REVIEWS

siRNA Conjugate Delivery Systems

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Small interfering RNA (siRNA) has been chemically conjugated to a variety of bioactive molecules, lipids, polymers, peptides, and inorganic nanostructured materials to enhance their pharmacokinetic behavior, cellular uptake, target specificity, and safety. To efficiently deliver siRNAs to the target cells and tissues, many different siRNA bioconjugates were synthesized and characterized, and their gene silencing efficiencies were tested in vitro and in vivo. In this review, recent developments of siRNA bioconjugates are summarized.

I. INTRODUCTION

Synthetic siRNAs have been considered a new class of nucleic acid therapeutics for treatment of various infectious and genetic diseases including cancer (*1*). RNA interference (RNAi), a cellular post-transcriptional gene silencing mechanism, can be induced by double-stranded siRNA consisting of 21−25 nucleotides that degrades a target mRNA in a highly sequence specific manner (*2–4*). Despite the high therapeutic potential of siRNA, its application in clinical settings is still limited mainly due to the lack of efficient delivery systems (*5*). Clinically acceptable siRNA delivery systems should be carefully designed to improve the stability of siRNA after administration into the body, to deliver siRNA specifically to the desired tissue site, and to facilitate the cellular uptake of siRNA within target cells (*6*)

A number of nonviral delivery carriers, including liposomes (*7, 8*), lipids (*9, 10*), polymers (*11, 12*), peptides (*13*), virus-based vectors (*14*), and pressurized hydrodynamic injection (*15*), have been suggested for improved intracellular delivery of siRNA. Various kinds of cationic species can form nanosized polyelectrolyte complexes with negatively charged siRNA by ionic interactions. The resulting complexes can provide excellent protection of siRNA from attack by extracellular nucleases and allow facile cellular uptake via an endocytic pathway. Among the carriers, a wide array of cationic polymers with different architectures and functionalities can also be molecularly engineered for further modifications to provide the carriers with targeted delivery, biodegradability, and prolonged circulation properties (*16*). However, many cationic agents used for condensing siRNAs have often exhibited severe cytotoxicity, limiting clinical applications.

Recently, it has been reported that direct conjugation of small drug molecules, aptamers, lipids, peptides, proteins, or polymers to siRNA could improve in vivo pharmacokinetic behavior of siRNA (*17*). Such siRNA bioconjugates, either with or without forming nanocomplexes with cationic carriers, could significantly enhance biological half-life with a concomitant increase of delivery efficiency to the target tissue while maintaining sufficient gene silencing activity. This short review focuses on the recent development of various siRNA conjugate delivery systems for in vitro and in vivo applications.

II. PREPARATION OF siRNA CONJUGATES

A. Site of Conjugation and Chemical Modification. Since siRNA is a hybridized product of two complementary strands (sense and antisense), there are four terminal ends for potential conjugation sites. After cellular uptake, an antisense strand of siRNA, a strand having a complementary sequence to a target mRNA, is incorporated into the RISC to initiate the RNAi mechanism. This characteristic strand bias could be greatly influenced by chemical modification or conjugation of siRNA. Previous observations showed that the integrity of the 5′-terminus of the antisense strand, rather than that of the 3′-terminus, is important for the initiation of an RNAi mechanism (*18–20*). Therefore, the 3′- and 5′-terminus of the sense strand and the 3′-terminus of the antisense strand are primarily considered as potential sites for conjugation with minimal influence on RNAi activity. Conjugation strategies of several siRNA conjugates in the literature are summarized in Table 1. Either the 3′- or 5′-terminus of the sense strand is generally used for conjugation. Also, most of the conjugates employ cleavable linkages including acid-labile and reducible bonds between siRNA and the conjugation partner for facilitating the release of intact siRNA inside cells. The acid-labile and disulfide linkages are expected to be cleaved in the acidic endosome compartments and the reductive cytosolic space, respectively. Some siRNA conjugates use the action of the endogenous Dicer, which can process a premature double-stranded RNA to generate an active siRNA (*17, 21*)

To improve the nuclease resistance of siRNA and enhance its stability in biological fluids, siRNA itself is chemically modified. Chemical modification of the phosphorothioate linkage (backbone phosphate group O → S) or the boranophosphate linkage (backbone phosphate group O → BH_3) has been popularly used, since it is considered a simple and effective

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Table 1. Strategies for Synthesis of siRNA Conjugates

