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Review Article

Theme: siRNA and microRNA: From Target Validation to Therapy
Guest Editor: Song Li

Delivery of siRNA Therapeutics: Barriers and Carriers

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Abstract. RNA interference is a naturally occurring endogenous regulatory process where short double-stranded RNA causes sequence-specific posttranscriptional gene silencing. Small interference RNA (siRNA) represents a promising therapeutic strategy. Clinical evaluations of siRNA therapeutics in locoregional treatment settings began in 2004. Systemic siRNA therapy is hampered by the barriers for siRNA to reach their intended targets in the cytoplasm and to exert their gene silencing activity. The three goals of this review were to provide an overview of (a) the barriers to siRNA delivery, from the perspectives of physicochemical properties of siRNA, pharmacokinetics and biodistribution, and intracellular trafficking; (b) the non-viral siRNA carriers including cell-penetrating peptides, polymers, dendrimers, siRNA bioconjugates, and lipid-based siRNA carriers; and (c) the current status of the clinical trials of siRNA therapeutics.

KEY WORDS: gene therapy; nanotechnology; siRNA; systemic delivery; vectors.

INTRODUCTION

RNA interference (RNAi) is a highly efficient regulatory process that causes posttranscriptional gene silencing in most eukaryotic cells (1). RNAi represents a promising new approach for producing gene-specific inhibition and knock-outs, producing transgenic animal models, and designing new therapeutics. This field is progressing at a very rapid pace. The mechanisms of RNAi were first reported in *Caenorhabditis elegans* in 1998 (2). The high significance and transformative values of this discovery were recognized by the award of the Nobel prize in Physiology or Medicine in 2006. The first clinical trial with short interfering RNA (siRNA) in patients suffering from age-related macular degeneration (AMD) commenced in 2004; the relatively good safety profiles of siRNA in humans have led to its clinical evaluation in other genetic and viral diseases (1,3).

There have been several excellent recent reviews on siRNA delivery, with in-depth discussion usually on a specific system (e.g., siRNA conjugates, lipid-based siRNA delivery, polymer nanocarriers, and peptide transduction domains and cell-penetrating peptide) (4–7) or specialized delivery techniques (e.g., hydrodynamic intravascular injection) (8). These earlier reviews are particularly useful for researchers working in the specific fields. The purpose of this report was to provide a broad and comprehensive overview on the multiple barriers, the diverse carrier systems, and the current status of the clinical development of siRNA therapeutics. This review contains four parts. Part 1 describes the mechanisms of RNAi. Part 2 describes the barriers to delivering siRNA therapeutics to their intended targets. Part 3 describes the non-viral carriers of siRNA. Part 4 provides the current status of clinical studies of siRNA therapeutics.

PART 1: MECHANISMS AND POTENTIAL APPLICATIONS OF RNAi

Mechanisms of Gene Silencing by siRNA

siRNAs are short stretch (19–30 nucleotides) dsRNAs capable of degrading the complementary mRNA in the cytoplasm. Figure 1 shows the mechanism of gene silencing by siRNA. In the cytoplasm, long dsRNAs are cleaved by the endoribonuclease Dicer into short dsRNA duplexes or siRNA. siRNA are loaded onto RNA-induced silencing complex (RISC). RISC contains Argonaute 2 (Ago-2) which cleaves and releases one strand from the dsRNA, resulting in an activated form of RISC with a single-strand RNA (guide siRNA) that directs the specificity of the target mRNA recognition through complementary base pairing (9). Ago-2 then cleaves the target mRNA between bases 10 and 11 relative to the 5′ end of the siRNA antisense strand, thereby causing mRNA degradation and gene silencing (10).

Comparisons of RNAi Therapeutics

The three types of RNAi are siRNA, short hairpin RNA (shRNA), and micro-RNA (miRNA) (10). miRNAs are non-coding single-stranded RNAs transcribed from either their own genes or from introns by RNA polymerase II. After transcription, the primary miRNA is first processed into pre-

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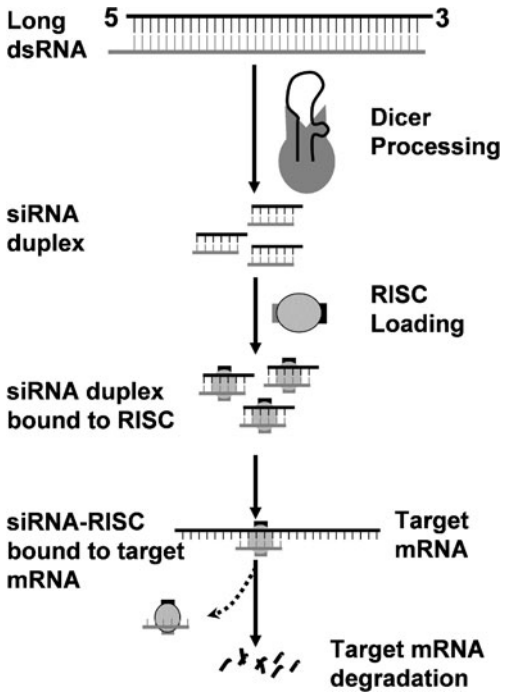

Fig. 1. siRNA-mediated gene silencing. In the cytoplasm, long dsRNAs are cleaved by the endoribonuclease Dicer into short dsRNA duplexes or siRNA. siRNAs are loaded onto and activate RISC, which contains Ago-2. Ago-2 cleaves and releases one strand, resulting in an activated form of RISC with a single-strand RNA molecule that directs the specificity of the target mRNA recognition through complementary base pairing. Ago-2 then cleaves the target mRNA, resulting in mRNA degradation and gene silencing

miRNA with a stem-loop structure (about 70 nucleotides) and then into a functional miRNA (21–23 nucleotides). Encoded within an expression vector, shRNA is a sequence of RNA with a tight hairpin turn. shRNA is transcribed by RNA polymerase III, and its expression is dependent on a promoter. The hairpin structure of shRNA is cleaved and processed into siRNA (11,12). All three types of RNAi in the end use the RISC for mRNA degradation and posttranscription gene silencing and have the potential to target any mRNA of interest, but have different efficiencies and specificities of gene knockdown.

