotein convertase subtilisin/kexin type 9 (PCSK9) siRNA called ALN-PCS02 for the treatment of hypercholesterolemia. the phase 1 stusy was completed with promisimg results of reduced levels of low-density lipoprotein [382].

A vitamin A-modified liposomal formulation called ND-L02-s0201 composed of O′-ditetradecanoyl-N-(α-trimethylammonioacetyl) diethanolamine chloride (DC-6–14), cholesterol, and DOPE (4:3:3, molar ratio) encapsulated siRNA targeting heat shock protein 47 (HSP47) was tested for hepatic fibrosis treatment. The phase 1 trials demonstrated the well-tolerability of this formulation (NCT02227459) [383].

Arbutus Biopharma Corporation employed the LNP formulation to encapsulate HBsAg siRNA (ARB1467) for treating HBV (NCT02631096) [384]. ARB1467 prevented HBV replication and reduced the antigens detected in the serum.

In 2009, Tekmira evaluated the effectiveness of siRNA loaded in a stable nucleic acid LNP targeting ApoB produced by hepatocytes to regulate the cholesterol level in the blood (PRO-040201). In a Phase 1 clinical trial, effective delivery of siRNA to the liver was demonstrated, resulting in reduced levels of low-density lipoprotein. However, flu-like symptoms were reported at the highest dose, leading the company to decide to stop the study in 2011 (NCT00927459) [46].

3.5.2. Challenges faced with clinical translation of siRNA-LNP

Although enormous efforts have been made in siRNA-based LNP development, the formulation of LNPs for RNA therapeutics must progress beyond research applications to demonstrate their potential for clinical translation and market entry. There are several obstacles that need to be addressed in order to successfully apply siRNA-LNPs in clinical practice.

One of the obstacles to why siRNA-LNPs do not stand at their deserved position in the clinical and market may be due to the low knowledge about their molecular and cellular mechanisms and how NPs deal with the body. Expanding our understanding of LNP structure, uptake, and release, as well as a thorough review of the properties of RNA molecules, provides an excellent opportunity for more precise rational design of LNP-formulations for siRNA and RNA drugs alike [385]. Animal studies cannot replace human trials, and their findings are not easily applicable to humans. Therefore, careful optimization of clinical trials appears to be essential to achieve more comprehensive and clinically desirable outcomes [380]. It is important to understand that producing large quantities of nanoparticles for human trials, rather than relying solely on animal studies, may alter the behavior and function of these nanoparticles. Thereby, some modifications, such as improved preparation techniques, physicochemical properties, and safety considerations of the nanoparticles, must be considered [386]. The majority of RNA-LNP candidates do not proceed with clinical trials or fail to complete them due to safety concerns and toxicity observed in non-human primates and humans. To overcome these challenges and improve the successful translation of RNA nanoparticles (RNA-NPs) into clinical applications, developing a suitable procedure for forming stable complex RNA-LNP with low surface charge is essential. Furthermore, RNA-NPs must exhibit high stability and maintain a diameter of less than 100 nm to ensure optimal cellular uptake and be produced through established large-scale, sterile processes [387].

The effective use of siRNA therapeutics in clinical settings will be enhanced by drug-loaded delivery systems that ensure stability and potency over extended periods under ambient conditions. It is important to determine the best method for storing LNPs after production in order to retain their efficacy. Various factors, such as temperature, pH, lyophilization, and excipients, may affect the stability of LNP delivery systems [388].

Initially, it was believed that mRNA-LNP COVID-19 vaccines needed to be stored at temperatures of −20 °C to −80 °C. However, a stability study has demonstrated that these formulations can remain stable at refrigerator temperatures (2 °C to 8 °C) for up to one month [389].

Although freeze-drying or lyophilization is the best known process to remove water during production of nanoparticle and improvement of their stability, but they have some boundaries associated with aggregation reduction in gen silencing efficacy upon reconstituted with DI water [388].

Freeze-drying has yielded paradoxical results in reducing gene silencing efficacy. In some instances, freeze-dried LNPs retained 80 % of their gene silencing activity, compared to 87 % for fresh LNPs [62].

Using lyoprotectants improved stability and reduced stresses on the LNPs during lyophilization's freezing and drying steps. Trehalose and sucrose, disaccharide lyoprotectants, maintained the gene silencing activity of lyophilized LNPs without requiring ethanol for reconstitution [388].

4. Conclusion

RNA-LNP therapy has been established as a reliable and effective treatment and preventive option for numerous diseases in clinical and pre-clinical settings, as exemplified by the approved medication Onpattro® and the two mRNA-based COVID-19 vaccines developed by Pfizer-BioNTech and Moderna. Regarding this perspective, enormous research has been centered on siRNA-LNPs as a salient option for treatment of various diseases such as cancer, liver, viral, inflammatory, and neurological disorders.

Lipid-based NPs as stable, safe and potent delivery vehicles successfully delivered siRNA to target regions, while stabilizing and entrapping siRNA in their structure and protecting them from the harsh environment. This review provides a comprehensive insight into the application of various lipid-based NPs such as liposomes, LCP, SLN, NLC, lipidoids and MEND-formulated siRNA molecules for therapeutic purposes.

Surface modification of LNPs with various ligands like aptamer, antibodies, peptides, and hydrophilic protective PEG layer not only provides specificity and stability against immune cells but also enhanced cellular uptake of siRNA through the process of receptor-mediated endocytosis, thereby resulting in enhanced transfection efficacy *in vitro* and *in vivo* compared with unmodified LNPs. Combination therapy has marked a novel chapter in the realm of siRNA-based therapies, heralding impressive levels of therapeutic efficacy that are difficult to attain with traditional treatment options. The significant progress in nanocarrier technology and the concurrent advancement in chemical modifications may enable the translation of increasing numbers of siRNA drugs from scientific research to clinical practice with greater feasibility. LNPs represent a promising prospect, as they have demonstrated satisfactory performance in pre-clinical trials while providing efficient nucleic acid protection and delivery capabilities. However, their translation to provide benefit to human patients has been limited, with only a fraction of formulations progressing to Phase II clinical trials. Thus, more investigations and researches are required to confirm their potential.

Authors' contributions

AA and FVL wrote the main manuscript text, and PK, SK, and AS revised the manuscript. PK and AS proofread and supervised during the writing of the original manuscript. All authors approved the manuscript.

CRediT authorship contribution statement

Anis Askarizadeh: Writing – original draft. **Fatemeh Vahdat-Lasemi:** Writing – original draft. **Sercan Karav:** Writing – original draft. **Prashant Kesharwani:** Writing – review & editing, Supervision, Conceptualization. **Amirhossein Sahebkar:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

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

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