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Amphiphilic Peptides With Arginine and Valine Residues as siRNA Carriers

Dong-Wook Ryu,[1] Hyun Ah Kim,[1] Jae Hwan Ryu,[1] Dong Yun Lee,[1,2,3] and Minhyung Lee[1,2,3]*

[1]*Department of Bioengineering, College of Engineering, Hanyang University, Seoul 133-791, Korea*
[2]*Institute for Bioengineering and Biopharmaceutical Research, Hanyang University, Seoul 133-791, Korea*
[3]*Institute of Aging Society, Hanyang University, Seoul 133-791, Korea*

ABSTRACT

An efficient and safe delivery carrier is required for the therapeutic application of siRNA. In this research, amphiphilic peptides with arginine and valine residues were evaluated as siRNA carriers. The peptides were composed of 1–4 arginine-blocks and 6 valine-blocks. In the aqueous solution, the arginine–valine peptides (RV peptides) formed micelles with hydrophobic cores comprised of a valine block and a cationic surface comprised of an arginine block. In a gel retardation assay, the RV peptides completely retarded siRNA at a 1:10 weight ratio (siRNA:peptide). A heparin competition assay suggested that the RV peptides formed more stable complexes with siRNA than they did with polyethylenimine (25 kDa, PEI25k). In an in vitro silencing assay, a dual luciferase expression (Renilla and firefly luciferases) vector, psiCHECK2, was co-transfected into human embryonic kidney 293 cells with Renilla-siRNA using the RV peptides. The specific silencing effect of Renilla luciferase was analyzed in reference to firefly luciferase. The results showed that the R3V6 peptide was more efficient than the R1V6, R2V6, and R4V6 peptides in silencing Renilla luciferase. In the flow cytometry and in vitro silencing studies, the R3V6 peptide delivered Renila-siRNA as efficiently as PEI25k. The siVEGF/R3V6 peptide also reduced endogenous vascular endothelial growth factor (VEGF) expression in CT27 cells as efficiently as PEI25k. A cytotoxicity assay showed that RV peptides did not cause any cytotoxicity. Therefore, RV peptides may be useful for the development of a safe and efficient delivery carrier of siRNA J. Cell. Biochem. 113: 619–628, 2012.

KEY WORDS: CYTOTOXICITY; GENE DELIVERY; GENE THERAPY; MICELLE; PEPTIDE

RNA interference (RNAi) has recently emerged as a powerful tool for biological research and specific gene silencing in gene therapy [Fire et al., 1998; Bumcrot et al., 2006; Gartel and Kandel, 2006]. A safe and efficient carrier is required for the clinical use of small interfering RNA (siRNA). To date, various siRNA carriers based on cationic polymers, liposomes, and peptides have been developed. Like DNA, siRNA has negative charges on its phosphodiester backbone. Therefore, polycations such as polymers, liposomes, and peptides can bind to siRNA via electrostatic interactions to form small particles. One of the most widely used siRNA carriers is polyethylenimine (PEI) [Aigner, 2006; Kim et al., 2007, 2008, 2009; Zintchenko et al., 2008; Jiang et al., 2009; Malek et al., 2009; Merkel et al., 2009; Biswal et al., 2010], with high positive charges on its primary amine groups. Various deravatives of PEI have been tested for the delivery of siRNA. For example, a choesterol conjugated low molecular weight PEI (2 kDa, PEI2k), which was named water-soluble lipopolymer (WSLP), was evaluated as a siRNA carrier as well as a DNA carrier [Kim et al., 2007]. In tumor-bearing mice models, WSLP delivered vascular endothelial growth factor (VEGF) siRNA and reduced tumor burden more efficiently than high molecular weight PEI (25 kDa, PEI25k). In another example, PEI25k formed electrolyte micelles with polyethylene glycol (PEG)-conjugated siRNA [Kim et al., 2006a, 2008]. The PEI25k/siRNA complex formed the core of the micelles. The PEG protruded outside of the micelles and protected the siRNA from non-specific interactions and degradation. However, one of the obstacles in the application of PEI in a clinic setting is its cytotoxicity [Han et al., 2001; Morimoto et al., 2003]. PEI has high cytotoxicity in various cells, which may be due to its high positive charge density [Fischer et al., 1999]. PEI may aggregate at the surface of the cellular

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*Correspondence to: Minhyung Lee, PhD, Department of Bioengineering, College of Engineering, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, South Korea. E-mail: minhyung@hanyang.ac.kr
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membrane and rupture the membrane. This suggests that the development of non-toxic and safe siRNA delivery carriers is an inevitable requirement for the clinical application of siRNA.

The cytotoxicities of cationic polymers, peptides, and liposomes are mainly due to high charge densities [Fischer et al., 1999; Kunath et al., 2003]. This fact suggests that low molecular weight carriers with less positive charges may be safer than high molecular weight carriers with higher positive charges. Indeed, PEI has varying cytotoxicity depending on the molecular weight [Fischer et al., 1999]. High molecular weight PEI, such as PEI25k, has much higher toxicity than low molecular weight PEI, such as PEI2k [Lee et al., 2003]. Therefore, many researchers have focused on the low molecular weight polycations. Oligoarginines have been used for siRNA delivery [Kim et al., 2006c; Kumar et al., 2007, 2008]. Oligoarginines demonstrate high siRNA delivery efficiency in various cell types. However, oligoarginines may form relatively unstable complexes with siRNA compared with a PEI/siRNA complex, possibly due to its low charge density.

In the current study, we evaluated RV peptides, which are composed of an arginine block and a valine block. The RV peptides are composed of 7–10 amino acids, and have low charge density. However, in aqueous conditions, the RV peptides form micelles with hydrophobic valine cores and cationic arginine surfaces. The RV peptides deliver plasmid DNA as efficiently as poly-L-lysine (PLL) [Ryu et al., 2011]. However, the plasmid DNA delivery efficiency was much lower than that with PEI, suggesting that the RV peptides could not form stable complexes with plasmid DNA. This inability to form a stable complex may be due to the fact that the size of the plasmid DNA was much larger than those of the RV peptides. However, siRNA has a similar molecular weight to RV peptides. Furthermore, RV peptides may locally increase charge density in aqueous solution through the formation of micelles, which may stabilize the peptides/siRNA complex and impart high siRNA delivery efficiency. In addition, the micelles are easily separated in intracellular conditions due to their interactions with intracellular components. Therefore, they may be non-toxic to cells. Based on this hypothesis, various experiments have been performed to evaluate the RV peptides as siRNA carriers.

