

Knocking down barriers: advances in siRNA delivery

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Abstract | In the 10 years that have passed since the Nobel prize-winning discovery of RNA interference (RNAi), billions of dollars have been invested in the therapeutic application of gene silencing in humans. Today, there are promising data from ongoing clinical trials for the treatment of age-related macular degeneration and respiratory syncytial virus. Despite these early successes, however, the widespread use of RNAi therapeutics for disease prevention and treatment requires the development of clinically suitable, safe and effective drug delivery vehicles. Here, we provide an update on the progress of RNAi therapeutics and highlight novel synthetic materials for the encapsulation and intracellular delivery of nucleic acids.

RNA interference

(RNAi). A fundamental pathway in eukaryotic cells by which a short piece of RNA is able to induce the destruction of mRNA containing a complementary sequence.

Small interfering RNA

(siRNA). RNA fragments approximately 21–23 nucleotides long that are capable of inducing the sequence-specific destruction of complementary mRNA.

RNA-induced silencing complex

(RISC). The protein complex responsible for the binding and unwinding of the double-stranded siRNA within the cytoplasm, and for the subsequent identification and destruction of the target mRNA.

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doi:10.1038/nrd2742

RNA interference (RNAi) gained international attention in 1998 when Fire, Mello and colleagues discovered the ability of double-stranded RNA to silence gene expression in the nematode worm *Caenorhabditis elegans*¹. Three years later, Tuschl and co-workers published their celebrated proof-of-principle experiment demonstrating that synthetic small interfering RNA (siRNA) could achieve sequence-specific gene knockdown in a mammalian cell line². The first successful use of siRNA for gene silencing in mice was achieved for a hepatitis C target shortly thereafter³. Since that time, the biotechnology sector has made considerable efforts in the advancement of siRNA therapeutics for the treatment of various disease targets, including viral infections^{4,5} and cancer^{6–8}.

RNAi is a fundamental pathway in eukaryotic cells by which sequence-specific siRNA is able to target and cleave complementary mRNA². RNAi is triggered by the presence of long pieces of double-stranded RNA, which are cleaved into the fragments known as siRNA (21–23 nucleotides long) by the enzyme Dicer⁹. In practice, siRNA can be synthetically produced and then directly introduced into the cell, thus circumventing Dicer mechanics (FIG. 1). This shortcut reduces the potential for an innate immune interferon response and the shutdown of cellular protein expression that can occur following the interaction of long pieces (>30 nucleotides) of double-stranded RNA with intracellular RNA receptors¹⁰.

Once siRNA is present in the cytoplasm of the cell, it is incorporated into a protein complex called the RNA-induced silencing complex (RISC)¹¹. Argonaute 2, a multifunctional protein contained within RISC, unwinds the siRNA, after which the sense strand (or passenger strand)

of the siRNA is cleaved¹². The activated RISC, which contains the antisense strand (or guide strand) of the siRNA, selectively seeks out and degrades mRNA that is complementary to the antisense strand¹³ (FIG. 1). The cleavage of mRNA occurs at a position between nucleotides 10 and 11 on the complementary antisense strand, relative to the 5'-end¹⁴. The activated RISC complex can then move on to destroy additional mRNA targets, which further propagates gene silencing¹⁵. This extra potency ensures a therapeutic effect for 3–7 days in rapidly dividing cells, and for several weeks in non-dividing cells¹⁶. Eventually, siRNAs are diluted below a certain therapeutic threshold or degraded within the cell, and so repeated administration is necessary to achieve a persistent effect.

Theoretically, when using appropriately designed siRNA, the RNAi machinery can be exploited to silence nearly any gene in the body, giving it a broader therapeutic potential than typical small-molecule drugs. Indeed, it has already been reported that synthetic siRNAs are capable of knocking down targets in various diseases *in vivo*, including hypercholesterolaemia¹⁷, liver cirrhosis¹⁸, hepatitis B virus (HBV)^{4,19}, human papillomavirus²⁰, ovarian cancer²¹ and bone cancer²². In order for these advances to be implemented in a clinical setting, safe and effective delivery systems must be developed. While 'naked', chemically modified siRNA has shown efficacy in certain physiological settings such as the brain²³ and the lung²⁴, there are many tissues in the body that require an additional delivery system to facilitate transfection. This is because naked siRNA is subject to degradation by endogenous enzymes, and is too large (~13 kDa) and too negatively charged to cross cellular membranes.