In contrast to siRNA which has perfect complementarity to the target mRNA, miRNA binds imperfectly to the target mRNA. This partial complementary binding allows each miRNA to potentially interact with many similar sets of target mRNA. In addition to mRNA degradation, miRNA may cause translational repression without endonucleolytic cleavage (13). Some miRNA-targeted genes may be regulated translationally without affecting the mRNA level of the target (14). The advantage of miRNA over siRNA is that one single miRNA transcript can be processed into multiple siRNAs (15).

Under *in vitro* conditions, siRNA-mediated gene silencing is relatively transient, lasting for only several days and usually of shorter duration compared to shRNA-expressing plasmid DNA (pDNA) (16). Under *in vivo* conditions, siRNA and shRNA were equally effective in luciferase knockdown from 3 h to 3 days, although shRNA was significantly more potent on a molar basis (17). From the standpoint of transfection efficiency and delivery, siRNA poses advantages over shRNA. For transfection, the activity of siRNA does not require interaction with chromosomal DNA, whereas shRNA-expressing pDNA requires suitably designed promoters. For delivery, siRNA acts in the cytosol and does not require transport into the nucleus, whereas shRNA acts in the nucleus. The latter poses an additional challenge, especially for quiescent cells that have low permeability across the nuclear envelope. For example, siRNA has higher transfection efficiency compared to shRNA in cells with low proliferating activity (16). The 100 times lower molecular weight of siRNA (~19–30 bp), compared to shRNA, also presents less obstacles for delivery and improves the ease of chemical modifications. The remainder of this review focuses on siRNA.

Factors Limiting the Utility of siRNA Therapeutics

Several factors limit the utility of siRNA. siRNA may compete with endogenous RNA for, and cause the saturation of, the miRNA processing pathways. The latter, in turn, can lead to toxicity, e.g., liver toxicity in mice receiving high doses of liver-directed AAV-encoded shRNA (18). RNA may stimulate innate immune responses. Certain GU-rich sequence motifs (e.g., 5′-GUCCUUCAA-3′) result in the secretion of inflammatory cytokines in a cell type- and sequence-specific manner. Longer dsRNA (>30 nucleotides) can rapidly induce interferons by activating the evolutionarily conserved mechanisms aimed at combating invading viral pathogens, whereas shorter dsRNA (<30 nucleotides) do not have this problem (19). Immune stimulation by synthetic siRNA can also be completely abrogated by selective incorporation of 2′-*O*-methyl (2′*O*Me) uridine or guanosine nucleosides into one strand of the siRNA duplex (20). siRNA may suppress off-targets due to partial nucleotide sequence match between the siRNA and off-target mRNA (21) and/or global degradation and inhibition of translation of mRNA caused by siRNA and its transfection agent (22,23).

The single most critical factor limiting the utility of siRNA as therapeutics is delivering siRNA to its intracellular target site due to their unfavorable physicochemical properties (negative charges, large molecule weight, and size) and instability with plasma half-lives of about 10 min (10,15,24). Furthermore, siRNA, after endocytosis, is transported to lysosomes where siRNA is degraded (5). The latter diminishes the activity of siRNA therapeutics. This review describes the barriers to systemic siRNA delivery and the strategies that may overcome these barriers.

PART 2: BARRIERS TO siRNA DELIVERY

Regional *vs.* Systemic Delivery

The site of action of siRNA therapeutics is the cytosol. The barriers to siRNA delivery are multiple and depend on the targeted organs and the administration routes. In general, locoregional delivery of siRNA has fewer barriers compared to systemic delivery. For example, intranasal inhalation of siRNA against respiratory syncytial virus, either naked or encapsulated in polycationic liposomes, was about equally

effective in reducing the viral infection (25). Readers are referred to the excellent reviews outlining the physical and immunologic barriers to siRNA delivery to the eye, skin, lung, and brain (26–29).

Systemic delivery, e.g., intravenous injection, of siRNA poses significantly greater challenges. Figure 2 outlines the steps for siRNA to travel from the site of administration to the site of action. After intravenous injection, siRNA is distributed to organs via the blood circulation and at the same time undergoes elimination. Within an organ, siRNA leaves the intravascular space within a blood vessel to enter the interstitium (i.e., extravasation). After entering the tissue

