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Amphiphilic and biodegradable hy-PEI-g-PCL-b-PEG copolymers efficiently mediate transgene expression depending on their graft density

Mengyao Zheng [1], Yu Liu [1], Olga Samsonova [1], Thomas Endres, Olivia Merkel, Thomas Kissel *

Department of Pharmaceutics and Biopharmacy, Philipps-Universität, Ketzerbach 63, D-35032 Marburg, Germany

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ABSTRACT

Novel biodegradable amphiphilic copolymers hy-PEI-g-PCL-b-PEG were prepared by grafting PCL-b-PEG chains onto hyper-branched poly(ethylene imine) as non-viral gene delivery vectors. Our investigations focused on the influence of graft densities of PCL-b-PEG chains on physico-chemical properties, DNA complexation and transfection efficiency. We found that the transfection efficiencies of these polymers increased at first towards an optimal graft density ($n = 3$) and then decreased. The buffer-capacity-test showed almost exactly the same tendency as transfection efficiency. Cytotoxicity (MTT-assay) depended on the cooperation of PEG molecular weight and graft density of PCL-b-PEG chains. With increasing the graft density, cytotoxicity, zeta-potential, affinity with DNA, stability of the polyplexes and CMC-values were reduced strongly and regularly. Increasing the excess of polymer over DNA was shown to result in a decrease of the observed particle size to 100–200 nm.

1. Introduction

Thanks to advances in molecular biology and genomic research, numerous diseases have been given a genetic identity for which gene therapy may provide a possible treatment (Pawliuk et al., 2001; von Laer et al., 2006; Wong et al., 2006). For viral gene delivery techniques, viruses can be transformed into gene-delivery vehicles by replacing parts of the genome of a virus with a therapeutic gene (Pack et al., 2005). But viral vectors could induce cancer (Check, 2002; Hacein-Bey-Abina et al., 2003), and initiate an immunogenic response which has led to a fatal outcome (Marshall, 2000). Instead of viral gene delivery techniques, non-viral gene delivery techniques have been investigated, which are considered as a safer, less pathogenic and immunogenic gene delivery alternative (Wong et al., 2007). Studies demonstrated that polymeric vectors might be advantageous over viruses and liposomes (Merdan et al., 2002) for gene delivery, regarding safety, immunogenicity, mutagenicity and production costs (Han et al., 2000).

Hyper-branched polyethylenimine (hy-PEI) is one of the most successful cationic polymers for gene delivery both in vitro and in vivo (Boussif et al., 1995; Godbey et al., 1999). hyPEI can condense DNA via electrostatic interactions to particles with sizes compatible with cellular uptake while providing steric protection from nuclease degradation (Bielinska et al., 1997). hyPEI has a unique property of endosomal buffering capacity due to protonable amino groups. This function causes osmotic swelling and subsequent endosomal disruption (Boussif et al., 1995), thus permitting the escape of endocytosed materials. However, PEI has a relatively high cytotoxicity due to the high density of cationic groups, especially at high molecular weights (Fischer et al., 1999; Kunath et al., 2003). PEI–PEG copolymers are less toxic than PEI (Sung et al., 2003). However they are not able to condense DNA into small particles less than 200 nm, which is critical for cell uptake and transfection efficiency (Park et al., 2005; Pun et al., 2004). Another drawback of PEI–PEG is the non-biodegradable bonds between PEI and PEG chains. Polymers that are not eliminated from the circulation may accumulate in tissues and cells. To resolve this problem, biodegradable hy-PEI-g-$(PCL\text{-}b\text{-}PEG)_n$ copolymers were synthesized (Shuai et al., 2003). Poly(caprolactone) (PCL) acts as a linker between PEI and PEG to increase the biodegradability of the copolymers. Hydrophobic PCL can additionally positively affect the hydrophilic–hydrophobic balance of the polymer and thus enhance the uptake of the complexes through the cell membranes. Another advantage of PCL is the shielding of the positive charges and the condensation of the DNA into small complexes of 100 nm (Han et al., 2001; Kono et al., 2005; Tian et al., 2007; Wang et al., 2002).

The copolymers hy-PEI-g-$(PCL\text{-}b\text{-}PEG)_n$ have been studied so far as potential gene delivery systems (Liu et al., 2009; Shuai et al., 2003). These investigations were limited to the discussion of the

* Corresponding author. Tel.: +49 6421 282 5881; fax: +49 6421 282 7016.
E-mail address: kissel@staff.uni-marburg.de (T. Kissel).
[1] These authors contributed equally.

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influence of PEI, PCL and PEG chain lengths. Nevertheless, the influence of graft density has not been studied systematically. In this report, a panel of hyPEI25K-(PCL570-PEG5k)$_n$ block copolymers was synthesized to elucidate the effect of graft density on physicochemical properties, DNA complexation and biological activities as non-viral gene delivery vectors. It is commonly believed that the molecular weight of PEI most suitable for gene transfer ranges between 5 and 25 kDa. Higher molecular weights lead to increased cytotoxicity (Fischer et al., 2003), presumably due to aggregation to huge clusters of the cationic polymer on the outer cell membrane, which thereby induce necrosis (Freshney, 2005). To study their physicochemical properties and complexation with DNA, measurements of size and zeta-potential, heparin-assay, critical micelle concentration (CMC) and buffer-capacity were performed. The physico-chemical properties of these complexes were then compared with in vitro results of cytotoxicity tests (MTT), confocal laser scanning microscopy (CLSM) and transfection experiments. These results provide a basis for the rational design of block copolymers as gene delivery systems.

2. Materials and methods

2.1. Materials

hy-PEI with molecular weight of 25 kDa was obtained from BASF. Poly(ethylene glycol) mono-methyl ether (mPEG) (5 kDa) and ε-caprolactone were purchased from Fluka (Taufkirchen, Germany), and all other chemicals were obtained from Sigma–Aldrich (Steinheim, Germany). hy-PEI–PCL–mPEG was synthesized as reported previously (Liu et al., 2009). DNA from herring testes (Type XIV, 0.3–6.6 MDa, 400–10 000 bp) was from bought from Sigma (Steinheim, Germany), and Luciferase-Plasmid (pCMV-Luc) (Lot No.: PF461-090623) was amplified by The Plasmid Factory (Bielefeld, Germany).