MATERIALS AND METHODS

PREPARATION OF RV-PEPTIDES AND siRNA COMPLEXES

The RV peptides were synthesized with 1–4 arginine residues and 6 valine residues and were purified via C18 reverse-phase chromatography (Peptron Co. Daejeon, Korea). The RV peptides were dissolved in distilled water at 5 mg/ml and were stored at −70°C. The renilla luciferase siRNA (siRluc)/RV-peptide complexes were prepared at various weight ratios in distilled water. The siRluc/PEI25k complex was prepared at a 1:1 weight ratio, based on the previous reports [Kim et al., 2006a; Jeong et al., 2010]. The siRluc/RV-peptide mixtures were formed as complexes for intracellular delivery via incubation at room temperature for 15 min. Vascular endothelial growth factor siRNA (siVEGF), enhanced green fluorescent protein siRNA (siEGFP), and fluorescein isothiocyanate (FITC)-labeled siRNA/RV peptide mixtures were also incubated at room temperature for 15 min.

SEQUENCES OF siRNAS

siRluc (sense, 5′-GGCCUUUCACUACUCCUACTT-3′; antisense, 5′-GUAGGAGUAGUGAAAGGCCTT-3′), siVEGF (sense, 5′-AUGUGAA-UGCAGACCAAAGAATT-3′; antisense, 5′-UUCUUUGGUCUGCAUU-CACAUTT-3′), siEGFP (sense, 5′-CAAGCUGACCCUGAAGUUCTT-3′; antisense, 5′-GAACUUCAGGGUCAGCUUGTT-3′), and FITC-labeled siRNA (sense, 5′-CUUACGCUGAGUACUUCGATT-3′; antisense, 5′-UCGAAGUACUCAGCGUAAGTT-3′) were synthesized, modified and purified by Samchunli Pharm Co., Ltd (Seoul, South Korea). For fluorescently activated cell sorting (FACS) analysis, siRNA was labeled with FITC at the 3′-terminal of the sense strand.

GEL RETARDATION ASSAY

The formation of a siRNA/RV peptide complex was verified through a gel retardation assay. A fixed amount of siRNA (0.7 μg) was mixed with increasing amounts of RV-peptides in distilled water. The complex mixtures were electrophoresed on a 4% agarose gel in the presence of ethidium bromide for 20 min at 100 V in 1× TBE buffer solution. The siRNAs were visualized using a UV transilluminator.

HEPARIN COMPETITION

The stabilities of the siRNA/RV peptide and siRNA/PEI25k complexes were evaluated through a heparin competition assay. The siRNA/R3V6 peptide and siRNA/PEI25k complexes were formed at 1:20 and 1:1 weight ratios, respectively, and were mixed with increasing amounts of heparin (Sigma, St. Louis, MO). The mixtures were analyzed on an acrylamide gel via electrophoresis at 100 V for 60 min. The siRNAs were visualized using a UV transilluminator.

IN VITRO SILENCING ASSAY

Human embryonic kidney 293 (HEK 293) cells and CT-26 colon adenocarcinoma (CT-26) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 1% penicillin and 10% fetal bovine serum at 37° in a 5% CO_2 atmosphere. The psiCHECK-2 vector (Promega, Madison, WI) is a renilla and firefly double luciferase expression vector and is useful for evaluation of the suppression efficiency of a luciferase expression relative to normalization with another luciferase expression. The complexes of RV peptides with siRluc were prepared at various weight ratios by mixing 1.0 μg of siRluc with increasing amounts of the RV peptides in 14 μl of water. HEK293 cells were transfected with psiCHECK-2 to prepare firefly and Renilla luciferase protein expressing cells. The cells were plated in 12-well plates at 50,000 cells per well and incubated for 24 h in a 5% CO_2 atmosphere. The siRluc/RV peptide complexes were added to each well and incubated for 48 h in a 5% CO_2 atmosphere. The siRluc/PEI25k complex was prepared at a 1:1 weight ratio [Lemkine et al., 1999; Turunen et al., 1999; Nguyen et al., 2000] and added to the psiCHECK-2 transfected HEK293 cells. After 24 h, the cells were washed with phosphate buffered saline, and passive lysis buffer (Promega) was added for cell lysis. The luciferase activities of double luciferases were measured in relative light units on 96-well plates using a luminometer. The evaluated values of luciferase activities were normalized through calculation as (firefly RLU/Renilla RLU) × 100 (%).

