Review Article

Self/co-assembling, cell penetrating peptides—a promising siRNAdelivery system

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Abstract

SiRNAs (short interfering RNAs) have revolutionized conventional drug therapy for treating many diseases. The clinical applications as anticancer drugsare still facing challenges, such as non-effective delivery systems, off-target effects, and immune stimulation. Many non-viral carriers have been developed so far to improve drug efficacy and biocompatibility. Among these delivery systems, a class of self/co-assembly, cell penetrating peptides (CPPs) is a most promising class of carriers. Recently, we designed and characterized a series ofself/co-assembly CCPs, C6 family. C6is highlycapable of forming stable and safe nanoparticles with siRNAs and efficiently delivering siRNA complexes into mammalian cells in vitro. In this short review, we will elucidate its physical and chemical characteristics and discuss its in vitro applications.

RNA interference (RNAi) is a cellular regulatory process, in which small, double-stranded RNA molecules that complement a specific gene (typically 21-25 nucleotides) are introduced into biological systems to suppress the expression of that gene [1-3]. Because the RNA molecule only interacts with mRNA to knock down the expression of the targeted gene without interacting with proteins and affecting chromosomal DNA, siRNA techniques greatly reduce possible adverse gene alterations in DNA-based gene therapy and the production of harmful proteins in conventional drug treatment. The effect is often potent: only a few siRNA molecules per cell are required to elicit effective gene silencing [4,5].

Due to RNAi's potency and specificity, siRNAhas revolutionized conventional drug therapy. Itcan also be widely utilized to dissect cell signaling pathways, discover important genes for embryonic development, and elucidate the function of novel genes in various fundamental biological processes, known as functional genomics [3, 6]. In theory, many proteins can be used asgene silencingtargets for treating diseases; thus, ansiRNA-based drug therapy offers versatility for targeting any sequences in mRNAs of interest, regardless ofthe cellular locations of their translated proteins [7]. For anticancer siRNA gene targeting, there are some popular choices, including anti-Bcl-2, anti-RRM2, anti-VEGF, anti-HER2, anti-Stat3, and surviving-targeted siRNA. Indeed, only a decade after its discovery, siRNA has been employed to treatcancer, HIVand other diseases.

In spite of the advantages of siRNAas a potential new therapeutic, there are still challenges to be overcome forfuture clinical applications, such as off-target effects, immune stimulation, andnon-effective delivery systems. Because they are anionic and hydrophilic, siRNAscannot enter cells by passive diffusion, andthey can berapidlydigested in the circulation and eliminated from the host liver and kidneys.Thus, developing an effective in vivo delivery system is essential.

Development of siRNA delivery systems

In search for effective siRNA delivery systems, the uses of many non-viral carriers have been attempted, including liposomes, polymeric complexes, micro emulsions, and nanoparticles. Among thesesiRNA carriers, liposomes or lipidic compounds arethe most studied. Cationic lipids can encapsulate negatively charged siRNA molecules and deliver them into biological cells. Although some satisfactory results were obtainedin certain in vitro systems, there are still difficulties with the lipidic strategies. First, there is inherent competition between vesicle assembly and intracellular unpacking of their molecular cargo.This phenomenonmarkedlyhinders efficient drug release intothe cytosol or cytoplasmic organelles [8]. Second, the relatively large amount of lipids required for siRNA transfection often induces severe cytotoxicitybecause cationic lipids can interfere with cellsignaling pathways that triggerimmune or anti-inflammatory responses [9]. Hence, these toxic/immunogenic features limit wide utilization of lipidic carriers in vivo. Although new approaches have beeninitiated to addresscytotoxicity and targeting specificity issues, the long-term in vivo safety and efficacy of this strategy remain in question.

Synthetic and natural polymers are another class of carrier systems that are a candidate for use insiRNAtherapeutics. Polymers consist of repeated units of covalently bonded monomers. Their structural and chemical properties are well established.Generally, cationic polymers bind to siRNAs through electrostatic interactions to facilitate crossing the cell membrane. A number of polymers, including polyethyleneimine (PEI), poly L-lysine (PLL), chitosan, L-lactide-co-glycolide (PLGA), poly dimethylaminoethylmethacrylate (PDMAEMA), gelatin, poly-D, and poly trimethylaminoethylmethacrylate (PTMAEMA), have been studied. Like lipidic carriers, polymeric delivery systems also suffer from cytotoxicity and immunogenicity; thus, their application in vivohas been hampered [10].

