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## Functional peptides for siRNA delivery☆



Wanyi Tai, Xiaohu Gao\*

Department of Bioengineering, University of Washington, William H Foegle Building N561, Seattle, WA 98195, USA

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### ABSTRACT

siRNA is considered as a potent therapeutic agent because of its high specificity and efficiency in suppressing genes that are overexpressed during disease development. For nearly two decades, a significant amount of efforts has been dedicated to bringing the siRNA technology into clinical uses. However, only limited success has been achieved to date, largely due to the lack of a cell type-specific, safe, and efficient delivery technology to carry siRNA into the target cells' cytosol where RNA interference takes place. Among the emerging candidate nanocarriers for siRNA delivery, peptides have gained popularity because of their structural and functional diversity. A variety of peptides have been discovered for their ability to translocate siRNA into living cells via different mechanisms such as direct penetration through the cellular membrane, endocytosis-mediated cell entry followed by endosomolysis, and receptor-mediated uptake. This review is focused on the multiple roles played by peptides in siRNA delivery, such as membrane penetration, endosome disruption, targeting, as well as the combination of these functionalities.

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### 1. Introduction

Short interfering RNAs (siRNAs), also known as small interfering RNAs, are short 21–23 base-pair RNA duplexes including two 3' 2-nucleotide overhangs. The antisense strand of siRNA with sequence

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\* Corresponding author.

E-mail address: [xgao@uw.edu](mailto:xgao@uw.edu) (X. Gao).

complementarity to a target mRNA can induce sequence-specific gene expression suppression (silencing), which is referred to as RNA interference (RNAi) [1]. RNAi was first discovered in *Caenorhabditis elegans*, and later was found in plant, animal and human cells. Naturally, endogenous RNAi is a process to help regulate gene expression and function as an innate immune system to help defend viruses and other foreign genetic materials [2]. Taking advantage of the high selectivity and efficiency of this natural mechanism, RNAi has become an indispensable research tool for analysis of gene functions. The programmable degradation of target mRNA by RNAi also opens new opportunities to suppress protein expressions involved in disease pathogenesis [3]. Indeed, siRNA has been demonstrated to be a potential drug candidate for many difficult-to-treat diseases such as viral infections and cancers [4]. Many clinical trials have also been initiated over the last decade, but only very limited siRNA treatments have been approved by the FDA (e.g., topical administration of naked siRNA in ocular and retinal diseases) [5].

The primary barrier to its clinical applications is cell type-specific cytosolic delivery. siRNAs are densely negatively charged macromolecules with a molecular weight of ~14 kDa. They generally can't cross the cell membrane by passive diffusion like many small molecules and ions [6]. Moreover, siRNA trapped in endocytic compartments is susceptible to rapid degradation during endosome-lysosome trafficking [7], since most highly charged macromolecules are taken up by cells via endocytosis.

In this context, carriers that enable efficient siRNA delivery are required. A number of delivery technologies have been developed based on cationic lipids, polymers, dendrimers and peptides [6,8,9]. Among these non-viral carrier materials, peptides have gained increasing popularity because of their sequence and function diversity. Various combinations of the 20 natural amino acids result in peptides with different 3-D conformations, electric charges, polarity, hydrophobicity, and hydrophilicity. These unique sequences, within a relatively small molecule-weight range, can display various functions including siRNA binding, membrane penetration, endosome disruption, and targeting, all of which are essential for targeted siRNA delivery. Furthermore, as natural biomolecules, peptides (and their larger counterparts, proteins) with known functionalities are highly abundant in human bodies and other organisms, offering the possibility of directly picking functional peptides of interest from the enormous proteome libraries. For example, some viral coat proteins naturally carry out the biological function of shuttling exogenous biomaterials into cells, which can be useful for drug delivery. In addition to natural proteins, sequences that do not exist in current organisms can also be designed computationally or identified through panning techniques (e.g., phage and bacteria display). In this context, the aim of this review is to systematically discuss the broad functionalities of peptides and their applications in siRNA delivery. We will begin the discussion with individual functionalities such as membrane penetration, endosome disruption, and targeting, and proceed to multifunctional designs and applications.

## 2. Cell penetrating peptides: siRNA intracellular uptake

Cell-penetrating peptides (CPPs) are a class of short peptides that can penetrate the cell membrane and translocate into the cytoplasm. By chemical conjugation or noncovalent complex formation with siRNA, CPPs can also bring siRNA inside cells. As of now, more than 100 peptides with this cell-penetrating ability have been identified, and they exhibit a great variety in terms of amino acid sequence, length, and polarity. It is important to point out that although CPPs, as their names indicate, can penetrate through the cell membranes and directly enter the cytosol, most CPPs enter cells through multiple pathways, such as combinations of hole punching in the cell membrane and endocytic uptake. siRNA diffusion through the punched holes and entry into the cytosol leads to RNAi; whereas the endocytic uptake requires additional mechanisms to facilitate siRNA escape from the

intracellular compartments. Sharing no sequence homology, CPPs can be divided into several categories (Table 1).

### 2.1. Hydrophilic cationic peptides

Hydrophilic cationic CPPs contain one or more stretches of positive charges that are critical for siRNA condensation and membrane penetration. Due to the high charge density, cationic CPPs are highly hydrophilic and often exhibit random coils in aqueous solutions. Among basic amino acids, arginine plays the key role in membrane penetration [10]. Studies indicate that the guanidine group of arginine has a strong affinity to the negatively charged phospholipids in the cell membrane. As a result, arginine-based peptides have higher cell penetrating ability than cationic peptides without arginines [11]. A minimum of eight positive charges is required for efficient penetration across the cellular membrane [12], and the charged side-chains can quickly insert into the cell lipid bilayer and nucleate a transient pore, followed by the translocation of CPPs through the pore [13]. Because the pores quickly close as the peptides cross the plasma membrane, the cytotoxicity of CPPs is low, often undetectable in their working range for siRNA delivery.

