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European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 134–148
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Methoxy poly(ethylene glycol) – Low molecular weight linear polyethylenimine-derived copolymers enable polyplex shielding

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Received 11 January 2007; accepted in revised form 1 October 2007
Available online 12 October 2007

Abstract

Targeted gene delivery relies on the development of materials that allow for the formation of small neutrally charged particles of sufficient colloidal stability preventing non-specific interactions with cells. In order to identify a copolymer composition that combines adequate plasmid DNA (pDNA) compaction with an efficient charge-shielding effect, we synthesized a series of copolymers by covalent linkage of activated 5 or 20 kDa linear methoxy poly(ethylene glycol) (mPEG) or 10 kDa two-arm-mPEG to non-toxic low molecular weight (2.6 and 4.6 kDa) linear polyethylenimine (lPEI) at different molar ratios (mPEG–lPEI copolymers). All of the copolymers condensed pEGFP-N1 pDNA to form nanoparticles with hydrodynamic diameters between 150 and 420 nm – sizes that were maintained for the entire duration of measurement. PEGylated complexes exhibited a reduced particle stability in comparison to the unmodified lPEI–pDNA polyplexes, determined by gel retardation assays and DNase I experiments. Copolymer–pDNA complexes exhibited a zeta potential between −4 and 6 mV, strongly depending on the dispersion medium applied (0.15 M NaCl or 5% glucose supplemented with serum-free cell culture medium). The transfection efficacy, determined in CHO-K1 (between $0.28 \pm 0.08\%$ and $1.92 \pm 0.46\%$) and HeLa (between $1.02 \pm 0.19\%$ and $3.53 \pm 0.30\%$) cells, was significantly reduced compared to lPEI–pDNA particles (between $3.2 \pm 1.3\%$ and $38.8 \pm 5.5\%$). The architecture of the copolymer, the molecular weight of the lPEI residue, and the supplementation of endosomolytic agents (saccharose, chloroquine) all failed to impact the efficacy of gene transfer. Uptake studies, based on Confocal Laser Scanning Microscopy (CLSM) imaging and flow cytometry analysis, suggest that the use of mPEG5/3–lPEI2.6, mPEG10/2–lPEI2.6, and mPEG20–lPEI4.6 lowers unspecific internalization of the corresponding transfection complexes. This provides an ideal basis for the development of transfection vehicles for targeted gene transfer.

Keywords: Linear polyethylenimine; Methoxy poly(ethylene glycol)-linear polyethylenimine; PEG–PEI copolymers; Non-viral transfection; Charge shielding

1. Introduction

Growing insight into the molecular pathology of many diseases has provoked a great interest for gene therapy using gene replacement or supplementation strategies [1,2]. Included in a class of materials showing particular promise are self-assembling polyethylenimine (PEI)–nucleic acid complexes. The rapid internalization of the cationic polyplexes and strong endosomolytic capacity of PEI allow for the transfection of a large variety of cells *in vitro* and *in vivo* [3].

We have recently shown that the use of low molecular weight linear polyethylenimine-plasmid DNA complexes (lPEI–pDNA) significantly improved the *in vitro* transfection efficacy and cell viability compared to commonly used

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doi:10.1016/j.ejpb.2007.10.006

vectors based on the commercially available 25 kDa branched (bPEI) and linear polyethylenimine (lPEI) [4]. Unfortunately, the low molecular weight lPEI–pDNA complexes rapidly aggregated into large clusters on the micrometer scale at physiological salt concentrations [5], making them inappropriate for *in vivo* transfection experiments.

Various efforts to increase the colloidal stability of PEI–DNA polyplexes and to prevent interaction with untargeted cells using pegylated PEI-derived vectors have been described in the literature [6–11]. Our strategy was based on the novel combination of linear low molecular weight PEI with mono-functional linear and two-armed methoxy-PEG (mPEG) resulting in a block-copolymer with improved charge-shielding efficacy.

In order to identify a copolymer composition that combines adequate pDNA compaction with an efficient charge-shielding effect, we synthesized a series of methoxy poly(ethylene glycol) – low molecular weight lPEI-based copolymers (mPEG-lmw lPEI). The pDNA condensing residue is based on non-toxic lPEI with a relative number average molecular weight (M_n) of 2.6 or 4.6 kDa. For charge shielding, 5 or 20 kDa linear mPEGs, as well as a branched PEG consisting of two 5 kDa α-amino-ω-methoxy PEG chains tethered to a glycerol-butanoyl linker, have been conjugated to the lPEI unit at various stoichiometric ratios (Tables 1 and 2).

In order to prove their applicability as gene carriers, we investigated the physico-chemical properties of the corresponding mPEG–lPEI–pEGFP-N1 complexes in media of different ionic strengths and determined the transfection efficacy and cell viability with and without supplementation of an endosomolytic agent using CHO-K1 and HeLa cells as *in vitro* models. We used flow cytometry analysis to quantify intracellular YOYO-1-labelled mPEG–lPEI–pDNA polyplexes and tracked their intracellular and nuclear localization by Confocal Laser Scanning Microscopy (CLSM) imaging.

2. Methods and materials

lPEIs with number average molecular weight of 2.6 and 4.6 kDa were synthesized by ring-opening polymerization of 2-ethyl-2-oxazoline followed by acid-catalysed hydrolysis of the resulting poly(2-ethyl-2-oxazoline) and characterized by ^{1}H-NMR and Gel Filtration Chromatography (GFC) [12].

The mPEG-active ester methoxy poly(ethylene glycol) succinimidyl-α-methylbutanoate was obtained from Nektar Therapeutics (AL, USA) in the 5 kDa (mPEG-SMB-5000) and 20 kDa (mPEG-SMB-20K) chain lengths, as was the 10 kDa 4-[1,3-bis(ω-methoxy-α-amino poly(ethylene glycol) carbonyloxy) propan-2-yloxy]-butanolyl-*N*-hydroxysuccinimide (mPEG2-NHS-10K) (Table 1).

Table 1
The methoxy poly(ethylene glycol)-linear polyethylenimine (mPEG–lPEI) copolymers were synthesized by coupling of linear or branched mPEG-NHS active esters with a molecular weight of 5, 20 or 10 kDa to lPEI with a number average molecular weight of 2.6 or 4.6 kDa

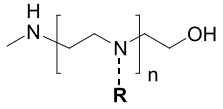
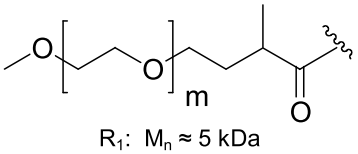
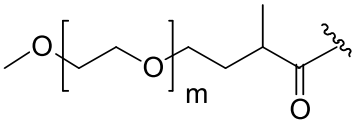
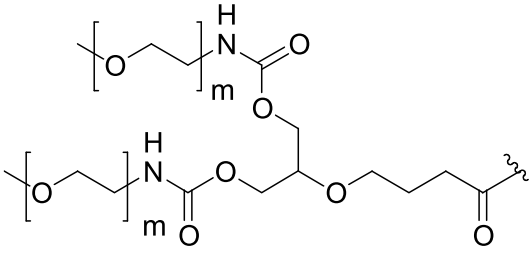
Linear polyethylenimine	mPEG component	PEGylation reagent
 Linear polyethylenimine 2.6 kDa or: 4.6 kDa (lPEI2.6 or lPEI4.6; M_n (GFC))	 R_1: $M_n \approx 5$ kDa mPEG5	mPEG-SMB-5000
	 R_2: $M_n \approx 20$ kDa mPEG20	mPEG-SMB-20K
	 R_3: $M_n \approx 10$ kDa mPEG10	mPEG2-NHS-10K

Table 2
Amounts and molar ratios of the starting materials used for the synthesis of the mPEG–lPEI2.6 and mPEG–lPEI4.6 copolymer series

Copolymer	lPEI 2.6 kDa (mg); (mmol)	lPEI 4.6 kDa (mg); (mmol)	PEGylation reagent (mg); (mmol)	lPEI: mPEG (mol/mol)
mPEG5/3–lPEI2.6	75; 0.0288	–	mPEG-SMB-5000: 500; 0.0868	1:3
mPEG5/3–lPEI4.6	–	145; 0.0315		1:3
mPEG20–lPEI2.6	63; 0.0242	–	mPEG-SMB-20K: 500; 0.0243	1:1
mPEG20–lPEI4.6	–	121.5; 0.0264		1:1
mPEG10/2–lPEI2.6	60; 0.0231	–	mPEG2-NHS-10K: 500; 0.0462	1:2
mPEG10/2–lPEI4.6	–	115.5; 0.0251		1:2

Analytical grade dichloromethane and acetone were purchased from VWR International, Germany. Branched polyethylenimine (high molecular weight, water free, M_W 25,000 Da (LS), M_n 10,000 Da (GPC)) was obtained from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. Linear polyethylenimine M_W ~25,000 Da (7–8%) poly(2-ethyl-2-oxazoline, mp. 73–75°) was purchased from Polysciences Inc., Warrington, PA, USA.

The nutrient mixture F-12 (HAM), ethidium bromide, and kanamycin were obtained from Sigma–Aldrich. pEGFP-N1 was purchased from Clontech (Heidelberg, Germany). The *Escherichia coli* JM109 bacterial strain was shipped from Promega. The Plasmid Maxi Kit was purchased from Qiagen (Hilden, Germany). CHO-K1 (ATCC No. CCL-61) and HeLa cells (ATCC No. CCL-2) were kindly provided by Prof. Buschauer's group at the Department of Medicinal Chemistry at the University of Regensburg (Germany). Fetal calf serum (FCS) was supplied by Biochrom KG Seromed (Berlin, Germany). Dulbecco's Medium, 1 mM sodium pyruvate, 2 mM L-glutamine, agarose, and LB broth medium were purchased from Invitrogen GmbH (Germany).