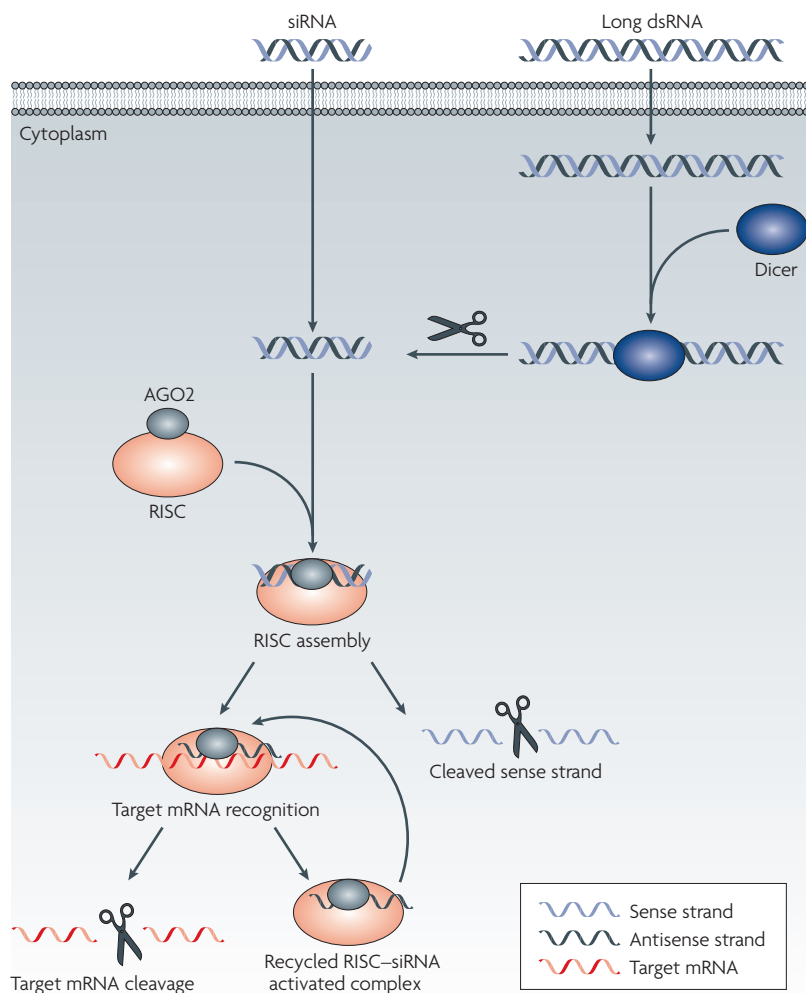


Figure 1 | The mechanism of RNA interference. Long double-stranded RNA (dsRNA) is introduced into the cytoplasm, where it is cleaved into small interfering RNA (siRNA) by the enzyme Dicer. Alternatively, siRNA can be introduced directly into the cell. The siRNA is then incorporated into the RNA-induced silencing complex (RISC), resulting in the cleavage of the sense strand of RNA by argonaute 2 (AGO2). The activated RISC-siRNA complex seeks out, binds to and degrades complementary mRNA, which leads to the silencing of the target gene. The activated RISC-siRNA complex can then be recycled for the destruction of identical mRNA targets.

The issue of effective and non-toxic delivery is a key challenge and serves as the most significant barrier between siRNA technology and its therapeutic application.

Modes of siRNA administration

The ease of siRNA delivery is partly dependent on the accessibility of the target organ or tissue within the body. Localized siRNA delivery — that is, application of siRNA therapy directly to the target tissue — offers several benefits, including the potential for both higher bioavailability given the proximity to the target tissue, and reduced adverse effects typically associated with systemic administration. By contrast, systemic delivery, meaning the intravenous injection of delivery particles that then travel throughout the body to the target organ or tissue, requires that particles have the ability to avoid uptake and clearance by non-target tissues (FIG. 2).

Antisense strand

The strand of the siRNA molecule that is complementary to the target mRNA, which activates RISC and has an important role in target mRNA identification and destruction.

Transfection

The process of delivering nucleic acid material into the cell.

There are several tissues that are amenable to topical or localized therapy, including the eye, skin, mucus membranes, and local tumours^{25–28} (TABLE 1). Local siRNA delivery is particularly well-suited for the treatment of lung diseases and infections. The direct instillation of siRNA into the lung through intranasal or intratracheal routes enables direct contact with lung epithelial cells. These cells play a part in a myriad of lung conditions and infections, including cystic fibrosis, asthma, influenza and the common cold²⁴. It has been reported that respiratory syncytial virus (RSV) replication can be inhibited by nasally administered siRNA formulated with or without transfection agents in mice^{29,30}. Progress in the treatment of RSV continues with Phase II clinical trials using an aerosolized siRNA delivery system³¹. Intratracheal administration of siRNA has also been reported to offer prophylactic and therapeutic effects in the treatment of severe acute respiratory syndrome³².

Another example of local delivery is direct intratumoral injection of siRNA delivery complexes into various mouse xenograft models. siRNA complexed with the delivery agent polyethyleneimine (PEI) was shown to inhibit tumour growth upon intratumoral injection in mice bearing glioblastoma xenografts²⁸. Niu and co-workers have also reported naked siRNA efficacy upon direct injection into a subcutaneous cervical cancer model in mice²⁰.

Barriers to systemic siRNA delivery in vivo

In contrast to the direct accessibility of localized targets, many tissues can only be reached through the systemic administration of delivery agents in the bloodstream. siRNA formulations for systemic application face a series of hurdles *in vivo* before reaching the cytoplasm of the target cell (FIG. 2). Post-injection, the siRNA complex must navigate the circulatory system of the body while avoiding kidney filtration, uptake by phagocytes, aggregation with serum proteins, and enzymatic degradation by endogenous nucleases³³.

Phagocytosis serves as a significant immunological barrier, not only in the bloodstream but also in the extracellular matrix of tissues. Phagocytic cells such as macrophages and monocytes remove foreign material from the body to protect against infection by viruses, bacteria and fungi. Unfortunately, phagocytes are also highly efficient at removing certain therapeutic nanocomplexes and macromolecules from the body, and steps must be taken to avoid opsonization when designing drug delivery vehicles³³.

Egress from the bloodstream and across the vascular endothelial barrier poses a significant challenge for delivery of siRNA to many tissues within the body. In general, molecules larger than 5 nm in diameter do not readily cross the capillary endothelium, and therefore will remain in the circulation until they are cleared from the body. There are certain tissues, however, that allow the entry of larger molecules, including the liver, spleen, and some tumours. These organs allow the passage of molecules up to 200 nm in diameter, which can accommodate a typical drug delivery nanocarrier³⁴.

