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A new synthesis method and degradation of hyper-branched polyethylenimine grafted polycaprolactone block mono-methoxyl poly (ethylene glycol) copolymers (hy-PEI-g-PCL-b-mPEG) as potential DNA delivery vectors

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ABSTRACT

Hyper-branched polyethylenimine grafted polycaprolactone block mono-methoxyl poly (ethylene glycol) copolymer (hy-PEI-g-PCL-b-mPEG) was obtained through the conjugation of mPEG–PCL with hyper-branched PEI (hy-PEI) based on the Michael addition. mPEG–PCL was synthesized by ring-opening polymerization of caprolactone using mPEG as the initiator. Compared earlier syntheses, this method offered a reduced number of reaction steps, milder reaction conditions, and a more efficient purification process. FTIR, ^{1}H NMR and ^{13}C NMR spectra proved the structure of the copolymers and controllability of this new synthesis method. Using ^{1}H NMR spectroscopy the degradation of these copolymers was evaluated. Cytotoxicity of copolymers and gene transfection efficiency of polyplexes displayed prominent composition dependence. Increasing the graft density of mPEG–PCL on hy-PEI and longer lengths of both PCL and mPEG within the copolymers investigated here reduced transfection and cytotoxicity on A549 cells. The hy-PEI-g-PCL-b-mPEG copolymers with very short PCL segments (342 Da and 570 Da) demonstrated 6-fold higher transfection efficiency than hy-PEI25k on A549 cells. The polyplexes of the most promising candidate, hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$, exhibited lower hemolysis compared to those of hy-PEI25k.

1. Introduction

Gene therapy has been promoted as a method for curing various inherited and acquired diseases [1–3]. The need for safe and efficient delivery methods has yet to be accomplished, and remains a formidable challenge. In the last three decades, both viral and non-viral gene delivery techniques have been investigated extensively. Viral vectors have posed problems with mutagenicity [4] and liposomal vectors showed negative serum interactions [2]. Recent studies demonstrated that polymeric vectors might be advantageous over viruses and liposomes for gene delivery, regarding safety, immunogenicity, mutagenicity and production costs [5]. For polymeric gene delivery systems, three major biological hurdles need to be considered: efficient cellular uptake of gene–vector complexes, rapid endosomal escape of gene into the cytosol, and efficient localization of gene in the nucleus [6,7]. Therefore, the ideal non-viral delivery systems should not only achieve higher gene expression as viral vectors, but also exhibit lower cytotoxicity, greater size flexibility and avoid the immune responses.

Hyper-branched polyethylenimine (hy-PEI) has been one of the most successfully used cationic polymers for gene delivery both in vitro and in vivo [8,9]. Every third atom of hy-PEI consists of a protonable amino group, which gives hy-PEI the unique property of endosomal buffering capacity or the proton-sponge effect, promoting endosomal escape. The high cytotoxicity of unmodified hy-PEI precludes its use as an in vivo transfection vector. Many factors influence the cytotoxicity of hy-PEI, including molecular weight, polydispersity, incubation time, and density of the cationic groups [10–13]. To address this cytotoxicity, hydrophilic segments, such as poly (ethylene glycol) (PEG), were grafted onto hy-PEI to form PEI–PEG block or graft copolymers.

It has been established that incorporation of PEG increases the hydrophilicity of polymers, as well as minimizes protein adsorption and decreases non-specific cell adhesion. When PEG–PEI copolymers were complexed with DNA in aqueous solution, the hydrophilic segments were assumed to reside on the surfaces of the formed complex to shield the high positive charges of hy-PEI, resulting in decreased aggregation and cytotoxicity of hy-PEI, while increasing the circulation time [14]. Potential drawbacks of the PEG–PEI copolymers could be the inability to condense DNA into small complexes of less than 200 nm, which is critical for cell endocytosis and in vivo transfection efficiency [15,16].