2.1. mPEG–lPEI copolymer synthesis

The anhydrous lPEI and the mPEG-derivative (specific amounts listed in Table 2) were each dissolved in 5 ml dichloromethane. The clear solutions were mixed and stirred overnight at room temperature. The solvent was evaporated and the residue was dissolved in dilute hydrochloric acid (pH 5). The crude white gel-like mPEG–lPEI copolymer was precipitated by the addition of a concentrated sodium hydroxide solution (pH 14). Precipitates containing lPEI4.6 were washed with water until the supernatant was neutral, yielding 20% to 60% of the corresponding water-soluble copolymer, separated from insoluble lPEI. Because of the water-solubility of the copolymers at neutral pH, the crude copolymer was alternatively purified by extracting the concomitantly formed NHS with acetone at low temperature. Diluted hydrochloric acid was added to all of the derivatives to yield the hydrochlorides of the mPEG–lPEI copolymers.

mPEG5/3–lPEI2.6: ^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 3.28–3.31 (s, CH_3O-PEG), 3.40–3.54 (m, -$^+$NH_2-CH_2-CH_2-), and 3.56-3.71 (m, -O-CH_2-CH_2-).

mPEG5/3–lPEI4.6: ^{1}H-NMR (400 MHz, D_2O): δ_H (ppm) = 3.28–3.30 (s, CH_3O-PEG), 3.38–3.52 (m, -$^+$NH_2-CH_2-CH_2-), and 3.56–3.66 (m, -O-CH_2-CH_2-).

mPEG20–lPEI2.6 and mPEG20–lPEI4.6: ^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 3.28–3.29 (s, CH_3O-PEG), 3.41–3.53 (m, -$^+$NH_2-CH_2-CH_2-), and 3.53–3.70 (m, -O-CH_2-CH_2-).

mPEG10/2–lPEI2.6: ^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 3.23–3.27 (s, CH_3O-PEG), 3.38–3.52 (m, -$^+$NH_2-CH_2-CH_2-), and 3.52–3.66 (m, -O-CH_2-CH_2-).

mPEG10/2–lPEI4.6: ^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 3.14–3.20 (s, CH_3O-PEG), 3.38–3.45 (m, -$^+$NH_2-CH_2-CH_2-), and 3.45–3.57 (m, -O-CH_2-CH_2-).

The ratio of PEG versus PEI was determined from ^{1}H-NMR spectra using integral values obtained for the -O-CH_2-CH_2- protons of PEG and -$^+$NH_2-CH_2-CH_2- protons of PEI. The total nitrogen content was determined by elemental analysis. lPEI as side-fraction was not detectable by TLC.

2.2. Amplification and purification of plasmid DNA [pDNA]

The pEGFP-N1 was transformed into the *E. coli* JM109 bacterial strain. The transformed cells were expanded in LB broth supplemented with kanamycin. The pDNA was isolated using the Quiagen Plasmid Maxi Kit according to the supplier's protocol. The concentration and purity of the pDNA was measured by UV absorption at 260 and 280 nm.

2.3. Preparation of polyplexes

Both lPEIs and all of the mPEG–lPEI copolymers were converted into the corresponding hydrochloride using 2 N HCl and the nitrogen content was determined by elemental analysis. Briefly, the polymers were dissolved in either 0.15 M NaCl or 5% glucose and the pH was adjusted to 7.0 with 0.01 N aqueous hydrochloric acid or 0.01 N aqueous sodium hydroxide. The concentrations of the corresponding polymer solutions were adjusted to produce an N/P ratio of 1 upon the addition of 1 μl polymer solution to 2 μg pDNA. The pDNA and the appropriate volume of polymer solution were each diluted with 0.15 M NaCl or 5% glucose to equal volumes corresponding to 25 μl per 1 μg pDNA. The pDNA and polymer solutions were

mixed by vortexing and the polyplexes were allowed to form for 20 min at room temperature.

2.4. Laser light scattering analysis and zeta potential measurements

Polyplexes prepared in 0.15 M NaCl or 5% glucose containing a total of 10 μg pEGFP-N1 were diluted to 2.5 ml with 0.15 M NaCl or HAM's F12 (serum-free transfection medium), respectively. The samples were thermostated to 25 °C and laser light scattering analysis was performed at 25 °C with an incident laser beam of 633 nm at a scattering angle of 90° using a Malvern ZetaSizer 3000 HSA, Malvern Instruments GmbH, Germany. The count rates for all dispersants were lower than 5 kcps, confirming their applicability in size measurements. The following parameters were used: viscosity of HAM's F12 and 0.15 M NaCl: 0.89 mm^2/s; the refractive indices of HAM's F12: 1.681 and 0.15 M NaCl: 1.33. The sampling time was set automatically.

Five measurements each with 10 sub-runs were performed for each of two independent samples ($n = 2$). Size measurements of polyplexes prepared at an N/P ratio of 18 were carried out for three independent samples ($n = 3$).

The zeta potential measurements of the same polyplex samples were performed in the standard capillary electrophoresis cell of the ZetaSizer HSA (Malvern Instruments GmbH, Germany), measuring the electrophoretic mobility at 25 °C. The sampling time was again set automatically ($n = 2$, polyplexes at an N/P ratio of 18: $n = 3$).

The measurements were analyzed statistically by one-way analysis of variance (ANOVA) and the Tuckey- or Dunnett's test.

2.5. Gel-retardation assay

Polyplexes (0.5 μg pEGFP-N1 in 5 μl 0.15 M NaCl or 5% glucose) were prepared with the appropriate amount of lPEI or mPEG–lPEI solution (always diluted to 8.5 μl) to produce N/P ratios of 0, 1, 1.5, 2, 3, and 4. After incubating for 10 min at room temperature, 5 μl HAM's F12 transfection medium was added to the polyplexes and the solutions were incubated for another 10 min at room temperature. After the addition of 1.5 μl loading buffer, the samples were loaded onto a 1% TAE agarose gel and run at 80 V for 50 min. The pDNA bands were visualized by ethidium bromide staining and detected on a Fisherbrand FT-20/312 UV transilluminator 312 (Herolab GmbH Laborgeräte, Germany).

2.6. DNase I experiments

PEI-based polyplexes were prepared in 0.15 M NaCl at an N/P ratio of 18 (2 μg pEGFP-N1/12 μl polyplex dispersion) and allowed to form at room temperature for 20 min. Thereafter, polyplexes or pEGFP-N1 (as positive control) were incubated in digestion buffer (20 mM $MgSO_4$, 0.4 M NaOAc, pH 8) with and without 2 U of DNase I (1 μl) at 37 °C. After 30 min incubation with DNase I, the digestion was halted by the addition of a stop solution (5 μl: 0.2 M EDTA pH 8, 0.7 N NaOH) and 10× loading buffer (2 μl) was added. The integrity of the plasmid was examined by loading the samples on a 1% alkaline agarose gel containing ethidium bromide. The gels were run for 90 min at 40 V.

2.7. Confocal laser scanning microscopy

A Zeiss Axiovert 200 M microscope coupled to a Zeiss LSM 510 scanning device (Carl Zeiss Co. Ltd., Germany) was used for imaging of the CHO-K1 cells. The inverted microscope was equipped with Plan-Apochromat 63× and Plan-Neofluar 100× objectives. Cells were plated in an 8-well Lab-Tek™ Chambered Coverglass (Nunc GmbH & Co., KG, Germany) at an initial density of 35,000 cells/chamber in 400 μl culture media. To maintain a pH of 7.4, 20 mM HEPES was supplemented in the media. After 18 h, polyplexes were added and the cells in each well were immediately imaged at 37 °C. The thickness of each optical section was set at 4 μm.

For the visualization of polyplexes, pDNA was labelled with YOYO-1. The intercalate was excited at 488 nm using an argon laser and the fluorescence was detected using a 505–530 nm band-pass filter.

2.8. In vitro transfection and cytotoxicity experiments

For gene transfer studies, CHO-K1 and HeLa cells were grown in 24-well plates at an initial density of 40,000 cells per well. Experiments were performed as described previously and evaluated by flow cytometry using a FACSCalibur (Becton–Dickinson, Germany) [13]. To investigate the effect of saccharose, a final concentration of 5 mM was supplemented to the cells during transfection. The cell population referred to the number of whole cells (dead or live) after the transfection process, but did not include the cellular debris. EGFP positive cells were detected using a 515–545 nm band-pass filter, whereas the propidium iodide (Sigma–Aldrich, Germany) emission was measured with a 670 nm longpass filter. The mean fluorescence intensity was determined using the EGFP positive cells. The transfection efficiency and cell viability were calculated as follows: first, the cell population of the samples was normalized to the cell population of untreated cells, as was the cell viability. In the next step, the number of EGFP positive or propidium iodide negative cells were referenced to the calculated cell population, expressing the transfection efficiency or cell viability, respectively.

2.9. Cellular uptake of polyplexes

YOYO-1-labeled nucleic acid was used to monitor polyplex delivery as described [13]. Cells were incubated with polyplexes for 6 h, followed by flow cytometry analysis of whole cells. The percentage of cells that had taken up polyplexes and their mean fluorescence intensity were determined by flow cytometry after excitation with a 488-nm argon laser and detection with a 515- to 545-nm band-pass filter.

3. Results

3.1. Particle size, surface charge, and efficacy of pDNA complexation

We used mPEG-NHS active esters with different molecular weights and architectures for conjugation with 2.6 and 4.6 kDa lPEIs (Tables 1 and 2). As with similar synthetic approaches, the potential for di-, tri- or multiblock copolymer side products has to be considered [11,14,15]. However, ^{1}H-NMR spectroscopy indicates that these did not appear to be dominant reaction products.

In order to prove that the synthesized copolymers are capable of condensing pDNA and to assess any particle aggregation, we determined the hydrodynamic diameters of copolymer-based polyplexes in 0.15 M NaCl in comparison to the corresponding unmodified lPEI–pDNA complexes.

