Pharm Res (2011) 28:2072–2085
DOI 10.1007/s11095-011-0412-y

EXPERT REVIEW

Self-Assembled and Nanostructured siRNA Delivery Systems

Ji Hoon Jeong • Tae Gwan Park • Sun Hwa Kim

Received: 6 January 2011 / Accepted: 25 February 2011 / Published online: 18 March 2011

ABSTRACT A wide range of organic and inorganic materials have been used in the development of nano-scale self-assembling gene delivery systems to improve the therapeutic efficacy of nucleic acid drugs. Small interfering RNA (siRNA) has recently been recognized as a promising and potent nucleic acid medicine for the treatment of incurable genetic disorders including cancer; however, siRNA-based therapeutics suffer from the same delivery problems as conventional nucleic acid drugs such as plasmid DNA and antisense oligonucleotides. Many of the delivery strategies developed for nucleic acid drugs have been applied to siRNA therapeutics, but they have not produced satisfactory *in vivo* gene silencing efficiencies to warrant clinical trials. This review discusses recent progress in the development of self-assembled and nanostructured delivery systems for efficient siRNA-induced gene silencing and their potential application in clinical settings.

KEY WORDS non-viral gene carriers · PEGylated polyplex · self-assembled nanostructures · siRNA medicines · siRNA-PEG conjugates

J. H. Jeong
School of Pharmacy, Sungkyunkwan University
Suwon 440–746, South Korea

T. G. Park
Department of Biological Sciences
Graduate School of Nanoscience and Technology
Korea Advanced Institute of Science and Technology
Daejeon 305–701, South Korea

S. H. Kim (✉)
Severance Integrative Research Institute for Cerebral & Cardiovascular Diseases, Yonsei University Health System
250 Seongsanno, Seodaemun-gu
Seoul 120-752, South Korea
e-mail: shkim77@yuhs.ac

INTRODUCTION

RNA interference (RNAi), a specific suppression of gene expression via breakdown of a target mRNA, has recently been recognized as a promising approach to treat a number of human genetic disorders and infectious diseases (1). Small interfering RNA (siRNA), 21~23 double-stranded nucleotides containing 2-nucleotide 3′-overhangs with 5′-phosphate and 3′-hydroxyl termini, is a key mediator of RNAi (2). As a therapeutic agent, siRNA has several advantages over conventional pharmaceuticals, including small molecules and other nucleic acid drugs, by providing efficient gene silencing at extremely low intracellular concentrations in a highly sequence-specific manner with few toxic effects (3). Despite the enormous therapeutic potential of siRNA drugs, their use in clinical settings is limited by the lack of an efficient delivery method (4,5).

For the development of optimal siRNA delivery methods, understanding the extracellular and intracellular barriers associated with siRNA delivery is an essential prerequisite. In general, siRNAs share most of the delivery hurdles that must be overcome for desirable delivery of other nucleic acid drugs such as plasmid DNA and antisense oligodeoxynucleotides (ODNs) (Fig. 1): (a) formation of compact nano-scale complexes between nucleic acid drug and carrier compounds, (b) choice of a route of administration, (c) systemic circulation, (d) target tissue localization, (e) intracellular transport, (f) escape from endocytic compartments such as endosomes and lysosomes, (g) nuclear transport, and (h) gene expression (6). Unlike plasmid DNA for gene expression, siRNA needs to rapidly escape from the intracellular organelles into the cytoplasm without being localized to the nucleus, where siRNA induces RNAi (7). Although a number of previously developed gene carriers have been used for siRNA, RNAi-based approaches for clinical therapy

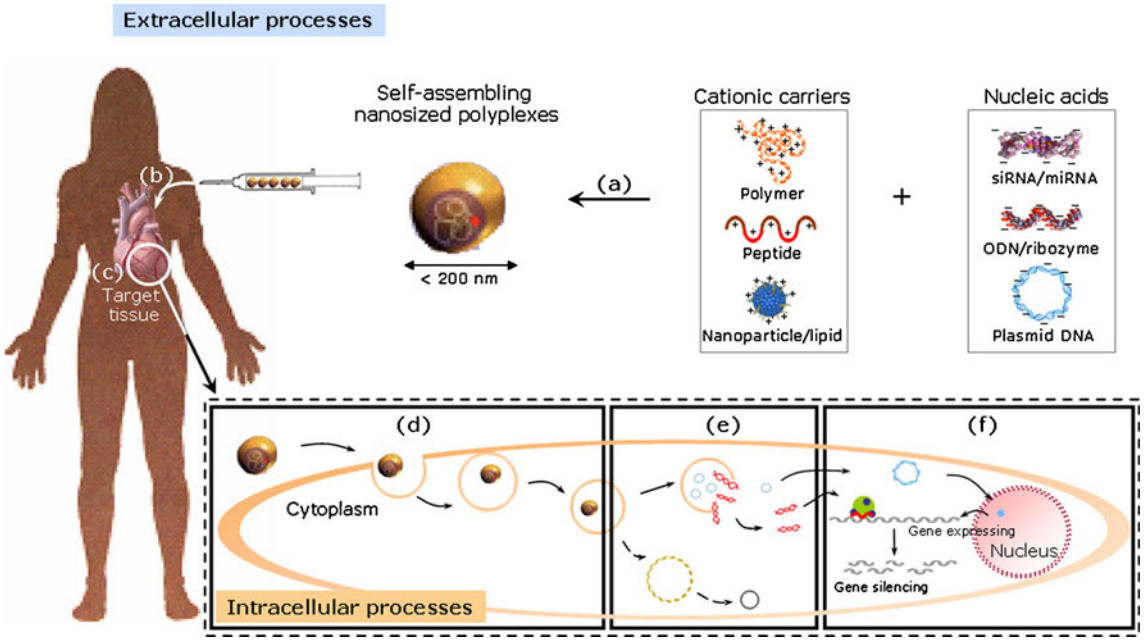

Fig. 1 Extracellular and intracellular barriers to non-viral gene delivery using various cationic carriers: (**a**) formation of condensed nanoparticles, (**b**) prolonged systemic circulation, (**c**) tissue- and cell-specific localizations, (**d**) intracellular uptake via endocytosis (or cell membrane penetration), (**e**) endosome escape, and (**f**) nuclear localization.

require more carefully designed delivery systems to achieve the maximum gene silencing effect.

This review focuses on current advances in self-assembled and nanostructured siRNA delivery systems using various organic and inorganic materials with a particular emphasis on various poly(ethylene glycol) (PEG) conjugates for systemic siRNA delivery.

SIZE AND STABILITY ISSUES OF CATIONIC POLYMER-BASED NUCLEIC ACID CARRIERS

Since nucleic acid drugs have relatively high molecular weights (mostly greater than 13 kDa) and intrinsic sensitivities to nuclease-mediated degradation, the physicochemical characteristics, including size and stability, of polyplexes formed from the interaction between nucleic acid and polymeric carrier are the key determinants for *in vivo* gene delivery (Fig. 2) (8). During the last few decades, a wide variety of cationic polymers with the ability to form self-assembled nanostructures with genetic materials have been extensively studied to achieve high transfection efficiency, biocompatibility, and low cytotoxicity (9). The polyplexes have a charge-neutralized and condensed structure with a diameter of less than 300 nm, which is required to protect their nucleic acid cargo from enzymatic degradation (6). The nucleic acid condensation and transfection efficiency of a carrier can be fine-tuned by controlling or modifying the composition and structure of a polycation. For instance, poly (L-lysine) (PLL) with a higher molecular weight tends to show more efficient nucleic acid condensation ability than PLL with a lower molecular weight, which often leads to the formation of more stable polyplexes (10). Low molecular weight PLLs (<3 kDa) produce less compact polyplexes with nucleic acid at the same polymer/DNA ratio. Depending on its backbone structure, polyethylenimine (PEI) shows different behaviors with regard to DNA condensation and polyplex formation. While polyplexes formed from branched PEI exist as highly condensed nanoparticles even after cellular uptake, linear PEI tends to form less condensed polyplexes, which dissociate more readily after cellular uptake than branched PEI (11). It should be noted that the less condensed nucleic acid fragments surrounding the central charge-neutralized core could be sites vulnerable to nuclease-mediated digestion, resulting in reduction of transfection efficiency. Although these polycation/nucleic acid polyplexes with positive surface charge are useful for *in vitro* gene transfer, when intravenously injected, they readily aggregate into larger structures via non-specific association with charged serum proteins, which leads to rapid clearance from the plasma through the reticuloendothelial system (RES) (12). To minimize inter-particular aggregation and non-specific absorption of serum-derived components, polyplex structures have been further tuned by conjugating blood-compatible functional polymers such as hydrophilic polyethylene glycol and dextran or by forming different nanostructures such as polymeric micelles, nanocapsules, and multi-shell nanoparticles.

