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Peptide-Based Nanomaterials for siRNA Delivery: Design, Evaluation, and Challenges

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9.1 Introduction

The use of small interfering RNA (siRNA) holds great promises for the development of gene-specific therapeutics. Similar to plasmid DNA transfection, cellular uptake of naked siRNA is difficult due to the lack of transport mechanisms to move negatively charged, large nucleic acids across the cell membrane. Double-stranded siRNA is more stable than single-stranded RNA, but unmodified siRNA is still prone to enzymatic degradation by nucleases in serum and can be destroyed within minutes (Layzer et al. 2004; Morrissey et al. 2005a). Also, unmodified siRNA might trigger Toll-like receptor 7 pathway and induce nonspecific activation of the immune system (Hornung et al. 2005; Judge et al. 2005). Therefore, efficient siRNA delivery systems have to be developed to protect siRNA from rapid degradation in serum, enhance cellular internalization, and reduce immunostimulatory activity. Different strategies have been employed to enhance siRNA delivery efficiency, including physical methods, chemical modification of siRNA, and non-covalent encapsulation of siRNA with lipids, polymers, or peptides.

Peptides are very attractive nanomaterials with significant potential for biomedical applications. The development of peptide nanomaterials is based on the biochemical understanding that the active sites of protein molecules, such as enzymes, receptor ligands, and antibodies, usually involve only 5–20 amino acid residues (Sparrow et al. 1998). With the rapid advances in structural biology and high-throughput genomics and proteomics, the identification of peptide motifs associated with biological functions has
been drastically accelerated (Saito et al. 2007). Thus, peptide materials offer a highly attractive feature of incorporating various natural or synthetic sequences with biological activities, for example, cell targeting domain and nuclear targeting domain. Peptides are relatively easy to be synthesized in large scale and can be characterized with well-established chemistry and instrumental operation. As biomaterials, peptides are generally less toxic and have low immunogenicity compared to high molecular weight (MW) polymers (Fabre and Collins 2006) and undergo degradation in the body to naturally occurring compounds. Different nanometric structures can arise from peptide-cargo or interpeptide interactions of electrostatic, hydrophobic, or aromatic nature. A good example is nanometric complex formed by peptides and nucleic acids. In this chapter, published studies using peptides to encapsulate siRNA non-covalently into nanoparticles will be reviewed and discussed in detail. A short summary of commonly used methods to deliver siRNA will also be presented.

9.2 RNA Interference

RNA interference (RNAi) is both an intrinsic and nearly universal mechanism of gene expression regulation and a means to control specific gene expression extrinsically. RNAi hinges on simple yet specific Watson–Crick base-pairing between small RNA and messenger RNA (mRNA), resulting in reduced gene expression at the posttranscriptional level. With its generally inhibitory effect on gene function, RNAi has greatly impacted the area of functional genomics and very rapidly entered the arena of therapeutic development in disease settings.

Double-stranded RNA (dsRNA) of various origins and lengths are the initiators of RNAi. They are processed into short dsRNAs, 21–28 nucleotides (nt), depending on species (Hutvagner and Zamore 2002), which then affect the sequence-specific degradation of complementary single-stranded RNAs. dsRNA can be of viral origin (converted from single-stranded form by RNA-dependent RNA polymerases), overlapping transcripts from repetitive sequences such as transposons (Waterhouse et al. 2001) or artificially introduced long dsRNAs. MicroRNAs (miRNAs) that form dsRNA hairpins via intramolecular complementarity are endogenous initiators of RNAi (Bartel 2004).

The use of short dsRNAs or siRNAs in mammalian cells as a direct mediator of RNAi is the method of choice for specific gene silencing in mammalian cells (Caplen et al. 2001; Elbashir et al. 2001). This overcomes the major hurdle in the use of long dsRNA-mediated RNAi that appears to cause nonspecific degradation of mRNAs and/or general toxicity in vertebrates, including zebrafish and mammalian cells (Tuschl et al. 1999; Caplen et al. 2000; Oates et al. 2000; Zhao et al. 2001). The reason for this toxicity is understood to be a dsRNA-induced interferon response (Manche et al. 1992; Kumar and Carmichael 1998; Stark et al. 1998). The discovery of endogenous miRNAs also led to the development of tools for the intracellular expression of RNAi triggers that mimic miRNAs in the form of short-hairpin RNAs (shRNA) (Paddison et al. 2004).

The canonical RNAi pathway begins with the excision of short dsRNA fragments from long dsRNA in the cytoplasm by the multidomain RNaseIII endonuclease Dicer. The dsRNA products of the Dicer activity are siRNA duplexes about 19–25 nt in length and have 5′ phosphates and 2-nucleotide 3′ overhangs (Bernstein et al. 2001; Elbashir et al. 2001; Macrae et al. 2006). Endogenous miRNAs are transcribed in the nucleus as primary structures that are cleaved by a nuclear RNaseIII enzyme Drosha to ∼70 nt precursor miRNAs (pre-miRNA). The pre-miRNAs are exported to the cytoplasm and are processed by Dicer to produce an miRNA duplex similar to the siRNA duplex (Zeng and Cullen 2004). In practice, synthetic siRNAs are designed to resemble Dicer cleavage products of 21–22 nt length duplexes with 2-nucleotide 3′ overhangs. The miRNA or siRNA duplex generated by Dicer is loaded into the RNA-induced silencing complex (RISC) by RISC-loading complex (RLC), a trimeric complex including Dicer (Maniataki and Mourelatos 2005). Synthetic siRNAs are most likely directly loaded to RISC, presumably independent of Dicer (part of RLC), at least in vitro in mammalian cells (Macrae et al. 2006; Carthew and Sontheimer 2009). The single-stranded siRNA guide strand, once loaded into RISC, guides RISC to mRNA targets that are perfectly complementary, orchestrating a sequence-specific degradation of targets. Although
siRNAs typically function to cleave target mRNA through perfect complementarity, mismatches in the siRNA/target duplex often result in cleavage or translational repression of unintended targets (Carthew and Sontheimer 2009). This aspect of siRNA function is nearly identical to that of miRNAs and is dictated largely by sequence complementarity between 7-nucleotide “seed” region at the positions 2–8 of antisense strand of siRNA and the target. This is summed up as the “off-target” effect of siRNAs (Jackson et al. 2003; Saxena et al. 2003). Chemical modification of siRNAs to limit such effects has been explored, and 2′-O-Me modifications and DNA substitutions have been demonstrated to be effective (Jackson et al. 2006; Ui-Tei et al. 2008). A summary of the RNAi pathway is illustrated in Figure 9.1.