While lPEI4.6–pDNA complexes in 0.15 M NaCl grew to hydrodynamic diameters between 1500 and 2500 nm, lPEI2.6 did not form discrete polyplexes with pDNA (aggregates were larger than 5 μm with polydispersity indices (PI) larger than 0.8, Fig. 3a). Using mPEG–lPEI copolymers as pDNA-condensing agents in 0.15 M NaCl, however, polyplexes between 150 and 300 nm with a broad particle size distribution were formed (PI between 0.4 and 0.7; Fig. 1).

Since these initial results indicated that the impact of the N/P ratio is negligible, we chose copolymer-based complexes formed at an N/P ratio of 18 to investigate whether the molecular weight of the lPEI-component or the mPEG-portion influences the particle size. We observed a subtle, but significant, reduction in the hydrodynamic diameter with an increasing molecular weight of the polycationic residue for mPEG20–lPEI–pDNA ($p < 0.01$). Considering the mPEG variants used, significant differences in the particles were only observed for mPEG5/3–lPEI2.6–pDNA and mPEG20–lPEI2.6–pDNA complexes ($p < 0.01$). mPEG–lPEI4.6-derived polyplexes all exhibited hydrodynamic diameters of about 150 nm, irrespective of the copolymer structure (Fig. 1).

When using 5% glucose as the pDNA complexation medium, we observed that polyplexes prepared with lPEIs of varying molecular weights doubled in size during less than 60 min incubation in serum-free cell culture medium (HAM's F12), irrespective of the N/P ratio (Fig. 3b). Polyplexes formed with the synthesized copolymers exhibited hydrodynamic diameters between 200 and 420 nm under these conditions and period of time (Fig. 2). The effect of the polymer concentration was negligible. At the mid-range N/P ratio of 18, we observed a reduction in the particle size with increasing molecular weight of the lPEI-component only for mPEG20–lPEI-derived polyplexes (between 220 and 250 nm).

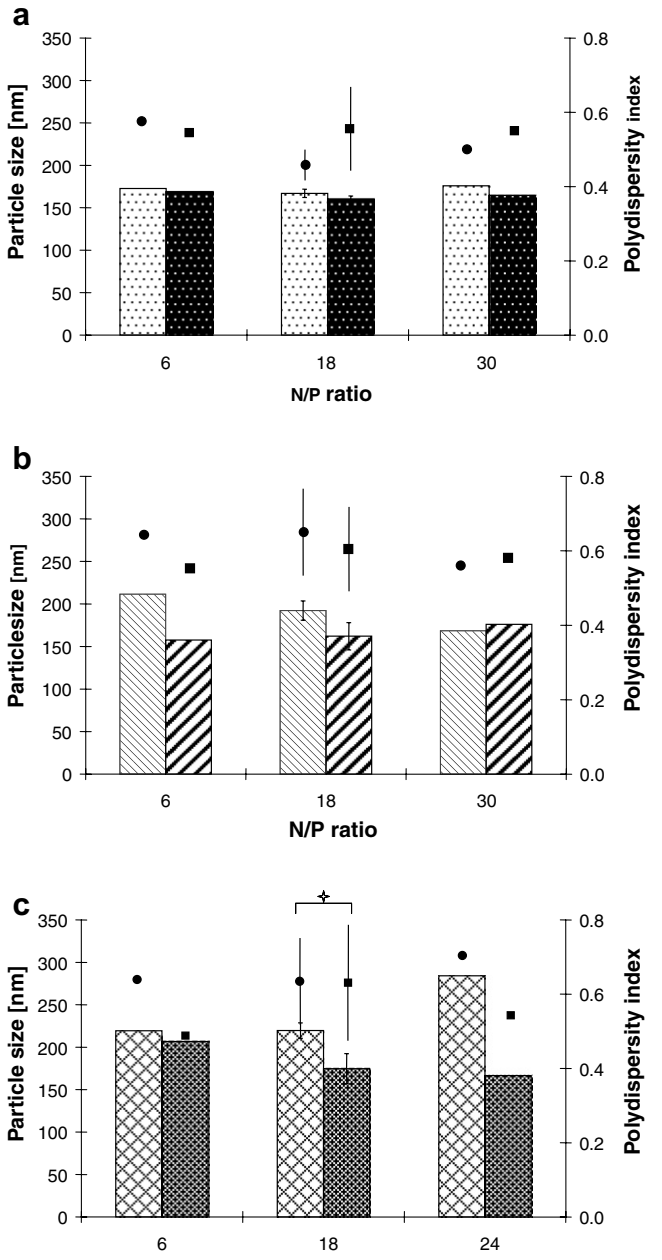


Fig. 1. The mean particle size/PI of (a) ▨/● mPEG5/3–lPEI2.6, ■/■ mPEG5/3–lPEI4.6–pDNA, (b) ▨/● mPEG10/2–lPEI2.6, ▨/■ mPEG10/2–lPEI4.6–pDNA, or (c) ▨/● mPEG20–lPEI2.6, and ▨/■ mPEG20–lPEI4.6–pDNA polyplexes, prepared in 0.15 M NaCl at N/P ratios of 6, 18 and 30 after approximately 45 min incubation in serum-free cell culture medium. (N/P 6 and 30: data represented as mean value ($n = 2$); N/P 18: data represented as mean value ± SD ($n = 3$). Significant differences in the particle sizes ($p < 0.05$) are denoted by ✧.)

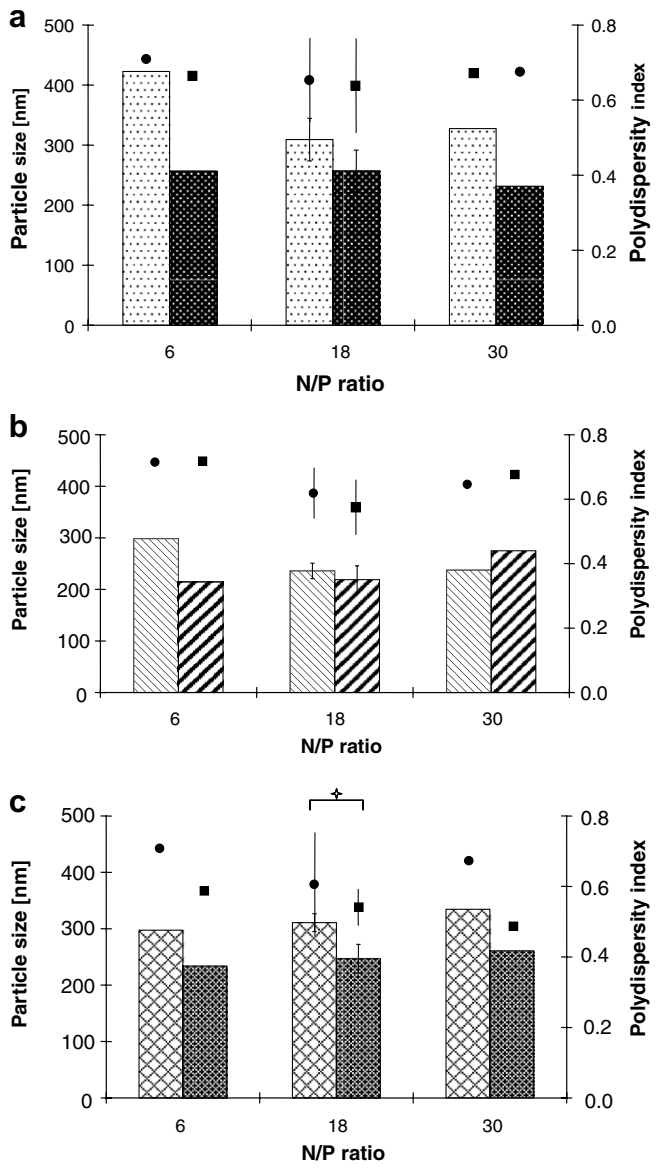


Fig. 2. The mean particle size/PI of (a) ⬚/● mPEG5/3–lPEI2.6, ■/■ mPEG5/3–lPEI4.6–pDNA, (b) ▨/● mPEG10/2–lPEI2.6, ▨/■ mPEG10/2–lPEI4.6–pDNA, and (c) ▨/● mPEG20–lPEI2.6, ■/■ mPEG20–lPEI4.6–pDNA polyplexes, prepared in 5% glucose at N/P ratios of 6, 18 and 30 after approximately 65 min incubation in serum-free cell culture medium. (N/P 6 and 30: data represented as mean value ($n = 2$); N/P 18: data represented as mean value ± SD ($n = 3$). Significant differences in the particle size ($p < 0.05$) are denoted by ✧.)

The mPEG content only appeared to induce a significant reduction in the particle size of the mPEG10/2–lPEI2.6–pDNA compared to mPEG5/3–lPEI2.6- and mPEG20–lPEI2.6–pDNA ($p < 0.05$).

In order to prove that polyplexes manufactured with the copolymers are stabilized against particle aggregation, we determined the polyplex size at multiple time points over at least 45 min in 0.15 M NaCl or 5% glucose/HAM's F12 (Fig. 3). The hydrodynamic diameter of the mPEG–lPEI–pDNA did not significantly increase during time ($p < 0.05$), irrespective of the copolymer used or the particle environment.

We used gel-retardation assays to investigate the efficacy of pDNA retention by the copolymers in comparison to the lPEI precursors. The capacity of mPEG5/3–lPEI- and mPEG20–lPEI-derived copolymers to complex pDNA in 5% glucose after incubation in HAM's F12 was remarkably reduced compared to unmodified lPEI2.6 and lPEI4.6 (Fig. 4).

pDNA was completely immobilized by lPEI2.6 and lPEI4.6 at all N/P ratios above 1. The lowest pDNA retardation efficacy was observed for mPEG5/3–lPEI2.6 and increased slightly with the molecular weight of the lPEI residue of the copolymer. pDNA was completely retarded by mPEG5/3–lPEI4.6 and mPEG5/3–lPEI2.6 at N/P ratios of 3 and 6, respectively. mPEG20–lPEI copolymers exhibited a slightly improved pDNA immobilization efficiency compared to the mPEG5/3–lPEIs, retarding pDNA dissociation even at an N/P ratio of 1.5. In contrast to the unmodified lPEI–pDNA complexes, most of the copolymer-based polyplexes with elevated N/P ratios were detected as pDNA bands localized on top of the gel pocket.

