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Synergistic effects of conjugating cell penetrating peptides and thiomers on non-viral transfection efficiency

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ABSTRACT

Nanoparticles generated by complex coacervation of plasmid DNA (pDNA) and modified chitosans namely chitosan–thioglycolic acid (TGA) conjugate and chitosan–HIV-1 Tat peptide conjugate were evaluated as gene delivery systems. In order to optimize transfection efficiency, chitosan–HIV-1 Tat peptide conjugate was combined with chitosan–TGA before its complexation with pDNA. Particle size and zeta potential measurements were performed to characterize the generated nanoparticles. The nanoparticles transfection efficiencies were assessed by exploitation of the green fluorescent protein (GFP) reporter gene. HEK293 cells were incubated for 24 h with the nanoparticles and the GFP positive cells were observed by fluorescence microscopy. The nanoparticles in the size range of 200–300 nm could transfect HEK293 cells as a model cell line with different transfection efficiencies. Unlike chitosan–TGA, chitosan–HIV-1 Tat peptide led to increased zeta potential of nanoparticles as compared to unmodified chitosan. The transfection efficiency of the nanoparticles generated by combination of chitosan–HIV-1 Tat peptide with chitosan–TGA was comparatively higher than that of the nanoparticles generated by either chitosan–TGA or the combination of chitosan–HIV-1 Tat peptide with unmodified chitosan. After 72 h of incubation, the combination of chitosan–HIV-1 Tat peptide with chitosan–TGA was found to be 7.12- and 67.37 times more efficient than unmodified chitosan and pDNA alone, respectively and showed a synergistic effect in transfection of pDNA into the cells. Moreover, none of the nanoparticles showed any severe cytotoxicity. Accordingly, this strategy might result in a potent carrier for gene delivery.

1. Introduction

The two main types of vectors that are used in gene therapy are based on viral and non-viral gene delivery systems. The viral gene delivery system shows a high transfection yield but it has many disadvantages such as oncogenic effects and immunogenicity. Non-viral gene therapy being based on nanoparticulate delivery systems using cationic polymers such as chitosan shows good biocompability, but still very low transfection efficiency in comparison with viral vectors [1].

On the one hand, chitosan, as cationic polymer can interact with DNA and its potential as vehicle for gene delivery can be improved by thiolation. Martien et al., for instance, reported that transfection studies with thiolated chitosan nanoparticles in Caco-2 cells during the exponential growth phase and differentiation growth phase of the cells led to a 5- and 2-fold increase, respectively, in gene expression than unmodified chitosan nanoparticles [2]. Both mucoadhesive and permeation enhancing properties of thiomers could lead to increased transfectability in vitro as well as in vivo [3]. Moreover, it is assumed that thiomers might undergo a thiol-disulfide exchange reaction in the presence of glutathione in the reducing environment of the cytoplasm. Accordingly, disulfide bonds within thiomers are cleaved in the cytoplasm resulting in the release of pDNA [4].

On the other hand, a promising strategy to enhance transfection of DNA into cells is the chemical conjugation of cell penetrating peptides (CPP) derived from proteins capable of crossing the plasma membrane directly to nanoparticulate delivery systems. A large number of different compounds such as oligonucleotides, peptides, proteins and liposomes have been delivered into cells by

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this strategy. One of these translocating peptides is derived from the HIV-1 Tat protein [5,6] exhibiting a positive charge in its transduction domain which extends from residues 47–57 (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) responsible for transduction ability [7]. Therefore, the conjugation of CPP to thiolated chitosan/pDNA complexes could offer the advantages of both systems for enhanced transfection efficiency.

So far, however, transfection using combination of chitosan–HIV-1 Tat peptide (GRKKRRQRRPPQC) with thiomer for non-viral gene delivery system has not been reported. It was therefore the aim of this study to combine both promising strategies – the HIV-1 Tat peptide and thiolated chitosan – in order to optimize a non-cytotoxic, non-immunogenic and potent non-viral gene delivery system.