The first CPP (Tat peptide, RKKRRQRRR) was discovered from the HIV-1 Tat protein in the late 1980s [14]. The Tat protein is a transcription regulator in the human immunodeficiency virus 1 (HIV-1) genome, and it can freely cross the plasma membrane [15]. Further study indicates that the transduction domain is confined in a small stretch of basic amino acids (amino acid 48–56) [16]. A chemically synthesized peptide of this stretch (the Tat peptide) can rapidly translocate across the plasma membrane and accumulate in the cell nucleus [16]. Since then, Tat peptide has been used as a universal carrier for intracellular delivery of a variety of cargo molecules. Chiu et al. covalently conjugated Tat peptide to the 3' end of siRNA antisense strand via a non-cleavable crosslinker SMPB (succinimidyl 4-(p-maleimidophenyl) butyrate) (Fig. 1A) [17]. After incubation with cells, the siRNA was quickly translocated inside cells as evidenced by a punctuate distribution of Cy3 labeled siRNA-Tat in the perinuclear region, which suggested that siRNA was primarily inside endosomes. Intriguingly, the siRNA-Tat conjugate was still able to induce targeted gene silencing (exogenously transfected eGFP and endogenous CDK9) likely due to partial siRNA release. An additional detailed study by Moschos et al., however, showed that siRNA-Tat conjugate purified with high-performance liquid chromatography (HPLC) was only able to induce 30% gene expression reduction at a siRNA concentration of 10  $\mu$ M, which was approximately two orders of magnitude higher than the concentration used by Chiu and co-workers [18]. Taking these results together, the silencing effect of the siRNA-Tat conjugate is likely induced by siRNA complexed with Tat instead of siRNA covalently linked to the Tat peptide since the peptide may cause steric hindrance for siRNA loading into the RNA-induced silencing complex (RISC), but systematic mechanistic studies are needed to confirm this hypothesis.

In general, conjugation of bulky chemical groups to siRNA can adversely affect siRNA incorporation into the RISC and consequently its gene silencing activity. In the RNAi pathway, internalized siRNA must be loaded into RISC, where the sense strand is cleaved during RISC activation and the antisense strand binds with the argonaute protein and directs gene silencing. Due to the compact space of siRNA binding pocket in RISC, terminal modification of siRNA often is intolerable, with the exception of some small chemical groups or cleavable bulky groups [19,20]. Considering the bulky structure of Tat peptide, peptide modification of siRNA terminus could dramatically limit RNAi activity.

Nonaarginine (R9) is an arginine oligomer and exhibits a higher arginine density than the Tat peptide [21]. Similarly, it can interact with siRNA through electrostatic interactions. In the presence of excess R9 peptides, the negative charges of siRNA backbone are neutralized and condensed by R9. Their final complexes are still highly positively charged due to the excess amount of R9, enabling membrane



(R8) with a long chain fatty acid promotes siRNA condensation, and the resulting highly condensed nanoparticle shows improved stability against particle disassembly and enzymatic degradation [26]. Furthermore, the complex formed using the modified R8 also exhibits 40–50 times higher cell uptake than the unmodified R8 [27].

## 2.2. Amphiphilic CPPs

The common feature of CPPs is that they are able to effectively cross the cellular membrane while carrying cargoes. In this process, the phospholipid bilayer in cell membrane prevents transportation of cargoes in and out of cells. Amphiphilic peptides, which share similar amphiphilic properties with phospholipids, can insert into the lipid bilayer and cross the cell membrane by formation of lipid rafts or transient channels. Many amphiphilic CPPs have been studied for intracellular delivery of siRNA. These amphiphilic CPPs are often classified into bipartite CPPs (based on their primary sequences) and  $\alpha$ -helical CPPs (based on surfaces or domains formed in the secondary structures).

### 2.2.1. Amphiphilic bipartite peptides

Amphiphilic bipartite CPPs are linear amphiphilic peptides with a hydrophobic domain at one end and a cationic domain at the other. The cationic end plays a major role in siRNA condensation, whereas the hydrophobic domain helps stabilize the CPP/siRNA complex through intermolecular hydrophobic interactions. Besides stabilizing the complex, the hydrophobic domain also tends to interact with the lipid bilayer, facilitating membrane fusion during cell entry. MPG (GLAFLGFLGAAGSTMGAWSQP~~KKRRKV~~) and Pep-1 (~~KEITWWETWWTE~~WSQP~~KKRRKV~~) represent two excellent examples of this type of peptide. The hydrophobic domain of MPG (underlined) is derived from the fusogenic sequence of the HIV glycoprotein 41 (gp 41), while that of Pep-1 (underlined) is a tryptophan-rich cluster that has high affinity to the lipid membrane [28]. Both MPG and Pep-1 contain a cationic nuclear localization signal (NLS) sequence (italic) that can bind with siRNA. A short peptide linker (bold) is inserted between the hydrophobic and hydrophilic domains to improve the flexibility of the bipartite peptides.

Among all the bipartite CPPs, MPG and its derivatives have been the major focus for siRNA delivery. MPG is originally designed for rapid and efficient delivery of plasmid DNA into the nucleus [29]. It was later optimized for siRNA delivery by a single mutation on the second lysine residue in NLS to serine (KK~~RRKV~~ to KSK~~RRKV~~, annotated as  $\Delta$ NLS), which abolishes the nuclear translocation function and enables rapid release of siRNA in the cytosol [30]. MPG $\Delta$ NLS and siRNA form complexes via electrostatic interactions, which are further stabilized by the gp41 hydrophobic domain, resisting enzymatic degradation (Fig. 1A2). siRNA intracellular delivery by MPG $\Delta$ NLS is enabled by direct membrane penetration, and the endocytic pathway only plays a minor role here [31]. Studies have shown that MPG $\Delta$ NLS interacts strongly with the phospholipid cell membrane through its hydrophobic gp41 sequence, which can adopt a transient  $\beta$ -sheet structure and insert into the plasma membrane. The  $\beta$ -sheet insertion temporarily changes cell membrane organization and creates a transient channel, allowing the siRNA/MPG $\Delta$ NLS complex to enter the cytoplasm [31–33]. MPG $\Delta$ NLS has been applied for siRNA transfection in a large panel of cells including primary cells and embryonic stem cells [34,35].

A 6-residue shorter version of the MPG $\Delta$ NLS, MPG-8, has also been developed to improve siRNA delivery efficiency [36]. Two hydrophobic residues (Phe<sup>7</sup> and Ala<sup>11</sup>) are mutated to Trp to promote membrane fusion. The siRNA delivery efficiency of MPG-8 has been compared with that of the parent MPG $\Delta$ NLS peptide by delivering anti-cyclin B1 (Cyc-B1) siRNA to HeLa and HS68 cells. At a similar peptide/siRNA molar ratio (20/1), MPG-8 condenses siRNA into nanocomplex with a diameter of  $120 \pm 50$  nm, significantly smaller than complexes formed with MPG $\Delta$ NLS (diameter:  $260 \pm 50$  nm), while its RNAi effect is 2-fold more potent than the original MPG $\Delta$ NLS. Further modification of this

peptide with cholesterol enhances its hydrophobicity, making the siRNA nanoparticles sufficiently stable in blood circulation. More importantly, the Chol-MPG-8 improves siRNA *in vivo* biodistribution under intravenous administration without activating the innate immune response [36].

