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Propoxylation of cationic polymers provides a novel approach to controllable modulation of their cellular toxicity and interaction with nucleic acids

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ABSTRACT

An effective chemical approach to modulation of biological interactions of cationic polymers was proposed and tested using polyethyleneimine (PEI) as a drug carrier. Branched 25 kDa PEI was modified in the reaction with propylene oxide (PO) to produce a series of propoxylated PEIs with NH groups grafted by single or oligomer PO units. Clear relationships between the propoxylation degree and biological effects, such as interaction with plasmid DNA, hemolytic, cytotoxic, and pro-apoptotic activities were revealed for PEIs modified upon PO/NH molar ratio of 0.5, 0.75, 1.0 and 3.0. The partial modification of available cationic centers up to 100% is predominantly accompanied by a significant gradual reduction in polycation adverse effects, while ability of complex formation with plasmid DNA is being preserved. Grafted PEI with 0.75 PO/NH ratio provides better protection from nuclease degradation and transfection activity compared with other modified PEIs. Revealed relationships contribute to the development of safe polymeric systems with controllable physicochemical properties and biological interactions.

1. Introduction

Synthetic cationic polymers are important polymeric systems which have been intensively studied as components of biomedical materials and drug formulations. Cationic polymers exhibit relatively strong interactions with anionic membrane constituents, nucleic acids and other macromolecules, which may result in beneficial antimicrobial effect [1,2] and promotion of cellular uptake [3], or undesirable (cyto-)toxicity [4].

Cationic polymers have been used as cell adhesives [5], controlled release systems [6,7], and oral drug formulations [8,9]. Non-viral gene delivery is one of the most promising applications of cationic polymers, which covers the direct gene therapy of multiple disease, modification of biomedical materials as well as cell biology research [10,11].

Basic amino acids and their derivatives have been utilized to produce biodegradable linear homopeptides and dendrimeric poly(amidoamine) peptides with a good ability for binding, condensation and cellular translocation of nucleic acids [10–14]. Among synthetic cationic carriers, polyethylenimines (PEIs) are considered as the 'gold standard' characterized by a high cationic charge, controllable structure, and robust synthesis techniques [15,16].

Toxicity issues of PEIs and other polycations have been reported previously in experimental studies [17–22] and reviews [3,16]. High membrane-damaging and hemolytic action of the polycations remains the main obstacle to their use as therapeutic gene carriers [4]. Adverse effects of cationic polymers are especially critical in vivo, compromising their therapeutic potential [23].

An appropriate chemical structure of cationic polymers should be developed to reduce side interactions, toxic outcome and improve pharmacokinetics of polymeric complexes with a substance delivered. Grafting of polymer backbone with small molecules and oligomers is a primary approach to improving biocompatibility of polycations, optimizing their cellular transport, and increasing selectivity. Attachment of saccharides, e.g. by cross-linking of PEI fragments with mannitol diacrilate to stimulate caveolae-mediated endocytosis [24], grafting of PEG molecules [19,25,26], and some combinative modifications [11,27] were recently proposed. Labeling of biodegradable PEI derivative with pyridoxal phosphate for specific gene delivery into cancer cells was reported [28]. Similarly, PEI–pullulan–folic acid conjugate has been recently developed for targeted delivery of plasmid DNA and short interfering RNA into folate receptor-overexpressing cells [29].

Among above approaches, PEGylation is the most accepted technology to modulate bio-interactions of macromolecular drugs and polymers [15,20,26,30]. In particular, structure-activity relationships of PEI modified with different PEG molecules of ~0.5–20 kDa were examined in [19,25]. The PEGylation allows for non-specific attenuation of side

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interactions of polycations by introduction of inert hydrophilic PEG component. However, this modification does not introduce any amphiphilic properties to polymers, which might impart them with relatively specific cellular effects to promote drug-induced activity and selectivity [31,32]. A promising alternative to PEGylation could be a technique to alter both biocompatibility and hydrophilic-lipophilic properties of macromolecules, thus providing more effective regulation of their biointeractions.

We propose the controllable propoxylation of cationic polymers as a new technique to modulate their interactions with cellular membranes and nucleic acids. In our study, branched PEI, a model cationic polymer, was modified with amphiphilic propylene glycol component in the reaction with propylene oxide. Structure-activity relationships for modified PEIs were revealed, and the potency of this modification approach was demonstrated.

2. Materials and methods

2.1. Reagents and materials

Branched polyethylenimine (PEI) 25 kDa was obtained from Sigma-Aldrich (CAS 9002-98-6). Propylene oxide was purchased from Acros (CAS 75-56-9). 2′-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride, (Hoechst 33342), chloroquine diphosphate, thiazolyl blue tetrazolium bromide (MTT), heparin sodium salt (from porcine intestinal mucosa), and ethidium bromide were purchased from Sigma-Aldrich. FITC-annexin V/propidium iodide dead cell apoptosis kit was supplied by BD Pharmingen. TurboFect transfection reagent and DNAse I were purchased from Fermentas. Pyrene was purchased from Acros.

Plasmid DNA (pDNA) pEGFP-N2 encoding enhanced green fluorescent protein was purchased from Clontech. GeneRuler™ DNA ladder mix (100–10,000 bp) was purchased from Thermo Scientific. Cell culture reagents were obtained from PAA Laboratories.

