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Expert Review

Efficient siRNA Delivery with Non-viral Polymeric Vehicles

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Abstract. Sequence-specific gene silencing using small interfering RNA (siRNA) provides a potent and specific method for gene expression, thus is now being evaluated in clinical trials as a novel therapeutic strategy. As a results, there has been a significant surge of interest in the application of siRNA in therapeutics as a means of silencing the specific gene function. However, for siRNA technology to be valuable and effective, the development of efficient siRNA delivery strategy is essential for improving biological activities such as stability, cellular uptake, sequence-specificity, devoid of nonspecific knockdown and toxic side effects. Accordingly, a number of delivery systems, both viral and nonviral, have been reported and some of them successfully used for the introduction of siRNA into cells both *in vitro* and *in vivo*. Here, we discuss the current understanding of synthetic siRNA delivery mechanism and strategies of siRNA delivery by non-viral polymeric vehicles which are currently used *in vitro* and *in vivo*.

KEY WORDS: cationic polymer; gene therapy; polymeric carrier; small interfering RNA.

INTRODUCTION

Since its discovery by Fire *et al*. in 1998, RNA interference (RNAi) represents a promising new approach towards the inhibition of gene expression in cell culture and *in vivo*, and has rapidly emerged as a promising therapeutic potential for human diseases (1–3). The RNAi process is occurred by the incorporation of specific siRNA to the specific target messenger RNA (mRNA) as a means of inhibiting the synthesis of the encoded protein. This siRNA is approximately 19–23 bp in length and contains two nucleotide 3'-overhangs. Once inside cells, siRNA associate itself with the multiprotein complex called the RNA induced silencing complex (RISC) (4). Upon incorporation into RISC, one of the RNA strands (sense strand) is cleaved while the rest of the single stranded RNA molecule (antisense strand) remains incorporated and directs RISC-mediated downregulation of specific mRNA. Following mRNA cleavage, antisense strand-loaded RISC is capable of multiple rounds of mRNA cleavage making RISC-mediated regulation of gene expression a powerful catalytic event.

Although the discovery of siRNA can lead to the revolution of therapeutic tools and has numerous potentials against diseases, the main limitation of siRNA technology is the inability of naked siRNA to successfully cross the cell membrane and induce RNAi response (5). So far, the technology of siRNA delivery has advanced rapidly due in part to the utilization of many delivery systems that have previously been shown to have success in the delivery of nucleic acids for gene therapy. It is now widely recognized that efficient intracellular siRNA delivery to target tissues following systemic administration is the most important hurdle for widespread use of siRNA *in vivo* (2,6).

Due to its large molecular weight (~13 kDa) and strong anionic charge of the siRNA phosphodiester backbone (~40 negative phosphate charges), naked siRNA does not freely cross the cell membrane. Cellular exclusion resulting from the inability of siRNA to passively diffuse through the cell membrane and electrostatic repulsion from the anionic cell membrane surface is one of the major obstacles to be overcome. Therefore, delivery systems are required to facilitate its access to intracellular sites of action as known in the case of oligonucleotide antisense delivery.

Numerous delivery strategies, both viral and nonviral, have been developed to circumvent this problem and some of them successfully used for the introduction of siRNA into cells both *in vitro* and *in vivo*. Viral vectors are highly efficient delivery systems for nucleic acids, but their clinical application is restricted by several drawbacks, including the potential of mutagenicity or oncogenesis, several host immune responses, the high cost of production, and as such, they are not covered further here. These concerns have made nonviral vectors an attractive alternative to viral vectors. Multiple nonviral delivery systems have been introduced in order to deliver siRNA efficiently including chemical modification of siRNA, cationic polymers, cationic lipids, cell-penetrating peptide, and targeted delivery (7–10).

This review will focus on the summarization of the current understanding of the siRNA delivery mechanism and discuss the strategies of delivery of exogenous, synthetic siRNA by non-viral polymeric vehicles which are currently used *in vitro* and *in vivo*.

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Chemical Modification of siRNA

Unmodified, naked siRNAs are relatively unstable in blood and serum, though they are more stable than single-stranded RNA. siRNAs are rapidly degraded by nucleases, indicating that they have short half-lives *in vivo*. In order to enhance biological stability without adverse effect on the gene-silencing activity, chemical modifications have been introduced into the siRNA structure. Various chemical modifications to the backbone, base, and sugar of the siRNA have been reported so far, which are mainly focusing on not only the increase of the stability but also enhancement of cellular uptake of siRNA. By these chemical modifications of siRNA, degradation of siRNA in serum and/or cells can be delayed from minutes to hours, and thus, gene-silencing activity *in vivo* can be sustained for several days in conjunction with an appropriate delivery system. Chemical modifications can also reduce off-target effects (11) and alter thermal stability within the various critical sequence regions of the siRNA duplex for improved activity of siRNA (12–14). However, these modifications must be designed and screened in such a manner that they do not inhibit the activity due to the structurally sensitive nature of siRNA. The introduction of phosphorothioate linkages into siRNA backbone has been reported as promising modifications (15,16) (Fig. 1a). This kind of backbone modification enhances the resistance of siRNA to nucleases. The siRNAs modified by phosphorothioate linkages showed similar activity to unmodified siRNA, while siRNAs with extensive phosphorothioate linkages are often toxic (17).

