Intracellular delivery of VEGF165 encoding gene therapeutic using trifunctional copolymers of ethylene oxide and propylene oxide

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ABSTRACT

New type of copolymers of propylene oxide and ethylene oxide was assessed for promoting delivery of plasmid DNA based gene therapeutics. Lipid-like trifunctional copolymers (TFCs), with both random or diblock structures and relatively low hydrophilic–lipophilic balance, were studied and compared with linear Pluronic™ L61. Structure-dependent relationships for micelle-forming, cytotoxic and hemolytic properties of these copolymers were revealed. The TFC with the mean number of propylene oxide and ethylene oxide units of 83.5 and 24.2, respectively, exhibited relatively low adverse effects in vitro. The latter TFC interacted with plasmid DNA–polyethyleneimine complexes and improved their intracellular delivery. Furthermore, this TFC efficiently promoted the transfection of dermal fibroblasts with VEGF165-encoding Neovasculgen® plasmid DNA, which has been clinically used for the therapeutic angiogenesis. Our findings demonstrated for the first time that TFCs are promising for the polymer-mediated delivery of gene therapeutics.

Keywords: trifunctional copolymers of propylene oxide and ethylene oxide; Pluronics; polymer-mediated gene therapy; plasmid DNA; Neovasculgen; VEGF165.

1. Introduction

Gene therapeutics are promising biologics which have been engineered to treat a host of monogenic and complex diseases including neurodegenerative, cardiovascular and cancer ones [1]. The main engineering platforms for gene therapeutics include synthetic oligonucleotides, viruses and plasmid DNAs (pDNA) [1,2], the two latter types are considered 'pro-drugs' that allow for a template-directed synthesis of a desired peptide within the cell. Compared to viruses, pDNA has a great advantage in clinical application due to its intrinsic safety, low cost and vector capacity. However, pDNA itself suffers from its poor intracellular penetration and stability [3].

Several pDNAs encoding bioactive peptides have been developed and studied as potential drugs in different applications, e.g. for: therapeutic angiogenesis, regeneration of peripheral nerves and bones (see review [4]), cancer treatment [5], and DNA vaccination [6]. However, the improvement of intrinsically low pDNA transfection efficiency remains relevant and unresolved biomedical task. The common approach exploits various cationic lipids and polymers, both of synthetic and natural origin, which bind to, condense and neutralize nucleic acids, thereby improving their stability and cellular pharmacokinetics [2,4].

While cationic carriers of pDNA exhibit a relatively high efficiency in vitro, their gene therapy applications are strictly restricted, due to membrane-damaging and cytotoxic properties of polycations [2,7]. To overcome this problem, new polymeric systems that deliver pDNA, both alone and combined with polycations, have been proposed. Ones of these, non-ionogenic amphiphilic copolymers, particularly promise the delivery of small drugs and nucleic acids into target cells [8].

In particular, block copolymers of ethylene oxide (EO) and propylene oxide (PO), known as Pluronics™ or Poloxamers™, were found to increase the transfection efficiency in vitro for pDNA complexes with polyethyleneimine (PEI) [9] and poly(N-ethyl-4-vinylpyridinium) [10]. The possible effects of amphiphilic polymers in pDNA formulation include: the prevention of aggregation of polyplexes in the presence of serum proteins [9]; promotion of endocytosis and/or

lysosomal escape [10] and promoter-specific regulation of gene expression [8,11]. The co-injection of 'naked' pDNA and some Pluronics was shown to allow for an enhancement of intramuscular vector expression [11–13]. Similar to Pluronics, another non-ionogenic block copolymer of poly(lactic-co-glycolic acid), PLGA, and polyethylene glycol, PEG (PLGA–PEG–PLGA) improved the transfection process in vivo [14].

While Pluronics are relatively simple linear polyethers which contain two terminal hydroxyl groups, their supramolecular analogs with a higher functionality and new characteristics are being developed [15]. Such polyfunctional polymers are also of particular interest for drug delivery applications in terms of their enhanced capacity for complex formation with biological components. We demonstrated recently that glycerol-based trifunctional block copolymers of EO and PO (TFCs) are promising analogues of Pluronics which exhibit high membranotropic activity and efficiently promote transmembrane transport of anticancer drugs [16]. Here we study the effect of several TFCs on the delivery of pDNA and its complexes into human cells. For the first time we assessed the ability of amphiphilic polymers to promote the delivery of Neovasculgen®, a vascular endothelial growth factor (VEGF) encoding pDNA, which has been recently developed and introduced in Russia by the Human Stem Cells Institute to treat chronic limb ischemia [17,18].