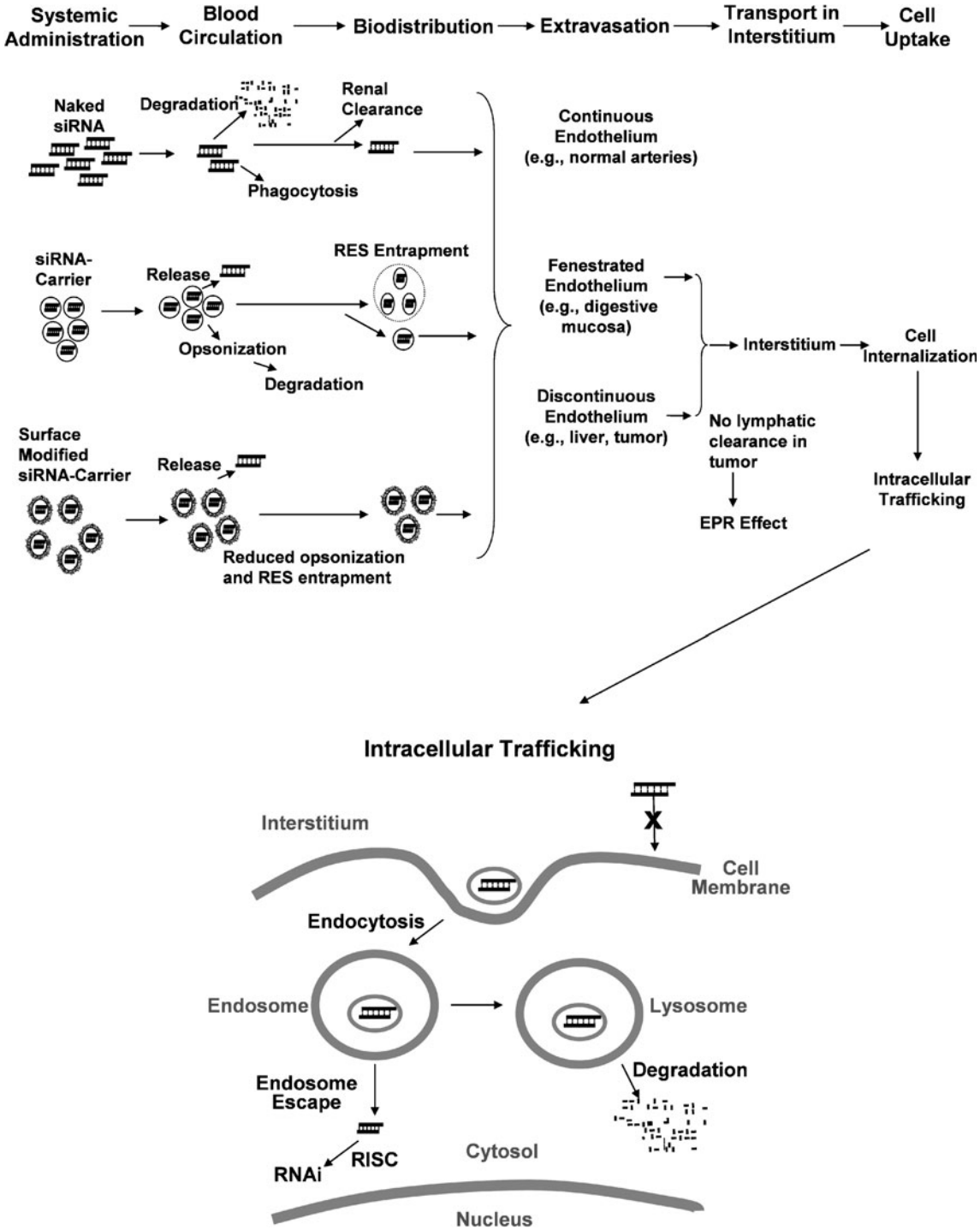

Fig. 2. Barriers to systemic siRNA delivery. Barriers to distribution to the target organs include the degradation of siRNA and carriers, protein absorption (opsonization) and phagocytosis by mononuclear phagocyte system, and entrapment in reticuloendothelial system (*RES*). Barriers to extravasation and penetration in extracellular matrix are dependent on the physiological structure of the target tissue (see text). Barriers to cellular internalization are dependent on the surface properties of siRNA and carriers (e.g., charge, pegylation, and specific binding antigen). The major barriers for delivering siRNA to its site of action are the endosomal entrapment and the lysosomal degradation of siRNA and carriers

interstitium, siRNA is transported across the interstitial space to the target cells. After reaching the target cell, siRNA undergoes internalization *via* endocytosis, a process that involves siRNA being encapsulated in endocytic vesicles that fuse with endosomes. After entering the cell, siRNA must escape from the endosomes and be released from its carrier to the cytosol in order to be loaded onto RISC (24).

Systemic Elimination of siRNA

From the drug delivery standpoint, siRNA molecules have unfavorable physicochemical properties including negative charges, large molecule weight and size, and instability. Naked siRNA is readily degraded by serum endonucleases and is efficiently removed by glomerular filtration, resulting in short plasma half-life of <10 min (30). These problems are partially overcome by chemical modifications of the RNA backbone and the use of nano-sized carriers (1).

Entrapment in the Reticuloendothelial System

Because naked siRNA is infrequently applied in systemic delivery, this section focuses on siRNA-loaded carriers. Such carriers include nanospheres, nanocapsules, liposomes, micelles, microemulsions, conjugates, and other nanoparticulates. These systems offer a suitable means to deliver small-molecular-weight compounds as well as macromolecules such as proteins, peptides, or siRNA. These carrier systems protect siRNA from undesirable interactions with biological milieu components and from metabolism or degradation and, in the case of cancer therapeutics, favorably improve the passive targeting of solid tumors due to the unique tumor features (i.e., leaky vasculature with capillary pore size of 100–800 nm and the absence of lymphatic drainage) (31).

Upon systemic administration, nano-sized carriers are rapidly distributed to organs in the reticuloendothelial system (RES) and phagocytosed by the mononuclear phagocyte system (e.g., macrophages and liver Kupffer cells). These clearance processes, mediated by the interaction of particles with blood components (e.g., immunoglobulins of the complement system), result in higher particle accumulations in RES organs, such as liver and spleen, relative to non-RES organs. This property has been used to target the siRNA delivery to the RES organs (32,33).

Several factors, including surface characteristics, surface charge, and size of the nanoparticles, may affect RES uptake and biodistribution. The general view is that a negative surface charge increases the clearance of particulates from systemic circulation relative to neutral or positively charged particles (34). Surface modifications using hydrophilic and flexible polyethylene glycol (e.g., pegylation) and other surfactant copolymers, e.g., poloxamers, polyethylene oxide, result in stealth particles that remain in the systemic circulation for a prolonged period of time (35,36). These modifications can limit the protein absorption on the particle surface and thereby protect the vectors against opsonization, reduce the complement activation, and promote the cargo stability. These stealth properties are effective for particles within the size range of 70–200 nm (37,38). On the other hand, pegylation may neutralize the positive surface charge that is required for siRNA uptake into cells. For example, increasing pegylation of siRNA–lipoplexes from 1–2 to 5 mol % PEG2000 completely abolished the siRNA-mediated gene silencing against PTEN protein *in vitro* (39).

Extravasation: Vascular Endothelial Barrier

Within an organ, siRNA or carriers undergo extravasation and enter the interstitium. Transport across vessel walls can occur *via* diffusion, convection through capillary pores, and transcytosis. Diffusion is driven by the concentration gradient. Transvascular fluid transport is driven by the difference in hydrostatic and osmotic pressures between the blood vessel and interstitial space. In general, capillary pore size poses the upper size limit for the extravasated particulates (31,40). Endothelium in blood vessels can be classified as continuous (e.g., normal arteries, arterioles, capillaries), fenestrated (e.g., digestive mucosa), and discontinuous (e.g., liver) (41). Abnormal neovascularization and enhanced vascular permeability are found in solid tumors, AMD, and retinopathy of prematurity and diabetic retinopathy (41). The following section discusses the effects of blood vessel permeability on drug delivery. More detailed discussions of this topic and the corresponding references can be found in our earlier reviews (31,40).