siRNA conjugate	site of conjugation (I)	site of conjugation (II)	linkage	conjugate chemistry	target siRNA
Cholesterol-siRNA	3′-end of sense strand (*33*)[a]	Cholesterol-aminocaproic acid-pyrrolidine linker	Noncleavable (pyrrolidone)	Solid-phase synthesis (phosphoramidite)	Apo-B-1 siRNA
	5′-end of sense strand (*49*)	Cholesterol phosphoramidite coupled to (CH2)−S-S-(CH2)6 linker	Reducible (disulfide)	Solid-phase synthesis (phosphoramidite)	p38 MAP kinase siRNA
α-tocopherol-siRNA	5′-end of antisense strand (*17*)[a]	α-tocopherol phosphoramidite	Cleavable (phosphodiester, cleavage site of Dicer)	Solid-phase synthesis (phosphoramidite)	Apo-B-1 siRNA
Lipid-siRNA	3′-end of sense strand (*34*)[a]	Hydroyprolinol-lipophile moiety	Noncleavable (trans-4-hydroxyprolinol)	Solid-phase synthesis (phosphoramidite)	Apo-B-1 siRNA
TAT-siRNA	3′-end amine of antisense strand (*46*)[b]	N-terminal Cys containing TAT	Noncleavable (amide bond)	Cross-linker (sulfo-succimidyl 4-(*p*-maleimidophenyl)-butyrate)	GFP siRNA/CDK9 siRNA
	5′-end thiol of sense strand (*49*)	C-terminal Cys containing TAT	Reducible (disulfide)	Disulfide exchange	p38 MAP kinase siRNA
Penetratin-siRNA	5′-end thiol of sense strand (*47*)[b]	N-terminal Cys containing penetratin	Cleavable (disulfide)	Oxidation	Luciferase siRNA
	5′-end thiol of sense strand (*49*)	C-terminal Cys containing penetratin	Cleavable (disulfide)	Disulfide exchange	p38 MAP kinase siRNA
Transportan-siRNA	5′-end thiol of sense strand (*47*)[b]	N-terminal Cys containing transportan	Cleavable (disulfide)	Oxidation	Luciferase siRNA
PEG-siRNA	3′-end amine of sense strand (*50, 55, 56, 81*)[b]	Thiol modified PEG	Cleavable (disulfide)	Disulfide exchange (SPDP)	VEGF siRNA
	5′-end thiol of sense strand (*65, 66*)	Acrylate modified PEG	Cleavable (β-thiopropionate)	Michael addition	Luciferase siRNA/RecQL1 siRNA
Peptide analogue of insulin-like growth factor 1-siRNA	5′-end amine of sense strand (*60*)	C-terminal carboxyl group of IGF1 analogue	Noncleavable (amide bond)	Carbodiimide chemistry (DCC/NHS)	Insulin receptor substrate 1 siRNA
Antibody-siRNA	3′-end biotin of sense strand (*63*)	Streptavidin-conjugated antibody	Noncleavable (tetra-ethyleneglycol)	Biotin-streptavidin	Luciferase siRNA
Aptamer-siRNA	5′-end of sense strand (*64*)	In vitro transcription	Cleavable (disulfide) or noncleavable	Biotin-streptavidin	Lamini A/C siRNA
	5′-end of sense strand (*21*)		Cleavable (phosphodiester)	RNA aptamer-siRNA chimera	Bcl-2 siRNA/PLK1 siRNA
Quantum dot-siRNA	5′-end thiol of sense strand (*69*)	Amine-modified quantum dot nanoparticle	Cleavable (disulfide) or noncleavable (thioether)	Disulfide exchange (SPDP) or Micheal addition	GFP siRNA
Iron oxide nanoparticle-siRNA	5′-end thiol of antisense strand (*70*)	MBS-activated iron oxide nanoparticle	Noncleavable (thioether)	Michael addition	GFP siRNA/survivin siRNA
Carbon nanotube-siRNA	5′-end thiol of sense strand (*71*)	Amine modified carbon nanotube	Cleavable (disulfide)	Disulfide exchange (SPDP)	Luciferase siRNA

[a] Chemical modification of backbone nucleotide: partial 2′-*O*-methyl sugar modification, phosphorothioate linkage. [b] Reaction with duplex siRNA -Cys: cysteine; rxn: reaction.

method to increase the nuclease resistance of siRNA (*22–25*). Modification of the 2′-hydroxyl group of the pentone sugar, such as 2′-*O*-methyl (*22, 26*), 2′-*O*-(2-methoxyethyl) (*26*), 2′-deoxy-2′floro (*27*), 2′-deoxy-2′floro-*β*-D-arabinonucleic acid (FANA) (*28*), and a methylene linkage between the 2′ and 4′ positions of the ribose (locked nucleic acid, LNA) (*29*), has also been employed for the enhanced nuclease resistance of siRNA, since the 2′ modifications apparently did not interfere with the action of the intracellular RNAi machinery (*18*). The replacement of oxygen linked to the 4′ carbon of the ribose with sulfur and the modification of terminal nucleotides of siRNA have also been attempted to enhance resistance to enzymatic degradation (*30, 31*). However, it should be noted that, in order to fully maintain its silencing activity of siRNA, extensive chemical modifications of siRNA are not desirable (*32*)