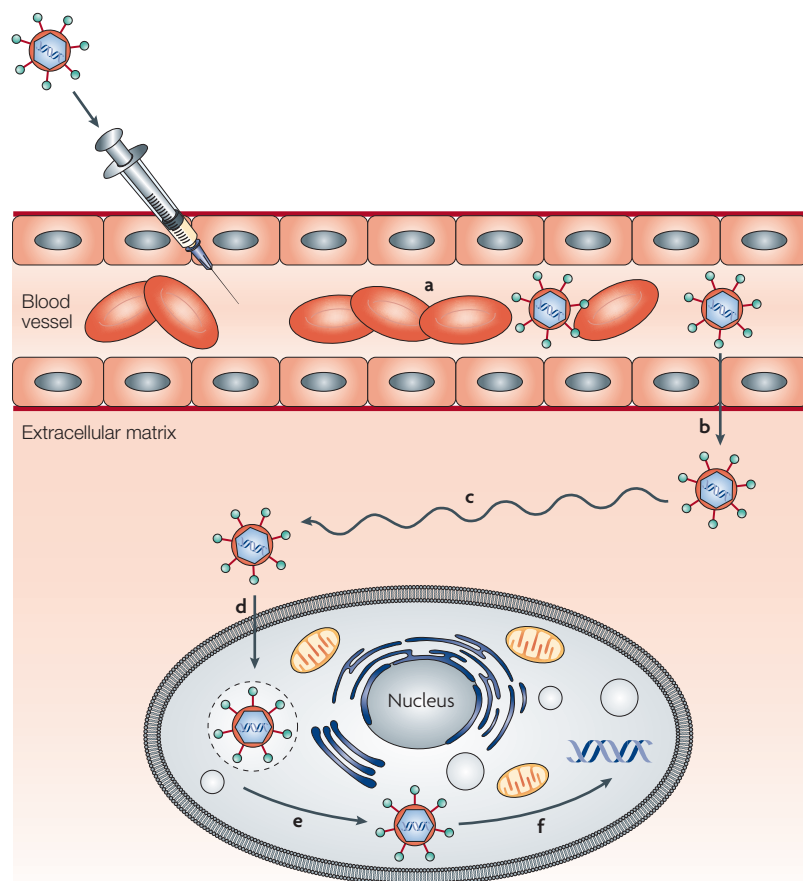


Figure 2 | Physiological barriers to the systemic delivery of small interfering RNA (siRNA) nanoparticles. An injected nanoparticle must avoid filtration, phagocytosis and degradation in the bloodstream (a); be transported across the vascular endothelial barrier (b); diffuse through the extracellular matrix (c); be taken up into the cell (d); escape the endosome (e); and unpackage and release the siRNA to the RNA interference (RNAi) machinery (f).

After an siRNA complex leaves the bloodstream, it must diffuse through the extracellular matrix, which is a dense network of polysaccharides and fibrous proteins that can create resistance to the transport of macromolecules and nanoparticles³⁵. This can slow or even halt the drug delivery process and create an additional opportunity for nanoparticles to be taken up by resident macrophages. Having been taken up by the target cell, particles must then escape the endosome to reach the cytoplasm³⁶. If the siRNA nanocomplex is unable to exit the endosome, it will be trafficked through endomembrane compartments of decreasing pH and be subject to degradative conditions in the lysosome³⁷. Finally, if formulated with delivery agents, siRNA must be released from the carrier to the cellular machinery.

Modified siRNA for improved delivery

Humans have evolved a number of host-defence mechanisms against siRNA, as it is a feature of certain viral infections. However, chemical modifications can be introduced into the siRNA molecule to evade immune defences *in vivo*. For example, many non-modified

siRNAs can induce nonspecific activation of the immune system through the Toll-like receptor 7 (TLR7) pathway^{38,39}. This effect can be reduced by the incorporation of 2'-O-methyl modifications into the sugar structure of selected nucleotides within both the sense and antisense strands^{38,40} (FIG. 3a). 2'-O-methyl modifications have also been shown to confer resistance to endonuclease activity⁴¹ and to abrogate off-target effects when incorporated into the seed region, which corresponds to nucleotides 2–8 on the antisense strand⁴². Other common modification approaches to mitigate enzymatic degradation include the introduction of phosphorothioate backbone linkages at the 3'-end of the RNA strands to reduce susceptibility to exonucleases. It is also possible to incorporate alternative 2' sugar modifications (for example, a fluorine substitution) to increase resistance to endonucleases⁴³.

Another strategy to improve the therapeutic efficacy of siRNA involves the conjugation of small molecules or peptides to the sense strand of the siRNA. Several small molecules have been reported to increase target-gene knockdown *in vitro*, including membrane-permeant peptides⁴⁴ and polyethylene glycol (PEG)⁴⁵. Of particular note are cholesterol-modified siRNAs, which have demonstrated increased binding to serum albumin, resulting in improved biodistribution to certain targets including the liver (FIG. 3b). Cholesterol-modified siRNA were capable of silencing apolipoprotein B (ApoB) targets in mouse liver and jejunum, and of ultimately reducing total cholesterol levels⁴⁶. Another study by DiFiglia and co-workers details the ability of a cholesterol-modified siRNA to knockdown a gene associated with Huntington's disease. A single intrastriatal injection was able to delay the abnormal behavioural phenotype observed in a rapid-onset mouse model of this disease²³.

Given the success of cholesterol-modified siRNA *in vivo*, Wolfrum and co-workers attempted to identify alternative lipid-like molecules to serve as RNA conjugates for improved delivery of siRNA⁴⁷. Specifically, fatty acids and bile-salt derivatives were conjugated to siRNA and injected into mice and hamsters in order to elucidate how modified siRNA conjugates interact with the high-density lipoprotein (HDL) and low-density lipoprotein (LDL) receptors that enable delivery to the liver. It was found that shorter fatty-acid chain lengths (<C₁₈) did not induce gene knockdown, whereas bile-salt derivatives and fatty-acid conjugates with longer chain lengths enabled potent silencing in hepatocytes via the HDL receptor⁴⁷.