One of the unique features of tumor microvessels is their leakiness due to endothelial discontinuity. The pore size of tumor microvessels ranges from 100 to 780 nm in diameter. In comparison, microvessels in most normal tissues are less leaky; the tight junctions between endothelial cells are usually <2 nm and the pore size in post-capillary venules is <6 nm, whereas fenestrated endothelium of the renal glomeruli and the sinusoidal endothelium of the liver and spleen show larger pore sizes of 40–60 and 150 nm, respectively. Due to vessel leakiness, the major pathway of drug transport across tumor microvascular wall is by extravasation *via* diffusion and/or convection through the discontinuous endothelial junctions, whereas transcytosis plays a relatively minor role. Leakiness in tumor vessels promotes siRNA/carrier extravasation, but also elevates interstitial fluid pressure and reduces transvascular fluid transport.

Transport in Tissue Interstitium

Transport of small molecules in the interstitial space is mainly by diffusion, whereas transport of large molecules is mainly by convection. Diffusion depends on the diffusivity and concentration gradient, and convection depends on the hydraulic conductivity and pressure difference. For tumors, due to the higher interstitial fluid pressure compared to normal tissues, the pressure-driven convective flow in tumor interstitium is outward from the core of a tumor into the surrounding normal tissues. The lack of a lymphatic system in solid tumors increases interstitial fluid pressure, thereby inhibiting the convective transport in tumor interstitial space (42).

Internalization of siRNA in Cells

Naked siRNA does not readily cross the anionic cell membrane through passive diffusion due to the high molecular weight, large size, and negative charges of the phosphate

backbone. The major mode of internalization is endocytosis, whereby the drug molecules are internalized together with a component of the cell membrane (Fig. 2). Coating of carriers with ligands and antibodies can promote the carrier-specific binding to cell membrane. The positively charged siRNA–carrier complex interacts with anionic proteoglycans on the cell surface, forms an endocytic vesicle, and enters the cells by endocytosis (43).

Intracellular Transport of siRNA

Following cellular internalization, the siRNA–carrier complex (in endocytic vesicles) is transported along microtubules to lysosomes that are co-localized with the microtubule organizing center. The endocytotic vesicles sequentially fuse with early endosomes which mature into late endosomes before fusing with lysosomes. The fate of the internalized molecules inside the vesicle depends on the specific type of receptors and includes the following: recycled to the cell surface, degraded inside lysosomes, or released to other intracellular compartments including the cytosol (44). The endosomal entrapment and lysosomal degradation of siRNA–carrier contributes to the low transfection efficiency and is a major impediment for non-viral carriers. Two recent reviews outline the strategies and mechanisms for siRNA carriers to escape the endosome–lysosome degradation axis (5,45).

PART 3: siRNA CARRIERS

Due to the similar physicochemical properties between DNA and siRNA, carriers developed for DNA have also been applied to siRNA. These carriers can be broadly divided into two categories, i.e., viral and non-viral. Readers are referred to a recent review on viral delivery systems for RNAi (1). In part due to the potential toxicities associated with viral vectors, non-viral vectors have become increasingly popular alternatives. Non-viral siRNA vectors typically involve complexing siRNA with a positively charged vector (e.g., cationic cell penetrating peptides, cationic polymers and dendrimers, and cationic lipids); conjugating siRNA with small molecules (e.g., cholesterol, bile acids, and lipids), polymers, antibodies, and RNAs; and encapsulating siRNA in nanoparticulate formulations (Fig. 3). Modification of the RNA backbone improves the stability of siRNA without affecting its RNAi efficiency (1). The selection of siRNA delivery systems depends on the properties of siRNA, the type of target cells, and the delivery routes for *in vivo* application.

Cationic Cell Penetrating Peptides

Cationic cell penetrating peptides (CPP) have been used for the intracellular delivery of macromolecules including proteins (e.g., antibodies), peptides, antisense oligonucleotides, and plasmid DNA (7). In addition to utilizing the traditional endocytotic pathways, CPP-mediated siRNA delivery systems may enter cells directly by crossing the cell membrane.

CPP and siRNA form non-covalent complexes (non-covalent CPP–siRNA) through electrostatic interactions; the resulting complexes are positively charged (46). The major advantage of this approach is that it does not require a chemical modification of siRNA, which in turn preserves the activity of siRNA and reduces the purification procedures. dsRNA can form a non-covalent complex with MPG, a peptide derived from the hydrophobic fusion peptide domain of HIV-1 gp41 protein and the hydrophilic nuclear localization sequence of SV40 large T antigen; the complexes enter cells using endosome-independent pathways followed by entry into the nucleus. The latter requires the SV40 nuclear localization sequence; mutations of such sequence prevented nuclear delivery and resulted in the rapid release of siRNA into the cytoplasm (47).

CPP and siRNA can also form covalent cross-links through disulfide bonds (covalent CPP–siRNA). Cleavage of the disulfide bonds in cells releases siRNA into the cytoplasm. For example, covalent conjugation of thiol-containing siRNA to the CPP penetratin or transportan via a disulfide bond knocks down the transient and stable expression of reporter genes in several mammalian cell types; the effects were equivalent or better compared to cationic liposome siRNA carriers (48). Rapid and highly efficient uptake of siRNA linked to the vector peptide Penetratin1 resulted in specific knockdown of the targeted proteins in cultured primary mammalian hippocampal and sympathetic neurons within hours without the toxicity associated with transfection (49).

Conjugation between siRNA and CPP affects cellular localization. For example, conjugation of siRNA with TAT peptide or TAT-derived oligocarbamate through chemical cross-linking enhances siRNA uptake in cells and the perinuclear localization of the conjugates, whereas the non-conjugated free TAT peptide was localized in the nucleus. In comparison, variations of the siRNA sequence and presence of the target mRNA had no effect on the subcellular localization of siRNA. These findings indicate that conjugation altered the localization of CPP (50), possibly in endosomes and lysosomes that are localized perinuclearly.