2.2. Polyplex formation

All complexes of DNA and polymer were prepared freshly before use. Luciferase-Plasmid and DNA from herring testes were stored at −20 °C. Before use, 5% glucose solution and other buffer solutions were filtered freshly through 0.20 μm pore sized filters (Nalgene® syringe filter, Sigma–Aldrich, Taufkirchen, Germany). The volume of a 1 mg/mL (based on hyPEI25k) polymer stock solution required for a certain N/P ratio (=nitrogen/phosphorus-ratios) was calculated as follows (Liu et al., 2009):

$$V_{DNA} = \frac{(C_{copolymer} \times V_{copolymer} \times 330)}{(C_{DNA} \times 43 \times N/P)}$$

where $C_{copolymer}$ = the concentration of the stock copolymer, C_{DNA} = the concentration of the stock DNA solution.

A certain amount of polymer stock solution was diluted with 5% glucose solution or other buffer solutions to a final volume of 50 μL, which was mixed with an equal volume of diluted DNA aliquots in microcentrifuge tubes by pipetting (IKA, Stauffen), and incubated for 20 min before use for complex and equilibrium formation.

2.3. Cell culture

Cells were seeded at a density of 3.5×10^3 cells/cm^2 in dishes (10 cm diameter, Nunclon Dishes, Nunc, Wiesbaden, Germany) and incubated at 37 °C in humidified 5% CO_2 atmosphere (CO_2-Incubator, Integra Biosciences, Fernwald, Germany). Medium (Dulbecco's modified Eagle's medium, supplemented with 10% serum) was exchanged every 2 days. Cells were split after 7 days, when confluence was reached.

2.4. Measurement of size and zeta-potential

Three buffer-solutions (5% glucose, pH 6.6; 10 mM TE-buffer, pH 9.0; 15 mM acetate-buffer, pH 5.5) were used for the measurement of size and zeta-potential, which were monitored with a Malvern Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK) at 25 °C. The measurement angle was 173° in backscatter mode. Following size measurements, zeta-potential measurements were performed with the same samples after diluting 50 μL of polyplexes with additional 500 μL of buffer solution to a final volume of 550 μL with a DNA concentration of 1.82 ng/μL. Three samples were prepared for each N/P-ratio and three measurements were performed on each sample. Each measurement of size consisted of 15 runs of 10 s. Each measurement of zeta-potential consisted of 15–100 runs, which was set to automatic optimization by the software.

2.5. Buffer-capacity

Titration studies were performed to determine the buffer-capacity of the studied polymers. Therefore, 0.4 mL of aqueous polymer solution with the concentration of 2.5 mg/mL (based on PEI) was titrated with standard 0.1 N HCl, until the pH of the polymer solutions decreased nearby pH = 2.5. The pH-value was detected with a pH-meter (Hanna PH210 Microprocessor pH Meter) and an electrode (Inlab, Mettler Toledo, Schwerzenbach, Switzerland) at 25 °C.

2.6. Critical micelle concentration (CMC)

In this experiment, the surface tension with increased polymer concentration in water was measured using a tensiometer (Krüss Tensionmeter Control Panel; K11-MK3) at 25 °C. Data correlation with the polymer structure was performed using "Origin 7.0" (Origin-Labsoftware, Northampton, USA).

2.7. Heparin-assay

The effect of heparin on the stability of complexes was evaluated by means of the change in fluorescence intensity obtained with the fluorescent intercalating probe sybr-gold (Creusat et al., 2010). To study the effect of pH on the stability of complexes, polyplexes were prepared in solutions with different pH and ionic strength. Heparin solution (150 000 IE/g, Serva, Pharm., USPXV2, Merck, Darmstadt, Germany) was diluted to a concentration of 0.521 mg/mL. Increasing amounts (0–17.5 μL) of heparin were added to the 96-well plate (Perkin Elmer, Rodgau-Jügesheim) where each well contained 200 μL of polymer/HT-DNA-complexes at N/P 10. Subsequently, 20 μL diluted sybr-gold-solution (Invitrogen, Karlsruhe, Germany) were added. After 20 min of incubation at 25 °C, fluorescence was directly detected with a fluorescence plate reader (BMG Labtech GMBH, Offenburg, Germany) at 495 nm excitation and 537 nm emission.

2.8. MTT-assay

In vitro cytotoxicity tests of the copolymers were performed by MTT-assays. Pure polymers were selected instead of DNA polyplexes to measure the cytotoxicity in a "worst case scenario" since it has been reported that the cytotoxicity was reduced when polymers were complexed with DNA (Godbey et al., 1999). The assays were performed as previously described (Liu et al., 2009). Briefly, L929 cells were seeded in 96-well cell culture-coated microtiter plates at the density of 8000 cells/well and incubated in DMEM low glucose (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (Cytogen, Sinn, Germany) in humidified atmosphere

with 5% CO_2 at 37 °C for 24 h prior to the treatment with polymer solutions of increasing concentration (from 9.77E−4 mg/mL to 0.5 mg/mL). After 24 h, the medium was replaced with 200 μL serum free medium and 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich, Germany) reaching a final concentration of 0.5 mg MTT/mL. Cells were incubated for another 4 h before 200 μL of dimethylsulfoxide (DMSO) was added to dissolve the purple formazane product. The absorption was quantified using a plate reader (Titertek plus MS 212, ICN, Germany) at wavelengths of 570 nm and 690 nm. The IC_{50} was calculated as the polymer concentration which inhibits growth of 50% of cells relative to non-treated control cells.

