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Supplementary Materials: Choosing an Optimal Solvent is Crucial for Obtaining Cell-Penetrating Peptide Nanoparticles with Desired Properties and High Activity in Nucleic Acid Delivery

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Table S1. CPPs used in the study, their sequences, charge and number of amino acid residues. Ac = acetyl, (R8) = (R)-2-(7-octenyl)alanine, O – ornithine.

CPP	Sequence	Charge (pH 7.4)	Length (aa)
PF14	Stearyl-AGYLLGKLLOOLAAAALOOLL-amide	+5	21
CADY	Ac-GLWRALWRLLRSLWRLLWRA-cysteamide	+4	20
hPep3	H-L(R8)KLAKA(R8)AKLLKA(R8)AKAL-NH_2	+6	16

Table S2. Characterization of particles forming from PF14 dissolved in different solvents by DLS. Nanoparticles were prepared by diluting 1 mM PF14 stock solutions in different solvent mixtures in water 100× and incubating them for 30 min before measurement with Zetasizer Nano. Each number represents a mean (± SEM) of at least three independent measurements.

Solvent	Hydrodynamic diameter (nm)	Polydispersity index (PDI)
MQ water	319.0 ± 109.1	0.59
DMSO	279.2 ± 67.7	0.58
MeOH	326.5 ± 84.9	0.52
EtOH	102.8 ± 11.1	0.33
iPrOH	112.9 ± 18.5	0.30
EtOH90/DMSO10	87.9 ± 15.2	0.26
EtOH90/DMSO9/TMC1	131.2 ± 35.1	0.21
EtOH90/DMSO9.4/TMC0.6	274.7 ± 206.1	0.49
EtOH90/DMSO9.5/TMC0.5	269.5 ± 156.6	0.63
EtOH90/DMSO9.6/TMC0.4	118.9 ± 11.3	0.26
EtOH90/DMSO9.7/TMC0.3	341.7 ± 95.1	0.32
EtOH90/DMSO9.9/TMC0.1	327.2 ± 67.8	0.60

Table S3. Hydrodynamic diameter, polydispersity index and zeta potential of nanoparticles forming upon complexation of PF14 with SCO (at MR 5) or siRNA (at MR 34) with addition of Ca^{2+} or Mg^{2+}. Nanoparticles were prepared by 10× dilution of the PF14-SCO-Ca^{2+}/Mg^{2+} or PF14-siRNA-Ca^{2+}/Mg^{2+} complexes in water and analysed by DLS. Each number represents the mean value (± SEM) of at least three independent measurements. For all solutions, PF14 stock in EtOH90/DMSO9.6/TMC0.4 was used.

Sample	Hydrodynamic diameter (nm)	Polydispersity index (PDI)	Zeta potential (mV)
PF14 + SCO	112.3 ± 20.9	0.58	21.9 ± 2.3
PF14 + SCO + $CaCl_2$	133.2 ± 11.6	0.80	28.8 ± 1.7
PF14 + SCO + $MgCl_2$	91.1 ± 12.0	0.49	25.8 ± 1.2
PF14 + siRNA	86.6 ± 23.4	0.76	18.9 ± 3.0
PF14 + siRNA + $CaCl_2$	69.3 ± 23.7	0.67	10.6 ± 0.4
PF14 + siRNA+ $MgCl_2$	335.5 ± 63.9	0.70	17.6 ± 0.2

Table S4. Average diameter of nanoparticles formed by differently dissolved PF14, that was calculated from HR-TEM images (see Figure 3) using the ImageJ software.

Sample	Average diameter from HR-TEM (nm)
PF14 in EtOH	67 ± 39
PF14 in EtOH90/DMSO10	33 ± 4
PF14 in EtOH90/DMSO9.6/TMC0.4	49 ± 11

Table S5. Average diameter of nanoparticles prepared of 100 nM SCO, 1 μM PF14 and $CaCl_2$ or $MgCl_2$, that was calculated from AFM, FE-SEM and HR-TEM images (see Figure 5) using the ImageJ software. For all solutions, PF14 stock in EtOH90/DMSO9.6/TMC0.4 was used.

Sample	Avg. diameter from AFM (nm)	Avg. diameter from FE-SEM (nm)	Avg. diameter from HR-TEM (nm)
PF14	73 ± 5	36 ± 3	44 ± 12
PF14 + SCO	51 ± 13	57 ± 7	37 ± 7
PF14 + SCO + $CaCl_2$	122 ± 31	276 ± 23	31 ± 3
PF14 + SCO + $MgCl_2$	35 ± 8	608 ± 99	28 ± 4

Table S6. Numerical results of EDX analysis of nanoparticles assembled from PF14 in EtOH90/DMSO9.6/TMC0.4, SCO and $CaCl_2$ or $MgCl_2$.