Due to its exposure on the hydrolysis of phosphodiester bond, protection at 2'-hydroxyl groups in siRNA structure has been utilized for enhancing the stability of siRNA. This chemical modifications include 2'-*O*-methyl, 2'-fluoro, and 2'-*O*-(2-methoxyethyl) modifications at 2'-hydroxyl groups as described in Fig. 1a (18–20). These modifications enhanced the affinity of the siRNA for complementary mRNA and increased its resistance to digestion by nucleases (21).

Modifications of terminal nucleotides of the siRNA (also called siRNA conjugate) have also been reported as an efficient delivery strategy. These include peptide modification such as TAT peptide, cholesterol conjugation, pegylation of siRNA, and aptamer conjugation as shown in Fig. 1b (22–24). Such modifications significantly enhanced resistance to enzymatic degradation in serum and tissue, thus increased the stability of siRNA. Chiu *et al.* conjugated cell penetrating peptide such as TAT peptide into siRNA for efficient delivery, thus silenced the expression of the target gene. They showed that TAT-conjugated siRNA is rapidly internalized by cells and becomes concentrated in nuclei (22). Soutschek *et al.* (23) reported multiply modified siRNA with partial phosphorothioate linkages, 2'-*O*-methyl sugars and a cholesterol moiety at the 3'-end of the sense strand. The RNA part of the conjugate contained phosphorothioate linkages and 2'-*O*-methyl RNA modifications having resistance to nuclease digestion. Attachment of the cholesterol moiety to the siRNA increased binding to human serum albumin and, thus enhanced the biodistribution to various organs including the liver, heart, kidney, adipose tissue, and lung.

To obtain the long blood circulation and high activity of tissue access, pegylation strategy has also widely been used in nucleic acids delivery because of its excellent characteristics including reducing cytotoxicity, increasing solubility, and shielding effect of ionic charges (25,26). Kim *et al.* (24) reported the enhanced potential of siRNA delivery by complexing the pegylated siRNA and cationic polymer. In their report, the interaction between PEG-conjugated siRNA and polyethylenimine (PEI) led to the spontaneous formation of polyelectrolyte complex micelles, having a siRNA/PEI complex inner core with a surrounding PEG shell layer. Moreover, intravenous as well as intratumoral administration of the micelles significantly inhibited siRNA specific VEGF expression at the tumor tissue and suppressed the tumor growth in an animal tumor model without showing any detectable inflammatory responses in mice.

Several other types of siRNA conjugate have also been investigated. Conventional antibody strategy was applied to siRNA for enhancing delivery efficacy and increasing the cell specificity. siRNAs have been mixed with antibodies engineered to display positively charged peptides on their surfaces (27). The siRNA is intended to be silencing agent and is linked to the positively charged antibody by non-covalent electrostatic interactions. The strength of this concept is its potential versatility and the fact that there is no need to synthesize chemical conjugate. Instead of conventional cell-specific antibody strategy, siRNA conjugated to cell specific aptamers have been reported to block specific protein expression in cell culture and animals (28). Aptamers are synthetic nucleic acids that are selected from random sequence libraries and optimized for their ability to bind to a specific target molecule. Aptamers are frequently viewed as non-protein based alternatives to antibodies and are thus also potential targeting reagents for the delivery of siRNAs (29). In addition to possessing high affinity and specificity for their targets, aptamers can be synthesized chemically and thus are attractive reagents for use in therapeutic and other applications. Aptamers targeting surface antigens as well as whole cells have previously been selected (30–32). Chu *et al.* (33) reported that an anti-prostate specific membrane antigen aptamer conjugated to siRNAs *via* a modular streptavidin bridge inhibited gene expression as efficient as observed with conventional lipid-based reagents, and it was dependent upon conjugation to the aptamer.

At this stage, work on aptamers is in its early stages, thus it is not possible to evaluate whether improved properties are obtained by aptamer-conjugated siRNA. For a better therapeutic application of aptamers, wide ranges of aptamers have to be screened as a cell specific siRNA delivery reagent.