The mechanistic understanding of RNAi has promoted rational siRNA design for experimental use. Thus, the following aspects should be taken into account: (1) structural requirements of siRNA duplex, having length of 19–21 nt, absence of 5′ overhangs, GC content of ~50%, and appropriate thermodynamics of duplex for guide strand selection; (2) target mRNA accessibility; and (3) “seed” region choice to minimize off-target effects (Reynolds et al. 2004). Interestingly, it has been shown that RISC programming is more efficient when a longer siRNA (27mers) is used, as they are incorporated into the Dicer-processing step and linked to RISC activation (Gregory et al. 2005; Kim et al. 2005; Siolas et al. 2005).

### 9.3 siRNA Delivery

Long-term stable gene silencing can be established with the use of viral delivery vectors for shRNA. Like miRNAs, shRNAs form hairpin structures and are Dicer substrates. The typical design uses small inverted repeats (19–29 nt) expressed from an RNA Pol III promoter that transcribes self-complementary shRNAs (Paddison et al. 2004). These are exported out to the cytoplasm and processed by Dicer.

One of the limitations associated with the use of shRNA is its inevitable interference with the endogenous miRNA pathway, given its nuclear phase. A study using a deno-associated virus, AAV/shRNA vectors for silencing luciferase transgene reported liver toxicity in ~50% of animals that were administered these shRNA vectors (Grimm et al. 2006). The saturation of the miRNA pathway, particularly nuclear export, resulted in downregulation of endogenous miRNAs, manifesting in outward toxicity, although optimizing shRNA dose and sequence may avoid the oversaturation. No similar toxicity and disruption of miRNA pathway was reported when synthetic siRNA duplexes were systemically administered to animals (John et al. 2007). In this sense, direct use of siRNA displayed a better safety profile even though silencing effect of siRNA might be transient.

#### 9.3.1 Physical Methods

Physical methods such as electroporation and hydrodynamic injection are the most direct methods to introduce foreign substance into cells. Electroporation utilizes externally applied electrical field to increase the permeability of cell membrane, create pores, and allow extracellular material to diffuse into cytoplasm. Electroporation has been demonstrated as a useful tool to facilitate in vitro siRNA delivery into primary cells and difficult-to-transfect cells such as human primary fibroblasts, human umbilical vein endothelial cells (HUVEC), and neuroblastoma cell lines (Jordan et al. 2008). The potential of electroporation for in vivo siRNA delivery has been reported in the rat brain (Akaneya et al. 2005), rats and mice muscle tissue (Kishida et al. 2004; Kong et al. 2004; Golzio et al. 2005; Takayama et al. 2009), and tumor xenograft (Takahashi et al. 2005; Takei et al. 2008). However, the use of electroporation is usually limited due to high rate of cell mortality caused by high-voltage pulses and the availability of other more effective delivery systems.

Another physical method is hydrodynamic injection, which involves the delivery of samples into tissue by intravascular injection of a relatively large volume of samples with high hydrostatic pressure (Liu et al. 1999). In fact, the first demonstration of RNAi in mammals such as mice was using siRNA delivered by hydrodynamic injection (Lewis et al. 2002; McCaffrey et al. 2002). While hydrodynamic injection is usually performed to deliver siRNA into the liver (Lewis et al. 2002; McCaffrey et al. 2002; Giladi et al. 2003; Klein et al. 2003; Song et al. 2003; Xu et al. 2005; Morrissey et al. 2005b), the uptake
FIGURE 9.1 The mechanism of RNAi. When exogenously introduced, the triggers of RNAi can be DNA-encoded shRNA, long dsRNA (Dicer-substrates), or chemically synthesized siRNA. In the nucleus, shRNAs are transcribed as hairpin-structures from a transgene and exported to the cytoplasm. The endogenous RNAi pathway begins with the transcription of primary miRNA structure, which undergoes nuclear processing by the enzyme Drosha into pre-miRNA. The pre-miRNA is exported to the cytoplasm. In the cytoplasm, the enzyme Dicer mediates processing of longer forms of dsRNAs (long dsRNA, shRNA, pre-miRNA) into 21–23 nt duplex siRNA or miRNA, with typical features such as 3′-overhangs and 5′-phosphate. The duplexes are loaded into RISC via an intermediate RLC. The passenger strand (—) is unwound and cleaved. The single guide strand (—) then directs target gene silencing based on sequence complementarity. Typically, the outcome of siRNA activity is target degradation (full complementarity). The outcome of miRNA activity could be translational repression (partial complementarity).
of siRNA by other organs such as the kidney (Hamar et al. 2004), pancreas (Bradley et al. 2005), and lung (Tompkins et al. 2004) were also possible. Hydrodynamic injection of siRNA is a relatively efficient method to deliver siRNA, but this method has limited application in humans due to the possible complications caused by rapid injection of large volume of fluid into the blood vessel.

9.3.2 Chemical Modification of siRNA

Ideally, chemical modifications of siRNA increase stability in serum, reduce immunostimulatory activity, increase silencing ability, and enhance cellular uptake. There are three commonly used chemical modifications for siRNA, including phosphodiester modification, such as phosphorothioate or boranophosphonate, at siRNA 3′-end (Braasch et al. 2004; Hall et al. 2004), modification of 2′-base sugar, such as 2′-O-methyl or 2′-deoxy-2′-fluoro, of selected nucleotides (Chiu and Rana 2003; Layzer et al. 2004), and the use of locked nucleic acid (LNA) in siRNA strand (Braasch et al. 2003; Elmen et al. 2005). Although these methods have been demonstrated to protect siRNA against nuclease degradation and reduce off-target effects, cytotoxicity and reduced gene-silencing activity associated with modifications were observed in some reports (de Fougerolles et al. 2007; de Paula et al. 2007; Rana 2007).

Besides chemically modifying nucleotide structure, covalent conjugation of biologically functional molecules is an attractive method to enhance siRNA delivery. The conjugation is normally done at the sense strand as it is less likely to affect the silencing effect of the siRNA. Cholesterol conjugation has previously been used to enhance delivery of antisense oligonucleotide and nucleic acid into liver cells (Biessen et al. 1999; Cheng et al. 2006). Injection of cholesterol-conjugated siRNA into mouse was able to silence in vivo mRNA expression of apolipoprotein B required for transport of cholesterol (Soutschek et al. 2004), and a mutated gene related to Huntington’s disease (DiFiglia et al. 2007). Another example of conjugation is to exploit peptides derived from cell-penetrating peptide (CPP) or protein transduction domain (PTD). CPPs are known to be capable of delivering different cargoes into cells efficiently (Stewart et al. 2008). Different CPPs, such as penetratin, transportan, or Tat, have been conjugated to siRNAs through reducible disulfide bond linkages (Chiu et al. 2004; Davidson et al. 2004; Muratovska and Eccles 2004; Moschos et al. 2007). In earlier reports, enhanced cellular uptake of the conjugated siRNA and successful inhibition of reporter gene were observed. However, because no purification after conjugation reaction between CPP and siRNA was done (Chiu et al. 2004; Davidson et al. 2004; Muratovska and Eccles 2004), the observed RNAi activity could be due to the complex formed by non-covalent encapsulation of siRNA with excess cationic peptide in the reaction mixture (Meade and Dowdy 2007). This assumption was confirmed by later findings showing that purified CPP-conjugated siRNA did not increase cellular uptake and distribution in vitro (Moschos et al. 2007; Meade and Dowdy 2008).