2. Experimental section

2.1. Materials

Bifunctional block copolymer of EO and PO (Pluronic™ L61) was purchased from Sigma-Aldrich. Trifunctional block copolymers of PO and EO (Laprol™ 6003-2B-18, Laprol™ 5003-2-15, Laprol™ 3603-2-12, structural analogs of Voranols, Dow Chemical) were produced by JSC Nizhnekamskneftekhim (Russia).

Branched 25 kDa polyethylenimine (PEI), Hoechst 33342 and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. TurboFect™ transfection reagent was purchased from Fermentas. NHS-rhodamine was obtained from Pierce. Pyrene, inorganic salts and solvents were produced by Acros Organics. Cell culture reagents were purchased from PAA Laboratories.

Plasmid DNA (pDNA) encoding enhanced green fluorescent protein (pEGFP-N2) was purchased from Clontech. Neovasculgen® pDNA was kindly provided by Dr. Roman Deev, the Human Stem Cells Institute (Russia). This preparation is a recombinant pDNA composed of a transcription regulating site, the minigene encoding VEGF isoform (165 amino acids), a splicing signal, a polyadenylation signal and transcription terminator SV40; molecular weight (MW) 2817092 (4559 b.p.).

Sandwich ELISA (enzyme-linked immunosorbent assay) kit for human VEGF was obtained from Vector-Best (Russia).

2.2. Characterization of pDNA–polymer complexes

pEGFP-N2 plasmid was isolated from transformed E.coli overnight culture with the use of Miniprep plasmid DNA isolation kit (Fermentas). The purity and integrity of pDNA was verified by measuring A_{260}/A_{280} ratio and agarose gel electrophoresis. The mass stoichiometry of pDNA–PEI complexes was determined by the agarose gel retardation assay.

Double complexes pDNA–PEI, pDNA–Turbofect and triple complexes pDNA–PEI–TFC were characterized by the dynamic light scattering (DLS) technique on a Zetasizer Nano ZS analyzer (Malvern Instruments). Hydrodynamic diameter and zeta potential of complexes were registered in 50 mM HEPES buffer (pH 7.0) at a working pDNA concentration of 10 μg/mL. Multi-modal (mean number) distribution based on non-negative least squares algorithm was utilized to evaluate DLS data. The measurements were performed in triplicates.

2.3. Determination of critical micelle concentration

The critical micelle concentration (CMC) of TFCs was determined with the use of pyrene probe as described earlier [19]. Briefly, 200 µL of serially diluted polymer solutions in phosphate buffered saline (PBS), pH 7.0, were pipetted into 96-well plate pre-covered with 2.5 nmol of pyrene from methanol solution. The plate was incubated for 1 hour at 37 ºC under agitation to allow pyrene to dissolve and redistribute into polymeric micelles. Emission spectra of pyrene were detected at RT using an Infinite 200 PRO multimode microplate analyzer (Tecan) in 365–410 nm wavelength range (λ_{ex} 339 nm). CMC was calculated through the relationship between the fluorescence intensity at λ_{max} 373 nm and the logarithm of copolymer concentration [19].

2.4. Cell isolation and culturing

Human skin fibroblasts (HSFs) were isolated from the skin explant according to the conventional protocol [20]. HEK 293 (human embryonic kidney) cells and A549 cells (human lung adenocarcinoma epithelial cells) were obtained from the ATCC collection. HSFs and HEK 293 cells were cultured in the minimum essential medium Eagle (α-MEM) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin under standard conditions (37 °C, 5 % CO_2 atmosphere). A549 cells were grown in the same conditions, but in the Dulbecco's modified Eagle's medium (DMEM). 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.

2.5. Assessment of cell viability and hemolysis

Cytotoxic concentrations (IC_{50}) of polymers were determined with the use of MTT assay. Cells were pre-seeded in 96-well plate at the density of 1000 cells per well and cultured with adding a series of diluted polymer solutions for 3 days under standard conditions. Culture

medium in the plate was then replaced by the fresh one supplemented with 0.5 mg/mL MTT and additionally kept for 4 hours to allow for reduction of MTT into colored product (formazan) by metabolically active cells. Optical absorbance of produced formazan, proportional to viable cell number, was registered on Infinite 200 PRO analyzer at 550 nm.