2.9. Confocal laser scanning microscopy (CLSM)

MeWo-cells were seeded in 8 well-chamberslides (Lab-Tek; Rochester, NY, USA) at 50,000 cells/well and incubated for 24 h in DMEM high glucose (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (Cytogen, Sinn, Germany) in humidified atmosphere with 5% CO_2 at 37 °C. The DNA labeling steps were performed at room temperature and in the dark to protect fluorescent markers. YOYO-1 stock solution (Invitrogen, Karlsruhe, Germany) was diluted 50-fold with TE-buffer and 30 μL of DAPI stock solution (6 μg/mL, Molecular Probes, Eugene, OR, USA) was diluted with 1 mL PBS. Plasmid-DNA (pDNA) was incubated with YOYO-1 solution for 30 min at a weight ratio of 1:15. Complexes of YOYO-1-labeled pDNA were formed as usual by incubation with polymer solution at N/P 15 in 5% glucose solution for 20 min followed by addition of 25 μL of polyplex solution, containing 0.5 μg plasmid-DNA, to 375 μL fresh culture medium with 10% FCS in each well. After incubation for 4 h, each chamber was washed twice with 0.5 mL PBS, and the cells were then fixed by incubating each chamber for 20 min with 0.5 mL of 4% paraformaldehyde in PBS (4% PFA). Subsequently, 100 μL DAPI-solution was added per chamber and incubated for another 20 min in the dark. The cells were washed three times with 0.5 mL PBS before being fixed with Fluorsafe (Calbiochem, San Diego, USA) and covered with a No. 1.5 thickness cover slip (Menzel Gläser, Braunschweig, Germany). YOYO-1 labeled DNA was excited with a 488 nm argon laser, while DAPI-stained chromosomal DNA was excited with an enterprise laser with an excitation wavelength of 364 nm, and CLSM was performed by using a 385 nm long pass filter and a band-pass filter of 505–530 nm in the single-track mode (Axiovert 100 M and CLSM 510 Scanning Device; Zeiss, Oberkochem, Germany).

2.10. In vitro transfection experiments with DNA

MeWo-cells were seeded in 48-well plates (Nunc, Wiesbaden, Germany) at the density of 60,000 cells/well (0.4 mL medium/well) 24 h before transfection. On the day of transfection, 175 μL medium (containing 10% serum) plus 25 μL polymer/DNA-complex were placed in each well (containing 0.5 μg pDNA per well). Polymer/DNA-complexes were prepared at different N/P-ratios. After 4 h of incubation at 37.0 °C, in humidified atmosphere with 5% CO_2, the medium was replaced with fresh medium containing 10% serum. Luciferase activity was assayed 44 h after transfection. Cells were lysed in 100 μL cell culture lysis buffer (Promega, Mannheim, Germany) for 15 min at 25 °C. Luciferase activity was quantified by injection of 50 μL luciferase-assay-buffer, containing 10 mM luciferin (Sigma–Aldrich, Taufkirchen, Germany), to 25 μL of the cell lysate. The relative light units (RLU) were measured with a plate luminometer (LumiSTAR Optima, BMG Labtech GmbH, Offenburg, Germany). Protein concentration was determined using a Bradford BCA assay (BioRad, Munich, Germany).

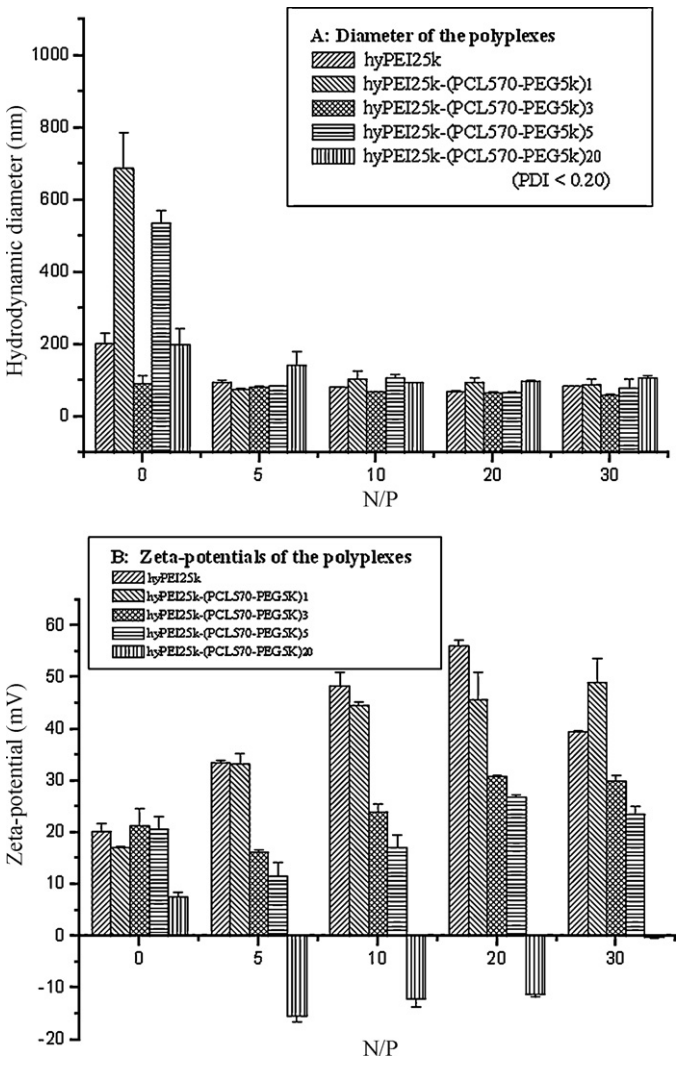


Fig. 1. (A) Diameter of the polyplexes in 5% glucose solution at different N/P ratios. N/P 0 = pure polymers in solution. (B) Zeta potentials of the polyplexes in 5% glucose solution at different N/P ratios.