9.3.3 Non-Covalent Encapsulation of siRNA

Difficulties in large-scale purification and characterization of siRNA covalently conjugated with functional molecules might raise concerns over the quality of siRNA-based therapeutics. Hence, non-covalent encapsulation of siRNA with cationic molecules could be a better alternative for siRNA delivery. One of the most commonly used encapsulation reagents is cationic lipid. Cationic lipid is an amphiphilic molecule composed of cationic hydrophilic amine groups and hydrophobic side chains. Cationic lipids are assembled into bilayer-structured liposomes and can interact with nucleic acids to form a complex termed lipoplex. A great variety of lipid-based products are commercially available for siRNA delivery, for example, Lipofectamine™ 2000 (Invitrogen), DharmaFECT™ set (Dharmacon), and siPORT™ NeoFX™ (Ambion). Despite their popularity and excellent in vivo results (Sioud and Sorensen 2003; Sorensen et al. 2003; Flynn et al. 2004; Ma et al. 2005), toxicity of some cationic lipids remains a safety issue for in vivo application (Ma et al. 2005; Lv et al. 2006; Akhtar and Benter 2007).

Many polymers originally developed for plasmid DNA delivery are also possible encapsulation reagents of siRNA, for instance, polyethylenimine (Urban-Klein et al. 2005; Werth et al. 2006;
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Zintchenko et al. (2008), chitosan (Howard et al. 2006; Katas and Alpar 2006), and cyclodextrin polymers (Hu-Lieskovan et al. 2005; Bartlett et al. 2007; Heidel et al. 2007; Bartlett and Davis 2008). Polyethylene-glycol (PEG) modified lipid (Zimmermann et al. 2006), stearyl octa-arginine (Tonges et al. 2006), and cholesteryl nona-arginine (Kim et al. 2006) have also been demonstrated to encapsulate and deliver siRNA efficiently in vivo.

9.4 Peptide-Based siRNA Delivery Vectors

Over the past few decades, different peptides have been designed and used for plasmid DNA delivery (Mann et al. 2008). Although both plasmid DNA and siRNA are double-stranded nucleic acids in nature, there are some differences that are worthy of being taken into account in designing peptide-based vectors. First of all, siRNA is more susceptible to hydrolysis by serum nucleases than DNA because of the hydroxyl group in the 2’ position of the pentose ring in RNA backbone. Secondly, in terms of size, plasmid DNA is often several kilo base pairs whereas siRNA is 19–21 base pairs. Thirdly, plasmid DNA has to be delivered into the nucleus where the functional gene can be expressed, whereas siRNAs usually induce degradation of target mRNAs in the cytosol, except for rare cases in which transcriptional gene silencing is involved in the nucleus (Kawasaki and Taira 2004; Morris et al. 2004). Therefore, peptides that are efficient in plasmid DNA delivery might not work similarly for siRNA. Keeping this in mind, some of the strategies developed for plasmid DNA delivery provide good starting points for new peptide vector design for siRNA delivery. Depending upon their design, peptide-based vectors involved in siRNA delivery can be categorized as (1) amphipathic peptides, (2) CPPs, (3) histidine-rich branched peptides, and (4) peptide-based reducible polymers (PRPs) (Table 9.1). In the following section, we highlight and discuss key features of each design.

9.4.1 Amphipathic Peptides

An amphipathic peptide is a peptide that possesses both hydrophobic (nonpolar) and hydrophilic (polar) properties. The amphipathicity characteristic could originate from either a primary structure that contains both hydrophobic domain and hydrophilic domain or a secondary structure that allows hydrophobic and hydrophilic amino acid residues to be positioned on the opposite side of peptide conformation (Fernandez-Carneado et al. 2004).

9.4.1.1 Primary Amphipathic Peptide

The most well-characterized primary amphipathic peptides are MPG-based peptides. These are a series of peptides composed of a hydrophobic domain derived from glycine-rich region of the membrane fusion sequence of HIV gp41 (Gallaher 1987; Rafalski et al. 1990) and a hydrophilic domain derived from the nuclear localization signal (NLS) of SV40 large T-antigen (Kalderon et al. 1984; Dingwall and Laskey 1992). The first generation of the MPG-based peptides, MPG-W, was designed to study intracellular localization of the peptides with different chemical modification (Vidal et al. 1996). Based on the MPG-W sequence, the MPG-mNLS peptide that contains a mutated NLS sequence was developed to deliver oligonucleotides (Morris et al. 1997). To derive the MPG-mNLS peptide, the following changes were made: (1) phenylalanine (F) residue at position 7 in the hydrophobic domain was restored; (2) the short linker that connects the hydrophobic and hydrophilic domains was changed to tryptophan-serine-glutamine (WSQ), allowing sensitive monitoring and quantification of interaction of peptide with oligonucleotides by measuring fluorescence quenching; (3) in hydrophilic domain, the proline (P) residue from the NLS of SV40 large T-antigen was included and the second lysine (K) residue was mutated to serine (S) residue; and (4) N- and C-termini were modified with acetyl group and cysteamide group, respectively, to improve the ability to cross the membrane (Mery et al. 1993). The MPG-mNLS peptide protects oligonucleotides from DNase degradation, suggesting that the peptide interacts with oligonucleotides strongly. At both 37°C and 4°C, peptide/nucleotide complexes prepared at molar ratio of 20,
TABLE 9.1  List of Peptides Tested for siRNA Delivery

Amphipathic Peptides

Primary amphipathic peptides
- MPG-W: X-GALFLGWLGAAGSTMGA-RKKRKV-Cya-X′
- MPG-mNLS: Ac-GALFLGFLGAAGSTMGA-WSQ-PKKRKV-Cya
- MPG-NLS: Ac-GALFLGFAAALSMLGL-WSQ-PKKRKV-Cya
- MPG-mNLS: Ac-GALFLAALSLMLG-WSQ-PKKRKV-Cya

Secondary amphipathic peptides
- KALA
- CADY: Ac-GLWRAIWLRLSRWRLWRA-Cya

Classical Cell-Penetrating Peptides

Penetratin-based peptide
- EB1: LIRLWSHLHIWFQNRRLKWKKK-amide

Oligoarginine-based peptide
- R

RVG
- RV-Mat: YTIWMPENPRGTCDFITNSRAGSKS
- RVG-9r: YTIWMPENPRGTCDFITNSRAGSKS
- RV-Mat-9r: YTIWMPENPRGTCDFITNSRAGSKS

Novel peptide
- POD: GGG(ARKKAAKA)_n

Histidine-Rich Branched Peptides
- H^+K^b: H^+K^+(+H)4b

Peptide-Based Reducible Polymers
- HIS6-RPC: (C-HK_H-C)_n
- cl-KALA: (C-WEAKLAALAKHLALAKALKACEA-C)_n