A hemolysis assay was performed according to ISO 10993-4:2002 recommendations and as described in [16].

2.6. Transfection study

HEK 293 cells and HSFs were transfected with pDNAs encoding green fluorescent protein or VEGF165 and their complexes with polymers. The cells were seeded into 24-well plate at the density of 50 000 cells per well. Next day, the culture medium was replaced with the fresh one. For cell transfection, pDNA–polymer complexes were added to the culture media at final concentrations 1 μg/mL for pDNA and PEI, and 20 μg/mL for TFC. Turbofect transfection reagent was diluted according to manufacturer's protocol (Fermentas). The cells were cultured in the presence of polyplexes for 4 hours in CO_2-incubator followed by replacement of the medium with the fresh one. The transfection efficiency was analyzed after 24 hours by registering green fluorescent protein-positive cells as described below and detecting VEGF165 protein secreted into the medium with the use of ELISA kit.

2.7. Fluorescent microscopy

Transfected cells were analyzed by means of inverted fluorescence microscopy on an Axio Observer.A1 microscope (Carl Zeiss) and an In Cell Analyzer 2000 imaging system (GE Healthcare). Cell culture medium was replaced with PBS supplemented with 1 μM Hoechst 33342 prior microscopy measurements. Green fluorescent protein and Hoechst 33342 fluorescence images were registered in appropriate channels.

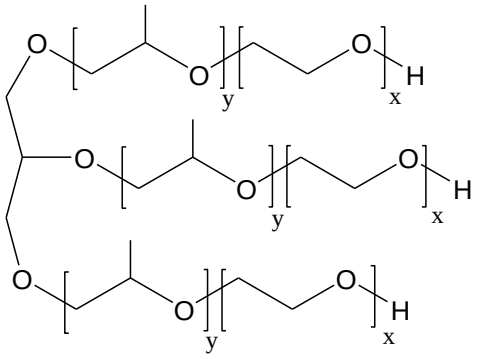
Quantification of stained cells was carried out in 24-well plate with the aid of In Cell Analyzer 2000 Workstation software (Multitarget Analysis mode). Briefly, 10 fields of view with 10 000 cells in each field were scanned in each well. Fluorescence threshold was adjusted manually to distinguish between positive cells and negative ones. The transfection efficiency (in %) was calculated as the number of green fluorescent protein-expressing cells versus total cell number.

In order to assess the cellular uptake of pDNA–PEI complexes, PEI was pre-labeled through primary amino groups with N-hydroxysuccinimide ester of rhodamine. One-step labeling of PEI was performed by mixing it with NHS-rhodamine (1 : 5 mole/mole) in 0.1 M sodium carbonate buffer solution (pH 8.5) for 2 hours followed by product dialysis. The PEI labeling was not accompanied by a decrease in its DNA-binding ability as revealed by agarose gel electrophoresis.

3. Results and discussion

3.1. Physicochemical properties of trifunctional copolymers

Trifunctional copolymers (TFCs) represent a series of non-ionogenic amphiphilic polymers synthesized by the alcoholate polymerization of glycerol with PO and EO to generate random or diblock copolymers with the general chemical structure:



Three TFCs with different physicochemical properties were used in this study: Laprol 3603 (L3), Laprol 5003 (L5), and Laprol 6003 (L6), where L3 and L5 are random copolymers and L6 is the block copolymer (Table 1). These TFCs possess relatively low hydrophilic-lipophilic balance (HLB) with theoretical values of about 6. This indicates the predominance of hydrophobic properties in copolymers studied which are expected to promote their interaction with cellular membranes. The physicochemical characteristics of TFCs are similar to those of Pluronic L61 (Table 1), which is the main polymeric component of several antitumor compositions proposed recently by Supratek Pharma Inc. (www.supratek.com, [21]).