Another example of the possibility of introducing beneficial modifications to nucleic acid therapeutics comes from the antisense drug mipomersen. Mipomersen is a 2'-O-(2-methoxyethyl)-modified single-stranded RNA molecule that is targeted to ApoB⁴⁸, a protein that has been implicated in cardiovascular disease. Isis Pharmaceuticals has reported promising Phase II safety and efficacy results, and a Phase III trial to assess efficacy in patients with a familial history of hypercholesterolaemia is currently underway in a joint venture with Genzyme⁴⁹.

Table 1 | Modes of siRNA delivery and potential targets

Mode of administration	Potential organ target	Potential disease target	Refs
Topical	Eye	Macular degeneration	102
	Skin	Atopic dermatitis	25
	Vagina	Herpes simplex virus	27
	Rectum	Inflammatory bowel disease	105
Local/direct	Lung	SARS	32
	Brain	Huntington's disease	23
	Spinal cord	Chronic pain	90
	Isolated tumour	Glioblastoma multiforme	28
Systemic	Liver	Hypercholesterolaemia	57
	Heart	Myocardial infarction	106
	Kidney	Kidney disease	61
	Metastasized tumours	Ewing's sarcoma	97

SARS, severe acute respiratory syndrome; siRNA, small interfering RNA.

Properties of synthetic delivery nanoparticles

For tissues and cells that are not amenable to the delivery of naked or chemically modified siRNA, delivery of nanoparticles that incorporate siRNA are used. In general, delivery vehicles are designed to both facilitate uptake into the target tissue of interest and, when used for systemic delivery, to protect siRNA payloads and inhibit nonspecific delivery. Below, we highlight several important characteristics of delivery nanoparticles and provide specific examples of their construction and use.

Surface properties. The surface charge of a delivery nanoparticle can significantly influence the way it interacts with the target cell and other physiological molecules. In the simplified *in vitro* setting, a positively charged delivery vehicle can facilitate uptake by associating with the negatively charged cellular membrane⁵⁰. A positive charge also promotes complex formation and compression with the polyanionic nucleic acids of the siRNA. The situation, however, becomes more complicated *in vivo* as negatively charged serum proteins in the bloodstream will often bind to a positively charged nanocomplex, therefore rendering it ineffective. The addition of PEG or other hydrophilic conjugates to the surface of a delivery vehicle can assist in mitigating this problem⁵¹. Additionally, PEG conjugation can control particle size and prevent particle aggregation in the presence of serum⁵¹.

Coating of the nanoparticle with hydrophilic molecules such as PEG can also play an important role in the ability of the siRNA delivery carrier to evade the immune system and associated phagocytes. PEG forms a barrier around nanoparticles that provides steric stabilization and protection from the physiological surroundings⁵². The length of the PEG chain can have a significant influence on its stabilization and protective properties, and chain length is typically optimized for each individual delivery system^{53,54}.

Biodistribution. The biodistribution of siRNA can be significantly influenced through formulation with a delivery vehicle. Systemic administration of synthetic delivery nanoparticles often results in accumulation in the organs of the reticuloendothelial system, including the liver, spleen, kidneys and lungs^{16,55,56}. It is no coincidence that much of the successful siRNA delivery seen in recent years has targeted disease within these organs^{4,29,30,57,58}.

Excretion through the kidney typically occurs for molecules less than 50 kDa in size⁵⁹. As such, naked siRNA experiences rapid renal clearance upon systemic administration⁴³. Several studies monitored the biodistribution of siRNA in mice after an intravenous injection and observed naked siRNA accumulation in the kidney and urine within 5 minutes of administration^{60,61}. By complexing siRNA with synthetic materials, the size of the delivery nanoparticle can be increased to avoid glomerular filtration through the kidneys and reserve the siRNA for alternative organ targets⁵⁹.

Additionally, it has been reported that certain siRNA formulations are capable of accumulation in subcutaneous tumours^{22,62}. This phenomenon has been attributed to the enhanced permeability and retention (EPR) effect. It has therefore become a common approach to exploit the leaky vasculature of tumours for the purposes of directed delivery^{7,62,63}. Others have also reported success in targeting tumours through conjugation to ligands such as antibodies^{64,65}.

Toxicity. Even the most efficacious siRNA delivery agents are rendered useless if they provoke unacceptable toxicity on either a cellular or systemic level. Viral vectors, which were among the first vehicles to be studied for siRNA delivery, can induce unacceptable levels of toxicity through the activation of immune responses⁶⁶. Therefore, synthetic lipids and polymers have been developed to offer alternatives to viral vectors for nucleic acid delivery applications, and are carefully formulated to avoid stimulation of the immune system³⁹. Clearance of larger molecular mass materials typically requires them to be biodegradable. The use of biodegradable, high molecular mass polycations and polymers containing linkages that can be cleaved inside the cell can help reduce cytotoxicity⁶⁷.

Synthetic materials for siRNA delivery

Synthetic materials have demonstrated potential as effective non-viral siRNA delivery carriers. Although many types of compound have been investigated as potential candidates, this Review focuses on synthetic materials that have successfully delivered nucleic acids *in vivo*. An overview of the delivery agents presented here can be found in TABLE 2.

