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Lipid-based nanotherapeutics for siRNA delivery

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RNA interference (RNAi) is a specific gene-silencing mechanism triggered by small interfering RNA (siRNA). The application of RNAi in the clinic requires the development of safe and effective delivery systems. Inspired by progress with lipid-based systems in drug delivery, efforts have been dedicated to the development of liposomal siRNA delivery systems. Many of the lipid-based delivery vehicles self-assemble with siRNA through electrostatic interactions with charged amines, generating multi-lamellar lipoplexes with positively charged lipid bilayers separated from one another by sheets of negatively charged siRNA strands. Internalization of lipid-based siRNA delivery systems into cells typically occurs through endocytosis; accordingly, delivery requires materials that can facilitate endosomal escape. The size of the carrier is important as carriers <100 nm in diameter have been reported to have higher accumulation levels in tumours, hepatocytes and inflamed tissue, whereas larger particles tend to be taken up by Kupffer cells or other components of the reticuloendothelial system (RES). To reduce RES uptake and increase circulation time, carriers have been modified on the surface with hydrophilic materials, such as polyethyleneglycol. Herein, we review the molecular and structural parameters of lipid-based siRNA delivery systems.

Keywords: cationic/anionic lipid, cellular uptake, cholesterol, endocytosis, nanoparticle, siRNA.

Introduction

The effects of RNA interference (RNAi) were first reported by Napoli *et al*. [1] in 1990, as a result of their attempt to overexpress chalcone synthetase (CHS), an enzyme largely responsible for plant colouration, in petunias. The authors were surprised to find that introducing the gene resulted in blocking pigment synthesis, and growth of white or partly white flowers instead of the purple ones [2, 3]. Although not fully understood at the time, an explanation for this result and other similar phenomena was revealed with the publication of Fire and Mello's seminal paper on RNAi in 1998. Fire, Mello and co-workers used double-stranded RNAs to manipulate gene expression in the nematode *Caenorhabditis elegans* and identified RNAi as a fundamental pathway in which sequence specific RNA strands are able to target and induce the silencing of complementary mRNA [4].

siRNA

Small interfering RNAs (siRNAs) are duplexes of 21–23 nucleotides, approximately 7.5 nm in length [5–7] and 2 nm in diameter [8]. siRNAs can be created intracellularly through cleavage of long double-stranded RNA by the enzyme Dicer [9, 10]. Once in the cytoplasm, the siRNA sense strand is cleaved and degraded, whereas the antisense strand is incorporated into the RNA-induced silencing complex (RISC) [11, 12]. RISC associates with and degrades complementary mRNA sequences; this prevents translation of the target mRNA into protein, 'silencing' the gene [12, 13]. As many diseases are caused by the overexpression of one

or multiple genes, the therapeutic potential of RNA silencing has been investigated for a number of diseases, including cancer [14, 15], infection and inflammation [16], respiratory diseases [17], neurological diseases [18] and autoimmune diseases [19].

Small interfering RNA delivery and the delivery of large DNA sequences for gene therapy differ in several respects. Some of these differences include the site of action in the cell, molecular stability and molecular size [20]. The destination of an siRNA molecule is the cytoplasm, whereas the delivery of a gene requires that the genetic material pass the nuclear membrane. In either case, it is thought that the nucleic acids must 'unpack' from the lipid complex to interact with the appropriate cellular targets. Once the siRNA has been delivered to the cell, the duration of expression knockdown is often between 3 and 7 days (in dividing cells) or up to 3–4 weeks (in nondividing cells) [21]. Transgene expression as a result of DNA-based gene therapy is variable, and can range from short-term to permanent [20].

The molecular weight of a double-stranded siRNA molecule is in the order of 13 kDa, whereas the molecular weight of a double-stranded DNA molecule for gene therapy (not antisense therapy) is often several hundred times greater. Accordingly, those materials suited for DNA delivery may not be ideal for siRNA delivery. In part this is because the size of lipoplexes and polyplexes is affected by the size of the genetic material and the carrier [22]. The phosphodiester backbone of RNA is more sensitive to hydrolysis than in DNA; RNA can be degraded *in vivo* by RNAses. This has prompted development of chemical strategies to improve stability, including various modifications to the backbone that do not affect RISC complexation, and hydrophobic conjugates that improve serum stability [23–25].

