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New generation of efficient peptide-based vectors, NickFects, for the delivery of nucleic acids

Piret Arukuusk [a,*], Ly Pärnaste [a], Nikita Oskolkov [a], Dana-Maria Copolovici [a], Helerin Margus [b], Kärt Padari [b], Kaidi Möll [c], Julia Maslovskaja [c], Radi Tegova [c], Gaily Kivi [c], Andres Tover [c], Margus Pooga [b], Mart Ustav [c], Ülo Langel [a,d]

[a] Laboratory of Molecular Biotechnology, Institute of Technology, University of Tartu, Nooruse 1, 50411 Tartu, Estonia
[b] Department of Developmental Biology, Institute of Molecular and Cell Biology, University of Tartu, Riia 23, 51010 Tartu, Estonia
[c] Icosagen Cell Factory OÜ, Nooruse 9, 50411 Tartu, Estonia
[d] Department of Neurochemistry, The Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-10691 Stockholm, Sweden

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ABSTRACT

Harnessing of a branched structure is a novel approach in the design of cell-penetrating peptides and it has provided highly efficient transfection reagents for intracellular delivery of nucleic acids. The new stearylated TP10 analogs, NickFects, condense plasmid DNA, splice correcting oligonucleotides and short interfering RNAs into stable nanoparticles with a size of 62–160 nm. Such nanoparticles have a negative surface charge (−11 to −18 mV) in serum containing medium and enable highly efficient gene expression, splice correction and gene silencing. One of the novel peptides, NickFect51 is capable of transfecting plasmid DNA into a large variety of cell lines, including refractory suspension and primary cells and in several cases exceeds the transfection level of commercially available reagent Lipofectamine™ 2000 without any cytotoxic side effects. Additionally we demonstrate the advantages of NickFect51 in a protein production system, QMCF technology, for expression and production of recombinant proteins in hardly transfectable suspension cells.

1. Introduction

A search for new compounds that selectively modulate gene expression in order to treat different human diseases, has been a hot topic for many years. Nucleic acids, such as double-stranded DNA in the form of expression plasmids (pDNA), short single-stranded splice-correcting oligonucleotides (SCO) and double-stranded small interfering RNAs (siRNA), are highly promising tools for the treatment of many diseases by gene therapy [1–6]. Clinical potential of these biomolecules remains restricted so far due to their poor stability in the presence of serum and low uptake into the cells due to their high molecular weight, negative charge and hydrophilic nature. Therefore, the development of efficient delivery systems that are non-toxic, effective at low doses, easy to manufacture and handle, and which promote rapid endosomal escape after intracellular delivery in order to reach the target, is vital [7–9].

Cell-penetrating peptides (CPP) is a class of non-viral delivery vectors that has been used for the intracellular delivery of various bioactive cargos, including nucleic acids. Numerous groups have reported their successful delivery of nucleic acids, such as SCOs [10–13]; siRNA [14–16] and pDNA [17,18], by CPPs in a large variety of cell lines without evoking toxic or immunogenic side effects. pDNA and other nucleic acids can be vectorized with CPPs by using a non-covalent complexation strategy, which in comparison to covalent conjugation is less laborious, cost-efficient and lower concentrations of oligonucleotides are required to achieve a biological response [19]. However, the limitation of this strategy is the entrapment of CPP–cargo complexes in endosomes after cellular uptake by endocytosis. To enhance endosomal escape of CPP–cargo complexes from intracellular compartments, several chemical modifications have been introduced into known CPPs [16,17,20]. Developing CPPs with increased endosomolytic properties is a necessary step towards achieving biological effects at low concentrations for future *in vivo* applications.

We have demonstrated that addition of hydrophobic stearyl-moiety to an amphipathic CPP, transportan 10 (TP10), improves the properties of the peptide as a transport vehicle for nucleic acids, albeit the transfection efficacy was limited due to the entrapment of CPP–cargo complexes in endosomal compartments [12,18]. In the current study we

Abbreviations: CPP, cell-penetrating peptide; CR, charge ratio; DLS, dynamic light scattering; DIEA, diisopropylethylamine; EGFP, enhanced green fluorescent protein; HBTU, O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate; HOBT, hydroxybenzotriazole; LF2000, Lipofectamine™ 2000; FACS, fluorescence activated cell sorter; NF, NickFects; pGL3, luciferase expressing plasmid; pDNA, plasmid DNA; SCO, splice-correcting oligonucleotide; siRNA, small interfering RNA; TP10, transportan 10; TEM, transmission electron microscopy

* Corresponding author. Tel.: +372 7 37 4867; fax: +372 7 37 4900.
E-mail address: piret.arukuusk@ut.ee (P. Arukuusk).

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further enhance the uptake and endosomal release of stearyl-TP10 by modifying its sequence at Lys7, which is the linker between the neuropeptide galanin motif and mastoparan residues. We designed and synthesized three new stearylated CPPs, named NickFects (NF), all of which upon complexation with nucleic acids formed small stable nanoparticles and had higher transfection efficacy than stearyl-TP10. Here we show that one of the peptides, NickFect51 (NF51) proved to be a surprisingly effective and versatile vehicle for pDNA, SCO and siRNA intracellular delivery into various adherent and suspension cells without revealing cytotoxic side effects. Furthermore, we demonstrate the applicability of NickFect51 in the mammalian protein production system named QMCF technology, that uses mammalian cells and appropriate plasmids for expression of biologically active substances, such as recombinant proteins, recombinant antibodies, virus like particles; and for generation of cell based assays for screening of active compounds for drug development.

Altogether our data suggest that NickFect51 has great potential as an efficient intracellular delivery vehicle for nucleic acid based pharmaceuticals and also in custom protein production applications.