Sample	Weight % of oxygen	Weight % of Ca	Weight % of Mg	Atomic % of oxygen	Atomic % of Ca	Atomic % of Mg
PF14 + SCO + $CaCl_2$	78.7 ± 7.6	21.3 ± 7.6	-	90.1 ± 4.0	9.9 ± 4.0	-
PF14 + SCO + $MgCl_2$	50.1 ± 9.4	-	30.6 ± 16.5	63.1 ± 8.6	-	25.2 ± 12.8

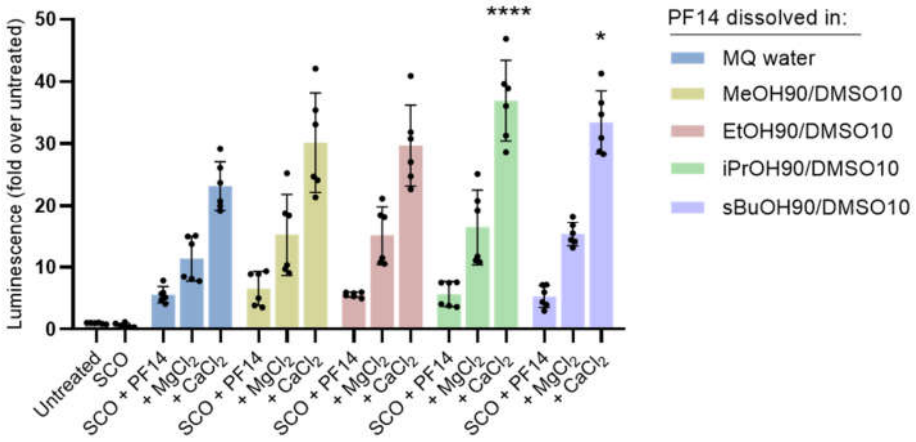

Figure S1. Effect of nanoparticles prepared from SCO-705 and differently dissolved PF14 on splicing correction. 1 mM stock solutions of PF14 were prepared by dissolving the peptide in different alcohols mixtures with DMSO (9/1, v/v). Nanoparticles of 1 μM SCO-705, 5 μM PF14 and 30 mM $MgCl_2$ or $CaCl_2$ were prepared by mixing the components in MQ water. After 30 min incubation, solutions were diluted 10-fold with cell culture medium and added to HeLa pLuc 705 cells. Luciferase activity was measured after 24 h of incubation. As a negative control, cells were incubated with a medium containing 10% (v:v) of MQ water („Untreated"). Data was analysed by one-way ANOVA with post-hoc Tukey's test. Asterisks indicate statistically significant difference, compared to the analogous solution from "MQ water" group, * p-value < 0.05, **** p-value < 0.0001.