Cationic Substances for siRNA Delivery

Several types of synthetic vectors have been investigated for siRNA delivery strategy, including the development of cationic polymers, cationic lipids, and cationic cell-penetrating peptides (CPPs). A common principle amongst these vectors is their net positive charge, which facilitates both complex formation with the polyanionic siRNA and interaction with the negatively charged cell membrane. Alternatively, these cationic lipids and polymers can form nanoparticles, and the nucleic acid is entrapped within the nanoparticle usually *via* ionic interactions, not by simple adsorption onto the surface. Although the exact mechanism of delivery has not been

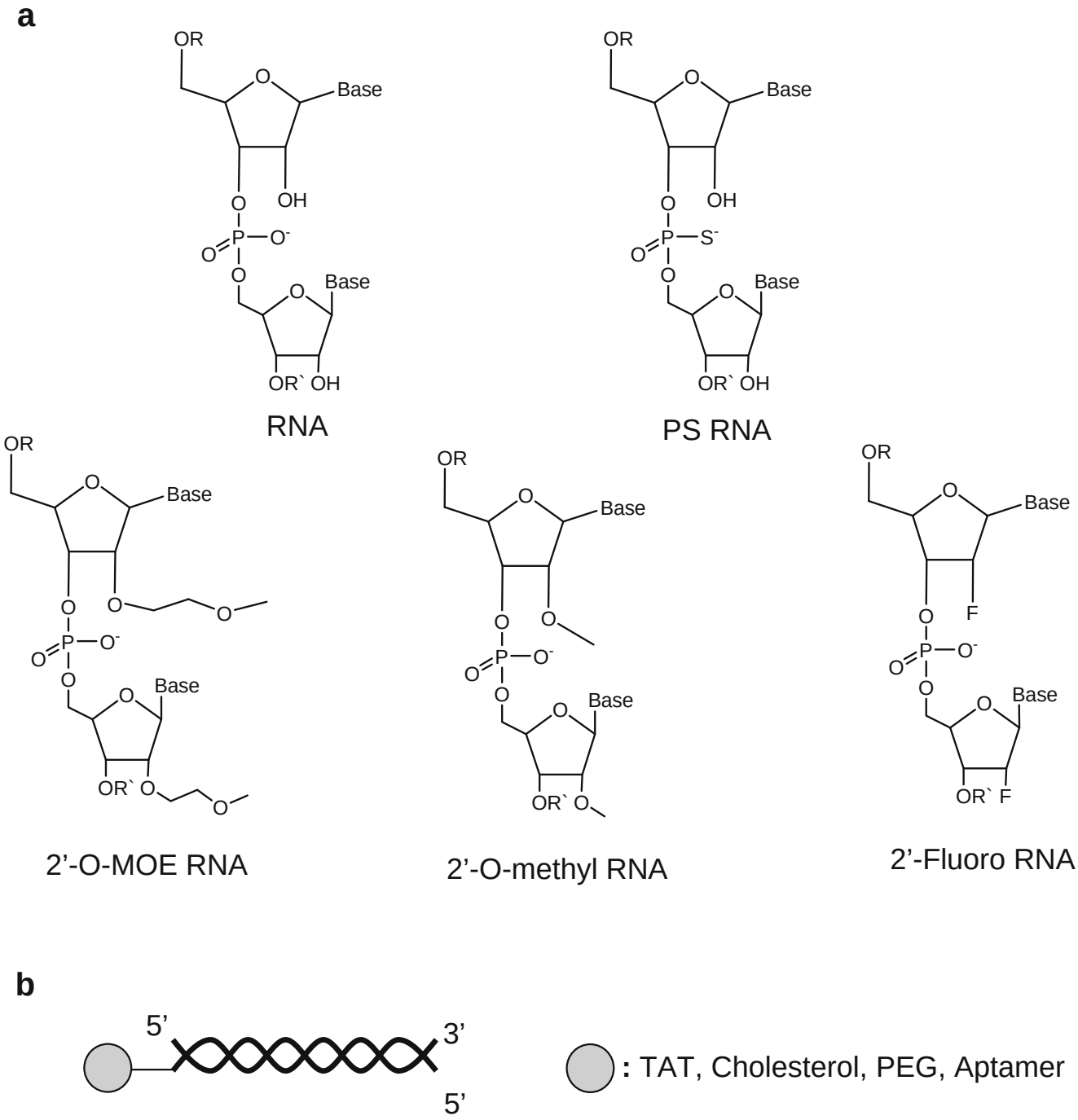


Fig. 1. **a** Chemically modified siRNAs, **b** siRNAs conjugated with peptide, cholesterol, PEG, and aptamer.

understood yet, these complexes and nanoparticles can provide siRNA stability within blood serum to varying degrees, leading to long blood circulation, and eventually reach target cells *via* formation of vesicular transport (endocytosis and/or macropinocytosis).