2. Materials and methods

2.1. Synthesis of peptides

Peptides were synthesized on an automated peptide synthesizer (Applied Biosystems, USA) using fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis strategy with Rink-amide methylbenzylhydrylamine resin (loading 0.41 mmol/g) to obtain C-terminally amidated peptides. The stearic acid was coupled manually to the N-terminus of the peptide overnight, at room temperature with 5 eq. stearic acid. For the synthesis of NF51 and NF53 tert-butyloxycarbonyl (Boc) monomers e.g. Boc-L-Orn(Fmoc)-OH, Boc-L-Lys(Fmoc)-OH, Fmoc-L-Orn(Boc)-OH (Iris Biotech., Germany) were used. Reaction was carried out using HOBT/HBTU as coupling reagents in DMF with DIEA as an activator base. The cleavage was performed with trifluoroacetic acid (TFA), 2.5% triisopropylsilane and 2.5% water for 2 h at room temperature. Peptides were purified by reversed-phase high-performance liquid chromatography on C4 column (Phenomenex Jupiter C4, 5 μm, 300A, 250 × 10 mm) using a gradient of acetonitrile/water containing 0.1% TFA. The molecular weight of the peptides was analyzed by matrix-assisted laser desorption-ionization/time of flight mass spectrometry (The Voyager-DE™ PRO Biospectrometry™ System, USA). The concentration of the peptides was determined based on dilutions of accurately weighted substances.

2.2. Cell culture

Chinese hamster ovary (CHO) cells were grown in Dulbecco's Modified Eagles Medium F12 (F12). HeLa pLuc705 was kindly provided by R. Kole, human embryonic kidney (HEK293), human osteosarcoma (U2OS), human glioblastoma (U87) cells, and mouse embryonal fibroblast (MEF) cells were grown in Dulbecco's modified Eagles medium (DMEM), and Jurkat and mouse B lymphoma (A20) cells in RPMI medium (PAA Laboratories Gmbh., Austria). All mediums were supplemented with glutamax, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (PAA Laboratories GmbH, Germany).

U2OSEBNALTD3 and CHOEBNALT85 are stable cell-lines derived from U2OS (ECACC, UK) and CHO-S (Invitrogen, USA) cell lines, respectively. The CHOEBNALT85 suspension cells are adapted to a serum-free suspension culture in QMix1 medium (Icosagen, Estonia). For adherent CHOEBNALT85 Ham's F-12 with stable glutamine (PAA E15-890 or equivalent) was used. U2OSEBNALTD3 cells were grown in DMEM supplemented with 10% FBS. All cells were grown at 37 °C in 5% CO_2 atmosphere.

2.3. Plasmid transfection

5×10^4 CHO, 6×10^4 HEK293, 2.5×10^4 U87 or U2OS cells and 5×10^4 MEF cells per well were seeded 24 h before the experiment into 24-well plates. In experiments with adherent modified cell-lines 6 well-plates and corresponding amount of cells were used. For experiments with suspension cells 1×10^5 Jurkat cells and 5×10^5 CHOEBNALT85 cells per well were seeded into 24 and 6-well plates, respectively.

For transfection luciferase expressing plasmid (pGL3) 4.7 kbp, Mw = 3.10 g/mol (Promega, Sweden) or enhanced green fluorescence protein expressing plasmid pQMCF-CMV-EGFP (EGFP) 6.4 kbp, Mw = 4.224 g/mol (Icosagen, Estonia) at concentration 1 μg/ml were used. The plasmid was mixed with CPPs at different charge ratios (CR 1–4) in milliQ H_2O in 1/10th of the final treatment volume and complexes were formed for 1 h at room temperature. Cells were treated with CPP/plasmid complexes for 4 h in serum-free or serum-containing medium. Lipofectamine™ 2000 (Invitrogen, Sweden) and Xfect (Clontech, US) were used according to the manufacturer's protocols. After 4 h treatment full growth media was added and incubated for 20 h. In experiments with chloroquine (Sigma, Germany), it was added to the cells to a final concentration of 100 μM directly prior to the treatment of the cells with preformed NF/pDNA complexes.

In experiments with pGL3 plasmid cells were washed twice with phosphate buffered saline (PBS), and lysed using 100 μl 0.1% Triton X-100 in PBS buffer for 30 min at +4 °C. Luciferase activity was measured in relative light units (RLU) using Promega's luciferase assay system on GLOMAX™ 96 microplate luminometer (Promega, Sweden). Data was normalized to protein content measured with DC protein determination kit (Bio-Rad Laboratories, Inc., USA).

In experiments with EGFP plasmid media was removed and cells were rinsed with PBS and detached from the plate using Trypsin/EDTA in PBS for 5 min at 37 °C, suspended in PBS containing 5% fetal bovine serum (FBS) and flow cytometry analysis (FACS) was carried out with a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser. Population of viable cells was determined from a scatter plot: forward scattered light (FSC) vs. side scattered light (SSC) plot. A minimum of 10,000 events from the viable cell population per sample were analyzed. Data was analyzed using BD FACS Diva software.

G418 selection was used as standard procedure in QMCF technology for selecting a plasmid-containing cell population. During the selection with antibiotics cell culture viability was kept as high as possible, in most cases it was close to 85% or higher. High cell culture viability allows fast expansion of the cell culture volume, and approximately 10–20 days after transfection, generation of an expression cell bank (1×10^7 cells/vial) was possible.

2.4. Splice correction assay

The delivery efficacy of NickFects for SCOs was evaluated by the splice-correction assay introduced by R. Kole [21]. 5×10^4 of HeLa pLuc705 cells per well were seeded in 24-well plates 24 h prior to experiments. 2′-OMe oligonucleotides (5′-CCU CUU ACC UCA GUU ACA) (Microsynth AG, Switzerland), final concentration 200 nM, were mixed with CPPs at different molar ratios (MR 5–10) in MilliQ H_2O. Complexes were formed and cells were treated as described before. In 24 h cells were lysed and luciferase activity was measured as mentioned above.