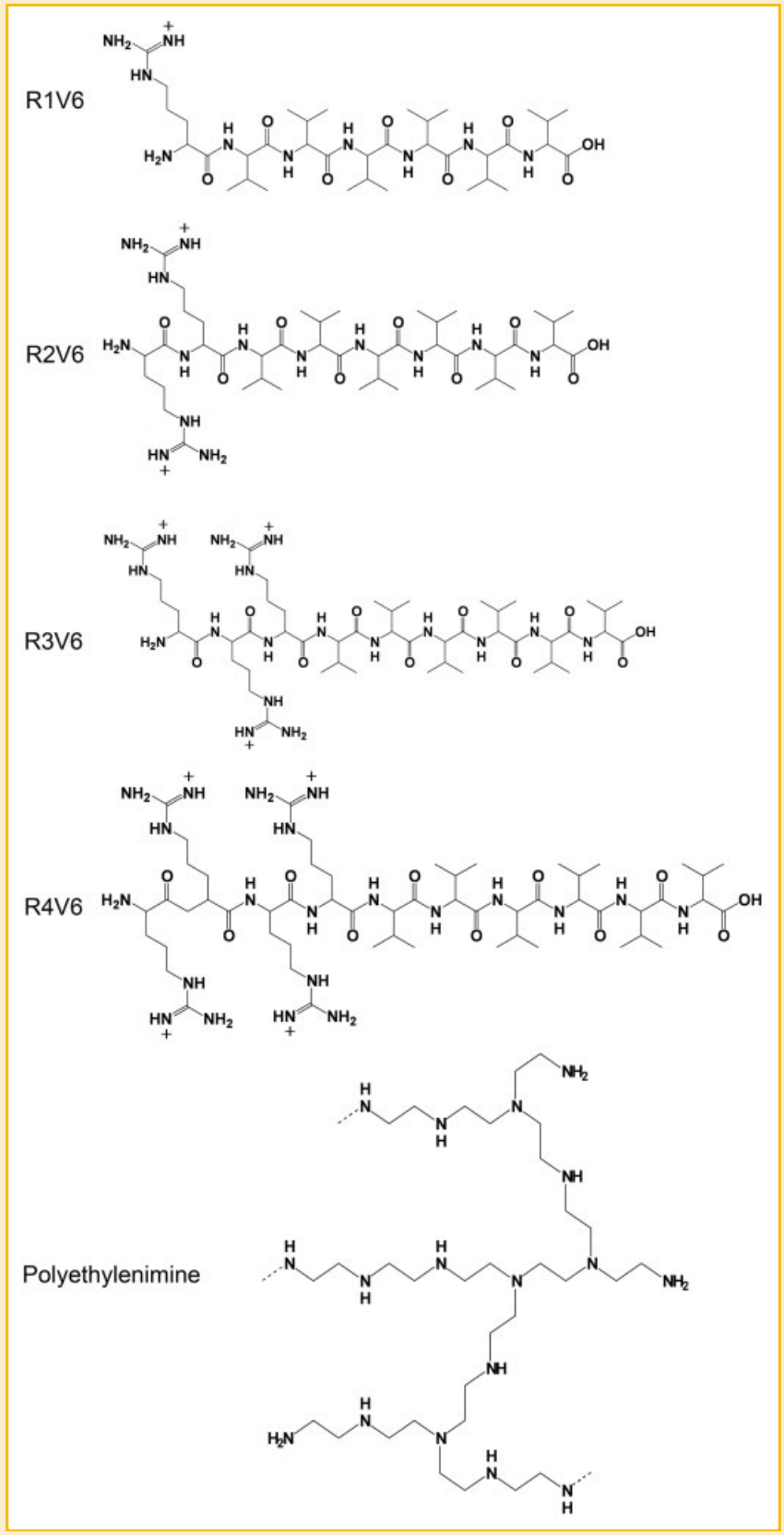

Fig. 1. Structures of the RV peptides and PEI25k.

FLOW CYTOMETRY ANALYSIS

The FITC-labeled siRNA/RV peptide complexes were added to HEK293 cells and incubated for 24 h at 37°C. After incubation, the cells were harvested and washed with PBS. The suspended cells were centrifuged at 2,000 rpm for 5 min. The supernatant was removed and the cells were re-suspended in FACS buffer (PBS with 0.02% NaN_3, 0.2% FBS). After centrifugation at 2,000 rpm for 5 min, the supernatant was removed and cells were re-suspended in fixing buffer (FACS buffer with 1% formaldehyde). The cells were transferred to FACS tubes, and flow cytometry was performed using the BD FACS Calibur (BD Biosciences Immunocytometry Systems, San Jose, CA).

VEGF SILENCING ASSAY

CT-26 mouse colon cancer cells were seeded 80,000 cells per well in 24-well plates and incubated at 37°C in a 5% CO_2 atmosphere. After 24 h, the si/RV peptide complexes were added to the cells. The cells were incubated for 48 h, and the media was collected for a determination of VEGF quantification using a VEGF ELISA kit (Peprotech Co., NJ).

EGFP SILENCING ASSAY

EGFP-expressing HEK 293 cells were seeded at 40,000 cells into a 24-well plate. After incubation for 24 h, either the siEGFP/R3V6 peptide or the siEGFP/PEI25k complex was transfected to the

