molecular pharmaceutics

Review

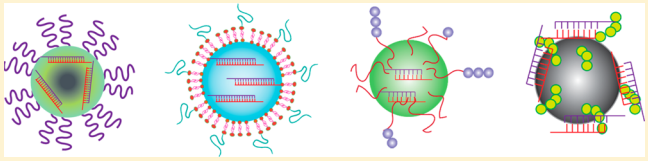
pubs.acs.org/molecularpharmaceutics

Peptides Used in the Delivery of Small Noncoding RNA

Ravi S. Shukla, Bin Qin, and Kun Cheng*

Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri—Kansas City, Kansas City, Missouri 64108, United States

ABSTRACT: RNA interference (RNAi) is an endogenous process in which small noncoding RNAs, including small interfering RNAs (siRNAs) and microRNAs (miRNAs), post-transcriptionally regulate gene expressions. In general, siRNA and miRNA/miRNA mimics are similar in nature and activity except their origin and specificity. Although both siRNAs and miRNAs have been extensively studied as novel therapeutics for a wide range of diseases, the large molecular weight, anionic surface charges, instability in blood circulation, and intracellular trafficking to the RISC after cellular uptake have hindered the translation of these RNAs from bench to clinic. As a result, a great variety of delivery systems have been investigated for safe and effective delivery of small noncoding RNAs. Among these systems, peptides, especially cationic peptides, have emerged as a promising type of carrier due to their inherent ability to condense negatively charged RNAs, ease of synthesis, controllable size, and tunable structure. In this review, we will focus on three major types of cationic peptides, including poly(L-lysine) (PLL), protamine, and cell penetrating peptides (CPP), as well as peptide targeting ligands that have been extensively used in RNA delivery. The delivery strategies, applications, and limitations of these cationic peptides in siRNA/miRNA delivery will be discussed.



KEYWORDS: *siRNA, miRNA, RNA delivery, cationic peptide, PLL, protamine, cell penetrating peptide*

1. INTRODUCTION

As one of the most remarkable findings over the past 15 years, RNA interference (RNAi) is an endogenous process in which small noncoding RNAs, including small interfering RNAs (siRNAs) and microRNAs (miRNAs), post-transcriptionally regulate gene expressions by binding to their complementary mRNAs.[1] Due to its unique roles in regulating the stabilities and functions of mRNAs, RNAi has emerged as a promising alternative for the treatment of various diseases and attracted substantial attention.[2−5]

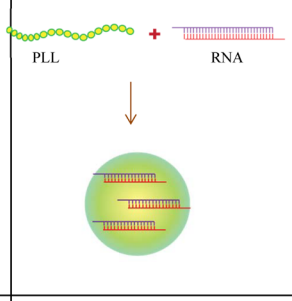
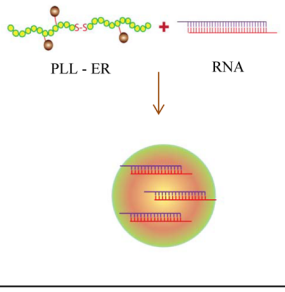
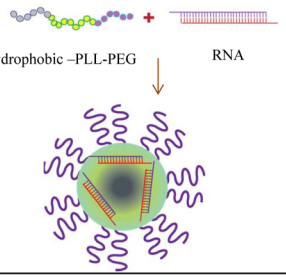
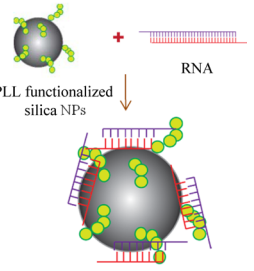
Once inside cells, siRNAs are unwound by an ATP-dependent helicase and the antisense strand is incorporated into the RNA-induced silencing complex (RISC). Subsequently, the antisense strand guides the RISC to its complementary mRNAs in a very specific way and triggers the degradation of target mRNAs. Unlike siRNAs, miRNAs encoded in the genome are transcribed into primary miRNAs (pri-miRNAs) in the nucleus. The pri-miRNAs are then processed by ribonuclease Drosha to form ∼75 nucleotide (nt) long hairpin precursor miRNAs (pre-miRNAs), which are translocated to the cytoplasm. The Dicer cleaves the pre-miRNAs to form mature miRNAs, which are duplex RNAs. The miRNA strand (also termed guide strand) is then separated from its complementary strand and incorporated into the RISC, followed by binding to its target mRNAs to suppress translation or trigger degradation of the mRNAs.

In general, siRNA and miRNA/miRNA mimics are similar in nature and activity except their origin and specificity. siRNAs are artificial double-stranded RNAs of 19−21 nt in length, while miRNAs are endogenous single-stranded RNAs of 21−25 nt. Endogenous miRNAs may be either downregulated or upregulated in a pathological condition and can be brought back to normal level by miRNA replacement therapy or miRNA inhibition therapy.[6] siRNAs are always exogenous and need to be delivered into cell cytoplasm to silence an overexpressed disease gene. Another major difference between siRNA and miRNA is their specificity to target mRNAs. A single siRNA forms a perfect match to its complementary mRNA and only induces the degradation of its target mRNA. On the contrary, a single miRNA may target hundreds of mRNAs that can form imperfect matches. Unlike exogenous siRNAs, endogenous miRNA cannot be used for therapeutic applications. Instead, synthetic miRNA mimics (RNA duplexes containing the guide strand of the miRNA) are always used in miRNA replacement therapy.

Although both siRNAs and miRNAs have been extensively studied as novel therapeutics for a wide range of diseases, the large molecular weight, anionic surface charges, instability in blood circulation, and intracellular trafficking to the RISC after cellular uptake have hindered the translation of these RNAs from bench to clinic.[2] As a result, a great variety of delivery systems have been investigated for safe and effective delivery of small noncoding RNAs.[7−9] Among them, cationic peptides have emerged as a promising type of carrier due to their inherent ability to condense negatively charged RNAs, ease of synthesis, controllable size, and tunable structure for tailoring physicochemical properties and targetability of the cargo. In

Received: June 17, 2014
Revised: August 20, 2014
Accepted: August 26, 2014
Published: August 26, 2014

Table 1. PLL-Based RNA Delivery Systems

	PLL Alone with RNA	PLL with Endosomolytic agents	Micelles	PLL-Silica nanoparticles
				
Advantage	• Positive charges on PLL allow good electrostatic interaction • Enhanced stability of encapsulated siRNA in the serum	• Endosomolytic agents enhance endosomal release of polyplexes • Enhanced stability of encapsulated siRNA in the serum	• Hydrophobic core reduces particle size • PEG in the outer corona enhances blood circulation time	• Well-defined structure • Stable, non-toxic, and biocompatible • Easy to adjust drug loading and release kinetics
Disadvantage	• Nonspecific binding to serum proteins	• Very few studies in siRNA delivery	• Low siRNA loading • Instability in systemic circulation	• Relatively new field in siRNA delivery • Safety of the inorganic materials is a concern

general, three approaches have been used for cationic peptide-mediated RNA delivery: covalent conjugation of cationic peptides to one strand of RNA duplex; noncovalent complexation of cationic peptides with RNA; and inclusion of cationic peptides as a condensing agent in a lipid or polymeric carrier. Moreover, peptides can also be used as targeting ligands in RNA delivery systems.

Direct covalent conjugation is the easiest strategy but less successful because of technical difficulties in synthesizing the cationic peptide−siRNA conjugates and neutralization of the positive charges, which are vital for membrane translocation and cellular uptake.[10] Some of the cationic peptide conjugated siRNAs fail to improve gene silencing effect *in vivo* compared to unmodified siRNA.[11] It is possibly due to insufficient amount of cationic peptides, which fail to effectively condense negatively charged siRNAs in this approach.[12]

In contrast, noncovalent complexation of cationic peptides with RNA exhibits a significant gene silencing effect *in vitro* and *in vivo*.[13−15] Peptides used in such a strategy are usually composed of two domains: a hydrophilic domain and a hydrophobic domain. The hydrophilic domain contains positively charged amino acids, such as ariginine (arg), lysine (lys), and histidine (his), to provide at least a net positive charge of +8.[15−17] The positive charge allows the condensation of RNA and enables multiple hydrogen bondings with anionic cell membrane to facilitate cellular uptake. The hydrophobic domain contains tryptophan (trp) and phenylalanine (phe) residues, which enhance interaction with the lipid bilayer of cells.[15]

Moreover, one terminal extreme of the cationic peptides can be modified with hydrophobic moieties, such as cholesterol, stearic acid, and cholic acid to enhance hydrophobicity.[9,18] The other terminal of the peptide can be conjugated with relatively hydrophilic moieties, such as polyethylene glycol (PEG). These constructs can form micelle-like structure after mixing with siRNA and efficiently deliver them to target cells.[9,18] To enhance stability and circulation time, a number of block copolymers of peptides, such as $mPEG_{2000}$-PLA_{3000}-*b*-R_{15},[19] PEI-*g*-(PLL-*b*-PEG),[18] and mPEG-PLGA-*b*-PLL [where PLGA is poly(lactic-*co*-glycolic acid)],[20] have been synthesized. Alternatively, cationic peptides such as protamine can be used as the condensing component of nanocomplex to encapsulate negatively charged RNAs.[21,22]

miRNA and siRNA can also be delivered using plasmid vector to achieve long-term activity.[23] However, delivery of these plasmid forms falls within the purview of DNA delivery and therefore beyond the scope of this article. In this review, we will focus on three major types of cationic peptides, including poly(L-lysine) (PLL), protamine, and cell penetrating peptides (CPP), as well as peptide targeting ligands that have been extensively used in RNA delivery. The delivery strategies, applications, and limitations of these cationic peptides in siRNA/miRNA delivery will be discussed. Although most of the RNA delivery systems mentioned in this article are initially designed for siRNA, they can also be used for miRNA delivery because of the similar size and chemical properties between siRNA and miRNA mimics.

2. POLY(L-LYSINE) IN RNA DELIVERY

Poly(L-lysine), also known as alpha poly(L-lysine) or PLL, is one of the first nucleic acid carriers reported back in 1989. PLL can be synthesized by a living polymerization of *N*-carboxyanhydrides (NCA), which provides narrow chain length distributions and the ability to obtain high molecular weight poly(peptide) polymers.[24] Various molecular weights ranging from 500 Da to more than 100 kDa of PLL are commercially available.[25,26] Among them, PLL ranging from 2.4 to ∼30 kDa have been exploited for siRNA delivery,[27] and PLL with the molecular weight >70 kDa is mainly recommended for enhancing cell adhesion to solid surface.[28] Naturally, polylysine appears as ε-poly-L-lysine (or EPL), which is produced by bacterial fermentation. The amide bond of EPL is formed between the carboxyl terminal and the epsilon-amino groups.[29] EPL contains similar positive charges as PLL and has been used in condensing nucleic acids.[28]

PLL is a well optimized cationic peptide for condensation of DNA molecules by electrostatic interaction between positively charged amino acids of PLL and the phosphate backbone of DNA. However, the molecular weight and topology of DNA molecules are very different from those of small noncoding

dx.doi.org/10.1021/mp500426r | *Mol. Pharmaceutics* 2014, 11, 3395−3408

RNA and other small oligonucleotides. Therefore, the findings from DNA complexation with cationic polymers, such as PLL, cannot be directly extrapolated to siRNA and miRNA. In fact, many factors, including polymer molecular weight, salt concentration, pH, charge ratio, and mixing order, can affect the complexation of siRNA with PLL.[30] For example, Zheng et al. have compared complexation characteristics of PLL with a long double-stranded DNA (dsDNA) and a 21nt double-stranded oligonucleotide (ds-oligo) which is structurally similar to a 21nt siRNA against firefly luciferase gene in the same condition.[30] The ds-oligo forms a compact rod shape structure with high scattering intensity in complexation with PLL. On the contrary, the dsDNA forms a coil conformation with PLL in aqueous media. These conformations are governed by charge density, rigidity, and chain length of the nucleic acids. The coiled structure of dsDNA prevents an ideal match of each base pair with PLL, leading to the formation of a loose structure. The linear, flexible, and shorter chain length of the ds-oligo interacts with PLL in a more ordered manner that allows the formation of a compact structure. However, even though the ds-oligo forms a dense complex, the scatter density gradually decreases with incubation time due to slow dissociation of the complex. Dissociation of the ds-oligo PLL complex exposes oligonucleotides to external medium, leading to reduction of the transfection efficiency of these complexes. By contrast, long chains of DNA entangle together and stabilize the complexes with PLL.[30]

Although PLL has been extensively used and characterized as a cationic carrier for siRNA delivery, there are few reports of successful siRNA delivery by simple complexation of siRNA with linear PLL alone. The potential reasons behind this may be toxicity, poor endosomal release, and nonspecific binding of linear PLL to serum proteins.[31,32] To circumvent these limitations, several derivatives of PLL have been developed to improve the efficacy of small noncoding RNA delivery (Table 1).