Cationic Polymeric Delivery Systems

Cationic polymers readily bind and condense polyanionic nucleic acids and have thus been widely used as transfection reagents for genes, oligonucleotides, and siRNAs (34–37). The high charge density of cationic polymers allows them to escape from endosomes and deliver nucleic acids into the cytosol through the so-called "proton-sponge" effect (38). During the intracellular trafficking, the polycationic nature of the polymers is thought to buffer low endosomal pH through enhanced influx of protons and water, maximizing in endosome rupture. Of the many cationic polymers, PEI has been widely studied for various types of nucleic acid delivery (39) (Fig. 2). PEI has become the prototype of non-viral gene delivery. PEI polymers with different molecular weights and degrees of branching have been synthesized and evaluated *in*

vitro as well as *in vivo*. Highly branched polymer (BPEI) such as the BPEI (25 kDa) is the most frequently used (40). BPEI is able to effectively complex with even large DNA molecules, leading to homogeneous spherical particles with a size of ~100 nm or less that are capable of transfecting cells efficiently *in vitro* as well as *in vivo* (41). They offer significantly more protection against nuclease degradation in comparison to other polycations, such as poly(L-lysine), possibly due to their higher charge density and more efficient complexation ability (42). The large amount of positive charge, however, results in a rather high toxicity and is one of the major limiting factors for its *in vivo* application. The efficacy of BPEI-derived vectors and their cytotoxicity effects depend to a remarkable extent on material characteristics like the molecular weight, the degree of branching, the cationic charge density, buffer capacity, and polyplex properties, such as the DNA content, particle size and zeta potential (43,44).

Recent reports in the literature show that PEI can deliver siRNA both *in vitro* and *in vivo* (8,45,46). In particular, Richards Grayson *et al.* (47) evaluated the efficacy of different PEI structures for siRNA delivery in a model system, and determined the biophysical and structural characteristics of PEI at different N/P ratios, i.e., the ratio of concentrations of total nitrogen atoms (N) of the polycation to the phosphate groups (P) of DNA, as the characteristic of the complex composition. According to the report, successful siRNA delivery with PEI was observed within a very narrow window of conditions, and only with the BPEI 25K at an N/P ratio of 6 and 8 with 200 nM siRNA. Further, they suggested that the complex stability may govern the transfection efficacy, while the zeta potential and size of PEI/siRNA complexes were correlated to transfection efficacy in some cases.

Modified PEI with several functional moieties was introduced and it was revealed that succinylation of BPEI resulted in up to tenfold reduction of polymer toxicity in comparison to unmodified PEI (48). Moreover, succinylated PEI/siRNA complexes were able to induce remarkable knockdown of target luciferase gene at the lowest tested siRNA concentration.

Polymers with high molecular weight BPEI (25 kDa, 800 kDa), for example, exhibit high transfection efficiencies, though the toxicity is extensive. Polymers (BPEI) with low molecular weight around 800 Da, display low toxicity, though transfection efficiency is very low. An approach to combine the advantages of high and low molecular weight BPEI has been taken recently by cross-linking small BPEIs *via* biodegradable bonds. This approach enhanced the transfection efficiency of cross-linked small PEIs, but with only a moderate increase in toxicity (49).

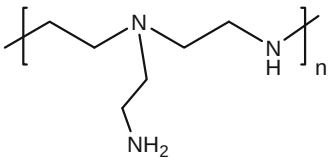
Combining of low molecular weight BPEI with lipid moiety has been reported by Kim's group. Water soluble lipopolymer (WSLP) has been synthesized by combining the cationic head group of BPEI (1.8 KDa) with a hydrophobic lipid anchor, cholesterol chloroformate and reported an efficient gene delivery carrier which has low cytotoxicity and enhanced transfection efficacy *in vitro* and *in vivo* (50–53) (Fig. 2). WSLP was also examined as a potential siRNA delivery vehicle (54). WSLP was complexed with siRNA designed to inhibit human vascular endothelial growth factor (VEGF) expression or scrambled siRNA as a control. It readily formed nano-sized complexes (~100 nm) with siRNA and protected siRNAs from enzymatic degradation in serum conditioned media. WSLP/siRNA complexes transfected in PC-3 cells derived from human prostate adenocarcinomas inhibited the VEGF production significantly, while complexes of WSLP with scrambled siRNA did not show this inhibitory effect. WSLP/siRNA complexes reduced the VEGF production by 40% when compared to unmodified branched polyethylenimine (bPEI, MW=1,800). Moreover, WSLP/siRNA complexes reduced tumor volume by 55% at 21 days, and by 65% at 28 days when compared to controls. These results indicate that WSLP has potential as a siRNA delivering agent and can be applied for anti-angiogenic tumor therapy.