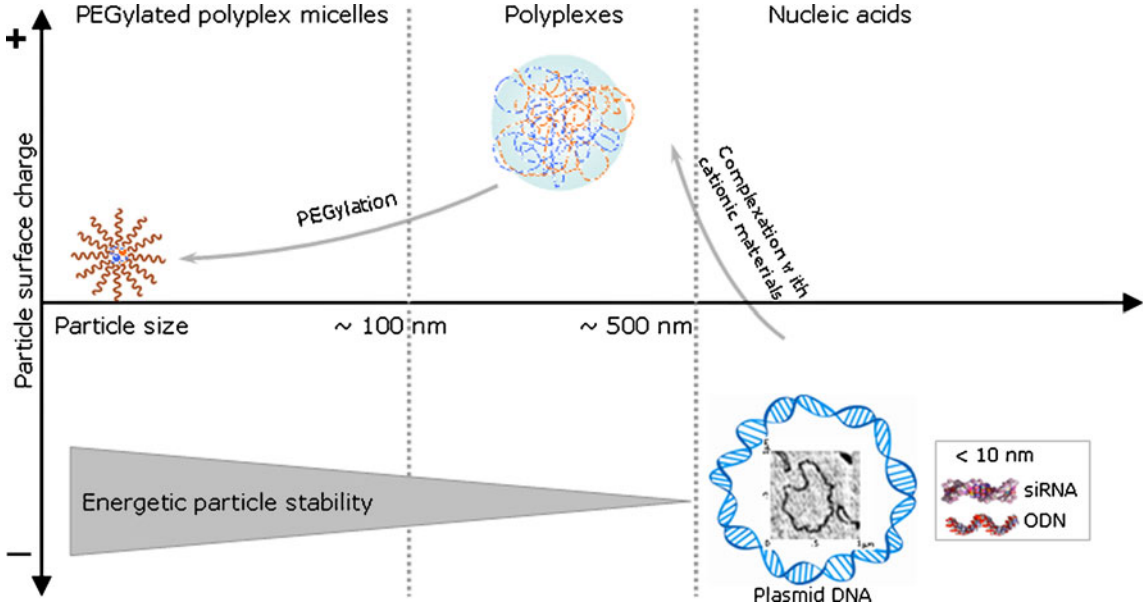

Fig. 2 Schematic illustration to explain the tendency for self-assembled nanoparticle formation. In an aqueous phase, micron-scale nucleic acids can self-assemble into nanosized polyplexes with various cationic materials. PEGylation can reduce particle size, neutralize surface charge, and sterically stabilize the nanoparticles.

In particular, the particle size of siRNA and antisense ODN complexed with polycations is generally larger than 300 nm in diameter, whereas their intact lengths under physiological conditions are typically less than 10 nm (13–15). In contrast to long plasmid DNA, double-stranded siRNA with short chain length and rigid structure is particularly difficult to form stable and highly condensed nanoparticles via an electrostatic interaction alone. Thus, there have been many approaches aimed to produce more stable and compact siRNA polyplexes through the modification of carrier systems themselves such as PEGylation of cationic condensing species. Interestingly, polymerization of siRNA monomers was recently conducted to form compact nanostructure via complex formation between polycations and the polymerized siRNA, which is mainly attributed to an increase in chain length and charge density leading to high chain flexibility and electrostatic affinity (16,17).

SELF-ASSEMBLING NANO-SIZED POLYPLEX MICELLES WITH PEGYLATED CONSTRUCTS

PEGylation, a process of covalent attachment of PEG chains to other molecules, has been commonly used to enhance the stabilities of pharmaceutical products. Since the first description of PEGylation in the 1970s by Davies and Abuchowski (18,19), PEGylation technology has been popularly used for the modification of numerous bioactive drugs such as proteins, peptides, antibody fragments, oligonucleotides, and other small molecules. In addition to acting as a physical stabilizer, PEGylation can also impart significant and distinct pharmacological advantages such as reduced toxicity, attenuated immunogenicity, improved bioavailability, and diminished aggregation (20,21).

Generally, physico-chemical properties of PEGylated products are mainly affected by the molecular weight and degree of PEG substitution. According to previous studies, a minimum PEG size of 2 kDa is required for stabilization and stealth effects, and the systemic circulation time increases with the degree of substitution of PEG molecules on the surface (22). In particular, PEGylated polyplexes (denoted as polyplex micelles) consist of two compartments: a charge-neutralized inner core containing anionic macromolecules such as nucleic acid drugs and a hydrophilic PEG corona layer serving as a sterically stabilizing barrier (23,24). Due to the unique core-shell structure, polyplex micelles can maintain colloidal stability under physiological conditions through the reduction of inter-particular aggregation. Thus, the polyplex micelles could reduce reticular-endothelial system (RES) clearance, extend systemic circulation time, and increase the chances to reach target sites (25). Compared to polyplexes without PEG, polyplex micelles produce smaller and more stable nanocomplexes having a diameter of less than 100 nm with a narrow size distribution (Fig. 2). For instance, PEI-PEG/DNA polyplex micelles have diameters as small as 60 nm, whereas PEI/DNA polyplexes have diameters of about 150 nm (26). It was also reported that the particle sizes of polyplex micelles decreased with increasing molecular weight of the conjugated PEG segment to a certain extent (27). The diameters of polyplex micelles vary from ca. 130 nm for 0.5 kDa PEG to ca. 50 nm for 20 kDa PEG. In addition, surface charges of polyplex

micelles are reduced with increasing grafting degree of the PEG chain, possibly due to the charge-shielding effect of PEG, which can only be observed with PEG greater than 5 kDa. The PEGylation strategies for cationic condensing materials have also been applied to construction of the polyplex micelles particularly useful for siRNA delivery (28–32). Several formulations of polyplex micelles containing various genetic drugs are listed in Table I.

SELF-ASSEMBLING NANOCARRIERS WITH NUCLEIC ACID-PEG CONJUGATES

Recently, an alternative method was introduced to obtain polyplex micelles by direct PEGylation of nucleic acids instead of using PEGylated polycation copolymers (56). The nucleic acid-PEG conjugate forms polyplex micelles by interacting with conventional cationic carriers, including polymers, peptides, liposomes, and nanoparticles. The resulting polyplex micelles have a characteristic core-shell structure consisting of charge-neutralized core surrounded by a hydrophilic PEG corona. Antisense ODN-PEG hybrid conjugates were synthesized and used as an alternative carrier forming polyplex micelles (23,57). Similar hybrid conjugates containing siRNA were also synthesized. The PEGylation of nucleic acid-based gene-silencing molecules such as antisense ODNs and siRNAs can also confer enhanced physical stability and biological activity. Although both antisense ODN and siRNA can specifically inhibit the expressions of complementary genes, they exhibit distinct

Table I Various PEGylated Polyplex-Based Nucleic Acid Delivery Systems

PEGylation	Type of nucleic acid	Cationic carrier	PEG molecular weight	Particle size[a] (nm)	Targeting moiety	Target gene	Ref.
Carrier modification	Plasmid DNA	PEI	3,400	100～200	RGD	sFlt01	(33)
			2,000	<200	–	Luciferase	(34)
			2,000	<100	Anti-GAD mAb	Luciferase	(35)
		PLL/KALA	5,000	<200	–	Luciferase	(36)
		Gelatin	5,000	100～500	–	β-galactosidase	(37)
		PEI-streptavidin	3,400	100～200	EGF	Luciferase	(38)
	Antisense ODN	PEI	3,400	94	Folate	GFP	(29)
		DOPE/DDAB/DOTAP	–	100～130 (TEM)	–	*Bcl*-2	(39)
		PLL	5,000	ca. 50	–	c-Ha-*ras*	(40)
		DSPE	2,000	100～200 (TEM)	–	CRF-R	(41)
	siRNA	Poly-L-glutamic acid/dextran	2,000～5,000	174	–	EFGP	(30)
		PEI	3,400	120	Folate	GFP	(29)
		PEAMA	8,400	110～120	–	Human survivin/luciferase	(31)
		Chitosan-PLR	8,200	ca. 300	–	Murine survivin/luciferase	(32)
Nucleic acid modification	Antisense ODN	PLL	4,400	–	Lactose	Luciferase	(42)
		PEI	3,400	92	Folate	*c-raf*	(43)
			4,900	120	–	Dopamine D2 receptor	(44)
			2,000	70	–	*c-raf*	(45)
		Lipofectamine	3,400	70～90	Folate	GFP	(46)
	siRNA	PLL	4,400	110 (TEM)	Lactose	RacQL1	(47)
		Hydroxyapatite	5,000	90～120	–	Luciferase	(48)
		PEI	3,400	142	LHRH	VEGF	(49)
			3,400	50～80 (AFM)	–	VEGF	(50,51)
		KALA	5,000	<200	–	VEGF	(52)
		Crosslinked KALA	3,400	100～200	–	GFP	(53)
		Amine-Au	5,000	<100 (TEM)	–	GFP	(54)
		Solid lipid nanoparticle	3,400	<100	–	VEGF/GFP	(55)