B. Lipophile−siRNA Conjugates. Cholesterol was covalently conjugated to siRNA for systemic delivery (*33*). Cholesterol was conjugated to the 3′-terminus of the sense strand of siRNA via a pyrrolidone linkage. The cholesterol−siRNA conjugate (chol-siRNA) could induce intracellular RNAi without the significant loss of gene silencing activity. In addition, the conjugate exhibited significantly higher cellular transfer efficiency in cultured cells without the aid of transfection agents. In animal experiments, the significant silencing of apoliporotein B (apoB) gene, which encodes a protein essential for cholesterol metabolism, was observed in the liver and the jejunum after intravenous administration of the chol-siRNA conjugate. The silencing of the apoB gene resulted in a decreased plasma apoB protein level and consequently a reduction in the total levels of cholesterol. The conjugation of cholesterol to siRNA improved in vivo pharmacokinetic behaviors of siRNA as well. Intravenously administered chol-siRNA was distributed to various tissues including the liver, heart, lungs, kidneys, and fat tissues and was detected in the tissues even 24 h after injection (*33*). In contrast, no detectable naked siRNA could be observed in the tissues 24 h after intravenous injection. In addition to the chol-siRNA conjugate, a series of lipophilic siRNA conjugates, including siRNA conjugates with bile acids and lipids, were synthesized (Figure 1) (*34, 35*). They interacted with lipoproteins in the blood serum, such as high-density lipoprotein (HDL) and low-density lipoprotein (LDL), lipoprotein receptors, and transmembrane proteins, influencing tissue distribution and uptake behaviors of the siRNA conjugates (*34*). The degree of hydrophobicity, which directly relates to the length of the alkyl chain, seemed to be a major determinant for the affinity of siRNA−fatty acid conjugates to lipoproteins. The siRNA conjugates with higher affinity to lipoproteins, i.e., the ones with longer fatty acid chains, showed enhanced gene silencing capabilities, suggesting that lipoproteins may facilitate the cellular uptake of the conjugates. When systemically administered, chol-siRNA bound to HDL demonstrated ca. five times higher cleavage of the target RNA transcript (apoB) in mouse, compared to unbound chol-siRNA at the same concentration. This suggests that the association of lipoproteins may further improve the pharmacokinetic properties of lipophilic siRNA conjugates. The association of lipoprotein also changed tissue distribution profiles of the conjugates. The lipophilic siRNA conjugates bound to LDL were mainly distributed to the liver by the action of LDL receptors, while the conjugates bound to HDL were taken up by various tissues, including the liver, gut, kidneys, and steroidogenic organs. The high affinity of HDL to the scavenger receptor SR-BI plays an important role in the uptake of HDL-bound siRNA conjugates. Further optimization may be possible by modifying strategies for the formation of preassembly between the lipophilic siRNA conjugates and lipoproteins or by identifying new lipophilic conjugate partners that lead to more favorable interaction with serum lipoproteins.

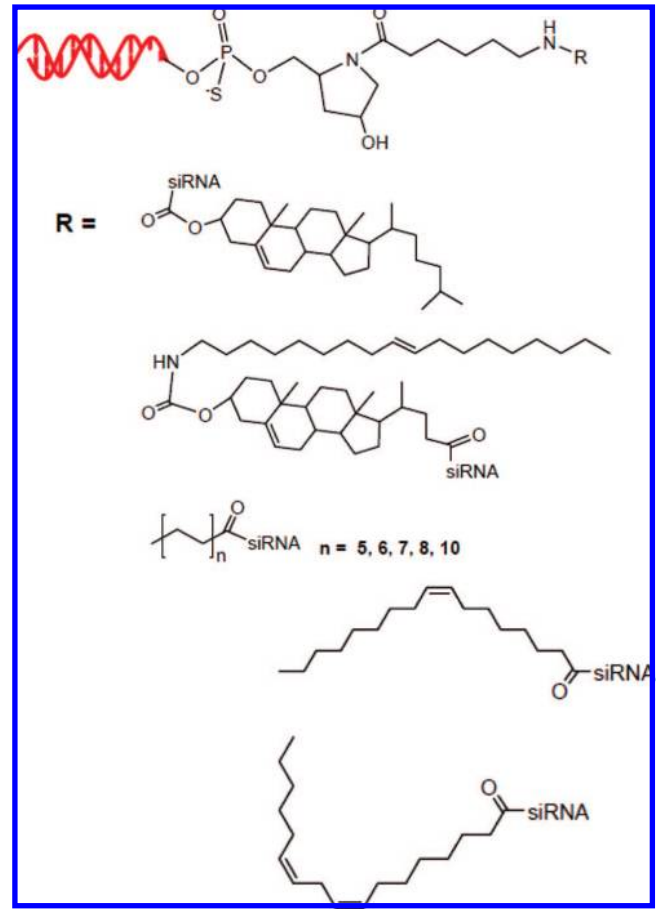

Figure 1. Structure of lipophile−siRNA conjugates. Various lipophiles (R), including cholesterol, bile acid, and a series of lipids with different chain lengths, were covalently conjugated to siRNA.

Although the animal results are encouraging, since the study showed the feasibility of using siRNAs as therapeutics, the requirement of a relatively high siRNA dose (50 mg/kg) remains to be solved for clinical applications.

Another lipophile−siRNA conjugate, α-tocopherol (vitamin E)-siRNA, was introduced for systemic siRNA delivery to the liver (*17*). Lipophilic vitamin E was covalently conjugated to the 5′-terminus of the antisense strand of 27/29-mer siRNA, which was partially modified with 2′-*O*-methylated ribose and phosphorothioate linkage. After intracellular delivery, the 27/29-mer siRNA was to be processed by the action of Dicer (*36*) to generate 21/21-mer siRNA, which caused the simultaneous release of the vitamin E moiety. The intravenous administration of the conjugate achieved a significant reduction of the target protein (apoB) in the liver without any induction of inflammatory interferons, such as interferon-α and interferon-*β* (*17*).