2.1. PLL Conjugates with Endosomal Disrupting Agents. One possible reason for the insufficient transfection efficacy of PLL is its inability to be released from endosomes (Table 1). Following endocytosis, a large proportion of the siRNA−PLL complex is retained in endosomes and then transported to lysosomes, where it is digested by hydrolase enzymes. This might be attributed to the absence of fusogenic groups to facilitate endosomal release of siRNA.[33] Consequently, various endosomal releasing residues, such as fusogenic peptides, chloroquine, and histidine, have been conjugated to PLL to overcome this problem.[31,33] The imidazole ring of histidine is a weak base that is positively charged at endosomal pH (∼6) and facilitates endosomal disruption by the proton sponge mechanism. Conjugation of histidine to PLL has shown significant improvement in transfection efficiency.[34,35] For example, a reducible copolypeptide (rCPP) composed of a histidine-rich peptide (HRP) and a nuclear localization sequence (NLS) peptide was developed for siRNA delivery.[36] In this system, three lysine residues were utilized to condense siRNA, and six histidine residues were inserted to promote endosomal release of the complex. In another study, branched histidine-lysine rich (HK) peptides containing four (H3K4b and H2K4b) or eight (H3K8b) terminal branches were synthesized and compared for effective delivery of siRNA targeting β-galactosidase (β-gal) expressed in SVR-bag4 cells. The siRNA complex made with the H3K8b peptide showed silencing activity up to ∼80%.[36]

2.2. PEGylated PLL. The presence of positive charges on the surface of PLL/siRNA complexes is believed to enhance cell internalization. However, interaction of the positively charged PLL/siRNA complexes with negatively charged serum proteins may cause undesired aggregation and RES uptake, which consequently decrease the therapeutic outcome of siRNA. Modification of PLL with polysaccharides, PEG, or other water-soluble polymers is the major strategy to enhance the systemic circulation profile of PLL/siRNA complexes by decreasing nonspecific interaction with blood components. PEGylation of PLL leads to formation of particles with a core−shell structure. Such particles contain a cationic fragment in the inner core that helps to condense nucleic acids. The uncharged hydrophilic PEG outer layer helps to reduce particle size, cytotoxicity, and nonspecific interaction with blood components, leading to prolonged systemic circulation.[18,37] However, shielding of the cationic core with PEG may inhibit the interaction of these complexes with the negatively charged cell membrane and eventually decrease cellular uptake and endosomal release.[38,39] To overcome these obstacles, siRNA delivery systems containing cleavable PEG spacers have been developed.[40] The cleavable spacer allows easy detachment of PEG from carriers at the site of destination. Cleavable linkers such as pH-sensitive, reduction-sensitive (disulfide), and enzyme-sensitive linkers have been used to tether cleavable PEG in polymeric and lipid based nanocarriers, and similarly can also be utilized for peptide-based siRNA delivery systems.[40−43]

2.3. PLL Amphiphilic Block Copolymers. In recent years, PLL-based triblock copolymers composed of a cationic core, a hydrophobic moiety, and an amphiphilic PEG chain have been designed for siRNA delivery (Table 1). The order of PLL, PEG, and hydrophobic core can be adjusted to achieve the desired property. For example, Guo et al. synthesized a PLL derivative in which the backbone was modified with cholic acid on one side and PEG on another side with a pH-sensitive linker, benzoic imine.[18] Cationic micelles were formed with a core−shell structure containing a hydrophobic cholic acid in the core and a hydrophilic segment, where siRNA is condensed on the shell surface hanging with PEG. The presence of a pH-cleavable linker allows the release of PEG and decreases shielding in the vicinity of tumor cells, where the pH is slightly acidic. The cationic micelles show significant inhibition of a reporter gene and tumor growth in a mouse model of prostate cancer.

Due to its amphiphilic nature, the PLL triblock copolymer (hydrophobic core-PLL-PEG) can self-assemble into micelles prior to siRNA encapsulation.[44] These micelles consist of three layers: a hydrophobic core, PLL/siRNA in the middle layer, and PEG in the outer corona. Because of the covalent conjugation of the hydrophobic core to PLL and PEG, a dense layer of the PLL/siRNA complex is formed around the compressed hydrophobic core, leading to relatively smaller particles (micelle-polyplex) as compared to an siRNA/PLL-PEG physical mixture without a hydrophobic core, which is called a polyplex. Moreover, formation of the hydrophobic core prior to siRNA addition allows the PLL/siRNA complex to localize on the surface of the hydrophobic core, and the PEG layer is squeezed out to form the third layer.[44] However, siRNA encapsulation in the micelle formulation is lower than that of the polyplexes. A limited thickness of the PEG layer on the corona of micelles exhibits a higher zeta potential of the complex that does not vary with N/P (the molar ratio of the amine groups of a cationic peptide to the phosphate groups of

an RNA) ratio. In contrast, simple physical mixing of PLL-PEG and siRNA shows an increase in zeta potential with increase in N/P ratios, probably due to the availability of siRNA/PLL complexes near the surface.[44]

To compare the advantage of the micellar structure over polyplexes, a triblock polymer of monomethoxy poly(ethylene glycol)-poly(ε-caprolactone)-poly(L-lysine) (mPEG-PCL-PLL, abbreviated "M") with variable length PCL and a diblock polymer without PCL block (mPEG-PLL, abbreviated "P") were synthesized to form micelle and polyplex structures, respectively. Both the polymers "M" and "P" contain the same PLL and PEG length. The study compared the effect of the hydrophobic segment (PCL and its different lengths) on particle size, zeta potential, stability, and siRNA delivery efficiency. "M" polymers show small particle sizes because the hydrophobic PCL core is more compressible. The particles size depends only on the length of the PCL core and not on the N/P ratio used for siRNA condensation. As a result, "M" polymers containing longer PCL chains exhibit large micelles. Moreover, the zeta potential of micelles made with these polymers was higher compared to "P" polymers due to the association of the siRNA/PLL complex on the PCL surface, which was covered uniformly with the mPEG layer. Both the "P" and "M" polymers showed similar siRNA condensation ability confirmed by gel electrophoresis, suggesting that only the PLL fragment is responsible for siRNA binding through electrostatic interactions. However, "M" polymers exhibited stronger gene knockdown effect than "P" polymers. It could be due to the loose architecture and large volume of the "P" polymer lipoplex, which hinder cellular uptake of the siRNA.[44] This finding is in accordance with a recent report in which cholesterol was conjugated to a lysine containing peptide to form a micelle structure before complexation with siRNA. The cholesterol modified peptide demonstrated much higher condensation capability and transfection efficacy compared to the peptide carrier without cholesterol.[9]

The length of PLL in PLL-PEG block polymers is also important in determining siRNA delivery efficacy. For instance, Ambardekar et al. investigated the effect of PLL molecular weight on a delivery system containing nuclease-resistant cholesterol-siRNA (3′ end of the sense strand is modified with cholesterol), and a block polymer of PLL-PEG (5 kDa) in which the PLL molecular weight was variable from 10 to 50 kDa. Increasing the PLL block length from 10 to 50 kDa decreased the minimum N/P ratio required to form complex with siRNA. Compared to unmodified siRNA, cholesterol modified siRNAs required lower N/P ratio at the same PLL block length. In addition, an increase in PLL chain length also enhanced siRNA loading and serum stability. Moreover, the PLL-PEG/siRNA complexes inhibited the target gene in a PLL length-dependent manner in primary breast tumors after iv administration. The reason behind these observations could be that longer PLL chain protects siRNA from nuclease degradation in systemic circulation and consequently enhances the bioavailability.[45]

PLL based amphiphilic polymers can also be used for codelivery of siRNA and hydrophobic anticancer drugs to synergetically impede tumor growth. For instance, codelivery of the anticancer drug docetaxel (DTX) and Bcl-2-targeting siRNA in a micelle made by a triblock copolymer of poly(ethylene glycol)-poly(L-lysine)-poly(L-leucine) (PEG-PLL-PLLeu) was investigated. PLLeu served as the hydrophobic core to entrap DTX, while siRNA were condensed with PLL via electrostatic interaction. The resulting formulation exerted enhanced antitumor activity with a small dose of DTX in MCF-7 xenograft murine model. The therapeutic effect of the codelivery system was significantly higher compared to an individual dose of siRNA or DTX alone.[46]

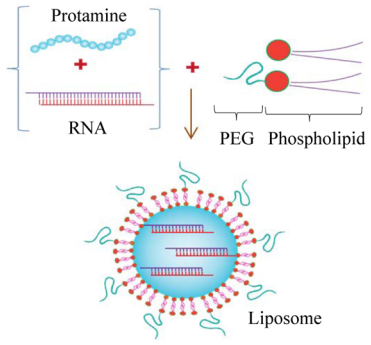
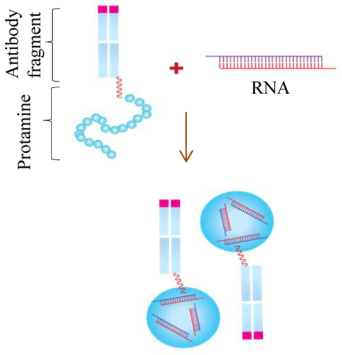
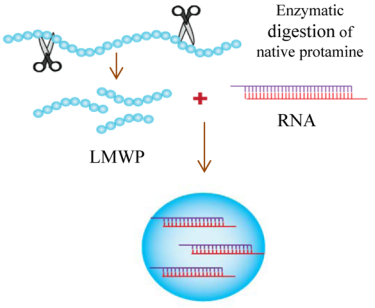
2.4. PLL Dendrimer. Dendritic PLLs have also been synthesized for efficient delivery of nucleic acids.[47] The earlier-developed G2 and G3 generation dendritic PLLs have shown efficient gene transfer into COS-7[48] and BHK[49] cells, respectively, and attracted scientists to develop higher generation dendrimers such as G5 and G6. These novel hyperbranched PLLs showed enhanced transfection efficiency similar to Lipofectin or SuperFact transfection reagent.[47] However, the absence of primary amine in the interior of these dendrimers minimizes the endosomal disruption property and consequently decreases the siRNA release in the cytoplasm. Therefore, dendritic PLL have been investigated in combination with endosome-disrupting agents to improve the transfection efficacy of siRNA. For example, Inoue et al. used Endo-Porter, a weakly basic amphiphilic peptide, to enhance the capability of KG6 (a sixth-generation PLL dendrimer) for efficient delivery of siRNA.[50] In addition, dendritic analogues of PLL can also be modified in terminal ends with histidine and arginine for effective endosomal disruption.[51]

2.5. Reducible PLL. High molecular weight PLLs exert prolonged cytotoxicity in clinical applications. As a result, several biodegradable PLL derivatives have been synthesized in recent years.[52,53] One strategy to improve the biodegradability and endosomal release of siRNA from PLL is to introduce reducible disulfide bonds that can cross-link low molecular weight PLL. The resulting PLL polymers can be degraded into small fragments via reduction of disulfide bonds in the cytoplasm. This enhances the release of nucleic acid cargo in the cytoplasm without addition of chloroquine or other endosomolytic agents and eventually increases the silencing effects. For instance, compared to a high molecular weight PLL (~20,900 Da), a 3200 Da reducible linear PLL showed significantly higher transfection efficiency with less cell cytotoxicity in several cell lines.[54]

Similarly, a reducible polycation consisting of histidine and polylysine (termed HIS RPC) was evaluated for siRNA delivery to avoid the use of endosomolytic agents, such as chloroquine, and enhance biodegradation.[55] Cys-His3-Lys3-His3-Cys (HIS3 RPC) and Cys-His6-Lys3-His6-Cys (HIS6 RPC) were synthesized by oxidative polycondensation (59 kDa and 113 kDa, respectively) and compared with Cys-Lys10-Cys (65 kDa). The presence of cysteine enabled the polymerization and intracellular degradation of the polymer. On the other hand, histidine residues enabled the buffering capacity of polymer in endosomal pH without the use of any endosomolytic agents. As a result, HIS6 RPC showed better results compared to its synthetic counterparts. Interestingly, when these HIS6 RPCs were evaluated for silencing of $p75^{NTR}$ gene, almost 100% knockdown was observed, akin to Oligofectamine but higher than PEI. In addition, HIS6 RPC mediated silencing of GFP gene was significantly higher than that of commercially available JetPEI.[55]