Liposomes and lipid-like materials. Unilamellar and multilamellar liposomes are commonly used as pharmaceutical delivery vehicles⁶⁸. In an aqueous environment, certain materials have the ability to form liposomes, in which a lipid bilayer forms a sphere with an aqueous core. For example, one set of polar head-groups can create the

Enhanced permeability and retention (EPR) effect
The effect by which macromolecules undergo increased accumulation in tumours. This is attributable to the quickly growing tumour vasculature, which is improperly formed and subsequently more permeable to large molecules.

Liposome
A type of drug delivery vehicle made of lipids. These nanocomplexes can be unilamellar (one set of head-groups) or multilamellar (two or more sets of head-groups). They can be used to deliver hydrophilic or hydrophobic payloads, depending on their structure.

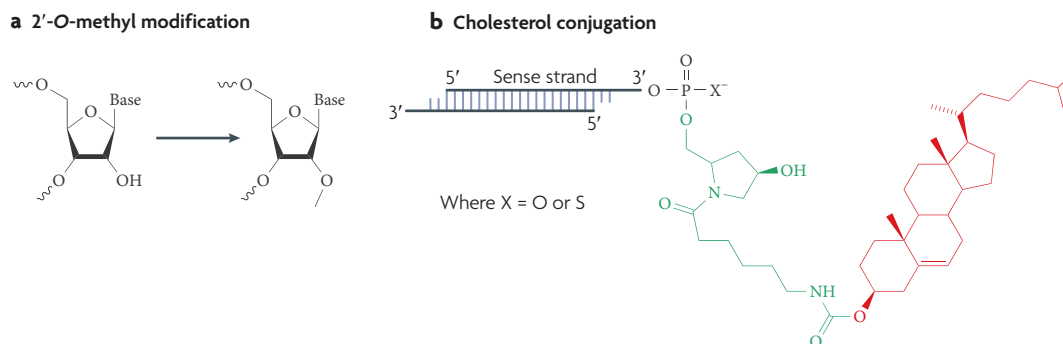


Figure 3 | **Two common small interfering RNA (siRNA) modifications used for therapeutic applications.** **a** | The 2'-O-methyl sugar modification prevents activation of the Toll-like receptor 7 immune response and confers enzymatic resistance to the siRNA molecule. **b** | The conjugation of cholesterol to the sense strand of an siRNA duplex improves delivery of naked siRNA to certain cellular targets, including hepatocytes. The cholesterol conjugate is shown in red, and the linker is shown in green.

outer surface of the nanocomplex, while another set of polar head-groups faces the interior hydrophilic core, which houses the nucleic acid payload⁶⁹ (FIG. 4). It is also possible for liposomes to be amorphous in structure, with the lipids and nucleic acids interspersed throughout. Liposomes can be created using single or multiple types of lipid, which allows for additional flexibility when optimizing the physical and chemical properties of the nanoparticle⁶⁸.

Liposomes have been used for the delivery of nucleic acids for over 20 years, originating with studies by Felgner and colleagues detailing the ability of the cationic lipid DOTMA (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride) to deliver both DNA and RNA into mouse, rat and human cell lines^{70,71}. More recently, stable nucleic acid–lipid particle (SNALP) formulations have demonstrated efficacy in several models *in vivo*. A study by Morrissey and co-workers indicated that HBV replication was inhibited through the delivery of an siRNA–SNALP complex that targeted HBV RNA. Three daily intravenous injections of 3 mg per kg per day reduced serum HBV levels by at least one order of magnitude, and the effect was specific, dose-dependent and lasted for up to 7 days after dosing⁴. In addition, Zimmerman and colleagues demonstrated the ability of SNALPs to enable knockdown of ApoB in the liver of cynomolgus monkeys⁵⁷. A single siRNA injection resulted in dose-dependent silencing of ApoB mRNA expression in the liver 48 hours after administration, with maximal silencing of more than 90%. Knockdown was confirmed to be caused by ApoB mRNA cleavage at precisely the site predicted for the RNAi mechanism, and persisted for 11 days at the highest administered dose of 2.5 mg per kg⁵⁷.

An additional study details the use of SNALP formulations to combat the Zaire strain of Ebola virus, which proves fatal to 90% of its victims via haemorrhagic fever⁷². When contained within the SNALP formulation, siRNA targeting of the polymerase gene of the virus completely protected guinea pigs against viraemia and death when administered shortly after an Ebola virus challenge. It is important to note that preliminary studies

in mice have indicated an activation of interferon α and β , suggesting that stimulation of the immune system may also have a role in efficacy⁷².

More recently, Sato and co-workers used vitamin-A-coupled Lipotrust liposomes to deliver anti-gp46 siRNA to fibrogenic hepatic cells for the treatment of liver cirrhosis. Five administrations of the siRNA lipocomplexes were reported to resolve liver fibrosis and prolong survival in rats that had otherwise lethal liver cirrhosis, in a dose- and duration-dependent manner. It was also shown that rescue was not related to off-target effects or associated with recruitment of the innate immune system¹⁸.

The use of lipid complexes for localized siRNA administration has also proved beneficial. Vaginal instillation of cationic liposomes comprised of Oligofectamine and siRNA targeting herpes simplex virus (HSV)-2 reportedly led to uptake by the epithelial and lamina propria cells in the vagina in mice and protected against lethal infection for up to 9 days. The siRNA complexes protected mice when administered before and/or after (otherwise) lethal HSV-2 challenge²⁷. In another study, intrathecal administration of complexes formulated by i-FECT and siRNA protected mice from fatal Japanese encephalitis virus and West Nile virus after intracranial administration, and reduced pain receptor expression following intrathecal administration in rats⁷³.