Table 1

Critical micelle concentration (CMC) of amphiphilic copolymers decreased in the order: Pluronic L61 > L3 > L5 > L6, indicating an enhancement of their micelle-forming properties with an increased amount of the PO component (Table 1). L3 was characterized by relatively weak micelle-forming properties when compared with L5 and L6, presumably, due to lower molecular weight and the random structure of the former copolymer, which may complicate the formation of hydrophobic core of micelles. In comparison with L3, CMC of L5 was almost 14-times lower (0.03 mg/mL), while it has only 1.4-fold higher number of PO units. Further extension of the PO component in L6 resulted in less promotion of its micelle-forming properties to the CMC value of 0.01 mg/mL (Table 1).

3.2. Cytotoxic and hemolytic activity of trifunctional copolymers

Comparative cytotoxicity study was carried out on A549 cells and primary human skin fibroblasts (HSFs) for amphiphilic polymers as well as 25 kDa PEI commonly used as a nucleic acid carrier [22,23]. Cytotoxic (inhibitory) concentrations (IC_{50}) of polymers, calculated from

dose-response curves, increased in the following order: L3 < Pluronic L61 < L5 < L6 for A549 cells and L3 < L5 < Pluronic L61 < L6 for HSFs (Table 2).

Table 2

All amphiphilic polymers were found to be more toxic for the cancer A549 cells than for HSFs. This could be explained by a relatively high proliferative potential and endocytic activity of cancer A549 cells, which therefore readily uptake polymeric molecules. Inside cells non-ionogenic amphiphilic polymers, e.g. Pluronics, disturb intracellular membrane structures and inhibit mitochondrial function, promoting the induction of apoptosis and decreasing the ATP level [24].

The hemolytic activity of amphiphilic copolymers at a concentration as high as 10 mg/mL decreased in the order: Pluronic L61 >> L6 > L5 > L3 (Table 2). Therefore, this activity was significantly lower for trifunctional copolymers compared to linear Pluronic L61 and did not correlate with the cytotoxicity of polymers. As shown previously, the direct damage of cell plasma membrane is substantially inhibited in serum-containing culture medium; under these conditions, the cytotoxicity of EO/PO copolymers apparently correlates with their cellular penetration and accumulation [19]. This helps to explain a relatively high cytotoxicity of L3 and Pluronic L61 as a result of their cellular uptake favored by their relatively low molecular weight and high CMC (high unimer concentration).

Our results show that among other polymers L6 exhibits moderate cytotoxicity towards human skin fibroblasts (IC_{50} ~ 135 μg/mL), which is much lower than for L5 copolymer with comparable to L6 characteristics as well as cationic PEI (Table 2). The higher cytotoxicity of L5 presumably arises from its random polymeric structure which could lead to less organized micelles and promote intracellular penetration of L5.

Unlike amphiphilic polymers, PEI has almost the same IC_{50} value of about 2 μg/mL for both A549 cells and HSFs (Table 2). Almost the same cytotoxic effect of PEI on cancer and 'normal' cells can be attributed to its non-specific damaging activity on cellular plasma membrane [25], which is not inhibited by the culture medium. Based on cytotoxicity data, we focused on L6 as a potential enhancer for PEI-mediated transfection of human cells.

3.3. Complex formation of plasmid DNA with polymers

Enhanced green fluorescent protein-encoding plasmid DNA (pEGFP) was used as a model pDNA to study the complex formation with polymers. According to an agarose gel retardation assay, the stoichiometry of electrostatic complexes of pEGFP with 25 kDa PEI was 10 : 8 by mass, and the corresponding nitrogen/phosphate (N/P) ratio was almost 6, which is consistent with the published data [26]. The electrophoretic mobility of pDNA was not altered in the presence of TFCs studied in the concentration range of up to 10 mg/mL, indicating the lack of strong interactions between polyanionic DNA and EO/PO based copolymers which exhibit a weak negative charge at physiological pH [19] and are not expected to condense nucleic acids.

As revealed by dynamic light scattering technique, pEGFP plasmid appears in the form negatively charged particles with a hydrodynamic diameter of 248 nm and polydispersity index (PDI) of 0.55 (Table 3). At the N/P ratio 6, PEI induced an almost two-fold decrease in both the hydrodynamic diameter of pDNA to 146 nm and its PDI to 0.29, apparently due to the condensation of negatively charged pDNA molecules by the polycation. Resulted complexes have a slightly positive zeta potential value of about +5 mV.