The challenge – siRNA delivery

One of the primary challenges of siRNA-based therapeutics is delivery [15]. Therapeutic applications of siRNA require the development of carriers that will: (i) protect siRNA from degradation during circulation [26]; (ii) deliver siRNA at the target cells and avoid delivery to nontarget cell types; (iii) facilitate cellular uptake and endosomal escape; (iv) release siRNA intracellularly so that it will be accessible to the cellular machinery.

In general, siRNA delivery carriers are designed to accumulate at the target site, while avoiding non-specific uptake in nontarget tissue. Many carriers are designed to avoid nonspecific interactions with blood and extracellular elements [27]. This can be achieved by introducing a hydrated steric barrier to surround the carrier using materials such as polyethyleneglycol (PEG) [28, 29]. When a carrier is injected into a peripheral vein, it enters the right side of the heart and is pumped out to the lungs; the lungs contain the first capillary beds and act as an initial mechanical filtration barrier [27]. If small enough, the carriers leave the lungs and enter the left side of the heart and are pumped into the systemic circulation. Given that the liver blood vessels contain fenestrae that are, on average, 100 nm in diameter [30], particles smaller than 100 nm are considered necessary to target hepatocytes [27]. Inclusion of targeting ligands, such as galactose derivatives (recognized by the asioglycoprotein receptor) [31] or peptides from the T7 phage [27, 32], have been reported to improve hepatocellular uptake of some delivery systems. In certain tumour types, passive targeting has been reported via the enhanced permeability and retention (EPR) effect [33, 34], in which increased permeability of blood vessels surrounding tumours [35] and inflamed tissue [36, 37] is used to target these tissue.

Penetrating the cell

Small interfering RNA is negatively charged and typically cannot cross the cell membrane by free diffusion [38]. A number of approaches have been developed to facilitate siRNA uptake, including: (i) conjugating siRNA to a ligand, such as a cell-penetrating peptide or small molecule to facilitate siRNA uptake; (ii) endocytosis of siRNA encapsulated within nanoparticles; or (iii) fusion of the carrier with the cell membrane, thereby releasing the carriers' content into the cytoplasm.

One study using siRNA lipoplexes generated from the commercially available cationic lipid DharmaFECT reported that ~95% of the lipoplexes enter cells through endocytosis [38]; ~50% of endocytosis was clathrin-mediated [38]. Typically, clathrin-mediated endocytosis is responsible for the uptake of many macromolecules from the extracellular medium. The vesicles generated by this pathway are about 100 nm in diameter and are decorated with a crystalline coat containing the protein clathrin [39]. In this same study about 20% of the remaining material delivered to the cytoplasm was internalized via lipid-raft/caveolin-mediated endocytosis [38]. Lipid-raft compartments are usually larger than 50 nm in diameter and consist of the cholesterol-binding protein caveolin and of liquid-ordered domains of cholesterol and glycosphingolipids [40, 41].

Xu and Szoka proposed that the release of nucleic acids from cationic lipid complexes may be facilitated by association of cellular *anionic* lipids with a carrier's *cationic* lipids, to form neutral ion pairs which 'free' the nucleic acid from the delivery system [27, 42]. Felgner *et al.* [43], discussing DNA delivery using liposomes composed of the cationic phospholipid DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane), suggested that positively charged liposomes adhere to the negatively charged DNA, forming a complex in which the DNA is surrounded by charged liposomes. This complex adheres to, and then fuses with, the negative surface of cells, enabling internalization of DNA into the cell. As siRNA is smaller than DNA, it can be loaded into a single lipoplex which is capable of fusing with the cell membrane and subsequently freeing siRNA into the cytosol. A schematic representation of siRNA internalization by fusion of the carrier with the cell membrane is presented in Fig. 1.