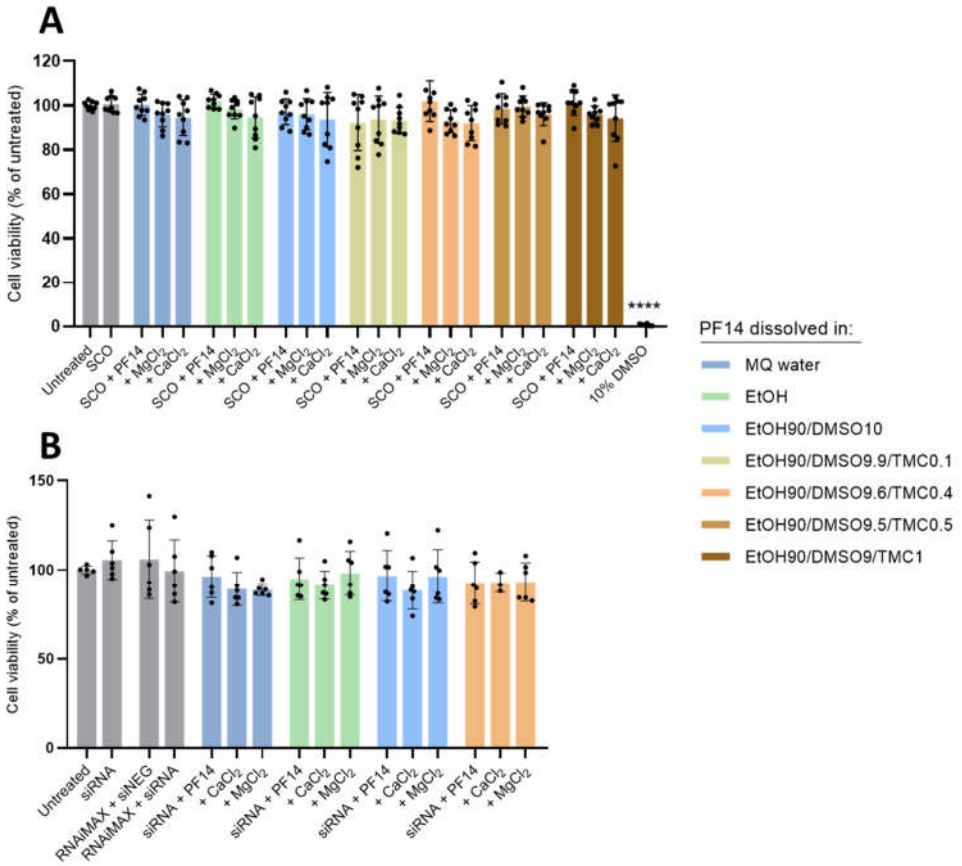

Figure S2. Viability of HeLa pLuc 705 (**A**) or U87 MG-Luc2 (**B**) cells after incubation with complexes of differently dissolved PF14 and SCO-705 (**A**) or siRNA (**B**). 1 mM stock solutions of PF14 were prepared by dissolving the peptide in different solvents and their mixtures, as indicated in the legend (proportions are given as v:v). (**A**) HeLa pLuc 705 cells were incubated for 24 h with solutions containing SCO alone (100 nM), nanoparticles of SCO and PF14 taken at MR 5 (PF14:SCO) with or without addition of 3 mM Ca^{2+} or Mg^{2+}. (**B**) U87 MG-Luc2 cells were incubated for 48 h with solutions containing siRNA alone (15 nM), nanoparticles of siRNA and PF14 taken at MR 34 (PF14:siRNA) with or without addition of 3 mM Ca^{2+} or Mg^{2+}. Viability of the cells was evaluated using WST-8 assay, and absorption of untreated cells was taken for 100%. Each dataset represents mean ± SD of technical replicates from three or two independent experiments, respectively. Data was analysed by one-way ANOVA with Dunnett's test.Asterisks indicate statistically significant difference, compared untreated cells, **** p-value < 0.0001.

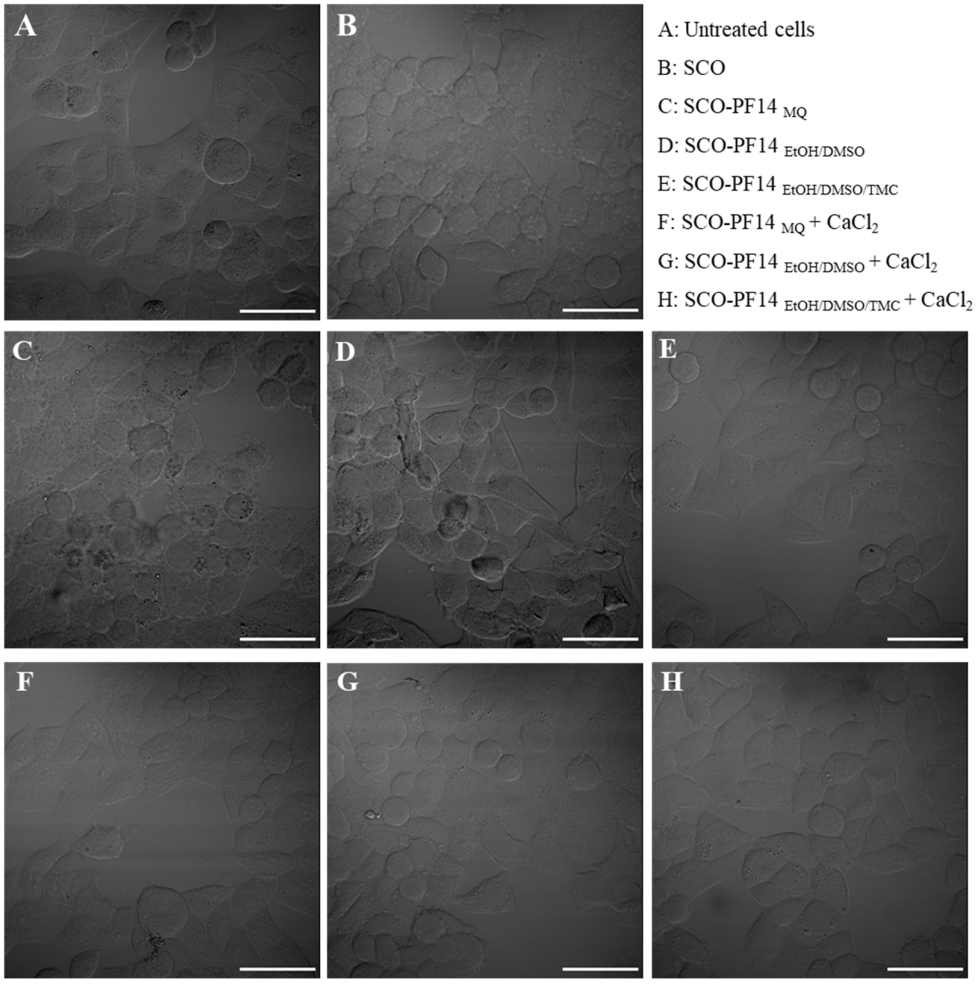

Figure S3. Bright field images of cells after incubation with nanoparticles containing SCO, differently dissolved PF14 and $CaCl_2$ (for fluorescent images, see Figure 4). HeLa EGFP 654 reporter cells were incubated with nanoparticles of 180 nM SCO-654, 20 nM Cy5-SCO-654, 1 μM PF14 and 3 mM $CaCl_2$ for 24 h. Cells were fixed and specimens were analysed with Olympus FluoView FV1000 confocal microscope. Cells were either left untreated (**A**), incubated with the two SCOs (**B**), the SCO-PF14 nanoparticles (**C**,**D**,**E**) or the SCO-PF14-Ca^{2+} nanoparticles (**F**,**G**,**H**). The following PF14 stocks were used: PF14 dissolved in MQ water (**C**, **F**); PF14 dissolved in EtOH90/DMSO10 mixture (**D**, **G**); and PF14 dissolved in EtOH90/DMSO9.6/TMC0.4 mixture (**E**, **H**). One confocal layer is presented. Scale bar 50 μm.

