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Oligoethylenimine-grafted polypropylenimine dendrimers as degradable and biocompatible synthetic vectors for gene delivery

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ABSTRACT

Several grafted polypropylenimine dendrimers were synthesized by modifying either polypropylenimine (PPI) dendrimer generation 2 (G2) or generation 3 (G3) via 1.6-hexandioldiacrylate with branched oligoethylenimine 800Da (OEI) or PPI dendrimer G2. The resulting derivatives were characterized (^{1}H NMR, GPC) and their biophysical properties such as DNA condensing ability, colloidal stability and hydrodynamic diameters were determined. All grafted dendrimers were able to efficiently compact DNA to nanosized polyplexes (100–200 nm) and exhibited an increased colloidal stability as compared to their unmodified counterparts. *In vitro*, grafted dendrimers resulted in much higher transfection levels as compared to the unmodified ones displaying alongside a clear structure–activity relationship regarding their transfection/toxicity profile. Transfection levels of OEI-grafted dendrimers were the highest, being similar or even higher as compared to standard polyethylenimines (linear and branched), demonstrating that the incorporation of ethylenimine moieties is the key factor contributing to this boosted transfection efficiency. None of the compounds resulted in polymer-induced erythrocyte aggregation. Upon i.v. injection of OEI-grafted dendrimer polyplexes into tumor-bearing mice transgene expression was predominantly found in the (subcutaneous) tumors. Importantly, the tumor gene expression levels significantly increased with the higher dendrimer core generation.

1. Introduction

Numerous synthetic gene delivery systems including cationic polymers, polypeptides and lipids have been synthesized in the past as alternatives to viral gene delivery devices [1]. Nevertheless, so far their clinical implementation and potential benefits remained limited [2,3], partially due to challenges of efficient systemic gene delivery.

Among synthetic gene carriers, dendrimers gained within the last years progressively the researchers' interest due to their well-defined structures and variable functionalities within their inner and outer parts [4–8]. Several modifications of the most investigated poly (amidoamine) (PAMAM), polylysine (PLL) and poly(propylenimine) (PPI) dendrimers including PEGylation, coupling of amino acids or ligands have been studied by a number of groups [9–16].

Recently, so called pseudodendrimers based on oligoethylenimine 800Da (OEI) as core unit were synthesized in a combinatorial approach. Structure–activity relationship studies within this class of polymers determined the constitutional requirements needed for successful gene delivery [8]. In order to obtain better defined polymers, the randomly branched non-toxic OEI core unit was replaced by non-toxic low generation polypropylenimine (PPI) dendrimers as central moiety. By the introduction of different PPI dendrimer generations within the core unit influences on physicochemical and biological properties of the resulting DNA polyplexes were investigated. Thus, generation 2 (G2) and generation 3 (G3) PPI dendrimers were grafted via ester-degradable branches with either OEI or PPI dendrimer G2 as surface modification. Both grafting units were studied in order to find out which of the two polymer chemistries (*ethylen*imine units: higher nitrogen density, but lower p*K* values, only partial protonation at neutral pH; *propylen*imine units: higher hydrocarbon content, lower nitrogen density but higher p*K* values) achieves the higher gene transfer efficiency at good biocompatibility—*in vitro* and *in vivo*.

In the current study the advantages of grafted PPI dendrimers with regard to their biophysical and biological properties were demonstrated. Their synthesis and characterization was described and investigations on biophysical properties, such as DNA condensation efficiency, colloidal stability of polyplexes as well as particle sizes and zeta-potentials were carried out. Furthermore, their transfection and toxicity profile under *in vitro* conditions was evaluated and the key factor contributing to enhanced transfection efficiency of the OEI-grafted dendrimers over the PPI-grafted ones was elaborated by bafilomycin A1 experiments. The biocompatibility of all conjugates was shown under *in vitro* and *in vivo* conditions. *In vivo* transfection studies resulted in efficient gene transfer to tumor tissue after intravenous injection of polyplexes in subcutaneous tumor-bearing mice.

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Importantly, these studies revealed a clear influence of the dendrimers' generation used within the core unit on the gene expression level resulting in significantly ($p<0.05$) higher expression levels if generation 3 (G3) dendrimer was used instead of generation 2 (G2) dendrimer.