Another derivative of the MPG $\Delta$ NLS peptide is MPG $\alpha$ , which differs from the parent peptide by six amino acids in the hydrophobic domain. The mutations in the gp41 domain allow this domain to adopt a helical conformation rather than a  $\beta$ -sheet structure when it approaches the phospholipid membrane [37]. Penetration experiments show that MPG $\alpha$  undergoes a conformational transition into  $\alpha$ -helix and can spontaneously insert into the phospholipid membranes. Further studies indicate that endocytosis is the main pathway for the uptake of MPG $\alpha$ /siRNA complexes, different from MPG $\Delta$ NLS. Despite the difference in cellular uptake mechanism, MPG $\alpha$  is equally effective in siRNA delivery when compared to MPG $\Delta$ NLS. Under optimal conditions, a model gene (Luciferase) was inhibited by up to 90% at a siRNA concentration of sub-nanomolar range [38]. A few other amphiphilic CPPs (such as BPrPp and Pep-1) have been reported, but only limited silencing has been achieved, likely due to their low binding affinity to siRNA [39,40].

### 2.2.2. Amphiphilic $\alpha$ -helical peptides

Besides the primary sequences (hydrophobic block), the amphiphilic property of a CPP can also be enabled by its secondary structure, such as the  $\alpha$ -helix structure. The amphiphilicity of  $\alpha$ -helix can be visualized through the helical wheel projection, in which hydrophilic and hydrophobic amino acids are shown in separate faces (Fig. 1B). **As membranolytic peptides, amphiphilic  $\alpha$ -helical CPPs generally have a cationic patch on one face for siRNA binding, whereas the other face is hydrophobic to embed into the lipid membrane. It is worth noting that most amphiphilic  $\alpha$ -helical CPPs are unstructured in aqueous solution, but spontaneously adopt the  $\alpha$ -helix configuration driven by interactions with anionic siRNA molecules or hydrophobic membrane lipids.**

Penetratin is the first amphiphilic  $\alpha$ -helical CPP discovered. The sequence of penetratin is derived from the third helix of the *Drosophila* Antennapedia protein homeodomain [41]. Penetratin adopts various secondary structures in different environments. It has been shown to adopt a  $\alpha$ -helix when in contact with a model membrane, become a random coil and  $\beta$ -strand in the cytoplasm, and switch to a  $\beta$ -sheet in the nucleus [42,43]. Penetratin binds with siRNA tightly at a relatively low peptide/siRNA molar ratio (10/1), resulting in high propensity of siRNA condensation and cell uptake. Despite the high uptake efficiency, penetratin/siRNA complex is mainly trapped inside the endosome/lysosome, resulting in no obvious gene silencing effect *in vitro* [39]. Surprisingly, penetratin has found to be able to transport exogenous siRNA into the cytoplasm when it is covalently conjugated with siRNA [44,45]. Muratovska and Eccles constructed a penetratin–siRNA conjugate via a disulfide bond formation (labile in the reducing environment of the cytoplasm). Using this approach, they were able to down-regulate luciferase reporter gene expression to a level equivalent or better than using cationic liposomes [44]. A similar result has also been reported by Davidson et al. [45]. In these studies, however, the penetratin–siRNA conjugates were not purified before testing on cells. Similar to the discussions above, there is a possibility that the RNAi effect is induced by siRNA complexed with penetratin (instead of conjugated). Indeed, when Moschos et al. used formamide as a disaggregant and purified the penetratin–siRNA conjugate by HPLC, only moderate suppression of p38 (20%) was observed using penetratin–siRNA conjugate at high concentration (10  $\mu$ M) [18]. Interestingly, the RNAi efficiency can be dramatically improved if the conjugation site to siRNA is switched from the 5'-end to 3'-end of the sense strand. 50% knockdown was obtained with a concentration of 1  $\mu$ M [46].

CADY is another amphiphilic  $\alpha$ -helical peptide designed for siRNA delivery. This 20-amino acid peptide can bind siRNA with high affinity through electrostatic and hydrophobic interactions. It is derived from two amphiphilic peptides: JST1 and PPTG1, but some residues are

mutated into Arg and Trp to improve siRNA binding ability and membrane fusion activity (Fig. 1B) [47,48]. The N- and C-termini of CADY are modified by acetyl and cysteamide groups to improve peptide stability and its cell membrane crossing efficiency, similar to the MPG peptides discussed above. Atomic force microscopy (AFM) studies have revealed that CADY molecules can self-assemble around siRNA and form a “raspberry”-like nanoparticle, in which each single complex constitutes a “drupelet” [49]. The penetrating mechanism of CADY/siRNA complex is not entirely clear yet, but a popular hypothesis is that the single complexes insert into the cell membrane through peptide conformational transition and induce membrane pinching, and translocate across the membrane through a transient channel [50]. The CADY/siRNA complexes can enter a wide variety of cell lines independent of the major endosomal pathway, leading to sustained knockdown of target genes at low siRNA concentrations (nanomolar range) with negligible toxicity and off-target effects [48].

Similarly, KALA is a cationic amphiphilic peptide that undergoes a pH-dependent conformational change from a random coil to amphiphilic  $\alpha$ -helical as the pH increases from 5.0 to 7.5 [51]. At physiological pH, one face of the  $\alpha$ -helix displays hydrophobic leucine residues, whereas the opposite face displays hydrophilic lysine residues (Fig. 1B). The cationic and amphiphilic properties facilitate siRNA condensation and cellular membrane penetration, which is useful for siRNA transfection. It has successfully transfected anti-GFP and -VEGF siRNAs into tumor cells and suppressed the target gene expressions by 80% [52,53]. Since KALA adopts a  $\alpha$ -helical structure at physiological pH, its toxicity can be a potential concern. It has been reported that KALA peptide exhibits strong hemolytic activity due to the nonspecific membrane disruption [54].

### 2.3. Targetable CPPs and activatable CPPs

The unique property of shuttling siRNA into virtually any cell makes CPPs potential carriers in RNAi therapy. However, this property is double-edged because CPPs enter cells without selectivity. For cell-type specific delivery, further developments are needed. A couple of strategies have been demonstrated recently by linking CPPs with other targeting ligands or making CPPs' cell entry capability activatable (initially masked, then activated by disease-associated enzymes).