2.5. Treatment with siRNA

4×10^4 of EGFP-CHO cells were seeded in 24-well plates 24 h prior to experiments. siRNA (5′-GGCUACGUCCAGGAGCGCACC, 3′-UGCGCUCCU GGACGUAGCCUU), was mixed with CPP at different molar ratios (MR 5–30) in MilliQ H_2O. Complexes were formed and cells were treated as described afore. After indicated time media was removed, cells

were rinsed with PBS, detached from the plate, suspended with PBS containing 5% FBS and FACS analysis was performed as described above.

2.6. Toxicity measurements

Cell viability was analyzed by CytoTox-Glo™ Assay (Promega, Sweden). 1×10^4 MEF, Jurkat, Hela pLuc 705 or EGFP-CHO cells were seeded into 96-well plates 24 h before the experiment. Complexes were formed and cells were treated as described before in serum-free medium for 4 h. Subsequently 100 μl of serum-containing medium was added to cells. 24 h after treatment the number of dead cells was measured on GLOMAX™ 96 microplate luminometer and normalized to the number of cells.

2.7. Dynamic light scattering (DLS) and zeta-potential measurements

Hydrodynamic mean diameter and ξ-potential of the nanocomplexes was determined by dynamic light scattering studies using a Zetasizer Nano ZS apparatus (Malvern Instruments, United Kingdom). Peptide/cargo complexes were prepared in MilliQ H_2O according to the protocols for transfection and diluted in Opti-MEM (Invitrogen, Sweden) with 10% fetal bovine serum. All results were based on three or four measurements from two independent samples. All data were converted to "relative by intensity" plots from where the mean hydrodynamic diameter was derived.

2.8. Transmission electron microscopy

For transmission electron microscopy (TEM) studies the nanogold-labeled 705 pLuc splice-correcting oligonucleotides (NG-SCO) were used. The thiol group at 5′ end of SCO was tagged with nanogold cluster (Monomaleimido Nanogold, Nanoprobes, NY, d 1.4 nm) by forming a covalent bond between the thiol group of oligonucleotide and the maleimide group of label. The non-covalent complexes of NF51 with NG-SCO were formed as for splice correction experiments described above. HeLa pLuc 705 were treated with NF51/NG-SCO complexes at MR 7 for 1 to 4 h at 37 °C. Cells were then fixed and the nanogold label was magnified by silver enhancement followed by stabilization with 0.05% gold chloride. After postfixation with osmium tetraoxide the cells were dehydrated and embedded in epoxy resin. Ultrathin sections were cut in parallel with the coverslip, post-stained with uranyl acetate a lead citrate, and examined with JEM-100S (JEOL, Japan) transmission electron microscope. The scanned TEM images were analyzed and processed with Adobe Photoshop CS4 software.

2.9. Statistics

Values in all experiments are represented as mean ± SEM of at least 3 independent experiments performed in duplicate.

3. Results

3.1. Design of NickFects

To improve the properties of stearyl-TP10 as a transport vehicle for intracellular delivery of nucleic acids we implemented modifications on Lys7, which is the linker between a neuropeptide galanin motif and mastoparan residues in TP10 peptide sequence. As a first modification, we replaced lysine7 with ornithine, to obtain NF53 (Fig. 1). Secondly, we used ε-NH_2 group of Lys7 for subsequent synthesis instead of α-NH_2 used ordinarily, yielding NF61. Thereafter, these above mentioned 2 modifications were implemented simultaneously. We obtained NF51 by replacing Lys 7 with ornithine and continuing synthesis by coupling Gly6 to δ-NH_2 group of ornithine.

3.2. NickFects form stable nanoparticles with nucleic acids

To assess whether NickFects form stable nanoparticles with nucleic acids (pDNA, SCO, siRNA) by simply mixing the substances in water, dynamic light scattering analysis (DLS) was performed. DLS measurements corroborated that NickFects packed plasmid DNA, splice correcting oligonucleotides and siRNA molecules in water into homogeneous, unimodal nanoparticles, with the sizes of 60–160 nm (Table 1). In the presence of serum NickFects/nucleic acid nanoparticles retained their size and displayed a negative surface charge, in the range from −11 to −18 mV.

3.3. NickFects deliver pDNA into different cell types and transfect the whole cell population

To study the efficacy of NickFects in conveying pDNA into cells, peptides were complexed with the luciferase-encoding plasmid (pGL3) and luciferase activity was measured 24 h after the treatment in CHO cells and compared with the efficacy of stearyl-TP10. NF53 and NF61 lead to 10–100 fold higher plasmid transfection than stearyl-TP10 (Fig. 2a) and NF51 yielded 1000-fold higher luciferase gene expression level in serum free medium. Next we evaluated the potential of NF51 to convey pDNA into different normal and cancer cells. Widely used lipid-based transfection reagent Lipofectamine™ 2000 (LF2000) was utilized as a positive control.

NF51 demonstrated high pDNA transfection efficiency in all tested adherent cell lines (Fig. S1a–d). In CHO cells NF51 lead to higher gene expression than LF2000 even in serum containing medium. In human cancer cell lines, U2OS and U87, in serum free medium NF51 reached the transfection efficacy of LF2000. However, in HEK cells, gene expression remained 10–100 fold lower than that achieved with LF2000. Moreover, NF51 was capable of delivering pDNA into hard-to-transfect cells, such as mouse embryonic fibroblast (MEF) cells, T and B lymphocyte cells, Jurkat and A20, respectively (Fig. 2b–d). In MEF cells NF51 mediated luciferase expression, which exceeded transfection level achieved by LF2000 in serum-free medium and resulted in 1000 fold improvement over control. In the presence of serum the transfection level achieved by LF2000 exceeded that of NF51 only 5 fold. In Jurkat and A20 cells NF51 mediated pDNA transfection in serum free medium was at the same level than expression achieved by LF2000 and in the presence of serum proteins NF51 mediated 10 fold higher gene expression compared to untreated cells.

An important feature for a delivery vector is the ability to transfect entire cell populations. The number of transfected cells was verified by delivering NF51/EGFP encoded plasmid complexes into different cell lines and quantitatively estimating the number of cells by flow cytometry analysis (Fig. S1e). 24 h after single treatment in serum-free medium more than 80% and in serum containing medium 45% of CHO, U87, and U2OS cell populations were transfected with reporter gene. Remarkably, the transfection efficacy was not dependent on cell confluency as verified in experiments with cells grown at different confluency (data not shown).