Table 3

A two-fold increase in PEI concentration (N/P ratio 12) was not further accompanied by the reduction of the size of pDNA complexes, but promoted their cationization (Table 3). The

interaction of pDNA with Turbofect transfection reagent generated smaller polyplexes of 62 nm in diameter and with zeta potential value of +25 mV. This shows that Turbofect condenses pDNA in a more efficient way, compared with PEI, while both these cationic polymers induce comparable cationization of resulted polyplexes.

In the presence of the trifunctional copolymer L6, the hydrodynamic diameter of pDNA–PEI complexes increased to almost 190 nm without notable change in polydispersity. The positive charge of polyplexes was partially suppressed by L6 (Table 3). This indicates that the TFC interacts with pDNA–PEI complexes presumably as a result of the reversible association of weakly anionic polymeric micelles on the surface of cationized pDNA particles.

3.4. Cellular uptake of plasmid DNA complexes in the presence of trifunctional copolymers

The L6-mediated penetration of pre-labeled pDNA–PEI complexes into HEK293 cells was analyzed as described in the 'Experimental' section. Cells were cultured in the presence of double complexes (pDNA–PEI) and triple complexes pDNA–PEI–L6 hourly, for 5 hours, followed by the analysis of intracellular fluorescence. The complexes appeared inside cells within the first hour, and the intensity of intracellular fluorescence gradually increased and reached maximum levels at 3 and 4 hours of incubation.

It was found that an addition of L6 to pDNA–PEI complexes noticeably increased the intracellular fluorescence, indicating the promotion of pDNA complexes delivery into cells in the presence of this TFC. The uptake of complexes by HEK293 cells is expected to occur through the clathrin-dependent endocytosis, which is a prevalent way of macromolecule transport into mammalian cells [27]. Based on existing reports on Pluronic-mediated transfection, one can suggest similar mechanisms for promoting cellular uptake in the presence of TFCs, in particular, the stabilization of pDNA complexes in serum-containing culture medium [9] and the stimulation of endocytic activity [10]. As shown previously, Pluronic P85 can internalize through clathrin-

dependent endocytosis (unaggregated 'unimer' form) and calveolin-dependent endocytosis (micellar form) depending on its aggregate form [28].

Fig. 1

We believe that the endocytosis stimulation might be responsible for the uptake-enhancing effect of the trifunctional copolymers. As we demonstrated earlier, L6 copolymer induced noticeable fluidization of the plasma membrane of mammalian cells attributed to its high affinity to membrane lipids [16]. Such a membrane fluidization effect, in turn, should promote endocytic activity of treated cells in accordance with the recent report, which reveals the correlation between endocytosis rate and membrane polarization and microviscosity [29].

3.5. Effect of trifunctional copolymers on transfection of human cells

After transfection with pEGFP, green fluorescent cells were detected and quantified automatically using In Cell Analyzer 2000 imaging system (see 'Experimental' section). Under experimental conditions, the transfection efficiency of HEK 293 cells with pDNA–PEI complexes was low. TFCs alone did not result in detectable transfection for uncomplexed 'naked' pDNA, however the copolymers noticeably increased the efficiency of pDNA–PEI complexes by almost 5 times (L3) and 6.5 times (L5 and L6) (Fig. 2).

Fig. 2

PEI / TFC system was less efficient than Turbofect reagent which resulted in almost a 40 % cell transfection (Fig. 2). A relatively high activity of Turbofect, which is poly(2-hydroxypropyleneimine) [30], could be explained by its ability to efficiently condense pDNA and promote its intracellular release from lysosomes due to their disruption by the cationic polymer

('proton sponge' effect). However, such characteristics of cationic polymers contribute to their high cytotoxicity [2,7], indicating the need to develop different polymeric systems for gene delivery. Our results demonstrate that TFCs can be used to enhance the transfection of human cells, presumably by promoting cellular uptake of pDNA complexes.

An effect of TFCs on the transfection was also studied on primary HSFs using Neovasculgen® plasmid encoding vascular endothelial growth factor – pVEGF (Fig. 3). Untreated HSFs produce this angiogenic factor at a relatively high level of about 200 pg/mL, reflecting the physiological role of fibroblasts as a producer of growth factors [31]. The treating of HSFs with 'naked' pVEGF (1 μg/mL) resulted in a small increase in VEGF expression by ~20 pg/mL, while the complexation of pVEGF with PEI did not affect the transfection level compared with 'naked' pVEGF, indicating a lack of the promoting effect of PEI on pVEGF delivery in experimental conditions.