We further investigated if PEGylation alters pDNA stability in the complex during incubation with DNase I in comparison to unmodified lPEI–pDNA polyplexes (Fig. 5). While pDNA appeared relatively stable after complexation with lPEI2.6 and lPEI4.6, at least partial degradation occurred when complexes were formed with the corresponding copolymers. Here, pDNA stability increased with increasing M_W of the lPEI component. With respect to the mPEG residue, pDNA reached maximum stability with mPEG10/2-, slightly reduced stability with mPEG20-, and was almost completely degraded in mPEG5/3-based polyplexes.

The zeta potential of the copolymer-based polyplexes spanned from low positive to slightly negative values, depending on the medium used during the measurement (Fig. 6). For polyplexes in 0.15 M NaCl, a surface charge between 1 and 6 mV was determined, while mPEG–lPEI–pDNA complexes prepared in 5% glucose and incubated in HAM's F12 exhibited a negative net charge between −4 and −1 mV. The PEG residue, the molecular weight of the lPEI component, and the N/P ratio had no significant impact on the zeta potential of the corresponding polyplexes.

3.2. Cell–polyplex interaction and transfection efficacy of mPEG–lPEI–pDNA complexes

To investigate differences in the cell–polyplex interactions, we prepared polyplexes with YOYO-1-labelled pEGFP-N1 reporter plasmids and either lPEI or mPEG–lPEI copolymers at an N/P ratio of 18. Using CLSM imaging (Figs. 7a and 8a), we observed large aggregates of unmodified lPEI2.6- and lPEI4.6–pDNA polyplexes, most of them dispersed extracellularly in the cell culture medium. The CLSM images suggest that the number of intracellularly localized polyplexes increased with the molecular weight of the lPEI used for complex formation.

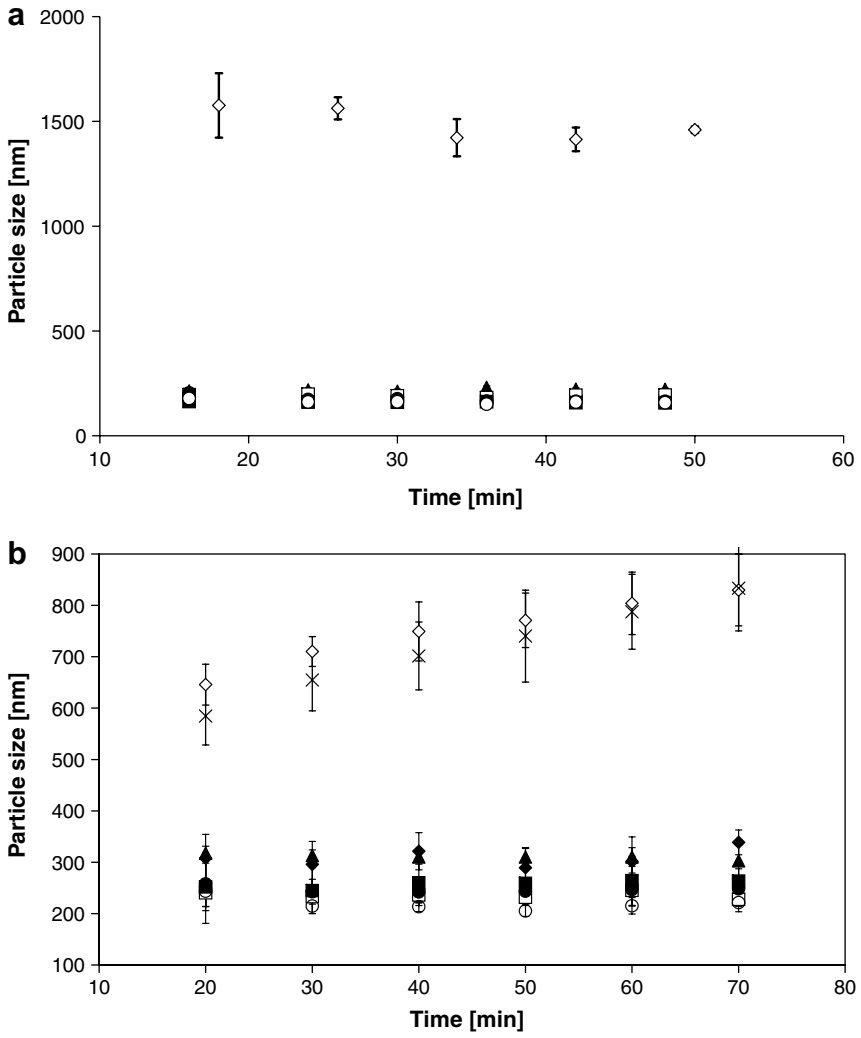


Fig. 3. Mean particle size values of lPEI2.6 (×) – pDNA, lPEI4.6 (◇) – pDNA, mPEG5/3–lPEI2.6 (◆) – pDNA, mPEG5/3–lPEI4.6 (■) – pDNA, mPEG10/2–lPEI2.6 (□) – pDNA, mPEG10/2–lPEI4.6 (○) – pDNA, mPEG20–lPEI2.6 (▲) – pDNA, and mPEG20–lPEI4.6 (●) – pDNA polyplexes prepared at an N/P ratio of 18, determined (a) over 45 min in 0.15 M NaCl or (b) over at 70 min in 5% glucose/HAM's F12. (Data represented as mean value ± SD ($n = 3$). For copolymer-derived nanoparticles no significance ($p < 0.05$) was detectable.)

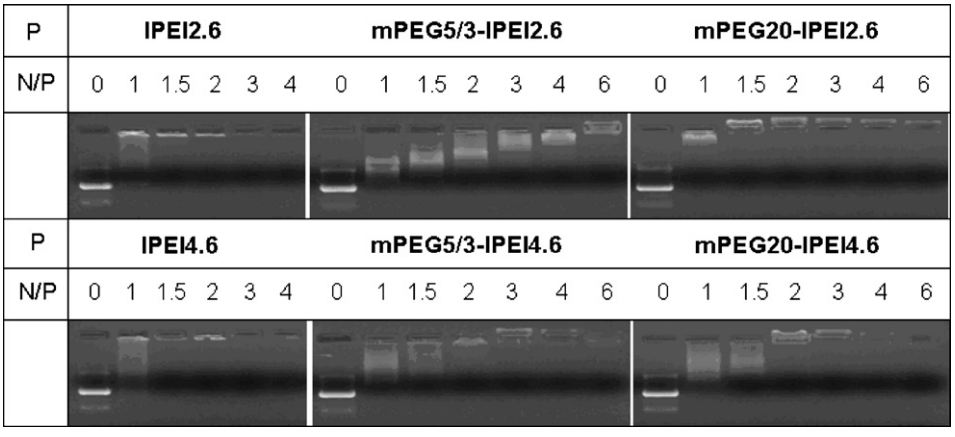


Fig. 4. In order to determine the capacity of the synthesized copolymers to retard pDNA migration in an electric field, gel-retardation assay was performed with lPEI2.6-, lPEI4.6-, mPEG5/3–lPEI2.6-, mPEG5/3–lPEI4.6-, mPEG20–lPEI2.6- or mPEG20–lPEI4.6–pDNA polyplexes, prepared in 5% glucose at N/P ratios between 1 and 6. The gel-retardation assays were performed after polyplexes were incubated in serum-free cell culture medium for 10 min at room temperature. pDNA complexation in 0.15 M NaCl led to comparable results (data not shown).

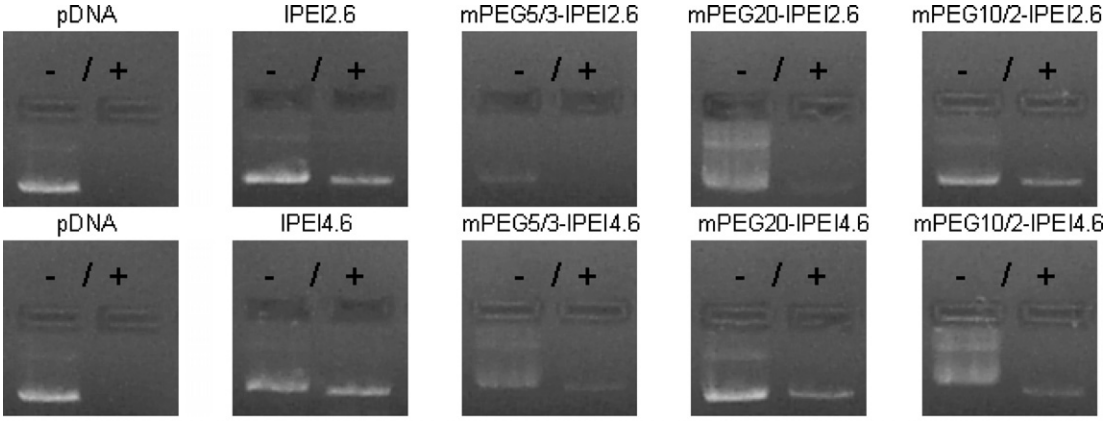


Fig. 5. In order to investigate if the synthesized copolymers stabilize pDNA against enzymatic degradation, polyplexes by the complexation of pDNA with lPEI2.6-, lPEI4.6-, mPEG5/3–lPEI2.6-, mPEG5/3–lPEI4.6-, mPEG20–lPEI2.6-, mPEG20–lPEI4.6-, mPEG10/2–lPEI2.6- or mPEG10/2–lPEI4.6–pDNA polyplexes were prepared in 0.15 M NaCl at N/P ratio of 18 and incubated with DNase I. The corresponding complexes were incubated with (+) or without (−) 2 U of DNase I at 37 °C to examine enzyme-dependent and independent degradation of pDNA. After 30 min the reaction was stopped and the integrity of the pDNA was examined using gel electrophoresis.