3.4. NF51 is an efficient transfection reagent for protein production

Uniqueness of the QMCF technology is the opportunity to generate a protein expressing cell line within a couple of weeks without a need for the integration of an expression cassette of the protein of interest into the genome and further time consuming clonal-selection. Cell lines used in QMCF technology stably express mouse Polyomavirus (Py) large T antigen and Epstein–Barr virus EBNA-1 protein, which bound to the specific DNA sequences. Py minimal origin of replication and family of repeat sequence in the expression plasmid assures thereby a stable replication and maintenance/segregation of the expression plasmid.

In the current work, we tested NF51 applicability in QMCF technology by delivering pQMCF-CMV-EGFP plasmid into adherent U2OSEBNALT D3,

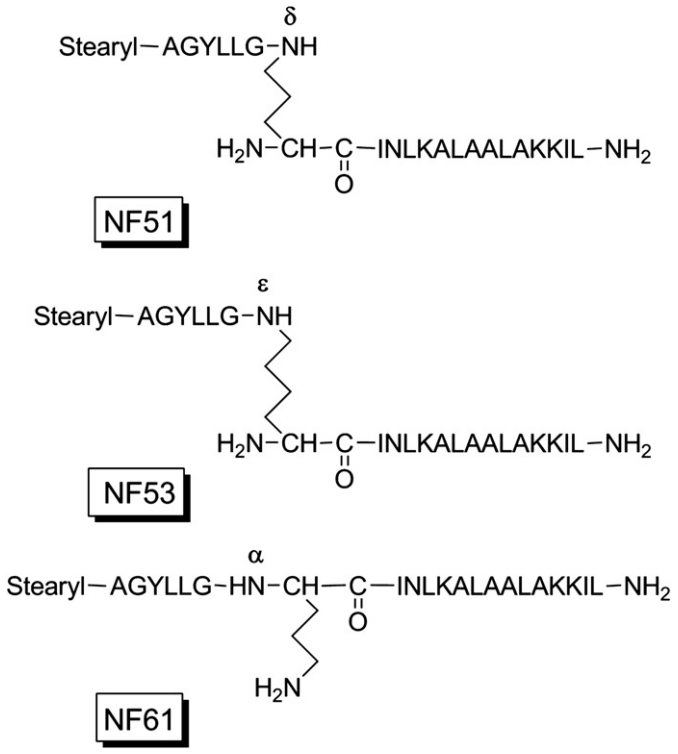

Fig. 1. Structures of NickFects: NF51, NF53, NF61 (stearyl moiety is CH_3–$(CH_2)_{16}$–CO–).

CHOEBNALT85A and suspension CHOEBNALT85 cells. After a single treatment of the cells with NF51/pDNA complexes for 24 h, approximately 50% of the adherent cell population was transfected with the reporter gene in both tested cell lines (Fig. 3a). More importantly, the percentage of EGFP positive cells reached the 95% at the 7th day of culturing transfected cells under selective conditions. NF51 exhibited no toxic effect and cell population recovered from the treatment rapidly. NF51 was also capable of transfecting suspension cells and in 40 h after treatment the gene expression was detectable in 70% of CHOEBNALT85 suspension cells (Fig. 3b). Such transfection level was achieved with NF51 at a very modest amount of pDNA (0.5 μg) while with the commercially available transfection reagent Xfect the same transfection level was reached only at a higher (2 μg) quantity of pDNA. With widely used liposome-based transfection reagent LF2000 only 45% of the cells were transfected with EGFP even at 2 μg of pDNA.

3.5. NickFects mediate effective splicing modulation

The delivery efficacy of splice-correcting oligonucleotides by NickFects was evaluated by a positive read-out splice-correction assay. NF61 induced SCO intracellular delivery similarly with stearyl-TP10, while NF53 mediated splice-correction at a modest level (Fig. 4a, b). Meanwhile NF51 induced 4–5 times higher splice correction levels than stearyl-TP10 and even exceeded the activity of LF2000, both in the absence and in the presence of serum.

The uptake of NF51/SCO nanocomplexes was also assessed by confocal microscopy analysis. NF51/SCO complexes internalized into to Hela pLuc705 cells effectively and 4 h after treatment the complexes were detectable inside the cells and in 24 h also in the cell nuclei (Fig. S2).

3.6. NickFects/siRNA nanoparticles induce gene knockdown

Subsequently, the ability of NickFects to facilitate siRNA intracellular delivery was tested. CHO cells stably expressing EGFP protein were treated with NF/EGFP-siRNA complexes at different molar ratios and the percentage of cells that underwent RNAi was measured by flow cytometry. NF53 and NF61 mediated EGFP gene downregulation more efficiently than Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX in serum-free medium, and they retained similar efficacy level as LF2000 in the presence of serum (Fig. 5a). NF51/siRNA nanoparticles mediated gene silencing of nearly whole cell population in serum-free medium and 60% of the cell population in the presence of serum. NF51 transfected siRNA into cells at low peptide concentrations, ranging from 0.5 to 1 μM, and for achieving efficient gene knockdown low amounts of siRNA (25–50 nM) were needed (Fig. 5b and Fig. S3c). The maximum silencing of the target population was achieved within 48 h after single treatment of cells with NF51/siRNA nanoparticles (Fig. S3a). In the absence of serum proteins the entire cell populations underwent RNAi within 48 h as determined by the complete shift in the FACS histograms (Fig. S3b). In contrast, a substantial fraction of the target population was not silenced by lipofection.