2.2. Propoxylation of polyethyleneimine

Branched PEI was modified in one- or two-step reactions with different quantities of propylene oxide (PO) per primary and secondary amino groups (NH) of PEI as described in [33]. The one-step modification was carried out by the reaction of 50 wt% PEI water solution with PO in a nitrogen atmosphere at +95–100 °C followed by the product drying on a rotary evaporator. Propoxylated PEIs with PO/NH group molar ratio of 0.5, 0.75, or 1.0 were thereby synthesized.

Further PO oligomerization was carried out by the reaction of propoxylated PEI (PO/NH ratio 1:1) with additional two moles PO in the presence of alkaline catalyst (KOH) to produce modified PEI with PO/NH ratio 1:3 [33]. The presence of PO units in modified PEIs was verified by an attenuated total reflectance FTIR spectroscopy on a Frontier spectrometer (Perkin Elmer). Spectra were recorded in 4000–400 cm^{-1} wavenumber region with the resolution of 1 cm^{-1}. ^1H NMR spectra of PEIs were recorded on a Bruker AVANCE 400 spectrometer at operating frequency of 400.17 MHz. Chemical shifts were registered relative to DMSO-d_6 solvent (2.50 ppm).

2.3. Preparation of plasmid DNA/polycation complexes

Serial dilutions of cationic polymers were prepared from their stock solutions of 5 mg/mL in PBS for cytotoxicity and transfection studies or in 50 mM HEPES buffer for DLS characterization (Section 2.4). An aliquot of the polymer solution was gently mixed with pDNA (pEGFP-N2) to obtain final concentration of pDNA of 10 μg/mL. The mixture was incubated at room temperature for 20 min for the formation of electrostatic pDNA/polycation complexes. The weight ratio of a polycation and pDNA in complexes and corresponding molar ratio of PEI amino groups to pDNA phosphate groups (N/P ratio) were determined.

2.4. Characterization of plasmid DNA/polycation complexes

pEGFP-N2 was isolated from transformed *Escherichia coli* overnight culture with the use of MaxiPrep plasmid DNA (pDNA) isolation kit (Thermo Scientific). The purity and integrity of pDNA was verified by spectrophotometric analysis (A_{260}/A_{280} ratio) and electrophoretic analysis in 1% agarose gel. DNA-binding concentrations of propoxylated PEIs and corresponding pDNA/PEI ratios were determined by the agarose gel retardation assay.

pDNA/polycation complexes were characterized by the dynamic light scattering (DLS) technique on a Zetasizer Nano ZS analyzer (Malvern Instruments). Hydrodynamic diameter and zeta potential were registered in 50 mM HEPES buffer (pH 7.0) at pDNA concentration of 10 μg/mL and the binding concentration of the polymer. Micelle-forming properties of modified PEIs were assessed with the use of pyrene fluorescent probe as described earlier [34].

2.5. Study of DNAse I resistance of plasmid DNA/polycation complexes

The ability of propoxylated PEIs to protect pDNA from degradation by DNAse I was studied. Naked pDNA and pDNA/polycation complexes (final pDNA concentration 10 μg/mL) were incubated with DNAse I (0.08 U/mL) in the digestion buffer (10 mM $MgCl_2$, 50 mM tris-HCl, pH 7.6) at 37 °C for 30 min. The reaction was stopped with 0.25 M EDTA and pDNA was released from the complex by adding heparin (160 μg/mL). Released pDNA was analyzed by 1% agarose gel electrophoresis.

2.6. Evaluation of polycations biocompatibility in vitro

2.6.1. Cell isolation and culturing

Human skin fibroblasts (HSFs) were isolated from the skin explant as described previously [35]. Briefly, a full-thickness postsurgical skin explant of ~1.5 cm^2 in area was rinsed with 70% ethanol, washed with sterile PBS solution, and then aseptically minced with surgical scissors. The minced explant was transferred into T75 culture flask, covered with a medium (α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin) and cultured in standard conditions (37 °C, humidified 5% CO_2 atmosphere). The medium was replaced once per week, until substantial number of fibroblasts was observed. HEK293 (human embryonic kidney) cells obtained from ATCC were cultured in supplemented DMEM in standard conditions. Adhered cells were collected from the culture flask by detaching them with trypsin-EDTA solution. Suspended cells were washed by centrifugation at 200 ×*g* in PBS.

2.6.2. Hemolysis test

The hemolytic activity of propoxylated PEIs was investigated according to ISO 10993-4:2002 [36]. Freshly collected human blood in heparinized tubes was centrifuged at 700 ×*g* for 10 min. The pellet containing red blood cells (RBCs) was washed 3 times with chilled PBS (pH 7.4) by means of centrifugation (700 ×*g*) and resuspended in the same buffer. Polymers solutions at different concentrations were added to RBC suspension (5×10^4 cells/mL) and incubated for 2 h at 37 °C under moderate shaking. After RBC sedimentation hemoglobin released was determined in the supernatant using a microplate analyzer Infinite 200 PRO (Tecan) at the wavelength of 540 nm. The hemolysis rate induced by 0.2% Triton X100 was assumed to be equal to 100%.