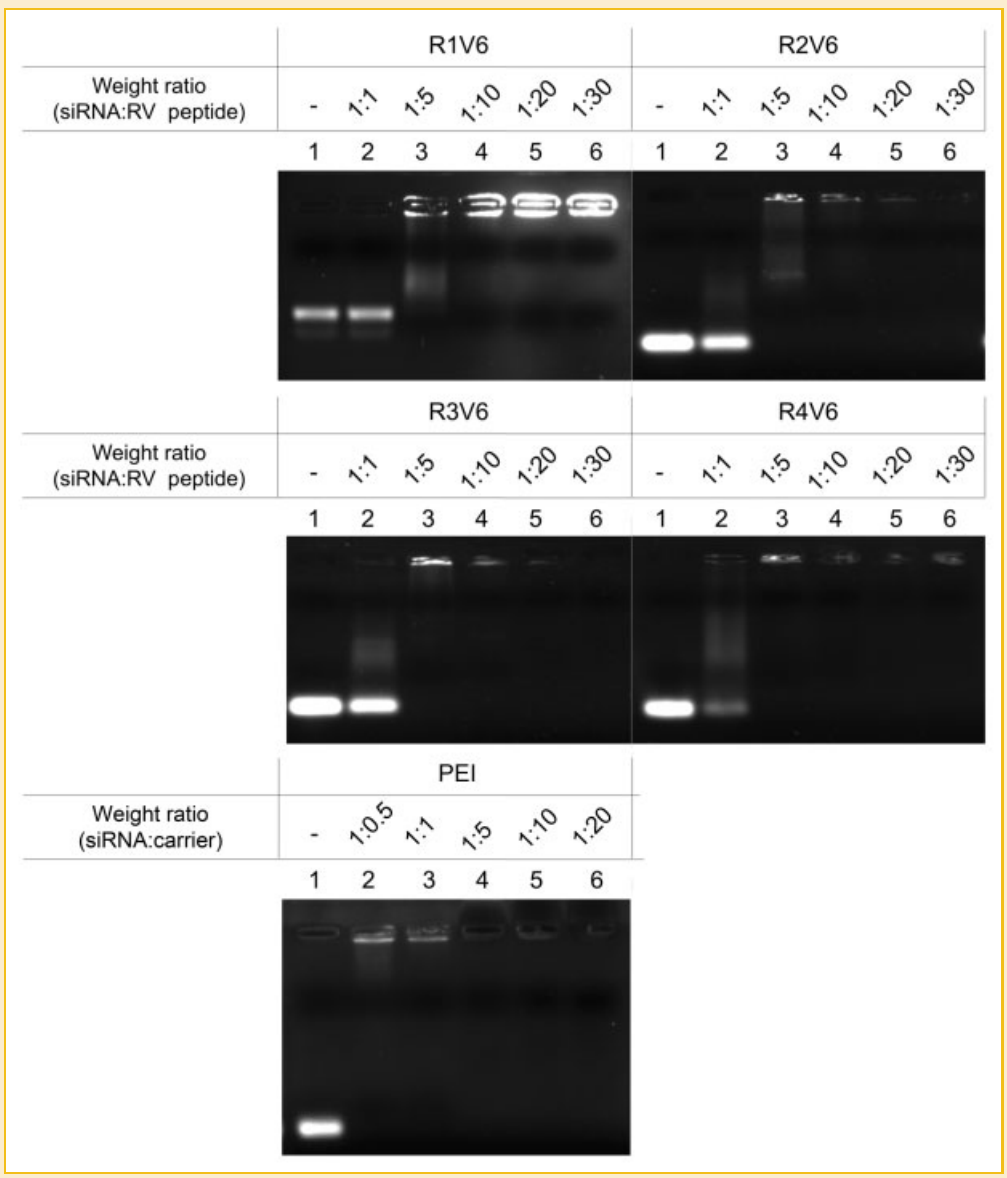

Fig. 2. Gel retardation assay. A fixed amount of siRNA was mixed with increasing amounts of RV-peptides or PEI25k for complex formation. The complexes were analyzed on a 4% agarose gel.

EGFP-expressing HEK 293 cells and incubated for 48 h. The cells were washed with PBS and lysed with lysis buffer (Promega). The quantitative analysis of EGFP expression was performed with a spectrofluorophotometer (excitation wavelength: 488 nm, emission wavelength: 525 nm).

The EGFP-expressed HEK293 cells were plated onto six-well plates at a density of 200,000 cells per well. Either the siEGFP (2.14 μg)/RV-peptide or PEI25k complexes were added to the cells in each well and then incubated for 48 h. EGFP was visualized using a TE 2000-E NIKON microscope.

MTT ASSAY

The cytotoxicity of the siRNA/RV peptide complexes was evaluated using a MTT assay. HEK293 cells were seeded at 35,000 cells per well in 24-well plates. The siRNA/RV peptides were added to the HEK293 cells. The siRNA/PEI25k complex was used as a control. After incubation for 48 h, 40 μl of 5 mg/ml MTT reagent in PBS was added. The plates were additionally incubated for 4 h at 37°C in a 5% CO_2 atmosphere. Cell culture medium was removed, and dimethyl sulfoxide (750 ml) was added to live cells in the wells to dissolve the formazan crystals. The absorbance was measured at 570 nm. Cell viability (%) was calculated according to the following equation: cell viability $(\%) = (OD_{570(sample)}/OD_{570(control)}) \times 100$, where the $OD_{570(sample)}$ represents the measurement from the wells treated with siRNA/RV peptide complex, and the $OD_{570(control)}$ represents the measurements from the wells treated with distilled water only.

STATISTICAL ANALYSIS

Statistical analysis was conducted using ANOVA followed by the Newman–Keuls test. All data are presented as average ± SEM, and $P < 0.05$ was considered statistically significant.

RESULTS

CHARACTERIZATION OF THE siRNA/RV PEPTIDE COMPLEX

Like biological lipids, RV peptides have hydrophilic heads with hydrophobic tails (Fig. 1). RV peptides are composed of 1–4 arginine residues, which have 2–5 positive charges, and 6 hydrophobic valine residues. The RV peptides form micelles in aqueous solution. However, PEI25k is hydrophilic and does not form micelle in aqueous solution (Fig. 1). The RV peptides, therefore, may behave as micelles just like larger molecules with more positive charges and may form tight complexes with DNA. Moreover, the RV peptides were designed to have low cytotoxicity with a small positive charge. To confirm that the RV peptides formed complexes with siRNA, a gel retardation assay was performed (Fig. 2). The R1V6, R2V6, and R3V6 peptides completely retarded siRNA at a 1:10 weight ratio (siRNA:RV peptide), while the R4V6 peptide completely retarded siRNA at a 1:5 weight ratio. The R4V6 peptides containing more positive charges more efficiently retarded siRNA than did the other tested RV peptides. PEI retarded siRNA completely at a 1:1 weight ratio, suggesting that PEI is much more efficient than the RV peptides in complex formation with siRNA. It may be due to higher charge density of PEI25k, compared with the RV peptides.

To evaluate the stability of the siRNA/RV-peptide complex, a heparin competition assay was carried out (Fig. 3). Since heparin is a highly negatively charged polysaccharide, it can competitively interact with the RV peptide complex. The siRNA/PEI25k complex released siRNA at low concentration of heparin (4 μg heparin), whereas the siRNA/R3V6 peptide complex began to release siRNA only above 10 μg heparin. These results suggest that the R3V6 peptide can form strong complexes with siRNA compared to a PEI25k-mediated complex.

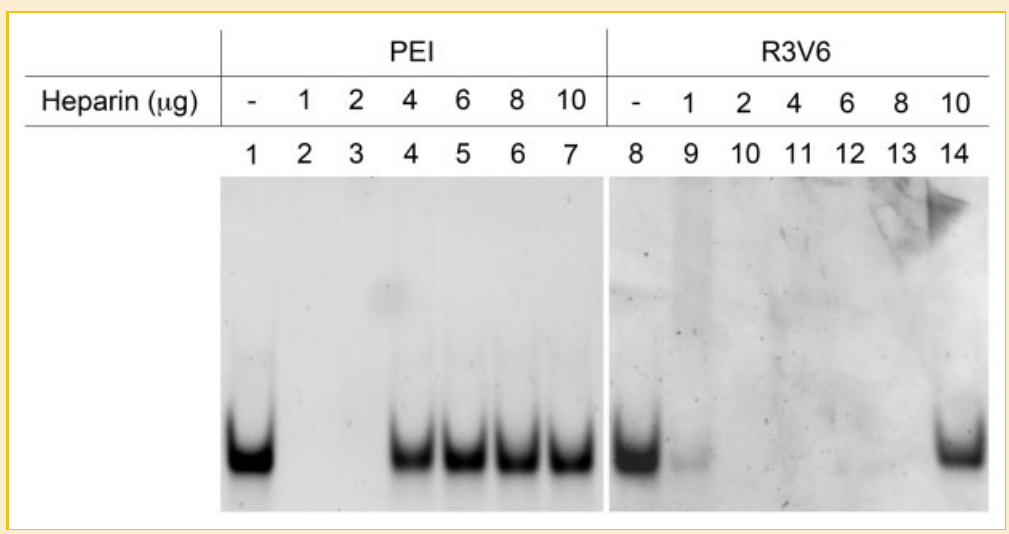

Fig. 3. Heparin competition assay. The stabilities of the siRNA/RV peptide complexes were evaluated using a heparin competition assay. The siRNA/R3V6 peptide and siRNA/PEI25k complexes were prepared as described in the Materials and Methods Section. Increasing amounts of heparin were added to the complexes, and the samples were analyzed in a 12% acrylamide gel.