Polymeric and Dendrimeric Carriers

Linear or branched cationic polymers are efficient DNA transfection agents. The structural and chemical properties of these polymers are well established. The positively charged polymers, through electrostatic interactions, form polyplexes with the negatively charged phosphates of DNA (51). This process results in DNA condensation and protects plasmids from nuclease digestion. Similar siRNA–polymer polyplexes have been used. Other polymeric carriers of siRNA include micelles, nanoplexes, nanocapsules, and nanogels (6). The properties of polyplexes (e.g., size, surface charge, and structure) are dependent on the ratio of the positive charges of cationic polymers to the number of phosphate groups of siRNA. A variety of polymers such as poly-L-lysine, polyethyleneimine (PEI), poly-D,L-lactide-co-glycolide (PLGA), poly(alkylcyanoacrylate), chitosan, and gelatin have been investigated. The following discussions focus on PEI and PLGA. PEI is the most widely investigated polymer for delivering DNA/siRNA/oligonucleotides *in vitro* and *in vivo*. PLGA is a well-studied, biodegradable, and biocompatible polymer that has been used for decades in pharmaceutical applications.

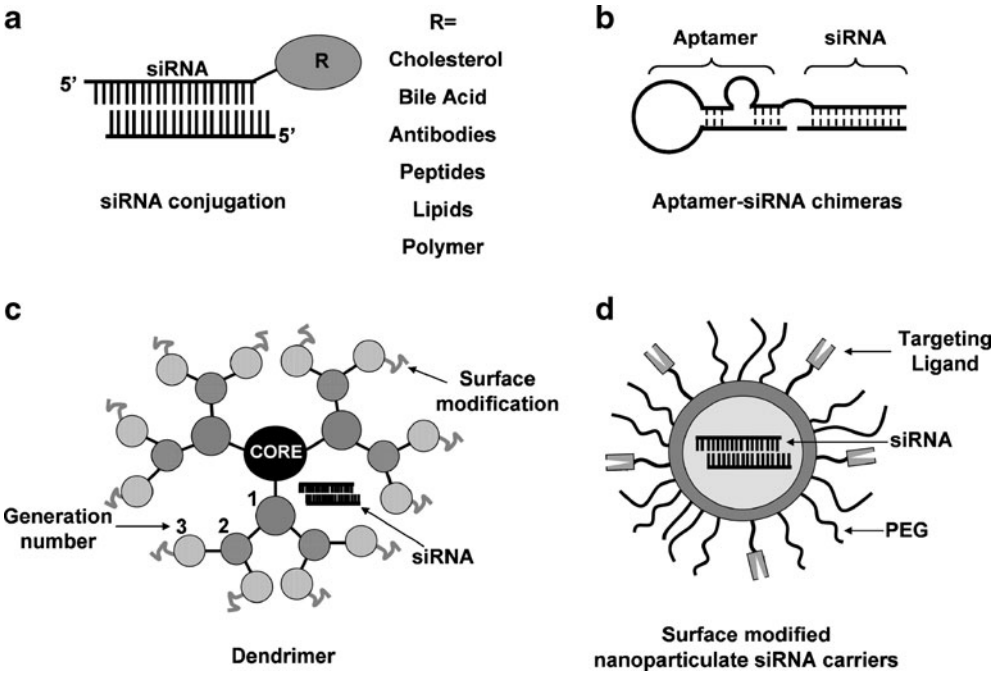

Fig. 3. Examples of siRNA delivery carriers. **a** siRNA bioconjugates: siRNA is conjugated with selected molecules through chemical cross-linking or disulfide bonds. **b** Aptamer–siRNA chimeras: the aptamer portion mediates the specific binding to the target cell and the siRNA portion mediates the gene silencing. **c** Dendrimer: the example shows the three-generation layer-by-layer nanostructure with interior encapsulation of siRNA. **d** Surface-modified nanoparticulate siRNA carriers: the surface of a nanoparticulate can be modified with pegylation to obtain stealth properties and/or attached to a targeting ligand to attain specific delivery to cells expressing the ligand receptors

Cationic polymers with high charge density present the "proton sponge" properties that stimulate escape from endosomes and protect oligonucleotides from degradation (5). PEI, by attracting and sustaining substantial proton influx, induces osmotic swelling and rupture of endosomes, leading to the release of DNA in the cytoplasm, and thereby prevents the transport of DNA to lysosomes and the subsequent degradation (52,53). Endosomal escape of polymeric carriers has also been observed with polymersomes which are polymer-based vesicular shells used for siRNA and protein delivery (54).

PEI are available as linear or branched structures with molecular weights ranging from 1 to 1,000 kDa; both properties can affect its transfection efficiency and toxicity. In general, a branched structure confers higher transfection efficiency, and a higher molecular weight leads to greater toxicity. Non-covalent complexation of synthetic siRNA with low-molecular-weight PEI stabilizes siRNA and enhances its intracellular delivery (55). The hydrolytic purity of PEI is important for both transfection efficiency and specificity (3,26,56). PEI–DNA or PEI–siRNA complex can be lyophilized in 5% glucose without loss of transfection efficacy and produces efficient transfection *in vivo* after systemic and regional delivery (26,57). For example, intrathecal injection of a branched PEI–siRNA complex targeting a pain receptor NMDA-R2B decreased the mRNA and protein levels of the receptor and abolished formalin-induced pain in rats (58). Intraperitoneal administration of PEI–siRNA targeting the c-erbB2/neu (HER-2) receptor resulted in siRNA delivery to the tumor, receptor downregulation, and significant reduction of tumor growth, whereas naked siRNA failed to produce these effects (55). PEI has also been used to construct ligand-targeted, sterically stabilized nanoparticles for systemic siRNA delivery; the pegylated nanoparticles were conjugated with an Arg-Gly-Asp peptide ligand attached at the distal end of PEG to target integrin-expressing tumor neovasculature. The resulting nanoparticles, upon intravenous administration to tumor-bearing mice, successfully delivered siRNA in a tumor-selective manner, inhibited vascular endothelial growth factor (VEGF) receptor-2 expression, inhibited tumor angiogenesis, and slowed tumor growth (59).

