Influence of electrostatic interactions on cell-penetrating peptide-small interfering RNA complex formation and intracellular delivery efficiency

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Abstract. Cell-penetrating peptides (CPP) are short positively charged biopolymers that can translocate through lipid membranes. Due to their unique properties CPPs are promising agents for intracellular drug delivery. In this work we studied potency of intracellular delivery of small interfering RNA (siRNA) by means of two CPPs, namely primary amphipathic MPG-NLS peptide and secondary amphipathic EB1 peptide. Optimal concentration conditions and peptide-to-siRNA ratios have been found for stable peptide-siRNA complex formation and delivery efficiency has been shown.

1. Introduction
Cell-penetrating peptides are able to cross lipid bilayer, enter into the cell and so they are considered as agents for intracellular drug delivery [1]. CPPs can carry as cargo a variety of different therapeutic molecules: small compounds, nucleotides, siRNA or proteins [1]. One common feature of cell-penetrating peptides is their positive net charge (+4 – +9) [2]. This property allows them to effectively bind cargo molecules with the opposite charge, resulting in nanocomplexes capable for intracellular delivery.

Small interfering RNAs (siRNAs) can inhibit expression of a specific genes, thus providing
potential for new target therapeutics [3]. The main obstacle for the clinical use of siRNA is that it cannot cross cell membranes, due to its high negative net charge, and thus auxiliary agents for the intracellular delivery are needed [4].

In this work we compare the ability for two peptides for siRNA intracellular delivery. The first peptide is cell-penetrating peptide EB1 [5], a Penetratin peptide derivative. It has net charge +8 and is secondary amphipathic, that is it shows amphipathic properties when acquires an alpha-helical secondary structure on a membrane. The second is MPG peptide variant (with ΔNLS amino acid mutation) [6], it has net charge +4 and is primary amphipathic, i.e. its amphipathicity is enclosed in the primary structure of its amino acid sequence.

We have found optimal conditions for cell penetrating peptide-siRNA complex formation, characterized these complexes and showed their ability for intracellular delivery.

2. Materials and methods

2.1 Peptides and siRNA
Two peptides had been compared: MPG-ΔNLS (ac-GALFLGFLGAAGSTMGAWSPKSKRKV-cya) and EB1 (LIRLWSHLIHIWFQNRRLKWKKK-NH₂). For the delivery tests siRNA Cy3 tagged duplex directed against the BCR-ABL was used (sense 5’-GCAGAGUUCAGGUCAAGCCCUUdTdT, antisense 5’-AAGGGCUUUGAAACUCUGCdTdT) [7].

2.2 Peptide-siRNA complex characterisation
The peptide-siRNA complex has been prepared by mixing equal volumes of peptide and siRNA water solutions, and subsequently incubation for 10 minutes at 37°C. Final siRNA concentration was 1μM in all cases, while amounts of peptide were chosen to get desired peptide-to-siRNA ratios. Size (hydrodynamic diameter) and zeta potential of the formed complexes were measured with ZetaSizer Nano ZS (Malvern, USA). siRNA binding into complexes was accessed with gel-electrophoresis on 20% polyacrylamide gel at 150V for 1 h in TBE (Tris-borate/EDTA) buffer containing ethidium bromide. Pictures were taken in Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA).

2.3 Intracellular delivery assay
For intracellular delivery tests we used CT26 cell line (Mus Musculus colon carcinoma, ATCC, CRL-2638). Cells were plated at a density 5 × 10⁴ cells in 400 μL RPMI 1640 medium (HyClone, US) containing 10% FBS (HyClone, US), 100 μM of streptomycin, and 100 U/mL of penicillin, in 24 well plate. 100 μL of prepared complex solutions were added to 400 μL of cells, resulting in total siRNA concentration of 200 nM in each well. For positive control HiPerFect liposomal transfection reagent (Qiagen, USA) was used according to the manufacturer protocol with same amount of siRNA. The intracellular delivery of siRNA tagged with Cy3 dye has been controlled after 24 hours using confocal microscopy Axio Observer Z1 (Zeiss, Germany), the cells have been washed before imaging. The transfection efficiency was examined using flow cytometry with Epics XL (Beckman Coulter, USA).
3. Results and discussion

Total net charge in physiological pH differs twice for the investigated peptides being +8 for EB1 peptide and +4 for MPG peptide. To ensure comparable influence of electrostatic interactions we studied complexes with the same peptide-to-siRNA charge ratios for both peptides. For complex formation peptide-to-siRNA molar ratios were 2.5/1, 5/1, 10/1 and 20/1 for EB1 peptide, while for MPG peptide we used values of 5/1, 10/1, 20/1 and 40/1.