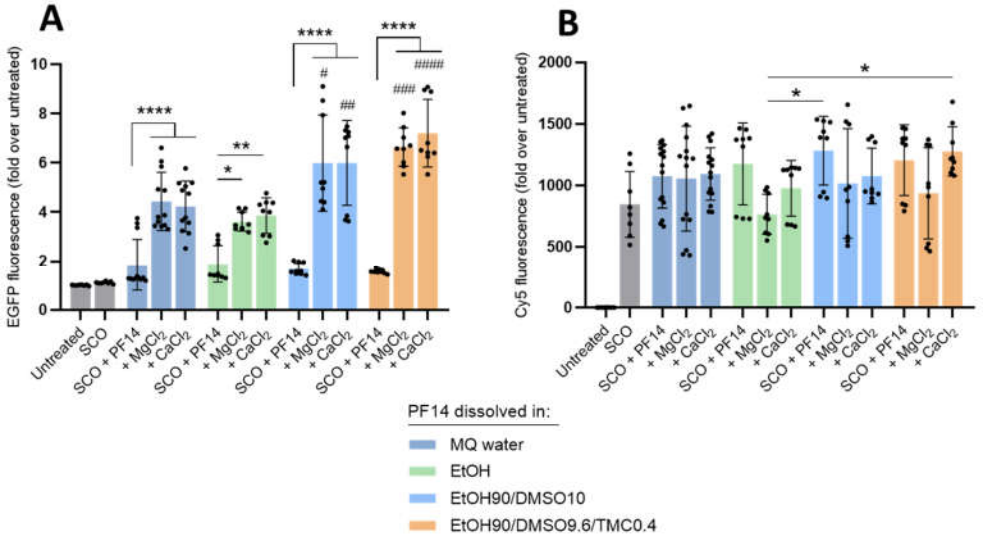

Figure S4. Transfection of and splicing correction in HeLa EGFP 654 cells with SCO-654 delivered with differently dissolved PF14. 1 mM stock solutions of PF14 were prepared by dissolving the peptide in MQ water and different organic solvents, as indicated in the legend (all ratios are given as v:v). HeLa EGFP 654 cells were seeded in 96-well plate and transfected with nanoparticles prepared of 90 nM SCO-654, 10 nM Cy5-SCO-654, 500 nM PF14 and 3 mM $CaCl_2$ or $MgCl_2$ next day. After 24 h of incubation, internalization of Cy5-labelled SCO (**B**) and splicing correction efficiency (**A**) were measured by Attune NxT flow cytometer. Each dataset represents mean ± SD of three independent experiments, with each dot corresponding to one technical replicate. The data was analysed by one-way ANOVA with post-hoc Tukey's test. Asterisks indicate statistically significant difference between two indicated datasets, * p-value < 0.05, ** p-value < 0.005, **** p-value < 0.0001. Hashes indicate statistically significant differences, compared to the analogous solution from "MQ water" group, # p-value < 0.05, ## p-value < 0.005, ### p-value < 0.0005, #### p-value < 0.0001.

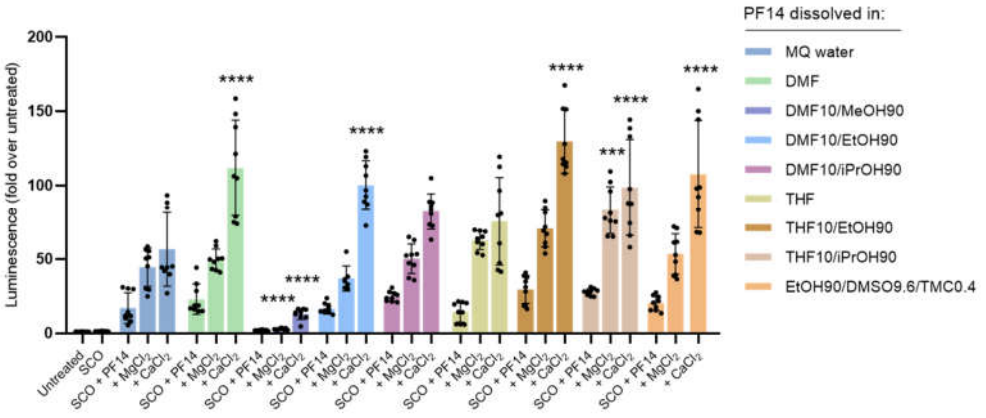

Figure S5. The effect of nanoparticles prepared of SCO-705, differently dissolved PF14 and Ca^{2+}/Mg^{2+} ions on splicing correction. 1 mM stock solutions of PF14 were prepared by dissolving the peptide in different solvents and their mixtures, as indicated in the legend (proportions are given as v:v). HeLa pLuc 705 cells were incubated for 24 h with solutions containing SCO alone (100 nM), nanoparticles of SCO and PF14 taken at MR 5 (PF14:SCO) with or without addition of 3 mM $CaCl_2$ or $MgCl_2$. Each dataset represents mean ± SD of technical replicates from three independent experiments. Data was analysed by one-way ANOVA with Tukey's test. Asterisks indicate statistically significant difference, compared to the analogous dataset from "MQ water" group, *** p-value < 0.0005, **** p-value < 0.0001.