In another study, Stevenson et al. investigated the effect of the molecular weight of HIS6 RPCs on the knockdown effect of siRNA. HIS6 RPCs with four different molecular weights (38, 44, 80, and 114 kDa) were synthesized by controlling the time of oxidative polycondensation reaction. Only the 80 kDa HIS6 RPC exerted a higher condensation of siRNAs with

Table 2. Protamine-Based RNA Delivery Systems

	Protamine RNA complex in liposome	Protamine-antibody fusion	Low Molecular Weight Protamine (LMWP)
			
Advantage	• Neutral phospholipids on the surface of protamine/RNA complex avoid nonspecific protein interactions and enhance stability • PEG on the outer corona enhance systemic circulation • Surface can be functionalized with targeting ligands	• Cell-specific delivery • Enhanced therapeutic effect	• LMWP is non immunogenic • Low production cost
Disadvantage	• The NLS sequence in protamine may decrease cytoplasmic availability of RNA • Side effects of protamine such pulmonary hypertension and thrombocytopenia can be harmful	• Difficulty in preparation • Potential stability and nonspecific interaction problems	• Aggregation and nonspecific interaction in the blood • Very few studies

smaller particle size of ~80 nm. The RPCs can efficiently silence stably expressed EGFP in liver cell lines. On the contrary, siRNA combined with nonreducible PLL showed negligible activity. Moreover, incorporation of a hepatocyte-specific peptide sequence derived from the *Plasmodium falciparum* circumsporozite protein (HNMPNDPNRNVD-ENANANSAYC) exhibited an enhanced knockdown effect of HIS6 RPCs in hepatocytes but not nonliver cells.[56]

2.6. PLL Modified Mesoporous Silica Nanoparticles (MSN). Mesoporous silica nanoparticles (MSNs) are attractive nanocarriers for nucleic acid delivery. The well-defined structures of MSNs allow for controlled loading and release of entrapped siRNAs. Other advantages include good chemical and physical stability, nontoxicity, biocompatibility, higher drug-loading efficiency, and controllable drug release. Loading and release kinetics of siRNA in MSNs can be adjusted by modulating the pore size, shape, surface properties, and surface area of the MSNs.[57]

Because MSNs are anionic in nature, surface modification with cationic peptides or polymers is therefore required to deliver small noncoding RNAs (Table 1), for example, a large pore mesoporous silica nanoparticle (LP-MSN) functionalized with PLL through covalent immobilization. Compared to unmodified or amino modified MSNs, the PLL modified MSNs show far more efficiency in delivering siRNA into cancer cells and silence the oncogenes.[58]

3. PROTAMINE IN RNA DELIVERY

Protamine is an FDA approved, naturally occurring peptide of ~5000 Da obtained from sperm of salmon and certain other species of fish. Protamine sulfate injection, USP, is a sterile, nonpyrogenic, isotonic solution of protamine sulfate used as heparin antagonist in humans. It acts as a heparin antidote by forming a stable salt with heparin, which results in loss of anticoagulant activity of both the protamine and heparin.[59] Protamine rapidly neutralizes heparin, which starts within 5 min after iv administration.[60] However, heparin reversal using protamine is associated with several adverse effects.[61] Numerous mechanisms have been proposed for adverse reactions caused by protamine, including thromboxane generation, inhibition of carboxypeptidase, histamine release, complement activation, and immunological reactions. In addition, increase in vasodilator factors, such as nitic oxide, and depression of myocardial function, including bradycardia, leads to hypotension in patients treated with protamine. Moreover, direct toxic effects of protamine on the phospholipid membranes result in thrombocytopenia and leukopenia.[62] The presence of high arginine content (~67%) in protamine and inherent characteristics to condense negatively charged DNA in sperm has been extensively exploited in gene delivery.[61] A peptide containing a higher content of arginine (R) promotes nucleus entry through nuclear pore complexes (NPC). In line with these observations, protamine shows DNA uptake in the nucleus due to the presence of six consecutive arginine residues in its backbone.[63,64] In recent years, to reduce immunological toxicity mediated by native protamine, several low molecular weight protamines have been synthesized for siRNA delivery.[65,66]

Recently, protamine has been utilized by our group to enhance siRNA condensation.[67] A complex containing streptavidin, siRNA targeting poly(rC) binding protein 2 (PCBP2), and cholesterol (SSC) was formed by noncovalent interaction between biotin (present in siRNA and cholesterol) and streptavidin. The resulting complexes were stable in the

dx.doi.org/10.1021/mp500426r | *Mol. Pharmaceutics* 2014, 11, 3395−3408

serum but unable to enter cells due to the absence of positively charged component to neutralize siRNA. The incorporation of protamine in the SSC complex results in smaller size and higher cellular uptake in hepatic stellate cells (HSCs).[67] In another study, we conjugated protamine to the LNCaP specific peptide KYL (KYLAYPDSVHIW) to condense a FITC-labeled siRNA and showed high cellular uptake in the cells.[68]

Protamine has been extensively used for siRNA delivery. Several approaches including siRNA/protamine complexed within liposomes,[21] and antibody-protamine fusions,[69], have been investigated for effective siRNA delivery (Table 2).

3.1. Liposomal Delivery of Protamine-Condensed siRNA. Liposome is the most commonly used nucleic acid delivery system. To efficiently entrap small RNAs in liposomes, cationic agents, such as protamine, are usually used to enhance the condensation and encapsulation of small RNAs in liposome-based systems (Table 2). For example, β-7 integrin-targeted liposomes were prepared for the delivery of cyclin D1 (CyD1) siRNA.[21] CyD1 governs the proliferation of normal and malignant cells, and its overexpression is observed in the colon during inflammatory bowel disease.[70,71] Liposomes were prepared using neutral phospholipid to avoid toxicity associated with cationic lipids. The liposomes were stabilized with surface decoration of hyaluronan, which was then conjugated with β-7 integrin-targeted antibodies. siRNA was encapsulated by rehydration of the liposomes in the presence of protamine/siRNA complex. The resulting nanocarriers showed more than 80% siRNA loading while maintaining nanoscale dimensions. *In vivo* administration of the liposomal formulations exhibited significant knockdown of CyD1 in leukocytes and reversed experimentally induced colitis.[21]

Similarly, a sigma receptor-targeted liposomal formulation of siRNA against human survivin was prepared. Sigma receptors are membrane-bound proteins overexpressed in various human tumor cells, including breast cancer, prostate cancer, and NSCLC, and exert higher binding affinity to benzamide derivatives such as anisamide.[72] To prepare a targeted delivery system, siRNA and calf thymus DNA (1:1 weight ratio) were mixed together with protamine to obtain electrostatic complex. These complexes were further coated with cationic liposomes consisting of cholesterol and DOTAP (1:1 molar ratio) to form liposome-polycation-DNA (LPD) nanoparticles. Consequently, to enhance the systemic circulation and targetability of the resulting LPD nanoparticles, PEG conjugated with anisamide on the terminal end was tethered on the nanoparticle surface. Calf thymus DNA in the LPD nanoparticles is essential to increase the delivery efficiency (20−80%) of particles while reducing particle size up to 10−30%. Higher protamine concentrations altered the net surface charge of protamine-DNA/siRNA complex to slightly positive and therefore decreased the interaction with cationic liposomes and lowered the encapsulation efficiency of siRNAs. Compared to LPD functionzlized with PEG alone, these anisamide functionalized nanocarriers showed enhanced siRNA uptake and survivin mRNA knockdown in sigma receptor-overexpressing cells H1299.[22]

In another study, an RGD-targeted LPD siRNA delivery system was prepared following the procedure developed by Li et al.[22] to knock down VEGFR-2 (also referred to as fetal liver kinase-1 (Flk-1) in angiogenic cells). To target angiogenic cells, a cyclic Arg-Gly-Asp (RGD) peptide that specifically binds to integrins expressed on tumor-associated endothelial cells was attached to the terminal end of PEG in LPD nanoparticles. The RGD peptide modified formulation showed enhanced uptake and silencing of VEGFR-2 in two endothelial cell lines.[73]

3.2. Antibody-Protamine Fusion Proteins. Tissue- and cell-specific delivery of small noncoding RNAs is a key obstacle to their therapeutic applications. One way to deliver small RNAs to target cells is to make fusion proteins of protamine with targeting antibodies (Table 2). Protamine in these fusion proteins helps in siRNA condensation because of its cationic nature, while antibodies allow cell-specific targeting.[74] Several proteins, antigens, and receptors, such as human integrin lymphocyte function associated antigen-1 (LFA-1),[75] epidermal growth factor receptor family member ErbB2 (HER2),[20] prostate-specific membrane antigen (PSMA),[76] and HIV envelope proteins,[69] are overexpressed in specific cells and have been utilized as targets for siRNA delivery using the protamine antibody fusion strategy. For instance, the recombinant fusion protein of protamine to HER-2 specific single-chain fragmented antibodies (ScFvs) (named F5-P) successfully delivered Polo-like kinase 1 (PLK1) siRNAs into Her2(+) breast cancer cell lines and primary human cancers in orthotopic breast cancer models. Silencing of the target gene induced apoptosis of Her2(+) breast cancer cell lines. When injected intravenously, the F5-P/PLK-1-siRNA complex showed significant accumulation in orthotopic Her2(+) breast cancer xenografts, leading to suppressed PLK1 gene expression and tumor cell apoptosis.[20]

In another study, a fusion protein containing the heavy chain of a Fab fragment (F105) specific for the HIV envelope protein gp160 and protamine (named F105-P) was constructed to deliver siRNA to HIV-infected cells or cells expressing exogenous HIV envelope glycoprotein gp160 (HIV *env*).[69] To investigate the targeting efficacy of the F105-P/siRNA complex in HIV *env* expressing Jurkat cells, FITC-siRNA were transfected either alone or with the unmodified F105 antibody or with F105-P. As a result, the F105-P/siRNA complex showed significantly higher uptake only in HIV *env* positive cells. Interestingly, siRNA alone or simple mixture with unmodified antibody (without protamine) showed negligible cellular uptake. Similarly, intravenous or intratumoral injection of the F105-P/siRNA complex was carried out in mice bearing HIV *env* expressing B16 melanoma cells. A cocktail of siRNAs targeting the cell cycle (c-myc), apoptosis (mdm2), and angiogenesis (VEGF) significantly reduced tumor growth only in HIV *env* positive tumors but not in normal tissue or in envelope-negative tumors. Intravenous injections of the complex also showed higher accumulation and inhibition of malicious genes in tumors.[69]

3.3. Low Molecular Weight Protamine (LMWP). Protamine, although effective in nucleic acid delivery, may have adverse effects such as mild hypotension to severe or ultimately fatal cardiac arrest and immunological responses. This has led researchers to develop nontoxic low molecular weight protamine (LMWP).[77−79] LMWPs are nontoxic arginine-rich peptides derived from native protamine by enzymatic digestion with thermolysin.[79−81] Briefly, digestion is carried out by incubation of thermolysin with protamine for 30 min at room temperature, and peptide fragments are separated using a heparin affinity column. Five peptides, thermolysin-digested segment of protamine (TDSP) 1−5, are obtained from the process.[82] Among them, TDSP4 containing a mixture of 2 tridecyl peptides with sequences of VSRR-RRRGGRRRR and ASRRRRRGGRRRR and TDSP5 (VSRR-RRRRGGRRRR) maintain the heparin-neutralizing ability.[66]

However, due to the similarity of their structures to TAT_{47-57} peptides, only TDSP5 has been used for siRNA and protein delivery.[82] (Table 2) In addition, these LMWPs have been suggested to be clinically safe delivery carriers, as neither an antigenic nor a mutagenic response was elicited when tested on a dog model.[79]

siRNA delivery using LMWPs was as effective as the TAT_{47-57} peptide, a known potent CPP. Choi et al. used these LMWPs for the delivery of siRNA against VEGF.[65] In an *in vitro* experiment on carcinoma cells, high cytoplasmic accumulation of fluorescently tagged siRNA was observed within a short period of time, leading to significant down-regulation of VEGF. Intraperitoneal injection of the LMWP/siRNA complexes also delivered the siRNA into tumors, knocked down VEGF expression, and eventually inhibited tumor growth. In addition, the LMWP/siRNA complex did not induce the expression of cytokines including interleukin (IL)-12 and interferon (IFN)-α, suggesting good safety in animals.[65]