3.7. NickFects are non-toxic delivery vectors

Another requirement for the use of a delivery vector in biological applications is the absence of cellular toxicity. Cell viability upon the treatment of the cells with NickFects/cargo nanocomplexes was assessed with CytoTox-Glo™ assay. Complexed with pDNA NF51 reduced viability of MEF and Jurkat cells less than 30% and NF53 nearly 15%, while LF2000 reduced cell viability 50% (Fig. S4a, b). All NickFects exhibited

Table 1
Average diameter and Zeta potential of NickFects/nucleic acids nanoparticles determined by DLS measurements. NickFects and nucleic acids were mixed at different charge ratios (CR) and molar ratios (MR). In 1 h nanoparticles' average size (size, nm ± SD) and surface charge (ξ-pot, mV ± SD) were measured in water and in Opti-MEM supplemented with 10% of fetal bovine serum (FBS).

		Peptide/pDNA CR3	Peptide/SCO MR 10	Peptide/siRNA MR10
NF51	Water	Size **62.0** ± 0.2 PdI 0.138	Size **86.0** ± 14.7 PdI 0.298	Size **74.2** ± 6.7 PdI 0.197
	Optimem + FBS	Size **121.4** ± 8.1 PdI 0.623 ξ-pot −**11.5** ± 2.5	Size **168.1** ± 12.2 PdI 0.637 ξ-pot −**11.1** ± 2.4	Size **94.4** ± 1.2 PdI 0.596 ξ-pot −**11.8** ± 0.5
NF53	Water	Size **74.3** ± 5.9 PdI 0.360	Size **135.3** ± 7.8 PdI 0.459	Size **68.6** ± 8.7 PdI 0.529
	Optimem + FBS	Size **86.6** ± 16.5 PdI 0.613 ξ-pot −**14.9**	Size **85.3** ± 13.4 PdI 0.582 ξ-pot −**8.8**	Size **78.9** ± 3.9 PdI 0.575 ξ-pot −**11.9**
NF61	Water	Size **68.7** ± 4.5 PdI 0.200	Size **60.5** ± 9.3 PdI 0.286	Size **159.4** ± 9.9 PdI 0.348
	Optimem + FBS	Size **177.9** ± 7.9 PdI 0.638 ξ-pot −**17.9**	Size **94.9** ± 9,8 PdI 0.666 ξ-pot −**10.2**	Size **110.9** ± PdI 0.608 ξ-pot −**13.6**

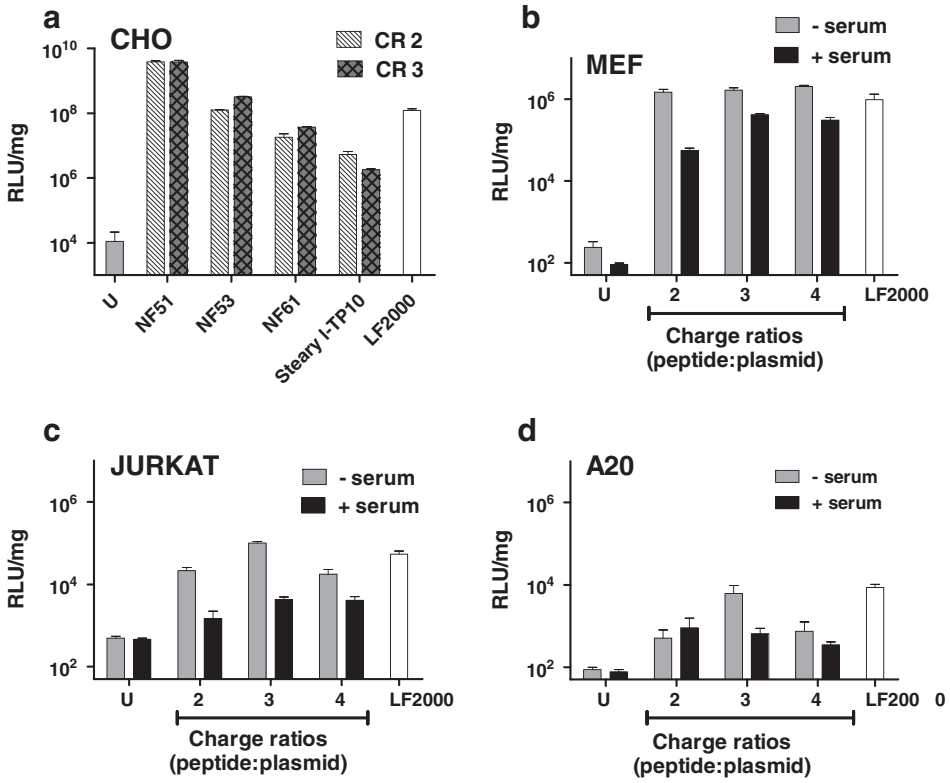

Fig. 2. Plasmid transfection efficacy of NickFects. a) NickFects and stearyl-TP10 mediated pDNA delivery in CHO cells in serum free medium. b–d) Delivery efficacy of NF51/pDNA nanoparticles in hard to transfect cell lines: MEF, Jurkat, A20. Concentration of peptides: 1.5 μM for CR2, 2.25 μM for CR3, 3 μM for CR4. Untreated cells (U) were used as negative control and Lipofectamin™ 2000 (LF2000) as a positive control.

very low cytotoxicity at concentrations used for SCO or siRNA delivery, while Lipofectamine™ 2000 reduced cell viability 35–45% (Fig. S4c, d).

3.8. NF51-cargo nanoparticles are able to escape from endosomes

In order to further characterize the NF51/nucleic acid nanoparticles and to gain better insight into their membrane interaction, cellular uptake and trafficking inside cells on the ultrastructural level we used transmission electron microscopy. The complexes of NF51/SCO were detected as small distinct particles located sparsely at the surface of HeLa pLuc705 cells (arrows in Fig. 6a and b). The particles were not very uniform and had sizes from about 80 to 170 nm (inset in Fig. 6a), and were rather loosely packed judging by the relatively low electron density of nanoparticles. Already after 30 min of incubation the number of the complexes in cells was markedly higher than on the plasma membrane suggesting a rapid cellular uptake. The NF51/SCO nanoparticles entered cells mainly by endocytosis and inside cells, the majority of complexes localized in large (400–500 nm) vesicles (Fig. 6c) and retained a nanoparticle-like structure. Often the nanoparticles were in close association with the endosomal membrane, and in some vesicles induced the disruption of endosomal membrane and escape to the cytosol (Fig. 6d). At 1 h of incubation many endosomes had lost intactness (arrowheads in Fig. 6c) and nanogold particles were detected in the cytoplasm (arrows in Fig. 6f) suggesting escape of SCO into the cytosol. Later, starting from 4 h, SCO had also reached the cell nuclei (inset in Fig. 6e).