[a] Particle sizes were measured by dynamic light scattering method unless mentioned

functional properties as a result of different silencing mechanisms (Fig. 3) (58,59). Antisense ODN hybridizes directly to target mRNA to form DNA-RNA double-strand that can be recognized and degraded by an endogenous enzyme called ribonuclease H (RNase H). Apparently, it is not necessary to remove the PEG chains covalently linked to antisense ODN after intracellular delivery. In the case of siRNA, intracellularly delivered siRNA is recognized and incorporated into the RNA-induced silencing complex (RISC), in which a guide strand is separated from the double-stranded siRNA and hybridized with a complementary sequence in a target mRNA. The siRNA-incorporated RISC eventually binds to and degrades target mRNA via an enzymatic process. RISC-mediated cleavage is constrained by the rate of conformational changes required for the formation of the active RISC containing the guide siRNA strand (60). Unlike antisense ODNs, the steric hindrance caused by the attached PEG block may restrict the siRNA from accessing the binding site in the RISC. However, there is still controversy over whether the linkage between the nucleic acid and PEG segments has to be cleaved after cellular uptake. Our recent study showed that both cleavable and non-cleavable linkages between siRNA and PEG exhibited similar RNAi activity when the molecular weight of the conjugated PEG is less than 10 kDa (61). Although there were no differences in gene-silencing activity between cleavable and non-cleavable siRNA-PEG conjugates, interestingly, only cleavable PEGylation of siRNA induced sequence-specific degradation of a target mRNA.

As shown in Fig. 4, four different PEGylation strategies to generate antisense ODN- or siRNA-PEG conjugates have been introduced: non-cleavable (amide) linkage, acid-cleavable (phosphoramidate) linkage, acid-cleavable (β-thiopropionate) linkage, and reductively cleavable (disulfide) linkage.

Nucleic Acid-PEG Conjugates with a Non-cleavable Linkage

Antisense *c-raf* ODN, having a primary amine group at the 5′-end, was directly conjugated to NHS-derivatized PEG via a non-cleavable amide bond (Fig. 4a) (23,45). The ODN-PEG conjugates formed polyplex micelles (ca. 70 nm) by condensing with branched PEI (25 kDa) at the N/P ratio of 1:2.5. The polyplex micellar formulation of antisense *c-raf* ODN exhibited up to 70% growth reduction in human ovarian cancer cells (A2780) compared with that of antisense ODN only (45). When folate was introduced to the distal position of the PEG segment and used as a cancer cell-targeting moiety, the polyplex micelles showed a two- to three-fold higher deposition in tumor xenografts from folate receptor over-expressing KB cells than in those from folate receptor-deficient A549 cells after systemic administration (43).

Nucleic Acid-PEG Conjugates with an Acid-Cleavable Linkage

To achieve endosomal release of ODN, acid-cleavable linkages such as phosphoramidate and β-thiopropionate were introduced between ODN and PEG for the synthesis of ODN-PEG conjugates (Fig. 4b). To synthesize PEGylated antisense *c-myb* ODN conjugates with an acid-cleavable phosphoramidate bond, the 5′-phosphate group of ODN was activated to generate active ODN-

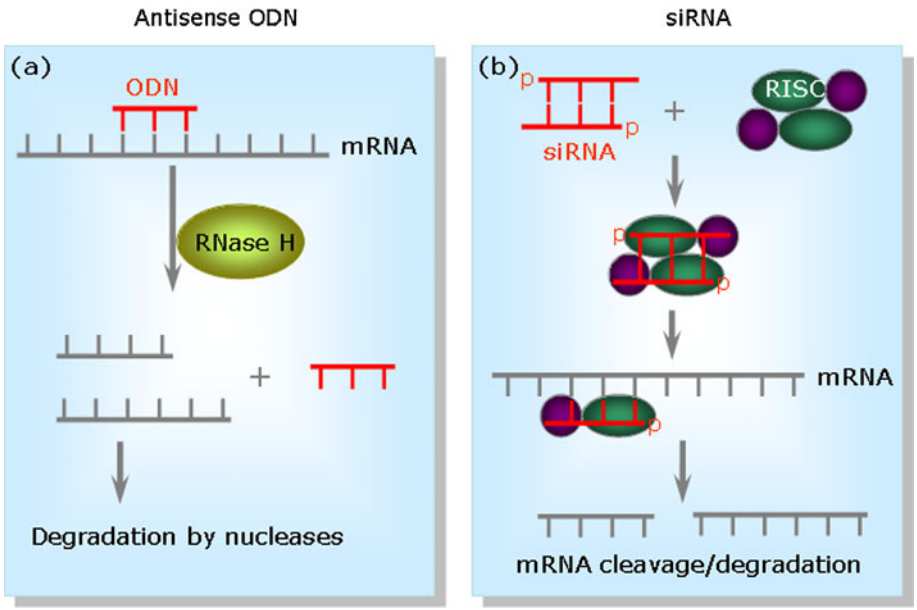

Fig. 3 Different post-transcriptional gene silencing mechanisms of (**a**) antisense ODN and (**b**) siRNA.

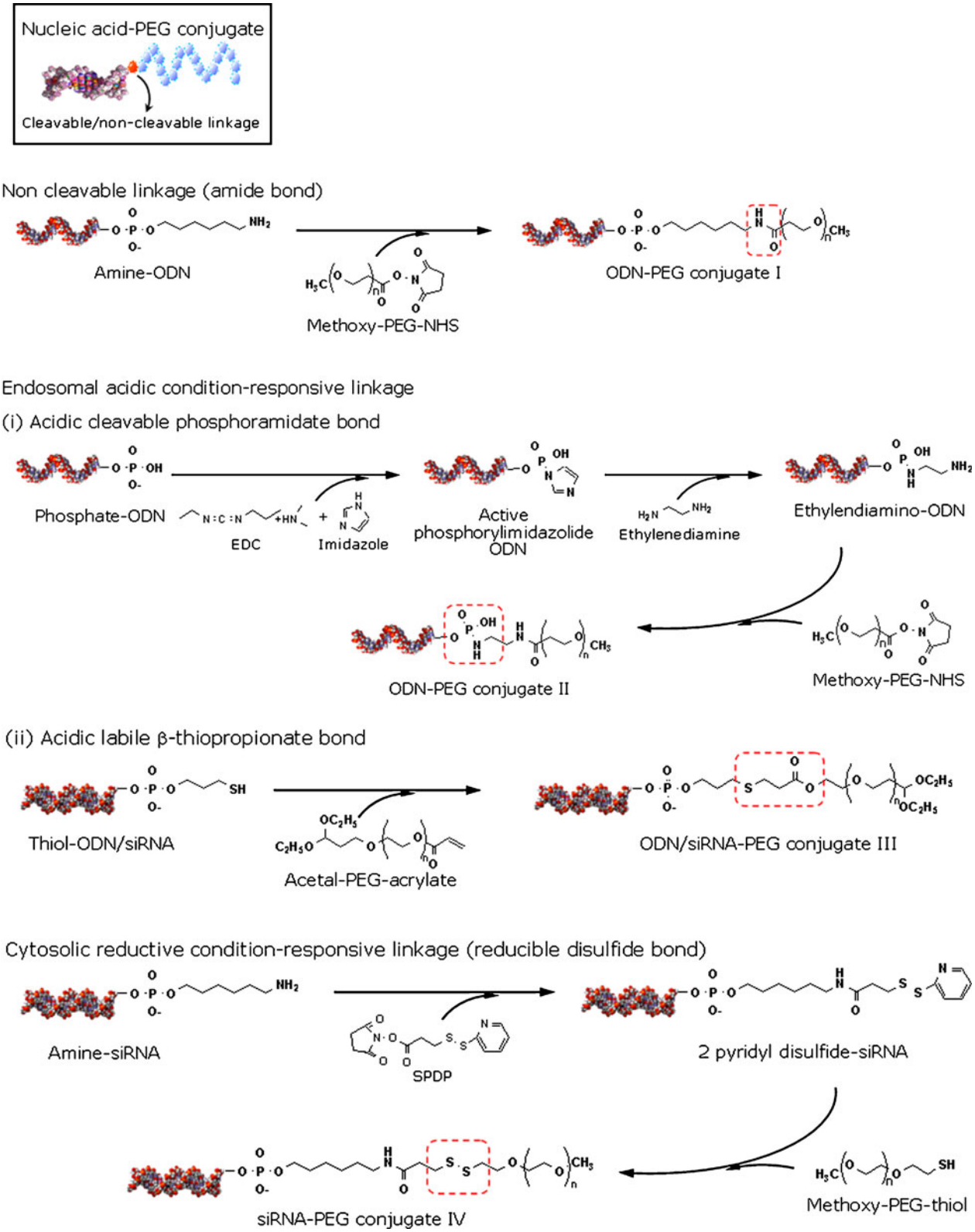

Fig. 4 Synthetic strategies to prepare nucleic acid-PEG conjugates with different linkages: (**a**) non-cleavable stable amide bond, (**b**) acidic-cleavable (i) phosphoramidate bond and (ii) β-thiopropionate bond, and (**c**) reducible disulfide bond. NHS: succinimidyl carboxymethyl ester; EDC: ethyldimethylaminopropylcarbodiimide; SPDP: N-succinimidyl-3-(2-pyridyldithio) propionate.