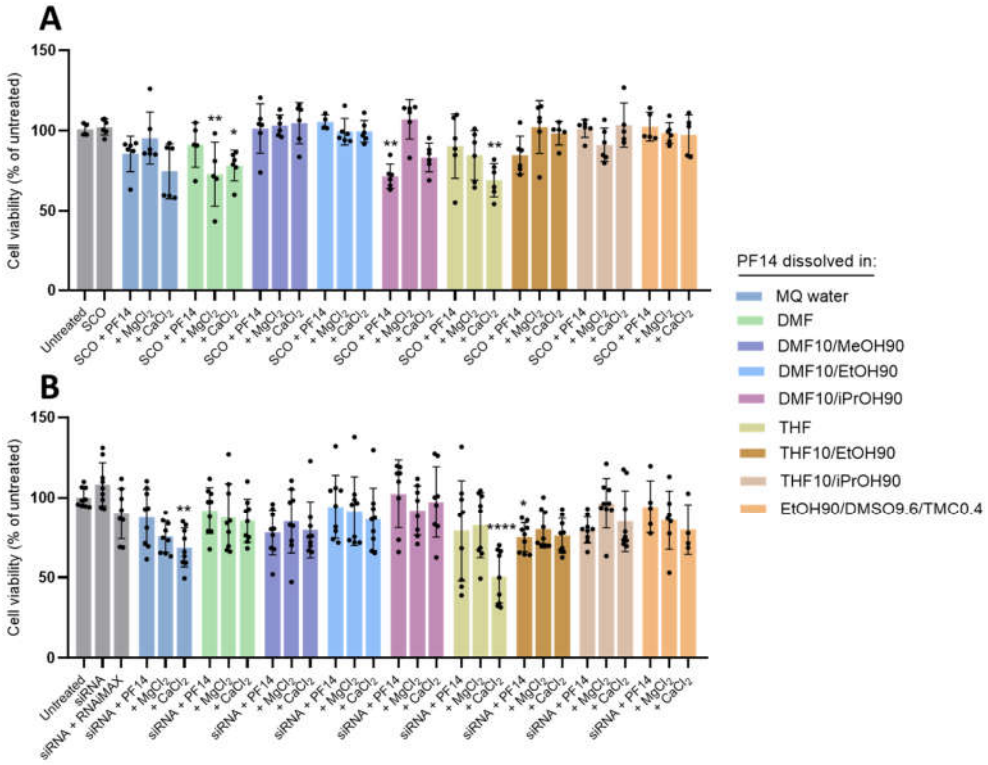

Figure S6. The effect of nanoparticles prepared of SCO (**A**) or siRNA (**B**), differently dissolved PF14 and Ca^{2+}/Mg^{2+} ions on cell viability. 1 mM stock solutions of PF14 were prepared by dissolving the peptide in different solvents and their mixtures, as indicated in the legend (proportions are given as v:v). (**A**) HeLa pLuc 705 cells were incubated for 24 h with solutions containing SCO alone (100 nM), nanoparticles of SCO and PF14 taken at MR 5 (PF14:SCO) with or without addition of 3 mM $CaCl_2$ or $MgCl_2$. (**B**) U87 MG-Luc2 cells were incubated for 48 h with solutions containing siRNA alone (15 nM), nanoparticles of siRNA and PF14 taken at MR 34 (PF14:siRNA) with or without addition of 3 mM $CaCl_2$ or $MgCl_2$. Viability of the cells was evaluated using WST-8 assay, and absorption of untreated cells was taken for 100%. Each dataset represents mean ± SD of at least one experiment, with each dot corresponding to one technical replicate. Data was analysed by one-way ANOVA with Dunnett's test. Asterisks indicate statistically significant difference, compared to untreated cells, * p-value < 0.05, ** p-value < 0.005, **** p-value < 0.0001. DMF – dimethylformamide, EtOH – ethanol, THF – tetrahydrofuran, MeOH – methanol, iPrOH – isopropanol, TMC – trimethylen carbonate.