In another proof-of-concept study, LMWP was used to enhance brain delivery of nanoparticles.[83] In this context, poly(ethylene glycol)-poly(lactic acid) (PEG-PLA) nanoparticles were modified with thiolated LMWP. The resulting nanoparticles exhibited significantly enhanced cellular accumulation in 16HBE140 cells without cytotoxicity. Further intranasal administration of coumarin-6-loaded LMWP surface functionalized nanoparticles showed significantly higher fluorescence accumulation in the rat cerebellum, cerebrum, olfactory tract, and olfactory bulb compared to nonfunctionalized nanoparticles. This study clearly suggested that brain delivery of nanoparticles can be enhanced by surface functionalization with LMWP. Moreover, this strategy can be employed for the brain delivery of siRNA and diagnostic and other therapeutic agents.[83]

LMWP was also investigated for the delivery of miR-29b to human stem cells to induce osteogenic differentiation.[84] Arginine rich LMWP (VSRRRRRRGGRRRR) was synthesized to condense human miRNA-29b sequence (sense: 5′-UAGCACCAUUUGAAAUCAGUGUU). The size of the resulting particles is small (30−50 nm) and dependent on the N/P ratios. To confirm the functional activity of the miR-29b on osteoblastic differentiation in hMSCs, real-time RT-PCR was employed to evaluate the expression of osteogenic gene markers such as COL1A1, ALP, Runx2, OPN, OCN, and TAZ. As a result, except COL1A1, mRNA levels of all osteogenic markers increased at 48 h, which was higher than that observed by using lipoplex delivery system of the same miRNA.[84]

4. CELL PENETRATING PEPTIDE (CPP) IN RNA DELIVERY

In 1988, two independent groups found that the transcription trans-activating (TAT) protein of HIV-1 can enter cells by crossing the cell membrane.[85,86] Later on, the first CPP, penetratin (pAntp, RQIKIYFQNRRMKWKK), was identified from the third helix homeodomain of the *Drosophila* Antennapedia protein.[87,88] The minimal TAT sequence (YGRKKRRQRRR) that mediates cellular uptake was also identified.[89] Since then, several cationic and/or amphiphilic CPP peptides containing 5−30 amino acids with the ability to cross the cell membrane and deliver attached cargo have been discovered. The most commonly used CPPs include transportan,[90] VP22,[91] model amphipathic peptide (MAP),[92] and synthetic arginine-rich peptides.[16,93] The classification and characteristics of individual CPPs have been extensively reviewed elsewhere.[94,95]

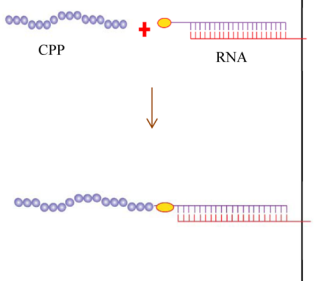
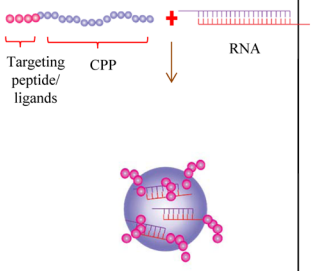
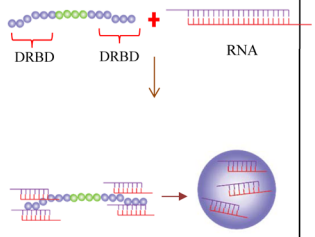
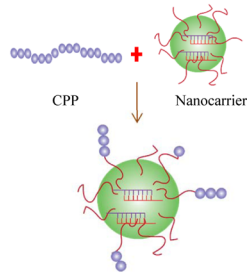
The uptake mechanism for cationic cell-penetrating peptides has been extensively studied but is not fully understood. Early studies demonstrated that fluorophore-conjugated CPP was predominantly taken up in a receptor-, energy-, and temperature-independent manner.[88,96] The methods used in these studies were later found to be inherently flawed. For example, when using confocal microscopy, the cell fixation protocol using methanol could cause artifactual redistribution of fluorescent signals from cell membrane to cytosol and nucleus. Furthermore, quantitative methods such as flow cytometry may have overestimated the uptake rate because they failed to distinguish between extracellular association and internalized fluorescence signals.[97] Since then, studies on internalization mechanism were mostly conducted in live cells instead of fixed cells, and a more thorough washing step, using trypsin or heparin, was introduced to remove membrane bound fluorescent CPPs prior to flow cytometry analysis.

Now it is widely accepted that both endocytotic and nonendocytotic pathways are involved in the cellular uptake of CPPs. In both cases, internalization begins with the interaction of peptides and extracellular matrix, such as electrostatic interactions, hydrophobic interactions, and hydrogen bonds. Positively charged CPPs strongly associate with the plasma membrane by binding to polysulfated and negatively charged cell-surface heparin proteoglycans, including syndecans and glypicans, which are commonly linked with specific core proteins via a GlcA-Gal-Gal-Xyl-Ser linkage.[98] Another common interaction is the electrostatic interaction between positively charged residues of the peptides and the anionic-charged phospholipid head groups. In the case of arginine-rich peptides, such as R9, the binding affinity of the peptides to heparin proteoglycan is greater than to phospholipid head groups.[99] Chemical modification of the peptide with stearic acid or cholesterol could increase the hydrophobicity and affinity to lipid bilayers.[9,100] It has also been postulated that peptides containing arginines are favorable for counterion-mediated membrane translocation. Guanidinium groups in arginine tend to form bidentate hydrogen bonds with anions to reduce charge repulsion with adjacent arginines.[101,102]

The cellular entry pathway of CPPs can be affected by their cargo. The features of cargo (size and type) and loading method (covalent or noncovalent binding) can influence the internalization mechanism. Many CPPs that are attached to a large cargo or complexed with nucleic acid are taken up via endocytotic pathway. Endo-Porter is a histidine- and leucine-containing, cationic amphipathic peptide that is able to deliver siRNA and morpholino-RNA into cells when noncovalently bound to these nucleic acids.[103,104] Although endocytotic pathway inhibitors, such as cytochalasin D and dynasore, do not block the Endo-Porter-mediated knockdown, lower temperature (4 °C) does abolish the gene silencing effect, suggesting that the internalization of Endo-Porter/siRNA complex is an energy-dependent process.[103] Similarly, gene silencing effect of the MPGα peptide/siRNA complex and cholesteryl peptide micelle/siRNA complex is completely or partially reversed at lower temperature (4 °C), indicating that endocytosis may be the primary pathway accounting for the functional delivery of siRNA.[9,105]

Some peptides, such as penetratin, can be translocated through the lipid bilayer of unilamellar vesicles without the assistance of any cell membrane proteins.[106−108] These reports

Table 3. CPP-Based RNA Delivery Systems

	CPP siRNA covalent conjugate	CPP siRNA noncovalent complex	Double stranded RNA binding domains (DRBD) siRNA complex	CPP modified nanocarriers
				
Advantage	• Well-defined conjugation with the flexibility of adding a cleavable linker • Ensures that the CPP-siRNA conjugate remains intact in the systemic circulation	• Easy to prepare • Targeting peptide ligands can be fused with CPP	• Only binds to double stranded RNA with high affinity but not to double stranded DNA • Fusion with TAT peptides allows transfection of primary cells	• Enhanced cellular uptake of nanocarriers
Disadvantage	• May reduce or completely abolish the effectiveness of CPPs by neutralizing positively charged amino acids	• Poor endosomal escape • High serum protein binding	• Protein expression and purification make this process tedious and expensive	• May exert serum protein binding and aggregation

support the existence of nonendocytotic pathway of peptides. Several hypothetical models have been proposed to explain the nonendocytotic pathway of CPP.[96,108−110] One of the most popular models is "inverted micelle formation". In this model, the cationic peptide first associates with the plasma membrane via electrostatic interaction to transiently form an inverted micelle. The inverted micelle structure allows CPPs to cross the hydrophobic environment of the phosphate lipid tail and release the cargo into the cytoplasm.[111] Alternatively, cationic peptide/siRNA complexes may insert into the membrane and form a transient transmembrane β-structure to allow the complex to pass through cells.[13,112]

In general, due to the highly dynamic structure of cell membrane, the interaction of CPPs with cell surface at the molecular level has been studied in the presence of a lipid system to mimic the cell membrane. In aqueous medium, CPP forms a negligible amount of secondary structures, which transforms into alpha- or beta-structures in the presence of lipid medium. These secondary structures in the presence of a lipid system (mimicking the cell membrane) are often oriented in such a way that favors CPP translocation through the cell membrane.[94,113] The inherent ability of CPPs to enhance cellular uptake and translocate to different intracellular compartments, such as the nucleus, mitochondria, and cytoplasm, has been utilized extensively in siRNA delivery. Approaches ranging from electrostatic noncovalent complexes to the synthesis of CPP−siRNA conjugates and CPP-functionalized nanoparticles have been developed for siRNA delivery (Table 3).

4.1. CPP−siRNA Conjugates. CPP−siRNA covalent conjugate enables well-defined one to one conjugation with the flexibility of incorporating a cleavable linker between the siRNA and CPP (Table 3). In addition, covalent conjugation also ensures that the CPP−siRNA conjugate remains intact in the systemic circulation. The direct conjugation approach may, however, minimize or completely abolish the effectiveness of CPP by neutralizing its positively charged amino acids, which are vital for cellular translocation. To avoid these obstacles to some extent, less negatively charged nucleic acid analogues, such as peptide nucleic acid (PNA) or phosphorodiamidate morpholino oligonucleotides (PMOs), have been investigated.[114] CPP's steric hindrance, the effective dose ratio between CPP and siRNA, the stability/cleavability of the linker, and the intracellular localization of siRNA after uptake are other important factors that need to be considered before using this approach.

Although the covalent conjugation approach is not widely used for siRNA delivery, there are a few studies showing effective siRNA delivery using this approach. For instance, Muratovska et al. conjugated penetratin and transportan to thiol-containing siRNAs targeting luciferase or green fluorescent protein (GFP) transgenes. The resulting disulfide bond containing conjugates showed reduction of the target genes in several mammalian cell lines. Moreover, the silencing effect of the CPP/siRNA conjugate was equivalent to or better than that of cationic liposomes.[115]

4.2. CPP/siRNA Noncovalent Complexes. At an optimal charge or molar ratio, the dense positive charges on CPP allow electrostatic condensation with siRNAs. The nanoscale CPP/siRNA complexes can enter cells by interacting with proteoglycans on the cell surface.[116] These noncovalent complexes are easy to prepare and therefore can be formed on a large scale in a cost-effective manner (Table 3).

For instance, the MPG peptide (GALFLGFLGAAGSTMGAWSQPKKKRKV) has been used for siRNA delivery using the noncovalent complexation approach.[13,117] MPG is a fusion peptide derived from the nuclear localization signal (NLS) of SV40 large T antigen and HIV-1 gp41 protein. The presence of the NLS in these peptides allows siRNA accumulation in the nucleus, but cationic charges enable siRNA condensation.[13,117] However, replacement of a single lysine with serine in the cationic domain of the peptide resulted in siRNA delivery only to the cytoplasm, which demonstrated a stronger silencing effect compared to the unmutated sequence.[13] In another study, the MPG peptide was utilized for the delivery of miRNA-122 (miR 122) mimic and inhibitor into primary mouse liver hepatocytes, liver cell lines and *Caenorhabditis elegans*. The resultant delivery systems demonstrated efficient miRNA

Table 4. Peptide Targeting Ligands for RNA Delivery

targeting peptide	peptide sequence	cargo	targeting destination	ref
cyclic RGD peptide	RGD	siRNA inhibiting vascular endothelial growth factor receptor-2 (VEGF R2) expression	integrin receptor expressing neuroblastoma	124
gastrin-releasing peptides (GRPs)	GRP1, CGGNHWAVGHLM; GRP2, CKMYPRGNHWAVGHLM	siRNA against survivin	GRP receptor expressing breast MDA-MB 231 cancer cells	125
rabies virus glycoprotein (RVG)	YTIWMPENPRPGTPCDIFTNSRGK RASNGC	PPIL2 siRNA for BACE1 mRNA knockdown	Neuro-2a cells (brain)	133
preS1 peptides	PAFGANSNNPDWDFNPNK DQWPAANQVGGG	siRNA delivery along with 9 Arg (RRRRRRRRR)	HepG2 cells (liver)	126

delivery to regulate cholesterol metabolism.[118] The targetability of CPPs can be improved by attaching a peptide targeting ligand.[119] For example, to achieve tumor selectivity, a six amino acid peptide (A1) with high affinity for vascular endothelial growth factor receptor-1 (VEGFR1) was fused with the TAT peptide (termed TAT-A1). The resulting TAT-A1 exhibited higher siRNA delivery efficacy in cancer cells compared to TAT alone.[119]