phosphoimidazolide intermediate (57). The active intermediate was reacted with ethylenediamine to generate a primary amine group via a phosphoramidate linkage, which was then conjugated to an NHS-modified PEG derivative. The resulting ODN-PEG conjugate bearing a phosphoramidate linker was further formulated with a cationic fusogenic peptide, KALA. While KALA/ODN polyplexes exhibited hydrodynamic diameters of approximately 200 nm, the KALA/ODN-PEG polyplex micelles were found to be around 70 nm in diameter. The PEGylated antisense *c-myb* ODN self-assembled with the KALA peptide was 1.6-fold more effective in suppressing murine smooth muscle cell proliferation than was unmodified-ODN condensed with KALA.

Another acid-cleavable linkage, a β-thiopropionate linkage, was also employed for the synthesis of ODN-PEG conjugate by using bifunctional PEG derivatives (acetal-PEG-acrylate, 5 kDa) combined with 3′-thiol-modified antisense ODN (42). The resulting ODN-PEG conjugates formed polyplex micelles with linear PEI that significantly increased the stability of ODN with respect to nuclease degradation and absorption of serum proteins. These acid-labile linkages between PEG and ODN segments can be cleaved corresponding to a pH decrease in the endosome after cellular uptake. After endosomal escape, the ODN released into the cytoplasm can hybridize to its complementary mRNA sequence to block the translation.

siRNA-PEG Conjugates with Cleavable Linkages

Recently, siRNA-based drugs have attracted much attention in the field of gene therapy. Therefore, PEG was also conjugated to siRNA for polyplex micelle-based delivery. Since the adjacent PEG chain may hinder the incorporation of siRNA into RISC, it would be important for the siRNA to be released from the siRNA-PEG conjugate for incorporation into RISC to activate the RNAi mechanism. Thus, siRNA-PEG conjugate may require a cleavable linkage between siRNA and PEG, which can be readily cleaved under intracellular conditions in order to facilitate the release of intact siRNA into the cytoplasm. To promote the physiological stability of siRNA without losing its RISC-incorporation property, siRNA-PEG conjugates were synthesized by employing pH-responsive (ester) and redox-responsive (disulfide) linkages that are expected to be cleaved in the acidic endosomal and reductive cytosolic environments, respectively (Fig. 4b,c) (46,49,62). It is noteworthy that the terminal structures of these modified siRNAs predominantly determine their selectivity and activity (63,64). Therefore, prior to siRNA-PEG synthesis, the conjugation site of siRNA should be carefully considered in order to minimize the reduction of RNAi activity. It has been suggested that the status of the 5′-terminus of the antisense strand of the siRNA duplex plays a crucial role in the induction of the RNAi mechanism, because the 5′-phosphate group of the antisense strand is required for the insertion of siRNA into the binding site of RISC (65). Among the four termini of the siRNA duplex (3′- and 5′-ends of both sense and antisense strands), the 3′- and 5′-termini of the sense and 3′-terminus of the antisense have generally been used for siRNA modification to minimize reduction in RNAi efficiency (66).

Recently, reducible disulfide linkages have been broadly applied to the chemical modification of siRNAs with various functional molecules such as cholesterol, peptides, and inorganic nanoparticles (67–70). To introduce disulfide linkages between PEG chains and siRNA molecules, a terminal primary amine group was derivatized at the 3′-end of the sense strand of siRNA duplex. The terminal amine was further modified with a pyridyl disulfide using hetero-functional coupling reagent SPDP. Thiol-modified PEG was then bound to the end-modified siRNA, resulting in siRNA-PEG conjugate containing a disulfide linkage between siRNA and PEG (Fig. 4c) (49,50). It has been generally accepted that the core-shell structures of self-assembling polyplex micelles mainly protect siRNA molecules from nuclease degradation. According to a previous study, siRNA-PEG itself also exhibited enhanced resistance to nuclease degradation even without complexation with cationic molecules, compared to unmodified siRNA (49). Similar to many other PEGylated therapeutic proteins that exhibit protease resistance in human plasma, siRNA-PEG conjugate alone helped to maintain the structural integrity of siRNA to some extent in the presence of serum.

CORE-FORMING MATERIALS FOR siRNA-PEG CONJUGATES

In aqueous medium, siRNA-PEG conjugates can be self-assembled to form polyplex micelles with various cationic core-forming agents, such as cationic polymers, peptides, and particles, as a result of complementary charge annealing. Like other PEGylated polyplexes, anionic siRNA segments and cationic counterparts form an inner core due to charge neutralization, while PEG chains surround the core to generate hydrophilic corona. This section describes organic and inorganic materials used as core-forming agents to form self-assembling micellar structures with the siRNA-PEG conjugates (Fig. 5).

Cationic Polymers and Peptides

PEI is one of the most widely used cationic polymer-based gene carriers due to its high transfection efficiency and consistent transfection in various cells (6). PEI and PEI-

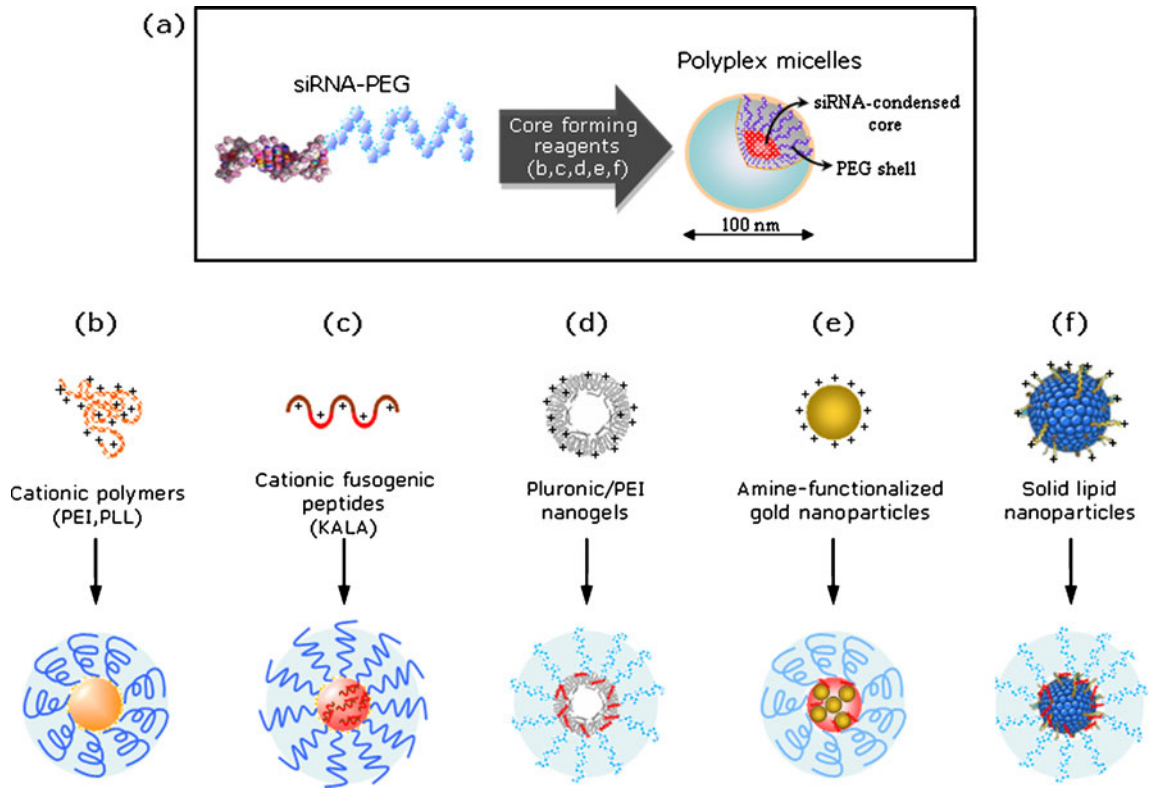

Fig. 5 (**a**) Schematic representation of polyplex micelle formation of siRNA-PEG conjugates with various types of cationic condensing reagents: (**b**) polymers, (**c**) peptides, (**d**) nanogels, (**e**) gold nanoparticles, and (**f**) solid lipid nanoparticles.