C. Peptide−siRNA Conjugates. A wide range of cell-penetrating peptides (CPPs), also called protein transduction domain (PTD), have been identified and employed as a vehicle for intracellular delivery of macromolecules such as proteins (*37, 38*). Well-known examples of CPPs are TAT trans-activator protein (48−60) from human immunodeficiency virus type-1 (HIV-1) (*39*), Penetratin from the homeodomain protein of *Antennapaedia* (*40*), and Transportan, a hybrid amino acid sequence from glanin and mastoparan (*41*). The intracellular delivery efficiency of antisense oligonucleotides (*42, 43*) and peptide nucleic acid (PNA) (*44, 45*) could be improved by direct conjugation of CPPs. The same strategies were applied for the enhanced intracellular delivery of siRNA (Figure 2). TAT (47−57) was conjugated to the 3′-terminus of the antisense strand of an siRNA using a heterobifunctional cross-linker (HBFC), sulfo-

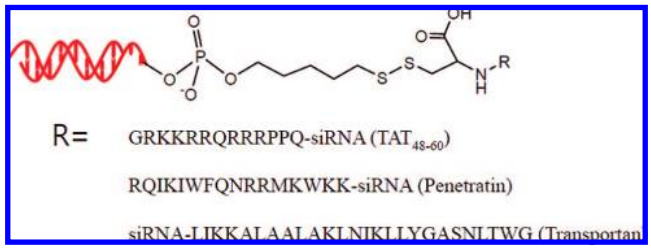

Figure 2. Structure of peptide−siRNA conjugates. Cell-penetrating peptides (TAT_{48-60}, penetratin, and transportan) were conjugated to siRNA through a terminal cysteine residue derivatized at either the N- or C-terminus of each peptide.

succinimidyl4-(*p*-maleimidophenyl)butyrate (*46*). The TAT−siRNA conjugate demonstrated a dramatic improvement in the intracellular delivery of siRNA. The extent of cellular uptake showed a direct relationship with the amount of conjugate used for the transfection and the time elapsed after transfection. Cellular uptake of siRNA by the conjugate was as highly efficient as the commercial Lipofectamine formulation. The TAT−siRNA conjugate also successfully elicited the effective silencing of the target gene. Although TAT was conjugated to the antisense strand of siRNA via a noncleavable thioether linkage, the presence of the peptide did not seem to seriously interfere with the downstream process for the induction of RNAi, including the incorporation of TAT−siRNA into the RNA-induced silencing complex (RISC). The 5′-terminus of the antisense strand of siRNA was considered crucial for induction of the RNAi mechanism (*19, 20*). Many research groups used a cleavable linkage, such as the disulfide linkage, between CPP and siRNA to minimize potential reduction of RNAi activity by the presence of highly charged peptides. The disulfide linkage was employed for the conjugation of CPPs, including Penetratin and Transportan, to the 5′-terminus of the sense strand of siRNA. This delivery system was designed to improve the intracellular delivery of siRNA by the action of CPP and to release intact siRNA by reductive cleavage of the disulfide linkage between the CPP and the siRNA in the reductive cytoplasmic environment. The reductive release of intact siRNA from the conjugate may further facilitate the incorporation of siRNA into RISC by eliminating the potential interference of CPP. The CPP−siRNA conjugates, in which the disulfide linkage between the CPP and siRNA was formed by oxidation of thiol groups in the presence of oxidant, diamide, exhibited efficient suppression of the target reporter gene expression, such as luciferase or green fluorescence protein (GFP), in a variety of cell lines (*47*). The gene silencing efficacy of the conjugates was comparable to those of a cationic lipid-based formulation. Similar conjugates of Penetratin 1 peptide to siRNA, which also contain a disulfide linkage between the two components, were used for the delivery of siRNA to primary mammalian neuronal cells (*48*). The conjugate showed efficient down-regulation of the target genes, including superoxide dismutase (SOD) variants and caspases, in the neuronal cells without apparent cytotoxicity. However, the conjugation of CPP to siRNA did not appear to improve siRNA stability. The conjugates of CPP and siRNA showed similar degradation profiles to those of naked siRNA after exposure to mouse bronchoalveolar lavage (BAL) fluid (*49*). Besides, some peptide−siRNA conjugates elicited undesirable immune responses. Although intratracheal delivery of siRNA using CPP−siRNA conjugates successfully suppressed the expression of a target gene, p38 MAP kinase, the siRNA conjugate of Penetratin but not TAT(48−60) caused elevated secretion of inflammatory cytokines, such as interferon-α, tumor necrosis factor-α, and interleukin-12, after intratracheal distillation, suggesting the induction of innate immunity (*49*)

D. PEG−siRNA Conjugates. Poly(ethylene glycol) (PEG), a biocompatible, hydrophilic, and nonionic polymer, was conjugated to siRNA via a reducible disulfide linkage (Figure 3A) (*50*). The PEG−siRNA conjugate could be further complexed with cationic polymers or peptides as core condensing agents to form colloidal nanoparticles, so-called polyelectrolyte complex micelles (PEC micelles) (Figure 3B). The formation of PEC micelles and their use as a delivery vehicle for oligonucleic acid therapeutics has also been studied using PEG conjugated antisense oligonucleotides and siRNA in our previous studies (*50–54*). The negatively charged siRNA part was completely buried inside the polyelectrolyte core by the addition of the core-forming polycation due to charge neutralization, while the hydrophilic PEG segment surrounded the charge-neutralized core, improving the solubility of the insoluble polyelectrolyte core in an aqueous environment. The micelle-like core−shell structure, which is thermodynamically favored, provides additional stability to the PEC micelles when they are administered into the bloodstream, leading to prolonged circulation. The surrounding PEG shell also effectively prevented interparticular aggregation and shielded the surface charges of the core to make the PEC micelles neutral nanoparticles with a hydrophilic surface, resulting in acceptable blood compatibility. The PEG−siRNA conjugate itself showed much higher levels of siRNA stability than the naked siRNA in the presence of 50% serum. The conjugate lasted up to 16 h without a significant loss of integrity (*50*). The integrity of siRNA was fully maintained even after 48 h in 50% serum, when the PEG−siRNA formed PEC micelles via interactions with polyethylenimine (PEI). The PEC micelles achieved significantly enhanced transfection efficiency. A fusogenic cationic peptide, KALA, which is known to destabilize the endosomal membrane, was also used as a core forming agent to facilitate the endosomal escape process (*55*). The PEG−siRNA/PEI PEC micelles were used for local and systemic treatments for tumors in an animal model (*56*). An siRNA targeting vascular endothelial growth factor (VEGF) was chosen for antiangiogenic cancer therapy. The local and intravenous administration of the PEC micelles achieved significant retardation of tumor growth in tumor-bearing mice. In situ fluorescence imaging directly showed enhanced accumulation of the PEC micelles in a solid tumor region after intravenous injection through a mouse tail vein, suggesting the possibility of passive tumor targeting of siRNA therapeutics using the PEC micelles. This property of PEC micelles may be due to the passive diffusion of nanoparticles through the loosened endothelial vascular structure in the vicinity of highly proliferative tumors, which is called the enhanced permeation and retention (EPR) effect (*57*)