THE SILENCING EFFECT OF THE RV PEPTIDE/siRNA COMPLEXES IN VITRO

The siRNA delivery efficiency of the RV peptides was evaluated using various siRNAs. To optimize the ratios between siRNA and RV peptides, the Rluc-siRNA/RV peptide complexes were prepared at various weight ratios and delivered to firefly- and Renilla luciferase-expressing HEK293 cells (Fig. 4). Compared with the control group, the Rluc-siRNA/R1V6 or R2V6 peptide complexes showed the highest silencing effect at a 1:30 weight ratio (siRNA:RV peptides), while the R3V6 and R4V6 peptides showed the highest silencing effect at a 1:20 weight ratio (Fig. 4). The silencing efficiencies of the RV peptides with Rluc-siRNA were compared with each other at their optimum conditions. Each complex at the optimal weight ratio of RV peptides was investigated for inhibitory effect compared with the PEI25k control (Fig. 5). The complexes of Rluc-siRNA with the R1V6, R2V6, or R4V6 peptides expressed Renilla luciferase at a level 75% greater than that of the control, while the complex of Rluc-siRNA with the R3V6 peptide showed approximately 50% of the control Renilla luciferase expression. These results demonstrated that the R3V6 peptide at a 1:20 weight ratio showed a similar silencing effect to that of PEI25k (Fig. 5).

The siRNA delivery efficiency of the RV peptide was confirmed with the EGFP gene. The siEGFP/R3V6 peptide complex was evaluated for the suppression of EGFP expression in EGFP-expressing HEK293 cells. The EGFP-expressing HEK293 cells were incubated for 48 h post-transfection of the siEGFP/R3V6 peptide complex (Fig. 6A,B). The siEGFP/PEI25k complex was employed as a control. The naked siEGFP without a carrier and scramble-siRNA with the R3V6 peptide complex did not reduce EGFP expression in the cells. In contrast, the siEGFP/R3V6 peptide

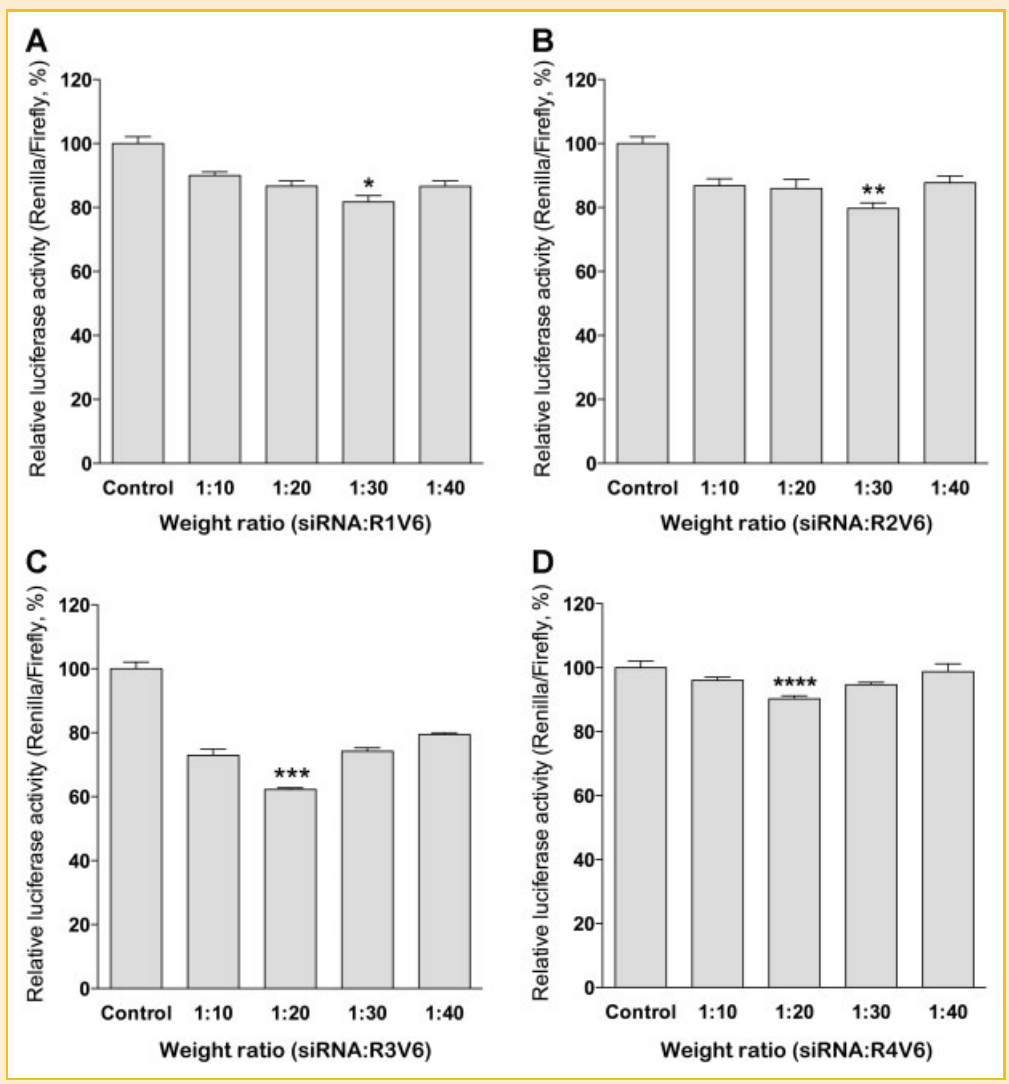

Fig. 4. Renilla luciferase silencing assay depending on weight ratio. The siRluc/RV peptide complexes were prepared at various weight ratios and delivered to the psiCHECK2-transfected HEK293 cells. The silencing efficiency was measured using a luciferase assay. A: R1V6, (B) R2V6, (C) R3V6, and (D) R4V6. The data are expressed as mean values (±SEM) of quadruplicate experiments. $^{*}P < 0.05$ compared with control and 1:10. $^{**}P < 0.01$ compared with control. $^{***}P < 0.01$ compared with control, 1:10, 1:30, and 1:40. $^{****}P < 0.05$ compared with control and 1:40.