based vectors have frequently been used for siRNA delivery (71,72). Branched PEI (25 kDa) was used to formulate self-assembling polyplex micelles with siRNA-PEG conjugates (Fig. 5a,b) (49). The PEI/siRNA-PEG micelles showed 3.5 times higher gene-silencing activity than PEI/siRNA polyplexes in medium containing 10% serum. Despite the high transfection efficiency, the major drawbacks of PEI as a gene carrier are high cytotoxicity and non-degradability. Although acute cell cytotoxicity of PEI caused by direct interactions with the plasma membrane may be reduced to some extent by the charge-shielding effect of the PEG layer, high molecular weight PEI (25 kDa) still has the potential to elicit long-term toxicity since it is hardly degradable in a physiological environment (6,73). PLL, which has been used for transfection due to its excellent DNA compaction ability, was utilized as a condensing agent for the formation of polyplex micelles with siRNA-PEG conjugates (47). Targeting hepatoma cells was also achieved by introducing a lactose moiety at the distal terminus of PEG in the siRNA-PEG conjugate. The lactosylated polyplex micelles with PLL suppressed hepatic tumor growth (62). Although PLLs are less cytotoxic than PEIs, they also have inherent cytotoxicity that may limit their use in clinical settings. Moreover, transfection efficiency of PLL-based carriers is usually lower than that of PEI, probably due to the lack of function for the endosomal escape, which is an essential step prior to the incorporation of siRNA into RISC.

A cationic fusogenic peptide, KALA, has an endosome-disrupting property mediated by pH-dependent conformational change (74). The cationic peptide has been employed in designing non-viral gene delivery systems. KALA peptide can condense nucleic acid drugs to form polyplexes via electrostatic interactions and generally exhibits a much lower toxicity than conventional cationic polymers such as PEI and PLL (75). KALA peptide was used as a core-forming agent for the formation of polyplex micelles with siRNA-PEG conjugate (Fig. 5a,c) (52). The KALA/siRNA-PEG polyplex micelles reduced VEGF gene expression by up to 90%. However, significant reduction in their gene silencing efficiency was observed in the presence of serum, which may be attributed to the low charge density of the KALA peptide. Since double-stranded siRNA has a rigid structure due to its short chain length, it may form loosely condensed complexes with KALA peptide, which may allow the interaction of the core complex with serum proteins. To increase the charge density of the KALA peptide, Mock *et al.* suggested crosslinking of KALA peptides through reducible disulfide linkages (53). The reducible crosslinked KALA/siRNA-PEG polyplex micelles showed particle sizes of less than 200 nm. The siRNA-PEG

formulated with the crosslinked KALA as a core-forming agent induced significant silencing of a target gene, green fluorescent protein (GFP), in a GFP-expressing established cell line (MDA-MB-435). In addition, the transfection efficiency was not influenced by the presence of serum.

Pluronic/PEI Nanocapsules

A variety of Pluronic-based nanocarriers have been proposed as drug and gene delivery systems (76,77). In particular, Pluronic-PEI (2 kDa) nanogel, which exhibits a sharp volume transition behavior in response to thermal stimuli, is an interesting candidate for delivery of siRNA-PEG conjugates (Fig. 5a,d) (78). The Pluronic-PEI nanogel was in a collapsed state with ca. 100 nm in diameter at 37°C, but became highly swollen (ca. 400 nm) following temperature transition to 15°C, leading to an over 40-fold increase in the volume of the nanogel particles. The temperature-induced volume transition property can be used for the disruption of the endosome by physical expansion of the endosomal membrane. The nanogel also exhibited significantly lower cytotoxicity than PEI 25 kDa. In addition, the Pluronic-PEI nanogel provides sufficient condensing ability to form tight polyplexes with nucleic acids. Since the site of action for siRNA molecules is the cytoplasm, the volume-phase transition property of the Pluronic-PEI nanogel allows it to be used as an effective endosome-disrupting reagent. This may facilitate further induction of RNAi in the cytoplasm. A brief cold shock after transfection resulted in significantly enhanced RNAi efficiency.

Gold Nanoparticles

Recently, gold nanoparticles (AuNPs) have been extensively investigated for their potential biomedical applications, such as drug delivery, diagnostics and image contrast enhancement (79,80). In particular, AuNPs with diameters of 5–20 nm are readily taken up by cells without exhibiting significant cytotoxicity and used as a carrier for drug and gene delivery (81). The surface of AuNPs can be conveniently functionalized with thiolated molecules; for example, Rosi *et al.* reported antisense ODN-loaded AuNPs using thiol-modified ODN (82). The AuNPs modified with amine groups exhibit strong tendencies to ionically interact with DNA with relatively high transfection efficiency (83,84). Primary amine-modified AuNPs have been used as another core-forming agent for siRNA-PEG conjugates (Fig. 5a,e) (67). The polyplex micelles formed from the siRNA-PEG conjugate and the positively charged AuNPs have an average diameter of ca. 100 nm. The core of the polyplex micelle contains approximately ten AuNPs. In contrast, complexes prepared with unmodified siRNA formed micron-sized aggregates. Since the AuNPs stabilized by negative charges are sensitive to alterations in ionic strength or pH that induce charge neutralization and then aggregation (83), the neutral PEG shell surrounding the AuNPs is especially crucial for steric stabilization after complexation with siRNA. The AuNPs/siRNA-PEG micelles reduced GFP gene expression by over 80%, with no detectable cytotoxicity.

Cationic Lipids

There are many commercially available cationic lipid formulations used as transfection reagents for *in vitro* gene delivery, including Lipofectamine (Invitrogen), Oligofectamine (Invitrogen) and Lipofectin (Qiagen). However, only a limited number of cationic lipids have been successfully used as carriers for *in vivo* siRNA delivery due to their poor colloidal stability and toxicological concerns (85). Recently, low density lipoprotein (LDL)-mimicking nanoparticles termed solid lipid nanoparticles (SLN), the surface of which was modified with PEG (Fig. 5a,f), significantly improved the pharmacokinetic behaviors of siRNA and targeted *apoB* gene silencing in mice and nonhuman primates (86,87). Since PEGylated liposomes are clinically approved for doxorubicin, they represent a feasible option for siRNA delivery in humans (88). The adsorption of siRNA-PEG conjugates onto the SLN surface resulted in SLN/siRNA-PEG polyplex micelles with high stability, compared to that of bare SLN or SLN/siRNA complexes (55). The presence of a PEG layer on the SLN surface could provide stabilization by preventing nonspecific adsorption of plasma proteins and by reducing inter-particle aggregation. In the presence of serum, the sterically stabilized SLN/siRNA-PEG polyplex micelles reduced GFP and VEGF expressions in cancer cells by 59% and 54%, respectively.

IN VIVO APPLICATION OF PEG-siRNA POLYPLEX MICELLES

Cancer has been one of the most popular targets for nanoparticle-based drug delivery systems, since it is one of the most frequent and hard-to-cure diseases and it has unique physiological characteristics. Much research on the development of siRNA delivery systems has also been conducted for cancer treatments (89–93). Cancer development commonly involves the abnormal over-expression of defined genes, making a gene silencing approach feasible in cancer treatment strategies. In addition, since tumors generally have highly proliferative characteristics, newly formed vasculature inside the tumor usually has a leaky structure that allows penetration of small nanoparticles into the tumor tissue, where the nanoparticles may accumulate

due to the lack of lymphatic drainage in highly proliferative tumor tissue. Nanoparticles with a diameter of less than 500 nm can achieve the passive tumor targeting via the hypothetical enhanced permeability and retention (EPR) effect (90). Recently, Kim *et al.* used siRNA-PEG polyplex micelles for both local and systemic cancer treatments in an animal model (51). The self-assembling nano-sized polyplex micelles were combined with a therapeutic siRNA targeting VEGF, which is known to play a crucial role in tumor angiogenesis (91). The intravenous administration of the polyplex micelles resulted in significant tumor growth retardation. The treatment suppressed intratumoral VEGF expression by about 40% in human prostate cancer (PC-3) xenograft. In particular, *in vivo* fluorescence imaging showed enhanced tumor accumulation of the polyplex micelles after systemic administration. This may be attributed to the prolonged circulation of the polyplex micelles in blood, which likely provide them with increased opportunity to reach and accumulate in the tumor via EPR effect. Recently, it was reported that PEI/siRNA-PEG polyplex micelles achieved efficient penetration through a three-dimensional solid tumor model *in vitro*. This may be attributed to the flexible and hydrophilic PEG layer localized outside the micelles with a high density, which allows smooth penetration of the nanoparticles into the artificial extracellular matrix, eventually facilitating their cellular uptake (92). The polyplex micelles containing VEGF siRNA showed at least 1.5-fold greater gene-silencing throughout the cell layers of the 3-D solid tumor model compared to VEGF siRNA alone.