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It is well known that hydrophobic segments in the copolymer influence the transfection activity of cationic polymers. The hydrophobicity of the polymer will influence the formation and stability of polymer/DNA complexes. The hydrophobicity enhances the interactions between polymers and the cell membranes [17–20]. For example, Wang et al. [18] introduced hydrophobic cholesterol molecules into hy-PEI structure to produce efficient vectors with improved transfection efficiency. Tian et al. [20] grafted hydrophobic amino acid segment, poly (γ-benzyl-l-glutamate), on hy-PEI molecules. The hydrophobic segments led to partial shielding of the positive charges and also condensed the DNA into small complexes of ~100 nm. The overall permeability of the complexes was improved through the cell membranes.

In the work presented here, polycaprolactone (PCL) was incorporated into the hy-PEI structure as hydrophobic segments. PCL is recognized for its biodegradability, biocompatibility, and its flexible backbone structure, where it has been used for sutures, bone fracture fixation, tissue engineering scaffolds, and drug delivery systems. The introduction of hydrophobic PCL and hydrophilic mPEG simultaneously was hypothesized to promote the permeation of DNA/polymer complexes into cells and further reduce the cytotoxicity of PEI.

We have previously synthesized the copolymer by conjugating succinimidyl carbonate mPEG–PCL with hy-PEI [21]. The copolymer was confirmed to be a promising gene delivery vector. Purification of the intermediates in this polymer synthesis was considered laborious, and a redesign of the synthesis strategy was desirable.

In this study, mPEG–PCL was coupled with hy-PEI by a Michael addition, which is known to generate structurally diverse polyesters containing tertiary amines in their backbones in a single step [22]. The purification was considered to be more straightforward as no byproducts were generated during the coupling. Moreover, acrylate groups were easily introduced to the terminal hydroxyl groups by the reaction with acryloyl chloride under mild conditions. Comparing the molecular structure, the copolymer conjugated by Michael addition would be identical with that synthesized by succinimidyl carbonate except for the linker between mPEG–PCL and hy-PEI. The correlation between copolymer compositions and cytotocicity as well as the degradation of copolymers with short PCL segments were evaluated, which have not yet been reported before. Additionally the application of the copolymers with short PCL segments in DNA transfection was also reported herein.

2. Materials and Methods

2.1. Materials

Poly (ethylene glycol) mono-methyl ether (mPEG) (2 kDa, 5 kDa) and ε-caprolactone were purchased from Fluka. Acryloyl chloride and Tin (II) 2-ethylhexanoate were from Sigma–Aldrich. Hy-PEI with molecular weight of 25 kDa was obtained from BASF. ε-caprolactone was distilled before use under vacuum over CaH_2. Other reagents were purchased from Acros and used without further purification. Endotoxine-free luciferase-encoding plasmid pCMV-luc (PF461) was purchased from Plasmid Factory (Bielefeld, Germany). The plasmid was obtained by standardized manufacturing technology and additional LPS endotoxin removal.

2.2. Synthesis of mono-hydroxyl terminated methoxyl poly (ethylene glycol)-co-poly(ε-caprolactone) (mPEG–PCL–OH)

mPEG–PCL–OH was synthesized by ring-opening polymerization of ε-caprolactone using mPEG as initiator and Tin(II)2-ethylhexanoate ($SnOct_2$) as a catalyst. Briefly, certain amount of mPEG 2000, ε-caprolactone and $SnOct_2$ (about 0.1% molar ratio of caprolactone) were added into a round-bottomed flask and sealed under dry argon. The mixture was heated at 120 °C under stirring for 24 h. The product was dissolved in chloroform and precipitated with cold methanol or methanol/ether (1/1, v/v) twice. The precipitate was dried under vacuum for 24 h (Yield: 80–95%).

2.3. Synthesis of acrylated methoxyl poly (ethylene glycol)-co-poly(ε-caprolactone) (mPEG–PCL-acrylated)

Hydroxyl end group in mPEG–PCL–OH coupling with acryloyl chloride was performed as follows. Briefly, 1 mmol mPEG–PCL–OH was dissolved in 40 ml benzene in a round-bottom flask. Then, 2 mmol triethylamine and 2 mmol acryloyl chloride were added and the mixture was stirred for 5–8 h at 80 °C. The mixture was cooled to room temperature and filtered to remove triethylamine hydrochloride. The filtered mixture was added into cold n-hexane to precipitate the product and then dried under vacuum for 24 h (Yield: 83–93%).