Also of note are several synthetic lipid-based materials that have demonstrated DNA transfection efficiency. Similar materials may also potentially serve as candidates for siRNA delivery, given the similarities involved in the two types of nucleic acid delivery⁷⁴. For example, Wheeler *et al.* used liposomes comprising a combination of the cationic lipid GAP-DLRIE (*N*-(3-aminopropyl)-*N,N*-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide) and the neutral co-lipid DOPE (dioleoylphosphatidylethanolamine) to transfect mouse lung with a gene that confers chloramphenicol resistance. After a single intranasal administration, gene expression was enhanced by more than 100-fold relative to plasmid DNA alone, followed by a gradual return to baseline levels by 21 days post-administration⁷⁵. Also of interest are transactivating transcriptional activator (TAT)-modified

Table 2 | **Selected synthetic materials for *in vivo* siRNA delivery**

Material	Model	Target	Route	Animal	Refs
Liposomes and lipids					
i-FECT	Japanese encephalitis virus (JEV) and West Nile virus (WNV)	JEV and WNV envelope	Intracranial	Mouse	70
Lipidoids	Dyslipidaemia	FVII/ApoB	Intravenous	Mouse, rat, monkey	30
	Dyslipidaemia	FVII/ApoB	Intravenous	Mouse, hamster	79
	Malaria	Haem oxygenase 1	Intravenous	Mouse	80
	Hypercholesterolaemia	PCSK9	Intravenous	Mouse, rat	17
LipoTrust	Liver cirrhosis	gp46	Intravenous	Rat	18
Oligofectamine	Herpes simplex virus 2 (HSV-2)	HSV-2-associated viral proteins UL27 and UL29	Intravaginal	Mouse	27
SNALP	Hepatitis B virus (HBV)	HBV	Intravenous	Mouse	4
	Dyslipidaemia	ApoB	Intravenous	Monkey	56
	Ebola (Zaire)	Polymerase L	Intravenous	Guinea pig	69
Cationic polymers					
Cyclodextrin	Ewing's sarcoma tumour xenograft	<i>EWS-FLI1</i>	Intravenous	Mouse	94
	Healthy monkey model	RRM2	Intravenous	Monkey	95
Dynamic PolyConjugate	Dyslipidaemia	ApoB/PPAR α	Intravenous	Mouse	98
Poly-ethyleneimine	Glioblastoma xenograft	PTN	Intratumoral	Mouse	28
	Formalin-induced pain	NMDAR2B	Intrathecal	Rat	87
	Cervical tumour xenograft	HPV E6/E7	Intratumoral	Mouse	20
	Ovarian tumour xenograft	HER2	Intraperitoneal	Mouse	88
Small interfering RNA (siRNA) conjugates					
Cholesterol	Dyslipidaemia	ApoB	Intravenous	Mouse	46
	Huntington's disease	Huntingtin gene	Intrastratial	Mouse	23
Fatty acids/bile salts	Dyslipidaemia	ApoB	Intravenous	Mouse, hamster	47

ApoB, apolipoprotein B; *EWS-FLI1*, Ewing's sarcoma–friend leukaemia virus integration 1; FVII, factor VII blood protein; gp46, a glycoprotein gene; HER2, human epidermal growth factor receptor 2; HPV E6/E7, human papillomavirus oncogenes; NMDAR2B, N-methyl-D-aspartate receptor type 2B; PCSK9, proprotein convertase subtilisin/kexin type 9; PPAR α , peroxisome proliferator-activated receptor α ; PTN, pleiotrophin (a secreted growth factor); RRM2, ribonucleoside-diphosphate reductase subunit M2; SNALP, stable nucleic acid–lipid particles.

liposomes, which, when complexed with a gene encoding green fluorescence protein (GFP) and injected locally, induced the expression of GFP in Lewis lung carcinoma tumour cells in mice⁵⁰.

Although liposomes are among the most popular nucleic acid delivery agents, some concerns regarding their safety for therapeutic use remain. Toxicity of certain cationic lipid particles has been reported both *in vitro* and *in vivo*^{76–78}, and certain synthetic agents have been found to induce a gene signature of their own that might increase the off-target effects of siRNA^{79,80}. Despite these issues, liposomes show promise for future clinical use, as evidenced by the approval of PEGylated liposomes for doxorubicin and amphotericin B delivery by the US Food and Drug Administration^{79,81}.

In another study, a combinatorial library of lipid-like materials was developed for use in siRNA delivery³⁰. These 'lipidoids' were synthesized through the conjugate addition of alkyl-acrylates and -amides to primary and secondary amines and then studied in cell culture. One leading candidate for *in vivo* gene knockdown was identified as 98N₁₂-5(1), which comprises five 12-carbon alkyl-acrylamide chains attached to an amine core (FIG. 4d). Formulations of this material with siRNA were capable of achieving potent and persistent silencing of various lung and liver targets in mice, rats and cynomolgus monkeys³⁰.