X—H, Ac, or methoxy coumarin; X′—H or lucifer yellow; Ac, Acetyl (–COCH_3), Cya, Cysteamide (–NH–CH_2–CH_2–SH); r, d-arginine; n, number of peptide monomers in the polymer.

corresponding to an amine/phosphate (N/P) ratio of 5, were rapidly localized in the nucleus of fibroblast cells (HS-68 and NIH-3T3) within 1 h, indicating that endosomal pathway was not involved in the internalization of peptide-mediated delivery. Even though both MPG-W and MPG-mNLS peptides adopted similar β-sheet structure in phospholipid solution (Chaloin et al. 1998; Vidal et al. 1998), alteration of the short linker to WSQ changed the localization pattern from membrane-associated to nucleus-associated localization. The authors reasoned that an additional arginine (R) residue in the MPG-W peptide possibly blocked the nuclear translocation property of NLS as suggested previously (Whitley et al. 1995).

In view of the success of oligonucleotide delivery, the potential use of the MPG-mNLS peptide was extended to plasmid DNA delivery (Morris et al. 1999). The MPG-mNLS peptide could bind to plasmid DNA through electrostatic interaction. Formation of peptide cage around DNA, rather than just charge...
neutralization, was hypothesized since large excess of the peptides was required for complex formation. The MPG-mNLS peptide was capable of efficiently delivering a plasmid vector expressing luciferase gene into various types of cell lines at an N/P ratio of 10, without exhibiting any cytotoxic effects. After delivering a plasmid DNA expressing antisense full-length cDNA human cdc25c (cell division cycle 25 homolog C) into late G1 phase human fibroblast (HS-68), efficient inhibition (70%) of entry into mitosis was observed, suggesting the loss of cdc25C protein that plays a key role in the regulation of cell division.

Following the discovery of RNAi, the possibility of using MPG-mNLS for siRNA delivery was explored (Simeoni et al. 2003). The authors first compared the difference between an MPG peptide with wild-type NLS (MPG-NLS) and MPG-mNLS in nuclear targeting ability. Plasmid DNA delivery efficiency of the MPG-mNLS peptide in HS-68, as measured by luciferase activity, was only about one-third of that offered by the MPG-NLS peptide. When fluorescently labeled siRNA was delivered by the two peptides, the delivery efficiencies were similar. However, siRNA localized to the nucleus when delivered by MPG-NLS but remained mostly in the cytoplasm when delivered by MPG-mNLS, although it has been previously demonstrated that oligonucleotide delivered by the MPG-mNLS peptide localized in the nucleus (Mery et al. 1993). The authors claimed that the cytoplasmic localization of siRNA was due to the reduced nuclear-targeting ability of the MPG-mNLS peptide. The discrepancy in intracellular distribution pattern could arise from the re-localization of free siRNAs released from the complex. The MPG-mNLS/siRNA complex might first localize in the nucleus, followed by early disassembly of the complex due to a weaker binding of the mutated NLS to siRNA. The free siRNA will then be actively excluded from the nucleus by Exportin-5 (Ohrt et al. 2006), resulting in cytoplasmic localization. Moreover, since the MPG-mNLS peptide was not fluorescently labeled, there is no evidence to demonstrate that the MPG-mNLS peptide was co-localized with siRNA cargo in the cytoplasm. Nonetheless, despite the uncertain effect of NLS mutation, MPG-mNLS-mediated siRNA delivery was effective in silencing target gene expression. In HeLa and COS7 cells pre-transfected with a luciferase plasmid, MPG-mNLS peptide/siRNA against luciferase at an N/P ratio of 10 reduced the luciferase activities by 90% and 95%, respectively. This silencing effect was comparable with that of commercial lipid-based delivery vector Oligofectamine™. Northern blot analysis showed that siRNA against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) delivered by the MPG-mNLS peptide was able to reduce 80% of protein expression in HS-68.

Based on results from conformational analysis (Deshayes et al. 2004a,b; 2006a,b), a cellular entry mechanism mediated by the MPG-mNLS and MPG-NLS peptides was proposed. The model consists of five steps: (1) formation of peptide/cargo complex, (2) electrostatic interaction between cellular membrane component and hydrophilic cationic domain of the peptides, (3) transient formation of β-sheet-related transmembrane channels leading to insertion of the peptide/cargo complex, (4) internalization of peptide/cargo complex, and (5) translocation to the nucleus.

A derivative of original MPG-NLS peptide, MPGα-NLS, was also used in a study that established techniques to analyze peptide-mediated siRNA internalization along with its biological effects (Veldhoen et al. 2006). The MPGα-NLS peptide, with a partial α-helical structure resulting from five mutations in hydrophobic domain, was originally designed to study the entry mechanism of the MPG-NLS peptide (Deshayes et al. 2004a). To test whether the MPGα-NLS peptide could mediate siRNA delivery, Veldhoen et al. first established two cell lines stably expressing firefly luciferase, HeLa-TetOff Luc (HTOL) and a derivative of human urinary bladder carcinoma cells (ECV304 GL3). These two cell lines were transfected with 50 nM of siRNA against luciferase complexed with either Lipofectamine 2000 (LF2000, 10 μg/mL) or MPGα-NLS (at an N/P ratio of 15). The MPGα-NLS/siRNA complexes reduced luciferase activity by 80%–90%, a silencing level similar to that offered by the LF2000/siRNA complexes. However, the apparent value of half maximal inhibition (IC50) of the MPGα-NLS/siRNA complexes was ~0.8 nM, which was about 20–40 times higher than that of the LF2000/siRNA complexes. The authors suggested that MPGα-NLS might be less efficient due to a lower rate of siRNA internalization. To quantify the amount of intracellular siRNAs, a sensitive liquid hybridization method developed previously (Overhoff et al. 2004) was adapted. Briefly, 4 h after transfection with peptide/siRNA complexes, the cells

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were treated with heparin (15 U/mL) three times to remove extracellularly bound complexes. The cells were incubated for another 24 h, followed by cell detachment with trypsin. Cellular RNA was extracted, hybridized with $^{32}$P-labeled sense-strand at 95°C for 10 min, followed by 1 h incubation at 37°C. The samples were resolved by polyacrylamide gel electrophoresis (PAGE) and blotted onto membrane for quantification. The authors discovered that to obtain 50% inhibition of maximum luciferase activity, 10,000 siRNA molecules were required in MPGα-NLS-mediated delivery. This value was 30-fold higher than the case of LF2000-mediated delivery. Based on microscopic observation of cellular distribution of fluorescently labeled siRNA and effects of various inhibitors/effectors of endocytosis, the authors concluded that accumulation in endosomes is the bottleneck of siRNA delivery mediated by the MPGα-NLS peptide. As opposed to what was reported in the case of MPG-mNLS (Simeoni et al. 2003), similar cellular localization and luciferase activity were observed when cells were transfected with siRNA complexed with either MPGα-NLS or MPGα-mNLS. This suggests that the mutation of lysine (K) residue in NLS might have a minor effect on nuclear-targeting ability of the peptide.