TARGETED DELIVERY OF PEG-siRNA POLYPLEX MICELLES

The use of a targeted drug delivery system is a desirable strategy to reduce adverse events in organs or tissues other than the disease site, primarily by decreasing the effective dose. Thus, a large number of targeting moieties, such as growth factors, antibodies, sugar molecules and peptides, have been widely investigated to develop tissue- or cell-specific delivery systems for drugs, genes, and diagnostic imaging agents (73,93). Although PEGylation is considered an attractive strategy for the stabilization and prolonged circulation of nanoparticles, PEGylation frequently reduces the cellular uptake of PEGylated nanoparticles by the steric hindrance effect, which is the same phenomenon observed in the decreased adsorption of blood components (94). Introduction of a targeting moiety on the surface of siRNA-PEG polyplex micelles improved cellular uptake via receptor-mediated endocytosis and increased target specificity. Lactosylated-PEG-siRNA (RecQL1 targeted) self-assembled with PLL exhibited eight times higher suppression of tumor growth in hepatic multi-cellular tumor spheroids, compared

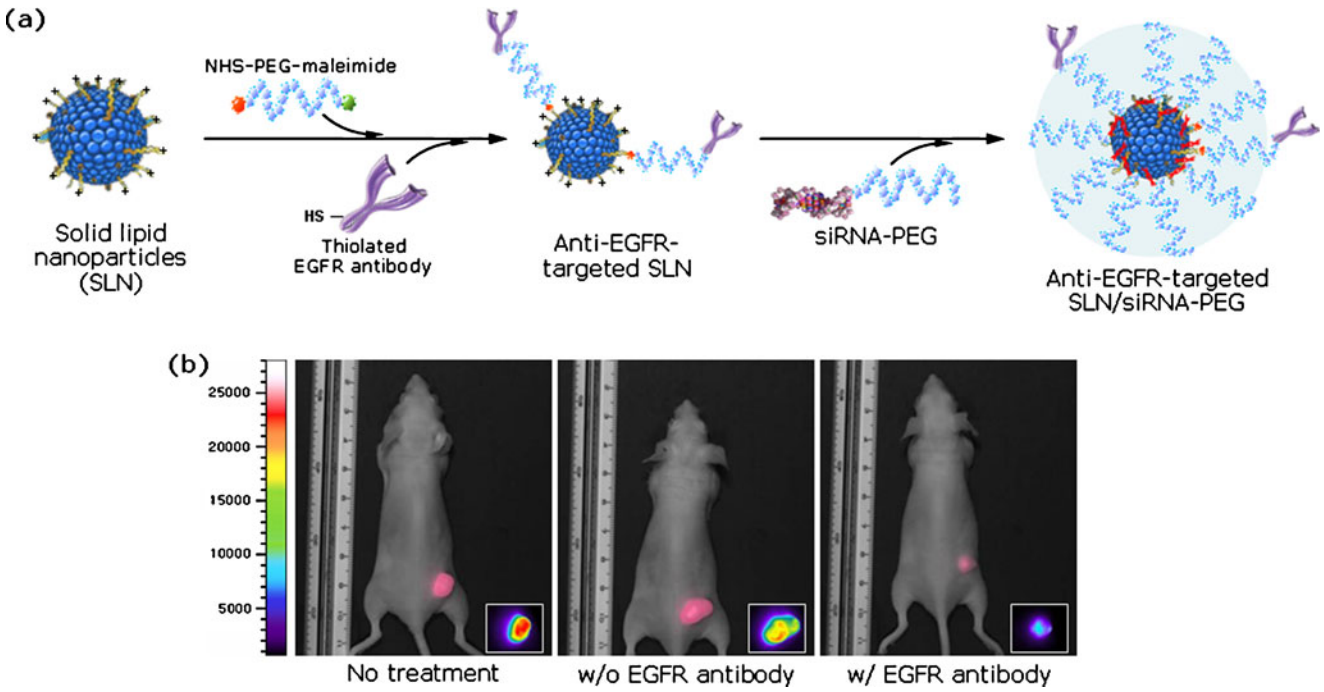

Fig. 6 (**a**) Schematic illustration of the preparation of anti-EGFR-targeted solid lipid nanoparticles (SLN) for cancer-targeted delivery of siRNA-PEG conjugates. (**b**) Representative bioluminescent images of luciferase-expressing human lung adenocarcinoma PC9 xenografts in nude mice. On day 14 after implantation, mice were injected intravenously with anti-EGFR-targeted SLN/luciferase siRNA-PEG. Inset panels show pseudo-color fluorescent images of tumor xenografts.

with siRNA formulated with Oligofectamine (47). When a luteinizing hormone-releasing hormone (LHRH) peptide was conjugated to the distal terminus of the PEG segment of siRNA-PEG (siRNA-PEG-LHRH), polyplex micelles generated from PEI and siRNA-PEG-LHRH exhibited enhanced delivery to cancer cells due to LHRH receptor-mediated endocytosis (49). As an alternative strategy for polyplex micelle-based siRNA delivery, PEGylated SLN with endothelial growth factor receptor (EGFR) antibody was used as a core-forming agent for siRNA-PEG (Fig. 6). The resulting polyplex micelles decorated with EGFR antibody exhibited remarkable gene silencing of a target gene after systemic administration in a mouse tumor xenograft model. The results suggest that targeted polyplex micelle-based siRNA delivery systems may be considered as candidates for cancer gene therapy.

CONCLUSIONS

There have been remarkable advances in RNAi techniques since the initial demonstration that siRNAs silence gene expression in mammalian cells (1). Now, siRNA has become a promising research tool for use in functional genomics and studying disease models *in vivo* without knockout animals, which are expensive and time-consuming. In addition, RNAi can be used for drug target validation in which siRNA may drastically accelerate the process of drug development. Thus, it was initially anticipated that siRNAs used for the target validation would be applicable in clinical settings with only minor modification. Although it only took three years to start the first clinical trials using siRNA-based therapeutics after the Elbashir *et al.* publication, these two major clinical trials of siRNAs for age-related macular degeneration have recently finished in failure. Despite the enthusiasm about a new class of therapeutics, many questions remain concerning the efficacy and duration of effect of siRNA drugs in the target disease. More recently, many studies on the development of siRNA delivery systems have provided theoretically important and commercially useful results. The current successes in siRNA delivery have certainly benefited from the scientific experiences acquired from existing gene delivery technologies for plasmid DNA and antisense ODNs. Although considerable progress has been made on developing and optimizing siRNA delivery, the major problems, such as off-target effects and immune stimulation, still remain unsolved. Moreover, pharmacokinetic and pharmacodynamic relationships of siRNA therapeutics have to be fully discussed and defined. In addition, there are typical characteristics of siRNA that are not observed with plasmid DNA or antisense ODN. For instance, short double-stranded siRNA provide a rigid structure that affects the integrity and stability of complexes formed by electrostatic interactions with cationic polymers (16). Therefore, for successful application of siRNA therapeutics in clinical settings, delivery systems should be carefully designed after considering the unique properties of siRNA in conjunction with essential requirements including high transfection efficiency, biocompatibility, and tissue- or cell-selective delivery.

ACKNOWLEDGMENTS

This study was supported by the Korea Healthcare Technology R&D Project from the Ministry for Health, Welfare & Family Affairs (A085136), Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2010–0022471), National Research Laboratory Basic Science Research Program (2010–0027955), and WCU Program from the Ministry of Education, and Science and Technology, Republic of Korea.

REFERENCES

1. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001;411:494–8.
2. Hannon GJ, Rossi JJ. Unlocking the potential of the human genome with RNA interference. Nature. 2004;431:371–8.
3. Durcan N, Murphy C, Cryan S. Inhalable siRNA: potential as a therapeutic agent in the lungs. Mol Pharm. 2008;5:566–99.
4. Kim SH, Jeong JH, Kim T, Kim SW, Bull DA. VEGF siRNA delivery system using arginine-grafted bioreducible poly(disulfide amine). Mol Pharm. 2009;6:718–26.
5. Dorsett Y, Tuschl T. siRNAs: applications in functional genomics and potential as therapeutics. Nat Rev Drug Discov. 2004;3:318–29.
6. Jeong JH, Kim SW, Park TG. Molecular design of functional polymers for gene therapy. Prog Polym Sci. 2007;32:1239–74.
7. Kim SH, Jeong JH, Ou M, Yockman JW, Kim SW, Bull DA. Cardiomyocyte-targeted siRNA delivery by prostaglandin E2-Fas siRNA polyplexes formulated with reducible poly(amido amine) for preventing cardiomyocyte apoptosis. Biomaterials. 2008;29:4439–46.
8. Smedt SCD, Demeester J, Hennink WE. Cationic polymer based gene delivery systems. Pharm Res. 2000;17:113–26.
9. Tomlinson E, Rolland AP. Controllable gene therapy-pharmaceutics of non-viral gene delivery systems. J Control Release. 1996;39:357–72.
10. Kwoh DT, Coffin CC, Lollo CP, Jovenal J, Banaszczyk MG, Mullen P, *et al.* Stabilization of poly-$_L$-lysine/DNA polyplexes for *in vivo* gene delivery to the liver. Biochim Biophys Acta. 1999;1444:171–90.
11. Gao X, Kim KS, Liu D. Nonviral gene delivery: what we know and what is next. AAPS J. 2007;9:E92–104.
12. Plank C, Mechtler K, Szoka Jr FC, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. Hum Gene Ther. 1996;7:1437–46.
13. Gary DJ, Puri N, Won YY. Polymer-based siRNA delivery: perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. J Control Release. 2007;121:64–73.
14. Shah SA, Brunger AT. A crystal structure of a statically disordered 17 base-pair RNA duplex: principles of RNA crystal