#### 2.3.1. CPP-targeting ligand bioconjugates

The targeting ability of CPPs can be enhanced by linking them to a wide variety of ligands such as antibodies, aptamers, homing peptides, and small molecules. Monoclonal antibodies and their fragments are a popular choice for targeted drug delivery in general because of their high specificity, strong binding avidity, and broad availability. Genetic fusion or chemical conjugation with cationic CPPs makes antibodies positively charged for electrostatic interaction with siRNA (Fig. 1A3). Song et al. used an antibody-protamine fusion protein to deliver non-covalently bound siRNA into target cells to treat HIV and breast cancer [55,56]. Shankar's group conjugated oligo-9-D-arginine (DR9) peptide with a single-chain fragment variable (scFv) to deliver siRNA for suppressing HIV-1 infection in humanized mice [57]. Ma et al. reported a siRNA delivery platform based on a Lewis-Y specific antibody conjugated to a nine-arginine peptide (R9) [58]. Although the results are highly encouraging, the binding between antibody-CPP and siRNA relies on electrostatic interactions, which are prone to aggregation, and the excess positive charge of the final complexes may also induce non-specific binding during systemic delivery. To assess and improve this delivery strategy, Schneider et al. constructed a bispecific antibody that binds to cell-surface antigens and digoxigenin (Dig). In parallel, siRNA was digoxigeninylated at its 3' end. When mixed together, the bispecific antibody and siRNA self-assembled at the single molecule level and entered cells efficiently, avoiding the aggregation and non-specific binding problems discussed above. However, RNAi effect was not observed, because the antibody-siRNA complex was not able to

escape from the endosomal compartments [59]. A similar result was also reported by Cuellar et al. [60]. Overall, such coupling methodology is capable of creating monomeric antibody-siRNA conjugates with retained antibody and siRNA bioactivities, but additional endosomolytic functionality is needed for efficient RNAi [60].

Homing peptides are another category of popular ligands that can be linked with CPPs. Compared to antibody-CPP fusion proteins, homing peptide-CPP conjugates are much smaller (generally <50 amino acids) and can be chemically synthesized by automated solid-phase reactions. Panning technologies such as phage display have led to the discovery of a large number of homing peptides targeting a wide variety of target molecules [61–63]. Some of them have been linked to CPPs to enable targeted delivery of various cargos, including siRNA [64–67]. For example, Tat peptide has been conjugated to a homing peptide A1, a 6-mer peptide showing high affinity to vascular endothelial growth factor receptor-1 (VEGFR1), for selective delivery of siRNA into tumor cells in the presence of co-cultured normal cells [68]. Successful *in vivo* delivery of siRNA was also reported by using a chimeric peptide consisting of an RVG peptide and an R9 CPP. The 29-aa short RVG peptide is derived from the rabies virus glycoprotein (RVG). It specifically binds to its natural target acetylcholine receptor (AChR) that has been reported to be expressed in dendritic cells, macrophages, and neuronal cells. The RVG-R9 chimera peptide was shown to bind and deliver siRNA selectively, resulting in efficient gene silencing in AChR expressing cells. Post-intravenous injection, RVG-R9 was able to deliver HMGB1 siRNA to macrophages and dendritic cells and enable specific gene knockdown in these immune cells, and help suppress inflammation in the brain and blood [69,70]. Another application of RVG-R9 for antiviral siRNA delivery led to robust protection against fatal viral encephalitis in mice [71, 72]. It is worth mentioning that the chirality of the arginines (L and D isomers) significantly affected the potency and duration of RNAi [73]. Microscopy study revealed that siRNA trapped in endosome can be slowly released when the arginines are the L-isomer due to enzymatic degradation, but not the protease-resistant D-isomer [73].

#### 2.3.2. Activatable CPPs

The first activatable CPP is a hairpin-structure peptide comprising a polycationic domain and a polyanionic domain connected by a cleavable peptide loop [74,75]. The positive charges of the cationic domain are neutralized by the intramolecular electrostatic interactions with the polyanionic domain, reducing cell uptake [76]. Cleavage of the linker loop by a disease-associated protease activates the cell penetrating function of the cationic domain (Fig. 1A4). Using far-red fluorescent dye as a model cargo, activatable hairpin CPP has shown a 10-fold increase of cargo uptake in cultured cells and 3.1-fold increase in xenograft tumors once it was activated by proteases [77]. For selective delivery of siRNA, Li et al. constructed a matrix metalloproteinase-2 (MMP2) activatable CPP and delivered hTERT siRNA into hepatocellular carcinoma cells (SMMC-7721) that over-express MMP2 [78]. For *in vivo* studies, it has been found that activatable CPPs can accumulate in cartilage non-specifically. To address this problem, Olson and coworkers appended CPPs with a long PEG chain to the polyanionic domain to improve blood circulation time and biodistribution in mice [74].

Another type of activatable CPPs is made by chemical modification of the key residues in CPP via cleavable bonds [79,80]. Most frequent modifications were made on lysines, while arginine's cationic charges were unmasked, making them currently not suitable for cell type-specific delivery of siRNA.

### 3. Endosome disrupting peptides facilitating siRNA endosomal escape

As aforementioned, most CPPs enter cells through more than one pathway. For example, they can directly penetrate through cell membranes and carry siRNA molecules into the cytosol where RNAi takes place. At the same time, majority of CPPs enter cells through the

endocytic pathway. Taking cationic CPP R9 as an example, R9/siRNA complexes can enter the cytosol directly, but majority tends to rapidly accumulate inside endosomes [73]. In general, the endosomolytic activity of CPPs is poor [81,82], showing the need of other peptide sequences for endosome/lysosome disruption.

### 3.1. Conformation-changing fusogenic peptides

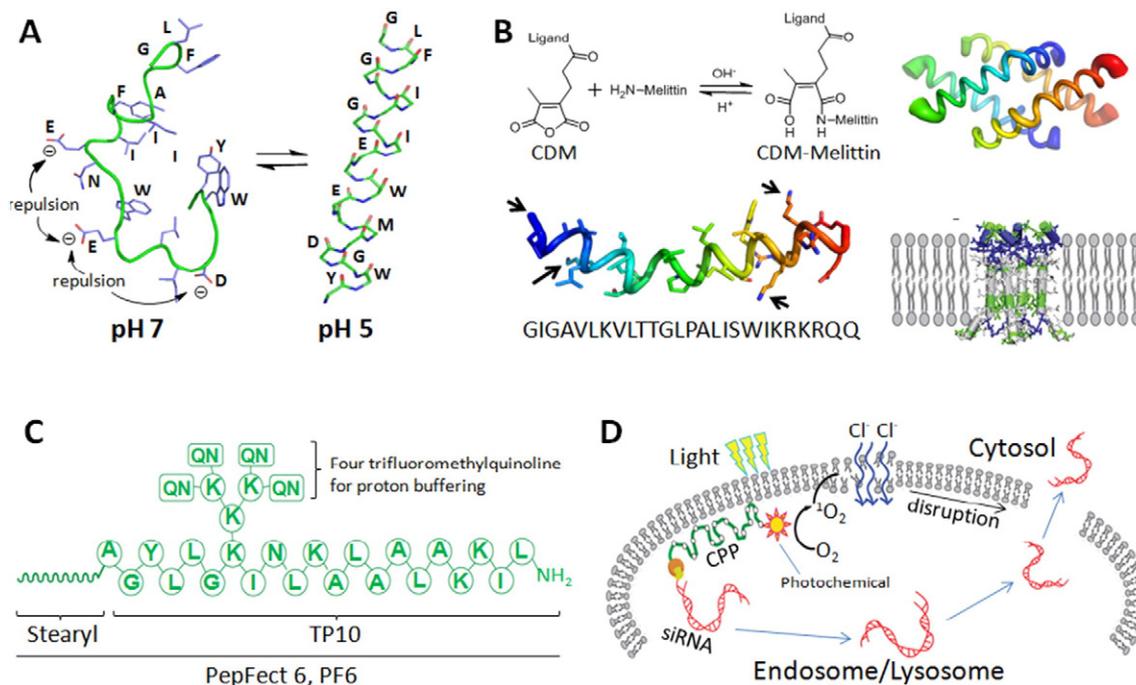
Fusogenic peptides are typically dormant at neutral pH, but its fusogenic functionality can be activated by lower pH. When the pH of surrounding environment drops (e.g., in acidic endosome and lysosome), they undergo a conformational change, such as forming an amphiphilic helix. The transformed peptides often self-associate into multimeric clusters and fuse with and disrupt the endosome membranes to aid in cargo release [83]. In contrast to the amphiphilic  $\alpha$ -helical CPPs, fusogenic peptides often have glutamic acid or histidine, because their side chains can be de-protonated or protonated in response to small changes in pH.