2.6.3. MTT assay

Cytotoxic concentrations (IC_{50}) of propoxylated PEIs were determined with the use of MTT assay [37]. HEK293 and HSFs were seeded in 96-well plate at the density of 1000 and 2000 cells per well, respectively. Next day, aliquots of PEI solutions were added at different concentrations, and the cells were cultured in the presence of polymers for 72 h under standard conditions. Then, the culture medium was

replaced by the fresh one with 0.5 mg/mL MTT reagent, which was reduced by metabolically active cells into water insoluble formazan. Formazan precipitate was dissolved in DMSO, and the optical absorption was registered on Infinite 200 PRO analyzer at the wavelength of 555 nm. IC_{50} values of polymers were calculated from dose-response curves as concentrations which inhibit cell growth by 50%.

2.6.4. Characterization of polycation-induced cell death

For apoptosis and necrosis analysis HEK293 cells were cultured in the presence of propoxylated PEIs at final concentrations of 0.01; 0.1 and 1.0 mg/mL for 4 h. Treated cells were collected, washed and resuspended in the annexin-binding buffer (BD Pharmingen) at the density of 2.5×10^5 cells/mL. The cells were stained with FITC-annexin V/propidium iodide probes (BD Pharmingen) and analyzed on a flow cytometer Guava easyCyte 8HT (Millipore) in green (FL-2) and red (FL-3) channels. FITC-annexin V positive and PI negative cells were considered early apoptotic, while cells positive for both FITC-annexin V and PI were considered late apoptotic/necrotic.

2.7. Transfection study

HEK293 cells were seeded in 24-well plate at the density of 50,000 cells per well. Next day pEGFP-N2 encoding enhanced green fluorescent protein (EGFP) and its complexes were added to the culture medium to obtain final concentration of pDNA of 1 μg/mL. TurboFect reagent was used according to manufacturer's protocol (Fermentas). Transfection was carried out for 4 h in DMEM/FBS or the serum-free medium, and then media were changed by the fresh DMEM/FBS. To assess the mechanism of polyplexes uptake, chloroquine was additionally supplemented to the medium at the concentration of 50 μM. Transfected cells were cultured for 48 h under standard conditions and analyzed on an Axio Observer.A1 fluorescent microscope (Carl Zeiss). The number of transfected cells was quantified using Guava easyCyte 8HT flow cytometer in the green (FL-2) channel. The experiments were done in triplicate. The transfection efficiency (in %) was calculated as the number of EGFP-expressing cells versus total cell number.

2.8. Statistical analysis

Data are presented as mean ± SD. The criterion level for determination of statistical significance was set as $p < 0.05$ for all comparisons.

3. Results and discussion

3.1. Structure characterization of propoxylated PEIs

Alkoxylation is based on the reaction of ethylene oxide (EO) or propylene oxide (PO) with hydroxyl and other functional groups containing the active hydrogen. Previously, ethoxylated and propoxylated polyethyleneimine (PEI) was synthesized as a component of colloidal systems and detergents [38] and a demulsifier agent [33], respectively. In our study branched 25 kDa PEI was reacted with PO to produce propoxylated PEI (poPEI) with their nitrogen atoms grafted with ring open PO units of general structure $–[CH_2CH(CH_3)O]_n–H$. The extent of grafting and the length of propoxylated chain were stoichiometrically controlled by using different molar ratio of PO and NH groups as described in Section 2 'Materials and methods'.

Branched PEIs are composed of primary, secondary and tertiary nitrogens in the ratio 1:2:1 [38]. In experimental conditions, NH groups in primary and secondary nitrogens undergo propoxylation. The reaction scheme and the chemical structure of propoxylated PEIs are presented in Fig. S1 (Supplementary material). Four modified PEI samples were synthesized using 0.5; 0.75; 1.0; 3.0 PO moles per NH groups, further denoted as poPEI(0.5); poPEI(0.75); poPEI(1.0); poPEI(3.0) (Table 1), which correspond to the expected PEI grafting of 50, 75, 100 and 300%. Propoxylated PEIs (0.5–1.0) contain predominantly single PO units in their backbone, while poPEI(3.0) should theoretically contain tri-propylene oxide chains.

Table 1
Stoichiometry of electrostatic complexes of unmodified PEI and propoxylated PEIs with plasmid DNA (pEGFP-N2).

No.	Polymer	Stoichiometry (wt/wt)	N/P ratio
I	PEI	1:1	6
II	poPEI(0.5)	1:1	6
III	poPEI(0.75)	1:1.5	9
IV	poPEI(1.0)	1:3	18
V	poPEI(3.0)	1:100	600

The structure of unmodified and propoxylated PEIs was characterized by FTIR spectroscopy (Fig. 1). Initial PEI spectrum contains a series of broad bands attributed to primary and secondary amines, e.g. at the wavenumber of ~1660; 1580 cm^{-1} and ~3280; 920; 870; 760 cm^{-1}, respectively. 1660 and 1580 cm^{-1} bands disappeared after PEI modification, indicating the reaction of terminal (primary) amines with PO.

The modification was also accompanied by the alteration in stretching vibrations around 1140/1050 cm^{-1} wavenumbers attributed to the appearance of CO bond. Furthermore, FTIR spectra of propoxylated PEIs contained a wave with the maximum at 3340–3360 cm^{-1} and a peak at ~2970 cm^{-1} (Fig. 1) which correspond to OH and CH3 groups, respectively. The results confirm the modification of NH groups of PEI with PO units upon propoxylation.