Since siRNA works in cytoplasm, the reducible polymers linked with disulfide bond are favorable for the delivery of siRNA due to its high ability of siRNA release into the cell cytoplasm. A reducible poly(amido ethylenimine) (SS-PAEI) synthesized by addition copolymerization of triethylenetetramine and cystamine bis-acrylamide (poly(TETA/CBA)) was used as a carrier for siRNA (55) (Fig. 2). Poly(TETA/CBA) efficiently condensed siRNA to form stable complexes under physiological conditions and performed complete release of siRNA in a reductive environment. When formulated with VEGF-directed siRNA, poly(TETA/CBA) demonstrated significantly higher suppression of VEGF than linear PEI (LPEI, 25 kDa) in PC-3. The triggered release of siRNA by reductive degradation of poly(TETA/CBA) in the cytoplasm may affect the RNAi activity by increasing cytoplasmic accessibility of siRNA. The efficacy of reducible polymeric system was also proved by Breunig *et al.* (56). They synthesized various PEI derivatives (SSPEI) linked by disulfide bond and evaluated their ability as siRNA carriers. It was observed that the siRNA release from the carrier, which was prompted by SSPEI, played an important role in the accessibility of siRNA for the gene silencing complex. They suggested that a combination of a high branching density and reductively cleavable bonds within the PEI-based carrier system could be one possible step towards improving siRNA delivery.

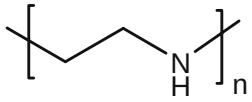
A short polycation containing cyclodextrin has been evaluated as a siRNA delivery carrier (57) (Fig. 2). The cyclodextrin-containing polycations (CDP) self-assemble with siRNA to form colloidal particles ~50 nm in diameter, and their terminal imidazole groups assist in the intracellular trafficking and release of the nucleic acids (58). CDP protects the siRNA from enzymatic degradation so that chemical modification of siRNA is unnecessary. The colloidal particles are stabilized for use in biological fluids by surface decoration with PEG that occurs *via* inclusion complex formation between the terminal adamantine (AD) and the cyclodextrins. They used cyclodextrin polycation delivery system in order to mask immune stimulation effects of siRNA (58,59).

Cationic Liposomal Delivery System

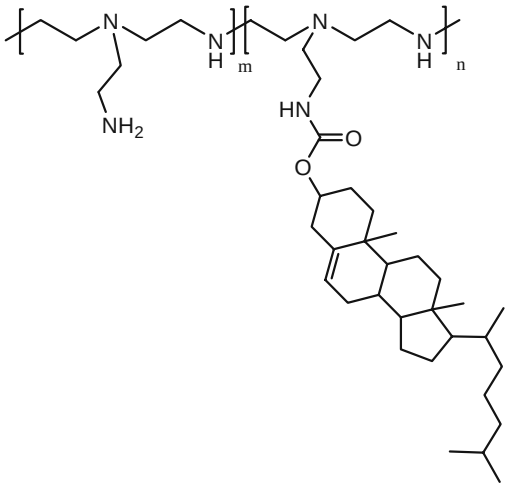
Cationic liposomes have been among the more efficient synthetic gene delivery reagents *in vitro* since the late 1980s (9). Cationic liposomes can condense nucleic acids into a cationic particle when mixed together. This cationic lipid/nucleic acids complex (also called lipoplex) can protect nucleic acids from enzymatic degradation and deliver nucleic acids into cells



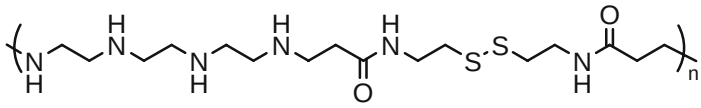
Branched PEI (BPEI)



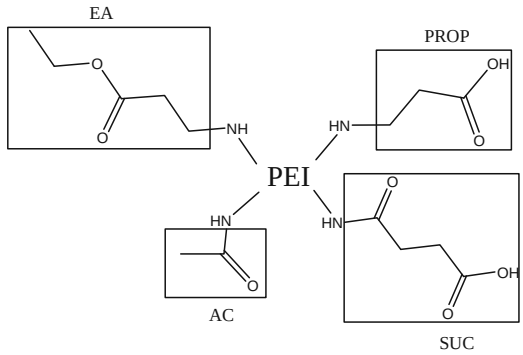
Linear PEI (LPEI)