packing and its effect on nucleic acid structure. J Mol Biol. 1999;285:1577–88.
15. Kebbekus P, Draper DE, Hagerman P. Persistence length of RNA. Biochemistry. 1998;34:4354–7.
16. Mok H, Lee SH, Park JW, Park TG. Multimeric small interfering ribonucleic acid for highly efficient sequence-specific gene silencing. Nat Mater. 2010;9:272–8.
17. Lee SY, Huh MS, Lee S, Lee SJ, Chung H, Park JH, *et al.* Stability and cellular uptake of polymerized siRNA (poly-siRNA)/polyethylenimine (PEI) complexes for efficient gene silencing. J Control Release. 2010;141:339–46.
18. Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem. 1977;252:3582–6.
19. Abuchowski A, van Es T, Palczuk NC, Davis FF. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J Biol Chem. 1977;11:3578–81.
20. Veronese FM, Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today. 2005;10:1451–8.
21. Veronese FM, Harris JM. Introduction and overview of peptide and protein pegylation. Adv Drug Deliv Rev. 2002;54:453–6.
22. Gursahani H, Riggs-sauthier J, Pfeiffer J, Lechuga-Ballesteros D, Fishburn CS. Absorption of polyethylene glycol (PEG) polymers: the effect of PEG size on permeability. J Pharm Sci. 2009;98:2847–56.
23. Jeong JH, Kim SW, Park TG. A new antisense oligonucleotide delivery system based on self-assembled ODNPEG hybrid conjugate micelles. J Control Release. 2003;93:183–91.
24. Jeong JH, Kim SH, Kim SW, Park TG. Intracellular delivery of poly(ethylene glycol) conjugated antisense oligonucleotide using cationic lipids by formation of self-assembled polyelectrolyte complex micelles. J Nanosci Nanotechnol. 2006;6:2790–5.
25. Osada K, Christie RJ, Kataoka K. Polymeric micelles from poly (ethylene glycol)–poly(amino acid) block copolymer for drug and gene delivery. J R Soc Interface. 2009;6:S325–39.
26. Petersen H, Fechner PM, Martin AL, Kunath K, Stolnik S, Roberts CJ, *et al.* Polyethylenimine-*graft*-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. Bioconjug Chem. 2002;13:845–54.
27. Kunath K, Harpe A, Petersen H, Fischer D, Voigt K, Kissel T, *et al.* The structure of PEG-modified poly(ethylene imines) influences biodistribution and pharmacokinetics of their complexes with NF-κB decoy in mice. Pharm Res. 2002;19:810–7.
28. Kataoka K, Itaka K, Nishiyama N, Yamasaki Y, Oishi M, Nagasaki Y. Smart polymeric micelles as nanocarriers for oligonucleotides and siRNA delivery. Nucleic Aicds Symp Ser. 2005;49:17–8.
29. Kim SH, Mok H, Jeong JH, Kim SW, Park TG. Comparative evaluation of target-specific GFP gene silencing efficiencies for antisense ODN, synthetic siRNA, and siRNA plasmid complexed with PEI-PEG-FOL conjugate. Bioconjug Chem. 2006;17:241–4.
30. Naeyea B, Raemdoncka K, Remauta K, Sproatb B, Demeestera J, De Smedta SC. PEGylation of biodegradable dextran nanogels for siRNA delivery. Eur J Pharm Sci. 2010;40:342–51.
31. Tamura A, Oishi M, Nagasaki Y. Efficient siRNA delivery based on PEGylated and partially quaternized polyamine nanogels: enhanced gene silencing activity by the cooperative effect of tertiary and quaternary amino groups in the core. J Control Release. 2010;146:378–87.
32. Noh SM, Park MO, Shim G, Han SE, Lee HY, Huh JH, *et al.* Pegylated poly-L-arginine derivatives of chitosan for effective delivery of siRNA. J Control Release. 2010;145:159–64.
33. Kim WJ, Yockmana JW, Lee M, Jeong JH, Kim Y, Kim SW. Soluble Flt-1 gene delivery using PEI-g-PEG-RGD conjugate for anti-angiogenesis. J Control Release. 2005;106:224–34.
34. Hong JW, Park JH, Huh KM, Chung H, Kwon IC, Jeong SY. PEGylated polyethylenimine for *in vivo* local gene delivery based on lipiodolized emulsion system. J Control Release. 2004;99:167–76.
35. Jeong JH, Lee M, Kim WJ, Yockman JW, Park TG, Kim YH, *et al.* Anti-GAD antibody targeted non-viral gene delivery to islet beta cells. J Control Release. 2005;107:562–70.
36. Lee H, Jeong JH, Park TG. PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity. J Control Release. 2002;79:283–91.
37. Kaul G, Amiji M. Tumor-targeted gene delivery using poly (ethyleneglycol)-modified gelatin nanoparticles: *in vitro* and *in vivo* studies. Pharm Res. 2005;22:956–65.
38. Lee H, Kim TH, Park TG. A receptor-mediated gene delivery system using streptavidin and biotin-derivatized, pegylated epidermal growth factor. J Control Release. 2002;83:109–19.
39. Meyer O, Kirpotin D, Hong K, Sternberg B, Park JW, Woodlei MC. Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides. J Biol Chem. 1998;273:15621–7.
40. Harada A, Togawa H, Kataoka K. Physicochemical properties and nuclease resistance of antisenseoligodeoxynucleotides entrapped in the core of polyion complex micelles composed of poly (ethylene glycol)–poly(L-Lysine) block copolymers. Eur J Pharm Sci. 2001;13:35–42.
41. Shi F, Wasungu L, Aomden A, Stuart MCA, Polushkin E, Engberts JBFN, *et al.* Interference of poly(ethylene glycol)–lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions. Biochem J. 2002;366:333–41.
42. Oishi M, Nagatsugi F, Sasaki S, Nagasaki Y, Kataoka K. Smart polyion complex micelles for targeted intracellular delivery of PEGylated antisense oligonucleotides containing acid-labile linkages. Chembiochem. 2005;6:718–25.
43. Jeong JH, Kim SH, Kim SW, Park TG. *In vivo* tumor targeting of ODN-PEG-folic acid/PEI polyelectrolyte complex micelles. J Biomater Sci Polym Ed. 2005;16:1409–19.
44. Oishi M, Sasaki S, Nagasaki Y, Kataoka K. pH-Responsive oligodeoxynucleotide (ODN)-poly(ethylene glycol) conjugate through acid-labile β-thiopropionate linkage: preparation and polyion complex micelle formation. Biomacromolecules. 2003;4:1426–32.
45. Jeong JH, Kim SH, Kim SW, Park TG. Polyelectrolyte complex micelles composed of *c-raf* antisense oligodeoxynucleotide-poly (ethylene glycol) conjugate and poly(ethylenimine): effect of systemic administration on tumor growth. Bioconjug Chem. 2005;16:1034–7.
46. Kim SH, Jeong JH, Mok H, Lee SH, Kim SW, Park TG. Folate receptor targeted delivery of polyelectrolyte complex micelles prepared from ODN-PEG-folate conjugate and cationic lipids. Biotechnol Prog. 2007;23:232–7.
47. Oishi M, Nagasaki Y, Itaka K, Nishiyama N, Kataoka K. Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile β-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. J Am Chem Soc. 2005;127:1624–5.
48. Zhang M, Ishii A, Nishiyama N, Matsumoto S, Ishii T, Yamasaki Y, *et al.* PEGylated calcium phosphate nanocomposites as smart environment-sensitive carriers for siRNA delivery. Adv Mater. 2009;21:3520–5.
49. Kim SH, Jeong JH, Lee SH, Kim SW, Park TG. LHRH receptor-mediated delivery of siRNA using polyelectrolyte complex micelles self-assembled from siRNA-PEG-LHRH conjugate and PEI. Bioconjug Chem. 2008;19:2156–62.