Escaping the endosome

As siRNA carriers typically enter cells via endocytosis [38], a means of endosomal escape is necessary. Although the precise mechanisms of endosomal escape by siRNA delivery systems remains unclear, one hypothesis is that certain materials can facilitate endosomal escape via 'the proton sponge effect' [44, 45]. The mechanism is proposed to act as follows: the endosome acidifies after internalization, and amine groups on delivery materials that have a pKa in this range (typically between 7 and 5) are protonated. This is followed by influx of additional protons as well as chloride ions. The uptake of ions creates an osmotic imbalance; water enters the endosome to counter this effect, causing the endosome to inflate until it ruptures. Rupture of the endosome releases its contents to the cytoplasm [44, 45]. There are a number of intracellular delivery materials that have amines with pKa values in the endosomal pH range of 5–7 [46],

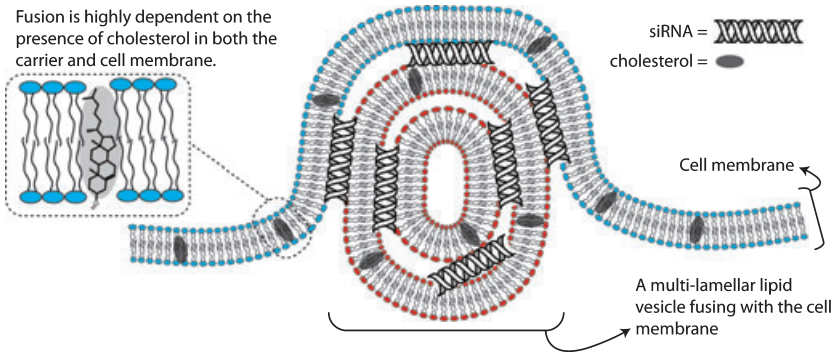
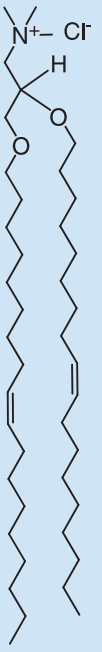
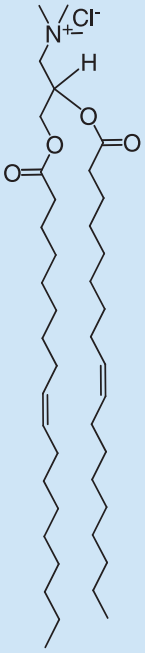
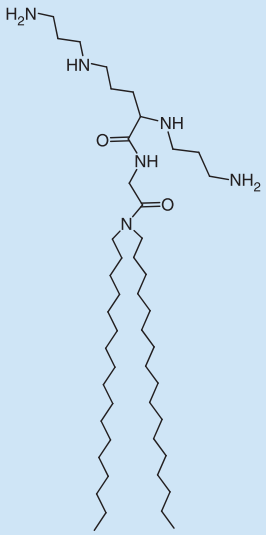
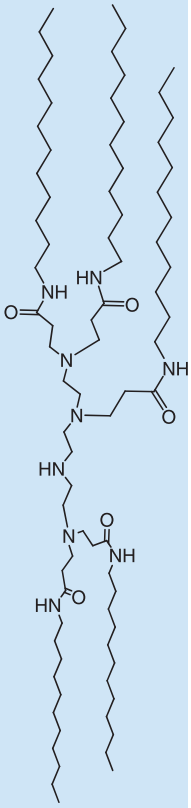


Fig. 1 A schematic representation of the fusion of a multilamellar small interfering RNA lipoplex with the cell membrane. The positively charged lipid bilayer adsorbs to the negatively charged surface of the cell, resulting in either an endocytosis process or by fusion of the lipoplex with the cell membrane, thereby releasing the nucleic payload into the cytosol [38]. During the process, the lipid membrane is stressed and lipids are freed to the intracellular and extracellular compartments.