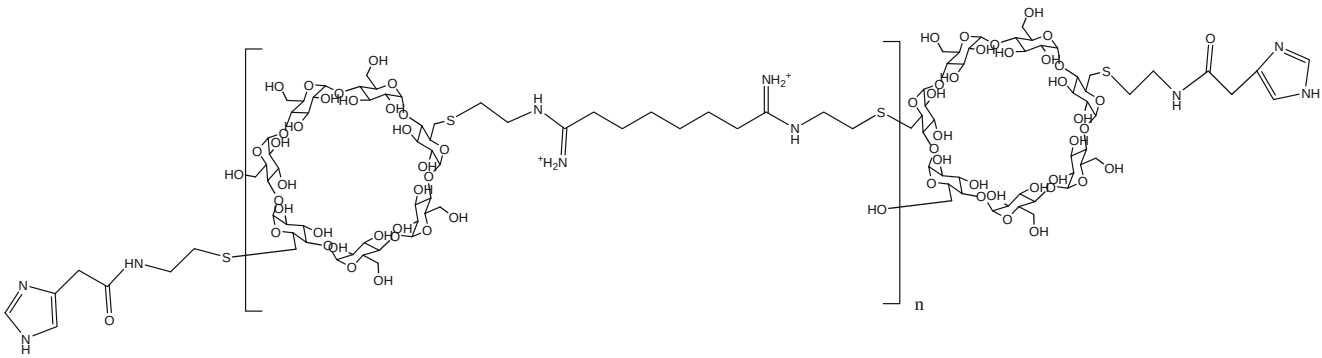
WSLP



Poly (TETA/CBA)



Multiply modified PEI



Cyclodextrin-containing polycations (CDP)

Fig. 2. Structure of polymeric siRNA carriers.

by interacting with negatively charged cell membrane. Cationic liposomes have also been applied with substantial success for the *in vitro* as well as *in vivo* delivery of siRNA.

Commercially available cationic lipid formulations such as lipofectin, RNAifect (Qiagen), Oligofectamine, Lipofectamine (Invitrogen), and TransIT TKO (Mirus) have all been investigated as potential reagents of siRNA delivery *in vitro*. DOTAP (*N*-[1-(2,3-dioleoyloxy)]-*N*-*N*-*N* trimethyl ammonium propane) is one of the first lipid formulations to be examined for the *in vivo* siRNA delivery and produced significant knockdown of specific protein in mice (60). Recently, Arnold *et al.* (61) showed that siRNA/DOTAP lipoplexes selectively inhibited β1-adrenoreceptor expression and markedly reduced blood pressure for up to 12 days.

Cationic liposomes termed "solid nucleic acid lipid particles" (SNALPs) that have been stabilized by pegylation for improved pharmacokinetics have also successfully been used to deliver siRNA systemically to silence the apoB gene in mice and nonhuman primates (62).

Protein Transduction Domain-mediated siRNA Delivery

PTD, also called cell penetrating peptides (CPPs) are short cationic peptide chains with a maximum of 30 amino acids that have been shown to bind the anionic cell surface through electrostatic interactions and rapidly induce cellular internalization through unknown mechanisms. The mechanism of internalization of PTD is not well understood and has recently been the subject of discussion. Most cellular uptake studies of PTD in the literature based on fluorescence microscopy of fixed cells and flow cytometry analysis report that internalization of PTD does not involve endocytosis (63,64). However, most current evidence shows that the internalization of PTD is an endocytosis-mediated process (65,66). Some of the most well characterized PTD are TAT peptide (10), penetratin (67), transportan (68), and oligoarginine (69). These cationic peptides have shown to enhance the cellular uptake of small molecules, making them attractive candidates for intracellular delivery of siRNA. PTD-medicated siRNA delivery strategies are summarized in Table I. TAT peptide was conjugated to the 3'-end of the antisense strand of siRNA through a stable thiomaleimide linkage and the sense strand carried a 5'-fluorescent label (22). It was revealed that siRNA cell uptake did correlate dose-dependently with EGFP gene silencing in a HeLa cell transient transfection reporter system. Transportan or Penetratin disulfide-linked to the 5'-end of the sense strand of siRNA were also shown to reduce expression of luciferase or EGFP mRNA reporters in cells (70,71). Turner *et al.* (72) studied the ability of multiple PTD including TAT, penetratin and transportan to enhance the cellular uptake of siRNA after covalent conjugation through a disulfide linkage. In HeLa cell assay with integrated plasmid reporters of TAT-dependent trans-activation at the TAR RNA target in the cell nucleus, it was not possible to obtain the inhibition of gene expression for conjugates of PTDs with a 12-mer oligonucleotide mixmer of 2'-*O*-methyl and locked nucleic acids units, while some reductions were shown in expression of P38α MAP kinase mRNA in HeLa cells using micromolar concentrations of penetratin or TAT peptides conjugated to the 3'-end of the sense strand of siRNA. Moreover, the most promising results have been observed with a 15-mer peptide nucleic acid (PNA) conjugated to the transportan or R6-penetratin, suggesting the possibility of development of PTD-PNA conjugates as anti-HIV agents.