Fig. 3

HSFs as well as other primary cells are known to be somewhat resistant to transfection [32], probably due to their relatively low endocytic activity and proliferative potential in culture. In contrast to PEI, Turbofect promoted VEGF expression in transfected HSFs to the concentration of almost 3 ng/mL, however at this concentration Turbofect induced the loss of cells, reflecting a poor 'pharmaceutical window' of this polymer.

The combination of PEI with L3, L5 as well as Pluronic L61 resulted in the similar increase in extracellular VEGF concentration up to 400–480 pg/mL which was about two-times higher of the background level of the growth factor (Fig. 3). L6 induced a significantly higher transfection of HSFs with pVEGF–PEI, stimulating VEGF expression to the concentration as high as 840 pg/mL without an increase in cell death. We believe that such promoting effect has similar mechanisms to those observed for HEK 293 transfection with pEGFP (Fig. 2). Among other

amphiphilic polymers examined, the L6 combines relatively low cytotoxicity (Table 2) and membranotropic properties [16] which favor the intracellular delivery and expression of pDNA upon transfection.

Altogether, our results support the use of polymeric systems containing polycations and amphiphilic polymers for delivery of pDNA-based gene therapeutics. An efficient system should contain a relatively safe amphiphilic component to allow for the modulation of physicochemical properties of cellular membranes and promotion of human cell transfection. Trifunctional copolymers of PO and EO (Table 1) are promising candidates for the development of new formulations of pDNA encoding bioactive peptides. Further in vivo studies of these formulations with different cationic polymers are required to reveal their efficiency and safety.

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4. Conclusions

Development of advanced polymeric systems for the therapeutic delivery of plasmid DNA requires the replacement of highly cytotoxic cationic polymers, generally designed for in vitro applications, by other safer carriers. Amphiphilic polymers are a promising platform for efficient and low toxic carriers of non-viral vectors.

The possibility of using new glycerol-based trifunctional copolymers (TFCs) of propylene oxide and ethylene oxide has been demonstrated in our study. Such polymers exhibit higher membranotropic and membrane-modulating activity compared with bifunctional Pluronics of similar hydrophilic-lipophilic balance. A comparative investigation of three TFCs with different structure and Pluronic L61 revealed the block copolymer with the number of propylene oxide and ethylene oxide units of 83.5 and 24.2, which had relatively lower cytotoxicity and higher promoting effect in intracellular delivery of condensed plasmid DNA. Such an effect presumably involves the fluidization of plasma membrane of living cells in the presence of TFCs, which is accompanied by the promotion of intracellular uptake of macromolecular complexes. The study of mechanism of TFC action on cellular pharmacokinetics of complexes of plasmid DNAs with different cationic ligands and the transfection efficiency in vivo will be carried out elsewhere. Our results indicate the superior properties of TFCs over bifunctional Pluronics as enhancers of intracellular delivery of plasmid DNA and suggest a route for designing safe polymer-based carriers of gene therapeutics encoding bioactive peptides, e.g. vascular endothelial growth factor.

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Table 1. Physicochemical characteristics of copolymers of propylene oxide (PO) and ethylene oxide (EO).

Polymer	MW*	PO unit number	EO unit number	HLB**	CMC*** (mg/mL)
Laprol 3603 (L3)	3600	53.2	9.6	6.5	0.41
Laprol 5003 (L5)	5000	71.9	16.7	6.0	0.03
Laprol 6003 (L6)	6000	83.5	24.2	6.7	0.01
Pluronic L61	2000	31	4.6	5.8	0.83

* Number average molecular weight.
** HLB – hydrophilic-lipophilic balance was calculated theoretically by the Davies method.
*** CMC was determined with the use of pyrene probe in PBS at 37 ºC [19].

Table 2. Cytotoxic and hemolytic activity of amphiphilic copolymers for human cells.

Polymer	IC_{50} ± SD (μg/mL)*		Hemolysis (%)**
	A549 cells	Skin fibroblasts	
L3	2.7 ± 0.1	6.0 ± 1.2	0
L5	11.7 ± 3.7	18.7 ± 3.4	2.3
L6	30.5 ± 10.9	135.4 ± 23.6	15.6
Pluronic L61	6.2 ± 0.3	37.4 ± 3.1	40
PEI	1.87±0.34	2.3±0.05	70.8

* MTT-assay for serially diluted polymers.
** Polymer concentration 10 mg/mL.