HA2 (GLFGAIAGFIENGWEGMIDGWYG) derived from hemagglutinin (HA) is a potent pH-responsive fusogenic peptide. The side chains of the two Glu and Asp residues are anionic, rendering the peptide to extend to a random coil structure at physiological pH. Inside the acidic endosome, however, the carboxyl groups of those side chains became protonated, reducing the negative charges. As a result, the peptide adopts a  $\alpha$ -helical conformation (Fig. 2A) [84], and fuses with and destabilizes the endosome membrane [85]. Because it is anionic, HA2 is often conjugated to cationic peptides for siRNA condensation. For example, HA2 has been linked with penetratin, nona(D-arginine), or cationic GFP to deliver siRNA to a broad range of cancer cells [39,86,87]. To enhance its fusogenic activity, HA2 has also been mutated into several derivatives. Common strategies include adding more glutamic acid to increase the pH sensitivity and incorporating hydrophobic amino acids such as Trp or Leu to improve the membrane fusion ability. The

HA2 derivatives such as INF7, E5, E5WYG and C6M1 have been applied for plasmid DNA and siRNA delivery (Table 2) [83,88–93].

Inspired by the natural HA2 fusogenic peptide, Francis C. Szoka's group has designed an artificial peptide with a glutamic acid–alanine–leucine–alanine (EALA) repeat. In this repeated sequence, the amino acid E is pH-responsive whereas ALA provides hydrophobicity. This peptide was named “GALA”. In the endosome, the helical GALA folds into a transmembrane peptide pore comprising an array of approximately 10 GALA  $\alpha$ -helical monomers [94]. As an anionic endosomolytic peptide, GALA is a popular choice to aid in endosomal escape of DNA/cationic polymer complexes (by simple mixing) [84,95]. Covalent conjugation is another common strategy, although it is known that modification of the peptide at the terminus compromises its pH sensitivity and endosomolytic activity. Nevertheless, it has been shown in several independent reports that GALA modified liposomes are significantly more efficient than liposomes alone in siRNA *in vitro* delivery [96–100].

Similar to glutamic acid, histidine is another pH sensitive amino acid commonly used in destabilizing endosomes. The imidazole group of histidine has a pKa around 6.0, falling in the range of pH transition in the endocytic pathway (from neutral to pH 6.0–6.5 in early endosomes and pH 4.5–5.5 in late endosomes). Naturally, histidine is a common component in modulating pH-dependent functions of proteins. For example, the pH-dependent binding activity of human prolactin and its receptor is believed to be linked to the presence of several histidines within the binding interface [101]. Taking advantage of histidine's natural pH sensitivity, Lundberg et al. replaced the two amino acids in penetratin by histidine and created a new pH-sensitive fusogenic peptide, EB1 (LIRLWSHLIHIWFQNRRLKWKKK-amide). This peptide does not disrupt the cell membrane (because outside cells the pH is neutral), but it adopts a membranolytic  $\alpha$ -helix upon protonation in the acidic endosome [39]. Similarly, histidine has also been utilized to control the  $\alpha$ -helix adoption in response to a pH change in many other fusogenic peptides such as LAH4, H5WYG, and Endoportor [102–105]. These peptides are widely used to facilitate endosome escape in



**Fig. 2.** Endosome disrupting peptides. A) Conformation-changing fusogenic peptide HA2 exhibits a random coil at neutral pH and adopts a  $\alpha$ -helical conformation at lower pH. B) A chemical structure-changing peptide made by CDM modification of the  $\text{NH}_2$  groups in melittin (the N-terminus and the Lys side chains indicated by arrows). The membrane activity of melittin is abolished after the modification, but can be recovered in a weakly acidic environment of endosome where it refolds into a homotetramer and disrupts the endosome membrane by pore formation. C) The chemical structure PF6 with a stearyl modified N-terminus and four trifluoromethyl quinoline moieties added onto the TP10 backbone. D) Photo-induced endosomal escape by modifying CPPs with photosensitizers. Light excitation of the sensitizer triggers the generation of singlet oxygen ( $^1\text{O}_2$ ), which lyses the endosome membrane for siRNA escape.

A) Adapted with permission from ref. [84]. Copyright 1994 National Academy of Sciences.

**Table 2**  
Endosome disrupting peptides for siRNA delivery.

Peptide	Mechanism of action	Sequence	Ref.
HA2	Conformation changing	GLFGAIAGFIENGWEGMIDGWYG	[39,86,87]
INF7	Conformation changing	GLFEAIEGFIENGWEGMIWDYG	[88]
E5	Conformation changing	GLFEAIAEFIEGGWEGLEG	[92]
E5WYG	Conformation changing	GLFEAIAEFIEGGWEGLEGWYG	[92]
C6M1	Conformation changing	Ac-RLWRLLWRLWRLLWRLLR-amide	[93]
GALA	Conformation changing	WEAALAEALAEALAEHLAEALAEALAA	[96–100]
EB1	Conformation changing	LIRLWSHLIHWFQNRRLKWKKK-amide	[39]
H5WYG	Conformation changing	GLFHAIHFHGGWHLIHWYG	[105,106]
Endoportter	Conformation changing	LHKLLHLLHLLHLLHLLHLLHLLHKL	[40,102,107]
Melittin	Chemical structure changing	GIGAVLKVLTTGLPALISWIKRKRQQ-amide (CDM modified)	[109]
H <sup>3</sup> K8b	Proton buffering	K <sub>3</sub> (HHHHNHHHH(HHHKHHKHHKHHKHH)) <sub>2</sub> <sub>4</sub>	[115,116]
PepFect 6	Proton buffering	Stearyl-AGYLLGK(K <sub>2</sub> QN <sub>4</sub> )INLKALAALAKKIL-amide	[118]
TatU1A-Alexa	Photo induced endosomolysis	RKKRRQRRR-U1A-Alexa Fluor 546	[125]

DNA and siRNA delivery [40,105,106]. For example, Endoportter appears to be a highly potent fusogenic peptide. Effective delivery of morpholino RNA (synthetic molecules with nucleic acid bases bound to morpholine rings instead of deoxyribose rings) into the cytoplasm has been demonstrated at a peptide concentration of 1  $\mu$ M in the presence of 10% FBS [107]. Similarly, peptides, proteins, siRNAs, antisense oligos and other high-molecular-weight cargos have been delivered into cells using the Endoportter peptide via the endocytic pathway [107].