PLGA is the most extensively investigated copolymer as carriers for controlled drug release. It has the advantage of lower toxicity compared to cationic lipids and cationic polymers. PLGA nanocapsules have been used to encapsulate DNA, antisense oligodeoxynucleotides, and siRNA in order to improve the stability and to provide sustained release (60). In mice, PLGA microspheres yielded sustained release of siRNA, antisense oligonucleotides, ribozymes, and DNAzymes for at least 7 days at the subcutaneous injection site (60). Recent efforts have focused on modifying PLGA to yield positively charged carriers in order to increase cellular uptake. For example, coating PLGA nanoparticles with chitosan improved the cellular internalization of antisense oligonucleotides and siRNA in cultured cells (61,62). Biodegradable polyester nanoparticles consisting of tertiary-amine-modified polyvinyl alcohol backbones grafted to PLGA yielded rapid degradation properties for siRNA release such that a minute amount (5 pmol) of anti-luc siRNA was sufficient to produce 80–90% knockdown of a luciferase

reporter gene in cultured cells (63). Incorporation of PEI into PLGA nanoparticles improved siRNA loading and activity; PEI–PLGA nanoparticles showed 100% siRNA loading, protected siRNA from nuclease degradation, and yielded greater gene silencing activity in cultured cells compared to PEI alone (64,65). Intratumoral injection of PEI–PLGA microspheres of anti-VEGF siRNA suppressed tumor growth (66). Addition of a cationic co-encapsulant (e.g., polyamines) to PLGA nanoparticles increased siRNA density and loading; the resulting nanoparticles, when applied intravaginally, penetrated the vaginal epithelial tissue, were internalized into cells, and yielded sustained siRNA release and sustained gene knockdown in the vaginal tract (67).

Dendrimers are synthetic macromolecules with well-defined and highly branched peripheral chain ends that are synthesized in an algorithmic step-by-step fashion; every repeated sequence represents a higher generation molecule. Dendrimers have been used as carriers of small molecule drugs and large biomolecules. Dendrimers with positively charged surface groups are used to deliver genes, antisense oligonucleotides, and siRNA. The precise core–shell nanostructures of dendrimers enable drug loading by interior encapsulation, surface adsorption, or chemical conjugation (Fig. 3). The biocompatibility of dendrimers is related to their structure, molecular size, and surface charge (68). Cytotoxicity and immunogenicity of dendrimers are related to its surface charge. Cationic polyamidoamine (PAMAM, amino-terminated surface) is more cytotoxic than anionic PAMAM (carboxylate-terminated surface) in Caco-2 cells (69). PAMAM-mediated siRNA delivery was found at both perinucleus and nucleolus locations (70,71). Similar to other nano-sized carriers, pegylation has been applied to improve the surface features of dendrimers (72,73). Poly(propylene imine) (PPI) is a highly branched dendrimer terminated with high-density amino groups. Modifications of PPI dendrimers at the exterior primary amine groups (e.g., with acetyl groups) and at the interior tertiary amines (e.g., with methyl iodide) improved the dendrimer-mediated intracellular uptake of fluorescein isothiocyanate (FITC)-labeled DNAzyme in cultured cells and, upon intravenous injection to mice, resulted in nuclear uptake in solid tumors (74). siRNA–PPI complexes were layer-by-layer caged with a dithiol containing cross-linker molecules, coated with PEG, and conjugated with a synthetic analog of luteinizing hormone-releasing hormone (LHRH) peptide to direct the specific siRNA nanoparticles delivery to LHRH receptor-positive cancer cells. The resulting siRNA–PPI nanoparticles (100–150 nm) showed specific intracellular delivery, release of siRNA in the cytoplasm, and efficient gene silencing *in vivo* (75).

Over the last decade, cyclodextrin-containing cationic polymers and dendrimers have received growing attention as gene carriers. Cyclodextrins (CD) are naturally occurring cyclic oligosaccharides composed of 6 (α-CD), 7 (β-CD), or 8 (γ-CD) D(+)-glucose units linked by α-1,4-linkages. CD and their derivatives are used in pharmaceutical formulations to enhance the solubility, stabilization, and absorption of small molecule drugs and proteins or peptides. Compared to its non-CD-containing polymer counterparts, CD-containing cationic polymers, including linear and branched PEI and PAMAM, have reduced cytotoxicity and are readily pegylated (76–79). Intravenous injection of a four-component carrier system, comprising CD-containing polycation, PEG, human transferrin ligand (to target the transferrin receptor on cell surfacse), and siRNA against EWS-FLI1 gene (transcriptional activator in the tumorigenesis of Ewing's family of tumors), dramatically inhibited tumor growth in a murine model of metastatic Ewing's sarcoma. The antitumor effect was transferrin ligand-dependent and was abolished by removal of transferrin (80,81). *In vivo* biodistribution, monitored using positron emission tomography and bioluminescent imaging, indicates similar biodistribution and tumor localization for non-targeted and transferrin-targeted siRNA nanoparticles. However, the transferrin-targeted formulation showed greater knockdown of the reporter gene luciferase, consistent with transferrin-mediated endocytosis. These data indicate the importance of cellular internalization after siRNA reaches the target site (82). The safety of a similar system was further tested in cynomolgus monkeys using a different siRNA, i.e., siRNA targeting the M2 subunit of ribonucleotide reductase. The toxicity of siRNA was dose-dependent; the treatments were well tolerated at lower doses (3 and 9 mg/kg) with kidney and liver toxicity and mild immune response at a higher dose of 27 mg/kg (83).

siRNA Bioconjugates

Conjugation of siRNA with a variety of molecules including small molecules (e.g., cholesterol, bile acids, and lipids), peptides (see "Cationic Cell-Penetrating Peptides"), polymers, proteins (e.g., antibody), and aptamers (e.g., RNAs) improves the stability, cellular internalization, or cell-specific active targeting delivery (4).