Synthetic oligoarginine has also been introduced as potential candidates for gene delivery agents. Nowadays, many studies utilize oligoarginine peptides of eight to ten residues instead of traditional PTD such as TAT or penetratin and in comparative studies they have shown an equal and sometimes enhanced ability for these peptides to translocate across the cellular membrane (73). Similar to other densely cationic PTD, oligoarginine conjugates to either cholesterol moieties or cell-targeting peptides increase the efficiency of siRNA uptake into cells in culture and tissues *in vivo*. The first study utilized a cholesteryl oligo-D-arginine (Chol-R9) peptide composed of nine arginine residues conjugated to an N-terminal cholesteryl group (74). While the domain composed of nine arginines offers efficient siRNA packaging and some cell-surface binding by ionic interaction, the cholesterol moiety is capable of binding into the hydrophobic residues of the extracellular membrane surface and enhances the delivery of the complexed siRNA. By using gel electrophoresis, it was possible to determine at what ratio the cationic nitrogen groups (N) were sufficient to complex the negatively charge phosphate groups (P). The N/P ratio that was shown to successfully remove all free siRNA from solution was 40 and this ratio was subsequently shown to be the favorable ratio for successful siRNA delivery. Using N/P ratios of 10, 20 and 40, Chol-R9 peptide *versus* R9 complexed VEGF siRNA were added to CT-26 cells for 4 h and assayed for VEGF secretion by ELISA 24 h later. After 24 h, VEGF production was suppressed by 55% for the Chol-R9/siRNA complexes formed from an N/P ratio of 40 compared to the untreated control and the unmodified R9/siRNA complex that displayed no VEGF inhibition. This observation further supports the argument that the cholesterol moiety plays a functional role in the delivery of these peptide/siRNA complexes. The authors then sought to validate this siRNA delivery system within an *in vivo* model system for RNA interference. CT-26 cells were injected into the flank of mice and allowed to form established tumors over a 2-week period. After 2 weeks, intratumoral administration of Chol-R9/VEGF siRNA complexes *versus* Chol-R9/scrambled VEGF siRNA complexes was performed with 50 μg of siRNA per mouse every 4 days for a total of 20 days. Tumor volume was measured throughout the duration of the experiment and showed a sevenfold reduction in tumor volume by day 17 after the start of siRNA treatments for the Chol-R9/VEGF siRNA compared to the Chol-R9/scrambled VEGF siRNA control. Importantly, no differences were observed with the untreated animals or the Chol-R9 packaged scrambled VEGF siRNA sequence. Additionally, the intratumoral concentration of VEGF was decreased by ~60% as assayed by ELISA at day 17. While the application of this system to the systemic administration was not investigated, this article is a clear

Table I. PTD-mediated siRNA Delivery

PTD	Target protein	Formulation	Reference
TAT peptide	EGFP, CDK9, MAP kinase	TAT-siRNA conjugate	(22,72)
Penetratin	GFP, luciferase, SOD, caspase, MAP kinase	Penetratin-siRNA conjugate	(70–72)
Transportan	GFP, luciferase	Transportan-siRNA conjugate	(70)
Oligoarginine	VEGF	Cholesteryl-R9/siRNA complex	(74)
TAT, Penetratin, Transportan	GL3 firefly luciferase	Peptide-siRNA conjugate	(73)

demonstration that these Chol-R9/siRNA particles can deliver siRNA *in vitro* and in a locally delivered *in vivo* model system.

Targeted siRNA Delivery

Numerous cell-targeting ligands, including glycosylated molecules, peptides, proteins and antibodies have been used for cell-specific delivery of nucleic acids. These targeting ligands have been conjugated to the various types of carriers, and thus showed the potency as a cell-specific siRNA delivery vehicle. The ligand-conjugated siRNA delivery systems are summarized in Table II. For example, the asialoglycoprotein receptors expressed on the surface of hepatocytes are used for hepatocyte-specific delivery. The receptor mediates endocytosis and subsequent internalization of proteins that are conjugated to galactose-terminated oligosaccharide moieties (75). Several groups have exploited this system for the delivery of genes to hepatocytes, using polymeric carriers with ligands, such as galactose and lactose (76–78). For siRNA delivery with lactose-conjugated carrier, Oishi *et al.* (79) have constructed a lactosylated PEG-siRNA conjugate with an acid-labile linkage. In this complex, siRNA is covalently linked to lactosylated PEG and under the slightly acidic conditions of the intracellular endosomal compartment, the covalent linkage is disrupted and free siRNA is released.