### 3.2. Chemical structure-changing peptides

In addition to fusogenic peptides switched on and off by pH-triggered confirmation change, peptides have also been developed by transiently masking the key residues critical for endosome disruption. Upon cell entry, the acidic environment of endosomes helps remove the masking moieties through chemical reactions, activating the membrane destabilization function. Carboxydimethylmaleic (CDM) modified melittin is an outstanding representative of this strategy. CDM is a maleic anhydride that reacts with nucleophiles such as primary amines in peptides, and this modification can be removed in acidic environments in as soon as 5 min [108]. With CDM modification, a carboxylic acid group is added to melittin. The anionic charge inhibits the membrane disruption activity of melittin (Fig. 2B). Microscopy studies show that unmodified melittin at a concentration of 10  $\mu$ g/mL can completely destroy cells in less than 10 min, whereas CDM-modified melittin has no cytotoxicity at concentration as high as 400  $\mu$ g/mL. Co-incubation of DNA plasmid and CDM-melittin results in simultaneous accumulation of both agents in the endosome, followed by their escape in approximately 35 min, and consequently a 10-fold enhancement in gene expression [108]. For *in vivo* targeted uses, a hepatocyte-targeted N-acetylgalactosamine ligand has been linked to CDM-melittin through the free carboxylic acid group of CDM. A simple co-injection of this pH-activatable peptide (named NAG-MLP) and a liver-tropic cholesterol-conjugated siRNA (chol-siRNA) targeting coagulation factor VII (F7) resulted in an efficient F7 knockdown in mice and nonhuman primates without changes in clinical chemistry biomarkers or induction of cytokines [109].

ARC-520 is also an NAG-MLP based RNAi therapeutic in development for the treatment of chronic hepatitis B virus (HBV) infection. It consists of a cocktail of two cholesterol-conjugated siRNAs against the highly conserved sequences of HBV genomes, and NAG-MLP that promotes endosomal escape of the HBV chol-siRNAs. A phase I study consisting of 36 subjects showed that ARC-520 produced no detectable side effects, except urticarial rash developed in one subject at the highest dose. Comparable to placebo, ARC-520 appears to be safe and well-tolerated even at a dose as high as 2 mg/kg [110]. A phase 2a clinical trial revealed that ARC-520 can dramatically reduce s-antigen, e-antigen, and core-related antigen in humans after a single dose, strong evidence that ARC-520 can enable reconstitution of the immune system (a pharmacological outcome of HBV protein suppression by siRNA)

[111]. A phase 2b clinical study is ongoing to evaluate its therapeutic consistency.

### 3.3. Proton buffering peptides

Accumulation of acid-absorbing peptides inside the endosome helps cargo molecules to escape the endosome through a process known as the proton sponge effect, which arises from a large number of weak bases with buffering capabilities at pH 5–6. Proton absorption in acidic organelles leads to osmotic pressure buildup across the organelle membrane and subsequent swelling and/or rupture of the acidic endosomes.

Histidine, with a pKa value of 6.0, has excellent proton absorbing capability. Histidine-rich polypeptides have been shown to enhance DNA transfection efficiency of liposomes by 100-folds more than using liposome alone [112]. Shorter linear peptides containing multiple histidines were also reported to increase DNA transfection efficiency [113], which can be further improved by using branched peptides where histidines are incorporated at higher density [114]. For siRNA delivery, an 8-arm peptide, H<sup>3</sup>K8b, was complexed with siRNA targeting  $\beta$ -galactosidase and applied to mouse endothelial cells that were stably expressing  $\beta$ -galactosidase. The branched peptide was capable of reducing  $\beta$ -galactosidase expression by 80% at a siRNA concentration of 300 nM. Combination of H<sup>3</sup>K8b with an integrin-targeting ligand (RGD peptide) further enhanced siRNA delivery efficiency [115]. Systemic delivery of anti-raf1 siRNA to tumor sites by the branched peptide has been tested in mice bearing MDA-MB-435 tumor cells. Despite significant accumulation in the kidney, siRNA tumor uptake was evident, and significant tumor shrinkage was observed after multiple tail-vein injections [116].

In addition to natural amino acid histidine, a proton-buffering agent, chloroquine, can be covalently conjugated to peptides for promoting cargo endosomal escape as well. PepFect6 (PF6) is designed this way (Fig. 2C), by modifying a CPP (stearyl-TP10) with four trifluoromethyl quinolines (a chloroquine analog) [117]. Unlike the parental TP-10, PF6 does not induce transient pores in the cell membrane. Instead, it enters cells via the endocytotic pathway. Trifluoromethylquinoline molecules neutralize excess protons and eventually break the endosomal compartment by osmotic swelling. Due to this endosome disruption capability, robust RNAi in various cell types, including primary cells, has been achieved. The stable PF6/siRNA nanoparticles are also suitable for systemic siRNA delivery. RNAi in mice following systemic injections was achieved without detectable toxicities [118].

### 3.4. Photo-induced endosomolysis

Besides chemical-triggered endosomolysis, endosome escape also can be achieved using external stimuli such as lights. When photosensitizers are included in the delivery systems, they can produce reactive oxygen species (ROS) upon light excitation. Common photosensitizers, such as sulfonated tetraphenyl porphyrins (TPPS2a or TPPS4a), sulfonated

aluminum phthalocyanines (ALPCs), and 5-(4-carboxyphenyl)-10,15,20-triphenyl-2,3-dihydroxychlorin (TPC) generate highly reactive singlet oxygen ( $^1\text{O}_2$ ), which damages endosomal membranes by reacting with membrane lipids, resulting in release of siRNA into the cytosol (Fig. 2D) [119]. For example, cationic CPPs Tat and hepta-arginine (R7) have been conjugated with photosensitizer ALPCs and TPC to promote endosome escape [120,121]. Although the mechanism remains unclear, it has been speculated that the cationic CPPs play an important role in this process by bringing the conjugated photosensitizers close to the endosome membrane through electrostatic interactions [122].