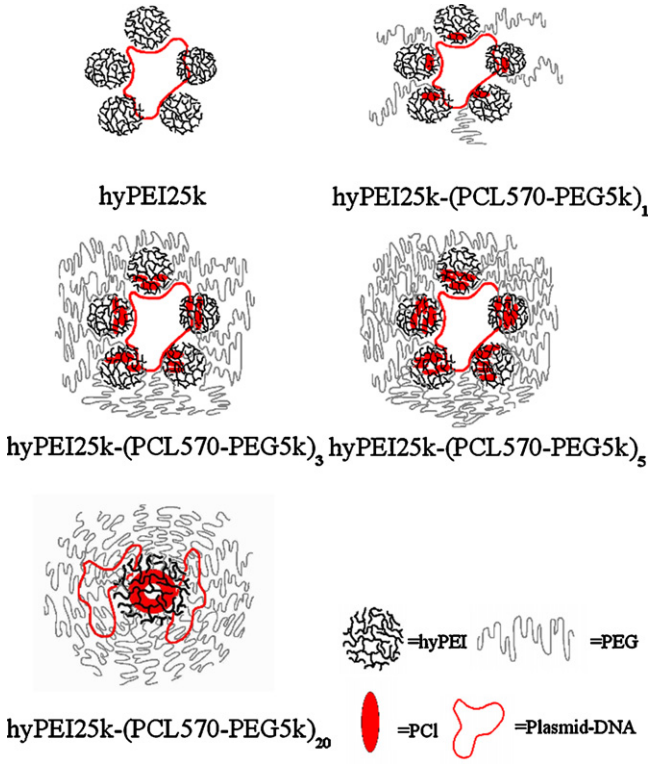
2.11. Statistics

All analytical assays were conducted in replicates of three or four, as indicated. Results are given as mean values ± standard deviation (SD). Two way ANOVA and statistical evaluations were performed using Graph Pad Prism 4.03 (Graph Pad Software, La Jolla, USA).

3. Results and discussion

3.1. Influence of polymer structure on the size and zeta-potential of polyplexes

All copolymers were able to condense DNA into particles with sizes of 100–200 nm (Fig. 1B). No obvious size decrease was observed in 5% glucose when N/P ratio increased from 5 to 30. Under high ionic strength conditions (10 mM TE-buffer, pH 9.0; 15 mM acetate-buffer, pH 5.5), all copolymers formed larger complexes as compared to 5% glucose solution (Table SM 1). This result can be explained by the shielding-effect of glucose molecules (Petersen et al., 2002). On the other hand, the polyplexes can aggregate in the buffer-solutions due to the reduced surface charge and lack of repulsion of the polymers (Petersen et al., 2002). The results of size



Scheme 1.

measurements yielded comparable values as recently reported (Liu et al., 2009). To investigate the influence of the degree of modification on the protonation of PEI, three buffer solutions with three different pH values (5.5, 7.0 and 9.0) were chosen. Due to the acidification in the endo-lysosomal compartment, PEI complexes undergo pH changes during the transfection process. The influence of these changes was evaluated by comparing trends over a broad pH range.

The zeta-potential was reduced with an increasing number *n* of PCL570-PEG5k segments, and was always highest in non-buffered glucose solution (Fig. 1A and Table SM 1). All polyplexes except hyPEI25k-(PCL570-PEG5k)$_{20}$ showed a positively charged surface at all buffer conditions. Due to the high graft density of hyPEI25k-(PCL570-PEG5k)$_{20}$, the polymer loses its ability to condense DNA into a stable polyplexes as most positive charges of hyPEI are shielded, resulting in negatively charged polyplexes. It is assumed that the DNA remains only on the surface of this polymer (Scheme 1).

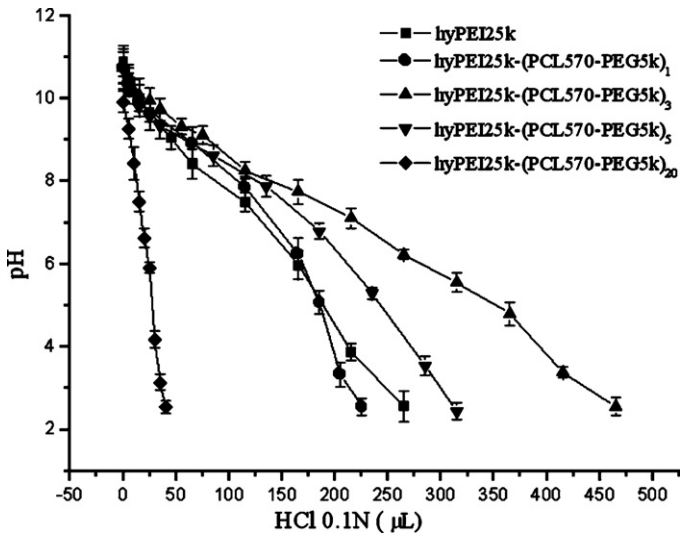


Fig. 2. Titration curve of aqueous polymer solution with standard 0.1 N HCl.

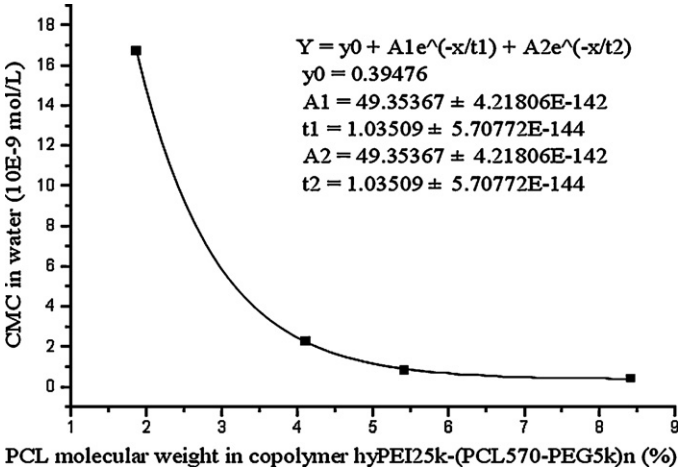


Fig. 3. The CMC-values decreased with the increase of the percentage of PCL molecular weight in the copolymers.

Interestingly, no further increase of the zeta-potential was observed when increasing the N/P ratio above 20, except for the polymer with graft density of 20. Therefore we hypothesize that for polymers with a graft density ranged between 1 and 5 at N/P 20 a polymer concentration is reached above which the additional polymer does not contribute to the condensation of DNA but is rather present as free polymer.