III. siRNA CONJUGATES FOR TARGETED DELIVERY

The lack of tissue- or cell-type specificity of siRNA is one of the most challenging problems in the development of therapeutic siRNAs. Targeted delivery of siRNA to the desired cells and tissues has been considered as an attractive way to bring siRNA drugs to clinical settings. The targeted siRNA delivery strategy not only minimizes the chance of potential adverse effects, but also reduces the amount of dose required to achieve a desired therapeutic effect. Cell-specific ligands, including aptamers, antibodies, sugar molecules, vitamins, and hormones, have been popularly employed to confer cell specificity on siRNA delivery systems (*58, 59*). Specific interaction between a specific ligand and its cellular membrane receptor generally enhances the cellular uptake by the aid of a mechanism called receptor-mediated endocytosis. Therefore,

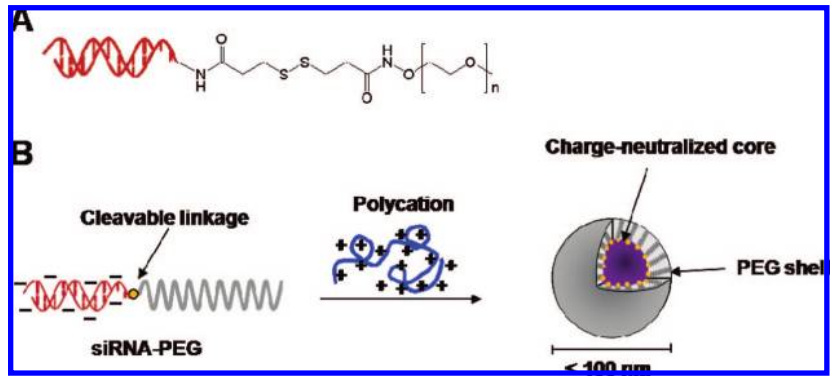

Figure 3. (A) Structure of PEG−siRNA conjugate. (B) Formation of PEC micelles from the interactions between PEG−siRNA conjugate and a polycation.

increasing specific cellular uptake by targeted delivery can significantly improve therapeutic efficacy with a much lower dose.

A. Ligand Peptide−siRNA Conjugates. For the receptor−ligand mediated delivery of siRNA, a ligand peptide for the targeted receptor was conjugated to siRNA. A carboxylic acid group of peptide mimetic of IGF1, D-(Cys-Ser-Lys-Cys) was activated and conjugated to an amine group of the 5′-sense strand of siRNA (*60*). After the annealing reaction, the gene silencing effect by an IGF1−siRNA conjugate was analyzed without the use of a transfection reagent. About 60% of the target IRS1 (insulin receptor substrate 1) gene expression was inhibited by the IGF1−siRNA conjugate, which was similar to that from the chol−siRNA conjugate.

B. Antibody−siRNA Conjugates. Antibody-mediated targeted drug delivery systems have attracted much attention due to their superior stability during systemic circulation and high selectiveness toward a target protein on the cell surface. A PEGylated immunoliposome-based brain targeting system was previously introduced, in which two antibodies were immobilized on the surface of liposomes (*61*). One monoclonal antibody was to target a transferring receptor expressed in the blood brain barrier (BBB), and the other antibody was to target insulin receptors expressed in brain cancer. The binding of transferrin receptor antibody may induce the transcytosis of the immunoliposome across the BBB. Similarly, the monoclonal antibody targeting the transferrin receptor at the BBB was directly conjugated to siRNA via a biotin−streptavidin linkage (*62*). The siRNA biotinylated at either terminus (3′ or 5′) of the sense strand was coupled to the streptavidin-conjugated antibody (Figure 5). The intravenous administration of the antibody−siRNA conjugate led to the efficient suppression of a reporter gene expression in a rat model bearing intracranially transplanted brain tumor.