Some cationic proteins contain canonical double stranded RNA (dsRNA) binding motifs, which can bind to dsRNA with high affinity but not to double stranded DNA.[120,121] Protein kinase R (PKR) is one of the well-studied dsRNA binding proteins. It contains two dsRNA binding domains (DRBD): an N-terminal domain (K_d = 3.8 × 10^{-7} mol/L) and a C-terminal domain (K_d = 2 × 10^{-7} mol/L).[121−123] Recently, PKR-DRBD was fused to the TAT peptide for siRNA delivery (named PTD-DRBD) (Table 3). The PTD-DRBD showed impressive siRNA delivery in many primary and transformed cells, including human embryonic stem cells, human umbilical vein endothelial cells, and T cells.[124] However, the propensity of TAT to interact with serum proteins such as glycosaminoglycans may retard their application in iv administration.[125] Moreover, nonspecific cell uptake by TAT may also induce several side effects.[126]

To overcome these hurdles, the TAT sequence was replaced with cell-homing peptides or receptor ligands to achieve cell-specific delivery of siRNA. Geoghegan et al. have developed a fusion protein consisting of two DRBD domains (2× DRBD) and three repeats of the B2 peptide sequence (GHKVKRPKG) in place of the TAT peptide.[127] The B2 peptide sequence, identified by phage display against recombinant transferrin receptor (TfR), showed enhanced TfR mediated uptake.[128] The resultant B2-2× DRBD/siRNA complexes significantly reduced the expression of a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), in HeLa cells. The silencing effect was further increased by the addition of chloroquine (an endosomal acidification inhibitor), suggesting endosomal entrapment of the B2-2× DRBD siRNA complex.[127]

4.3. CPP Modified Nanocarriers. CPPs, along with fusogenic and membrane-disruptive peptides, have been linked to the surface of nanoscale siRNA systems, including lipid nanoparticles, polymer nanoparticle, and inorganic material-based nanoparticles, to enhance their cellular uptake.[93,95] Kanazawa et al. prepared a micelle using the amphiphilic block copolymers poly(ethylene glycol) (MPEG)/polycaprolactone (PCL) conjugated with TAT peptide via a disulfide linkage (MPEG-PCL-SS-TAT) for effective delivery of a VEGF siRNA. In this system, TAT was used for siRNA condensation, while the disulfide bond was introduced for rapid dissociation by glutathione in the cell cytoplasm. The resulting 100−200 nm MPEG-PCL-SS-Tat/siRNA complexes were safe and exerted good silencing activity *in vitro* (S-180 sarcoma cells) and *in vivo*. Intravenous injection of these micelles (MPEG-PCL-SS-Tat/siRNA) exhibited a significantly stronger antitumor effect in S-180 tumor-bearing mice compared to MPEG-PCL-SS-TAT/control.[129] In another study, TAT peptide was conjugated to chitosan via a PEG linker. The resulting covalent conjugate was utilized for siRNA delivery to neuronal cells (Neuro-2a). These nanoparticles showed safe and effective siRNA delivery with higher reduction of the target ataxin-1 gene compared to nanoparticles made only with chitosan.[130]

5. PEPTIDES AS TARGETING LIGANDS

Apart from RNA condensation, peptides have also been used as targeting ligands in RNA delivery systems.[131−133] Several approaches, such as phage display technology and one-bead one-compound (OBOC) combinatorial bead library method, have been employed to identify peptide targeting ligand.[134] For example, Qin et al. identified a LNCaP specific peptide using the M13 phage display peptide library (Ph.D.-12). Four rounds of biopanning were carried out with LNCaP cells after precleaning on PC-3 cells to remove nonspecific peptides. As a result, a LNCaP cell specific peptide ligand KYLAYPDSV-HIW (also termed KYL peptide) was identified. The KYL peptide conjugated protamine successfully delivered FITC-labeled siRNA into LNCaP cells.[68] Table 4 summarizes some of the peptide targeting ligands that have been adopted for RNA delivery.

Cyclic RGD (cRGD) is a widely utilized peptide targeting ligand for various therapeutic agents including small noncoding RNAs. cRGD is the targeting ligand of the $\alpha_v\beta_3$ integrin, which plays important roles in the regulation of cell differentiation, progression, proliferation, and apoptosis. More importantly, the $\alpha_v\beta_3$ integrin is overexpressed in various cancer cells and promotes cancer cell growth and metastasis.[43,135] As a result, cRGD can be used to specifically deliver RNA carriers to cancer cells. For example, a cRGD decorated poly(lactic-*co*-glycolic acid) (PLGA) nanoparticle containing microRNA-132 (miR-132) was developed to transfect cultured endothelial cells before transplantation, thereby sensitizing the cells to endogenous growth factors.[136] In another study, a RGD-PEG decorated polycation liposomes (PCLs) containing tetraethylenepentamine (TEPA) was developed for efficient siRNA delivery. Gene silencing of the nanocomplexes was first optimized using a luciferase siRNA (siLuc2) in B16F10-luc2 murine melanoma cells stably expressing the luciferase 2 gene. Later on, the silencing activity was improved by grafting cholesterol on the 3′ end of the siRNA sense strand that allows better retention in the liposomes. This improved delivery system exhibited higher efficiency against metastatic B16F10-luc2 tumors in a mouse model.[137]

Peptides have also been used as brain-specific targeting ligands for siRNA delivery. For example, Manjunath et al. demonstrated that a 29 amino acid peptide derived from rabies virus glycoprotein (RVG) can specifically bind to the nicotinic acetylcholine receptor (AchR) on neuronal cells. The chimeric peptide consisting of the RVG peptide and nine arginines was mixed with siRNA and successful delivered the siRNA to neuronal cells in a dose-dependent manner. Moreover, the same chimeric peptide cannot deliver siRNA into AchR-negative HeLa cells, indicating the specificity of the chimeric peptide for neuronal cells.[138] In another study, the RVG peptide was decorated on the surface of the siRNA/trimethylated chitosan (TMC) complexes through bifunctional PEG for brain delivery of siRNA. The RVG peptide modified siRNA/TMC−mPEG complexes showed significantly higher uptake in AchR-positive Neuro-2a cells as well as in mouse brain compared to unmodified complexes. Moreover, siRNA encapsulated in these complexes exhibited potent knockdown of the BACE1 gene, a therapeutic target in Alzheimer's disease.[139]

6. SAFETY PROFILE OF PEPTIDE-BASED RNA CARRIERS

One major challenge in the clinical transition of RNA therapeutics is the development of an efficient RNA delivery system with a broad therapeutic window.[140] Therefore, low toxicity of peptide-based carriers is critical for its successful application in RNA therapy because the carrier peptide is mainly responsible for both efficacy and toxicity. The toxicity of a peptide-based carrier depends on the amine type, arrangement, molecular weight, and number of cationic charges per monomer unit.[141] In general, polypeptides are safe because of the presence of polyamide backbone that can be degraded in the body by proteolytic enzymes. Since L-amino acids are the components of naturally occurring polypeptides, the cellular proteolytic machinery does not recognize polypeptides made of D-amino acids. It has been observed that the CPP sequences containing D-amino acids induce higher toxicity than parent L-peptides due to enhanced stability against proteolytic enzymes present in intracellular environment.[142]

Barrett et al. recently synthesized numerous poly(amide)-based polymers using the *N*-carboxy anhydride (NCA) polymerization method and studied the structure−activity relationships (SAR) of these siRNA delivery carriers.[140] The fully D-isomer polymers PA Block (D-Orn:D-Phe) and poly(D-ornithine) homopolymer (PDO) were stable up to 2 h in the presence of protease cocktail, indicating negligible degradation of D-isomer polymers. On the other hand, the PA Block (D-Orn:L-Phe) showed little degradation, while the PA Block (L-Orn:D-Phe) showed modest degradation, indicating that L-Orn has better degradability as compared to L-Phe. The PA Block (L-Orn:L-Phe) and PLO (L-Orn) exhibited the highest degradation rate in the presence of protease cocktail. Similarly, a significant difference was observed in the plasma PK of the ^{14}C-mesyl conjugated nondegradable D-isomer PDO versus the degradable L-isomer PLO. A very slow elimination rate of PDO from the plasma was observed as compared to PLO.[140]

Amino acid residues can also alter the toxicity profile of polypeptides. For example, peptide nucleic acids (PNA) show nephrotoxicity after being conjugated to amphipathic peptide containing Ala, Leu, and Lys. However, addition of Arg sequence in the peptide does not show such toxicity.[143] Moreover, tissue distribution profiles of these peptides are dramatically different. Arg containing peptides show good splicing redirection in targeted adipose tissues even at a low dose of 2.5 mg/kg.[114,143]

7. CONCLUSION

Despite the great promise of small noncoding RNAs in treating various diseases, the effort of translating RNA therapeutics from bench to bedside has been hampered by several obstacles, such as formulation variations, aggregation in systemic circulation, nonspecific binding, and endosomal entrapment. A great variety of lipids, polymers, and peptides have been investigated in RNA delivery. Among them, peptides have attracted unique attention due to their ease of synthesis, controllable size, multiple functionalities, and tunable structure. Peptides can be used in an RNA delivery system as a cationic component, a cell penetrating component, a targeting ligand, or the hydrophobic portion of an amphiphilic carrier. While using peptides alone as a carrier may not be enough to efficiently deliver RNAs into cells, peptides can definitely be used as an essential part of a multicomponent RNA delivery system. For example, cationic peptides can be utilized as the condensing component to form a nanocomplex with RNAs. Peptide targeting ligands can also be used to modify an RNA delivery system to achieve targeted delivery. Therefore, combination of different strategies targeting each of the barriers is necessary to explore the safe and effective delivery of RNAs using peptide-based carriers. Nonspecific binding and stability of peptides in systemic circulation could be a potential hurdle for any peptide-based delivery systems. As a result, careful fabrication of the delivery system and even PEGylation are needed to guarantee the effectiveness of peptides. Possible immune response is another potential problem for some of the peptides used in RNA delivery. However, these are general problems associated with any peptide-based drug delivery systems, and therefore many strategies have been developed to overcome these problems.

Toxicity or therapeutic window of RNA therapeutics is another important issue that scientists need to consider during drug development. It is critical to use the minimum amount of peptide or polymer carriers in RNA therapeutics to avoid any possible toxicity associated with these carriers, thus leading to a broad therapeutic window.

Moving forward, we believe that peptides will continue playing critical roles in a significant portion of RNA delivery systems.

AUTHOR INFORMATION

Corresponding Author

*Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri—Kansas City, 2464 Charlotte Street, Kansas City, MO 64108. Phone: (816) 235-2425. Fax: (816) 235-5779. E-mail: chengkun@umkc.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work has been supported by Grant 1R01AA021510 from the NIH.

REFERENCES

(1) Cheng, K.; Mahato, R. I. Biological and therapeutic applications of small RNAs. *Pharm. Res.* **2011**, *28* (12), 2961−5.