3.2. Influence of polymer structure on the buffer-capacity

The most commonly used pH-sensitive excipients for gene delivery that exhibit the so-called "proton-sponge effect" are polymers such as PEI with protonable amino groups with $5 < pK_a < 7$ (Behr, 1997). The polymers with high buffer-capacity increase the ion concentration in the endosome and ultimately cause osmotic swelling and rupture of the endosome membrane, which releases the polyplexes into the cytosol. Fig. 2 shows the buffer-capacity of the polymers which decreased in the following order: hyPEI25k-(PCL570-PEG5k)$_3$ > hyPEI25k-(PCL570-PEG5k)$_5$ > hyPEI25K-(PCL570-PEG5k)$_1$ = hyPEI25k > hyPEI25k-(PCL570-PEG5k)$_{20}$. With these results, it was shown that a low grafting degree of PEI with PCL–PEG segments can increase the accessibility of amines to be protonated, thereby increasing the buffer capacity. Thus, stability of the complexes could additionally be increased.

3.3. Influence of polymer structure on the CMC

The critical micelle concentration (CMC) is defined as the concentration where the interfacial tension reached a minimum. It is extremely valuable not only to predict the micelle-forming capac-

Table 1
CMC-values in water.

polymer	CMC in water (pH 7.0) (10E−9 mol/L)
hyPEI25k	None
hyPEI25k-(PCL570-PEG5k)$_1$	16.76 ± 0.14
hyPEI25k-(PCL570-PEG5k)$_3$	2.30 ± 0.11
hyPEI25k-(PCL570-PEG5k)$_5$	0.87 ± 0.09
hyPEI25k-(PCL570-PEG5k)$_{20}$	0.46 ± 0.02

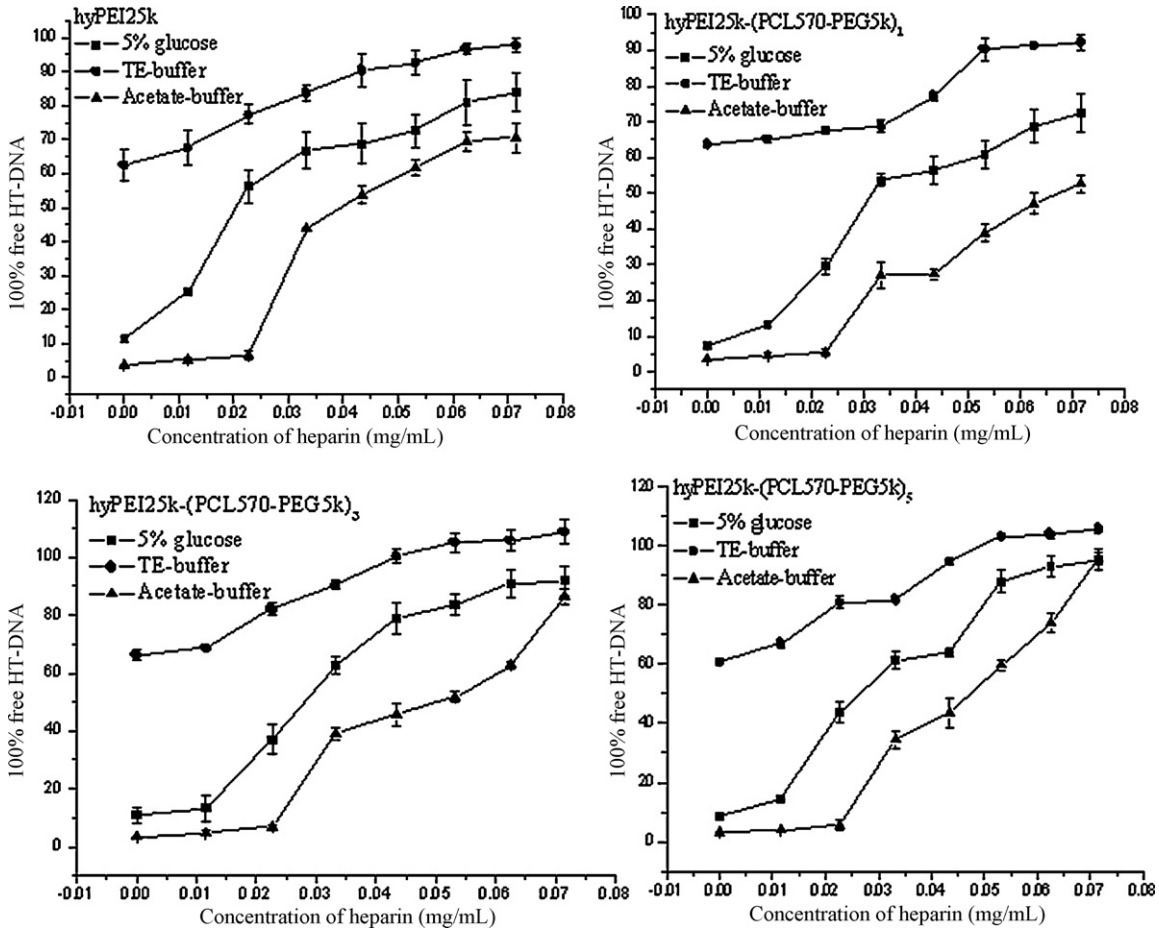


Fig. 4. DNA dissociation from complexes by heparin competition in different buffers.

ity but also to determine the stability of the polymeric micelles, which was believed to play a crucial role in DNA transfection. In general, all amphiphilic polymers were able to form micelles at low concentrations (Table 1). For the copolymers with graft densities of 5 and 20, the CMC was reached at lower concentrations ($8.7 \times E{-}10$ mol/L and $4.6 \times E{-}10$ mol/L) in water. The results of CMC measurements in water were expected to be a function of the PCL molecular weight. Generally, increasing the amounts of hydrophobic segments decreases the CMC of copolymers. This principle could clearly be observed in our study where CMC-values decreased exponentially with the increase of PCL molecular weight, as shown in Fig. 3. The tendency towards aggregation may also be affected by the presence of shielding components, for example glucose molecules or PEG chains in the copolymers, which may also decrease interactions between individual complexes as well as interactions between complexes and blood components in the systemic circulation (Petersen et al., 2002). Besides the shielding-effects of the PEG–PCL chains, the faster degradation of the polymers with higher graft density can also change the CMC tendency in base (Liu et al., 2010).