C. Aptamer−siRNA Conjugates. Aptamers, modified oligonucleotides having selective affinities toward target proteins, are characterized by their globular structure, like the shape of tRNA. Generally, aptamers have intermediate molecular weights between small peptides (∼1 kDa) and single chain antibody fragments (∼25 kDa) (*63*). An aptamer targeting prostate-specific membrane antigen (PSMA), a cellular receptor abundantly expressed in prostate cancer cells, was directly conjugated to siRNA for prostate cell-specific delivery. Chu et al. used the biotin−streptavidin interaction for the conjugation of siRNA to the aptamer (Figure 4A) (*64*). Biotinylated siRNA and aptamer were joined together by using streptavidin via streptavidin−biotin interactions. The resulting conjugate was readily taken up by PSMA overexpressing cells without using transfection agents and successfully mediated specific RNAi, comparable to silencing activities by conventional lipoplexes. Another group proposed similar but more sophisticated targeting

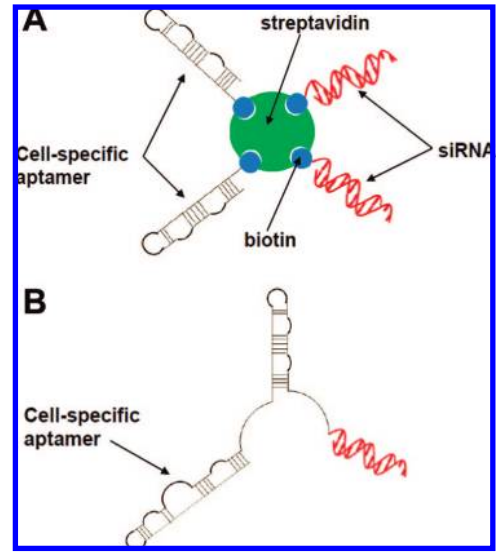

Figure 4. Schematic diagrams of aptamer−siRNA conjugates. (A) Conjugation of siRNA to a PSMA-targeting aptamer. The end group of the aptamer and siRNA was derivatized with biotin. Streptavidin was used as a core for the association of the biotinylated aptamer and siRNA. (B) Structure of chimeric conjugate of RNA aptamer and double-stranded siRNA. After intracellular delivery, the conjugate can be further processed by Dicer to induce RNAi.

system using a chimeric double-stranded RNA−aptamer hybrid (Figure 4B) (*21*). The chimera was synthesized by the in vitro runoff transcription method from a double-stranded DNA template containing a T7 RNA polymerase promoter. The dsRNA−aptamer hybrid was selectively bound to the cells overexpressing the target receptor, PSMA, and was taken up by the cells via receptor-mediated endocytosis. After the cellular uptake, the hybrid molecule could be further processed by endogenous Dicer, producing siRNA which target survival genes, such as polo-like kinase 1 (PLK1) and Bcl-2. Cell-type specific inhibition of tumor growth and tumor regression were achieved in a mouse tumor xenograft model by intratumoral administration of the chimeric siRNA delivery system. Although both proof-of-concept studies suggested the potential use of aptamers for the targeted delivery of siRNA drugs, nuclease susceptibility of aptamer ligands may limit their application in systemic treatments and the possibility of premature degradation of siRNA in the endolysosomal compartment due to the lack of endosomal escape machinery may require additional functionality.

D. Targetable Polyelectrolyte Complex Micelles. Polyelectrolyte complex (PEC) micelles consisting of PEG-conjugated siRNA and a polycation as a core forming agent can be formed by electrostatic interactions between negatively

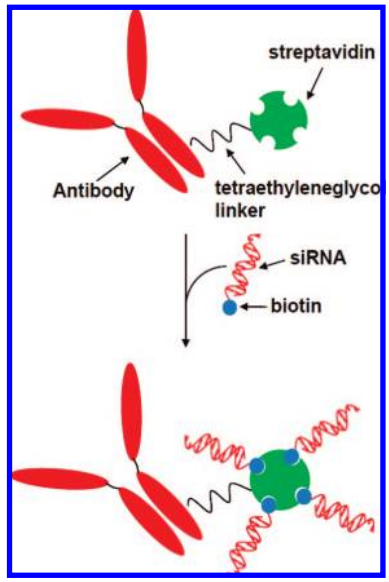

Figure 5. A schematic representation of antibody−siRNA conjugate. Biotinylated siRNA was coupled to streptavidin-conjugated antibody.

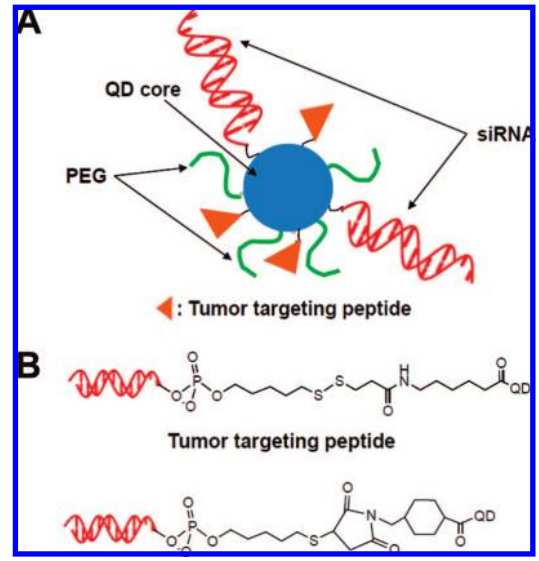

Figure 6. Quantum dot (QD)−siRNA conjugate. (A) A schematic diagram of a targetable QD−siRNA conjugate. Quantum dot modified with PEG and primary amine was used for further conjugation of siRNA and tumor targeting peptide. (B) The conjugate of QD−siRNA was synthesized through a reducible disulfide linkage or a nondegradable thioether linkage.