9.4.1.2 Secondary Amphipathic Peptide

As an essential protein for influenza viral entry, hemagglutinin (HA) mediates a low pH-dependant membrane fusion through the exposure of its amphipathic α-helix (Skehel et al. 1982). Based on the idea of mimicking the α-helical structure associated with the membrane fusion feature of viral proteins, several secondary amphipathic peptides have been designed to function as a membrane destabilization agent or a DNA delivery vector (Fernandez-Carneado et al. 2004). Among them, KALA peptide is known to undergo conformational change from pH 5.0–7.5 (Wyman et al. 1997) and has been chosen to condense and deliver siRNA conjugated with PEG via a disulfide linkage (Lee et al. 2007). The studies suggested that the self-assembled polyelectrolyte complex micelle has an inner core with KALA peptide-condensed siRNA, surrounded by protective PEG corona. The average size of KALA/siRNA-PEG complexes was around 200 nm at an N/P ratio of 6, a size favorable for cellular uptake. Vascular endothelial growth factor (VEGF) expression in KALA/siRNA-PEG complex-transfected prostate carcinoma (PC-3) cells was reduced to 20% of that in the untransfected control. Although cytotoxicity of the KALA peptides was not observed in this study, it was previously reported that the KALA peptides exhibited hemolytic activity at a physiological pH due to nonspecific membrane destabilization (Wyman et al. 1997). Further cell viability tests with a prolonged incubation time and an increased amount of the KALA peptides should be performed to evaluate the cytotoxicity.

CADY peptide is another secondary amphipathic peptide tested for siRNA delivery. The peptide was designed based on two amphipathic peptides called JTS1 (Gottschalk et al. 1996) and ppTG1 (Rittner et al. 2002). To improve interaction with siRNA and cell membrane, arginine (R) and tryptophan (W) were included in the CADY peptide (Crombez et al. 2009). Similar to MPG-based peptides, N- and C-termini of the CADY peptide were modified by acetyl group and cysteamide group, respectively. The CADY peptide formed complex with siRNAs and protected them from serum nuclease degradation with increasing molar ratio of peptide to siRNA, most significantly at a molar ratio of 80. Flow cytometry analysis showed that cellular uptake of fluorescently labeled siRNA was improved by increasing the molar ratio. To investigate CADY peptide-mediated siRNA delivery, functional siRNAs targeting GAPDH or p53 were used to monitor silencing effects. After transfection of CADY/siRNA-GAPDH complexes at a molar ratio of 40, the silencing effect was >80% after 24 h in human osteosarcoma cells (U2OS) and primary HUVEC. When the CADY peptide was used to deliver siRNA against p53 into U2OS cells at a molar ratio of 40, the inhibitory effect was maintained for at least 5 days, with 97% and 60% knockdown at day 2 and 5, respectively. The pretreatment of cells with different inhibitors of the endocytosis pathway had no significant effect in the cellular uptake and silencing effect mediated by the CADY/siRNA complexes. Unlike the JTS1 peptide, the CADY peptide adopts α-helical structure independent of pH. Hence, it was hypothesized that the cellular uptake of the CADY peptide was due to the direct interaction of aromatic tryptophan (W) residues with cell membrane components.
9.4.2 Classical Cell-Penetrating Peptides

Classical CPPs are usually rich in arginine (R) and lysine (K) that are highly positively charged. Some CPPs, like penetratin (Derossi et al. 1994) and Tat (Vives et al. 1997), are directly derived from natural proteins, while others have a designed sequence, such as oligoarginine (Mitchell et al. 2000) and transportan (Pooga et al. 1998). Many different cargoes have been successfully delivered into cells by CPPs, mainly by covalent conjugation and also after non-covalent complex formation (Stewart et al. 2008).

9.4.2.1 Penetratin-Based Peptide

Although classical CPPs are able to cross cell membrane effectively, endosomal escape of cargos carried by classical CPPs remains as the limiting step for efficient intracellular delivery. To furnish the penetratin peptide with endosomolytic property, an analogue peptide called EB1 was designed, in which certain amino acids were replaced with histidine to adopt $\alpha$-helical structure upon protonation in acidic endosomes (Lundberg et al. 2007). Six amino acid residues were also added at N-terminus to provide length required to span endosomal membrane. Both EB1 and penetratin peptides contain seven cationic amino acid residues, but ethidium bromide exclusion assay showed that EB1 had better siRNA-binding efficiency than penetratin. This indicates that hydrophobic interactions might be involved in EB1 peptide/siRNA complex formation. However, no conformational studies were performed to support the assumption.

The cellular uptake of fluorescently labeled siRNA delivered by EB1 or penetratin was compared in HeLa cells. In agreement with ethidium bromide exclusion assay, siRNA delivered by EB1 was enhanced by at least 2.5-fold, depending on the molar ratio used for complex formation. When delivering siRNA against luciferase into HeLa cells transiently transfected with luciferase plasmid, no silencing effect was observed for penetratin. By contrast, luciferase activity was reduced by 45% by EB1 peptide-mediated siRNA delivery. This silencing effect was similar to that provided by the MPG-mNLS peptide, a control included in the study. The authors hypothesized that endosomal pH change would induce conformational change of the EB1 peptide, although the cellular uptake pathway of EB1 peptide/siRNA complexes was not investigated. Moreover, no experiment was performed using inhibitor of vacuolar proton pump, such as bafilomycin A1 (Bowman et al. 1988), to confirm the pH-dependant conformational change.

9.4.2.2 Oligoarginine-Based Peptide

DNA/RNA-binding protein domains are often found to be rich in arginine residues (Tan and Frankel 1995), suggesting that oligoarginine peptides could be a potential nucleic acid carrier. It was also discovered that to achieve efficient internalization, the guanidinium group and the number of arginine residues are more important than the presence of positive charge or backbone structure (Mitchell et al. 2000; Futaki et al. 2002; Rothbard et al. 2002). Accordingly, a nona-arginine (R$_9$) peptide was demonstrated to have the capability to deliver siRNA into mammalian cells. Gel retardation assay revealed that the R$_9$ peptides could form complex with siRNA at low N/P ratios. When human gastric carcinoma cells stably expressing enhanced green fluorescent protein (GC-EGFP) were exposed to R$_9$ peptide/siRNA against EGFP complexes, the EGFP intensity was reduced to 57% of the untransfected cell control after 48 h incubation. The distribution of fluorescently labeled siRNA was found to be cytoplasmic.