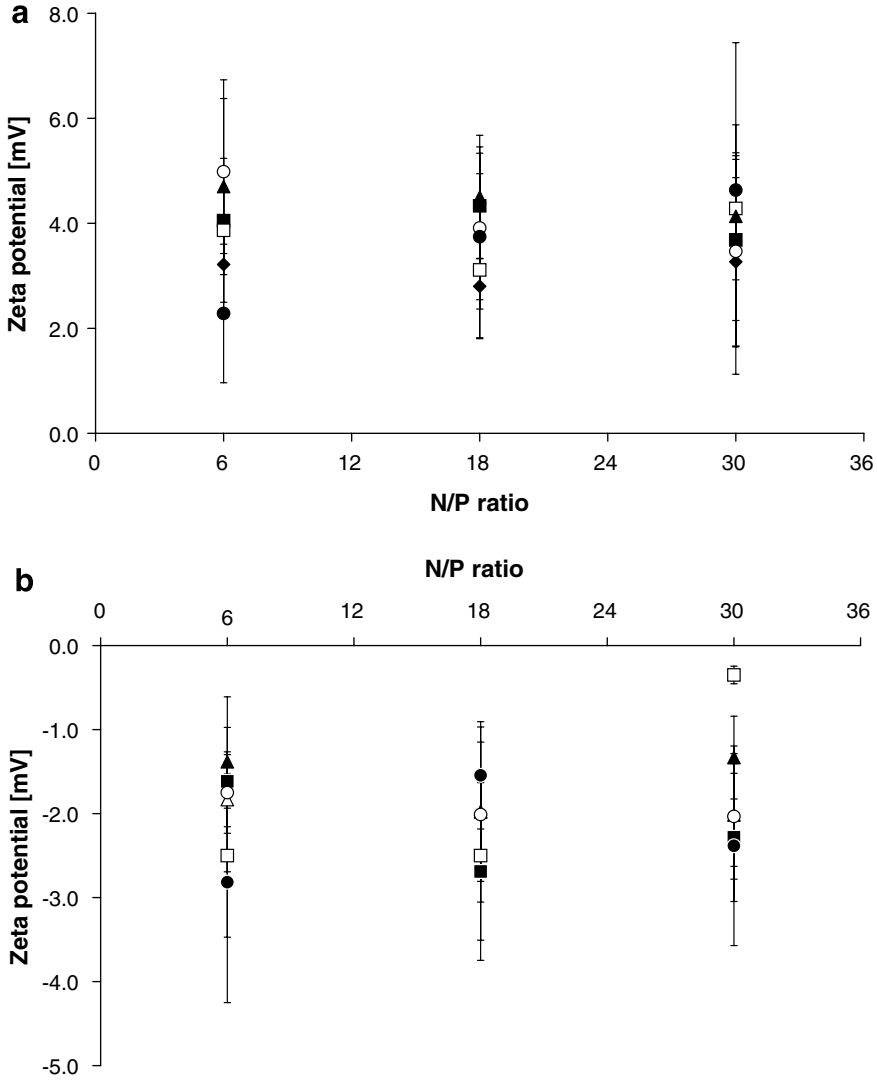


Fig. 6. Zeta potential of mPEG–lPEI–pDNA polyplexes was determined as a measure of the polyplex surface charge. mPEG5/3–lPEI2.6 (◆) – pDNA, mPEG5/3–lPEI4.6 (■) – pDNA, mPEG10/2–lPEI2.6 (□) – pDNA, mPEG10/2–lPEI4.6 (○) – pDNA, mPEG20–lPEI2.6 (▲) – pDNA, and mPEG20–lPEI4.6 (●) – pDNA polyplexes were prepared at N/P ratios of 6, 18 and 30. The zeta potential was determined (a) after polyplex formation in 0.15 M NaCl or (b) for polyplexes prepared in 5% glucose after at least 30 min incubation in serum-free cell culture medium. N/P 6 and 30: data represented as mean value ($n = 2$); N/P 18: data represented as mean value ± SD ($n = 3$). No significant differences ($p < 0.05$) were detected between the samples tested.

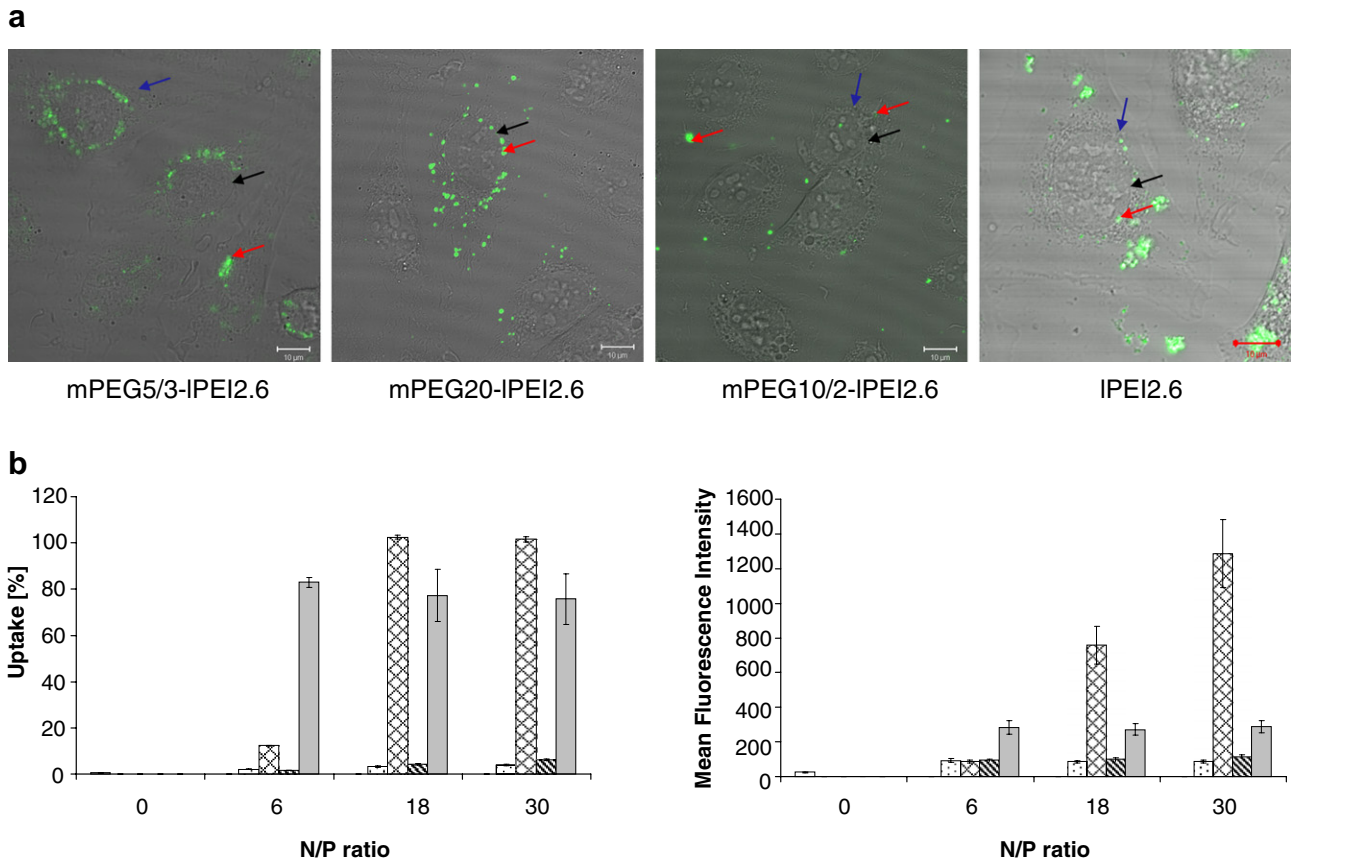


Fig. 7. (a) Confocal microscope images of adherent CHO-K1 cells in serum-free cell culture medium after 4 to 5 h incubation with mPEG–lPEI2.6-YOYO-1-labelled pEGFP–N1 complexes. The red arrows indicate individual polyplexes or large polyplex aggregates, the blue and the black arrows are exemplarily pointed at a cell membrane and nuclear membrane of a CHO-K1 cell, respectively. (b) The cell uptake and the mean fluorescence intensity of CHO-K1 cells were quantified after incubation with ⬚ mPEG5/3–lPEI2.6, ▨ mPEG20–lPEI2.6, ▧ mPEG10/2–lPEI2.6, and ▢ lPEI2.6 polyplexes using flow cytometry analysis.

It seemed that lPEI4.6–pDNA complexes almost flooded the cytoplasm of CHO-K1 cells, while a few were localized in the nucleus (Fig. 8b).

With the exception of the mPEG20–lPEI-derived polyplexes, CLSM images indicated a similar trend for mPEG–lPEI–pDNA complexes. Even though the particle size and the number of particle aggregates seemed to be reduced compared to unmodified lPEI–pDNA particles, the internalization of polyplexes into the cytoplasm was enhanced with increasing molecular weight of the lPEI residue in the copolymer. The corresponding polyplexes seemed, thereby, to be homogeneously distributed in the cytoplasm, partially assembling at the nuclear membrane. In contrast to lPEI4.6–pDNA complexes, we did not observed nuclear localization of pegylated polyplexes, irrespective of the copolymer used for particle formation. Polyplex internalization was at a minimum with mPEG10/2–lPEI2.6-polyplexes or mPEG20–lPEI4.6–pDNA, within the group of mPEG–lPEI2.6- or mPEG–lPEI4.6-derived polyplexes, respectively.