Cholesterol is covalently conjugated to the 3′-terminus of the sense strand of siRNA *via* a pyrrolidone linkage. The cholesterol–siRNA conjugate showed greater stability and, upon intravenous injection, was detected in the liver, heart, lungs, kidneys, and fat tissues at 24 h, silenced the expression of an endogenous gene encoding apolipoprotein B (apoB, which encodes a protein for cholesterol metabolism) in the liver and the jejunum, decreased plasma apoB protein level, and reduced the total cholesterol level (33). In comparison, intravenous injection of the naked siRNA did not result in a detectable siRNA activity in tissues at 24 h.

siRNA was conjugated to the endosomolytic agent amphipathic poly(vinyl ether) PBAVE to enhance endosomal escape after endocytosis. The conjugation was through a disulfide linkage which was then reversibly modified with maleic anhydride derivatives containing PEG (to reduce nonspecific interactions) and *n*-acetylgalactosamine ligand (to enhance hepatocytes specific targeting) (84,85). The resulting siRNA had a small particle size (10 nm) and was negatively charged and, upon intravenous injection, was effective to knock down two endogenous genes in mouse liver, i.e., apoB-1 and apoB-2 and peroxisome proliferator-activated receptor alpha without altering the levels of serum liver enzymes (ALT and AST) and cytokines (TNF-α and IL-6) (85). In theory, hepatocytes are attractive targets for siRNA due to the relatively easy delivery secondary to the passive targeting features of nano-sized drug carriers to liver.

Conjugation or complexation of siRNA to antibody increases the specificity of siRNA delivery and minimize the off-target effects. Fusion of protamine to the C terminus of the heavy chain Fab fragment (F105) against HIV-1 envelope

yielded the fusion protein F105-P that binds FITC-labeled siRNA in a 1:6 molar ratio. The F105-P–siRNA conjugate was selectively delivered to HIV-infected Jurkat cells, did not trigger interferon responses, and reduced the HIV replication in the difficult to transfect HIV-infected CD4 T cells. Under *in vivo* conditions, intratumoral injection of F105-P–siRNA showed selective cellular delivery to the HIV envelope-expressing B16 melanoma cells without delivery to the adjacent normal tissue or the envelope-negative B16 cells. Similarly, intratumoral and systemic injection of F105-P–siRNA targeting c-myc, MDM2, and VEGF inhibited the growth of envelope-expressing subcutaneous B16 tumors in mice. These data indicate the therapeutic potentials of the antibody–siRNA complex (86).

Cell type-specific delivery can also be achieved by linking siRNA to an RNA aptamer or aptamer–siRNA chimeras. Aptamers are oligonucleic acids or peptides that bind to a specific target molecule. These chimeras have the theoretical advantage that their relatively small molecular weights may improve the transport in tissue interstitium. An aptamer–siRNA chimera where the aptamer portion (A10) provided specific binding to prostate-specific membrane antigen (PSMA, a receptor overexpressed in prostate cancer cells) was internalized in PSMA-expressing cells, but not in PSMA-negative cells, and the repeated intratumoral injections of the A10-PLK1 chimera targeting the prosurvival gene PLK1 promoted tumor regression in the PSMA-positive human prostate cancer LNCap xenograft model, but had no benefits in the PSMA-negative prostate cancer PC3 xenograft model (87). The structure of aptamer–siRNA chimeras was further optimized by adding 2-nucleotide 3′-overhangs and PEGylation, which resulted in a significant regression of PSMA-expressing tumors in athymic mice after systemic administration (88).

Lipid-Based Carriers

Lipid-based siRNA delivery systems include liposomes, micelles, microemulsions, and solid lipid nanoparticles (89). Liposomes are globular vesicles with an aqueous core and phospholipid bilayer, comprise natural body constituents (e.g., lipids, sterols), and are biocompatible and biodegradable. Furthermore, due to their relative simplicity and well-known pharmaceutical properties, liposomes are popular siRNA carriers. The amphipathic nature of liposomes allows the incorporation of a wide variety of hydrophilic and hydrophobic drugs. Hydrophilic molecules show greater affinity between the hydrophilic head groups of phospholipid bilayers and the aqueous core of the liposomes, whereas hydrophobic molecules tend to be intercalated into the fatty acyl chains of the lipid bilayer. Several liposomal carriers of cancer drugs have shown good safety records in humans, and one (Doxil) has received FDA approval for human use.

While neutral liposomes have been successfully used to deliver siRNA under *in vitro* and *in vivo* situations (90), cationic lipids remain the key components of lipid-based siRNA delivery systems. A number of cationic lipids, e.g., 1,2-dioleoyl-3-trimethylammonium-propane and *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride, have been investigated. Commercially available formulations such as Lipofectamine 2000 are used for *in vitro* transfection. Cationic lipids consist of three parts, a cationic head group, a lipophilic tail group, and a connecting linker. Cationic head groups include quaternary ammonium salt lipids, lipoamines, combinations of lipoamines and quaternary amines, and amidinium salt lipids. Lipophilic tail groups usually consist of saturated or unsaturated alkyl chains (12–18 carbons in length) or cholesteryl groups. Linkers are usually ethers and esters, amides, or carbamates. The structure of the cationic head group, the carbon chain length of the tail group, and the nature of the linker affect the transfection efficiency and the toxicity of siRNA carriers. Lipids with small hydrophilic head group and bulky alkyl chains favor the endosomal escape and enhance the transfection efficiency (5). The cationic lipid spontaneously forms multilamellar structures with the anionic siRNA upon mixing. The formation of such lipoplexes protects siRNA from degradation, facilitates cellular uptake through endocytosis, enhances the release of siRNA from endosomal/lysosomal entrapment, and thereby promotes siRNA accumulation in the cytosol. The ratio of lipid and siRNA can be optimized to generate a positive zeta potential for the lipoplex to facilitate its interaction with the cell membrane and the subsequent cellular internalization. A recent review discusses the toxicogenomics of cationic lipids including the initiation of immune response and changes in the expression of non-target genes (3).

Lipid-based carriers have been successfully used to deliver siRNA to target sites in the endothelium, RES organs (e.g., liver), and solid tumors. For example, intravenous injection of siRNA loaded in liposomes of cationic and fusogenic lipids yielded sustained siRNA presence and decreased the mRNA and protein levels of the two targeted genes, CD31 and Tie2, in the vasculature endothelium in different organs, whereas the injection of naked siRNA resulted in localization in the kidney (39). Intraperitoneal injection of cationic liposomal anti-TNF-alpha siRNA to mice inhibited lipopolysaccharide-induced TNF-alpha gene expression and prevented the development of sepsis induced by a subsequent lipopolysaccharide injection (91). In cynomolgus monkeys, a single intravenous injection of ApoB siRNA encapsulated in pegylated solid nucleic acid lipid particles resulted in dose-dependent reductions of ApoB mRNA and protein in the liver and reductions of serum cholesterol and low-density lipoprotein levels lasting for 11 days (92). Intravenous injection of siRNA to mice, where siRNA was complexed with the positively charged peptide protamine and coated with cationic liposomes with surface pegylation, resulted in selective delivery to solid tumors and silencing of the targeted epidermal growth factor receptor gene (93). These various findings indicate liposomes as a viable option for siRNA carriers.

