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Divalent Metal Ions Boost Effect of Nucleic Acids Delivered by Cell-Penetrating Peptides

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Introduction

Nucleic acids can be used as therapeutic agents for various medical purposes, starting from treatment of genetic diseases and finishing with vaccination [1,2]. Therapeutic nucleic acids include short non-coding single- or double-stranded nucleic acids, e.g. antisense oligonucleotides (ASOs) [3] and small interfering RNAs (siRNAs) [4], as well as longer coding nucleic acids, e.g. plasmid DNA (pDNA) [5] and messenger RNA (mRNA) [6,7]. The key issue with successful use of nucleic acids in live systems is their insufficient stability and efficiency. Being large and negatively charged molecules, nucleic acids cannot effectively penetrate cell membrane, thus providing insufficient effect in the case of intracellular targets [8]. Furthermore, unmodified nucleic acids are rapidly degraded by extra- and intracellular nucleases [9,10].

For cellular delivery of nucleic acids, numerous delivery systems have been developed, including liposomes [11], polyplexes [12], dendrimers [13], and peptides [14]. Among them, lipid formulations are currently most widely used in clinics, including those developed for delivery of mRNA-based vaccines that helped to fight the COVID-19 pandemic overworld [1,2]. However, it has been shown that cell-penetrating peptides (CPPs), up to 30 amino acid residues long peptides with ability to cross biological membranes, can be considered a promising alternative to lipids, often showing comparable efficiency, at the same time displaying lower cytotoxicity [15].

However, the efficiency of peptide-mediated delivery is still generally lower than that of the commercial lipid-based transfection systems. One of the most significant hindrances that can reduce productive delivery is inefficient endosomal escape of the CPP-nucleic acid complexes, which leads to eventual degradation of cargo molecules [16,17]. There are different ways to increase endosomal escape of peptide particles, e.g. adding endosomolytic moieties or strengthening proton sponge effect. In the current research, we were looking for novel safe and effective approaches for enhancing transfection efficiency of complexes formed from CPP and nucleic acids, without increasing concentration of the peptide.

Calcium has been known for a long time as a compound that is able to condense nucleic acids, facilitating transfection of viral DNA [18] and has also been recently used to condense siRNA to nanoparticles, providing more efficient transfection [19]. Recently, calcium has been used to facilitate transfection of CPP-nucleic acid nanoparticles. In the current study, we added Ca2+ as well as a range of other metal ions with different valencies to non-covalently formed complexes of CPP and nucleic acids and tested the efficiency of transfection in cell culture conditions. We used PepFect14 (PF14), a stearylated amphipathic CPP (stearyl-AGYLLGKLLOOLAAAALOOLL, where O = ornithine) that has been developed in 2011 by Ezzat *et al.* [20] and has been shown to efficiently deliver different types of cargo molecules [15,20,21].

We have found that adding calcium and magnesium chloride to the complexes during their preparation can greatly enhance efficiency of CPP-mediated transfection of splice-correcting ASO into cultured cells. Similar effects were also achieved in the case of pDNA, siRNA and mRNA, and in different cell lines. We also observed significant changes in intracellular distribution and trafficking of complexes prepared with the addition of calcium chloride [22], with ion-supplemented particles showing more extensive nuclear localization and, most likely, more efficient endosomal escape. Interestingly, also some salts of mono- and trivalent metals provided an increase in the expression of the reporter protein similar to that of CaCl2 and MgCl2. Importantly, particles containing CaCl2 and MgCl2 did not have any cytotoxic effect on cell lines that we used. Altogether, our results indicate that efficiency of CPP-mediated transfection can be greatly enhanced by the addition of calcium, magnesium and some other cations.

Results and Discussion

First, we tested the effect of adding $CaCl_2$ and $MgCl_2$ to the complexes of CPP and nucleic acid in the luciferase-based reporter system. For this, a reporter cell line with aberrant splicing, HeLa pLuc 705, was used. This cell line contains firefly luciferase gene that is interrupted by the second intron of beta-globin. The intron contains a mutation in the position 705 of the respective gene that prevents normal splicing, so that a part of the intron is retained, causing beta-thalassemia in humans or lack of functional luciferase in the reporter cell line. When splice-correcting oligonucleotide (SCO-705) is added, an aberrant splicing-inducing site is blocked, splicing occurs normally and functional luciferase is produced. Thus, luminescence intensity can be used to estimate productive transfection of the SCO. For formation of nanoparticles, CPP and SCO (final concentration 100 nM) were mixed at a molar ratio 5 in Milli-Q water, and after 15 min incubation, $CaCl_2$ or $MgCl_2$ solution was added to preformed complexes. After 15 min, solutions were diluted with pre-warmed growth medium 10-fold to reach the final volume, applied to the HeLa pLuc 705 cells and incubated for 24 h.