A class of self/co-assembling, cell penetrating peptides (CPPs) has been developed recently. In our opinion, they area most promising class of carriers.These CPPs,such as ionic-complementary peptides (ICPs) and amino acid pairing peptides (AAPs), are small in size and

typically contain fewer than 30 amino acids. Most CPPs are designed to include both hydrophilic and hydrophobic moieties. Through electrostatic interaction, the hydrophilic side of the peptide interacts with both the hydrophilic heads of the lipid bilayer as well as hydrophilic drugs and siRNAs. Meanwhile, the hydrophobic side is anchored in the hydrophobic core of the bilayer for switching on endocytosis or assisting the direct peptide-cargo translocation to the cytosol [11, 12]. By altering the physical and chemical properties of the peptides, small CPPs can be constructed to promote siRNA cellular uptake and facilitate intracellular complex dissociation to release siRNAs and turn on the RNAi machinery. Furthermore, through the targeting ligand valence and the peptide charge, the peptide-cargo complexes can be transported to the desired intracellular compartments to elicit target-specific RNA delivery and further enhance overall drug delivery efficiency[13]. In comparison, CPPs offer great advantages in flexibility and versatility as efficient and safe drug/siRNA delivery vehicles. In fact, peptide-based drug carriers have been designed and employed in research recently, and their role as siRNA delivery vehicles has been demonstrated in both *in vitro* and *in vivo* systems [14-17].

Design and characterization of a cell penetrating peptide-C6

We have been exclusively working on self/co-assembling CPPs for peptide-assisted anticancer drug/siRNA delivery systems. The first series of peptides we generated was the C6 family, which is derivatives of C6, an 18-mer amphipathic and amino-acid-pairing peptide (Ac-RLLRLLLRLWRRLLRLLR-NH$_2$) [18]. Strategically, three types of amino acids were incorporated and particularly arranged in our design to achieve (1) ionic interactions between positively charged arginine residues and negatively charged phosphate groups on the siRNA backbone as well as negatively charged cell surface proteoglycans for initiating cellular uptake [12, 19, 20]; (2) amphiphilic and helix structures for facilitating peptide translocation across the cell membrane through interactions between the hydrophobic tails of the lipid bilayer and leucine residues [11,14]; and (3) an intrinsic fluorescence probe for tracking intracellular siRNA localization by inclusion of an aromatic tryptophan residue in the middle of the sequence

The nanostructure of C6 and C6-siRNA complexes was characterized exclusively with various methods. Initially, C6 formed self-assemblies/aggregates in water. When siRNA was added in the peptide solution, C6 electrostatically formed coassemblies with siRNA. Based on Dynamic Light Scattering (DLS), the majority of C6-siRNA co assemblies showed a size of 50 nm. This structure was further confirmed with Atomic Force Microscope (AFM). Since 7 positively charged arginine groups in the peptide C6 and 21 pairs of negatively charged nucleotides in a siRNA molecule, 6 molecules of peptide are theoretically needed to neutralize the negative charge of a siRNA molecule. But, a molar ratio (MR) of 10:1 was required practically and an excess of peptide molecules (MR=15:1) was needed in order to stabilize the peptide-siRNA complex based on gel shift assay result [18]. The excess peptide molecules can provide a shield to protect siRNA molecules against degradation and interact with cell membrane to initiate the peptide-siRNA cellular uptake [18]. At a molar ratio of 15:1, C6 completely covered the surface of complex to generate a zeta potential of +30 mV. With increasing C6 concentration at a constant

siRNA concentration, the positive value of the surface charge of the complex rose to +60 mV (MR= 40:1).

Cellular uptake and cytoplasmic localization of C6-siRNA

To investigate the capability of C6 facilitating siRNA internalization, Cy3-labeled GAPDH siRNA (50 nM), as an extrinsic fluorescence probe, was complexes with C6 and then transfected into CHO-K1 cells. Cellular uptake of C6-Cy3-siRNA complexes was measured with Fluorescence-Activated Cell Sorting (FACS) after 3 hours of incubation. Uptake of C6-Cy3-siRNA correlated well with the molar ratio between C6 and Cy3-siRNA. At a molar ratio of 15:1, C6-Cy3-siRNA uptake raised to over 90% compared with the non-treated control group. Intracellular fluorescence intensified with increasing molar ratios from 25:1 to 40:1. Moreover, it was noticed under fluorescence microscope that Cy3-siRNA itself was unable to enter the cells due to the negative charge and lack of an appropriate delivery vehicle while C6-Cy3-siRNA was clearly visualized intracellularly. C6-Cy3-siRNA complexes localized to regions in close proximity to the nuclear membrane in the cells. Cy3-siRNA delivered by C6 displayed a nonhomogeneous distribution pattern around the periphery of the nucleus inside the cells, indicating the possibility of endocytosis pathway.