50. Kim SH, Jeong JH, Lee SH, Kim SW, Park TG. PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. J Control Release. 2006;116:123–9.
51. Kim SH, Jeong JH, Lee SH, Kim SW, Park TG. PEG Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. J Control Release. 2008;129:107–16.
52. Lee SH, Kim SH, Park TG. Intracellular siRNA delivery system using polyelectrolyte complex micelles prepared from VEGF siRNA-PEG conjugate and cationic fusogenic peptide. Biochem Biophys Res Commun. 2007;357:511–6.
53. Mok H, Park TG. Self-crosslinked and reducible fusogenic peptides for intracellular delivery of siRNA. Biopolymers. 2008;89:881–8.
54. Lee SH, Bae KH, Kim SH, Lee KR, Park TG. Amine-functionalized gold nanoparticles as non-cytotoxic and efficient intracellular siRNA delivery carriers. Int J Pharm. 2008;364:94–101.
55. Kim HR, Kim IK, Bae KH, Lee SH, Lee Y, Park TG. Cationic solid lipid nanoparticles reconstituted from low density lipoprotein components for delivery of siRNA. Mol Pharm. 2008;5:622–31.
56. Jeong JH, Mok HJ, Oh Y, Park TG. siRNA conjugate delivery systems. Bioconjug Chem. 2009;20:5–14.
57. Jeong JH, Kim SW, Park TG. Novel intracellular delivery system of antisense oligonucleotide by self-assembled hybrid micelles composed of DNA/PEG conjugate and cationic fusogenic peptide. Bioconjug Chem. 2003;14:473–9.
58. Scanlon KJ. Anti-genes: siRNA, ribozymes and antisense. Curr Pharm Biotechnol. 2004;5:415–20.
59. de Martimprey H, Vauthier C, Malvy C, Couvreur P. Polymer nanocarriers for the delivery of small fragments of nucleic acids: oligonucleotides and siRNA. Eur J Pharm Biopharm. 2009;71:490–504.
60. Brown KM, Chu C, Rana TM. Target accessibility dictates the potency of human RISC. Nat Struct Mol Biol. 2005;12:469–70.
61. Jung S, Lee SH, Mok H, Chung HJ, Park TG. Gene silencing efficiency of siRNA-PEG conjugates: effect of PEGylation site and PEG molecular weight. J Control Release. 2010;144:306–13.
62. Oishi M, Nagasaki Y, Nishiyama N, Itaka K, Takagi M, Shimamoto A, *et al.* Enhanced growth inhibition of hepatic multicellular tumor spheroids by lactosylated poly(ethylene glycol)-siRNA conjugate formulated in PEGylated polyplexes. ChemMedChem. 2007;2:1290–7.
63. Braasch DA, Jensen S, Liu Y, Kaur K, Arar K, White MA, *et al.* RNA interference in mammalian cells by chemically-modified RNA. Biochemistry. 2003;42:7967–75.
64. Prakash TP, Allerson CR, Dande P, Vickers TA, Sioufi N, Jarres R, *et al.* Positional effect of chemical modifications on short interference RNA activity in mammalian cells. J Med Chem. 2005;48:4247–53.
65. Shah S, Friedman SH. Tolerance of RNA interference toward modifications of the 5′ antisense phosphate of small interfering RNA. Oligonucleotides. 2007;17:35–43.
66. Amarzguioui M, Holen T, Babaie E, Prydz H. Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res. 2003;31:589–95.
67. Moschos SA, Jones SW, Perry MM, Williams AE, Erjefalt JS, Turner JJ, *et al.* Lung delivery studies using siRNA conjugated to TAT(48–60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. Bioconjug Chem. 2007;18:1450–9.
68. Muratovska A, Eccles MR. Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. FEBS Lett. 2004;558:63–8.
69. Akerman ME, Chan WC, Laakkonen P, Bhatia SN, Ruoslahti E. Nanocrystal targeting *in vivo*. Proc Natl Acad Sci USA. 2002;99:12617–21.
70. Meong F, Hennink WE, Zhong Z. Reduction-sensitive polymers and bioconjugates for biomedical applications. Biomaterials. 2009;30:2180–98.
71. Werth S, Urban-Klein B, Dai L, Hobel S, Grzelinski M, Bakowsky U, *et al.* A low molecular weight fraction of polyethylenimine (PEI) displays increased transfection efficiency of DNA and siRNA in fresh or lyophilized complexes. J Control Release. 2006;112:257–70.
72. Urban-Klein B, Werth S, Abuharbeid S, Czubayko F, Aigner A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA *in vivo*. Gene Ther. 2005;12:461–6.
73. Mok H, Park TG. Functional polymers for targeted delivery of nucleic acid drugs. Macromol Biosci. 2009;9:731–43.
74. Wagner E. Application of membrane-active peptides for nonviral gene delivery. Adv Drug Deliv Rev. 1999;38:279–89.
75. Wyman TB, Nicol F, Zelphati O, Scaria PV, Plank C, Szoka FC. Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. Biochemistry. 1997;36:3008–17.
76. Choi SH, Lee SH, Park TG. Temperature-sensitive pluronic/poly (ethylenimine) nanocapsules for thermally triggered disruption of intracellular endosomal compartment. Biomacromolecules. 2006;7:1864–70.
77. Choi SH, Lee J, Choi S, Park TG. Thermally reversible pluronic/heparin nanocapsules exhibiting 1000-fold volume transition. Langmuir. 2006;22:1758–62.
78. Lee K, Bae KH, Lee Y, Lee SH, Ahn C, Park TG. Pluronic/polyethylenimine shell crosslinked nanocapsules with embedded magnetite nanocrystals for magnetically triggered delivery of siRNA. Macromol Biosci. 2010;10:239–45.
79. Storhoff JJ, Elghanian R, Mucic RC, Mirkin CA, Letsinger RL. One-pot colorimetric differentiation of polynucleotides with single base imperfections using gold nanoparticle probes. J Am Chem Soc. 1998;120:1959–64.
80. Otsuka H, Akiyama Y, Nagasaki Y, Kataoka K. Quantitative and reversible lectin-induced association of gold nanoparticles modified with α-lactosyl-ω-mercapto-poly(ethylene glycol). J Am Chem Soc. 2001;123:8226–30.
81. Gannon CJ, Patra CR, Bhattacharya R, Mukherjee P, Curley SA. Intracellular gold nanoparticles enhance non-invasive radiofrequency thermal destruction of human gastrointestinal cancer cells. J Nanobiotech. 2008;6:2.
82. Rosi NL, Giljohann DA, Thaxton CS, Lytton-Jean AK, Han MS, Mirkin CA. Oligonucleotide-modified gold nanoparticles for intracellular gene regulation. Science. 2006;312:1027–30.
83. Sandhu KK, McIntosh CM, Simard JM, Smith SW, Rotello VM. Gold nanoparticle-mediated transfection of mammalian cells. Bioconjug Chem. 2002;13:3–6.
84. Niidome T, Nakashima K, Takahashi H, Niidome Y. Preparation of primary amine-modified gold nanoparticles and their transfection ability into cultivated cells. Chem Commun. 2004;10:1978–9.
85. Behlke MA. Progress towards *in vivo* use of siRNAs. Mol Ther. 2006;13:644–70.
86. Zimmermann TS, Lee ACH, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, *et al.* RNAi-mediated gene silencing in non-human primates. Nature. 2006;441:111–4.
87. Pal A, Ahmad A, Khan S, Sakabe I, Zhang C, Kasid UN, *et al.* Systemic delivery of RafsiRNA using cationic cardiolipin liposomes silences Raf-1 expression and inhibits tumor growth in xenograft model of human prostate cancer. Int J Oncol. 2005;26:1087–91.
88. Coleman RE, Biganzoli L, Canney P, Dirix L, Mauriac L, Chollet P, *et al.* A randomized phase II study of two different schedules of pegylated liposomal doxorubicin in metastatic breast cancer (EORTC-10993). Eur J Cancer. 2006;42:882–7.

89. Zhu C, Jung S, Luo S, Meng F, Zhu X, Park TG, *et al.* Co-delivery of siRNA and paclitaxel into cancer cells by biodegradable cationic micelles based on PDMAEMA-PCL-PDMAEMA triblock copolymers. Biomaterials. 2010;31:2408–16.
90. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release. 2000;65:271–84.
91. Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell. 1994;79:185–8.
92. Al-Abd AM, Lee SH, Kim SH, Cha J, Park TG, Lee SJ. Penetration and efficacy of VEGF siRNA using polyelectrolyte complex micelles in a human solid tumor model *in-vitro*. J Control Release. 2009;137:130–5.
93. Bareford LM, Swaan PW. Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev. 2007;59:748–58.
94. Merdan T, Callahan J, Petersen H, Kunath K, Bakowsky U, Kopeckova P, *et al.* Pegylated polyethylenimine-Fab' antibody fragment conjugate for targeted gene delivery to human ovarian carcinoma cells. Bioconjug Chem. 2003;14:989–96.