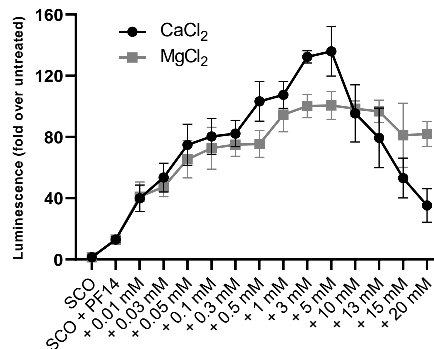

Fig. 1. Effect of $CaCl_2$ and $MgCl_2$ on rescue of luminescence in HeLa pLuc 705 cells by the SCO-PF14 complexes.

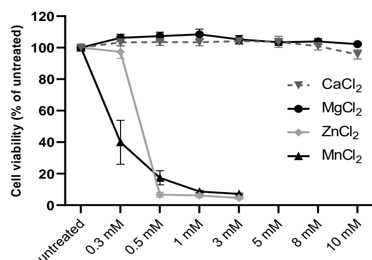

Fig. 2. Viability of HeLa pLuc 705 cells in the presence of $CaCl_2$, $MgCl_2$, $ZnCl_2$ and $MnCl_2$ at various concentrations.

Titration of $CaCl_2$ and $MgCl_2$ resulted in a concentration-dependent increase of biological effect of the SCO-PF14 complexes, reaching 130-fold increase, compared to untreated cells (Figure 1). Addition of other divalent metal ions that are biologically relevant – Zn^{2+}, Mn^{2+}, Fe^{2+} and Cu^{2+} – did not result in any positive change in splicing correction.

While $CaCl_2$ and $MgCl_2$ were not toxic for HeLa pLuc 705 cells, some other divalent ions were highly cytotoxic already at sub millimolar concentrations, as measured with WST-1 assay (Figure 2).

Endosomal entrapment is the major bottleneck that reduces productive delivery of CPP complexes, so that only a small proportion of cargo reaches cytosol and can be biologically active. To investigate whether endosomal trapping is a significant hindrance to the activity of ion-complemented PF14-SCO complexes, we used an endosome-destabilizing compound chloroquine (Figure 3). HeLa pLuc 705 cells were incubated with nanoparticles in the growth medium containing 100 µM chloroquine for 4 h. After that, the solutions were exchanged to fresh growth medium and luciferase activity was measured the next day. In line with what was expected, chloroquine enhanced the efficiency of PF14-SCO complexes (over 9-fold increase of luminescence). However, the positive effect of chloroquine decreased at higher calcium ion concentrations, indicating that calcium-complemented complexes are less dependent on additional endosome destabilizing, being more successful at endosomal escape.

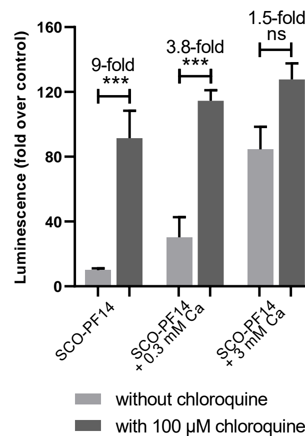

Fig. 3. Change in the effect of SCO-705 transfected into HeLa pLuc 705 cells with PF14 particles formed with or without addition of $CaCl_2$, upon addition of an endosome-destabilizing compound chloroquine (B).

Next, we wanted to know if the same effect could be achieved with other types of nucleic acids. For that, we prepared complexes

of PF14 and pDNA (Figure 4A), siRNA targeting luciferase expressed by U87-MG Luc2 cells (Figure 4C), and mRNA encoding luciferase (Figure 4B). As a result, we observed very similar positive effects of calcium and magnesium chloride in the case of all nucleic acid types tested. In most cases, adding $CaCl_2$ resulted in a more significant increase in effect than $MgCl_2$, in line with what was observed for SCO. Interestingly, adding $CaCl_2$ alone, i.e. without the peptide, is sufficient to facilitate transfection of siRNA, which has been shown also by other research groups. The most prominent effect on the activity of cargo was observed in the case of mRNA, reaching several hundred fold difference in luminescence between complexes with and without $CaCl_2$.