The ability of NF51/cargo complexes to escape from endosomes was also confirmed by pDNA transfection experiments in the presence and absence of chloroquine (Fig. S5). Lysosomotropic agent chloroquine did not influence the gene expression mediated by

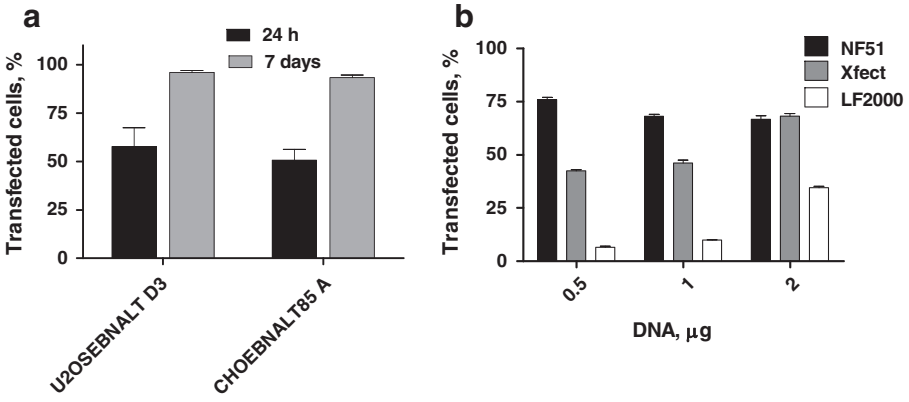

Fig. 3. Application of NF51 in protein production system. Number of transfected cells with pQMCF-CMV-EGFP plasmid with NF51/pDNA complexes in a) adherent U2OSEBNALTD3 and CHOEBNALT85A cells in 24 h and 7 days after single treatment; b) suspension CHOEBNALT85 cells at different plasmid concentrations per well (0.5–2 μg), with NF51 at charge ratio 2 in 48 h after single treatment compared to Xfect and LF2000.

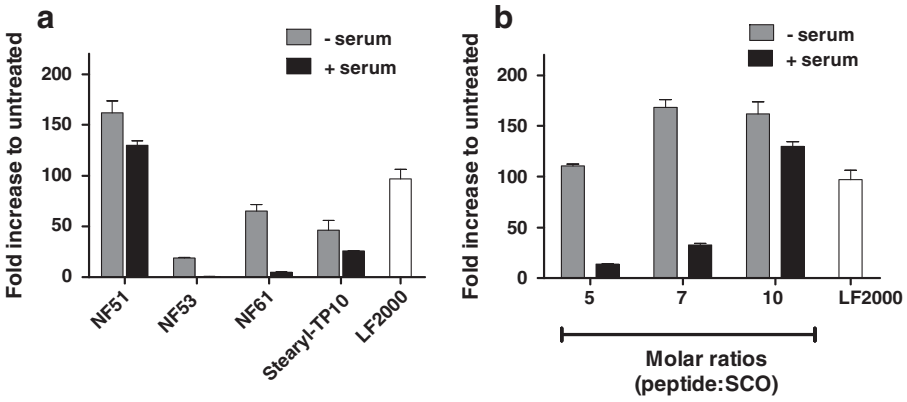

Fig. 4. Transfection of splice-correcting oligonucleotides by NickFects. Resque of luciferase expression by a splice correction in Hela pLuc 705 cells induced by SCO complexed with a) NickFects and stearyl-TP10 at molar ratio 10, and b) NF51 in a dose dependent manner. Concentration of SCO was 200 nM. Lipofectamine™ 2000 was used as a positive control. Results are presented as fold-increase over untreated cells.

NF51 neither in the absence nor in the presence of serum. In a contrast 10-fold increase in pDNA transfection level was induced by chloroquine in the case of stearyl-TP10/pDNA complexes.

4. Discussion

Triggering of mRNA degradation by short interfering RNAs or delivering pDNA, which codes for a protein that is insufficiently or aberrantly expressed, offers a great potential to treat various diseases, e.g. viral and genetic diseases or cancer [22,23]. In addition, numerous genetic diseases in humans are caused by mutations affecting alternative pre-mRNA splicing amenable to splice switching by oligonucleotides that restore correct splicing as well as rescue the function of the defective gene [6]. The key factor for the application of nucleic acids as therapeutics is to overcome the problems concerning delivery of these active biomolecules to intracellular targets.

CPP are non-viral vectors facilitating the delivery of various biomolecules across biological barriers. CPPs have been shown to condense nucleic acids via electrostatic and hydrophobic interactions [24], but despite the effective ability of nanoparticle formation the relative transfection efficiencies have usually remained lower than that of lipofection [17]. The majority of CPPs and their cargos are taken up by different endocytic pathways [25]. In order to become bioactive the complexes must ultimately escape from the endosomes and nucleic acids should reach their site of action in the cytosol or nucleus. The main drawback while using CPPs is the entrapment of peptide–cargo complexes in the endosomal compartments [26] and to overcome this limitation, different approaches have been utilized [16,27].