2. Experimental part

2.1. Materials

The plasmid DNA (pEGFP) was kindly donated by Gottfried Baier, Department for Medical Genetics, Molecular and Clinical Pharmacology, Medical University Innsbruck. PureLink TM HiPure Plasmid DNA Megaprep Kits and N-hydroxysulfosuccinimide (NHSS) were purchased from Invitrogen™, Life Tech. (USA). Low viscous chitosan, penicillin-streptomycin, MES hydrate, RPMI-1640 medium, Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic cid)), 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dialysis tubing (molecular weight cutoff 12 kDa, cellulose membrane) and thioglycolic acid (TGA) were obtained from Sigma–Aldrich (Vienna, Austria), whereas HIV-1 Tat peptide (GRKKRRQRRPPQC) was purchased from piCHEM, Graz, Austria. All other salts and chemicals were of analytical grade.

2.2. Cell culture conditions

Human embryonic kidney cells (HEK293) were maintained at 37 °C under 5% CO_2 and 90% relative humidity in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.3. Amplification and purification of plasmid DNA

The plasmid DNA (pEGFP) was maintained and propagated in *Escherichia coli*. It was isolated and purified using PureLink™ HiPure Plasmid DNA Megaprep Kits in accordance with the manufacturer's instructions, resuspended in distilled water and stored at −20 °C. The purity was assessed with 1% w/v agarose gel electrophoresis and pDNA concentration was measured by UV absorption at 260 nm (UV-1202 SHIMADZU spectrophotometer).

2.4. Synthesis of the chitosan–TGA conjugate

The synthesis of the chitosan–TGA was carried out as described previously [8]. Briefly, TGA was attached covalently to chitosan. First, 500 mg of low viscous chitosan was hydrated in 4 ml of 1 M HCl and dissolved by the addition of demineralized water to obtain a 1% solution of chitosan hydrochloride. Thereafter, 500 mg of TGA was added. After TGA was completely dissolved in the chitosan hydrochloride solution, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was added in a final concentration of 125 mM in order to activate the carboxylic acid moieties of TGA. The reaction mixture was incubated at pH 4 for 3 h at room temperature under stirring. Samples prepared in exactly the same way but omitting EDAC during the coupling reaction served as controls for the analytical studies. In order to eliminate unbound TGA and to isolate the polymer conjugates, the reaction mixtures were dialyzed five times in dialysis tubing (molecular weight cutoff 12 kDa, cellulose membrane) for 3 days in total at 10 °C in the dark. In detail they were dialyzed one time against 5 mM HCl, then two times against the same medium but containing 1% NaCl to quench ionic interactions between the cationic polymer and the anionic sulfhydryl compound. Then, the samples were dialyzed exhaustively two times against 1 mM HCl to adjust the pH of the polymer to 4. Thereafter, samples and controls were lyophilized by drying frozen aqueous polymer solutions at −70 °C and 0.01 mbar (Benchtop 2K, VirTis, NY, USA) and stored at 4 °C until further use.

2.5. Synthesis of the chitosan–HIV-1 Tat peptide (GRKKRRQRRPPQC) conjugate

HIV-1 Tat peptide was conjugated to low viscous chitosan via EDAC/NHSS chemistry according to procedure described by Liu et al. [9] and Kafedjiiski et al. [10] with slight modification. Typically, 1 ml of 1% chitosan in 1% HCl solution containing 0.1 M MES buffer pH 6 were mixed with 200 mM EDAC and 200 mM NHSS at room temperature. In the following, 5 mg of HIV-1 Tat peptide was added to the chitosan solution and incubated for 4 h at room temperature. Afterward, 1 mg of tris(2-carboxy-ethyl)phosphine hydrochloride was added and incubated for 30 min. Samples prepared in exactly the same way but omitting EDAC during the coupling reaction served as controls for the analytical studies. Unreacted HIV-1 Tat peptide and other reagents were removed by exhaustive dialysis (cellulose membrane, molecular weight cutoff 12 kDa) against deionized water. Samples and controls were harvested by freeze-drying at −70 °C and 0.01 mbar (Benchtop 2K, VirTis, NY, USA) and stored at 4 °C until further use.