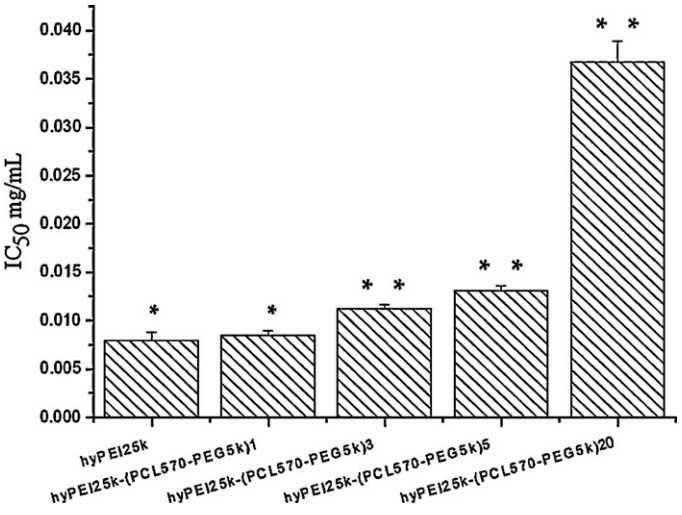


Fig. 5. IC_{50}-values of each polymer as determined by MTT-assays in L929-cells.

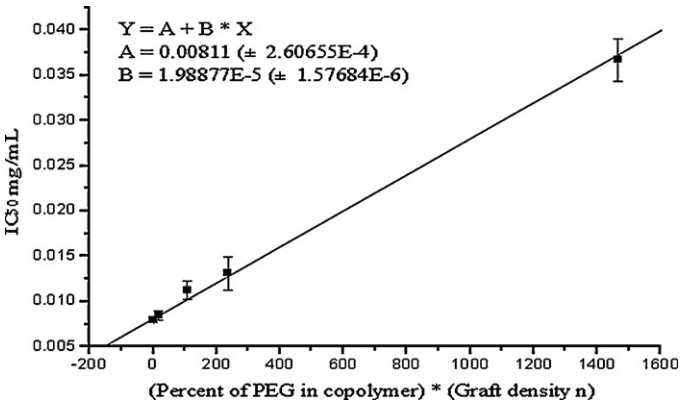


Fig. 6. PEG molecular weight and graft density of PCL–PEG chains both influence the cytotoxicity.

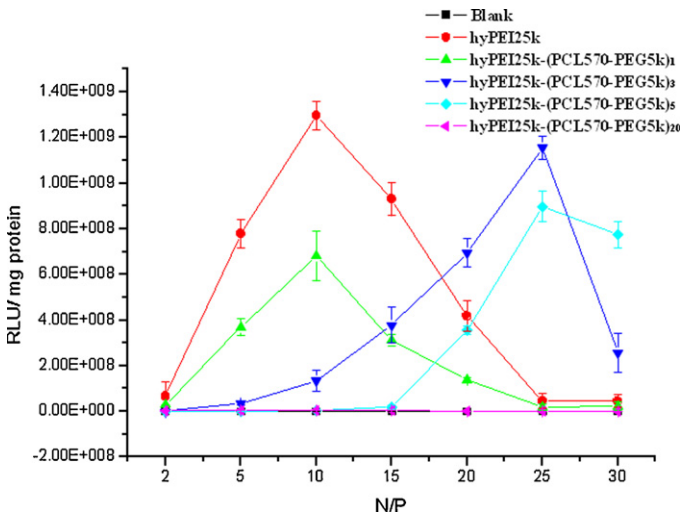


Fig. 7. Result of transfection of MeWo-cells in presence of serum in 48 well-plates.

3.4. Influence of polymer structure on the stability of polymer–DNA-complexes

Polymer/DNA-complexes, which are built due to electrostatic interaction, can be dissociated easily with the competing polyanion heparin (Moret et al., 2001). The release of DNA from complexes in the presence of heparin is summarized in Fig. SM 1. The dissociation profiles all exhibit significant dependency on the heparin concentration. Another general trend is that all polymers display more stable complexes in acetate buffer as compared to other solutions. Interestingly, hy-PEI25k-(PCL570-mPEG5k)$_1$, released only 50% of the DNA load at 0.075 mg/mL heparin, the highest concentration tested. However, unstable complexes were obtained in TE buffer for all polymers. This can be explained by the fact that the amino groups in the polymers were protonated under acidic conditions, while deprotonated in TE buffer. Comparing the diagrams in Fig. 4, no obvious difference was observed amongst the polymers in 5% glucose and TE buffer, while the stability of complexes was increased with decreasing PEG–PCL-grafting in acetate buffer at low heparin concentrations. This may be caused by the shielding-effect of PCL–mPEG and a resulting inaccessibility for heparin. However, a very high grafting degree as found in polymer hy-PEI25k-(PCL570-mPEG5k)$_{20}$, leads to decreased interaction of PEI with DNA and thus to less stable complexes. We found that the affinity between hyPEI25k-(PCL570-PEG5k)$_{20}$ and DNA is very weak and that this polymer therefore only hardly protects DNA. As shown by result of gel electrophoresis (Fig. SM 1), only 21% DNA can be condensed at N/P10. The structure of the copolymer hyPEI25K-g-(PCL570-b-PEG5k)$_{20}$ is very different from all the other polymers studied here. With a graft density of 20, 73.3% of the molecular weight of this polymer is PEG, and only 18.3% is hyPEI25K. Due to the large part of PCL–PEG segments, most of the positive charges of hyPEI are shielded, and this copolymer is assumed to form micelles by itself. Therefore, the topology of its polyplexes is different, the complexes are negatively charged and the polymer loses the ability to condense DNA into stable polyplexes. Although our data emphasize the importance of protonation of the polymer for stable interaction with DNA, a certain amount of lipophilic segments can even increase the stability as shown by hyPEI25k-(PCL570-PEG5k)$_1$. Indeed, slightly reduced complexation ability of copolymers as found for hyPEI25K-g-(PCL570-b-PEG5k)$_1$ may additionally facilitate the unpacking of the vector inside the cell, and a balance between DNA-complexation and DNA-release is necessary. Many reports described similar observations of increased transfection efficiency with reduction of positive charges (Banaszczyk et al., 1999; Schaffer et al., 2000).