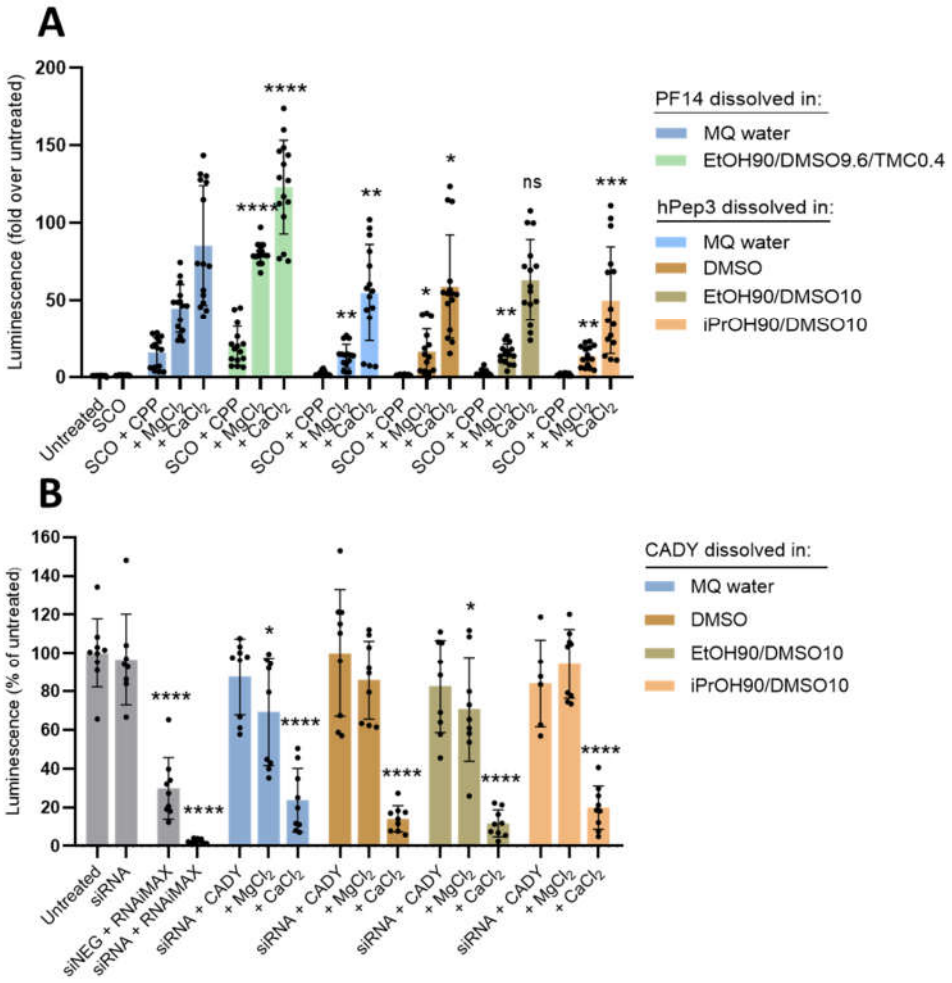

Figure S7. Effect of cosolvent on tranfection of SCO and siRNA by hPep3 and CADY. (**A**) HeLa pLuc 705 cells were incubated for 24 h with solutions containing SCO alone (100 nM), nanoparticles of SCO and PF14 or hPep3 [1] taken at MR5 with or without addition of 3 mM of $CaCl_2$ or $MgCl_2$. (**B**) U87 MG-Luc2 cells were incubated for 48 h with 17 nM siRNA, nanoparticles of siRNA and CADY [2] taken at MR34 with or without addition of 3 mM of $CaCl_2$ or $MgCl_2$. Each dataset represents as mean ± SD of at least three independent experiments, while each datapoint represents one technical replicate. Data was and analysed by one-way ANOVA with Tukey's (**A**) or Dunnett's (**B**) test. In the panel (**A**), asterisks show statistically significant differences, compared with the analogous solution from „PF14 dissolved in MQ water" group, and in the panel (**B**), compared with untreated cells, * p-value < 0.05, ** p-value < 0.005, *** p-value < 0.0005, **** p-value < 0.0001, ns – not significant.