Our previous studies demonstrated that stearylation of amphipathic peptide TP10 at N-terminus refines its carrier properties and stearyl-TP10 was able to deliver SCO and pDNA into different cell-lines by using a non-covalent co-incubation strategy [12,18]. In the current work we further developed stearyl-TP10 as a delivery vehicle for nucleic acids by improving its ability to form particles, endosomolytic properties and the stability in the presence of serum. We implemented two new chemical modifications in stearyl-TP10 sequence. The first modification was inspired by poly-L-ornithine's higher ability to condense DNA and therefore its superior transfection efficiency compared to equivalent poly-L-lysine-based systems [28,29]. Moreover, ornithine, as a non-coded amino acid is less susceptible to serum proteases and increases the stability of the peptide in a serum containing environment. The rationale behind the second modification was the higher transfection efficacy of ε-poly-L-Lys compared to α-poly-L-Lys due to the ability of the latter to form smaller particles with pDNA and to promote efficient endosomal escape [30].

Our results emphasize that both introduced modifications were well-justified and all new peptides surpassed the efficacy of stearyl-TP10 for intracellular delivery of nucleic acids [18]. NickFects condensed nucleic acids into 60–160 nm sized stable complexes that retained constant size in the presence of serum, suggesting that they should meet the size criteria for *in vivo* applications [31]. Remarkably high transfection level was achieved with NF51, which enabled highly efficient pDNA intracellular delivery in all tested cell lines, including hard to transfect cell lines, e.g., T and B lymphocytes, and mouse embryonic fibroblast cells. In several cell lines the gene expression obtained with NF51/pDNA complexes even exceeded the transfection level achieved by LF2000. Moreover, NF51 enabled the

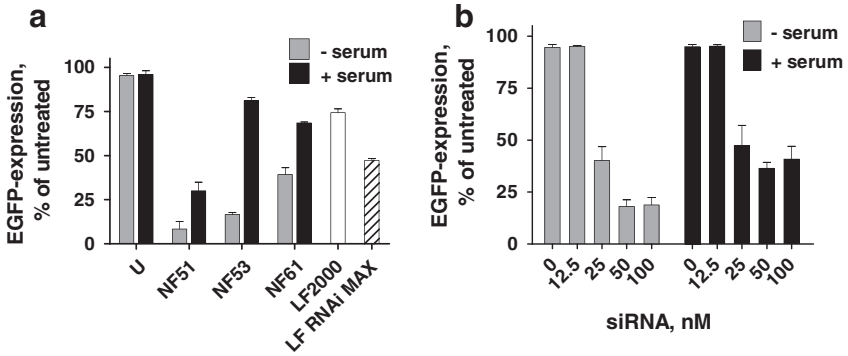

Fig. 5. Gene knockdown by NickFects/siRNA nanoparticles. RNAi response in EGFP-CHO cells measured by FACS analysis 48 h after single treatment with a) NickFects and 100 nM siRNA, molar ratio 10, controls Lipofectamine™ 2000and Lipofectamine™ RNAiMAX were used according to manufacturer's protocol and b) NF51/siRNA complexes, molar ratio 10, at different siRNA concentrations (12.5–100 nM).