The propoxylation of amino groups in initial PEI was also proved by ^1H NMR spectroscopy. ^1H NMR spectra of modified PEIs contain signals from both propylene glycol and polyethyleneimine protons at different chemical shifts. In particular, the signal at 1.2 ppm attributed to methyl protons of propylene glycol adjacent to methine protons was detected. The modification of PEI was accompanied by an increase in the intensity of methyl protons (1.2 ppm) and a decrease in the intensities of signals at 2.25–2.55 ppm which correspond to –N–CH2–CH2–N– (Fig. S2, Supplementary material).

Taking into account a relatively hydrophobic nature of PO component, which is temperature-dependent [31], we assessed micelle-forming properties of modified PEIs using pyrene fluorescent probe. No increase in pyrene fluorescence was observed for propoxylated PEIs at a range of concentrations and temperatures, indicating the lack of aggregation of polymeric molecules in water solution. Therefore, the introduction of single to tri-propyleneoxide units into PEI backbone, leading to a partial HLB decrease, is not accompanied by the appearance of micelle-forming properties of modified polymers.

3.2. DNA-binding and protective properties of propoxylated PEIs

The complex formation between propoxylated PEIs and a model plasmid DNA (pDNA) pEGFP-N2 (4.7 kb) encoding an enhanced green fluorescent protein (EGFP) was assessed using agarose gel retardation and dynamic light scattering (DLS) techniques. The electrophoretic mobility of supercoiled pDNA (10 μg/mL) mixed with serially diluted polycations were analyzed, and binding concentrations of polymers to completely retard pDNA were established. Corresponding electrophoregrams presented in Fig. S3 (Supplementary material) contain DNA ladder (lane 1), naked pDNA (lane 2), and polycation/pDNA complexes at different ratios (starting from lane 3). Based on these results, the weight stoichiometry and N/P ratio of polycation/pDNA complexes were calculated (Table 1).

Unmodified PEI binds pDNA at almost 1:1 weight ratio and at the N/P ratio of 6. PoPEI(0.5) had the same ratios to those of PEI, indicating that propoxylation of 50% cationic centers in PEI does not alter its ability to bind pDNA. Further modification of PEI with 0.75 and 1.0 mol PO resulted in a gradual increase in its DNA-binding concentration by 1.5 and 3 times (N/P 9 and 18, respectively). In contrast to modified polymers poPEI(0.5–1.0), poPEI(3.0) weakly interacted with pDNA with the

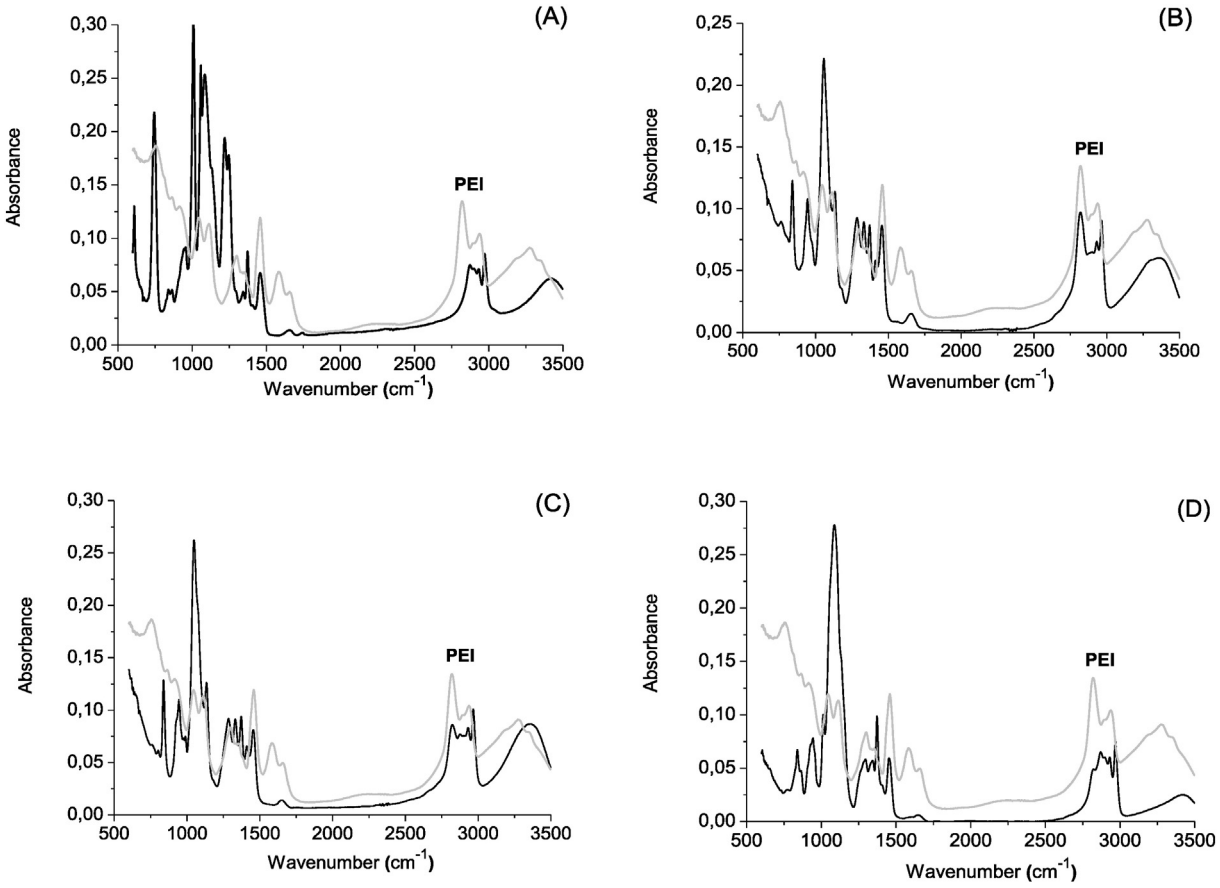

Fig. 1. FTIR/ATR spectra of branched 25 kDa PEI (grey line) and propoxylated PEIs (black line) with different modification extent: (A) poPEI(0.5), (B) poPEI(0.75), (C) poPEI(1.0), and (D) poPEI(3.0).