3.5. Influence of polymer structure on the cytotoxicity

To evaluate the cytotoxicity of hy-PEI-g-PCL-b-mPEG copolymers in L929 cells, MTT-assays were performed. Considering the IC_{50} values, the cytotoxicity was clearly reduced with increasing of the graft density of the PCL570-PEG5k segments (Fig. 5). PEG is very hydrophilic and considered to be safe by the FDA (Sung et al., 2003). By grafting PEG onto PEI, the toxicity of hyPEI25k is known to be reduced (Petersen et al., 2002). At the same time, the PCL segment is hydrophobic and shields the positive charges from hyPEI25K. On the other hand, the addition of PCL-segments increases also the degradation of the copolymers (Liu et al., 2010). It was interestingly found in Fig. 6 that the IC_{50}-values increased proportional as a function of the parameter: (percent of PEG molecular weight in copolymer) × (graft density n). This result provides a basis for the rational design of block copolymer with low cytotoxicity. But on the other hand, with the decrease of positive charges on the polymers, the stability of the polymer/DNA-complex and the interaction with negatively charged cell membranes can be reduced. It was therefore hypothesized that a low graft density would be advantageous for transfection.

3.6. Transfection experiments with plasmid-DNA

From Fig. 7, we found that the transfection efficiencies of these polymers increased at first towards an optimal graft density (n = 3) and then decreased. hyPEI25k and hyPEI25k-(PCL570-PEG5k)$_1$ showed the same tendency of transfection efficiency: the highest transfection was reached at the N/P-ratio of 10, but the absolute transfection efficiency of hyPEI25k-(PCL570-PEG5k)$_1$ was still lower than that of hyPEI25k due to the fact that higher concentrations of copolymers are needed for stable complexes with copolymers. Polymers with graft densities of 3 and 5 also showed comparable tendencies of transfection efficiency: the best N/P-ratio of transfection was 25. In case of N/P 25, the polymer with graft density 3 showed a 25.4 fold higher transfection efficiency than hyPEI25k. Due to the decrease of toxicity with increasing graft density, the optimal N/P-ratio was shifted for hyPEI25k-(PCL570-PEG5k)$_3$ and hyPEI25k-(PCL570-PEG5k)$_5$. This shift additionally led to the hypothesis that polymers with little PEI content exhibit low transfection efficiency at low N/P-ratios but higher efficiencies than PEI at high N/P ratios. These properties rendered the two polymers with grafting densities of 3 and 5 promising candidates for in vivo transfection. However, polymer hyPEI25k-(PCL570-PEG5k)$_{20}$ was, despite very low toxicity, always inefficient, and no obvious transfection was registered at any N/P-ratio. If the low transfection efficiency of hyPEI25k-(PCL570-PEG5k)$_{20}$ was due to the low buffer capacity described above, a treatment with chloroquine could increase the endosomal escape, and also the transfection of the polyplexes (Luthman and Magnusson, 1983). During the 4 h of incubation with the hyPEI25k-(PCL570-PEG5k)$_{20}$/DNA-complex, the cells were treated with 50 μM, 100 μM, and 150 μM chloroquine. However, no increase in transfection efficiency was observed (data not shown). The low transfection efficiency of hyPEI25k-(PCL570-PEG5k)$_{20}$ can, however, be explained mainly by the negative charged surface of hyPEI25k-(PCL570-PEG5k)$_{20}$/DNA-complexes. The buffer capacity did in fact play an important role for complexes that were efficiently taken up. Copolymer hyPEI25k-(PCL570-PEG5k)$_3$ displayed a much higher buffer capacity than hyPEI25k or the other hyPEI-polymers, and the tendency of the buffer-capacity profiles was exactly the same as the one observed for transfection efficiency. These results are comparable with those of other authors. For instance, Jong et al. reported that polymers possessing