To extend the potential use of CPPs, cell-targeting sequence can be fused with CPPs for cell type-specific delivery. In a particular study, a short peptide sequence derived from rabies virus glycoprotein (RVG) was fused with nona-$\alpha$-arginine to deliver siRNA specifically to neuronal cells with nicotinic acetylcholine receptor (AchR) (Kumar et al. 2007). The authors first investigated the in vitro binding specificity of the RVG peptide and confirmed that the peptide bound only to AchR-expressing Neuro2a cells but not to receptor-negative HeLa cells. The snake-venom toxin $\alpha$-bungarotoxin (BTX), which specifically binds to AchR (Lentz 1990), inhibited the in vitro RVG peptide binding in a dose-dependent manner. After tail vein injection of biotinylated RVG peptides, primary neuronal cells in mice brains were stained positive for the peptide, indicating that the RVG peptides were able to cross the blood brain barrier (BBB). To use the RVG peptide for siRNA delivery, a chimeric peptide (RVG-9r) was designed...
by addition of a spacer and nona-D-arginine at C-terminus of the RVG peptide. A peptide derived from rabies virus matrix protein, RVG-Mat-9r, was used to serve as a control peptide. Both RVG-9r and RVG-Mat-9r peptides bound to siRNA at a molar ratio of 10:1, but only the RVG-9r peptide could deliver fluorescently labeled siRNA into Neuro2a cells. In Neuro2a cells stably expressing green fluorescence protein (GFP), RVG-9r/siRNA targeting GFP complexes silenced GFP expression up to 70%, similar to that offered by Lipofectamine 2000 transfection. When challenged by serum nuclease, the RVG-9r peptide partially protected siRNA for up to 8 h, indicating the potential of the peptide for siRNA transvascular delivery to brain cells. Indeed, after intravenous injection of RVG-9r/FITC-siRNA complexes into mice, fluorescein isothiocyanate (FITC) fluorescence was detected in the brain, but not in the liver or spleen. To test brain-specific gene silencing, RVG-9r/siRNA against GFP complexes were injected into GFP transgenic mice for three consecutive days. Two days after final injection, GFP expression in brain was reduced 30% while the expression in the liver or spleen was not affected. Furthermore, treatment with multiple intravenous injections of RVG-9r/antiviral siRNA complexes improved the survival of mice challenged by fatal Japanese encephalitis virus (JEV). The presence of antiviral siRNA in the brain tissue was confirmed by northern blot analysis. This remarkable study is the first one reporting a peptide-based approach for noninvasive siRNA delivery into mammalian brain without significant toxicity. Use of chemically modified siRNAs or liposomal nanoparticles decorated with the RVG-9r peptide might enhance complex stability in blood circulation and make significant impact for future targeted brain delivery. It is also possible to replace the RVG sequence with other cell-targeting sequences to achieve tissue or cell type-specific delivery in other organs.

9.4.2.3 Peptide for Ocular Delivery

To improve delivery of small molecules into ocular tissues, a novel peptide with a sequence of GGG(ARKKAAKA)4 and named as peptide for ocular delivery (POD) was designed (Johnson et al. 2008). Lissamine-conjugated POD peptide (L-POD) was taken up by human embryonic retinal (HER) 911 cells with cytoplasmic distribution. Cellular uptake of L-POD was inhibited by chondroitin sulfate and heparan sulfate, suggesting that cell-surface proteoglycans were involved in binding and internalization. The L-POD peptide could penetrate retinal or ocular tissues through delivery into subretinal space or topical application, respectively. An N-terminus cysteiny1 POD peptide (C-POD) was able to deliver plasmid DNA and streptavidin-coated quantum dots into HER 911 cells. When HER 911 cells were co-transfected with plasmid encoding EGFP and C-POD/siRNA against EGFP complexes, the percentage of EGFP-positive cells was reduced twofold when compared to that of the control without siRNA delivery. Unfortunately, the authors did not attempt any in vivo siRNA delivery. Nevertheless, the C-POD peptide inhibited bacterial growth on Lysogeny Broth (LB) agar plate in a concentration-dependent manner, a property useful for treatment of eye infection.

9.4.3 Histidine-Rich Branched Peptides

Cationic peptides bind electrostatically to nucleic acids to form nanoparticles, but they are incapable of mediating the endosomal escape of the delivered cargos into the cytoplasm. Thus, endosomolytic agents, such as chloroquine, are often used to enhance in vitro transfection efficiency (Wattiaux et al. 2000). The use of these agents for in vivo application might not be a feasible approach due to possible toxicity. To overcome the problem, histidine residues are commonly incorporated into the design of peptide-based vectors. pH-dependant liposome fusion in the presence of poly(L-histidine) was found to correlate with the protonation of imidazole group of histidine residues (Wang and Huang 1984; Uster and Deamer 1985). Linear peptides containing multiple histidine residues (pKₐ = 6.0) were also demonstrated to enhance plasmid DNA transfection efficiency by buffering acidic endosomes (Midoux et al. 1998; Kichler et al. 2003; Lo and Wang 2008). Another interesting histidine-rich peptide design is a series of branched peptides consisting of histidine-rich branches emanating from an uncharged lysine core (Chen et al. 2001; Chen et al. 2002). Depending on the degree of branching, these branched
peptides could enhance transfection efficiency in combination with cationic liposomes. To work without liposomes, the design of these branched peptides was improved by increasing histidine contents in the branches (Leng and Mixson 2005a). In cell transfection with luciferase plasmid DNA, the branched peptides with histidine-rich tail were more effective than their counterparts without the tail. One of the designs, H^2K4bT, could deliver gene into cells more effectively than two commercial transfection agents, Lipofectamine and SuperFect. After replacing the histidine-rich tail of the H^2K4bT peptide with other peptide sequences, the transfection efficiencies were reduced significantly, indicating that histidine-rich tail is essential for endosomal escape.