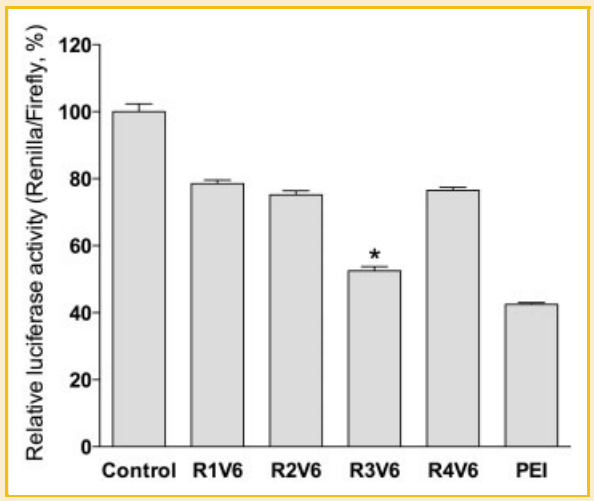

Fig. 5. Comparison of renilla luciferase silencing efficiencies of RV peptides. The siRluc/RV peptide complexes were prepared at the optimal weight ratios and were compared to one other in psiCHECK2-transfected HEK293 cells. The silencing efficiency of the R3V6 peptide was compared with those of the other RV peptides and PEI25k. Silencing efficiency was measured via a dual luciferase assay. The data are expressed as mean values (±SEM) of quadruplicate experiments. $^{*}P < 0.01$ compared with control, R1V6, R2V6, R4V6, and PEI25k.

complex reduced EGFP expression to a lesser extent than the siEGFP/PEI25k complex.

It has been reported that siVEGF with a specific sequence could suppress the expression of VEGF in CT-26 cells [Kim et al., 2006b]. The relative silencing of the VEGF expression was assayed through ELISA. To evaluate the efficiency of siVEGF delivery to CT-26 cells, siVEGF was delivered with the R3V6 peptide complex (Fig. 7). As expected, siVEGF without a carrier and the complex of scramble-siRNA (scr-siRNA) with the R3V6 peptide did not show any silencing effect. However, the siVEGF/R3V6 complex suppressed the VEGF expression by 35% compared with that of the untreated control. In addition, the suppression efficiency of the complex of the R3V6 peptide with siVEGF was similar to that of the siVEGF/PEI25k complex.

INTRACELLULAR UPTAKE EFFICIENCY OF RV-PEPTIDE/siRNA COMPLEXES AND CELLULAR TOXICITY

The cellular uptakes of the peptide/siRNA complexes were examined with FACS using FITC-labeled siRNA (Fig. 8). The FITC-siRNA/R3V6 peptide complex had higher intracellular uptake efficiency than the other RV–peptide complexes at their optimum weight ratios. As mentioned above, the siVEGF/R3V6 peptide complex demonstrated a similar level of VEGF silencing to that of the complex of PEI25k

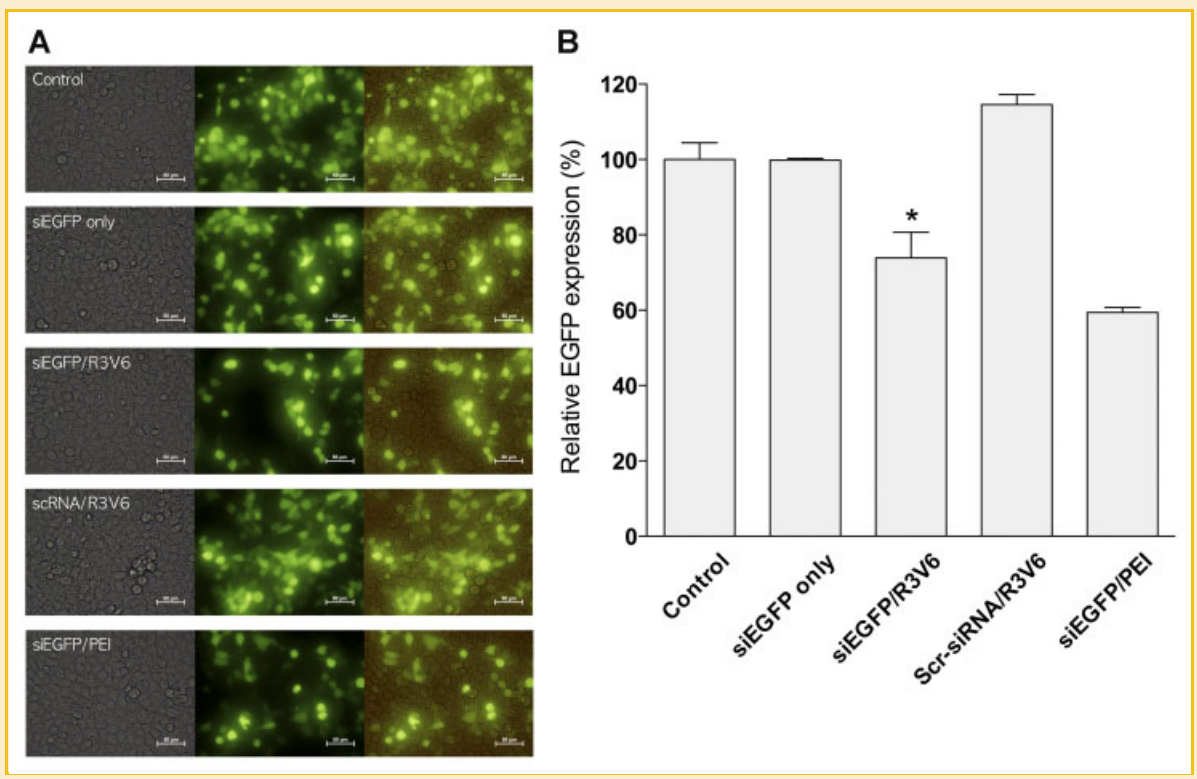

Fig. 6. Comparison of the EGFP silencing efficiencies of the RV peptides. The siEGFP/RV peptide complexes were prepared at the optimal weight ratios and were compared to one other in pEGFP-transfected HEK293 cells. The silencing efficiency of the R3V6 peptide was compared with those of the other RV peptides and PEI25k. Silencing efficiency was visualized using (A) fluorescence microscopy and measured using (B) fluorometry. The data are expressed as mean values (±SEM) of quadruplicate experiments. $^{*}P < 0.01$ compared with control, R1V6, R2V6, and R4V6, but there was no statistical significance compared with PEI25k. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