Size properties of obtained complexes differs for two investigated peptides (see Fig. 1a). As is seen for EB1 complex sizes are around 100 nm, while it seems that MPG peptide-siRNA complex tend to aggregate into large micron-sized structures.

Zeta potential values of the resulting complexes demonstrate same trend for both peptides with the increase of peptide-to-siRNA ratio. However, it is seen from Fig. 1b that for the same peptide-to-siRNA charge ratios zeta-potential is always lower for MPG-siRNA complex comparing with EB1-siRNA complex, and this difference is more pronounced for larger peptide-siRNA ratios.

From gel-shift experiments (Fig. 2) it is seen that siRNA is completely bind into complexes for the same charge peptide-to-siRNA ratios. Absence of band on electrophoresis represents the total capture of siRNA in complex, so we conclude that there is no free siRNA in complex solution for 10 and 20 EB1 peptide-to-siRNA ratios and 20 and 40 MPG peptide-to-siRNA ratios. This correlates with low or positive zeta potential of corresponding complexes (see Fig. 1b).

Figure 1(a, b). (a) Mean peptide-siRNA complex size (hydrodynamic diameter) measured with dynamic light scattering for different peptide-to-siRNA ratios for MPG and EB1 peptides; (b) Zeta-potential value of peptide-siRNA complex for different peptide-to-siRNA ratios for MPG and EB1 peptides.
Figure 2. Electrophoresis of peptide-siRNA complexes in different peptide-to-siRNA ratios showing siRNA capture in complexes.

Figure 3. Confocal microscopy image of transfected cells, 24 hours after addition of transfection agents with siRNA. Three columns correspond to brightfield microscopy, Cy3 fluorescence and both channels merged. The first line corresponds to cells with naked siRNA in water solution without transfection agents, the second and the third line corresponds to siRNA transfection by peptide-siRNA complexes in given peptide-to-siRNA ratios for EB1 and for MPG peptides, the fourth line corresponds to siRNA transfection by commercially available transfection reagent HiPerFect.
For EB1 and MPG peptides we identified optimal 20/1 and 40/1 ratios respectively, as for this ratios all siRNA is captured into complexes and they have positive values of zeta potential, which is important for intracellular delivery [2]. Results of the delivery efficiency of this complexes compared with HiPerFect transfection reagent (HPF) are shown on Fig. 3 and Fig. 4.

Transfection efficiency for the investigated peptide complexes was almost the same as for liposomal delivery agent HiPerFect, as it is seen from Fig. 4b. Interestingly, while the size of complex is about 100 nm for EB1 peptide, it was up to 1.5 μm for MPG peptide as it is seen from Fig. 1a. Though it is known that micron-sized complexes are less effective for intracellular delivery than nanometer-sized ones [8], large MPG-siRNA complex showed the ability for intracellular siRNA delivery (see Fig. 3 and Fig. 4 (a, b)). A possible explanation is that such a large peptide-siRNA complexes come into contact with cells leading to increase of peptide and siRNA concentration near the cell surface, thus giving a way to peptide collective behaviour-induced translocation.

**Figure 4(a, b). (a) Histogram of fluorescence distribution of cells measured by flow cytometry; (b) Transfection efficiency calculated from flow cytometry measurements. The first column corresponds to control cells, second – shows the transfection of naked siRNA, third and fourth columns represent the transfection siRNA complexed with peptide in peptide-to-siRNA ratios 20-1 and 40-1 for EB1 and MPG respectively, fifth column – siRNA transfection by HiPerFect.**

**4. Conclusions**

Our findings demonstrate that charge peptide-to-siRNA ratio in complex formation is more important than molar ratio. Charge ratio determines siRNA capturing into complex and correlates with its zeta potential – one of the main physico-chemical property that influence internalization into the cell. Also, we found that efficiency of intracellular delivery of siRNA is high enough for complexes with both peptides. Interestingly, micron-size structure of MPG-siRNA complex does not degrade delivery. MPG carry twice less charge than EB1, and thus complex with MPG contains twice as much peptide as
complex with EB1, however delivery efficiency is comparable in both cases. This indicates that charge ratio may have a greater effect on peptide delivery properties than total amount of cell-penetrating peptide in the probe.

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