charged siRNA and polycation. The PEG segment acts as a hydrophilic shell protecting the charge-neutralized PEC core. In previous studies, PEC micelles could be passively targeted to a solid tumor region through the loosened endothelial junctions via the EPR effect (*56*). Because of the lack of cell specificity of the PEC micelles, the main route of cellular entry is expected to be adsorptive endocytosis. However, the cellular contact of PEC micelles may be hampered by the PEG shell that prevents nonspecific adsorption of blood components owing to the steric-hindrance effect of flexible PEG chains. Therefore, decoration of cell-specific ligands on the micelle surface would not only facilitate the cellular uptake via receptor-mediated endocytosis, but also reduce it by nontargeted cells. The lactose moiety was coupled to the distal end of the PEG−siRNA conjugate and used as a targeting ligand for the asialoglycoprotein receptors on hepatoma cells (*65, 66*). An siRNA was conjugated to the PEG segment via an acid-cleavable β-thiopropionate linkage and expected to be cleaved in the acidic environment of the endosomal compartment. The lactosylated-PEG−siRNA conjugate was used for the formation of PEC micelles by interacting with poly(L-lysine). The ligand installed PEC micelles containing siRNA targeting RecQL5 helicase demonstrated significant growth inhibition of the spheroids formed from human hepatocarcinoma cells (*66*). In our recent study, a luteinizing hormone-releasing hormone (LHRH) peptide analogue was attached to the end of PEG segment of the PEG−siRNA conjugate for the targeted siRNA delivery to ovarian cancer cells (Figure 7A) The system was designed to release siRNA in the cytoplasm by the reductive cleavage of the disulfide linkage between PEG and siRNA. The PEC micelles formed from the interaction between LHRH-PEG-siRNA and PEI (Figure 7B) showed selectiveness toward the ovarian cancer cells expressing LHRH receptors, leading to enhanced cellular uptake and suppression of the target gene expression. Surface installment of cell-specific targeting moieties onto the carriers gives selectivity toward target cells, which may increase the therapeutic effect in a target tissue with minimal risk of potential adverse effect by reducing the amount of the dose.

IV. siRNA−NANOPARTICLE CONJUGATES

Recently, various nanosized imaging agents (quantum dot nanoparticles, iron oxide nanoparticles, gold nanoparticles, carbon nanotubes, etc.) have been utilized for the development of multifunctional nanoparticles for dual therapeutic and diagnostic purposes (*67, 68*). QDs are fluorescent semiconductor nanocrystals with tunable emission properties in a wide range of colors (*69*). PEGylated QDs with surface amine groups were used for the conjugation of a tumor targeting peptide (F3), which binds to cell-surface nucleolin, and siRNA targeting a reporter gene encoding green fluorescence protein (GFP) (Figure 6) (*67*). The peptide and siRNA was attached to the QD using sulfo-LC-SPDP (sulfosuccinimidyl 6-(3′-[2-pyridyldithio]-propionamido) hexanoate) and sulfo-SMCC (sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate) as heterofunctional cross-linkers. Sulfo-LC-SPDP generates a disulfide linkage that can be cleaved in the reductive cytoplasm, while sulfo-SMCC generates a stable thioether linkage. The QD−siRNA/F3 conjugate successfully delivered to HeLa cells without transfection agents presumably by receptor-mediated endocytosis. However, the gene silencing effect could not be observed due to the lack of an endosomal escape function (*67*). The addition of cationic liposomes after transfection of the QD conjugate led to significant reduction of the target gene expression in HeLa cells. Significantly higher silencing efficiency was observed with the conjugate containing a disulfide linkage between QD and siRNA, suggesting that the release of siRNA is required for the induction of RNAi due to the potential hindrance of large QD in the incorporation of siRNA into RISC.

Magnetic nanoparticles have been intensively investigated for their potential biomedical applications, such as magnetic resonance imaging (MRI), hyperthermia, magnetofection, and drug delivery (*70*). For siRNA delivery with concomitant visualization of its localization in vivo, siRNA was immobilized onto the surface of magnetic nanoparticles (*68*). Primary amine groups on the iron oxide nanoparticles were activated with a heterofunctional cross-linker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), and then conjugated to the thiol group of the antisense strand of siRNA. Near-infrared Cy5.5 dye (NIRF) and membrane translocation peptides were also coated onto magnetic nanoparticles. Tumor tissues were successfully visualized by MRI and NIRF image due to the accumulation of the resultant magnetic nanoparticle−siRNA conjugate at the tumor tissue after intravenous injection. The

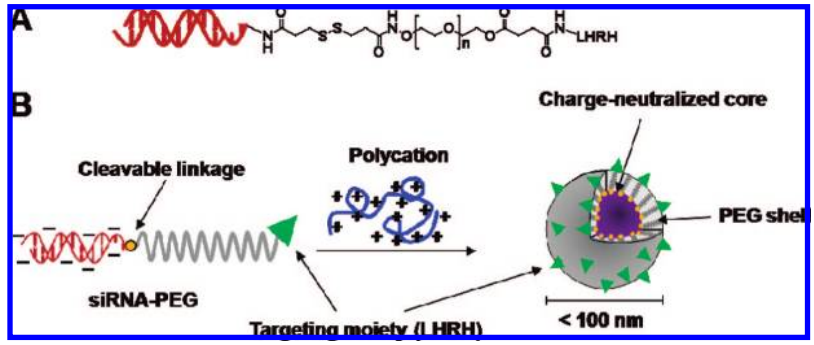

Figure 7. (A) Structure of an LHRH-tethered PEG−siRNA for targeting ovarian cancer cells. (B) The formation of PEC micelles from the interaction between LHRH-PEG-siRNA conjugate and a polycation (*81*).