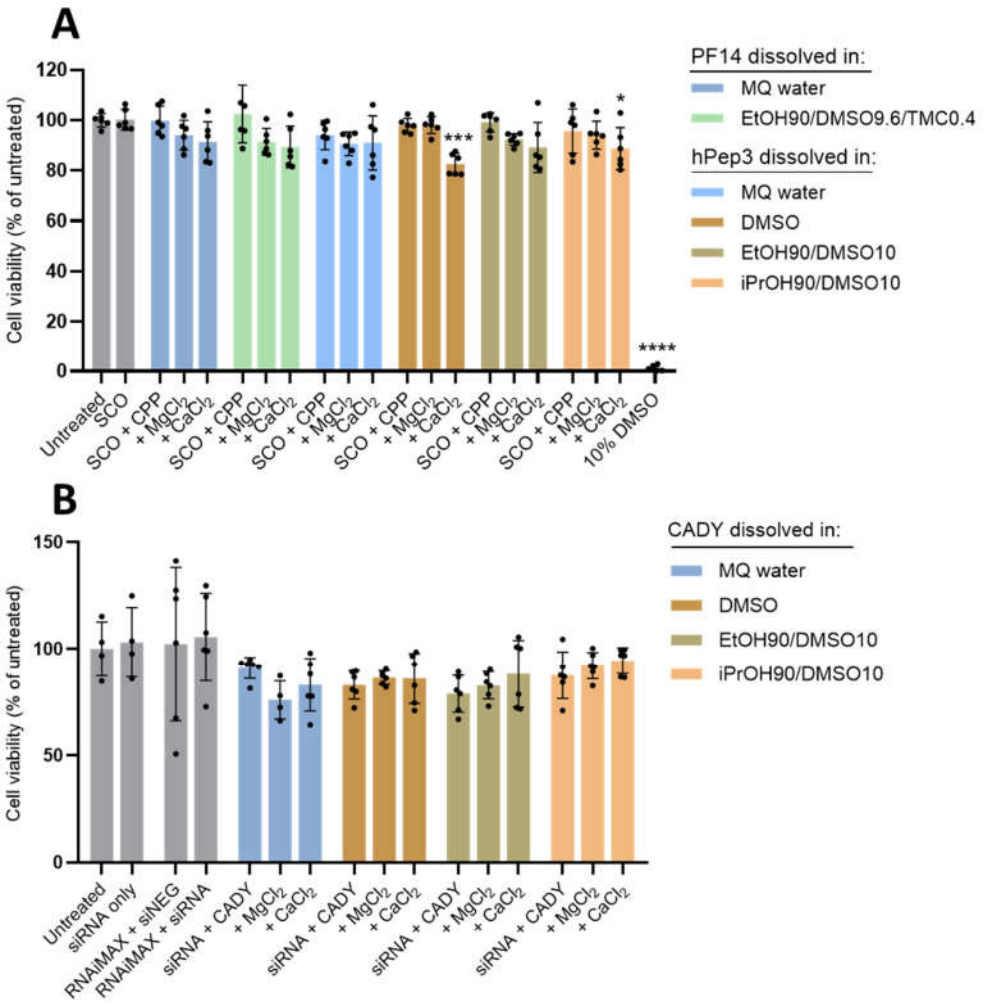

Figure S8. Effect of nanoparticles prepared of SCO and hPep3 (**A**) or siRNA and CADY (**B**), on viability of HeLa pLuc 705 or U87 MG-Luc2 cells, respectively. (**A**) HeLa pLuc 705 cells were incubated for 24 h with solutions containing SCO alone (100 nM), nanoparticles of SCO and PF14 or hPep3 taken at MR5 with or without addition of 3 mM of $CaCl_2$ or $MgCl_2$. (**B**) U87 MG-Luc2 cells were incubated for 48 h with 17 nM siRNA, nanoparticles of siRNA and CADY taken at MR34 with or without addition of 3 mM of $CaCl_2$ or $MgCl_2$. Each dataset represents as mean ± SD of two independent experiments. Data was analysed by one-way ANOVA with post-hoc Dunnett's test. Statistically significant differences compared to untreated cells are shown with asterisks, * p-value < 0.05, *** p-value < 0.0005, **** p-value < 0.0001.

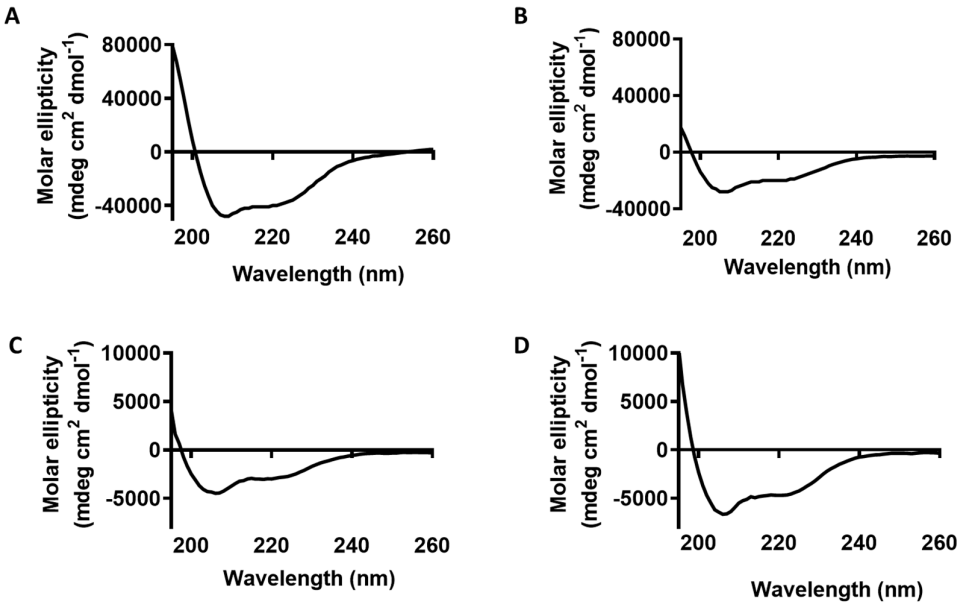

Figure S9. Circular dichroism (CD) spectra reflecting the secondary structure of PF14 dissolved in MQ water (**A**), EtOH (**B**), EtOH90/DMSO10 (**C**), and in EtOH90/DMSO9.6/TMC0.4 (**D**) after dilution into MQ water. Each 1 mM PF14 stock solution was diluted to 60-80 μM concentration in MQ water for the measurement.