Table 1 Some commonly used cationic lipids, their molecular structure and tail configuration. Molecules were drawn using ChemBioDraw Ultra 11.0.1 (CambridgeSoft, Cambridge, MA, USA)

Lipid	DOTMA	DOTAP	Transfectam®	$98N_{12}$-5(1)
Chemical name	1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt)	1,2-dioleoyl-3-trimethylammonium-propane (chloride salt)		
Structure				
Tail length : unsaturated bonds	18 : 1	18 : 1	17 : 0	11 : 0

such as polyethylenimine (PEI) and β-amino esters [44, 47–50].

Despite the fact that there is some controversy as to what extent endosomal escape affects transfection [38, 51, 52], we hypothesize that the influx of protons into the endosome may facilitate unpackaging of siRNA from some carriers. Internalization within acidified endosomes may facilitate siRNA release from the lipoplex prior to its release in the cytoplasm [27].

pH-sensitive bonds

Another strategy to improve nucleic acid delivery from lipid-based systems is the incorporation of pH-sensitive groups. These groups can induce phase or structural transformations that can promote unpackaging of

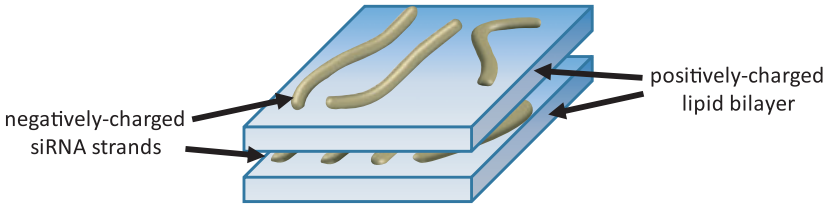


Fig. 2 Multilamellar structure of cationic lipid and small interfering RNA (siRNA) lipoplexes. SiRNA double strands adsorb to the positively charged surfaces of lipid bilayers, to form a multilamellar structure in which, ~3.7-nm thick [65] lipid bilayers are separated ~2 nm apart from each other by siRNA strands [68].

siRNA from the complex [53, 54]. This approach has been used to also trigger drug release in tumours [55].

The pH level of the extracellular matrix and of blood is 7.4, whereas intracellularly the pH is 7.2 [56]. However, in a majority of tumours pH levels are lower both extracellularly and intracellularly [57, 58], reaching 5.7 in some cases [56]. This is primarily because of a higher rate of glycolysis in tumours [59]. An example of a pH-responsive phospholipid is citraconyl-DOPE (1,2-dioleoyl-3-phosphatidylethanotamine) modified by citraconic anhydride [60]. This lipid degrades under acidic conditions, destabilizing the siRNA lipid complex and promoting release of the siRNA. Degradation also releases a fusogenic entity, which can disrupt the endosome capsule, thereby releasing the free siRNA to the cytosol [46].

Cationic lipids as building blocks of siRNA delivery systems

The development of siRNA delivery systems has been influenced by the studies on intracellular DNA delivery [38]. However, there are significant differences between siRNA and DNA, including that: (i) the overall size and charge of siRNA is less than that of DNA, and (ii) siRNA needs to reach the cytosol for therapeutic effect, whereas DNA must enter the nucleus to be effective. As with DNA, siRNA carriers can be composed of polymers [61] (to form polyplexes), peptides, lipids (to form lipoplexes or liposomes) and their combinations [62]. This review will focus on lipid-based siRNA delivery systems.

Cationic lipids were introduced as carriers for DNA and RNA over 20 years ago [63, 64]. Cationic lipids interact with negatively charged nucleic acids through electrostatic interactions forming complexes called lipoplexes [27]. The proposed [65] mechanism of formation of lipoplexes is that negatively charged nucleic acids bind to positively charged lipid vesicles. Additional positively charged vesicles adsorb to the solvent-exposed nucleic acids. This process causes formation of a multilamellar structure of positively charged lipid bilayers [66, 67] ~3.7 nm thick [65], spaced ~2 nm apart from each other by negatively charged nucleic acids [68]. A schematic representation of this structure appears in Fig. 2.