binding concentration of almost 1 mg/mL and N/P ratio as high as 600 (Table 1).

According to these data, the relationship between propoxylation extent of PEI and N/P ratio of polymer/pDNA complexes could be represented as a sigmoid function (data not shown).This relationship suggests that the modification extent of PEI, which preserves 50% pDNA-binding activity, lays between 1.0 and 2.0 mol PO per NH groups. The modification of PEI with single PO units is accompanied by a relatively weak decrease in electrostatic interaction with pDNA, indicating low steric effect of the grafted component, while it results in the significant reduction in cellular toxicity (Table 3, Figs. 2 and 3). Together this shows that the propoxylation allows for improving the safety of cationic polymers without losing their ability of complex formation with nucleic acids.

Table 2 shows DLS characteristics of complexes of polycations with pDNA. Initial and propoxylated polymers poPEI(0.5–1.0), but not poPEI(3.0), were found to condense pDNA into nanosized particles. The hydrodynamic diameter of resulting complexes increased gradually from 130 to 290 nm in the range of polymers: PEI, poPEI(0.5), poPEI(0.75), poPEI(1.0).

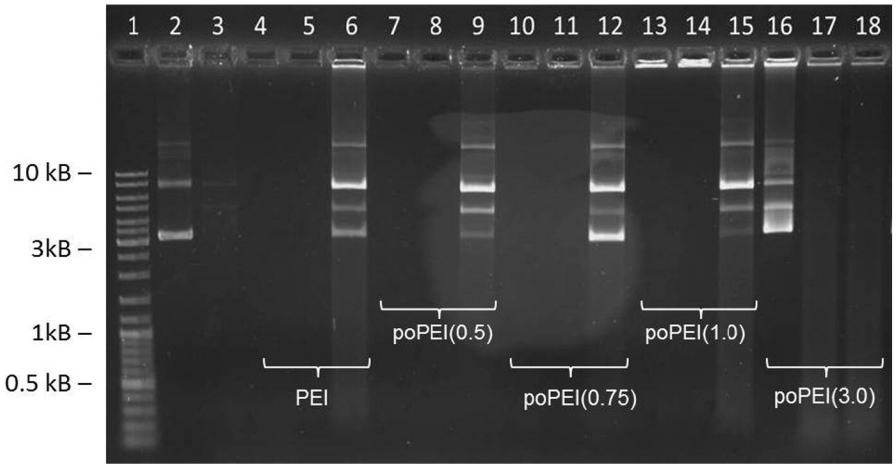

Fig. 2. Integrity of plasmid DNA released from polyplexes after 30 min incubation with 0.08 U/mL DNAse I. Lanes: 1 – DNA ladder; 2 – pDNA; 3 – pDNA + DNAse; 4 – pDNA/I (PEI); 5 – pDNA/I + DNAse; 6 – pDNA/I + DNAse + heparin; 7–9, 10–12, 13–15, 16–18 – the same as in 4–6 for poPEI(0.5) - II, poPEI(0.75) - III, poPEI(1.0) - IV, poPEI(3.0) - V, respectively.

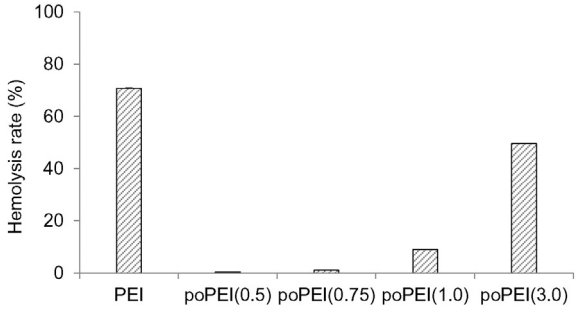

Fig. 3. Hemolytic activity of unmodified PEI and propoxylated PEIs. Hemolysis rate of human RBCs (5×10^4 cells/mL) was calculated as a percent of total hemolysis induced by Triton X100 (100%). Values are presented as mean ± SD.

PoPEI(0.75) and poPEI(1.0) based complexes had a relatively low particle dispersity index (0.21 and 0.25, respectively) compared with unmodified PEI (PDI 0.36). This could be explained by an aggregation preventing effect of the propoxylated component to produce more uniform and stable particles. Furthermore, the complexes of propoxylated PEIs had a relatively high positive zeta potential values within +25.6 and +28.5 mV, which were somewhat lower compared with initial PEI (+37.1 mV, Table 2), apparently due to a partial decrease in cationic properties of modified polymers.