(2) Leng, Q.; Woodle, M. C.; Lu, P. Y.; Mixson, A. J. Advances in Systemic siRNA Delivery. *Drugs Future* **2009**, *34* (9), 721.
(3) Mahato, R.; Qin, B.; Cheng, K. Blocking IKKalpha expression inhibits prostate cancer invasiveness. *Pharm. Res.* **2011**, *28* (6), 1357−69.
(4) Qin, B.; Cheng, K. Silencing of the IKKepsilon gene by siRNA inhibits invasiveness and growth of breast cancer cells. *Breast Cancer Res.* **2010**, *12* (5), R74.
(5) Tai, W.; Qin, B.; Cheng, K. Inhibition of breast cancer cell growth and invasiveness by dual silencing of HER-2 and VEGF. *Mol. Pharmaceutics* **2010**, *7* (2), 543−56.
(6) Karolina, D. S.; Jeyaseelan, K., microRNAs as therapeutic agents and targets. In *Advanced delivery and therapeutic applications of RNAi*; Cheng, K., Mahato, R. I., Eds.; John Wiley and Sons Ltd.: 2009; pp 439−82.
(7) Meyer, M.; Dohmen, C.; Philipp, A.; Kiener, D.; Maiwald, G.; Scheu, C.; Ogris, M.; Wagner, E. Synthesis and biological evaluation of a bioresponsive and endosomolytic siRNA-polymer conjugate. *Mol. Pharmaceutics* **2009**, *6* (3), 752−62.
(8) Wagner, E. Polymers for siRNA delivery: inspired by viruses to be targeted, dynamic, and precise. *Acc. Chem. Res.* **2012**, *45* (7), 1005−13.
(9) Qin, B.; Chen, Z.; Jin, W.; Cheng, K. Development of cholesteryl peptide micelles for siRNA delivery. *J. Controlled Release* **2013**, *172* (1), 159−68.
(10) Laufer, S., Detzer, A.; Sczakiel, G.; , and Restle, T., Selected Strategies for the Delivery of siRNA In Vitro and In Vivo. In *RNA Technologies and Their Applications*; Erdmann, V. A., Barciszewski, J., Eds.; Springer: Berlin, Heidelberg, 2010; pp 29−58.
(11) Moschos, S. A.; Jones, S. W.; Perry, M. M.; Williams, A. E.; Erjefalt, J. S.; Turner, J. J.; Barnes, P. J.; Sproat, B. S.; Gait, M. J.; Lindsay, M. A. Lung delivery studies using siRNA conjugated to TAT(48−60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. *Bioconjugate Chem.* **2007**, *18* (5), 1450−9.
(12) Turner, J. J.; Arzumanov, A. A.; Gait, M. J. Synthesis, cellular uptake and HIV-1 Tat-dependent trans-activation inhibition activity of oligonucleotide analogues disulphide-conjugated to cell-penetrating peptides. *Nucleic Acids Res.* **2005**, *33* (1), 27−42.
(13) Simeoni, F.; Morris, M. C.; Heitz, F.; Divita, G. Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. *Nucleic Acids Res.* **2003**, *31* (11), 2717−24.
(14) Crombez, L.; Morris, M. C.; Dufort, S.; Aldrian-Herrada, G.; Nguyen, Q.; Mc Master, G.; Coll, J. L.; Heitz, F.; Divita, G. Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth. *Nucleic Acids Res.* **2009**, *37* (14), 4559−69.
(15) Fang, B.; Guo, H. Y.; Zhang, M.; Jiang, L.; Ren, F. Z. The six amino acid antimicrobial peptide bLFcin6 penetrates cells and delivers siRNA. *FEBS J.* **2013**, *280* (4), 1007−17.
(16) Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* **2001**, *276* (8), 5836−40.
(17) Tunnemann, G.; Ter-Avetisyan, G.; Martin, R. M.; Stockl, M.; Herrmann, A.; Cardoso, M. C. Live-cell analysis of cell penetration ability and toxicity of oligo-arginines. *J. Pept. Sci.* **2008**, *14* (4), 469−76.
(18) Guo, J.; Cheng, W. P.; Gu, J.; Ding, C.; Qu, X.; Yang, Z.; O'Driscoll, C. Systemic delivery of therapeutic small interfering RNA using a pH-triggered amphiphilic poly-L-lysine nanocarrier to suppress prostate cancer growth in mice. *Eur. J. Pharm. Sci.* **2012**, *45* (5), 521−32.
(19) Zhao, Z. X.; Gao, S. Y.; Wang, J. C.; Chen, C. J.; Zhao, E. Y.; Hou, W. J.; Feng, Q.; Gao, L. Y.; Liu, X. Y.; Zhang, L. R.; Zhang, Q. Self-assembly nanomicelles based on cationic mPEG-PLA-b-Polyarginine(R15) triblock copolymer for siRNA delivery. *Biomaterials* **2012**, *33* (28), 6793−807.
(20) Yao, Y. D.; Sun, T. M.; Huang, S. Y.; Dou, S.; Lin, L.; Chen, J. N.; Ruan, J. B.; Mao, C. Q.; Yu, F. Y.; Zeng, M. S.; Zang, J. Y.; Liu, Q.; Su, F. X.; Zhang, P.; Lieberman, J.; Wang, J.; Song, E. Targeted delivery of PLK1-siRNA by ScFv suppresses Her2+ breast cancer growth and metastasis. *Sci. Transl. Med.* **2012**, *4* (130), 130ra48.
(21) Peer, D.; Park, E. J.; Morishita, Y.; Carman, C. V.; Shimaoka, M. Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science* **2008**, *319* (5863), 627−30.
(22) Li, S. D.; Huang, L. Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. *Mol. Pharmaceutics* **2006**, *3* (5), 579−88.
(23) Dai, L.; Wang, W.; Zhang, S.; Jiang, Q.; Wang, R.; Dai, L.; Cheng, L.; Yang, Y.; Wei, Y. Q.; Deng, H. X. Vector-based miR-15a/16-1 plasmid inhibits colon cancer growth in vivo. *Cell Biol. Int.* **2012**, *36* (8), 765−70.
(24) Kowtoniuk, R. A.; Pei, T.; DeAngelo, C. M.; Waldman, J. H.; Guidry, E. N.; Williams, J. M.; Garbaccio, R. M.; Barrett, S. E. Optimization of an α-(Amino acid)-N-carboxyanhydride polymerization using the high vacuum technique: Examining the effects of monomer concentration, polymerization kinetics, polymer molecular weight, and monomer purity. *J. Polym. Sci., Part A: Polym. Chem.* **2014**, *52* (10), 1385−91.
(25) Poly-L-lysine hydrobromide mol wt 500−2000, Sigma-Aldrich, 2013. Available from: http://www.sigmaaldrich.com/catalog/product/sigma/P8954?lang=en&region=US.
(26) Poly-L-lysine hydrobromide mol wt 40,000−60,000, Sigma-Aldrich, 2013. Available from: http://www.sigmaaldrich.com/catalog/product/sigma/P3995?lang=en&region=US.
(27) Yogasundaram, H.; Bahniuk, M. S.; Singh, H.-D.; Aliabadi, H. M.; Uludağ, H.; Unsworth, L. D. BSA Nanoparticles for siRNA Delivery: Coating Effects on Nanoparticle Properties, Plasma Protein Adsorption, and In Vitro siRNA Delivery. *Int. J. Biomater.* **2012**, *2012*, 10.
(28) Togo, Y.; Takahashi, K.; Saito, K.; Kiso, H.; Huang, B.; Tsukamoto, H.; Hyon, S.-H.; Bessho, K. Aldehyded Dextran and μ-Poly(L-lysine) Hydrogel as Nonviral Gene Carrier. *Stem Cells Int.* **2013**, *2013*, 634379.
(29) Moccia, M.; Roviello, G. N.; Bucci, E. M.; Pedone, C.; Saviano, M. Synthesis of a l-lysine-based alternate alpha,epsilon-peptide: a novel linear polycation with nucleic acids-binding ability. *Int. J. Pharm.* **2010**, *397* (1−2), 179−83.
(30) Zheng, C.; Niu, L.; Yan, J.; Liu, J.; Luo, Y.; Liang, D. Structure and stability of the complex formed by oligonucleotides. *Phys. Chem. Chem. Phys.* **2012**, *14* (20), 7352−9.
(31) Martin, M. E.; Rice, K. G. Peptide-guided gene delivery. *AAPS J.* **2007**, *9* (1), E18−29.
(32) Dash, P. R.; Read, M. L.; Fisher, K. D.; Howard, K. A.; Wolfert, M.; Oupicky, D.; Subr, V.; Strohalm, J.; Ulbrich, K.; Seymour, L. W. Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin. *J. Biol. Chem.* **2000**, *275* (6), 3793−802.
(33) Patil, M. L.; Zhang, M.; Minko, T. Multifunctional triblock Nanocarrier (PAMAM-PEG-PLL) for the efficient intracellular siRNA delivery and gene silencing. *ACS Nano* **2011**, *5* (3), 1877−87.
(34) Benns, J. M.; Choi, J. S.; Mahato, R. I.; Park, J. S.; Kim, S. W. pH-sensitive cationic polymer gene delivery vehicle: N-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer. *Bioconjugate Chem.* **2000**, *11* (5), 637−45.
(35) Midoux, P.; Monsigny, M. Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjugate Chem.* **1999**, *10* (3), 406−11.
(36) Leng, Q.; Scaria, P.; Zhu, J.; Ambulos, N.; Campbell, P.; Mixson, A. J. Highly branched HK peptides are effective carriers of siRNA. *J. Gene Med.* **2005**, *7* (7), 977−86.
(37) Mannisto, M.; Vanderkerken, S.; Toncheva, V.; Elomaa, M.; Ruponen, M.; Schacht, E.; Urtti, A. Structure-activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. *J. Controlled Release* **2002**, *83* (1), 169−82.