mRNA level of target genes (green fluorescence protein and human survivin) was suppressed to below 15% by systemic injection of the magnetic nanoparticle−siRNA conjugate, compared to saline-treated controls. Although siRNA was conjugated onto magnetic nanoparticles via a noncleavable bond (thioether) in this experiment, surprisingly significant RNAi activity was achieved in vivo. The carbon nanotube−siRNA conjugates was also reported (*71*). The gene silencing effect by cleavable nanotube−siRNA conjugates was more enhanced about 20%, compared to that by lipofectamine/siRNA complexes and noncleavable nanotube−siRNA conjugates in vitro.

V. REDUCING OFF-TARGET EFFECTS BY siRNA CONJUGATES

Several recent papers have suggested that false-positive gene silencing could also be generated by siRNA due to its unexpected off-target effects. Recent evidence demonstrated that the off-target effects are mainly mediated by host innate immune response against the administered double-stranded RNA (siRNA) and the hybridization of siRNA-loaded RISC to undesirable mRNAs.

Synthetic siRNA can be recognized by cytoplasmic proteins, including RNA-dependent protein kinase, retinoid-acid-inducible gene 1 (RIG-1), and melanoma differentiation-associated gene 5 (MDA-5), to trigger downstream signal transduction required to elicit inflammatory responses (*72, 73*). In addition, a series of transmembrane toll-like receptors (TLRs) are also known to play a crucial role in the recognition of synthetic siRNAs, which trigger innate immune responses (*74*). The siRNA-directed immune responses may be modulated by varying the sequence and backbone chemistry of siRNA and the type of delivery vehicle (*74, 75*) The siRNA-dependent immune stimulation could be reduced by a certain degree of chemical modification in the backbone, including 2′-fluoro, 2′-*O*-methyl substitution and *N*-benzyl-2′-deoxyguanosine modification (*76, 77*). However, the effect of siRNA conjugates on the inflammatory responses remains to be investigated. Previous reports suggest that the inflammatory responses can be induced depending on the property of a conjugation partner of siRNA. Intratracheal administration of Penetratin−siRNA conjugate could induce the expression of IFN-α and TNF-α, while TAT−siRNA and cholesterol−siRNA conjugate did not show any detectable immune responses (*49*). In the case of PEG−siRNA conjugate, the conjugation of PEG to siRNA seems not to further induce the expression of an inflammatory cytokine, interferon alpha (IFN-α) both in vitro and in vivo (*56*). It may be possible to reduce immune responses by conjugating appropriate materials to siRNA, which can hinder the interaction of siRNA with cellular proteins involved in the inflammatory signaling process.

Sometimes, silencing of unwanted genes by siRNA can occur. These false positive hits can be frequently observed in transcripts with ∼7 nucleotides of sequence complementarity at the 5′ end of the siRNA antisense strand (*78, 79*). In addition, due to structural symmetry of siRNA strands, the incorporation of siRNA sense strand to RISC could lead to the off-target silencing of genes with sequence complementarity of the siRNA sense strand (*78*). Although improved selection of siRNA sequence may reduce the chance of off-target silencing, such a method cannot completely remove the off-target phenomenon, due to the lack of an efficient algorithm for the selection process (*79*). Recent study demonstrated that 5′-*O*-methylation at 5′ end of the siRNA sense strand could enhance biased incorporation of siRNA antisense into RISC, leading to reduction of the sense strand-directed off-target effect (*80*). This result suggests that the conjugation of proper materials to the 5′ terminus of the siRNA sense strand may further facilitate the biased strand selection process to reduce undesirable off-target effects. Reducing the off-target effect of siRNA is one of the challenging barriers that should be addressed before clinical applications of siRNA therapeutics. The conjugation of foreign materials to siRNA may be a double-edged sword, the benefit and risk, in terms of off-target effect. However, it is worth further investigation to determine whether the critical problem of siRNA can be avoided with siRNA bioconjugates.

VI. CONCLUSIONS

Therapeutic use of RNAi has gained a lot of attention because of its high specificity and broad therapeutic potential to target proteins that are not easily accessed and modulated by conventional small molecular weight or protein drugs. Synthetic siRNA is considered a major player in the induction of RNAi. Development of an efficient delivery system is one of the most challenging hurdles to turn siRNAs into clinically acceptable therapeutic drugs. A wide variety of siRNA conjugate delivery systems have been proposed, and some of them have demonstrated very promising preclinical results. Direct cleavable and noncleavable conjugation of various functional and nonfunctional molecules to siRNAs could not only improve their pharmacokinetic behaviors and cellular uptake efficiencies, but also promote cell specificities. To maximize delivery efficiency of siRNA bioconjugates, several crucial conjugation parameters, such as the conjugation site of siRNA, cleavability, the length of spacer, and the nature of molecules to be conjugated (charge, molecular weight, and hydrophobicity), should be carefully considered. A new class of siRNA bioconjugates integrating multiple functions in a single conjugate delivery system would be further required to solve various challenging barriers such as stability, prolonged circulation, target-specific cellular uptake, endosomal escape, and off-target property of siRNA.

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