Some conventional fluorescent dyes have also been explored following a similar mechanism. For example, fluorescein-labeled arginine-rich CPPs can be released from endosomes upon irradiation with a laser at 488 nm [123]. The similar endosomal release was also observed using another organic fluorophore, Alexa Fluor 633 [124]. Ohtsuki and co-workers explored siRNA delivery using Alexa Fluor 546-labeled TatU1A peptide (TatU1A-Alexa) [125]. The endosomal release of TatU1A-Alexa and its cargo U1A-siRNA was observed 10 s post photo-irradiation. Once in the cytoplasm, U1A-siRNA induced gene silencing of eGFP in CHO cells and the endogenous epidermal growth factor receptor (EGFR) gene in A431 cells. In contrast, no silencing effect was observed in areas where cells were not irradiated by laser.

Despite the fast response time of photochemical-induced endosomolysis, the experimental conditions must be precisely controlled because photostimulation of photosensitizers and fluorophores can cause cell death for light overdose [126]. Apoptosis and necrotic cell damage have been observed after laser excitation of R7-TPC and Tat-TMR [120,127]. Cytoplasm membrane permeabilization and blebbing, and cell shrinkage were seen shortly after photo-illumination of TMR-TAT at 560 nm [127]. Another potential limitation of photochemically induced endosomolysis is the difficulty of *in vivo* light delivery. Similar to optical-based imaging technologies, light penetration depth is limited to millimeters, including near-infrared (NIR) light [128].

#### 4. Targeting peptide-siRNA bioconjugates

A large number of peptides with specific affinity to disease-associated biomolecules have been identified through biopanning, site-specific mutagenesis of natural peptide sequences, and computer-aided design. Some peptides, such as BBN [7–14], exhibit affinity and specificity to antigens comparable to therapeutic antibodies [129], while being much more compact in size and cheaper to make. Because extensive reviews exist in the literature, here, we will only highlight a few that have been directly conjugated to siRNAs.

An IGF1-targeting cyclic peptide D-(Cys-Ser-Lys-Cys) has been conjugated to the sense strand of a siRNA at its 5' end by an ester bond. Without the use of cationic lipids or electroporation, the peptide-siRNA conjugate was shown to knock down the target gene by 35–55% in MCF-7 cells that overexpress IGF1 receptor at a siRNA concentration of 100 nM [130]. Similarly, an integrin-binding peptide cRGD was conjugated to anti-VEGFR2 siRNA and applied to integrin-positive human umbilical vein endothelial cells (HUVEC). The cRGD-siRNA conjugate can enter HUVEC selectively and silence targeted genes both *in vitro* and *in vivo*. Systemic delivery of cRGD-siRNA through multiple administrations resulted in down-regulation of the corresponding mRNA by 45 to 55% and of corresponding protein by 45 to 65% in tumor tissues. Impressively, the overall tumor volume was also reduced by 70–90% [131]. The integrin targeting efficiency of RGD peptide can be further enhanced by engineering a multivalent version. Alam et al. constructed a multivalent cRGD (mCRGD) with higher affinity and specificity than the monomeric peptide, and conjugated it to an anti-luciferase siRNA. Integrin-positive human melanoma cells M21 + showed strong receptor-mediated uptake of the mCRGD-siRNA conjugate, but the cargo siRNA was primarily accumulated in the endosome. Despite the endosome trapping problem, the mCRGD-siRNA conjugate produced dose-dependent luciferase reduction markedly, whereas the monovalent version had little effect

[132]. A similar endosome trapping problem has also been observed by Park's group when they tested a luteinizing hormone-releasing hormone (LHRH) peptide-siRNA conjugate (siRNA-PEG-LHRH) on an ovarian cancer cell line, A2780. Despite strong cellular uptake, siRNA-PEG-LHRH exhibited limited gene silencing compared to siRNA-PEG-LHRH delivered by polyethyleneimine (PEI) [133]. All of these studies point to the significance of combining targeting ligands with peptides that can get siRNA out of intracellular compartments.

Detzer et al. covalently attached a localization signal peptide to facilitate intracellular delivery [134]. Although phosphorothioate (PS) modified siRNA can stimulate cellular uptake via the caveosomal uptake pathway [135], most of the siRNA is trapped in the ER-specific perinuclear sites. A localization signal peptide TQIENLKEKG has been shown to promote siRNA release because it is recognized by the trans-membrane transporter and transferred into the cytoplasm from the perinuclear sites [134].

#### 5. Multifunctional peptides

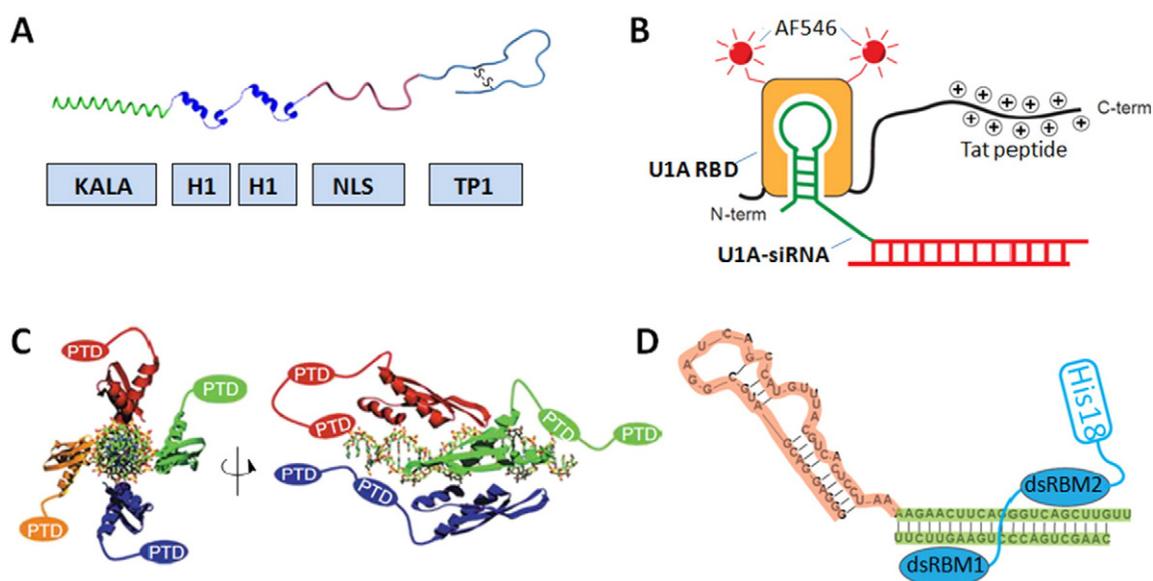
Besides the two strategies discussed above where siRNAs are either covalently linked to peptides or complexed with cationic peptides through electrostatic interactions, another emerging approach is to utilize double-strand RNA (dsRNA) binding peptides. Many dsRNA binding peptides exist in nature. For example, the first 172 amino acids in human protein kinase R (PKR) is a double-stranded RNA binding domain (dsRBD). It is composed of a tandem repeat of double-stranded RNA binding motif 1 and 2 (dsRBM1 and dsRBM2) linked by a flexible unstructured peptide linker (33 amino acids). Once mixed with siRNA, dsRBM1 and dsRBM2 wrap around the siRNA backbone with an affinity as low as 50 nM ( $K_d$ ) [136]. Therefore, it can be used as an adaptor to link siRNA molecules with other functional peptides. Compared with cationic peptides and polymers, dsRBD does not introduce excessive positive charges, avoiding non-specific binding and aggregation. Compared with covalent linkage, the plug-and-play preparation is much simpler than running chemical reactions.