In addition, we broadened our selection of metal salts, including also mono- and trivalent metals, such as sodium, potassium and aluminum, and combining them with various anions. We observed a positive effect on splicing switching by PF14-SCO complexes in the case of many salts tested (Figure 5). Interestingly, aluminum salts generally started being efficient already at low concentrations, while efficiency of sodium and potassium salts usually increased with raising concentration. Some salts, like $AlCl_3$ and $BiCl_3$, showed cytotoxicity and, as a result, reduced efficiency.

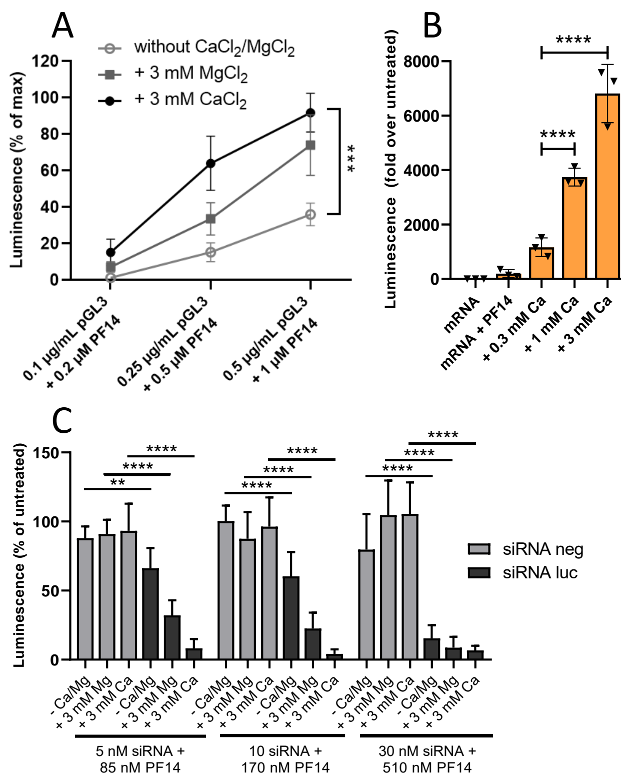

Fig. 4. Effect of adding $CaCl_2$ and $MgCl_2$ to the complexes of PF14 and (A) luciferase-encoding plasmid pGL3 in HeLa cells; (B) luciferase-encoding mRNA in HaCaT cells; and (C) luciferase-targeting siRNA in U87-MG Luc2 cells.

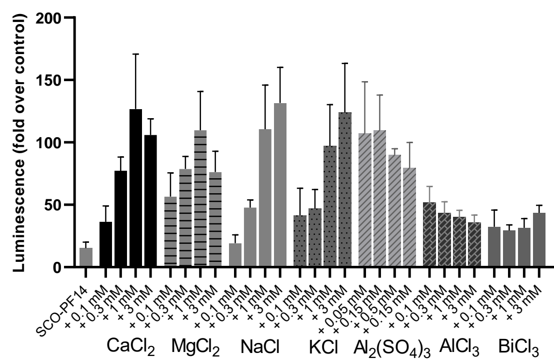

Fig. 5. Effect of adding various salts to the complexes of PF14 and SCO-705 in HeLa pLuc 705 cells.

Altogether, our data shows a significant impact of various salts that dissociate in water on the efficiency of CPP-mediated transfection of nucleic acids of different sizes and chemistries. The resulting biological effect of a cargo molecule can be increased over 10-fold by simply adding a salt solution to CPP-nucleic acid complexes during preparation. The resulting complexes are non-cytotoxic and are efficient in different cell lines. Still, the exact mechanism of this effect remains not fully understood, although some of our results indicate that divalent metal ions might act by facilitating endosomal escape of nanocomplexes. Describing CPP-nucleic acid complexes prepared with addition of a full panel of biocompatible salts and investigating mechanism of such transfection in more detail is of high interest and a subject of further research.

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