2.6. Determination of free thiol group content

The amount of free thiol groups immobilized on the conjugates was determined by using Ellman's reagent, whereas disulfide content was determined using $NaBH_4$ and Ellman's reagent [4].

2.7. Formulation of nanoparticles

Unmodified chitosan (low viscous), chitosan–TGA and chitosan–HIV-1 Tat peptide were prepared in 5 mM acetic acid solution with stirring at final concentration of 0.02%, and the pH of solutions was adjusted to 4 with 1 M NaOH. All solutions were sterile filtered through a 0.2 μm filter as the polymer working solution. The pDNA (25 μg) dissolved in 250 μl of 50 mM sodium sulfate and the polymer solutions were heated at 50 °C for 10 min. The pDNA and polymer solutions were quickly mixed together by vortexing at 2500 rpm for 20 s. The volume of each polymer solution which is mixed with the pDNA solution is given in Table 1.

2.8. Nanoparticle characterization

Particle size and zeta potential of nanoparticles were measured with a NICOMP® ZPW 388 submicron particle sizer (Nicomp Particle Sizing Systems, Santa Barbara, USA). The particle size measurement was performed at room temperature at a scattering angle of 90°. Each measurement was run for 10 min. For the zeta potential measurements, electrodes were directly dipped in the nanoparticles suspension to provide a quantitative measure of the charge by using the electric field strength set to 1 V/cm [2].

2.9. Electron microscopic characterization of nanoparticle

A 10 μl drop of freshly prepared nanoparticle suspension was placed on a carbon-coated copper grid and air-dried for 15 min. The rest of sample on the grids were dispunged with paper and dried under a lamp for 15 min. Samples were examined with a ZEISS LIBRA® 120 transmission electron microscope with an in-column energy filter (EFTEM). The morphology and size of particles was studied by applying selected contrast. Thus, nanoparticles were analyzed at 0, or 50–70 eV energy loss, depending on their density. Digital micrographs were obtained from a ProScan Slow Scan CCD camera system using iTEM® 5.0 Software from Soft Imaging System GmbH [4].

2.10. In vitro transfection assay

HEK293 cells were seeded on a 12-well plate at an initial concentration of 5×10^4 cells per well and incubated for 24 h before addition of the nanoparticle suspension. The condition of incubation set up was the same as described previously. The generated nanoparticles (Table 1) were tested for gene delivery using pDNA expressing a green fluorescent protein (GFP). The culture medium was removed and substituted with fresh medium containing either 10 μg/ml pDNA or 260 μl of each tested nanoparticles suspension (Table 1). After incubation for 24 h, culture medium was changed with fresh medium. The culture cells were harvested for FACS analysis after 72 h of incubation.

2.11. Determination of transfection efficiencies

After 72 h of incubation, HEK293 cells were washed twice with 1 × PBS, trypsinized for 5 min. The cells were then centrifuged at 12,000 rpm at room temperature for 5 min and the supernatant was discarded. Pellets were resuspended in 1 × PBS and cell-associated fluorescence was determined using FACScalibur™ flow

Table 1
Formulation of nanoparticles.

Formulation	pDNA solution (25 μg)	Unmodified chitosan (0.02%)	Chitosan–TGA (0.02%)	Chitosan–HIV-1 Tat (0.02%)
F1	250 μl	400 μl	–	–
F2	250 μl	–	400 μl	–
F3	250 μl	–	–	400 μl
F4	250 μl	200 μl	–	200 μl
F5	250 μl	–	200 μl	200 μl