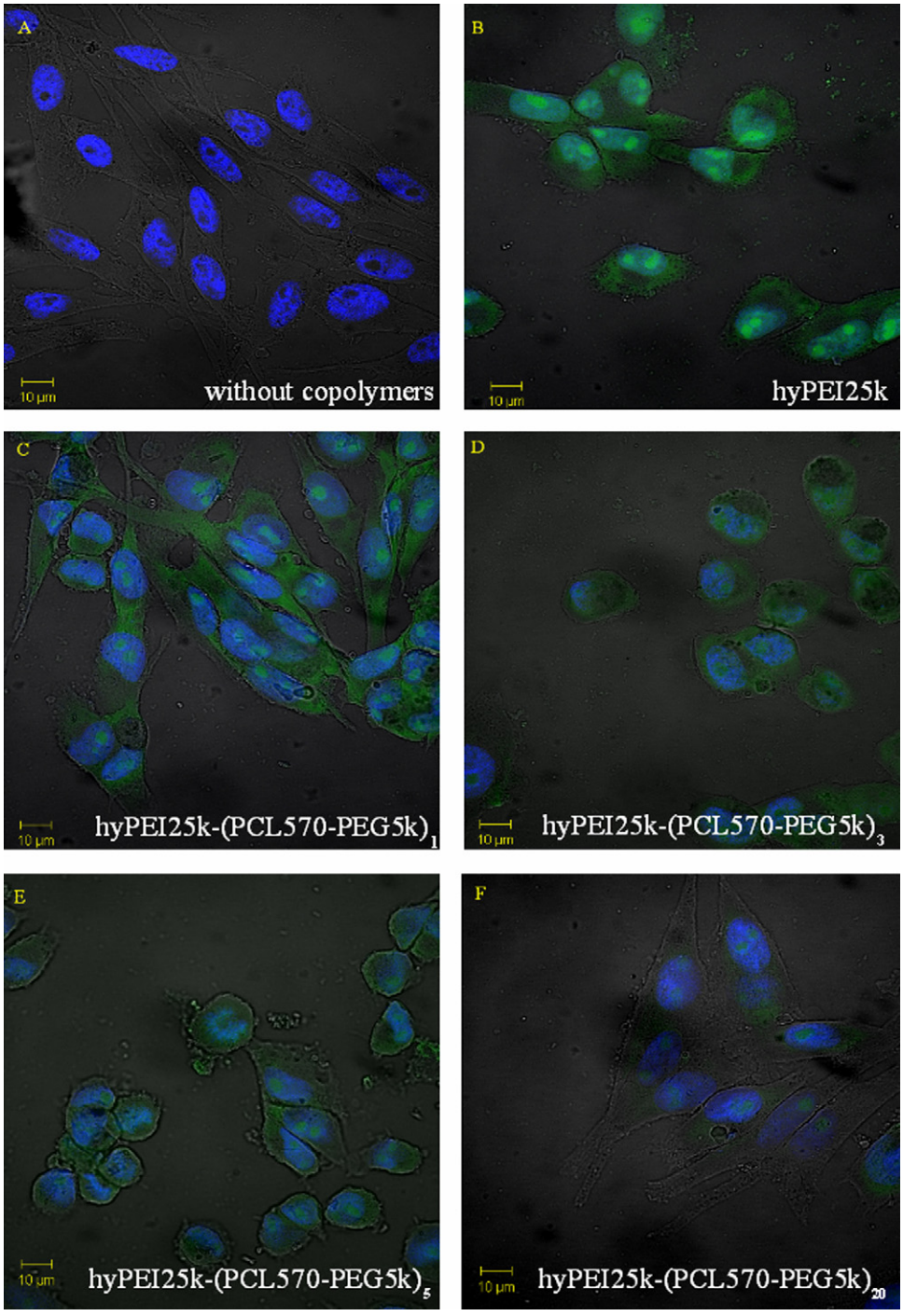


Fig. 8. Results of confocal laser scanning microscopy with MeWo-cells. (A) Only MeWo-cells, without treatment with copolyplexes (blue: nuclear; green: plasmid-DNA) and cellular uptake of (B). pDNA complexed by polymer hyPEI25k, (C) pDNA/hyPEI25k-(PCL570-PEG5k)$_1$ complexes, (D) pDNA/hyPEI25k-(PCL570-PEG5k)$_3$ complexes, (E) pDNA/hyPEI25k-(PCL570-PEG5k)$_5$ complexes, and (F) pDNA/hyPEI25k-(PCL570-PEG5k)$_{20}$ complexes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

better buffering capacity yield higher transfection efficiency (Jong, 2009).

3.7. Confocal laser scanning microscopy (CLSM)

The CLSM-micrographs showed a clear trend of the cellular uptake efficiency from hyPEI25k to hyPEI25k-(PCL570-PEG5k)$_{20}$ (Fig. 8). hyPEI25k clearly yielded more uptake of plasmid-DNA into the nucleus, the site of action, than into the cytosol. Cellular uptake of plasmid-DNA complexed by hyPEI25k-(PCL570-PEG5k)$_1$ was also clearly observed, but the fluorescence intensity of plasmid-DNA in nucleus was not as strong as observed with hyPEI25k. In case of hyPEI25Kk-(PCL570-PEG5k)$_3$ and hyPEI25k-(PCL570-PEG5K)$_5$, the most pDNA remained in the cytosol, and only a low amount of pDNA could enter into the nucleus. After transfection with hyPEI25k-(PCL570-PEG5k)$_{20}$, only very weak fluorescence of the pDNA was observed. We can conclude that the cell uptake was reduced clearly with an increasing number n of PCL570-

PEG5k segments. This tendency of cell uptake agreed perfectly with the results of zeta-potential measurement. Due to their positive zeta-potentials, polyplexes could easily enter the cells. And the negatively charged polyplexes hardly entered the cells, as shown with hyPEI25k-(PCL570-PEG5k)$_{20}$/DNA-complexes (Wong et al., 2007).

4. Conclusions

A general observation of our study was that with increasing graft density, toxicity, buffer-capacity and transfection efficiency increased at first until the graft density of 3, and then decreased. Cytotoxicity, zeta-potential, CMC-values, affinity with DNA and stability of the polyplexes were reduced upon increasing graft density. However, no correlation was shown between the sizes of polyplexes and transfection efficiencies. The results of the transfection experiments could be explained only by a combination of physicochemical and biological parameters. Buffer-capacity, cytotoxicity and zeta-potential turned out to be the key factors for the explanation of the results of the gene transfer experiments. Whereas strong cytotoxicity is disadvantageous, a higher buffer-capacity was generally assumed to be advantageous for in vitro gene delivery since it enhances the endosomal escape of polyplexes. Of all the experimental results, buffer-capacity has almost exactly the same tendency as transfection efficiency. We therefore assume that in all processes of DNA transfection, the endosomal escape has a really important and rate-limiting role. The zeta-potential of the complexes is also very important for the uptake of polyplexes. The high surface charges enhance the adhesion of the cationic complexes to the negatively charged cell membrane. Polymers with high buffer-capacity and zeta-potential but with low cytotoxicity showed high transfection activities and seemed to be most efficient as in vitro gene transfer vectors (e.g., hyPEI25k-(PCL570-PEG5k)$_3$). In vivo experiments and the evaluation of the described polymers for siRNA delivery, such as elucidation of the structure of polyplexes are currently under way. Efficient transfection efficiency is expected with polymer hyPEI25K-(PCL570-PEG5K)$_3$ and polymer hyPEI25K-(PCL570-PEG5K)$_5$.

Our results provide a basis for the optimization of the molecular structure of gene delivery vectors with higher buffer-capacity in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.05.017.

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