(38) Choi, Y. H.; Liu, F.; Kim, J. S.; Choi, Y. K.; Park, J. S.; Kim, S. W. Polyethylene glycol-grafted poly-L-lysine as polymeric gene carrier. *J. Controlled Release* **1998**, *54* (1), 39−48.
(39) Oupicky, D.; Ogris, M.; Howard, K. A.; Dash, P. R.; Ulbrich, K.; Seymour, L. W. Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol. Ther.* **2002**, *5* (4), 463−72.
(40) Oumzil, K.; Khiati, S.; Grinstaff, M. W.; Barthélémy, P. Reduction-triggered delivery using nucleoside-lipid based carriers possessing a cleavable PEG coating. *J. Controlled Release* **2011**, *151* (2), 123−30.
(41) Nakabayashi, H.; Ito, T.; Igawa, T.; Hiraiwa, Y.; Imamura, T.; Seta, T.; Kawato, M.; Usukura, N.; Takeda, R. Disopyramide induces insulin secretion and plasma glucose diminution: Studies using the in situ canine pancreas. *Metabolism* **1989**, *38* (2), 179−83.
(42) Suzawa, T.; Nagamura, S.; Saito, H.; Ohta, S.; Hanai, N.; Kanazawa, J.; Okabe, M.; Yamasaki, M. Enhanced tumor cell selectivity of adriamycin-monoclonal antibody conjugate via a poly(ethylene glycol)-based cleavable linker. *J. Controlled Release* **2002**, *79* (1−3), 229−42.
(43) Barve, A.; Jin, W.; Cheng, K. Prostate cancer relevant antigens and enzymes for targeted drug delivery. *J. Controlled Release* **2014**, *187*, 118−32.
(44) Qi, R.; Liu, S.; Chen, J.; Xiao, H.; Yan, L.; Huang, Y.; Jing, X. Biodegradable copolymers with identical cationic segments and their performance in siRNA delivery. *J. Controlled Release* **2012**, *159* (2), 251−60.
(45) Ambardekar, V. V.; Wakaskar, R. R.; Sharma, B.; Bowman, J.; Vayaboury, W.; Singh, R. K.; Vetro, J. A. The efficacy of nuclease-resistant Chol-siRNA in primary breast tumors following complexation with PLL-PEG(5K). *Biomaterials* **2013**, *34* (20), 4839−48.
(46) Zheng, C.; Zheng, M.; Gong, P.; Deng, J.; Yi, H.; Zhang, P.; Zhang, Y.; Liu, P.; Ma, Y.; Cai, L. Polypeptide cationic micelles mediated co-delivery of docetaxel and siRNA for synergistic tumor therapy. *Biomaterials* **2013**, *34* (13), 3431−8.
(47) Sheikhi Mehrabadi, F.; Fischer, W.; Haag, R. Dendritic and lipid-based carriers for gene/siRNA delivery (a review). *Curr. Opin. Solid State Mater. Sci.* **2012**, *16* (6), 310−22.
(48) Toth, I.; Sakthivel, T.; Wilderspin, A.; Bayele, H.; O'DONNELL, M.; Perry, D.; Pasi, K.; Lee, C.; Florence, A. Novel cationic lipidic peptide dendrimer vectors in vitro gene delivery. *STP Pharma Sci.* **1999**, *9* (1), 93−9.
(49) Shah, D. S.; Sakthivel, T.; Toth, I.; Florence, A. T.; Wilderspin, A. F. DNA transfection and transfected cell viability using amphipathic asymmetric dendrimers. *Int. J. Pharm.* **2000**, *208* (1), 41−8.
(50) Inoue, Y.; Kurihara, R.; Tsuchida, A.; Hasegawa, M.; Nagashima, T.; Mori, T.; Niidome, T.; Katayama, Y.; Okitsu, O. Efficient delivery of siRNA using dendritic poly(L-lysine) for loss-of-function analysis. *J. Controlled Release* **2008**, *126* (1), 59−66.
(51) Okuda, T.; Sugiyama, A.; Niidome, T.; Aoyagi, H. Characters of dendritic poly(L-lysine) analogues with the terminal lysines replaced with arginines and histidines as gene carriers in vitro. *Biomaterials* **2004**, *25* (3), 537−44.
(52) Ahn, C. H.; Chae, S. Y.; Bae, Y. H.; Kim, S. W. Synthesis of biodegradable multi-block copolymers of poly(L-lysine) and poly-(ethylene glycol) as a non-viral gene carrier. *J. Controlled Release* **2004**, *97* (3), 567−74.
(53) Jeong, J. H.; Park, T. G. Poly(L-lysine)-g-poly(D,L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. *J. Controlled Release* **2002**, *82* (1), 159−66.
(54) Nounou, M. I.; Emmanouil, K.; Chung, S.; Pham, T.; Lu, Z.; Bikram, M. Novel reducible linear L-lysine-modified copolymers as efficient nonviral vectors. *J. Controlled Release* **2010**, *143* (3), 326−34.
(55) Read, M. L.; Singh, S.; Ahmed, Z.; Stevenson, M.; Briggs, S. S.; Oupicky, D.; Barrett, L. B.; Spice, R.; Kendall, M.; Berry, M.; Preece, J. A.; Logan, A.; Seymour, L. W. A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. *Nucleic Acids Res.* **2005**, *33* (9), e86.
(56) Stevenson, M.; Ramos-Perez, V.; Singh, S.; Soliman, M.; Preece, J. A.; Briggs, S. S.; Read, M. L.; Seymour, L. W. Delivery of siRNA mediated by histidine-containing reducible polycations. *J. Controlled Release* **2008**, *130* (1), 46−56.
(57) Malmsten, M. Inorganic nanomaterials as delivery systems for proteins, peptides, DNA, and siRNA. *Curr. Opin. Colloid Interface Sci.* **2013**, *18* (5), 468−480.
(58) Hartono, S. B.; Gu, W.; Kleitz, F.; Liu, J.; He, L.; Middelberg, A. P. J.; Yu, C.; Lu, G. Q. M.; Qiao, S. Z. Poly-L-lysine functionalized large pore cubic mesostructured silica nanoparticles as biocompatible carriers for gene delivery. *ACS Nano* **2012**, *6* (3), 2104−17.
(59) Dehmer, G. J.; Lange, R. A.; Tate, D. A.; Pirwitz, M.; Daniel, W.; Fisher, M.; Bonnem, E. M. Randomized trial of recombinant platelet factor 4 versus protamine for the reversal of heparin anticoagulation in humans. *Circulation* **1996**, *94* (9 Suppl.), II347−52.
(60) *RxMed: Pharmaceutical Information - PROTAMINE SULFATE INJECTION*. 2002. Available from: http://www.rxmed.com/b.main/b2.pharmaceutical/b2.1.monographs/CPS-%20Monographs/CPS-%20(General%20Monographs-%20P)/PROTAMINE%20SULFATE%20INJECTION.html.
(61) Chang, L. C.; Lee, H. F.; Yang, Z.; Yang, V. C. Low molecular weight protamine (LMWP) as nontoxic heparin/low molecular weight heparin antidote (I): preparation and characterization. *AAPS PharmSci* **2001**, *3* (3), E17.
(62) Wakefield, T. W.; Lindblad, B.; Stanley, T. J.; Nichol, B. J.; Stanley, J. C.; Bergqvist, D.; Greenfield, L. J.; Bergentz, S.-E. Heparin and protamine use in peripheral vascular surgery: A comparison between surgeons of the Society for Vascular Surgery and the European Society for Vascular Surgery. *Eur. J. Vascular Surgery* **1994**, *8* (2), 193−198.
(63) Delgado, D.; del Pozo-Rodriguez, A.; Solinis, M. A.; Rodriguez-Gascon, A. Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: the importance of the entry pathway. *Eur. J. Pharm. Biopharm.* **2011**, *79* (3), 495−502.
(64) Tsuchiya, Y.; Ishii, T.; Okahata, Y.; Sato, T. Characterization of Protamine as a Transfection Accelerator for Gene Delivery. *J. Bioact. Compat. Polym.* **2006**, *21* (6), 519−37.
(65) Choi, Y. S.; Lee, J. Y.; Suh, J. S.; Kwon, Y. M.; Lee, S. J.; Chung, J. K.; Lee, D. S.; Yang, V. C.; Chung, C. P.; Park, Y. J. The systemic delivery of siRNAs by a cell penetrating peptide, low molecular weight protamine. *Biomaterials* **2010**, *31* (6), 1429−43.
(66) Chang, L. C.; Liang, J. F.; Lee, H. F.; Lee, L. M.; Yang, V. C. Low molecular weight protamine (LMWP) as nontoxic heparin/low molecular weight heparin antidote (II): in vitro evaluation of efficacy and toxicity. *AAPS PharmSci* **2001**, *3* (3), E18.
(67) Shukla, R. S.; Tai, W.; Mahato, R.; Jin, W.; Cheng, K. Development of streptavidin-based nanocomplex for siRNA delivery. *Mol. Pharmaceutics* **2013**, *10* (12), 4534−45.
(68) Qin, B.; Tai, W.; Shukla, R. S.; Cheng, K. Identification of a LNCaP-specific binding peptide using phage display. *Pharm. Res.* **2011**, *28* (10), 2422−34.
(69) Song, E.; Zhu, P.; Lee, S. K.; Chowdhury, D.; Kussman, S.; Dykxhoorn, D. M.; Feng, Y.; Palliser, D.; Weiner, D. B.; Shankar, P.; Marasco, W. A.; Lieberman, J. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* **2005**, *23* (6), 709−17.
(70) van Dekken, H.; Wink, J. C.; Vissers, K. J.; Franken, P. F.; Ruud Schouten, W.; WC, J. H.; Kuipers, E. J.; Fodde, R.; Janneke van der Woude, C. Wnt pathway-related gene expression during malignant progression in ulcerative colitis. *Acta Histochem.* **2007**, *109* (4), 266−72.
(71) Yang, R.; Bie, W.; Haegebarth, A.; Tyner, A. L. Differential regulation of D-type cyclins in the mouse intestine. *Cell Cycle* **2006**, *5* (2), 180−3.
(72) Aydar, E.; Palmer, C. P.; Djamgoz, M. B. A. Sigma Receptors and Cancer: Possible Involvement of Ion Channels. *Cancer Res.* **2004**, *64* (15), 5029−35.
(73) Vader, P.; Crielaard, B. J.; van Dommelen, S. M.; van der Meel, R.; Storm, G.; Schiffelers, R. M. Targeted delivery of small interfering

RNA to angiogenic endothelial cells with liposome-polycation-DNA particles. *J. Controlled Release* **2012**, *160* (2), 211−6.
(74) Vornlocher, H. P. Antibody-directed cell-type-specific delivery of siRNA. *Trends Mol. Med.* **2006**, *12* (1), 1−3.
(75) Peer, D.; Zhu, P.; Carman, C. V.; Lieberman, J.; Shimaoka, M. Selective gene silencing in activated leukocytes by targeting siRNAs to the integrin lymphocyte function-associated antigen-1. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (10), 4095−100.
(76) Su, Y.; Yu, L.; Liu, N.; Guo, Z.; Wang, G.; Zheng, J.; Wei, M.; Wang, H.; Yang, A. G.; Qin, W.; Wen, W. PSMA specific single chain antibody-mediated targeted knockdown of Notch1 inhibits human prostate cancer cell proliferation and tumor growth. *Cancer Lett.* **2013**, *338* (2), 282−91.
(77) Levy, J. H.; Zaidan, J. R.; Faraj, B. Prospective evaluation of risk of protamine reactions in patients with NPH insulin-dependent diabetes. *Anesth. Analg.* **1986**, *65* (7), 739−42.
(78) Weiler, J. M.; Gellhaus, M. A.; Carter, J. G.; Meng, R. L.; Benson, P. M.; Hottel, R. A.; Schillig, K. B.; Vegh, A. B.; Clarke, W. R. A prospective study of the risk of an immediate adverse reaction to protamine sulfate during cardiopulmonary bypass surgery. *J. Allergy Clin. Immunol.* **1990**, *85* (4), 713−9.
(79) Lee, L. M.; Chang, L. C.; Wrobleski, S.; Wakefield, T. W.; Yang, V. C. Low molecular weight protamine as nontoxic heparin/low molecular weight heparin antidote (III): preliminary in vivo evaluation of efficacy and toxicity using a canine model. *AAPS PharmSci* **2001**, *3* (3), E19.
(80) Park, Y. J.; Chang, L. C.; Liang, J. F.; Moon, C.; Chung, C. P.; Yang, V. C. Nontoxic membrane translocation peptide from protamine, low molecular weight protamine (LMWP), for enhanced intracellular protein delivery: in vitro and in vivo study. *FASEB J.* **2005**, *19* (11), 1555−7.
(81) Byun, Y.; Singh, V. K.; Yang, V. C. Low molecular weight protamine: a potential nontoxic heparin antagonist. *Thromb Res.* **1999**, *94* (1), 53−61.
(82) Park, Y. J.; Chang, L.-C.; Liang, J. F.; Moon, C.; Chung, C.-P.; Yang, V. C. Nontoxic membrane translocation peptide from protamine, low molecular weight protamine (LMWP), for enhanced intracellular protein delivery: in vitro and in vivo study. *FASEB J.* **2005**, *19* (11), 1555−7.
(83) Xia, H.; Gao, X.; Gu, G.; Liu, Z.; Zeng, N.; Hu, Q.; Song, Q.; Yao, L.; Pang, Z.; Jiang, X.; Chen, J.; Chen, H. Low molecular weight protamine-functionalized nanoparticles for drug delivery to the brain after intranasal administration. *Biomaterials* **2011**, *32* (36), 9888−98.
(84) Suh, J. S.; Lee, J. Y.; Choi, Y. S.; Chung, C. P.; Park, Y. J. Peptide-mediated intracellular delivery of miRNA-29b for osteogenic stem cell differentiation. *Biomaterials* **2013**, *34* (17), 4347−59.
(85) Frankel, A. D.; Pabo, C. O. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **1988**, *55* (6), 1189−93.
(86) Green, M.; Loewenstein, P. M. Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* **1988**, *55* (6), 1179−88.
(87) Heitz, F.; Morris, M. C.; Divita, G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br. J. Pharmacol.* **2009**, *157* (2), 195−206.
(88) Derossi, D.; Joliot, A. H.; Chassaing, G.; Prochiantz, A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* **1994**, *269* (14), 10444−50.
(89) Vives, E.; Schmidt, J.; Pelegrin, A. Cell-penetrating and cell-targeting peptides in drug delivery. *Biochim. Biophys. Acta* **2008**, *1786* (2), 126−38.
(90) Pooga, M.; Hallbrink, M.; Zorko, M.; Langel, U. Cell penetration by transportan. *FASEB J.* **1998**, *12* (1), 67−77.
(91) Elliott, G.; O'Hare, P. Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **1997**, *88* (2), 223−33.
(92) Oehlke, J.; Scheller, A.; Wiesner, B.; Krause, E.; Beyermann, M.; Klauschenz, E.; Melzig, M.; Bienert, M. Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically. *Biochim. Biophys. Acta* **1998**, *1414* (1−2), 127−39.
(93) Koren, E.; Torchilin, V. P. Cell-penetrating peptides: breaking through to the other side. *Trends Mol. Med.* **2012**, *18* (7), 385−93.
(94) Trabulo, S.; Cardoso, A. L.; Cardoso, A. M.; Morais, C. M.; Jurado, A. S.; Pedroso de Lima, M. C. Cell-penetrating peptides as nucleic acid delivery systems: from biophysics to biological applications. *Curr. Pharm. Des* **2013**, *19* (16), 2895−923.
(95) Wang, F.; Wang, Y.; Zhang, X.; Zhang, W.; Guo, S.; Jin, F. Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery. *J. Controlled Release* **2014**, *174*, 126−36.
(96) Derossi, D.; Calvet, S.; Trembleau, A.; Brunissen, A.; Chassaing, G.; Prochiantz, A. Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* **1996**, *271* (30), 18188−93.
(97) Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **2003**, *278* (1), 585−90.
(98) Poon, G. M.; Gariepy, J. Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. *Biochem. Soc. Trans.* **2007**, *35* (Part 4), 788−93.
(99) Goncalves, E.; Kitas, E.; Seelig, J. Binding of oligoarginine to membrane lipids and heparan sulfate: structural and thermodynamic characterization of a cell-penetrating peptide. *Biochemistry* **2005**, *44* (7), 2692−702.
(100) Anko, M.; Majhenc, J.; Kogej, K.; Sillard, R.; Langel, U.; Anderluh, G.; Zorko, M. Influence of stearyl and trifluoromethylquinoline modifications of the cell penetrating peptide TP10 on its interaction with a lipid membrane. *Biochim. Biophys. Acta* **2012**, *1818* (3), 915−24.
(101) Schmidt, N. W.; Lis, M.; Zhao, K.; Lai, G. H.; Alexandrova, A. N.; Tew, G. N.; Wong, G. C. Molecular basis for nanoscopic membrane curvature generation from quantum mechanical models and synthetic transporter sequences. *J. Am. Chem. Soc.* **2012**, *134* (46), 19207−16.
(102) Gooding, M.; Browne, L. P.; Quinteiro, F. M.; Selwood, D. L. siRNA delivery: from lipids to cell-penetrating peptides and their mimics. *Chem. Biol. Drug Des.* **2012**, *80* (6), 787−809.
(103) Bartz, R.; Fan, H.; Zhang, J.; Innocent, N.; Cherrin, C.; Beck, S. C.; Pei, Y.; Momose, A.; Jadhav, V.; Tellers, D. M.; Meng, F.; Crocker, L. S.; Sepp-Lorenzino, L.; Barnett, S. F. Effective siRNA delivery and target mRNA degradation using an amphipathic peptide to facilitate pH-dependent endosomal escape. *Biochem. J.* **2011**, *435* (2), 475−87.
(104) Summerton, J. E. Endo-Porter: a novel reagent for safe, effective delivery of substances into cells. *Ann. N.Y. Acad. Sci.* **2005**, *1058*, 62−75.
(105) Veldhoen, S.; Laufer, S. D.; Trampe, A.; Restle, T. Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: quantitative analysis of uptake and biological effect. *Nucleic Acids Res.* **2006**, *34* (22), 6561−73.
(106) Spinella, S. A.; Nelson, R. B.; Elmore, D. E. Measuring peptide translocation into large unilamellar vesicles. *J. Visualized Exp.* **2012**, No. 59, e3571.
(107) Terrone, D.; Sang, S. L.; Roudaia, L.; Silvius, J. R. Penetratin and related cell-penetrating cationic peptides can translocate across lipid bilayers in the presence of a transbilayer potential. *Biochemistry* **2003**, *42* (47), 13787−99.
(108) Binder, H.; Lindblom, G. Charge-dependent translocation of the Trojan peptide penetratin across lipid membranes. *Biophys. J.* **2003**, *85* (2), 982−95.
(109) Deshayes, S.; Gerbal-Chaloin, S.; Morris, M. C.; Aldrian-Herrada, G.; Charnet, P.; Divita, G.; Heitz, F. On the mechanism of non-endosomial peptide-mediated cellular delivery of nucleic acids. *Biochim. Biophys. Acta* **2004**, *1667* (2), 141−7.
(110) Schwarze, S. R.; Ho, A.; Vocero-Akbani, A.; Dowdy, S. F. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **1999**, *285* (5433), 1569−72.
(111) Trehin, R.; Merkle, H. P. Chances and pitfalls of cell penetrating peptides for cellular drug delivery. *Eur. J. Pharm. Biopharm* **2004**, *58* (2), 209−23.