We quantified polyplex internalization by flow cytometry, determining the portion of CHO-K1 cells that incorporated YOYO-1-labelled polyplexes as a measure of cell uptake, and the mean fluorescence intensity of these cells (MFI) to estimate the number of polyplexes internalized per cell. For all experiments, the cell uptake and the mean fluorescence values showed the same trend. While cell uptake was significantly reduced after treatment with mPEG5/3–lPEI2.6- and mPEG10/2–lPEI2.6–pDNA, the number of cells that internalized polyplexes increased in the case of mPEG20–lPEI2.6-derived particles, compared to lPEI2.6–pDNA complexes. However, the inverse tenor was found for the mPEG–lPEI4.6–pDNA polyplex uptake studies.

As another parameter for the polyplex–cell interactions, we determined the transfection efficacy of mPEG–lPEI–pDNA polyplexes in comparison to the unmodified ones using the CHO-K1 (Fig. 9) and HeLa (Fig. 10) cell models.

In CHO-K1 cells the transfection efficacy reached values between $0.28 \pm 0.08\%$ and $1.85 \pm 0.25\%$ using mPEG–lPEI2.6–pDNA (Fig. 9a) or 0.69 ± 0.08 and $1.92 \pm 0.46\%$ with mPEG–lPEI4.6–pDNA polyplexes (Fig. 9b). The number of transfected cells was significantly reduced compared to transfection with the corresponding lPEI–pDNA

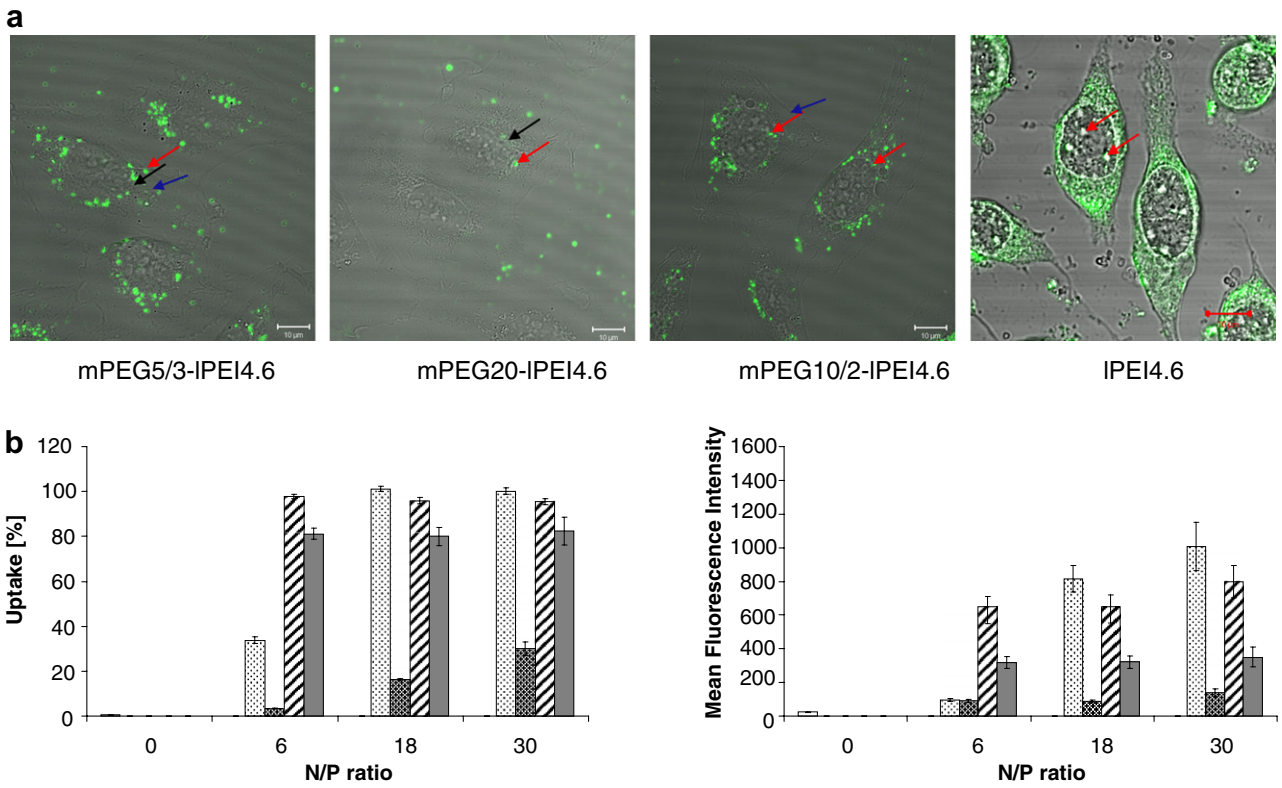


Fig. 8. (a) Confocal microscope images of adherent CHO-K1 cells in serum-free cell culture medium after 4 to 5 h incubation with mPEG–lPEI4.6-YOYO-1-labelled pEGFP–N1 complexes. The red arrows indicate individual polyplexes or large polyplex aggregates, the blue and the black arrows are exemplarily pointed at a cell membrane and nuclear membrane of a CHO-K1 cell, respectively. (b) CHO-K1 cells were incubated with polyplexes, prepared with YOYO-1-labelled pDNA and mPEG5/3–lPEI4.6 (⬚), mPEG20–lPEI4.6 (▩), mPEG10/2–lPEI4.6 (▨), and lPEI4.6 (■) at an N/P ratio of 18. The cell uptake and the mean fluorescence intensity of these cells were determined by flow cytometry analysis.

particles (lPEI2.6–pDNA, N/P 30: $3.2 \pm 1.3\%$; lPEI4.6–pDNA, N/P 30: $38.8 \pm 5.5\%$) for mPEG10/2–lPEI2.6 at N/P ratios of 6 and 18, mPEG5/3–lPEI2.6, and mPEG20–lPEI2.6 as well as all mPEG–lPEI4.6-derived polyplexes at any polymer concentration ($p < 0.05$).

We observed a significant increase in gene transfer efficacy with the molecular weight of the lPEI residue for mPEG5/3–lPEI–pDNA and mPEG20–lPEI–pDNA polyplexes at N/P 18 and 30 ($p < 0.05$). Polyplexes of mPEG10/2–lPEI variants led to significantly higher transfection efficacies compared to mPEG5/3–lPEI- and mPEG20–lPEI-derived particles, irrespective of the polymer concentrations applied (Fig. 9, $p < 0.05$).

In HeLa cells the transfection proceeded with an efficacy between $1.02 \pm 0.19\%$ and $1.44 \pm 0.29\%$ using mPEG–lPEI2.6–pDNA (Fig. 10a) or $1.24 \pm 0.10\%$ and $3.53 \pm 0.30\%$ with mPEG–lPEI4.6–pDNA polyplexes (Fig. 10b). In comparison to transfection with the corresponding unmodified lPEI–pDNA complexes, the number of GFP-positive cells was significantly reduced using mPEG–lPEI2.6–pDNA polyplexes at an N/P ratio of 30 or mPEG–lPEI4.6–pDNA complexes at any N/P ratio ($p < 0.05$). We observed a significant increase in gene transfer efficacy with molecular weight of the lPEI residue for mPEG5/3–lPEI–pDNA and mPEG10/2–lPEI–pDNA polyplexes at N/P 18 and 30 ($p < 0.05$). The use of mPEG20–lPEI4.6-derived particles reduced transfection significantly compared to mPEG5/3–lPEI4.6–pDNA and mPEG10/2–lPEI4.6–pDNA complexes at all applied N/P ratios. All mPEG–lPEI2.6–pDNA variants reached comparable transfection efficacy, irrespective of the N/P ratio applied ($p < 0.05$).

The toxicity of the copolymers and the unmodified lPEIs seemed comparable or slightly improved for lPEI4.6-derived polyplexes. The cell viability of CHO-K1 and HeLa cells after exposure to mPEG–lPEI copolymers remained between $90.47 \pm 1.67\%$ and $94.1 \pm 0.54\%$ or $93.68 \pm 2.34\%$ and $101.49 \pm 2.41\%$, respectively (Fig. 11, $p < 0.05$).

In order to investigate if gene expression was reduced due to a lowered endosomolytic capacity of the copolymers, we determined the transfection efficacy of the mPEG–lPEI–pDNA polyplexes in CHO-K1 cells with or without the supplementation of saccharose as a lysosomotropic agent (Fig. 12, $p < 0.05$). However, we observed no significant differences in transfection efficacy. A similar setup using chloroquine as endosomolytic agent gave comparable results (data not shown).