One of the first cationic lipids to be used for DNA delivery is DOTMA [63, 64], see Table 1. Upon hydration, DOTMA will form liposomes either alone, or in presence of other lipids. These liposomes can be downsized into small unilamellar vesicles (SUVs) <100 nm in diameter. Liposomes differ from micelles; liposomes are spherical vesicles in which a single or several continuous lipid bilayers/s separate the external aqueous medium from the intraliposomal aqueous core, whereas micelles have an inner oil core. Based on the efficacy of DOTMA and other cationic lipids, Ren *et al.* [69] proposed structural features common to those lipids most effective for DNA delivery *in vivo*. These features, schematically represented in Fig. 3, include: (i) a cationic head group and its neighbouring aliphatic chain being in a 1,2-relationship on the backbone; (ii) an ether bond for bridging the aliphatic chains to the backbone; and (iii) paired oleyl chains as the hydrophobic anchor into the lipid assembly.

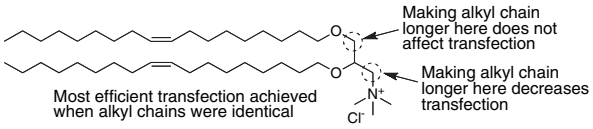


Fig. 3 Lessons learned from structural modifications of DOTMA acting as a transfection agent.

More recently [70], a combinatorial library of lipid-like molecules, termed lipidoids, was developed for siRNA delivery. The performance of the lipidoids was compared with different structural motifs including alkyl chain length and the degradability of the linker between amine and alkyl groups. Highest levels of knockdown were achieved using lipidoids with the following properties: (i) more than two amines per head unit; (ii) amide bonds between the amine 'core' and acyl tails; (iii) greater than two acyl chains; (iv) acyl chains between 8 and 12 carbon atoms; and (v) at least one secondary amine [70]. An example of a lipidoid, called $98N_{12}$, together with other commonly used cationic lipids, is presented in Table 1.

Cholesterol: common component in siRNA carriers

Cholesterol plays a role in many cellular membrane-related events such as membrane fusion, macropinocytosis and caveolin and lipid-raft-mediated endocytosis [38, 71, 72]. Introducing cholesterol as a component of certain DNA/RNA carriers has been reported to improve transfection *in vivo* in comparison with carriers not containing cholesterol [73–75]. When formulated in delivery vehicles at more than 25 mol%, cholesterol can decrease carrier permeability, increase carrier circulation time [76, 77] and increase structural rigidity and stability of the carrier [71]. Furthermore, cholesterol is reported to protect nucleic acids from extraliposomal degradative entities such as RNases [73, 74]. The importance of cholesterol for internalization of siRNA into cells has been exemplified by extracting cholesterol from cell membranes, and then exposing the cells to siRNA lipoplexes. In the cholesterol-depleted cells siRNA uptake and transfection were totally abolished [38].

Conjugating cholesterol to siRNA improves cellular uptake and transfection, and decreases siRNA degradation in serum [12, 15, 75]. Wolfrum *et al.* [78] showed that introducing cholesterol-conjugated siRNA into plasma resulted in association of these particles with either high-density lipoproteins (HDLs), which *in vivo* targets the liver, gut, kidney and steriodogenic organs, or with low-density lipoproteins (LDLs), which targeted the liver primarily [78]. siRNA conjugated to other hydrophobic molecules, with more than 22 carbons, also showed HDL and LDL association [78]. The association of the conjugated siRNA with HDL/LDL may protect siRNA from being degraded by plasma components.

Cholesterol may play a dual role in the delivery of siRNA. When incorporated in the carrier, cholesterol may help facilitate cell fusion or endosomal internalization of the carrier. When conjugated to siRNA, cholesterol seems to act as a targeting entity.

Derivatives of cholesterol have also been shown to improve the performance of cationic liposomes. Han *et al.* [79], showed that cationic liposomes enriched with an amine-based cholesterol derivative, cholesteryloxypropan-1-amine, increased delivery efficiency of siRNA in serum, in comparison with ordinary cholesterol.