Cytotoxicity of C6-siRNA complexes

While C6 demonstrated high capabilities of protecting siRNAs and delivering them into mammalian cells, cytotoxicity evaluation also showed promising result. In this experiment, CHO-K1 cells were treated either with C6 or with C6-siRNA complexes and cell viability was determined thereafter with a cell counting kit (CCK-8). After 48 hours of treatment; there was no obvious cell death in the treated cells compared with non-treated cells. From molar ratios of 10:1 to 40:1, the treated cells all retained viability over 85%, indicating only a minimum cytotoxicity with C6-siRNA application. In agreement with this result, similar group of CPPs did not cause significant hemolytic activation and acute immune stimulation in our recent *in vitro* tests as well as *in vivo* animal studies.

Conclusions

Among the delivery candidates, small self/co-assembling CPPs are one of the most promising carriers for delivering anticancer siRNAs and drugs. From *in vitro* studies, these CPPs reveal several advantages in targeted gene delivery efficiency and minimal cytotoxicity. While thorough *in vitro* evaluations on drug efficacy and biocompatibility are still necessary, research on acute and long-term biocompatibility should be emphasized. In particular, investigation of cancer treatment efficiency, off-target effects, and long-term immune stimulations/suppressions in animal models should be focused for the future.

References

1. SchmidtCW. Therapeutic interference. Modern drug discovery. 2003; Jul: 37-42.

2. FireA, Xu SQ, Montgomery MK, Kostas ST, Driver SE, et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditiselegans. Nature.1998; 391: 806-811.

3. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001; *411*: 494-498.

4. Bumcrot D, Manoharan M, Koteliansky V, Sah DW. RNAi therapeutics: apotential new class of pharmaceutical drugs. Nat. Chem. Biol. 2006; 2: 711–719.

5. Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. Nat. Rev. Genet. 2007; 8: 173–184.

6. DykxhoornDM, PalliserD, Lieberman, J. The silent treatment: siRNAs as small molecule drugs. Gene Therapy. 2006; 13: 541-552.

7. de Fougerolles A, Vornlocher HP, Maraganore J, Lieberman J. Interfering with Disease: a progress report on siRNA-based therapeutics, Nat. Rev. Drug Discov.2007; 6: 443–453.

8. Schiffelers RM, Ansari A, Xu J, Zhou Q, Tang Q, et al.Cancer siRNA therapy by tumour selective delivery with ligand-targeted stearically stabilized nanoparticle. Nucleic Acids Research. 2004; 32: 1-10.

9. Novina CD, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, et al. siRNA directed inhibition of HIV-1 infection. Nature Medicine. 2002; 8: 681-686.

10. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNAi. Nature. 2002; 418: 435-438.

11. Langel, Ü. Handbook of Cell-Penetrating Peptides. Taylor & Francis: Boca Raton, FL, 2007.

12. Zeigler A. Nervi P., Durrenberger M., and Seelig J. The cationic cell-penetrating peptide CPP [TAT] derived from the HIV-1 protein Tat is rapidly transported into living fibroblasts: optical, biophysical and metabolic evidence. Biochemistry. 2005; 44:138-148.

13. Ren Y, Hauert S, Lo JH, Bhatia SN. Identification and Characterization of Receptor-Specific Peptides for siRNA Delivery. ACS Nano.2012; 6: 8620-8631.

14. Schroeder JA, Bitler BG. Anticancer therapies that utilize cell penetrating peptides. Recent Pat. Anticancer Drug Discov. 2010; 5:99-108.

15. Jarver P, Langel U. The use of cell-penetrating peptides as a tool for gene regulation. D.D.T. 2004; 9: 395-402.

16. Brooks NA, Pouniotis DS, Tang CK, Apostolopoulos V, Pietersz GA. Cell-penetrating peptides: application in vaccine delivery. Biochim, Biophys. Acta. 2010; 1805: 25-34.

17. Andaloussi SE, Lehto T, Oprea I, Smith CE, Langel U. Application of endosomolytic cell-penetrating peptides for nucleic acid delivery—applications and potential implication for cancer treatment. B.T.C.C Journal. 2010; 2:

18. Jafari M, Xu W, Naahidi S, et al. A new amphipathic, amino-acid-pairing (AAP) peptide as siRNA delivery carrier: physicochemical characterization and *in vitro* uptake. Journal of Physical Chemistry B. 2012; 116: 13183-13191.

19. Nakase I, Tadokoro A, Kawabata N, Takeuchi T, Katoh H, et al. Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. Biochemistry. 2007; 46: 492-501.

20. Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, et al. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J. Biol Chem. 2001; 276: 5836-5840.

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