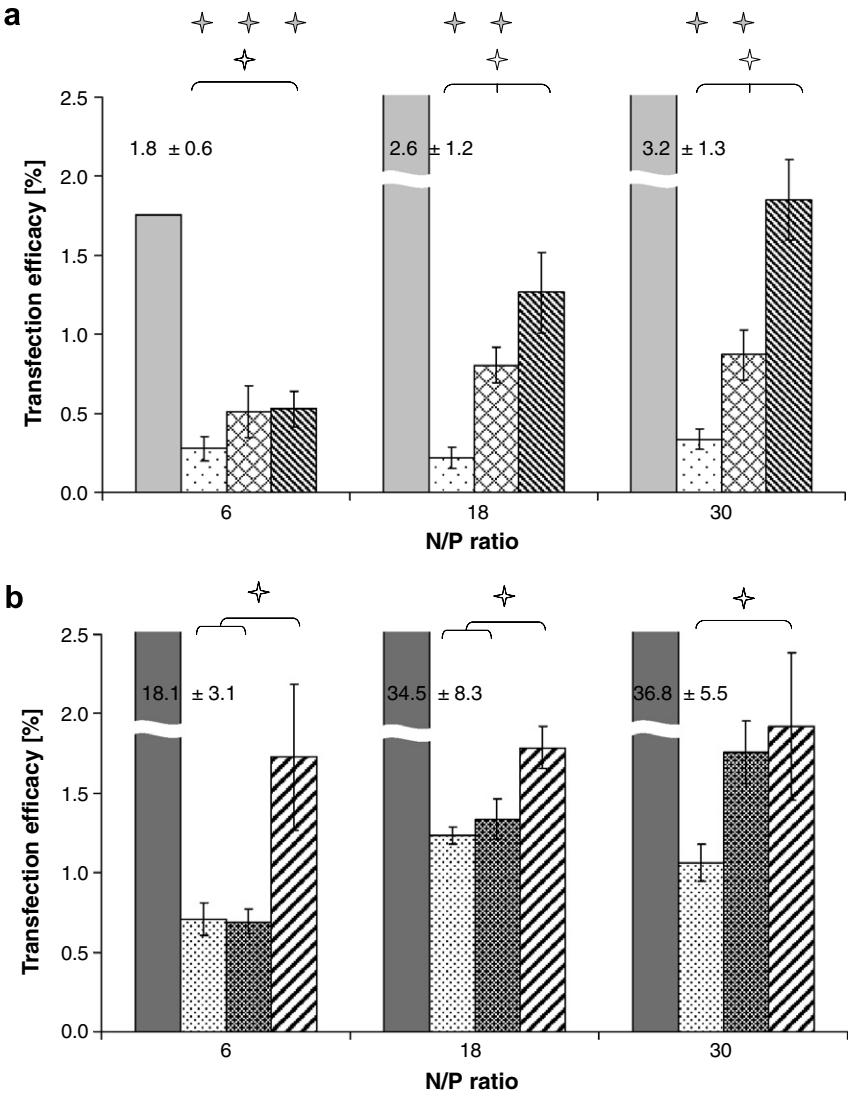


Fig. 9. The transfection efficacy of copolymer-based polyplexes was determined in CHO-K1 cells after 4 h incubation with: (a) ▢ lPEI2.6, ⁘ mPEG5/3–lPEI2.6, ▨ mPEG20–lPEI2.6 or ▧ mPEG10/2–lPEI2.6 pDNA, as well as (b) ■ lPEI4.6, ⁘ mPEG5/3–lPEI4.6, ▩ mPEG20–lPEI4.6, or ▨ mPEG10/2–lPEI4.6–pDNA in serum-free cell culture medium. The polyplexes were prepared in 0.15 M NaCl at N/P ratios between 6 and 30 and the transfection efficacy was determined after 48 h by flow cytometry analysis. Data are represented as mean value ± SD ($n = 3$); differences with significance of $p < 0.05$ are depicted by ✧.

4. Discussion

The efficacy and biocompatibility of PEI-based vectors is limited due to the low colloidal stability as well as non-specific interaction with blood components and untargeted cells [6,16–18]. In efforts to stabilize the polyplexes for a biological environment, PEGylation was applied to introduce hydrophilic non-charged layers to the particle surface, reducing particle aggregation and non-specific interactions through steric hindrance and shielding of the excess positive net charge [7–9,19,20]. As the PEGylation of preformed PEI–DNA polyplexes would result in non-uniform and poorly characterized polyplexes [6,10], we focused on particle stabilization using PEG–PEI copolymers. While other groups used PEG–PEI copolymers based on PEI of higher molecular weight [21–23], formed PEI–PEG–PEI triblock copolymers [20,24], or introduced PEG as a crosslinker to short chain PEIs [25,26], we used a simple synthetic approach that enables the individual combination of non-toxic low molecular weight lPEIs and PEG-starting blocks, in order to induce the maximum shielding effect (Table 2).

We performed the conjugation of mPEG in an aprotic solvent to reduce NHS-ester hydrolysis. The purification of the corresponding amine-base with water allowed for the removal of unmodified mPEG and lPEI, but also reduced the yield of mPEG–lPEI, due to a remarkably improved water-solubility [11,27].

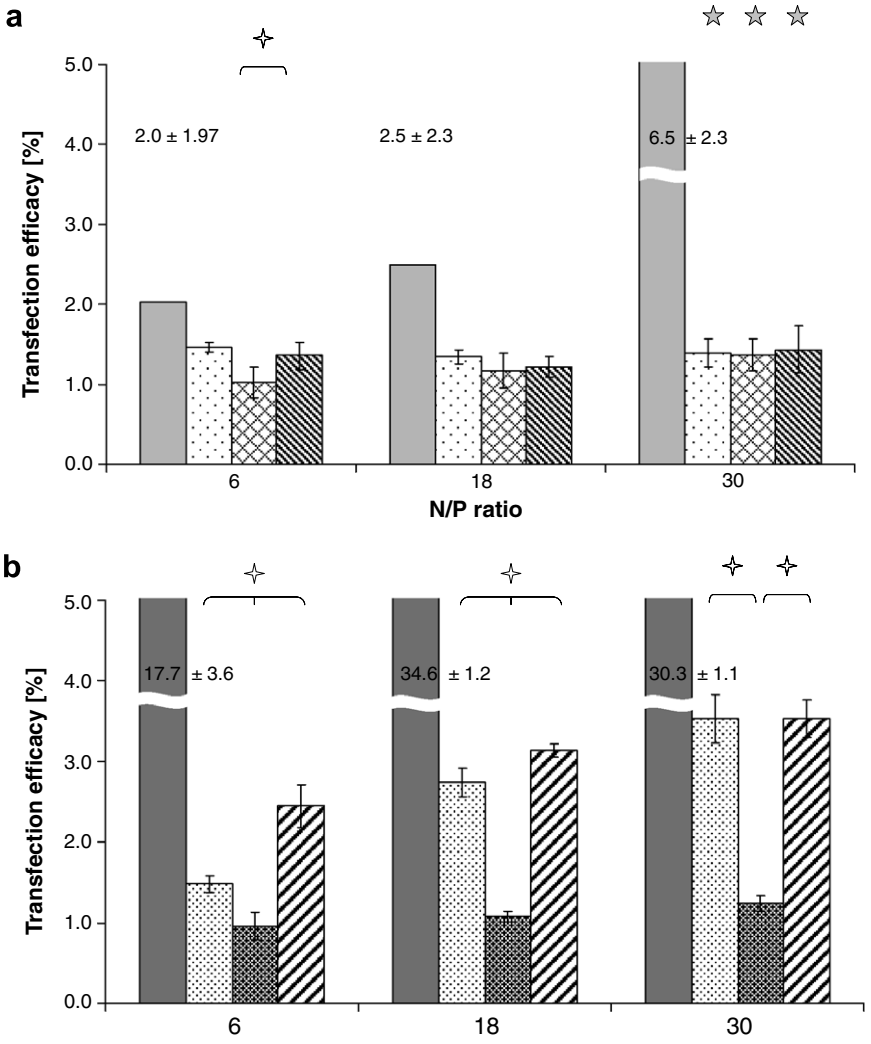


Fig. 10. The transfection efficacy of copolymer-based polyplexes was determined in HeLa cells after 4 hours incubation with: (a) lPEI2.6, mPEG5/3–lPEI2.6, mPEG20–lPEI2.6 or mPEG10/2–lPEI2.6 pDNA, as well as (b) lPEI4.6, mPEG5/3–lPEI4.6, mPEG20–lPEI4.6, or mPEG10/2–lPEI4.6–pDNA in serum-free cell culture medium. The polyplexes were prepared in 0.15 M NaCl at N/P ratios between 6 and 30 and the transfection efficacy was determined after 48 h by flow cytometry analysis. Data represented as mean value ± SD ($n = 3$); differences with significance of $p < 0.05$ are depicted by ✧.

It has been described that the charge-shielding effect of the PEG component often counteracts efficient pDNA complexation [6,7,17,19]. We have shown that, despite the dominating charge-shielding component, all of the synthesized copolymers condensed pDNA at moderate N/P ratios, resulting in the formation of nanoparticles between 150 and 420 nm (Figs. 1–3). In contrast to the unmodified lPEI–pDNA polyplexes, the copolymer-based particles did not show further particle growth in any dispersion medium tested during the entire time of measurement.

According to the literature [6,7,17,19], we observed that all mPEG–lPEI copolymers exhibited a reduced capacity to bind pDNA to the complex, compared to the unmodified polyamines [22,23] (Fig. 4). This effect was compensated by using the mPEG–lPEI derivatives based on the higher molecular weight lPEI (lPEI4.6) or elevated N/P ratios. Concomitantly, with a reduced pDNA complexation the stability of incorporated pDNA against the digestion by DNase I dropped (Fig. 5).

The zeta potential of copolymer-based polyplexes was remarkably reduced, compared to unmodified lPEI2.6- or lPEI4.6–pDNA polyplexes (lPEI2.6- and lPEI4.6–pDNA in 0.15 M NaCl: 28–32 mV, in 5% glucose at least 30 min of incubation in serum-free cell culture medium: 25–27 mV). We have shown that the dispersion medium had thereby a major impact on particle net charge, which ranged from slightly positive to slightly negative values (Fig. 5), an aspect that has not yet been addressed.