Structural and physiological effects of carrier charge

A number of reports have addressed the relationship between lipid charge and lipid-to-RNA ratio in formulations on carrier shape, trafficking and efficacy. Pitard *et al.* [66] examined the morphology of siRNA lipoplexes prepared using lipids synthesized from aminoglycosides. In formulations with low lipid-to-siRNA ratios, the resulting lipoplex particles were small (<200 nm), stable and had overall negative charge. Increasing the concentration of lipids in the formulations neutralized the overall charge, but resulted in the formation of large

(~700 nm) unstable aggregates [66]. Increasing the lipid/siRNA ratio further induced formation of small (<200 nm) stable particles with overall positive charge [66]. The ratio of lipid-to-siRNA strongly influences the shape, size and behaviour of a lipoplex. However, this effect depends on the lipid structure; different lipids at similar lipid/siRNA ratios will spontaneously form complexes of different sizes [80].

Safinya *et al.* [67] tested the effect of cationic lipid/siRNA charge ratio on particle uptake and gene knockdown in mammalian cells. They found that increasing the charge ratio had little effect on the total knockdown whereas it did however increase nonspecific knockdown. Interestingly, they found that multivalent (five-charge) lipids exhibited lower toxicity, higher total knockdown and lower nonspecific knockdown in comparison with a similar charge ratio carried by several univalent cationic lipids [67].

The overall charge of the carrier can affect its destination *in vivo*. Jain *et al.* [81] using 150-nm cationic liposomes composed of DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), DOPC (1,2-dioleoyl-3-phosphatidylcholine), cholesterol and PEG-DSPE, showed that a majority (>55%) of liposomes accumulated in the liver. Increasing carrier charge (by introducing additional DOTAP to the carrier) reduced accumulation in the spleen and blood, and increased accumulation in the liver [81]. Although tumour uptake was not affected by particle charge, imaging analysis revealed that increasing positive charge on the particles did increase accumulation of the carriers in tumour vasculature. Litzinger *et al.* [82], using ~2 μm diameter cationic liposomes, showed that biodistribution and cellular uptake were not affected by charge. In this case, a majority of liposomes accumulated in the Kupffer cells of the liver. Interestingly, they found that above a certain liposomal dose, the liver became saturated with liposomes, causing an 'overflow' of liposomes to accumulate in the spleen [82]. It should be noted that in this case accumulation in Kupffer cells (i.e. specialized macrophages located in the liver) may be owing to the large size of the carrier and not related to charge [83].

Anionic lipids

Although a majority of siRNA carriers are based on cationic lipids or polymers (e.g. DOTMA, DOTAP, poly-L-lysine, PEI and poly(2-(dimethylamino)ethyl methacrylate), several studies have tested the ability to deliver nucleic acids using combinations of cationic and *anionic* lipids. In one example, the negatively charged nucleic acid is complexed with an amine-based polypeptide (such as poly-L-lysine), generating a particle with a net positive charge. The particles are then treated with negatively charged lipids [84]. Mastrobattista *et al.* were able to improve transfection by preparing positively charged polyplexes coated with an anionic lipid [85]. The purpose of this strategy was to protect nucleic acids from deactivation by polyanions present in tumour ascitic fluid, such as hyaluronic acid (HA). Despite these reports, negatively charged complexes are uncommon in nucleic acid delivery as they can induce immunogenic responses, and are less likely to penetrate the negatively charged surface of the cells [27, 62, 86].

Effect of the carriers' size on biodistribution

The size of the carrier seems to have a great effect on its biological fate and activity. Szoka *et al.* suggest that a size cutoff of 100 nm is highly important to overcome *in vivo* barriers for systemic gene delivery: blood components, RES uptake, tumour access, extracellular matrix components and intracellular barriers [83].