As shown in [25], the grafting of 25 kDa PEI with 0.55 or 5 kDa PEGs does not noticeably alter the hydrodynamic diameter of PEI/pDNA complexes (~100 nm at N/P ratio 9), while the attachment of single 20 kDa PEG molecule results in small and compact particles (~70 nm). Grafting of PEI molecule with ten 2 kDa PEGs produces pDNA complexes of bigger size of 127 nm but neutrally charged [19], apparently due to the ability of PEG to effectively shield PEI/pDNA particles. This suggests that propoxylated PEI have somewhat lower ability of pDNA condensation than PEGylated ones, however the former polymers generate less disperse complexes which preserve their positive charge, indicating the possibility of using propoxylated polycations for gene delivery.

A protecting effect of propoxylated PEIs on pDNA stability upon enzymatic degradation was studied as a characteristic for gene carriers. PEI/pDNA complexes (Table 1) were treated with bovine DNAse I followed by the electrophoretic analysis of pDNA products (Fig. 2). Naked pDNA was rapidly digested by DNAse I to undetectable products (Fig. 2, lane 3). No cleavage products of pDNA appeared after DNAse I treatment of complexes of initial PEI and propoxylated polymers poPEI(0.5–1.0) (lanes 5, 8, 11, 14) except poPEI(3.0) (Fig. 2, lane 17). The latter polymer did not protect pDNA from degradation due to its weak pDNA-interacting properties (Table 1, Fig. S3).

To better compare the protective effect of initial and modified PEIs, the integrity of pDNA forms was estimated after heparin-induced release of pDNA from its complexes (Fig. 2). Among polycations, poPEI(0.75) was found to preserve pDNA integrity more efficiently as cleavage products predominantly contained an intact supercoiled form and a relaxed form of pDNA at the similar ratio (Fig. 2, lane 12). In the same conditions, cleavage products of other pDNA complexes were characterized by increased amount of relaxed (circular) and linearized pDNA forms.

Our results suggest the possibility of modulation of cationic polymers pDNA protection activity against nuclease degradation by their controlled propoxylation. The inhibition of nuclease interaction with pDNA in complexes with propoxylated polycations apparently occurs due to the reduction of protein adsorption by PO component similarly to the effect of PEGylation [41].

3.3. Hemolytic and cytotoxic activity of propoxylated PEIs

We carried out a comparative study on membrane-damaging properties and cytotoxicity of propoxylated PEIs and unmodified PEI. The hemolytic activity of cationic polymers was observed only at the concentration as high as 10 mg/mL (Fig. 3), but not at lower concentrations (0.1; 1.0 mg/mL). PEI was found to induce severe hemolysis rate of 70.8 ± 0.2% due to its high membrane-damaging action on red blood cells (RBCs). In the same conditions, poPEI(0.5) and poPEI(0.75) did not have any hemolytic activity, similarly to poPEI(1.0), which induced the permissible level of hemolysis below 10% (Fig. 3). Unlike the modified PEIs(0.5–1.0), poPEI(3.0) with highest propoxylation extent had a relatively high hemolytic activity of almost 49.5 ± 0.03% (Fig. 3).

Our results indicate that grafting of PEI with single PO units (50–100%) dramatically decreases its hemolytic and membrane-damaging activity. This could be explained by a shielding of positively charged PEI nitrogens by PO units, resulting in softening of electrostatic interactions of modified polymers with anionic cellular membranes. Extension of PO chain in poPEI(3.0) restores the hemolytic action to the cationic polymer. Apparently, oligomeric PO component of the modified PEI, unlike single PO units, is capable of hydrophobic interactions with PBC membrane and inducing its damage.

Cytotoxicity of propoxylated PEIs was evaluated on human HEK293 cells and primary skin fibroblasts (HSFs) using the MTT assay. Half-maximal inhibitory concentrations (IC_{50}) of polymers increased in the order: PEI < poPEI(0.75) ≈ poPEI(0.5) ≈ poPEI(1.0) < poPEI(3.0) for HEK293 cells and PEI < poPEI(0.5) < poPEI(0.75) < poPEI(1.0) < poPEI (3.0) for HSFs (Table 3). The results show that PEI propoxylation is accompanied by a significant reduction in its cytotoxicity for human cells proportionally to the modification extent. IC_{50} values observed for poPEI(3.0) approach ~1.4 mg/mL (HEK293) and ~1.7 mg/mL (HSFs), which are almost 3-order higher than that for unmodified PEI (Table 3).

Considering a relatively low membrane-damaging action of propoxylated PEIs (Fig. 3), which should be further decreased in cell culture medium due to membrane-protecting effect of serum [34], we suppose that cytotoxic activity of modified PEIs (Table 3) affects their ability of intracellular trafficking. To characterize adverse intracellular effect of cationic polymers, HEK293 cell death was examined after 4-h exposition using flow cytometry. Polymer-induced cell death was found to occur mainly due to necrosis (Fig. 4), which is typical for cationic polymers including PEI [4,10]. Propoxylation of PEI was followed by significant decrease in the number of necrotic/late apoptotic cells and increase in the number of viable cells proportionally to the modification extent; both poPEI(1.0) and poPEI(3.0) had the lowest cytotoxic action (Fig. 4).

This shows that the modification with PO allows for controllable reduction of adverse effects of the polycation on human cells. Total

Table 2
Hydrodynamic diameter and zeta potential of electrostatic complexes of unmodified PEI and propoxylated PEIs with plasmid DNA.