It is expected that due to the nearly neutral polyplex net charge, non-specific interactions of the complexes with negatively charged components at the cell surface are reduced, which in turn limits the non-specific internalization of polyplexes [28–30]. However, both CLSM images and flow cytometry analysis data (Figs. 7 and 8) suggest that the

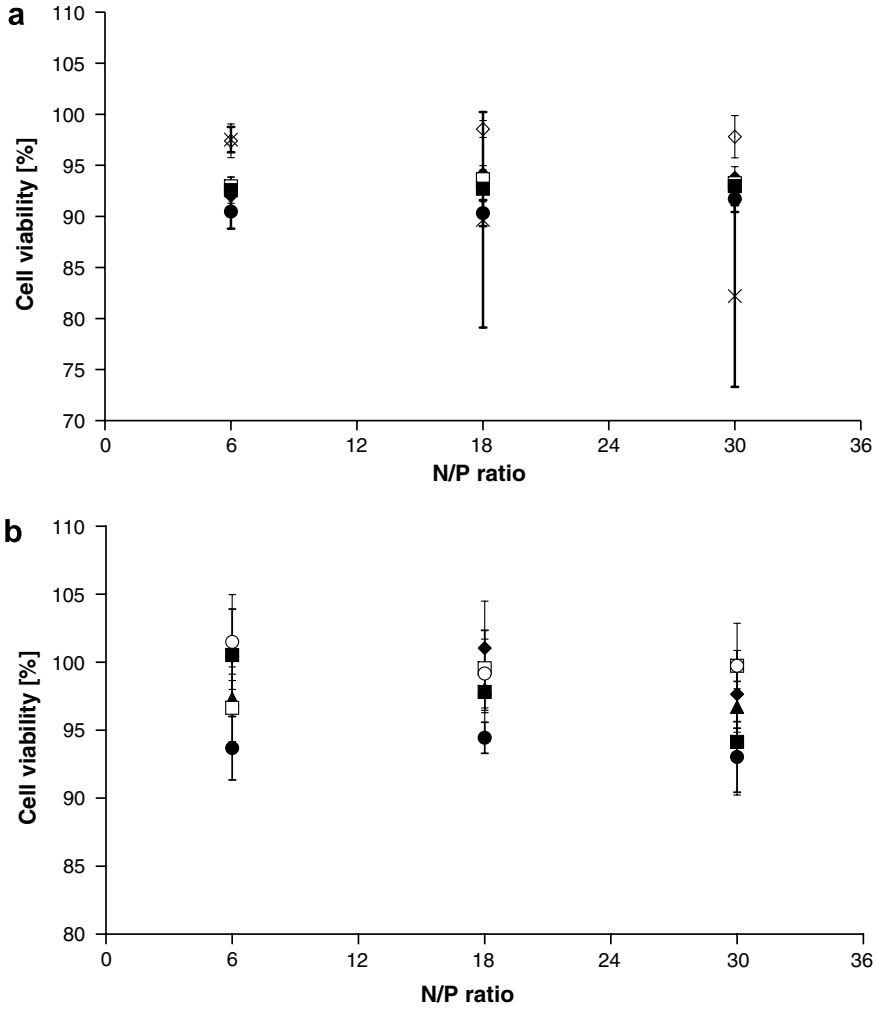


Fig. 11. lPEI2.6 (◇)-, lPEI4.6 (×)-, mPEG5/3–lPEI2.6 (◆)-, mPEG5/3–lPEI4.6 (■)-, mPEG20–lPEI2.6 (▲)-, mPEG20–lPEI4.6 (●)-, mPEG10/2–lPEI2.6 (□)-, and mPEG10/2–lPEI4.6–pDNA (○) prepared in 0.15 M NaCl at N/P ratios between 6 and 30. The polyplexes were added to CHO-K1 cells under serum-free conditions for 4 h and the relative cell viability was determined after 48 h by flow cytometry analysis. Data represented as mean value ± SD ($n = 3$). Differences with significance of $p < 0.05$ or $p < 0.01$ are depicted by ✧ and ☆, respectively.

inhibition of particle internalization was dependent on the copolymer used for pDNA compaction. The application of mPEG20–lPEI4.6, mPEG5/3–lPEI2.6, and mPEG10/2–lPEI2.6 reduced the number of internalized polyplexes, while mPEG5/3–lPEI4.6-, mPEG20–lPEI2.6- and mPEG10/2–lPEI4.6–pDNA showed an increase of particle uptake in comparison to unmodified lPEI–pDNA polyplexes. In contrast to the lPEI4.6–pDNA polyplexes, we did not observe the nuclear localization of PEG-shielded particles.

Irrespective of the extent of particle internalization, we obtained comparable levels of transgene expression with all applied copolymer-based polyplexes. The transfection efficacy was reduced by several orders of magnitude (Figs. 9 and 10) at a comparable or even increased cell viability compared to the unmodified lPEI–pDNA polyplexes (Fig. 11). This effect was documented in both the CHO-K1 and HeLa cell models and independent of the molecular weight of the lPEI residue and the copolymer architecture. In order to elucidate if PEGylation reduces endosomolytic activity compared to unmodified PEI, we determined the transfection efficacy in the presence and absence of saccharose or chloroquine as lysosomotropic agents (Fig. 12). We could show that the supplementation of saccharose or chloroquine did not enhance the transfection efficacy, suggesting that the release of polyplexes from endo/lysosomes was not limiting for gene transfer using the pegylated complexes.

5. Conclusion

We have shown that mPEG-derived copolymers based on non-toxic 2.6 and 4.6 kDa low molecular weight lPEI were still capable of compacting pDNA, resulting in the formation of small nanoparticles between 150 and 420 nm. Despite the different architecture of the copolymers, the corresponding mPEG–lPEI–pDNA polyplexes exhibited a comparable endosomolytic activity to unmodified lPEI–pDNA complexes, while the net charge and tendency to particle growth were remarkably reduced. The efficacy of

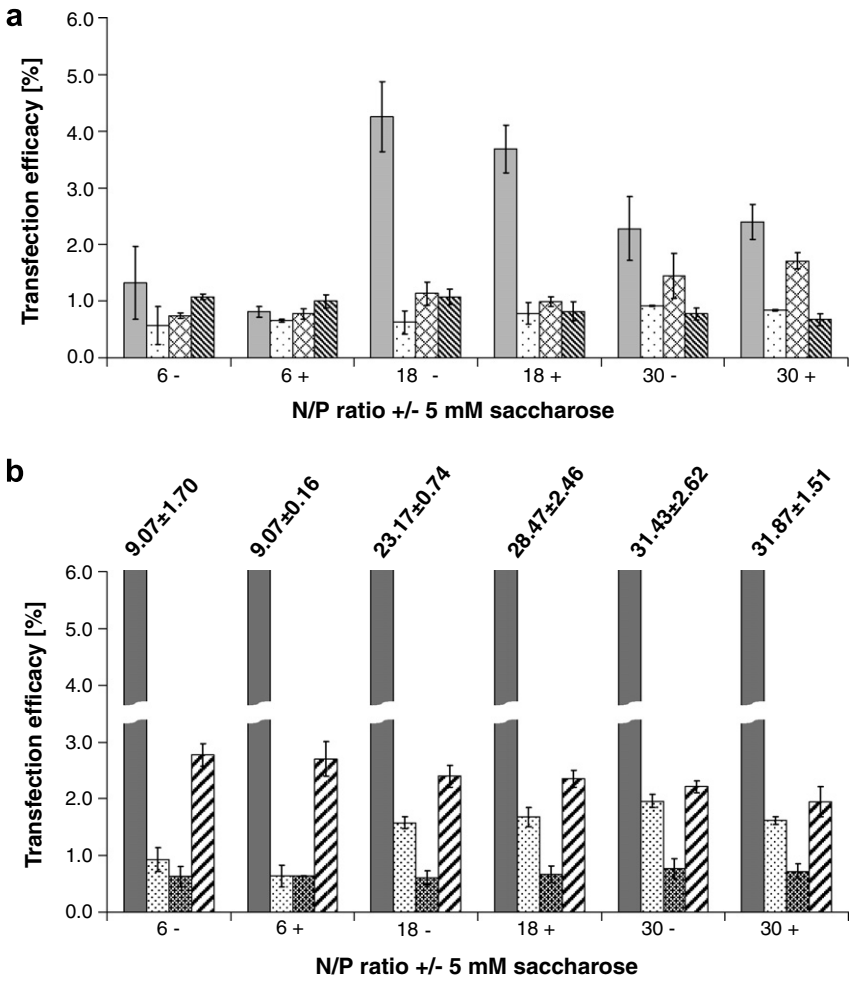


Fig. 12. The transfection of CHO-K1 cells was performed with (+) or without (−) the supplementation with a 5 mM saccharose solution using: (a) lPEI2.6, mPEG5/3–lPEI2.6, mPEG20–lPEI2.6, or mPEG10/2–lPEI2.6 pDNA, and (b) lPEI4.6, mPEG5/3–lPEI4.6, mPEG20–lPEI4.6, or mPEG10/2–lPEI4.6–pDNA polyplexes, prepared in 0.15 M NaCl at N/P ratios of 6, 18, and 30. The transfection efficacy was determined after 48 h by flow cytometry analysis. Data represented as mean value ± SD ($n = 3$).

pDNA retardation and pDNA stability against DNase I digestion decreased compared to the unmodified lPEIs.

Cell uptake was enhanced for polyplexes based on a long PEG chain linked to a comparably short lPEI residue, or several low molecular weight PEGs (5 kDa) tethered to lPEI4.6, conceivably due to an improved solubility of the complexes, while the polycationic residue still mediated internalization. These complexes failed to mediate transgene expression, however, most likely due to DNA digestion during their residence in endo/lysosomes and suppression of nuclear localization. It is yet unknown whether vector unpacking might be altered due to PEGylation, which also might derogate transfection, mediated by copolymer-based polyplexes.

The number of intracellular complexes was reduced when copolymers consisting of 3 to 4 PEG 5 kDa linked to lPEI2.6, or a high molecular PEG (20 kDa) tethered to lPEI4.6, were used for pDNA compaction, indicative of a sufficient suppression of non-specific interactions with cells. Further experiments with mPEG5/3–lPEI2.6-, mPEG10/2–lPEI2.6- and mPEG20–lPEI4.6–pDNA complexes would be necessary to investigate if the tagging of a targeting sequence to the complex can recover or even enhance the efficacy of transfection in comparison to unmodified lPEI–pDNA polyplexes, before an *in vivo* application can be considered.

Acknowledgement

We thank Allison Dennis from the Georgia Institute of Technology in Atlanta for the careful revision of this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2007.10.006.

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