Kizelsztein *et al.* [87] showed that ~1% of the injected dose of neutral PEGylated liposomes ~80 nm in diameter will cross the blood–brain barrier and accumulate in brains of healthy mice. This number is tripled when the mice are suffering from a multiple sclerosis model disorder. Similarly, Avnir *et al.* [29], using ~80 nm PEGylated liposomes to treat an autoimmune arthritis model, showed that the levels of liposome accumulation in an arthritic joint were similar, or even higher, than those found in the liver, kidney or spleen. In these cases, increased accumulation is explained by the high permeability of vasculature surrounding the inflamed tissue.

Garbuzenko *et al.* [17, compared the pharmacokinetics of ~120 nm in diameter DOTAP-based liposomes containing siRNA that were administered either intravenously (IV) or intratrachealy. When administered IV, gradual accumulation of the liposomes in the liver, kidney and spleen were noticed over a period of 24 h; during this time, liposome concentration in the lungs increased for the first hour but declined thereafter [17]. Conversely, liposomes administered intratrachealy remained in the lungs for at least 72 h postadministration, with low levels in other organs [17]. In a different study by Ishiwata *et al.* [88] the majority of positively charged liposomes, ~150 nm in diameter, resided in the lung for the first hour post-IV administration, after which they mostly accumulated in the liver.

Chan *et al.* [89, 90] examined endocytosis of gold nanoparticles of varying sizes. They showed that uptake efficiency versus particle size followed a bell-shaped pattern with the most efficient uptake occurring in ~50-nm diameter particles. Although different from lipid-based carriers, studies with these particles provide insight into the optimal size of carriers that can enhance their efficiency and effectivity *in vivo.*

Carriers' shape may affect delivery

The effect of the size of carriers on delivery has been studied for many years. However, limited *in vitro* and *in vivo* studies focused on the behaviour of carriers with regard to their shape and configuration. Discher *et al.* [91–94] formed worm-like micelles from degradable copolymers, with dimensions several nanometres wide and several microns long. These flexible filaments, named filomicelles, were shown to persist in rodent circulation for up to 1 week after IV injection, 10 times longer than spherical counter-particles, and were internalized by A549 human lung cancer cells. Sailor *et al.* [95] showed that dextran-coated magnetic iron oxide nanoparticles, elongated along one dimension, had longer circulation time, higher accumulation levels in murine MDA-MB-435 tumours and improved cellular uptake in comparison with the spherical ones. Similarly, it was shown [96] that single-walled carbon nanotubes coated with PEG-2000 accumulated at high levels in U87MG human glioblastoma tumours in mice. Champion and Mitragotri [97], using alveolar macrophages as model phagocytes and polystyrene particles of various sizes and shapes as model targets, showed that target shape at *the point of first contact* by macrophages, and not size, decisively determines whether cells will proceed with phagocytosis or simply spread on the particle. While, prolate ellipsoids (major axis 2–6 μm, aspect ratio 1.3–3) and elliptical discs (major axis 3–14 μm, aspect ratio 2–4, thickness 400–1000 nm) were internalized by cells with great effectivity, spheres (radius 1.0–12.5 μm) or oblate ellipsoids (major axis 4 μm, aspect ratio 4) were covered by the cells and not internalized.

From the aforementioned studies it appears that carriers with a 'pinhead' format may be somewhat advantageous in penetrating cells, hence intracellular delivery. Recently, attempts to form microtubes and nanotubes from lipids have been reported [98–104], which are promising for use in nucleic acid delivery.

Current status and future prospective of clinical applications of siRNA nanotherapeutics

Since the first demonstrations of RNAi in *C. elegans* and mammalian cells about a decade ago, the development of RNAi therapeutics has progressed rapidly with a growing number of siRNA-based therapeutics currently in clinical trials (see Table 2). Early siRNA therapeutics for the treatment of age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) [105] were administered locally using unmodified or chemically modified siRNA (in saline). More recently, formulations for systemic administration of siRNA packaged using polymers [106] or lipids have begun to be evaluated in the clinic. For example, a study conducted by Silence Therapeutics is testing a siRNA-liposomal formulation aimed at targeting protein kinase N3. This approach has proven to significantly inhibit tumour growth in prostate and pancreatic cancer models in mice [107], and is being tested in humans with advanced solid tumours. Alnylam Pharmaceuticals is investigating a lipid-based nanoformulation containing two different siRNA molecules aimed at targeting the kinesin spindle protein (KSP) and the vascular endothelial growth factor