##### 5.1. DNA binding peptides for gene delivery

The strategy was first demonstrated for gene delivery by using a DNA-binding peptide. Histone H1 is a natural protein that can bind to DNA and fold linear DNA into a higher-order structure. By using a histone H1-based recombinant fusion peptide, Hatefi's group constructed a multi-domain peptide for targeted delivery of genes to ZR-75-1 breast cancer cells (Fig. 3A) [137]. The multifunctional peptide was made with two tandem repeat units of truncated histone H1 for DNA condensation, a short peptide KALA for promotion of endosome escape, a cyclic peptide TP1 for ZR-75-1 cell targeting, and an NLS peptide (from human immunodeficiency virus) for enhancement of DNA translocation toward the cell nucleus. A gel shift assay proved that the multifunctional peptide efficiently binds to DNA and protects it from nuclease degradation, and a cell transfection study indicated that both the KALA and NLS motifs maintain their functions after being chained together with the other peptide domains, as evidenced by efficient luciferase transfection. His lab further developed the gene delivery system by inserting a cathepsin D substrate linker between the targeting motif and histone H1 peptide [138]. The protease substrate facilitates the dissociation of the targeting motif in endosome where cathepsin D is abundant.

##### 5.2. dsRNA binding peptides for siRNA delivery

Endoh and coworkers constructed the first sequence-specific siRNA binding system using a U1A RNA-binding peptide. siRNAs containing a short 5'-extension of the U1A (U1A-siRNA) can be specifically recognized by the U1A RNA-binding peptide (U1A RBD) and form a biomolecular complex (Fig. 3B). In their experiments, the U1A peptide was labeled with a Tat peptide and a fluorophore [125]. When the



**Fig. 3.** Gene and siRNA delivery by multifunctional peptides. A) Schematic of the trifunctional peptide for targeted gene delivery. The KALA domain promotes endosome escape; the tandem H1 repeats enable DNA condensation; the NLS sequence enhances nucleus translocation; and the TP1 block serves as a ligand for cell-specific homing. B) Sequence-specific RNA binding peptide U1A-RBD-Tat forms a complex with U1A modified siRNA, which can escape endosome aided by photo-induced endosomolysis. AF546: Alexa Fluor 546. C) Schematic drawing of a siRNA associated with PTD modified dsRBD. D) dsRBD-His<sub>18</sub> chimeric peptide as a general platform for targeted delivery of aptamer-siRNA chimeras. Orange: PSAM targeting aptamer; green: siRNA. C) Adapted with permission from ref. [139]. Copyright 2009 Nature Publishing Group.

peptide-siRNA complex enters cells through endocytosis, the fluorophore was excited with UV light for photo-induced endosome destabilization. Similarly, Eguchi et al. reported a siRNA delivery approach that uses dsRBD, which binds with siRNA in a sequence-independent fashion (Fig. 3C). The recombinant dsRBD was fused with several repeats of cationic peptides (Tat peptide) to mask the negative charges of siRNA and promote cell uptake. Rapid gene knockout was observed without significant toxicity, off-target effect, or immune response [139]. This approach, although effective in the cultured cells, is likely to have problems under *in vivo* conditions, because the cationic peptides could induce interactions with serum proteins and cells non-specifically. Gopal's group has extended this approach by fusing the dsRNA binding peptide with target homing peptides such as BTP (binding to GM1 and GT1b) and Her2 affibody (binding to Her2 receptor). By replacing the cationic peptides with homing ligands, the new constructs have a better chance to reach the target cells [140,141]. Similarly, Geoghegan et al. constructed a tandem repeat of dsRBD for higher binding affinity to siRNA. However, without an endosome destabilization mechanism, the majority of the delivery siRNA was trapped without eliciting the desired RNAi effect [142]. Most recently, our group designed a dsRBD-polyhistidine recombinant protein. This simple dual-block protein complements the siRNA-aptamer chimera structures reported by Dassie et al. [143], because the complexes exhibit cell targeting, endosome escape, and therapeutic functionalities simultaneously, without introducing excessive positive charges (Fig. 3D) [144].

## 6. Conclusions

siRNA has gained considerable interest in biology and medicine because it can elicit potent, target-specific knockdown of virtually any mRNA, creating a useful tool in basic sciences to study gene functions as well as a therapeutic option in pharmaceuticals. Unfortunately, as with other antisense approaches, clinical translation of the powerful siRNA technology has proven challenging. Problems include enhancing siRNA stability, minimizing off-target effects, identifying sensitive sites in the target RNAs, and particularly ensuring efficient delivery. For RNA molecule stability, they are inherently less stable. Chemical modifications have been extensively explored to improve their serum

stability and *in vivo* pharmacokinetics. The chemical modification of siRNA is often performed at the phosphodiester backbone and ribose 2'-OH group [145]. For example, the ribose 2'-OH group can be substituted by 2'-Ome or 2'-F group, which can help extend the half-life of siRNA in serum beyond 48 h [146,147]. For *in vivo* therapeutics uses, despite limited success in some niche applications (e.g., deliver to the liver), a general platform for *in vivo* cell type-specific delivery does not exist. To address the delivery problem, recent advances in bio-engineering and nanotechnology have produced a number of non-viral siRNA carriers including liposomes, inorganic nanoparticles, cationic polymers, and the focus of this review, peptides. However, the *in vivo* delivery problem remains because these current delivery technologies all suffer from one or another shortcoming, preventing efficient, specific delivery of siRNA, and consequently calling for truly innovative solutions to this difficult problem. A 'perfect' delivery platform should simultaneously achieve a compact size, low immunogenicity, low cytotoxicity, selective targeting (free of excessive positive charges), *in vivo* stability, high payload, efficient endosomolysis, ease of production, flexibility in siRNA and target ligand selection, and affordability. In this regard, peptides are of great promise to achieve this goal because of their diverse functionalities, biocompatibility, and the recent development and maturation of the recombinant protein technique that allows various peptides and proteins to be assembled into precisely controlled nanostructures. Thus, multifunctional peptides are of great excitement and promise to fill in the long-standing gap of RNAi clinical translation.

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