In a more recent primate study, 98N₁₂-5(1) was also used to deliver siRNA against PCSK9, a protein that regulates LDL receptor protein levels and function¹⁷. Liver-specific siRNA silencing of PCSK9 reduced PCSK9

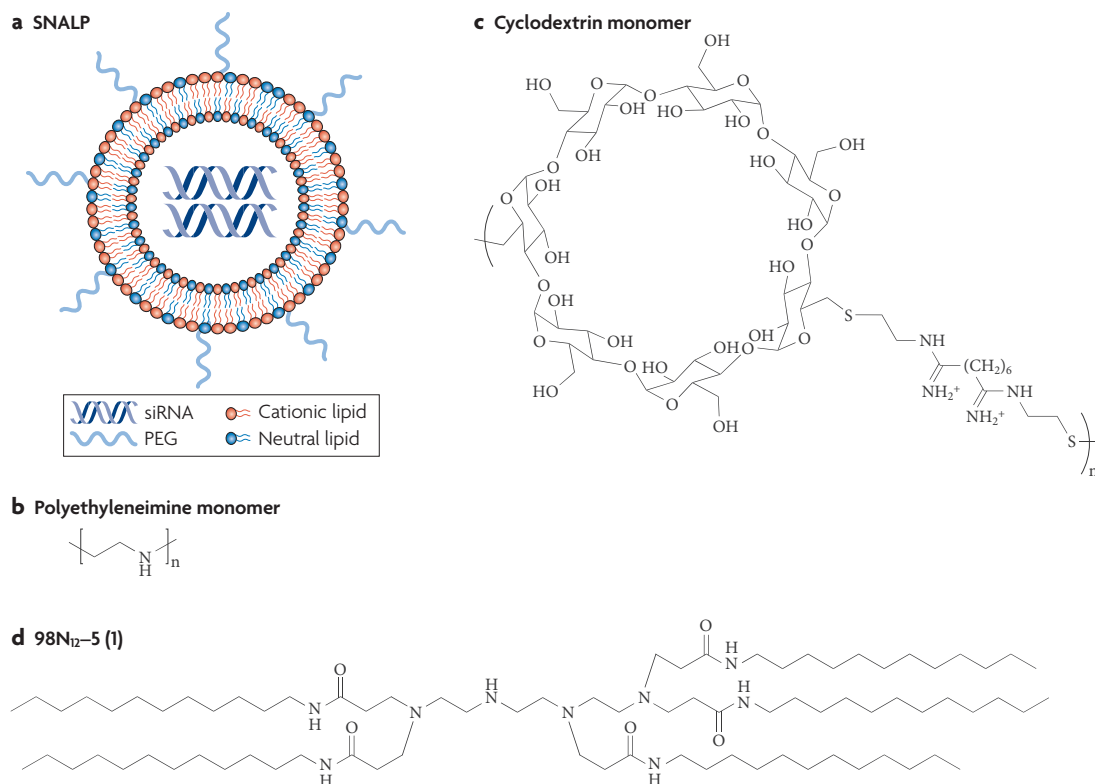


Figure 4 | Synthetic materials for small interfering RNA (siRNA) delivery. a | A representation of stable nucleic acid–lipid particles (SNALPs), which are liposomes comprising cationic lipids, non-ionic lipids and polyethylene glycol (PEG). siRNA is contained in the hydrophilic interior of the particle. **b** | Polyethyleneimine is used to fabricate both linear and branched polymeric delivery agents. **c** | A cyclodextrin-based delivery agent. **d** | The lipidoid 98N₁₂-5(1).

mRNA levels by 50–70% in mice and rats, as well as reducing human PCSK9 transcript levels in transgenic mice by more than 70%. Silencing persisted for up to 3 weeks after a single intravenous dose, indicating that anti-PCSK9–lipidoid complexes may serve as a potent and effective treatment for hypercholesterolaemia¹⁷. Importantly, lipidoid materials were shown to facilitate siRNA delivery without disrupting endogenous micro-RNA processing⁸². Finally, *Hmox1*, a gene expressed in the liver encoding the protein haem oxygenase 1, has also been silenced in mice by siRNA nanoparticles formulated with lipidoids. Knockdown of *Hmox1* may represent a potential approach for the treatment of malaria infection and disease progression⁸³.

Polymers. Cationic polymers with a linear or branched structure can serve as efficient transfection agents because of their ability to bind and condense large nucleic acids into stabilized nanoparticles^{84,85}. Such materials have also been shown to stimulate nonspecific endocytosis as well as endosomal escape⁷⁹. A proposed mechanism for this is the ‘proton-sponge’ effect⁸⁴, whereby buffering of the endosome leads to an accumulation of ions within this compartment and an osmotic pressure that eventually bursts the endosome^{86,87}.

PEI is a broadly investigated delivery carrier for the administration of a wide range of nucleotide-based therapies, including DNA, siRNA and oligonucleotides^{88,89}.

It has also been frequently used for various local siRNA delivery applications. Intrathecal administration of PEI–siRNA complexes was reported to selectively knockdown a pain receptor in rats. Maximal effect occurred on day 3 for mRNA levels and day 7 for associated protein levels following an injection of 5 mg of siRNA targeting a subunit of the NMDA (*N*-methyl-D-aspartate) receptor NR2B⁹⁰. PEI has also shown efficacy in a subcutaneous mouse tumour model. The intraperitoneal administration of complexed siRNA led to the delivery of the intact siRNA into the tumours and a marked reduction of tumour growth through siRNA-mediated downregulation of human epidermal growth factor 2 (HER2; also known as ERBB2)⁹¹. There has been significant concern regarding the toxicity of PEI at higher molecular masses and high doses^{92,93}. However, strategies to modify the structure of PEI to reduce toxicity while retaining its potent ability to transfect cells are in development^{94–96}.