Complex	Hydrodynamic diameter	Particle dispersity index	Zeta potential(mV)
pDNA (10 μg/mL)	331.9 ± 24.6	0.52 ± 0.04	−31.7 ± 1.4
pDNA/I[a]	129.5 ± 12.8	0.36 ± 0.01	+37.1 ± 0.9
pDNA/II[a]	168.9 ± 48.9	0.41 ± 0.05	+28.5 ± 0.6
pDNA/III[a]	255.2 ± 15.9	0.21 ± 0.01	+25.9 ± 0.2
pDNA/IV[a]	288.1 ± 29.5	0.25 ± 0.01	+25.6 ± 0.3
pDNA/V[a]	1028.3 ± 229.5	0.51 ± 0.06	+13.1 ± 0.6

[a] I–V are indicated in Table 1.

Table 3
IC_{50} cytotoxic concentrations (μg/mL) for unmodified PEI and propoxylated PEIs (MTT assay).

Polymer	HEK293 cells	HSFs
PEI	1.9 ± 0.3	2.3 ± 0.1
poPEI(0.5*)	33.1 ± 5.1	7.6 ± 0.8
poPEI(0.75*)	30.0 ± 3.2	21.3 ± 5.1
poPEI(1.0*)	37.3 ± 4.3	130.6 ± 14.0
poPEI(3.0*)	1400.0 ± 47.6	1733.8 ± 38.1

Asterisks show moles of 1.2-propylene oxide per 1 mol NH in PEI.

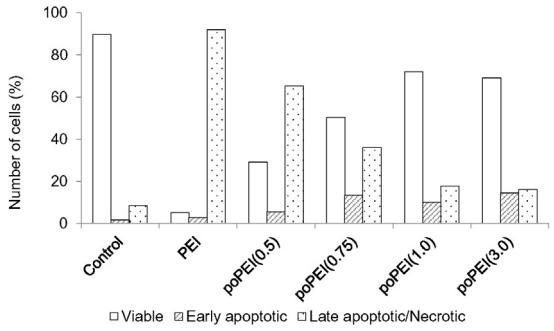

Fig. 4. Pro-apoptotic and necrotic activity of unmodified PEI and propoxylated PEIs towards HEK293 cells. Cells were cultured in the presence of 0.5 mg/mL polymers for 4 h, stained with FITC-annexin V/propidium iodide and analyzed by flow cytometry.

polycation grafting with PO provides the highest cell viability similar to that for untreated cells. Increased cellular compatibility of modified polymers could be explained by the inhibition of polymer–plasma membrane interaction by PO component and concomitant reduction of cellular uptake of polymeric molecules. The inhibiting effect of propoxylation slows down rapid polycation-induced necrosis of cells, which can also result in a partial increase in the number of early apoptotic cells as observed for poPEIs (Fig. 4).

The mechanism by which propoxylation improves cytocompatibility of PEI seems to differ from that of proposed techniques based on the attachment of polysaccharides [24,29,38], PEGs [19,25,26] or Pluronics [40,41,48], where polycation is grafted with one or few extended polymeric chains. The latter modification should result in the inhibition of biological interactions of the polycation by its wrapping with hydrophilic polymers; it should not affect cationic centers in polymeric backbone, and therefore not allow for fine tuning physicochemical and biological properties of polycations in contrast to propoxylation. Proposed modification can be used to improve safety of pharmaceutically relevant cationic polymers, which intrinsically possess high membrane-damaging and cytotoxic activity [10,16]. Considering gene therapy as one of the most promising applications of such polymers, we further studied the effect of propoxylation of PEI on its DNA-binding and delivery properties.

3.4. Transfection activity of propoxylated polyethyleneimines

HEK293 cells were cultured with pEGFP-N2 based complexes in DMEM with and without 10% FBS and analyzed after 48 h using flow cytometry. Fig. S4 shows typical EGFP fluorescence histograms in FL2 channel for (A) control cells and (B) transfected cells. The latter histogram appears as a highly spread curve attributed to EGFP-expressing cells with different fluorescent intensities, indicating that propoxylated polycations are capable of delivering exogenous pDNA to the cells (R2 region, Supplementary material). Initial PEI exhibited the highest transfection efficiency of 48 and 35% in DMEM/FBS and DMEM, respectively, which was 1.5–2 times lower compared with poly(2-hydroxypropyleneimine) reagent, TurboFect® (Fig. 5).

An addition of 50 μM chloroquine, the lysosomotropic agent which promotes endosomal release [43], did not enhance and even somewhat decreased both PEI and TurboFect activity [45]. Above polyalkylimines exhibit the “proton sponge” effect, which is responsible for endosome disruption, pDNA release and its enhanced expression [17,42,44]. Therefore, their transfection activity is not promoted by chloroquine. Almost 3% of HEK293 cells were transfected by naked pDNA in serum-containing DMEM in the presence of chloroquine, but not in any other conditions (Fig. 5). This indicates that cellular uptake of naked pDNA is promoted by serum proteins, and the endosomal release is required to reach measurable transfection level [39].