Folate receptors are overexpressed in many human tumors, while their distribution in normal tissues is minimal (80). Therefore, attachment of folic acid, the ligand for the folate receptor, to various macromolecules allowed their binding to folate receptors and subsequent internalization by endocytosis (81,82). For tumor targeting gene delivery, the folate-PLL conjugate was evaluated as a gene carrier by *in vitro* transfection into tumor cells, resulting in higher transfection efficacy than PLL (83). Kim *et al.* (84,85) successfully delivered synthetic siRNA as well as plasmid DNA that encoded siRNA by incorporating folate-modified PEI into the plasmid DNA. Other receptors that have received significant attention for tumor targeting are the integrins. Integrins are heterodimeric cell-adhesion receptors that are composed of alpha and beta subunits. The $\alpha v\beta 3$ and $\alpha v\beta 5$ intergrins are overexpressed on angiogenic endothelial cells within tumors (86,87). Therefore, the localized expression of the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins enables targeting gene delivery by using a RGD peptide (88,89). Schiffelers *et al.* (90) attached a circularized RGD peptide to the end of PEG-conjugated forms of BPEI and used the nanoparticle to deliver siRNA inhibiting VEGF receptor 2 expression and thereby suppressed tumor angiogenesis. Cell delivery and activity of pegylated PEI was found to be siRNA sequence specific and dependent on the presence of RGD peptide and could be competed by free peptide. Moreover, intravenous administration into tumor-bearing mice gave selective tumor uptake, siRNA sequence-specific inhibition of protein expression within the tumor and inhibition of both tumor angiogenesis and growth rate.

Transferrin is a glycoprotein that delivers iron to cells (91). Tumor cells often overexpress transferrin receptors on their surface, and this protein has also been studied as a ligand for tumor-targeted delivery. Lieskovan *et al.* have constructed a multicomponent delivery system that included short polycationic cyclodextrins for condensing and protecting siRNA, adamantine-PEG for stabilization of the particle, and transferrin. It consists of a cyclodextrin core to which is attached PEG and transferrin. Using this delivery system, effective gene silencing of the oncogene EWS-FLI1 was reported in a metastatic model of Ewing sarcoma. More recently, a multidosing study of this delivery system in cynomolgus monkeys has suggested that the delivery system is well tolerated and that even repeat doses failed to elicit a significant delivery system-specific antibody response (58,59). Another tissue-specific delivery of siRNAs has been achieved using fusion protein (F105-P) composed of cationic protamine and tumor-specific antibodies. In this system, siRNAs were bound by the basic fusion protein and then targeted to tumor cells *via* tumor-specific antibodies. Intratumoral or intravenous injection of F105-P-complexed siRNAs into mice targeted HIV envelope-expressing B16 melanoma cells, but not normal tissue or envelope-negative B16 cells; injection of F105-P with siRNAs targeting c-myc, MDM2 and VEGF inhibited envelope-expressing subcutaneous B16 tumors. Furthermore, an ErbB2 single-chain antibody fused with protamine delivered siRNAs specifically into ErbB2-expressing cancer cells (27).

Other Delivery Systems

In addition to these polymeric siRNA delivery systems there are few other systems such as chitosan, PLGA, and PAMAM dendrimer which are less effective in siRNA delivery. Cationic natural polymer, chitosan is a well-tolerated biodegradable polymer that forms polyelectrolyte complex with nucleic acids. Effective *in vivo* siRNA delivery was achieved by chitosan/siRNA complexes both in lung epithelial cells of transgenic enhanced green fluorescent protein (EGFP) mice after intranasal administration and in subcutaneously implanted breast cancer cells after intravenous administration (92,93). Poly(lactic-co-glycolic acid) (PLGA) nanoparticles and polyaminoamine (PAMAM) dendrimers, are also being investigated as vehicles for siRNA delivery

Table II. Ligand-conjugated siRNA Carrier for Targeted Delivery

siRNA carriers	Formulation	Target cell	Reference
Lactose-PEG-siRNA/PLL	Polyion complex micelles	HuH-7	(79)
Folate-PEG-PEI/siRNA	Polyion complex	KB	(84)
RGD-PEG-PEI/siRNA	Polyion complex	HUVEC	(90)
Tf-PEG-AD/CDP/siRNA	Polyion nanoparticle	EFT	(58,59)
Fab-Protamine fusion protein/siRNA	Polyion complex	B16	(27)
Aptamer-siRNA	Conjugate	LNCaP	(28,33)

(94,95). The entrapment of siRNA within biodegradable PLGA microparticle improved its stability and reduced the doses required for efficacy. However, the conjugation of PAMAM dendrimer to TAT peptide was poorly effective for delivery of siRNA.

CONCLUSIONS

RNAi technology has quickly been advanced from research discovery to clinical trials as an effective gene-silencing strategy since its discovery in 1998. The principal challenge that remains in magnifying the benefit of RNAi therapeutics is the hurdle of delivery. As described in above, a number of strategies have been reported as efficient siRNA delivery technology such as chemical modification or conjugation of siRNA, cationic polymer-based delivery, PTD-assisted delivery, and targeted siRNA delivery. These strategies have a potential for improving biological properties such as stability, cellular uptake, and delivery efficacy of siRNA. With the remarkable advancement of siRNA delivery, it will be possible to advance siRNA therapeutics rapidly into clinical studies for many diseases, including some which remain untreatable or poorly treated by conventional drugs, though there are many factors to be refined and tuned for optimized system.

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