Table 2 Current clinical trials for small interfering RNA therapeutics

Disease	Target	Formulation	Delivery mode	Company	Status	Preclinical reference
Age-related macular degeneration; diabetic macular oedema	Vascular endothelial growth factor pathway	Saline	Topical; intravitreal injection	Opko Health; Allergan	Phases I–III	[109, 110]
Acute kidney injury	I5NP		IV	Quark	Phase I	
Hypercholesterolemia		Liposome	IV	Tekmira	Phase I	[111, 112][a]
Cancer (solid tumour)	Ribonucleotide reductase subunit R2	Cyclodextrin, PEG, transferrin- targeted	IV	Calando	Phase I	[106]
Cancer (solid tumour)	Protein kinase N3	Liposome	IV	Silence Therapeutics	Phase I	[107]
Melanoma	Immunoproteasome	Transfected dendritic cells	Intradermal	Duke University	Phase I	[113]
Pachyonychia congenital	K6a keratin	–	Topical	Pachyonychia Congenita Project	Phase I	[114, 115]
Respiratory syncytial virus	RSV-P	Saline	Intranasal	Alnylam	Phase II	[105]
Cancer	Advanced solid tumours with liver involvement	Lipoplex	IV	Alnylam	Phase I	[70, 108][a]

[a]Other RNAi studies conducted by the company (US National Institute of Health, http://www.clinicaltrials.gov).
IV, intravenous.

(VEGF) for their potential antiliver tumour activity. VEGF and KSP are upregulated in many tumour cells and play an important role in tumour proliferation and survival. Preclinical studies show that upon IV administration, the KSP/VEGF siRNAs in the lipid carrier target both KSP and VEGF messenger RNAs (mRNAs) [70, 108]. The results of these trials with lipid and formulated materials will provide important information regarding the translatability of delivery systems developed in rodents and primates.

Conclusions

Lipid-based carriers are promising candidates for therapeutic siRNA delivery. When designing carriers, consideration of both the molecular and meta-molecular scales must be taken into consideration. On the molecular scale, the building blocks, i.e. the lipids, must be able to assemble into stable delivery systems, which may or may not be affected by the nucleic acid payload. Complexation with siRNA often occurs via electrostatic interactions; therefore, the polar head of the lipid should contain a positive charge during siRNA complexation, carried in most cases by the amine groups. Electrostatic interactions must be stable enough to sustain the nucleic payload in the carrier *en route*, but must allow dissociation, to execute therapeutic activity, at the delivery site. Molecules containing several amines per head group, in which slight spacing exists between one amine to the other, are able to adhere to the negatively charged backbone of siRNA in a better manner than several lipids containing a single positive charge per headgroup. When assembling carriers from positively charged lipids, stability may be enhanced by addition of neutral lipids (sometimes referred to as helper lipids) to reduce repulsion between similar charges in the bilayer. Adding cholesterol, which resides in the hydrophobic region of the bilayer, improves carrier stability, and seems to play an important role in facilitating cellular

uptake of siRNA. PEG lipids, which extend out of the lipid bilayer, presenting a highly hydrated corona surrounding the carrier, enhance circulation time and reduce carrier uptake by RES components. To enable carrier uptake and permeation across fenestrae, a size limit of less than 100 nm should be maintained.

Conflict of interest statement

R. Langer is a shareholder and member of the Scientific Advisory Board of Alnylam. D.G. Anderson is a consultant with Alnylam Pharmaceuticals. R. Langer and D.G. Anderson have sponsored research grants from Alnylam. Alnylam also has a license to certain intellectual property invented at Massachusetts Institute of Technology by Dr Anderson, Langer and colleagues.

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