Cyclodextrin polymers have also been developed as siRNA delivery agents. Tumour growth in a mouse model of metastatic Ewing’s sarcoma was shown to be inhibited by the systemic delivery of nanoparticles formed by cyclodextrin, the targeting ligand transferin, and siRNA specific for the *EWS–FLI1* fusion gene commonly associated with the condition. Knockdown was not observed upon removal of the targeting ligand, nor was there any evidence of immune stimulation or toxicity⁹⁷. In another study, Heidel and co-workers used

Table 3 | Current clinical trials for siRNA therapeutics

Company	Disease	Mode of administration	Status
Allergan	Age-related macular degeneration	Topical	Phase II
Alnylam	Respiratory syncytial virus	Local/direct	Phase II
Nucleonics	Hepatitis B virus	Systemic	Phase I
Quark Pharmaceuticals/Pfizer	Acute renal failure	Systemic	Phase I
Opko Health	Age-related macular degeneration	Topical	Phase III
Silence/Quark/Pfizer	Diabetic macular oedema	Topical	Phase II
Transderm	Pachyonychia congenita	Topical	Phase Ia/b

siRNA, small interfering RNA.

cyclodextrin–transferrin nanoparticles (FIG. 4c) to study the effects of siRNA delivery on the immune system of cynomolgus monkeys⁹⁸. Specifically, an siRNA targeting the M2 subunit of ribonucleotide reductase was delivered intravenously in escalating doses, and it was established that multiple, systemic doses of targeted nanoparticles containing non-chemically modified siRNA can safely be administered to non-human primates⁹⁸. In addition, cyclodextrin–transferrin nanoparticles demonstrated efficacy in knocking down luciferase and ribonucleotide reductase genes in mice^{99,100}.

Polymer–siRNA conjugates have also shown potential for applications in systemic siRNA delivery. Rozema *et al.* have developed a polymer-conjugated delivery system called Dynamic PolyConjugates to facilitate delivery of siRNA to hepatocytes¹⁰¹. Key features of the Dynamic PolyConjugate technology include a membrane-active cationic polymer, the ability to reversibly mask the activity of this polymer until it reaches the acidic environment of the endosome, and the ability to target this modified polymer and its siRNA cargo specifically to hepatocytes after intravenous injection. Dynamic PolyConjugates were capable of inducing knockdown of two mouse liver genes. Analyses of serum liver enzyme and cytokine levels in treated mice indicated that siRNA complexes formed with this synthetic polymer were well tolerated¹⁰¹.

Clinical trials

Today, siRNA therapeutics are progressing in the clinic (TABLE 3). Many of the most advanced trials rely on forms of localized delivery, although several ongoing clinical trials involve the use of delivery agents. Not included in TABLE 3 are many formulations that are in preclinical or animal-study phases at the major pharmaceutical companies developing siRNA therapeutics.

Several of the most advanced clinical trials focus on the treatment of age-related macular degeneration (AMD), which is a leading cause of blindness. AMD arises from excessive blood-vessel growth and rupture within the cornea. Naked siRNA targeted to genes for vascular endothelial growth factor (VEGF) and its receptor

(VEGFR), has shown therapeutic potential in its inhibition of the excessive vascularization of the eye that leads to AMD²⁶.

In 2004, the first clinical trial involving siRNA was carried out by Acuity Pharmaceuticals for the treatment of AMD. The completed Phase II trials reported that all doses were well tolerated with a lack of adverse systemic effects. Testing has now moved into Phase III trials, which have been taken over by Opko Health. Allergan is currently conducting a Phase II clinical trial on an siRNA for AMD, with completed Phase I results indicating minimal side effects and improved vision in some of the patients. Although Silence Therapeutics also had an siRNA product for AMD in the pipeline, they have now refocused their Phase II clinical study on the treatment of diabetic macular oedema, which is another condition caused by leaky vasculature within the eye. Importantly, a recent study has reported that anti-VEGF siRNA efficacy in the eye is not due to specific gene silencing, but is instead caused by nonspecific stimulation of the TLR3 pathway, which can reduce angiogenesis¹⁰². Although this study calls into question the nature of the anti-angiogenic effect reported in AMD clinical trials, it does not explain the therapeutic effects observed in other applications of siRNA in which appropriate controls have been performed.

Also of note is Alnylam's RSV01 formulation, which targets the nucleocapsid N gene of RSV — a major cause of respiratory illness in infants and young children. The RSV01 formulation completed Phase I trials and was found to be well tolerated in healthy adults. Since then, Phase II trials have demonstrated significant antiviral efficacy of this formulation in adults¹⁰³.

Future directions and conclusions

Moving forward, we expect that synthetic nanoparticles composed of polymers, lipids, lipidoids or conjugates will have a key role in the systemic application of siRNA in the clinic. The incorporation of tissue-specific ligands into these particles may enable targeting, which will assist with *in vivo* biodistribution and delivery. We also anticipate that future developments will require attention to nonspecific activation of the immune system by siRNA, including TLR3 and TLR7 pathways^{102,104}. Given the potential for nonspecific effects, it is important that the therapeutic mode of action be validated when possible, such as through direct measurement of target mRNA levels *in vivo*. Chemical modification of siRNA — such as 2'-O-methyl substitutions — can minimize nonspecific effects⁴⁰, and we expect that additional improvements in both activity and delivery will be mediated by direct chemical modification of the siRNA sequence.

In summary, the field of RNAi therapeutics has made significant progress since the first demonstration of gene knockdown in mammalian cells. siRNA-based formulations offer significant potential as therapeutic agents to induce the potent, persistent and specific silencing of a broad range of genetic targets. Delivery remains the most significant barrier to the widespread use of RNAi therapeutics in a clinical setting, and future work focusing on the development of safe and effective delivery materials is needed to ensure the broadest application of RNAi in the clinic.

Age-related macular degeneration (AMD). This disease of the eye is caused by excessive growth and rupture of blood vessels within the cornea and is a leading cause of blindness. Several early siRNA clinical trials have targeted this disease, primarily due to the ease of local delivery to the eye.

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Acknowledgements

The authors would like to thank A. de Fougères, R. Alvarez and E. Deguns for their advice and assistance in the preparation of the manuscript.

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