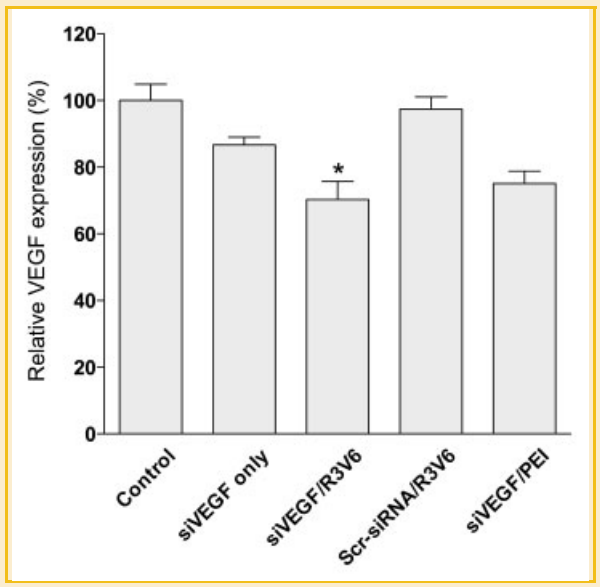

Fig. 7. Comparison of the VEGF silencing efficiencies of the RV peptides. The siVEGF/RV peptide complexes were prepared at the optimal weight ratios and were compared to one other in CT26 cells. The silencing efficiency of the R3V6 peptide was compared with that of PEI25k. VEGF expression was measured using ELISA. The data are expressed as mean values (±SEM) of quadruplicate experiments. $^{*}P < 0.05$ compared with control, siVEGF only, scr-siRNA/R3V6, but there was no statistical significance compared with PEI25k.

with siVEGF. In addition, the FITC-labeled siRNA/R3V6 peptide complex showed similar levels of cellular association to those of the FITC-labeled siRNA/PEI25k complex (Fig. 8).

To measure the cytotoxicity of the siRNA/R3V6 peptide complexes, an MTT assay was performed on HEK293 cells (Fig. 9A). As expected, the RV peptide complexes were non-toxic to cells, while PEI25k showed in vitro toxicity with approximately 70% viability. Although PEI25k demonstrates effective delivery, its high cationic density and high molecular weight are toxic to cells [Moghimi et al., 2005]. On the other hand, the R3V6 peptide has small positive charge. Therefore, we assumed that the lower charge density of the R3V6 peptides would be less toxic to live cells. The cytotoxicity of the R3V6 peptide was compared with that of PEI25k, depending on dose. Various amounts of the R3V6 peptides and PEI were added to the HEK293 or CT-26 cells. The results showed that the R3V6 peptide was less toxic to HEK293 and CT-26 cells (Fig. 9B,C).

DISCUSSION

In this study, we showed that the R3V6 peptide is a useful carrier for siRNA delivery. The RV peptides have some advantages as siRNA carriers. First, the RV peptides have low cytotoxicity. As shown in Figure 9A, none of the siRNA/RV peptide complexes demonstrated any cytotoxicity to HEK293 cells, while treatment with the siRNA/PEI25k complexes resulted in an approximate 60% cell viability. When PEI25k and R3V6 were added to cells without siRNA, R3V6 did not show any cytotoxicity in HEK293 and CT-26 cells (Fig. 9B,C). It was previously reported that high positive charge density may induce cytotoxicity [Fischer et al., 1999]. Positively charged PEI25k and DNA/PEI25k complexes may interact with negatively charged cell membranes and aggregate on the surface of the membrane, resulting in membrane rupture. Therefore, reduction and shielding of charge density may reduce the cytotoxicity of these carriers.

The conjugation of PEG to PEI25k reduced the cytotoxicity of PEI25k by shielding the positive charges of PEI25k [Petersen et al., 2002; Sagara and Kim, 2002; Sung et al., 2003]. Low molecular weight PEIs such as PEI2k also have lower cytotoxicities [Lee et al.,

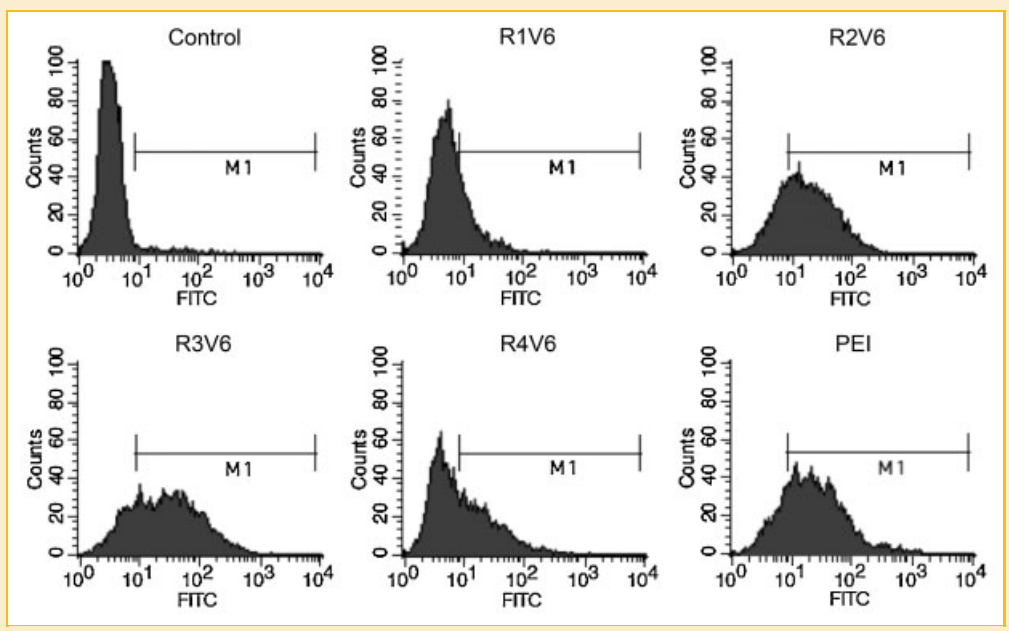

Fig. 8. Cellular uptake of siRNA by the RV peptides. The complexes of RV peptides with FITC-labeled siRNA were delivered to HEK293 cells and incubated for 24 h at 37°C. PEI25k was used as a control carrier. The efficiency of cellular uptake was measured using flow cytometry.