The above branched peptides, although effective in gene delivery, were unable to deliver siRNA effectively (Leng et al. 2005). To study the effect of the number of terminal branches and the histidine contents in the branches on siRNA delivery, siRNAs targeting β-galactosidase were complexed with different peptides and delivered into mouse endothelial cells stably expressing β-galactosidase (SVR-bag4). The authors discovered that the H^3K4b peptide with fewer lysine residues in the branches was more effective than the H^2K4b peptide with more lysine residues in the branches, suggesting that strong binding between siRNA and branched peptides might not be favorable for efficient siRNA delivery. A peptide with eight terminal branches, H^3K8b, was able to reduce β-gal activity to 80% of the untransfected control. Addition of integrin-binding ligand arginine-glycine-aspartic acid (RGD) to the H^3K8b peptide (H^3K8 + RGD) further increased the silencing effect by 20% when an optimal weight/weight ratio of 4:1 was used for in vitro siRNA delivery. However, the in vitro results could not translate to in vivo application (Leng and Mixson 2005b). In an attempt to deliver siRNA targeting Raf-1 (activated substrate of oncogenic Ras) intratumorally to inhibit subcutaneous tumor growth in adult nude mice, the H^3K8b peptide was less efficient than the H^2K4b peptide with the lowest lysine:histidine ratio. Since the H^2K4b peptide was easier and cheaper to be synthesized, the authors recently focused on the modification of H^3K4b for systemic delivery of siRNAs (Leng et al. 2008). A new branched peptide H^3K(+H)4b, with one additional histidine residue in each of the branches of H^3K4b, was designed to improve endosomal escape. Nanoparticles of around 230 nm arose from mixing the H^3K(+H)4b peptide with siRNA at weight/weight ratio of 4:1. In comparison to the earlier design of the H^3K8b peptide, the H^3K(+H)4b peptide was slightly more efficient (10%–20%) in inhibiting growth of cancer cell lines by delivering siRNA targeting Raf-1. Systemically delivered H^3K(+H)4b/fluorescently labeled siRNA complexes into adult nude mice were observed inside tumor xenograft and other tissues, with the greatest accumulation in the kidney. Seven injections of H^3K(+H)4b/siRNA against Raf-1 complexes significantly reduced tumor growth, confirmed by histological and immunohistochemical studies. Even though no toxicity was observed in other organs, the H^3K(+H)4b peptide was moderately toxic in in vitro studies. Further evaluation of the toxicity of these peptide/siRNA complexes might be required. Alternatively, cell-targeting sequence or PEG could be used to address the biocompatibility issue of the H^3K(+H)4b peptide.

### 9.4.4 Peptide-Based Reducible Polymers

A PRP is prepared by connecting cysteine-containing peptide monomer through formation of inter-peptide disulfide bonds, either by auto-oxidation or chemical oxidation. Cargos carried by PRP will be released in the cytoplasm due to the cleavage of disulfide bonds by reductive glutathione species in the cellular environment. Findings from previous studies suggest that the environment in the endosomal and lysosomal compartments does not permit efficient cleavage of disulfide bonds (Feener et al. 1990; Austin et al. 2005; Yang et al. 2006). Therefore, endosomolytic properties would be an essential requirement in the design of PRP monomers. After incorporating histidine residues in the peptide design, it has been proven that the resulting polymers could mediate endosomal escape without chloroquine (McKenzie et al. 2000a,b; Read et al. 2005; Manickam and Oupicky 2006; Lo and Wang 2008).

A particular reducible polycation (RFC) with 12 histidines (HIS6-RPC, MW of 113 kDa) obtained by oxidation with dimethyl sulfoxide (DMSO) is a highly versatile nucleic acid delivery vector (Read et al. 2005). HIS6-RPC mediated high levels of transfection with DNA or mRNA, encoding GFP...
at weight/weight (HIS6-RPC/nucleic acid) ratio of 40. HIS6-RPC/siRNA complexes formed at weight/weight ratio of 24 also successfully suppressed expression of EGFP in a human prostatic cell line (PC-3) transiently expressing EGFP. While plasmid DNA requires large MW PRPs for efficient condensation, low molecular weight (LMW) PRPs might be an appropriate alternative for siRNA delivery. Hence, a series of HIS6-RPC with different MW ranging from 38 to 162 kDa were synthesized by varying the time of oxidative polymerization (Stevenson et al. 2008). HIS6-RPC 162 kDa was not able to retard siRNA mobility in agarose gel, whereas LMW HIS6-RPCs (38, 44, 80, and 114 kDa) retained siRNA at weight/weight ratio of 10. HIS6-RPC 80 kDa formed 90 nm nanoparticles with siRNA, approximately half the size of those formed with HIS6-RPC 162 kDa. Consistent with these observations, only siRNA delivered by LMW HIS6-RPCs could provide up to 30% decrease in fluorescence intensity in human hepatocyte carcinoma cells (HepG2) stably expressing EGFP. LMW HIS6-RPCs' terminally incorporated cell-targeting domain, derived from the circumsporozoite protein of malaria parasite, improved cell-specific siRNA delivery into hepatocytes. However, HIS6-RPCs were generally less efficient than commercially available lipid-based vectors such as N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP). Interestingly, a primary miRNA (pri-miRNA-23a), delivered by a similar histidine-rich polymer consisting of a nuclear localization sequence, was processed into mature miRNA in HeLa cells, indicating successful nuclear-targeted delivery (Rahbek et al. 2008).

A KALA PRP (cl-KALA) was developed recently for siRNA delivery (Mok and Park 2008). Considering that individual KALA peptide with fusogenic activities would only be produced from cl-KALA after cleavage of disulfide bonds intracellularly, it has been speculated that the cytotoxicity of the KALA peptide associated with nonspecific membrane destabilization would be reduced. However, the reduction of gene expression in GFP over-expressing cells transfected by cl-KALA/siRNA complexes was less than 25%, even when an N/P ratio of as high as 64 was used. This inefficient silencing activity could be related to reduced endosomal escape. Unlike histidine-rich peptides that can buffer acidic endosomes, a significant amount of the KALA peptides might be required to perturb endosomal membrane for efficient cargo release, since it was previously demonstrated that the cleavage of disulfide bonds in endosomes is not efficient. On the other hand, the polydispersity of the polymers could be another contributing factor that affects the delivery efficiency.

### 9.5 Methods for Evaluating Peptide-Based Vectors

In this section, important experimental methods that are useful in evaluating the efficiency of peptide-based vectors for siRNA delivery will be discussed. Although some of the methods are not designed or performed using siRNA as a cargo, it is always possible to adapt the protocols for this purpose.

The most commonly used method to investigate the interaction between siRNA and peptide is electrophoretic mobility shift assay (EMSA), also known as gel retardation assay. Mobility of siRNA through an agarose gel will reduce after its binding to the peptide. This is visible after staining with a nucleic acid stain like ethidium bromide. Using the same method, it is also possible to find out whether peptide binding could prevent siRNA degradation after being subjected to nuclease treatment. The rate of binding and binding constants can be estimated using fluorescence resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FCS) methods (Ayame et al. 2008). Both methods will require fluorescent labeling of siRNAs and peptides. Hence, additional experiments might be necessary to show that the binding of peptide to siRNA will not be affected by the attachment of fluorophores. Other possible methods to study interactions between siRNA and peptides include size-exclusion chromatography (SEC) (Morris et al. 2001), fluorescence titration (Morris et al. 1997), UV-vis absorbance spectroscopy (Law et al. 2008), and circular dichroism (Law et al. 2008).