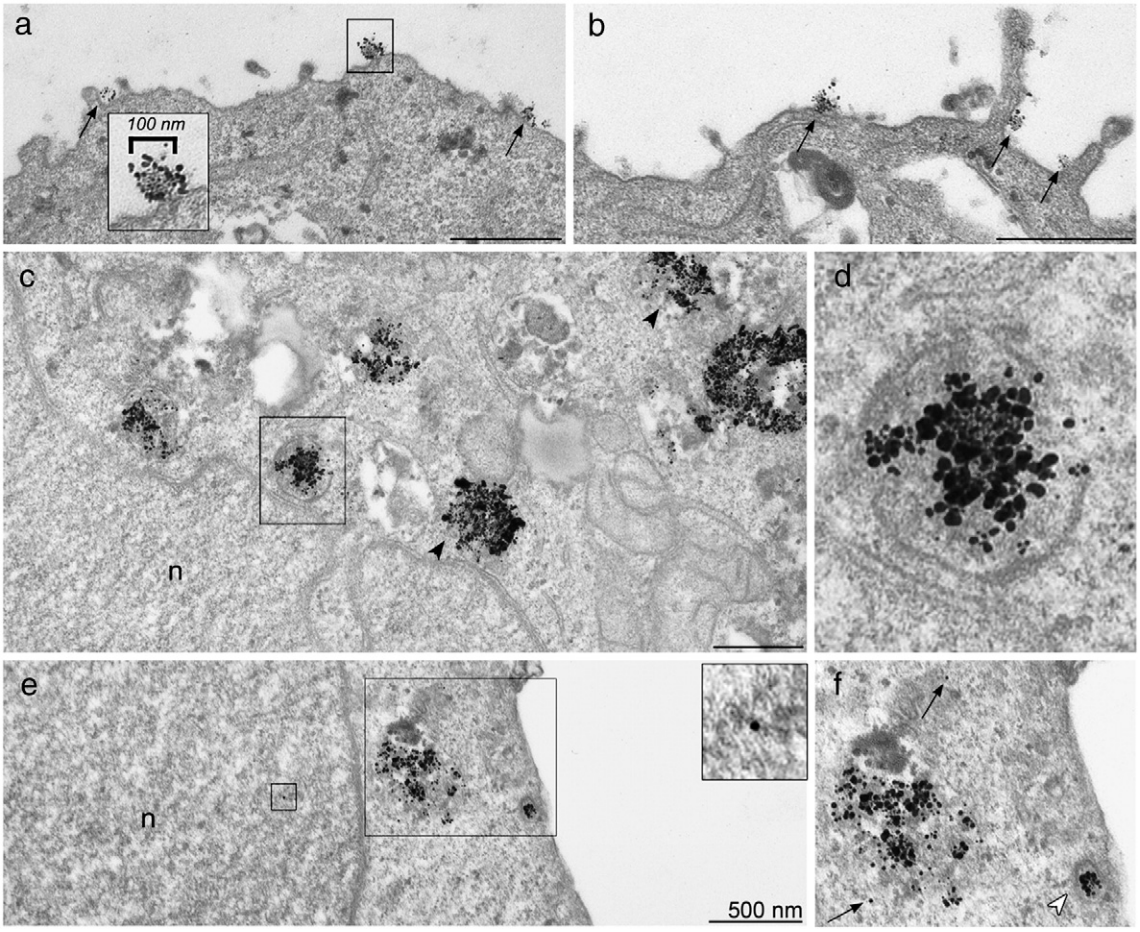

Fig. 6. Electron microscopy images of the interaction with plasma membrane and intracellular localization of NF51/SCO complexes. HeLa pLuc 705 cells were incubated with nanogold-labeled SCO/NF51 complexes for 30 min (a, b) or 4 h (c–e). Interaction of NF51/SCO nanoparticles with cell surface (arrows on a and b, enlarged inset on a) and localization in endosomal structures (c). (d) NF51/SCO complexes in vesicle with disrupted membrane (magnified image from c) and inside nucleus (enlarged inset on e). Arrow on (e) indicates cellular uptake of NF51/SCO complexes in endocytic vesicle. Scale bar 500 nm; n, nucleus.

transfection of almost a whole cell population, an effect that was not feasible by using lipofection.

Aberrant pre-mRNA splicing is involved in various diseases, and the blockage of aberrant splice sites by SCO has a significant therapeutic potential. Performing confocal and transmission electron microscopy analysis and positive read-out splice correction assay results all corroborated NF51-mediated SCO uptake to the cells and cell nucleus where splicing takes place. Even in the presence of serum NF51 induced higher splice correction than Lipofectamine™ 2000.

Despite great progress in the use of siRNA as therapeutics, only a handful of papers have reported on successful CPP-facilitated siRNA delivery [15,16,32–35]. NF51 formed small 74 nm sized nanoparticles with siRNA which entered quickly into the cells and transfected the whole cell population, whereas the target gene population was silenced by lipofection only partially. Maximal silencing in the target population was achieved in 48 h after a single treatment, and importantly, for effective gene downregulation very low concentrations of peptide (0.5–1 μM) and siRNA (25–50 nM) were needed.

As demonstrated in several studies, the high transfection efficacy is often associated with increased cellular toxicity, e.g. widely used reagent Lipofectamine™ 2000 significantly reduces cell viability at concentrations suggested by the manufacturer [36]. None of the NickFects revealed substantial cytotoxic effects to cells at used concentrations. Although NF51 yielded a transfection efficacy comparable to or even higher to that of LF2000, the high transfection efficacy of the peptide was not accompanied by increased cytotoxicity.

In parallel with the quantitative analysis of NF51/nucleic acid nanoparticles by DLS we assessed by electron microscopy, whether all sizes of nanoparticles are engulfed by cells or preferentially only a certain fraction of them is internalized. The nanogold labeled SCO complexed with NF51 formed small nanoparticles with diameters about 80 to 170 nm, which were by electron microscopy rarely detected on the cell surface but abundantly in cells. Both DLS and TEM reported the same size for NF51/nucleic acid nanoparticles, suggesting that despite of being less uniform or homogenous than stearyl-TP10/SCO nanoparticles, they are sufficiently stable in serum containing medium and on the cell surface. However, NF51 seems to organize SCOs into less tightly packed nanostructures since the electron density of particles was relatively low and single gold clusters are located at a distance from each other. The number of nanoparticles on the cell surface was very small compared to the amount of complexes inside cells, suggesting rapid and efficient cellular uptake. Most of the internalized complexes were detected in endososomal structures, but importantly, a considerable fraction of them had still escaped to the cytosol and reached the nucleus. It is reasonable to speculate that less densely packed NF51/oligonucleotide particles might dissociate into smaller clusters more easily and better translocate into the nucleus.

The main limiting step in the bioavailability of nucleic acids as therapeutics is the endosomal entrapment of CPP/cargo complexes. The lysomotropic agent chloroquine induces osmotic swelling and promotes disruption of endosomal compartments and subsequently endosomal release of CPP/cargo complexes. Both chemical modifications introduced

in the sequence of stearyl-TP10, significantly augmented the endosomlytic properties of NF51, as chloroquine did not increase the biological response obtained with NF51 as supposed to when the particles were entrapped in the endosomes.

Based on our findings that NF51 is a highly efficient and non-toxic transfection reagent for nucleic acids both in adherent and suspension cells, we estimated the applicability of NF51 in the mammalian protein production system, a QMCF technology, established by Icosagen Cell Factory. Comparing with transient protein production technologies where the expression of the protein of interest decreases in a few days after transfection, the copy number of QMCF expression plasmids and the expression of the protein of interest remains stable during a period of two months or longer of continuous culturing under selective conditions. The main advantage of QMCF technology is the possibility to generate the expression cell banks within 2–3 weeks after transfection.

There are certain requirements for the transfection reagent to be applicable in QMCF technology: it has to be non-toxic and gentle to delicate suspension and adherent modified cells, and it has to transfect the whole cell population. NF51 effectively facilitated pDNA intracellular delivery and 7 days after a single treatment almost all cell populations were transfected with the gene of interest. Our data corroborates that NF51 is a highly efficient peptide-based transfection reagent for application in protein production and thus it can be an easy to use alternative to electroporation that needs specific equipment.

5. Conclusions

In the current study, we demonstrated the advantage of two novel modifications implemented to a stearyl–TP10 sequence that leads to a highly efficient transport vector for intracellular delivery of nucleic acids. NickFect51 delivered plasmid DNA into a large variety of cell lines, including refractory suspension and primary cells and in several cases exceeded the transfection level of commercially available reagent Lipofectamine™ 2000 without any cytotoxic side effects. Besides that NickFect51 was applicable in spice correction and gene silencing. Furthermore, we demonstrated that NickFect51 can be utilized in a protein production system, QMCF technology, for expression and production of recombinant proteins in hardly transfectable suspension cells.

Altogether our data suggests that versatile transfection reagent NickFect51, which efficiently targets intracellular machineries both in the nucleus and cytoplasm, has a great potential in gene therapy applications and in protein production.

Conflicts of interest statement

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.01.011.

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