PART 4: CURRENT STATUS OF siRNA IN CLINICAL EVALUATION

Several siRNA therapeutics have advanced to clinical trials, mostly for locoregional treatments such as intravitreal and intranasal administration (Table I). siRNA therapeutics have been evaluated in diverse diseases, including AMD, diabetes, hypercholesterolaemia, respiratory disease, hepatitis, human immunodeficiency virus infection, and cancer.

Table I. Current Clinical Trials of siRNA-Based Therapeutics

Target	Disease	siRNA (carrier)	Delivery routes	Status as of October 2009
Human p53	Acute kidney injury; prophylaxis of delayed graft function	I5NP	Intravenous	Phase I/II[a]
RTP801	Wet AMD	REDD14NP	Intravitreal	Phase I[a]
VEGF	Wet AMD	Bevasiranib	Intravitreal	Phase III[b]
VEGF Receptor 1	Wet AMD	AGN211745 (Sirna-027, chemically modified siRNA)	Intravitreal	Phase II[b]
N-protein of RSV	Respiratory syncytial virus infection	ALN-RSV01	Intranasal	Phase II[c]
Pachyonychia congenita keratins, K6a (single nucleotide mutation)	Pachyonychia congenita	TD101	Callus injection	Phase I[c]
M2 subunit of ribonucleotide reductase (R2).	Solid tumor refractory to standard-of-are therapies	CALAA-01	Intravenous	Phase I[d]
Protein kinase N3	Advanced solid tumor	Atu027 Lipoplex	Intravenous	Phase I[a]
Apolipoprotein B	Hypercholesterolemia	PRO-040201 (SNALP liposome)	Intravenous	Phase I[a]
Immunoproteasome subunits LMP2, LMP7, and MECL1	Metastatic melanoma	Proteasome siRNA	Ex vivo[e]	Phase I[a]

Data are obtained from ClinicalTrials.gov
HBV hepatitis B virus, *VEGF* vascular endothelial growth factor, *AMD* age-related macular degeneration, *RSV* respiratory syncytial virus
[a] Currently recruiting patients
[b] Terminated
[c] Completed
[d] Active
[e] Isolated monocytes are transfected with siRNA and then differentiated into dendritic cells *in vitro*. After maturation, dendritic cells are transfected with RNA encoding defined melanoma antigens and used to vaccinate subjects with metastatic melanoma

Several clinical trials focus on the intravitreal treatment of AMD. The first and most advanced clinical study was the phase III trial of bevasiranib (siRNA to VEGF) as maintenance therapy following anti-VEGF therapy with Lucentis (antibody to VEGF). This trial was terminated early because the preliminary data indicated that the primary endpoint is unlikely to be met. The two remaining trials are with VEGFA165b siRNA that targets VEGF A165 isoform and with AGN211745 (Sirna-027), a chemically modified siRNA that targets VEGF receptor-1. A recent study raised questions on the mechanism of anti-VEGF siRNA therapeutics for treating AMD; this study showed that intravitreal injections of all siRNA of 21-nucleotides or longer, irrespective of the nucleotide sequences or their intended targets (i.e., whether or not the siRNA targets VEGF or VEGFR), were able to suppress neovascularization in mice without causing off-target RNAi or interferon-alpha/beta activation. This study further showed that this general siRNA-mediated suppression of neovascularization was due to nonspecific stimulation of the cell surface Toll-like receptor TLR3 pathway, its adaptor TRIF, and induction of interferon-gamma and interleukin-12 (94). A different siRNA, REDD14NP, has recently entered phase I trial in patients with blinding choroidal neovascularization secondary to AMD. REDD14NP is a synthetic chemically modified 19-mer siRNA molecule targeting hypoxia-inducible gene RTP801 (or DDIT4) that is involved in disease progression. REDD14NP does not activate TLR3 (95).

Two siRNA therapeutics, CALAA-01 and Atu027, entered clinical evaluation for the treatment of solid tumors; both are administered by intravenous injection. CALAA-01 is an siRNA targeting the M2 subunit of ribonucleotide reductase where siRNA is formulated in self-assembled cyclodextrin nanoparticles with surface pegylation and conjugation with the transferrin ligand (the latter is to target the transferrin receptors on tumor cells) (96). The first in human phase I trial of intravenous injection of CALAA-01 in patients with solid tumors refractory to standard-of-care therapies was initiated in May 2008. The results indicated successful delivery of nanoparticles to intracellular localizations and reduction of corresponding mRNA and protein levels in tumor biopsies. This is the first evidence of specific gene inhibition by siRNA in three patients after systemic administration (97). Atu027 is an siRNA lipoplex that targets protein kinase N3. Preclinical studies have shown that repeated intravenous administrations of Atu027 resulted in specific, RNAi-mediated silencing of protein kinase N3 expression in mice, rats, and nonhuman primates, significant inhibition of tumor growth, and inhibition of the formation of lymph node metastasis in orthotopic mouse models of prostate and pancreatic cancers (98). The phase I trial of Atu027 in patients with advanced solid cancer was recently opened, with the first patient enrolled in July 2009. These encouraging clinical results pave the way for transitioning RNAi from a research tool into clinic evaluation and future applications.

CONCLUSIONS

Posttranscriptional gene silencing by RNAi represents a promising new gene therapy approach. The development of carriers, e.g., polymers, lipids, and conjugates, will be critical

for the systemic delivery of siRNA therapeutics. The incorporation of surface pegylation and cell-specific targeting ligands in the carriers may improve the pharmacokinetics, biodistribution, and selectivity of siRNA therapeutics. The safety, effectiveness, and ease of production and manufacturing are important considerations for selecting the appropriate siRNA carriers. Evaluation of the efficacy of siRNA therapeutics requires considerations of off-target effects and innate immune response.

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