Table 3. Dynamic light scattering data for complexes of plasmid DNA with polymers in HEPES buffer (pH 7.0).

Complexes*	Hydrodynamic diameter (nm)	Polydispersity index	Zeta potential (mV)
pDNA	248 ± 16	0.55	–9 ± 6
L6	77 ± 14	0.31	–4 ± 4
pDNA + PEI (10)	146 ± 15	0.29	+5 ± 4
pDNA + PEI (20)	142 ± 1	0.25	+28 ± 1
pDNA + Turbofect	62 ± 16	0.28	+25 ± 18
pDNA + PEI (20) + L6	189 ± 7	0.30	+10 ± 6

* Plasmid DNA and L6 concentrations are 10 and 200 μg/mL, respectively. PEI concentration is shown in parenthesis (μg/mL).

Figure captions

Fig. 1. Microphotographs of HEK 293 cells after 4-hour culturing in the presence of (A) plasmid DNA–PEI and (B) plasmid DNA–PEI–L6 complexes. DNA, PEI and L6 concentrations in culture medium are 1, 1 and 20 μg/mL, respectively. PEI was pre-labelled with rhodamine.

Fig. 2. Effect of trifunctional copolymers on the transfection of HEK 293 cells with pEGFP–PEI complexes. DNA, PEI and copolymer concentrations in culture medium are 1, 1 and 20 μg/mL, respectively.

Fig. 3. Effect of trifunctional copolymers on the transfection of human skin fibroblasts with VEGF165-encoding plasmid DNA–PEI complexes. DNA, PEI and copolymer concentrations in culture medium are 1, 1 and 20 μg/mL, respectively. BG – background (untreated cells).