In the absence of chloroquine, propoxylated PEIs (0.5–1.0) exhibit low transfection level around 1–2% both in serum-free and serum-

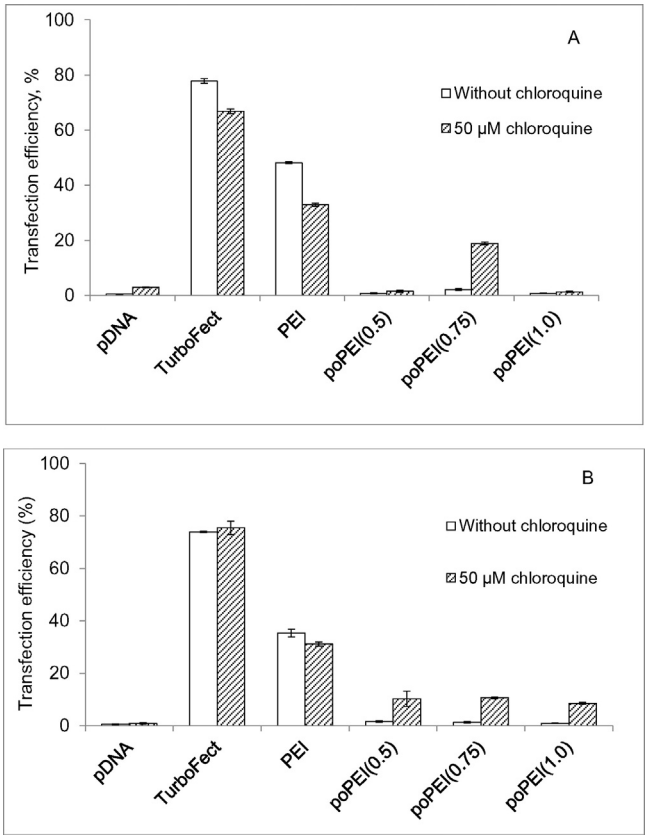

Fig. 5. Transfection efficiency of HEK293 cells by pEGFP-N2 complexes in (A) DMEM containing 10% FBS and (B) DMEM without FBS. White columns – no chloroquine; hatched columns – with 50 μM chloroquine. DNA concentration – 1 μg/mL; PEI/pDNA ratios are indicated in Table 1. Values are presented as mean ± SD.

containing media (Fig. 5). Co-treatment of above polyplexes with chloroquine resulted in 5–10-fold increase of the transfection efficiency up to ~10% similarly for all above propoxylated PEIs in serum-free medium (Fig. 5B). Upon serum supplementation, only propoxylated PEI(0.75) based polyplexes provided an increased transfection level of 18.8%, which is just 1.8-times lower than that for unmodified PEI in the same conditions (Fig. 5A).

This demonstrates that, in contrast to unmodified PEI, poPEI-mediated transfection requires the co-treatment with chloroquine, apparently due to lower capacity of modified PEIs to endosomal disruption. Similar enhancing effect of chloroquine was observed for PEGylated poly(amidoamine) based carrier of pDNA [20]. Furthermore, PEGylation of PEI through endosomal pH-sensitive linkers was shown to restore the transfection activity of the modified polymer to that of unmodified one [46].

Altogether, these data support that limited endosomal escape is responsible for low transfection activity of alkoxylated cationic polymers. As shown recently in [47], an endosomal release and transfection efficacy of poly(amidoamine) and poly(propylenimine) dendrimers can be increased by their modification with nitrogen-containing heterocycles.

Relatively high transfection activity of poPEI(0.75), which correlates with its pDNA protection activity to DNAse I (Fig. 2), could be explained by better balance between DNA-binding and protein resistance properties of the modified polymer. This is expected to promote transfection by inhibiting nuclease interaction with pDNA complexes as well as reducing their aggregation in cell culture medium, which is often responsible for decrease in transfection level of polycations [43,46].

Altogether the results demonstrate that transfection activity of PEI can be modulated by the controllable grafting of its cationic centers with single PO units (up to 100%), but not with oligomeric molecules which inhibit DNA binding and transfection. Though our in vitro study

did not reveal an enhancement of pDNA delivery after PEI propoxylation (Fig. 5), the modification resulted in dramatic decrease of polycation adverse effects on human cells (Table 3, Figs. 3 and 4) and preserved its transfection activity. Considering already established therapeutic effects of naked pDNA based formulations, e.g. growth factor-encoding plasmids [49], the biological safety of polymeric carriers should be the primary issue upon their development [50].

Highly cationic systems with high in vitro transfection efficiency are not often active in vivo, in contrast to some less cationic polymers which do not form positively charged complexes with pDNA [24]. Improved biological interactions of propoxylated PEIs make them promising candidates for the delivery of non-viral genes. In vivo study of safety and efficacy of propoxylated PEIs as pDNA carriers will be carried out elsewhere. The proposed approach can be extended to improve biocompatibility of other cationic macromolecules for biomedical and pharmaceutical applications.

4. Conclusions

We demonstrate for the first time that the grafting of cationic polymers with propylene oxide provides an effective approach to tuning their interactions with biological components. Introduction of single propylene oxide units into cationic centers allows for the significant reduction of membrane-damaging and cytotoxic activities of polycations, but preserves their ability for binding, condensing and intracellular delivery of plasmid DNA. Our study reveals structure/biocompatibility relationships of propoxylated cationic polymers and shows the potential route to developing advanced drug delivery systems. Our results suggest that propoxylation can be implemented as a powerful technique to alter bio-interaction and compatibility of macromolecules.

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Appendix A. Supplementary data

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