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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