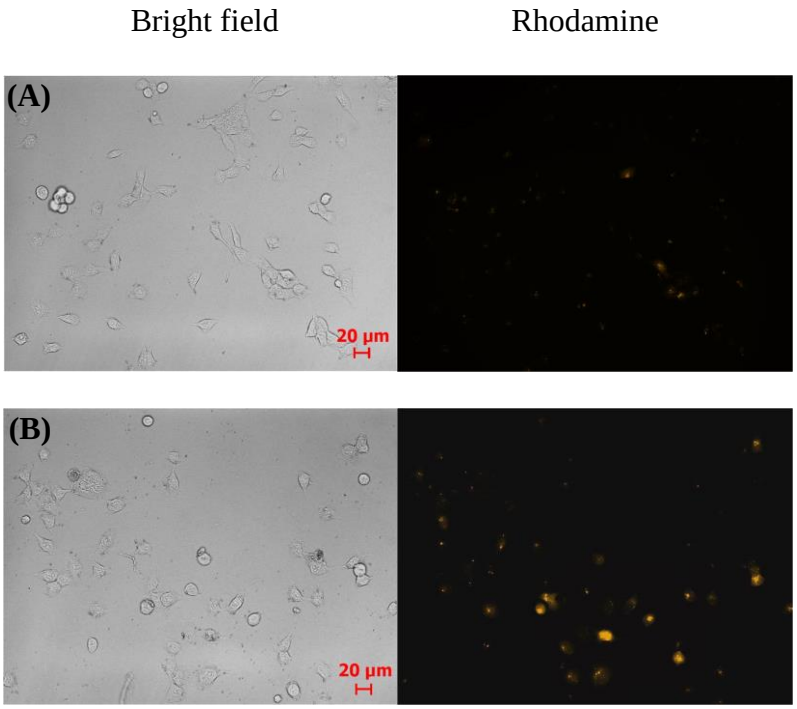

Fig. 1

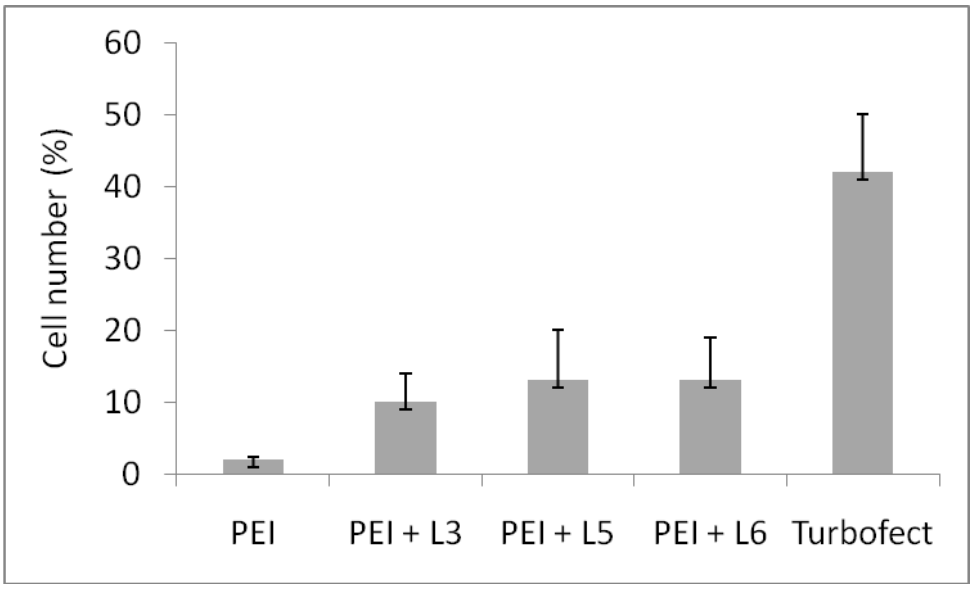
60
50
40
30
20
10
0
Cell number (%)
PEI
PEI + L3
PEI + L5
PEI + L6
Turbofect

Fig. 2

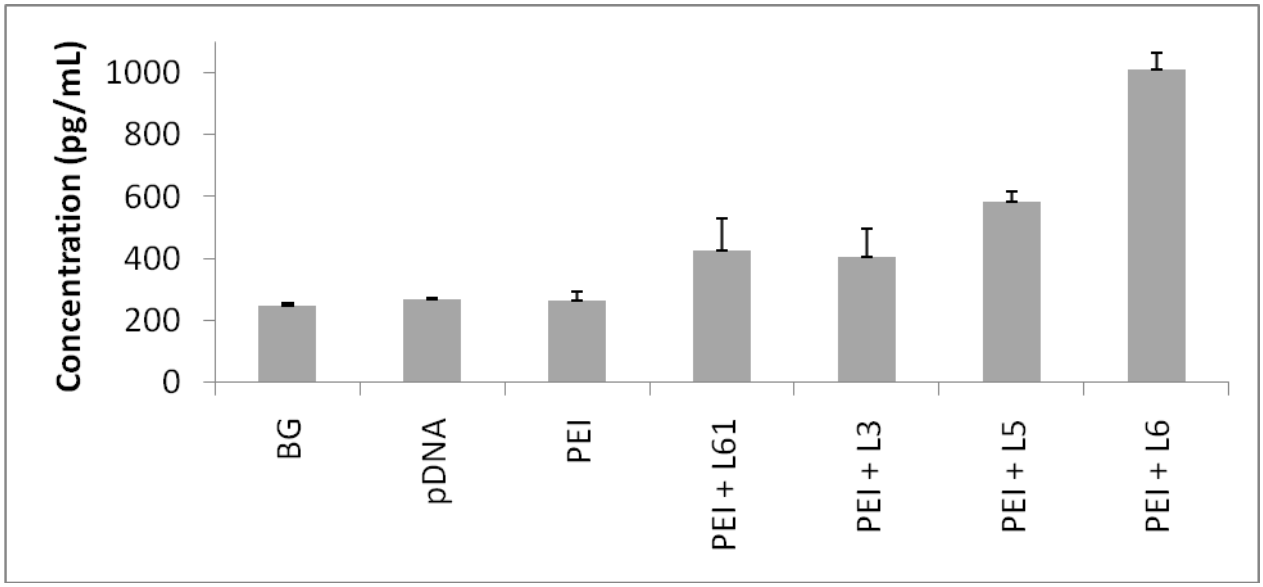
Concentration (pg/mL)
1000
800
600
400
200
0
BG
pDNA
PEI
PEI + L61
PEI + L3
PEI + L5
PEI + L6

Fig. 3