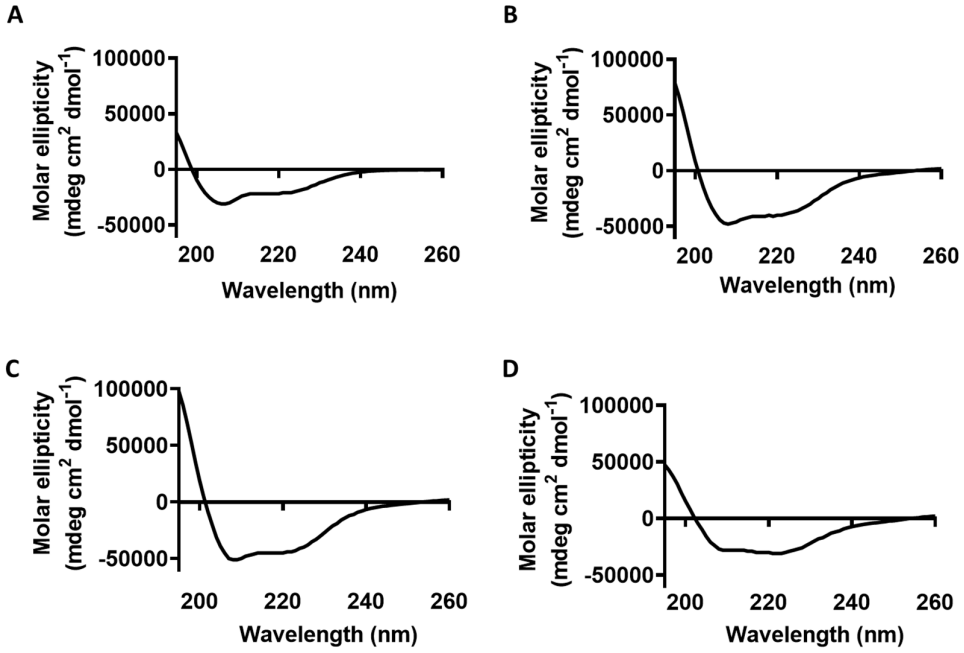

Figure S10. Circular dichroism (CD) spectra reflecting the secondary structure of PF14 alone (**A**); complex of PF14 with SCO (**B**); complex of PF14 with SCO and 3 mM $CaCl_2$ (**C**); and complex of PF14 with SCO and 3 mM $MgCl_2$ (**D**). PF14 was dissolved in EtOH90/DMSO9.6/TMC0.4 and each solution was diluted to 60-80 μM concentration of peptide in MQ water for the measurement.

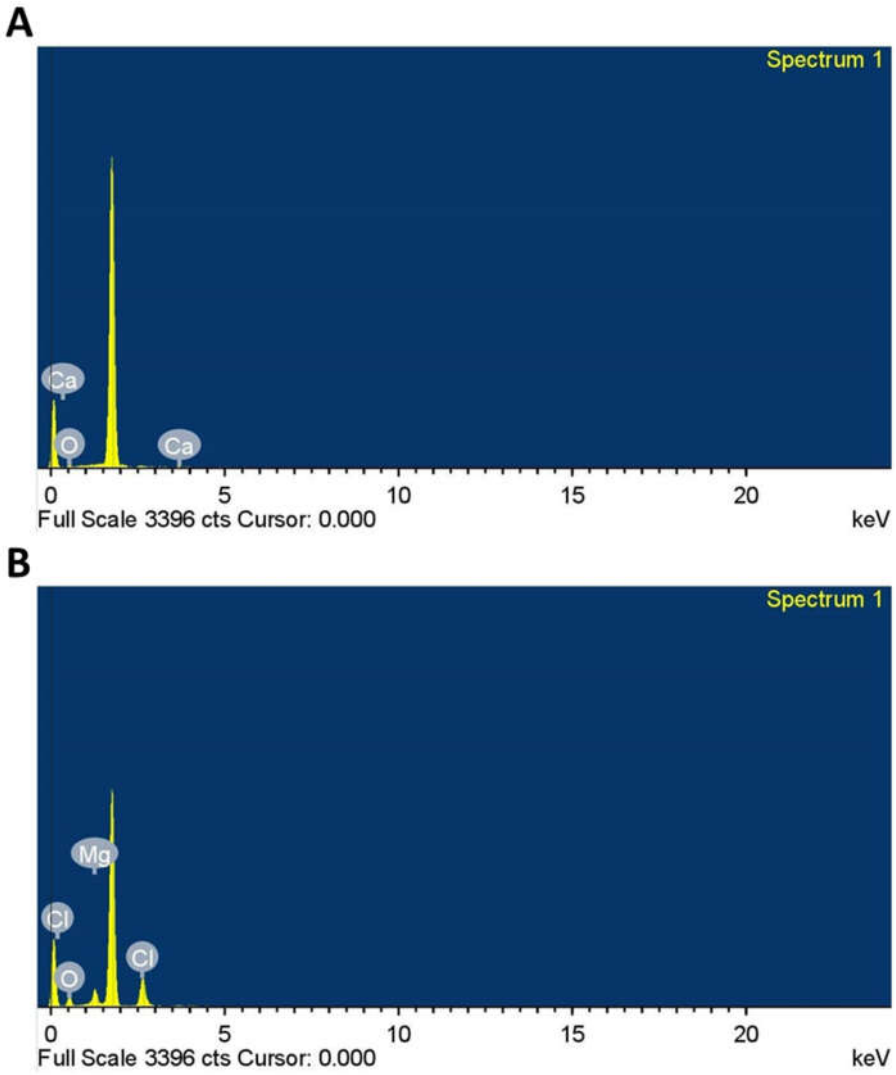

Figure S11. EDX spectra confirming the presence of Ca^{2+} (**A**) and Mg^{2+} (**B**) ions in nanoparticles assembled from PF14, SCO and $CaCl_2$ or $MgCl_2$, respectively.

References

1. Bazaz, S.; Lehto, T.; Tops, R.; Gissberg, O.; Gupta, D.; Bestas, B.; Bost, J.; Wiklander, O.P.B.; Sork, H.; Zaghloul, E.M.; et al. Novel Orthogonally Hydrocarbon-Modified Cell-Penetrating Peptide Nanoparticles Mediate Efficient Delivery of Splice-Switching Antisense Oligonucleotides In Vitro and In Vivo. *Biomedicines* **2021**, *9*, 1046. https://doi.org/10.3390/biomedicines9081046.
2. Crombez, L.; Aldrian-Herrada, G.; Konate, K.; Nguyen, Q.N.; McMaster, G.K.; Brasseur, R.; Heitz, F.; Divita, G. A New Potent Secondary Amphipathic Cell–Penetrating Peptide for SiRNA Delivery Into Mammalian Cells. *Mol. Ther.* **2009**, *17*, 95–103. https://doi.org/10.1038/mt.2008.215.