2. Materials and methods

2.1. Materials

DAB-Am-8, polypropylenimine-octaamine dendrimer, generation 2.0 (PPI G2), DAB-AM-16, polypropylenimine-hexadecaamine dendrimer, generation 3.0 (PPI G3), low molecular weight polyethylenimine 800 Da waterfree (oligoethylenimine, OEI), 1.6-hexandioldiacrylate (HD), methylthiazolyldiphenyl-tetrazolium bromide (MTT), deuterium oxide (D_2O), dimethyl sulfoxide puriss. waterfree (DMSO) were purchased from SIGMA-Aldrich (Steinheim, Germany). PPI G2 and PPI G3 are also available from SyMO-Chem (Eindhoven, The Netherlands). Bafilomycin A1 was obtained from Alexis Biochemicals (Lausen, Switzerland). Linear PEI 22 kDa (LPEI) was synthesized by acid-catalysed deprotection of poly(2-ethyl-2-oxazoline) (50 kDa, Aldrich) in analogous form as described [17] and is also available from Polyplus Transfections (Strasbourg, France). All reagents were used without further purification. LPEI and BPEI were adjusted to pH 7 using 1 M HCl prior to use. Cell culture media, antibiotics and fetal calf serum (FCS) were purchased from Life Technologies (Karlsruhe, Germany). Luciferase cell culture lysis buffer 5×reagent and D-luciferin sodium salt were obtained from Promega (Mannheim, Germany). Plasmid DNA pCMVLuc for *in vitro* studies (Photinus pyralis luciferase under control of the CMV enhancer/promoter) described in Plank et al. [18] was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany). pEGFPLuc as used for *in vivo* studies (a fusion of enhanced green fluorescent protein (EGFP) and Photinus pyralis luciferase under control of the CMV enhancer/promoter) was obtained from Clontech Laboratories (Mountain View, CA) and was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany). Water was used as purified, deionized water.

2.2. Synthesis of grafted PPIs

Grafted dendrimers were synthesized in two steps, similarly as previously described [8]. In brief, G2 or G3 was coupled with an excess of 1.6-hexandioldiacrylate (HD) to generate the core unit. In a typical experiment, G2 (Mw 773) (77.3 mg, 0.1 mmol) or G3 (Mw 1687) (75.9 mg, 0.045 mmol) and HD (452.5 mg, 2 mmol) were separately dissolved in DMSO (1.0 mL) under argon (stock solutions). 100 µl G2 stock (0.01 mmol) or G3 stock solution (0.0045 mmol) was mixed with 800 µl DMSO and subsequently 100 µl HD stock solution (0.2 mmol) was added. The reaction occurred at 45 °C for 24 h under constant shaking (1000 rpm, Eppendorf Thermomixer). The resulting mixture was used for further synthesis without isolation of the product. Grafting the acrylate terminated cores proceeded for each oligoamine (G2 or OEI) using a 0.6 M stock solution (in DMSO, 0.5 mL for G2 core unit; 1.1 mL for G3 core unit). Reaction occurred for 24 h at 22 °C (1000 rpm, Eppendorf Thermomixer). The obtained products were diluted 1:10 in water and the pH was immediately adjusted to pH 7 using 1 N HCl. Purification was done via dialysis using a Spectra/Por membrane against 4 L of water at 4 °C for 24 h (MWCO 1K for G2 core derivatives; MWCO 3.5 K for G3 core derivatives) (Spectrum Medical Industries, Inc, CA). Water was exchanged twice. The dialysates were freeze-dried. Yields of obtained products were listed in Table S1.

2.3. Characterization of grafted PPIs

Resulting products were characterized by ^{1}H NMR spectroscopy. Spectra in D_2O (10 mg/mL) were recorded on an Eclipse +500 spectrometer (JOEL, Tokyo, Japan) operating at 500 MHz. Chemical shifts are reported in ppm.