(112) Crombez, L.; Charnet, A.; Morris, M. C.; Aldrian-Herrada, G.; Heitz, F.; Divita, G. A non-covalent peptide-based strategy for siRNA delivery. *Biochem. Soc. Trans.* **2007**, *35* (Part 1), 44−6.
(113) Eiriksdottir, E.; Konate, K.; Langel, U.; Divita, G.; Deshayes, S. Secondary structure of cell-penetrating peptides controls membrane interaction and insertion. *Biochim. Biophys. Acta* **2010**, *1798* (6), 1119−28.
(114) Jarver, P.; Coursindel, T.; Andaloussi, S. E.; Godfrey, C.; Wood, M. J.; Gait, M. J. Peptide-mediated Cell and In Vivo Delivery of Antisense Oligonucleotides and siRNA. *Mol. Ther. Nucleic Acids* **2012**, *1*, e27.
(115) Muratovska, A.; Eccles, M. R. Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. *FEBS Lett.* **2004**, *558* (1−3), 63−8.
(116) Sandgren, S.; Cheng, F.; Belting, M. Nuclear targeting of macromolecular polyanions by an HIV-Tat derived peptide. Role for cell-surface proteoglycans. *J. Biol. Chem.* **2002**, *277* (41), 38877−83.
(117) Meade, B. R.; Dowdy, S. F. Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. *Adv. Drug Delivery Rev.* **2007**, *59* (2−3), 134−40.
(118) Wang, L.; Tang, W.; Yan, S.; Zhou, L.; Shen, T.; Huang, X.; Dou, L.; Wang, M.; Yu, S.; Li, J. Efficient delivery of miR-122 to regulate cholesterol metabolism using a non-covalent peptide-based strategy. *Mol. Med. Rep.* **2013**, *8* (5), 1472−8.
(119) Fang, B.; Jiang, L.; Zhang, M.; Ren, F. Z. A novel cell-penetrating peptide TAT-A1 delivers siRNA into tumor cells selectively. *Biochimie* **2013**, *95* (2), 251−7.
(120) Bevilacqua, P. C.; Cech, T. R. Minor-groove recognition of double-stranded RNA by the double-stranded RNA-binding domain from the RNA-activated protein kinase PKR. *Biochemistry* **1996**, *35* (31), 9983−94.
(121) Schmedt, C.; Green, S. R.; Manche, L.; Taylor, D. R.; Ma, Y.; Mathews, M. B. Functional characterization of the RNA-binding domain and motif of the double-stranded RNA-dependent protein kinase DAI (PKR). *J. Mol. Biol.* **1995**, *249* (1), 29−44.
(122) Tian, B.; Mathews, M. B. Functional characterization of and cooperation between the double-stranded RNA-binding motifs of the protein kinase PKR. *J. Biol. Chem.* **2001**, *276* (13), 9936−44.
(123) Geoghegan, J. C.; Gilmore, B. L.; Davidson, B. L. Gene Silencing Mediated by siRNA-binding Fusion Proteins Is Attenuated by Double-stranded RNA-binding Domain Structure. *Mol. Ther.—Nucleic Acids* **2012**, *1*, e53.
(124) Eguchi, A.; Meade, B. R.; Chang, Y. C.; Fredrickson, C. T.; Willert, K.; Puri, N.; Dowdy, S. F. Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. *Nat. Biotechnol.* **2009**, *27* (6), 567−71.
(125) Wadia, J. S.; Stan, R. V.; Dowdy, S. F. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* **2004**, *10* (3), 310−5.
(126) Tünnemann, G.; Cardoso, M. C., Cell-Penetrating Peptides—Uptake, Toxicity, and Applications. *Membrane Active Peptides: Methods and Results on Structure and Function*; Castanho, M. A. R. B., Ed.; IUL Biotechnology Series 9; International University Line: La Jolla, CA, 2010; Chapter 14.
(127) Geoghegan, J. C.; Gilmore, B. L.; Davidson, B. L. Gene Silencing Mediated by siRNA-binding Fusion Proteins Is Attenuated by Double-stranded RNA-binding Domain Structure. *Mol. Ther. Nucleic Acids* **2012**, *1*, e53.
(128) Xia, H.; Anderson, B.; Mao, Q.; Davidson, B. L. Recombinant human adenovirus: targeting to the human transferrin receptor improves gene transfer to brain microcapillary endothelium. *J. Virol.* **2000**, *74* (23), 11359−66.
(129) Kanazawa, T.; Sugawara, K.; Tanaka, K.; Horiuchi, S.; Takashima, Y.; Okada, H. Suppression of tumor growth by systemic delivery of anti-VEGF siRNA with cell-penetrating peptide-modified MPEG-PCL nanomicelles. *Eur. J. Pharm. Biopharm.* **2012**, *81* (3), 470−7.
(130) Malhotra, M.; Tomaro-Duchesneau, C.; Prakash, S. Synthesis of TAT peptide-tagged PEGylated chitosan nanoparticles for siRNA delivery targeting neurodegenerative diseases. *Biomaterials* **2013**, *34* (4), 1270−80.
(131) Schiffelers, R. M.; Ansari, A.; Xu, J.; Zhou, Q.; Tang, Q.; Storm, G.; Molema, G.; Lu, P. Y.; Scaria, P. V.; Woodle, M. C. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* **2004**, *32* (19), e149.
(132) Sioud, M.; Mobergslien, A. Efficient siRNA targeted delivery into cancer cells by gastrin-releasing peptides. *Bioconjugate Chem.* **2012**, *23* (5), 1040−9.
(133) Huang, W.; Li, X.; Yi, M.; Zhu, S.; Chen, W. Targeted delivery of siRNA against hepatitis B virus by preS1 peptide molecular ligand. *Hepatol. Res.* **2013**, *44* (8), 897−906.
(134) Aina, O. H.; Sroka, T. C.; Chen, M. L.; Lam, K. S. Therapeutic cancer targeting peptides. *Biopolymers* **2002**, *66* (3), 184−99.
(135) Sutherland, M.; Gordon, A.; Shnyder, S. D.; Patterson, L. H.; Sheldrake, H. M. RGD-Binding Integrins in Prostate Cancer: Expression Patterns and Therapeutic Prospects against Bone Metastasis. *Cancers (Basel)* **2012**, *4* (4), 1106−45.
(136) Devalliere, J.; Chang, W. G.; Andrejecsk, J. W.; Abrahimi, P.; Cheng, C. J.; Jane-wit, D.; Saltzman, W. M.; Pober, J. S. Sustained delivery of proangiogenic microRNA-132 by nanoparticle transfection improves endothelial cell transplantation. *FASEB J.* **2014**, *28* (2), 908−22.
(137) Yonenaga, N.; Kenjo, E.; Asai, T.; Tsuruta, A.; Shimizu, K.; Dewa, T.; Nango, M.; Oku, N. RGD-based active targeting of novel polycation liposomes bearing siRNA for cancer treatment. *J. Controlled Release* **2012**, *160* (2), 177−81.
(138) Kumar, P.; Wu, H.; McBride, J. L.; Jung, K. E.; Kim, M. H.; Davidson, B. L.; Lee, S. K.; Shankar, P.; Manjunath, N. Transvascular delivery of small interfering RNA to the central nervous system. *Nature* **2007**, *448* (7149), 39−43.
(139) Gao, Y.; Wang, Z. Y.; Zhang, J.; Zhang, Y.; Huo, H.; Wang, T.; Jiang, T.; Wang, S. RVG-peptide-linked trimethylated chitosan for delivery of siRNA to the brain. *Biomacromolecules* **2014**, *15* (3), 1010−8.
(140) Barrett, S. E.; Burke, R. S.; Abrams, M. T.; Bason, C.; Busuek, M.; Carlini, E.; Carr, B. A.; Crocker, L. S.; Fan, H.; Garbaccio, R. M.; Guidry, E. N.; Heo, J. H.; Howell, B. J.; Kemp, E. A.; Kowtoniuk, R. A.; Latham, A. H.; Leone, A. M.; Lyman, M.; Parmar, R. G.; Patel, M.; Pechenov, S. Y.; Pei, T.; Pudvah, N. T.; Raab, C.; Riley, S.; Sepp-Lorenzino, L.; Smith, S.; Soli, E. D.; Staskiewicz, S.; Stern, M.; Truong, Q.; Vavrek, M.; Waldman, J. H.; Walsh, E. S.; Williams, J. M.; Young, S.; Colletti, S. L. Development of a liver-targeted siRNA delivery platform with a broad therapeutic window utilizing biodegradable polypeptide-based polymer conjugates. *J. Controlled Release* **2014**, *183*, 124−37.
(141) Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **2003**, *24* (7), 1121−31.
(142) Holm, T.; Raagel, H.; Andaloussi, S. E.; Hein, M.; Mae, M.; Pooga, M.; Langel, U. Retro-inversion of certain cell-penetrating peptides causes severe cellular toxicity. *Biochim. Biophys. Acta* **2011**, *1808* (6), 1544−51.
(143) Wancewicz, E. V.; Maier, M. A.; Siwkowski, A. M.; Albertshofer, K.; Winger, T. M.; Berdeja, A.; Gaus, H.; Vickers, T. A.; Bennett, C. F.; Monia, B. P.; Griffey, R. H.; Nulf, C. J.; Hu, J.; Corey, D. R.; Swayze, E. E.; Kinberger, G. A. Peptide nucleic acids conjugated to short basic peptides show improved pharmacokinetics and antisense activity in adipose tissue. *J. Med. Chem.* **2010**, *53* (10), 3919−26.