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Characterization of reducible peptide oligomers as carriers for gene delivery

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ABSTRACT

The stability of DNA-polyplexes and intracellular DNA release are important features of gene delivery systems. To study these features, we have evaluated reducible cysteine-flanked linear lysine and arginine-rich peptides, modified with histidine residues. The reducible disulfide bonds in cysteine flanked peptides and histidine residues should augment DNA release from the peptide–DNA complexes upon disintegration of the reducible bonds. Template polymerization and oxidative polycondensation were applied to obtain peptide oligomers used for DNA-polyplex preparation. The peptides and DNA–peptide complexes were investigated with physical, chemical and transfection measurements. Physicochemical and transfection properties of DNA-polyplexes depended on the amino acid sequence of the peptidic polymers and type of the polymerization. MALDI-TOF analysis of oxidatively polycondensed products revealed several forms of peptide oligomers corresponding to 5–8 amino acid monomers. DNA–peptide particles based on template-polymerized complexes were more resistant to relaxation by negatively charged heparan sulfate than polyplexes formed with oxidatively condensed peptides. Complexes of DNA with the polycations prepared by oxidative polycondensation exhibited a 100–1000-fold higher level of gene expression compared to DNA/template-polymerized peptide complexes. The most efficient transgene expression was shown with arginine-rich polyplexes. Transfection efficacy of the arginine-rich polyplexes was even 10-fold better than that of DNA/PEI complexes. On average, polyplexes based on cysteine-flanked peptide oligomers showed lower cytotoxicity than non-reducible high molecular weight polylysine/DNA particles. We conclude that reducible peptide oligomers provide efficient DNA transfection and have the potential as vehicles for gene delivery.

1. Introduction

A variety of synthetic vehicles have been developed and studied for effective gene delivery. Vectors based on polycations such as polyethylenimine (Boussif et al., 1995), polyamidoamine dendrimers (Tang et al., 1996), lysine containing polypeptides and dendrimers (Wagner et al., 1990; Kiselev et al., 2007) have been widely used to transfer nucleic acids into cells. These polycations bind, condense, and protect plasmid DNA from enzymatic degradation. Polymer–DNA-complexes (i.e. polyplexes) show cellular gene transfer in vitro, but the efficacy of in vivo transfection is often poor. On the other hand, these vehicles are considered to be safer than viral vectors. Alternate biophysical methods (e.g. ballistic transfection and electroporation) can provide efficient in vivo gene delivery only locally (Zelenin et al., 1998; Mir, 2009).

Transfection efficacy of DNA polyplexes is determined by the cellular access of the polyplexes, their intracellular distribution, vehicle toxicity, and ability to protect DNA from degradation (Ledley, 1995; Ward et al., 2001). It was previously demonstrated, that high molecular weight polycations were toxic for cells (Brown et al., 2001), while small cationic peptides usually exhibit lower cytotoxicity (Plank et al., 1996). However, polyplexes based on low-molecular-weight polycations are unstable under physiological conditions (Ward et al., 2001). For example, extracellular glycosaminoglycans (GAG) may cause premature DNA release and/or relaxation from the polyplexes (Ruponen et al., 1999).

To circumvent these problems, short peptides may be cross-linked in order to generate more stable vehicle for DNA delivery. Trubetskoy et al. (1998) described sulfhydryl-reactive and crosslinker-assisted template polymerization of cysteine-flanked cationic peptide simultaneously with peptide/DNA complex formation. Further studies were devoted to cross-linking of peptides with disulfide bridges between cysteine residues without sulfhydryl-reactive crosslinkers. Two approaches for forming DNA-polyplexes containing reducible polycations (RPC) include oxidative

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polycondensation that depends on dimethylsulfoxide (DMSO)-mediated oxidation and polymerization on DNA template (McKenzie et al., 2000; Oupicky et al., 2002). Most of RPC studied to date have been generated to reach high-molecular weights, but there is not enough information concerning physico-chemical and transfection properties of their oligomeric forms (Oupický and Diwadkar, 2003). Parker et al. (2007) produced the lowest molecular weight product of 24 kDa ($CK_{16}C$ peptide) by oxidative polycondensation.

According to current knowledge cysteine-flanked peptides form small, stable DNA complexes that can be cleaved in the intracellular reducing environment. Relative stability of the disulfide bonds in the extracellular space makes this approach attractive for in vivo use (Trentin et al., 2006). Read et al. (2003) and Parker et al. (2007) developed synthetic vectors based on a linear reducible polycations prepared by oxidative polycondensation, but these vehicles required additional fusogenic agents to reach high level of transfection. In another study, transfection properties of DNA polyplexes based on arginine-rich TAT peptides were studied for complexes prepared both by template polymerization and oxidative polycondensation. Both approaches resulted in 100-fold higher transfection activity compared to low molecular weight forms of TAT/DNA conjugates, but transfection efficacy was chloroquine dependent (Manickam et al., 2005). Ability to escape from endosomes is an important requirement for successful gene delivery mediated by most of non-viral vectors. "Proton sponge" mechanism of endosomolysis is associated with PEI and PAMAM-dendrimer mediated transfections. This approach can also be used in peptide carriers (Cho et al., 2003), by including Lys for His residues in peptide-based vehicles. This resulted in enhanced in vitro gene expression due to the enhanced buffering capacity of imidazole groups at endosomal pH (Read et al., 2005). Other modifications of cross-linking peptide carriers structure (e.g. conjugation with fatty acid) also resulted in increase of transfection efficacy. Recently Tanaka et al. (2010) demonstrated suppressive effect in sarcoma bearing mice by means of anti-VEGF siRNA delivered by arginine-rich cross-linking peptides modified with stearoyl residues.