The major cellular uptake pathways in eukaryotic cells are phagocytosis, macropinocytosis, clathrin-mediated endocytosis, and caveole-mediated endocytosis. Surface charge and size of peptide/siRNA complexes are two parameters that affect cellular uptake. The surface charge of the complexes can be measured as zeta potential. Complexes with large positive zeta potential are usually more favorable for cellular uptake due to interaction with cellular membrane and a less possibility to aggregation. A good
estimation of particle size can be achieved by measuring hydrodynamic diameter using dynamic light scattering method. Particle size can be further confirmed by microscopic method such as atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

Study on the cellular uptake of peptide/siRNA complexes often relies upon fluorescence imaging or flow cytometry analysis using peptides and siRNAs labeled by different fluorophores. Importantly, only living cells, but not fixed cells, should be used in these studies. Fixation procedures can result in artifacts, leading to overestimation of cellular uptake, especially for membrane-bound CPP, that are internalized upon fixation (Richard et al. 2003). When using living cells for localization studies, extra washing steps with trypsin or heparin are important to remove the extracellularly bound complex (Richard et al. 2003; Veldhoen et al. 2006). Additionally, confocal laser scanning microscopy should be routinely used to demonstrate the intracellular localization of the complexes. Combined with these methods, it is possible to investigate the cellular uptake pathway of peptide/siRNA complexes by using different inhibitors or effectors listed in Table 9.2 (Simeoni et al. 2003; Veldhoen et al. 2006; Ayame et al. 2008). Sensitive methods developed for quantification of siRNA delivered by peptides, such as liquid hybridization method and competitive quantitative PCR (qPCR) (Veldhoen et al. 2006; Liu et al. 2009), should be very useful in optimizing peptide design and complex formulation.

Following studies of cellular uptake, the next step is to investigate silencing effects in the cells transfected with peptide/siRNA complexes. The cells usually carry different siRNA-targeted reporter genes expressed transiently or stably, for example, luciferase, GFP, or β-galactosidase. Housekeeping genes like GAPDH are convenient targets useful to siRNA silencing study (Simeoni et al. 2003). At the protein level, expression of reporter genes can be confirmed by Western blot and quantified by available ELISA methods. At the mRNA level, Northern blot and qPCR can quantify the extent of silencing of the target gene. When a dose–response curve is plotted based on reduction of expression levels, the apparent value of maximal inhibition (IC$_{max}$) and half maximal inhibition (IC$_{50}$) can be identified to compare the effectiveness of peptide/siRNA complexes with different compositions (Veldhoen et al. 2006). With the safety profile of a delivery vector being of utmost concern, in vitro and in vivo toxicity of individual components of the complexes should also be evaluated.

### 9.6 Conclusion

With increasingly extensive application of siRNA in disease treatment, the bottleneck for developing siRNA-based therapeutics will be efficient delivery. As the use of physical methods and chemically modified siRNAs might be limited, a delivery vector that can encapsulate siRNA molecules non-covalently

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<th>TABLE 9.2 List of Different Inhibitors or Effectors That Are Commonly Used to Study the Cellular Uptake Pathway of Peptide/siRNA Complexes</th>
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<td>Treatment</td>
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<td>Chloroquine, monensin</td>
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<td>Cytochalasin B</td>
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<td>Amiloride</td>
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<td>Filipin complex, nystatin</td>
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<td>Okadaic acid</td>
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<tr>
<td>Sucrose, ikarugamycin</td>
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<td>Wortmannin</td>
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<td>Bafilomycin A1</td>
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appears to be more desirable. An ideal siRNA delivery vector should fulfill the following criteria: (1) synthesized easily in large scale; (2) relatively stable for a long shelf life; (3) biodegradable and biocompatible; (4) low toxicity, as well as low immunogenicity that allows multiple administrations; (5) flexibility to be functionalized for tissue or cell type-specific delivery; (5) high drug-loading capacity; (6) protection of siRNA from nuclease degradation; and (7) ability to release siRNA efficiently within target cells.

FIGURE 9.2 Schematic diagram on delivery of siRNA by peptide-based vectors. Peptide/siRNA complexes of nanometer size are usually formed by incubating a mixture of peptide and siRNA. These complexes will be internalized by cells either through direct membrane penetration or endocytosis. In the cytoplasm, the complexes will dissociate and the released siRNAs will then silence gene expression. If the peptide contains nuclear localization sequence, it is possible that the complexes are transported into the nucleus and free siRNAs can be actively excluded from the nucleus into the cytoplasm.
Taking these into consideration, peptides are emerging as attractive nanomaterials for siRNA delivery. In fact, a wide range of peptides with different properties have been tested and utilized in in vitro studies, as reviewed here. The delivery of siRNA using peptide-based vectors is graphically summarized in the schematic diagram in Figure 9.2.

Before the applications of peptide-based vectors can be translated from in vitro to in vivo and from the bench to the bedside, several concerns need to be addressed. First, it is highly unlikely to have only one single entry pathway for different peptides and peptide/siRNA complexes, due to differences in peptide sequence, conformation, surface charge, and particle size. In order to have better understanding on cellular entry mechanisms, the characterization of relevant biophysical properties of the complexes under various conditions is desirable. Second, silencing effect on a reporter gene system like luciferase or GFP expression might be insufficient to illustrate the transfection efficiency of peptides. Sensitive quantification methods to quantify siRNA should be employed to enable comparison of the amount of internalized siRNA mediated by different vectors. Third, 10% serum-containing medium has been widely used to study the stability of peptide/siRNA complexes. Nonetheless, it is arguable whether this trustfully reflects in vivo condition, where many components are present in blood stream with higher concentrations that can potentially result in inactivating effects on the complexes in a dose-dependent manner. Intravenous administration of the complexes into animal models might be essential to justify whether tested peptides will be able to protect and deliver siRNA efficiently in the body. Fourth, the immunogenicity of peptide, siRNA, and complexes should be investigated carefully when moving toward in vivo application. A combined use of siRNA with high specificity and peptide with cell-targeting sequence might minimize the possibility of stimulating the immune system and lower the dosage to be used for therapeutic purpose.

In conclusion, recent progress provides increasing evidence that peptides are promising nanomaterials for siRNA delivery. With inputs from different research disciplines from molecular biology, genetics, chemistry, physics, materials sciences to nanoscience, we will have a much better understanding of siRNA mechanism, peptide chemistry, biophysical properties of peptide/siRNA complex, pharmacodynamics, and pharmacokinetics, thereby accelerating the development of peptide-based vectors for in vivo therapeutic applications of siRNAs.

References


Peptide-Based Nanomaterials for siRNA Delivery: Design, Evaluation, and Challenges


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