



UNIVERSITY OF WATERLOO
FACULTY OF ENGINEERING

Notice of PhD Oral Defence

CANDIDATE: Baoling Chen

DEPARTMENT: Chemical Engineering

THESIS TITLE:

*Characterization and Evaluation of Amphipathic, Cationic Peptides for
Small Interfering RNA delivery*

SUPERVISOR(S): Pu Chen, Chemical Engineering

ORAL DEFENCE DATE:

Tuesday May 19, 2015 at 9:00am in E6 2022

The thesis of the above candidate is on deposit in the Engineering Graduate Office (PHY 3003).

The thesis is available for overnight perusal and may be signed out until the defence date.

Date Submitted: Tuesday April 14, 2015

Engineering Graduate Studies Office
PHY 3003

THESIS ABSTRACT ATTACHED

Abstract

RNA interference (RNAi) is a highly efficient and specific posttranscriptional gene silencing process, which can be triggered by small interfering RNA. The efficiency and specificity of this process makes siRNA a powerful tool for gene therapy. However, the use of siRNA is hampered by its rapid degradation and poor cellular uptake. A variety of carriers for example, virus, lipid, polymer, peptides, and nanoparticles, have been developed to protect siRNA and maximize its therapeutic effects. Among these carriers, peptides have emerged as a promising candidate due to their intrinsically rapid and highly efficient internalization and their typically low toxicity.

The amphipathic, cationic peptide C6 (sequence: Ac-RLLRLLLRLWRLLRLLR-NH₂) was previously designed and studied in our group. C6 achieved significant siRNA cellular uptake but induced low gene silencing, possibly due to siRNA entrapment in endosomes. In order to enhance endosomal release of siRNA, two strategies were incorporated to design a new peptide: (1) adding more pore-formation moieties, such as tryptophan; (2) including pH-sensitive moieties, such as histidine. The resulting peptide C6M3 (sequence: Ac-RLWHLWRLWRRLHRLLR-NH₂) incorporated three tryptophan residues and two histidine residues that could induce the so called "proton sponge effect". Similar to C6, C6M3 could encapsulate all the free siRNA (siRNA: 5 μ M) at minimal molar ratio 10/1. Moreover, C6M3 alone adopted a defined structure- α -helical secondary structure that could enhance peptide-cell membrane interaction, while C6 could not form a secondary structure. C6M3-siRNA complexes displayed a particle diameter of 80-100 nm, which was smaller than that of C6-siRNA complexes. Compared to C6 (K_a : $9.23 \times 10^6 \text{ M}^{-1}$), C6M3 had stronger binding affinity with siRNA (K_a : $8.59 \times 10^8 \text{ M}^{-1}$) in water at room temperature, which was confirmed by isothermal titration calorimetry (ITC). Moreover, in CHO-K1 monolayer cell culture, the GAPDH gene silencing efficiency induced by C6M3-siRNA complexes was significant ($68\% \pm 2$). Effective in vivo RNAi was achieved in a human non-small lung tumor xenograft model through intratumoral injection of the C6M3-Bcl-2 siRNA complexes

(molar ratio: 60/1). This treatment induced a marked tumor suppression, with an inhibition rate of $55\% \pm 3$, through suppression of antiapoptotic Bcl-2 protein in mice.

CADY (sequence: Ac-GLWRALWRLLRSLWRLWRA-cysteamide), a reported amphipathic peptide, has been shown to deliver nucleic acids, peptides, and proteins into various cells. The high potency of this peptide is strongly related to its tryptophan rich property, helical conformation, and amphipathic character. The segment GLWRA seems to play a role in the potency of CADY. This information, associated with the sequence of C6M3, and also alanine amino acid being reported to facilitate helical conformation, led to the design of GL family peptides. After pre-screening for transfection activity, a promising candidate GL1 was identified and selected for further evaluation. Compared to C6M3, GL1 (sequence: Ac-**GLWRAW**LWKAFLASNWRLLRLLR-NH₂) included a higher amount of tryptophan (4 vs. 3) to enhance peptide-membrane interaction, and three alanine (A) amino acids to contribute to helical conformation. GL1 could encapsulate all the siRNA (siRNA: 5 μ M) at minimal molar ratio 15/1, displaying a spherical shape with a particle diameter of 80-100 nm. GL1 adopted α -helical secondary structure in water and exhibited strong binding affinity with siRNA (K_a : $4.47 \times 10^8 \text{ M}^{-1}$) confirmed by circular dichroism (CD) spectroscopy and ITC respectively. In monolayer culture of CHO-K1 cells, GL1 could deliver siRNA to 95% of the cells even at molar ratio 20/1 (siRNA: 50 nM), while C6M3 required a higher molar ratio (40/1) to achieve the same result. This difference may be due to the higher amount of tryptophan and the GLWRAW segment in the GL1 sequence. Interestingly, GL1 induced a lower GAPDH gene silencing efficiency ($49\% \pm 2$) than C6M3, possibly because of lack of efficient endosomal release of siRNA. Both GL1 and C6M3 achieved minimal cytotoxicity ($<10\% \pm 2$) with siRNA concentration of 50 nM. As monolayer cell culture lacks the 3D features of an in vivo model, multicellular tumor spheroids (MCTS), an intermediate model between traditional monolayer cell culture and in vivo model, are developed. CHO-K1 MCTS were established utilizing a hanging drop method and used for investigating the efficacy of GL1-siRNA complexes. This approach induced markedly suppression of GAPDH gene expression ($50\% \pm 2$) with minimal cytotoxicity ($<5\% \pm 2$). Following procedures of in vivo studies mentioned above, GL1-Bcl-2 siRNA complexes (molar ratio:

60/1) caused a tumor inhibition rate of 54%±4 through downregulation of antiapoptotic Bcl-2 protein, which was similar to that of C6M3-Bcl-2 siRNA complexes.

Recently, stearylation has proven to be successful in increasing the transfection efficiency of cell penetrating peptides (CPPs) for DNA delivery or that of polyethyleneimine (PEI) for siRNA delivery. A stearyl moiety is believed to play an essential role in enhancing compaction of DNA, cellular uptake, and endosomal escape. Therefore, a new design strategy incorporating stearic acid conjugation in peptide sequence was applied. STR-HK (sequence: C₁₈-HHHPKPKRKV-NH₂) consisted of a stearyl moiety enhancing peptide-siRNA/cell membrane interaction, three histidine inducing “proton sponge effect”, a cytoplasm localization sequence (PKPKRKV) contributing to cytoplasm localization of the complexes, and the cationic amino acids (K & R) facilitating the peptide-siRNA binding through electrostatic interaction. STR-HK required minimal molar ratio 15/1 to fully encapsulate siRNA (siRNA: 5 μM) and the complexes displayed a spherical shape with a size distribution of 80-140 nm in diameter. Note that STR-HK adopted β sheet secondary structure in water and exhibited strong binding affinity with siRNA (K_a : $3.73 \times 10^5 \text{ M}^{-1}$) confirmed by CD and ITC respectively. Interestingly, at molar ratio 20/1 (siRNA: 50 nM), STR-HK could deliver siRNA to ~20% of the CHO-K1 cells, which was significantly lower than that of C6M3 and GL1. Increasing the molar ratio to 40/1, STR-HK achieved similar cellular uptake as C6M3. The possible reason was that a high amount of stearyl moieties could enhance cellular uptake. In monolayer culture of CHO-K1 cells, STR-HK achieved higher GAPDH gene silencing efficiency (75%±2) than C6M3 and GL1 with minimal cytotoxicity (<10%±2). Following similar procedures of in vivo treatment mentioned above, STR-HK-Bcl-2 siRNA complexes (molar ratio: 60/1) resulted in a tumor inhibition rate of 63%±3 via downregulation of Bcl-2 protein in mice. This tumor inhibition rate was higher than that of C6M3/GL1-Bcl-2siRNA complexes.

This thesis focuses on characterization and evaluation of the three newly designed peptides as potential siRNA delivery systems. These three peptides were designed according to the following strategies: (1) modification of the amphipathic, cationic peptide C6 with tryptophan and histidine in the sequence; (2) adoption of parts of peptides CADY and C6M3

along with addition of an amphipilic segment; (3) conjugation of a stearyl moiety to peptide sequence. Among the three peptides, C6M3 could encapsulate all the free siRNA (siRNA at 5 μ M) with the lowest molar ratio (10/1) and exhibited the strongest binding affinity with siRNA (K_a : $8.59 \times 10^8 \text{ M}^{-1}$). The three peptides adopted secondary structures: C6M3 and GL1 adopted helical structure and STR-HK adopted β sheet structure. The peptide-siRNA complexes all assumed a spherical shape and induced minimal cytotoxicity in CHO-K1 cells ($<10\% \pm 3$). To achieve optimum gene silencing efficiency, the peptide/siRNA molar ratio needed to be optimized. STR-HK-siRNA complexes at molar ratio 60/1 achieved the highest GAPDH gene silencing efficiency ($75\% \pm 2$) in monolayer culture of CHO-K1 cells. In the human non-small cell lung tumor xenograft model, the STR-HK-siRNA complexes induced the highest tumor inhibition rate ($63\% \pm 3$). These data indicated the advantage of the peptide STR-HK. However, the potency of these three peptides could be further evaluated in other cell lines and in vivo models through systemic administration of the peptide-siRNA complexes.