In the present study, we evaluated influence of amino acid sequence, length, and polymerization method of cysteine-flanked peptides on their ability (1) to bind DNA; (2) to protect DNA against nucleases and cell surface glycosaminoglycans; (3) to enhance transfection efficacy without toxicity. We studied peptide Cys-His-(Lys)6-His-Cys (designated K6) with enhanced buffering capacity in vitro in cells. Previously it was shown that minimal chain length of six cationic amino acids is required for the formation of DNA complexes (Plank et al., 1999). The peptide Cys-His-$(Lys)_6$-His-His-$(Lys)_6$-His-Cys (K12) was designed as duplicated variant of previous one in order to elucidate role of molecular weight of the cross-linking monomer. Substitution of Lys residues with Arg in the third peptide monomer Cys-His-$(Arg)_6$-His-Cys (R6) was based on data concerning cell-penetrating activity of arginine-rich TAT peptide (Ignatovich et al., 2003). The DNA-polyplexes prepared both by template polymerization and oxidative polycondensation were investigated. Oxidative polycondensation has resulted in generation of oligomeric forms of RPC. Oxidatively polymerized peptides were coded as K6p, K12p, and R6p, respectively.

2. Materials and methods

2.1. Cell lines

A Chinese hamster ovary (CHO) carcinoma cell line was obtained from the University of Eastern Finland (Kuopio, Finland). Human cervical carcinoma HeLa cell line was obtained from the Cell Collection of Institute of Cytology RAS (Saint-Petersburg, Russia). The cell lines were maintained under mycoplasma-free conditions in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal calf serum and 2 mM glutamine (referred to as culture medium) with the addition of 50 U/ml of penicillin, 50 μg/ml of streptomycin and 1 mM sodium pyruvate.

2.2. Peptide design and synthesis

K6, K12, R6 peptides were synthesized using a solid-phase Boc-chemistry by NPF Verta Ltd. (Saint-Petersburg, Russia), were supplied as a dry powder, and stored desiccated at −20 °C. Quantities of 1–2 mg were dissolved in 0.1% TFA at 2 mg/ml and stored in small aliquots at −20 °C. The purity of the peptides, as determined by high-performance liquid chromatography, was in the range 90–95%. K6p, K12p, and R6p carriers were synthesized by DMSO-mediated oxidation that was performed in 30 mM concentration of the peptides. Peptides were dissolved in 30% DMSO in 30 μl total volume. The reaction mixture was incubated at ambient for 96 h. Resulting polymers were dissolved in water at 2 mg/ml and stored in small aliquots at −20 °C. No centrifugal concentrating was done to prevent removal of low molecular weight oligomers. Subsequent analysis included size exclusion chromatography and MALDI-TOF.

2.3. Reporter plasmid, preparation of DNA complexes, DNA-binding assay and DTT destabilization

The pCLUC4 plasmid contained the firefly luciferase gene under the control of the cytomegalovirus promoter (gift from Dr Francis Szoka, UCSF, San Francisco, CA, USA). The plasmid was amplified from overnight bacterial cultures by alkaline lysis and purified using a Qiagen Plasmid Giga kit under endotoxin-free conditions (Qiagen, Dorking, UK). The purified DNA was diluted in TE buffer pH 8.0 to 1 mg/ml and stored in small aliquots at −20 °C. DNA/peptide complexes were prepared at various N/P ratios (peptide nitrogen/DNA phosphorus ratio) in the range 0.1–20. All positively charged amino acids were taken into account for the N/P charge ratios. The required amount of plasmid DNA was diluted to 20 μg/ml in Hepes-buffered mannitol (HBM) (5% (w/v) mannitol, 5 mM Hepes, pH 7.5). The appropriate volume of peptides at 2 mg/ml in HBM was added to the DNA solution and vortexed. Complexes were allowed to stand at room temperature for 30 min.

Polyethyleneimine (branched PEI 25 kDa; Sigma–Aldrich) was used as 0.9 mg/ml (pH 7.5) aqueous stock solution, stored at +4 °C. The ratio of PEI to DNA was 8:1. Hyperbranched polylysines were used as described previously (Egorova et al., 2009). Histidine-modified HbpKH1 and HbpKH2 vehicles were used as controls at N/P ratio 3/1. Peptide binding to DNA was monitored using ethidium bromide (EtBr) fluorescence quenching method. EtBr displacement was assayed following the decrease in emission fluorescence at 590 nm (544 nm excitation) after addition of peptides to a plasmid DNA solution (0.0025 mg/ml) prelabeled with the intercalating agent EtBr (400 ng/ml). Fluorescence measurements were performed in a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific Oy, Vantaa, Finland). Displacement was calculated as $(F - F_f)/(F_b - F_f)$, where Ff and Fb are the fluorescence intensities of EtBr in the absence and presence of DNA, respectively.

2.4. DNase I protection assay

The peptide/DNA complexes were prepared as described above and incubated with 0.2 units of DNase I (Ambion) for 30 min at 37 °C in a 1× DNase I buffer (Ambion). Then DNase I inactivation reagent (Ambion) was added for 2 min to inactivate DNase I. To release DNA the complexes were treated with trypsin (0.0625%)