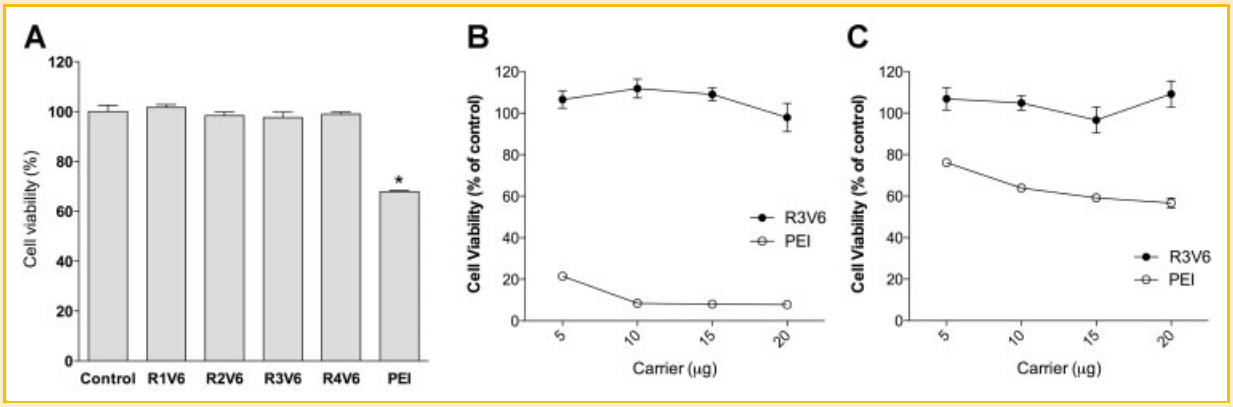

Fig. 9. Cytotoxicities of the RV peptides. A: Cytotoxicities of the siRNA/RV peptides. The siRNA/RV peptides and siRNA/PEI25k complexes were transfected into HEK293 cells. After delivery, cell viability was measured using the MTT assay. The data are expressed as mean values (±SEM) of quadruplicate experiments. $^{*}P < 0.01$ compared with control, R1V6, R2V6, R3V6, and R4V6. B: Cytotoxicity of the R3V6 peptide in HEK293 cells. Various amounts of the R3V6 peptide and PEI25k were added to HEK293 cells without siRNA. After 24 h, cell viability was measured using the MTT assay. The data are expressed as mean values (±SEM) of quadruplicate experiments. C: Cytotoxicity of the R3V6 peptide in CT-26 cells. Various amounts of the R3V6 peptide and PEI25k were added to HEK293 cells without siRNA. After 24 h, cell viability was measured using the MTT assay. The data are expressed as mean values (±SEM) of quadruplicate experiments.

2003]. However, the reduction of PEI molecular weight also resulted in reduced transfection efficiency. For example, PEI2k demonstrates a much lower transfection efficiency compared to that of PEI25k [Lee et al., 2003]. The RV peptides also have fewer positive charges than PEI25k. However, the RV peptides form micelle structures in aqueous solution, behaving like higher molecular weight peptides. After delivery or in the process of delivery, the RV peptide micelles may be dissociated through interactions with other intracellular or extracellular molecules. After dissociation, the RV peptides have low charge density, resulting in little cytotoxicity.

Second, the RV peptides form stable complexes with siRNA. In the heparin competition assay, siRNA was released from PEI25k more easily than from the R3V6 peptides (Fig. 3). PEI and its derivatives have been used as siRNA delivery carriers. In a previous report, it was shown that siRNA/PEI25k complexes are not stable in the presence of serum and easily release siRNA, suggesting that PEI25k is not a good carrier for the systemic delivery of siRNA [Merkel et al., 2009]. Therefore, the R3V4 peptide may be more useful than PEI25k for the systemic delivery of siRNA.

Third, the R3V6 peptide was as efficient as PEI25k in terms of siRNA delivery. Luciferase-, GFP-, and VEGF-silencing assays showed that the R3V6 peptide reduced gene expression as efficiently as PEI25k (Figs. 5, 6, and 7). Furthermore, flow cytometry showed that FITC-labeled siRNA entered the cells as efficiently as PEI25k (Fig. 8). In a previous report, the R3V6 peptide was also evaluated as a plasmid DNA carrier [Ryu et al., 2011]. The results showed that the R3V6 peptide could deliver plasmid DNA into the cells as efficiently as PLL but much less efficiently than PEI25k. Considering that PLL does not have high transfection efficiency, the R3V6 peptide was not useful for plasmid DNA delivery. Plasmid DNA has a much higher molecular weight compared with that of siRNA. The R3V6 peptides may not form stable plasmid DNA/peptide complexes because of large differences between the molecular weights of plasmids and R3V6 peptides. Because of its low molecular weight, the R3V6 peptide may not form a tight complex with plasmid DNA. On the contrary, the molecular weight of the R3V6 peptide is similar to that of siRNA, suggesting that a much smaller amount of peptides is required for the formation of a complex between the siRNA and the peptide. In addition, the siRNA/R3V6 complex is tighter than the siRNA/PEI25k complex. Therefore, the R3V6 peptide is more suitable for the delivery of small nucleic acids such as siRNA than large plasmid DNAs.

In a gel retardation assay, R3V6 retarded siRNA completely at a 1:5 weight ratio (Fig. 2). However, R3V6 had the highest siRNA delivery efficiency at a 1:20 weight ratio (Fig. 4). R3V6 might neutralize negative charge at a 1:5 weight ratio and therefore, siRNA/R3V6 complex did not have mobility in the gel. However, siRNA/R3V6 complex at a higher weight ratio may have positive surface charge. The positive surface charge of the complex may facilitate the interaction with negatively charged cell membrane, which is beneficial to the delivery of siRNA into the cells.

We demonstrated that short peptide micelles, such as those formed by the R3V6 peptide, can efficiently transfer siRNA to the mammalian cells. As our results have demonstrated, the silencing efficiency of the R3V6 peptide complex with siRNA is comparable to that of the siRNA/PEI25k complex. More importantly, unlike PEI25k, R3V6 peptides are non-toxic to cells. Over all, as water-soluble self-assembled amphiphilic peptide micelles, the R3V6 peptides have potential as an efficient siRNA delivery carrier.

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