^{1}H NMR (D_2O) of **G2-HD-OEI**: δ 4.0 (COOCH_2, ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HOCH_2, mono-ester, 2H), δ 2.5–3.4 (136H NCH_2 of OEI ethylenes, N$CH_2CH_2CH_2$ of PPI propylenes, NCH_2CH_2COO linker), δ 1.7–2.0 (28H of NCH$_2CH_2$CH$_2$ of PPI propylenes), δ 1.6 (COOCH$_2CH_2$, 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2CH_2$ mono-ester); δ 1.35 (4H–OCH$_2$CH$_2CH_2$ linker). **G2-HD-G2**: δ 4.0 (COOCH_2, ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HOCH_2, mono-ester, 2H), δ 2.8–3.4 (64H N$CH_2CH_2CH_2$ of PPI propylenes, NCH_2CH_2COO linker), δ 1.65–2.2 (28H of NCH$_2CH_2$CH$_2$ of PPI propylenes), δ 1.65 (COOCH$_2CH_2$, 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2CH_2$ mono-ester); δ 1.35 (4H–OCH$_2$CH$_2CH_2$ linker). **G3-HD-OEI**: δ 4.0 (COOCH_2, ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HOCH_2, mono-ester, 2H), δ 2.5–3.3 (200H NCH_2 of OEI ethylenes, N$CH_2CH_2CH_2$ of PPI propylenes, NCH_2CH_2COO linker), δ 1.75–2.1 (60H of NCH$_2CH_2$CH$_2$ of PPI propylenes), δ 1.65 (COOCH$_2CH_2$, 4H di-ester, 2H mono-ester), δ 1.55 (2H HOCH$_2CH_2$ mono-ester); δ 1.4 (4H–OCH$_2$CH$_2CH_2$ linker). **G3-HD-G2**: δ 4.0 (COOCH_2, ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HOCH_2, mono-ester, 2H), δ 2.5–3.3 (184H N$CH_2CH_2CH_2$ of PPI propylenes, NCH_2CH_2COO linker), δ 1.75–1.9 (88H of NCH$_2CH_2$CH$_2$ of PPI propylenes), δ 1.65 (COOCH$_2CH_2$, 4H di-ester, 2H mono-ester), δ 1.55 (2H HOCH$_2CH_2$ mono-ester); δ 1.4 (4H–OCH$_2$CH$_2CH_2$ linker).

Hydrolytic degradation of the conjugates was investigated in aqueous solution (10 mg/mL; 1 mL, D_2O) at pH 7 at 37 °C using ^{1}H NMR spectroscopy. Remaining ester content in solution was expressed as a percent relative to determined ester content after synthesis ($t=0$ d). The percent of remaining ester content was calculated from the integrals of the signals attributed to the methylene protons neighbouring to the ester (δ 4.0 COOCH_2) and hydroxyl groups (δ 3.5 HOCH_2), respectively.

Molecular weights of grafted dendrimers were determined by gel permeation chromatography (GPC). GPC analysis was performed using an Agilent 1200 series HPLC system (Morges, Switzerland) equipped with a refractive index detector, a NOVEMA 10 µm precolumn and a NOVEMA 300 analytical column (10 µm, 8×300 mm) (PSS, Mainz, Germany). The mobile phase was maintained in formic acid and sodium chloride (0.1% (V/V) HCOOH, 0.1 M NaCl, pH 2.8) at a flow rate of 1 mL/min. 10 µl of methanol were added to 1 mL of sample (5 mg/mL) as internal standard. Results were evaluated using PSS WinGPC Unity software. Molecular weights (Table S1) were measured relative to Pullulan molecular weight standards that were used for preparing a standard calibration curve.

2.4. Ethidium bromide exclusion assay

DNA condensation ability was evaluated in HBG (20 mM HEPES, 5% (V/V) glucose, pH 7.4) and HBS (20 mM HEPES, 0.15 M NaCl, pH 7.4) using an ethidium bromide exclusion assay as described elsewhere [19]. Results are given as relative fluorescence intensities to plain DNA resulting in 100% fluorescence intensity. All experiments were run three times. Mean values±SD are indicated.

2.5. Polyplex formation

Plasmid DNA was condensed with polymers at indicated conjugate/plasmid (c/p)-ratios (weight/weight). In general, HBG was used for polyplex formation unless otherwise indicated. In brief, polyplexes were prepared by adding the polymer solution at varying concentrations to DNA solution. Polyplexes were allowed to incubate for 20 min at room temperature prior to use.

2.6. Stability of complexes against NaCl

The stability of polyplexes against an increasing amount of NaCl was studied using a Cary eclipse fluorimeter (Varian Deutschland GmbH, Darmstadt, Germany) at $\lambda_{ex}=\lambda_{em}=600$ nm following a procedure as described by Oupický et al. [20]. A significant reduction