2.4. Synthesis of hyperbranched polyethylenimine grafted poly-(ε-caprolactone) block monomethoxyl poly (ethylene glycol) copolymer (hy-PEI-g-PCL-b-mPEG)

The reaction was performed according to a Michael addition. Hy-PEI (1 mmol) was dissolved in chloroform, and the chloroform solution of mPEG–PCL-acrylated (1 mmol or 8 mmol) was added drop wise at 45–50 °C. The mixture was stirred at 45–50 °C for 24 h and then cooled to room temperature. The product was collected by solvent replacement. Briefly, 5 ml of hy-PEI-g-PCL-b-mPEG dissolved in chloroform was transferred to a 25 ml conical bottom flask. 10 ml of methanol was added to the solution and the mixture was reduced to ~1 ml on a rotary evaporator (100 Torr with 40 °C water bath). 10 ml of methanol was again added and reduced to 200–500 μl on the rotary evaporator. The solution was diluted to 1 ml using methanol and added all at once to 10 ml of aqueous solution and immediately frozen in −20 °C freezer.

2.5. Nomenclature

The copolymers consist of three different component. The source-based IUPAC nomenclature for these polymers suggests the following designation, e.g., Hyper-branched polyethylenimine grafted polycaprolactone block mono-methoxyl poly (ethylene glycol) copolymer (hy-PEI-g-PCL-b-mPEG). As abbreviation hy-PEIα and hy-PEIα-g-(PCLβ-b-mPEGγ)n are used. Herein n represents the graft density of mPEG–PCL on PEI while α, β and γ stand for the molecular weight of PEI, PCL and mPEG segments respectively.

2.6. Characterization of copolymers

Fourier transformation infrared (FTIR) spectroscopy and Nuclear magnetic resonance (NMR) were used to characterize the compositions of the copolymers. FTIR was performed on Nicolet 510P FTIR spectrometer in the range between 4000 and 400 cm^{-1}. The powder sample was mixed with KBr and pressed into tablets for measurement. The chloroform solution of liquid samples was coated on the surface of KBr plates for measurement after being dried. NMR experiments were carried out on JEOL GX 400D spectrometer in either $CDCl_3$ or deuterated water or methanol-d_4. Gel permeation chromatography (GPC) was used to determine the molecular weight and molecular weight distribution of the copolymers. The GPC system consisted of a Duratec 7505 degasser, Merck–Hitachi HPLC system (L6000 pump, AS-2000A auto-sampler, T-6300 column

thermostat), Wyatt Dawn-EOS multi-angle laser light scattering detector (calibrated with toluene and normalized with 22K pullulan standard) and an Optilab DSP refractometer. The columns used for sample separation were PSS 10 μ Novema precolumn, PSS 10 μ Novema 30 8 × 300 mm, and PSS 10 μ Novema Linear 8 × 300 mm. The measurements were performed at 35 °C column temperature using 1% formic acid as eluent with a flow rate of 1.0 ml/min (laser wavelength: 690 nm, cell type: K5). Molecular weight analysis was determined using the Wyatt Astra software V5.19.

2.7. Degradation of copolymers

The degradation of copolymer was carried out in water, PBS buffer (0.1 M, pH7.4), disodium hydrogen phosphate–potassium dihydrogen phosphate buffer (0.1 M, pH 5.2) and borate buffer (0.1 M, pH 9.0) respectively. Polymer samples of ca. 200 mg were dissolved in 1 ml of the respective buffer and then transferred into dialysis bags (MWCO = 25,000, Spectrum laboratories, Inc. Rancho Dominguez, CA, USA). The bags were placed into 500 ml buffer solutions, which were replaced daily and maintained at 37 °C under 200 rpm stirring for 3 days. The solutions in dialysis bags were collected into a conical bottom flask. Water was removed using a rotary evaporator under vacuum (65 mbar) in a 100 °C water bath. Methanol was added to dissolve the polymer and crystalline under −20 °C overnight. The salts were removed by the filtering from cold methanol quickly. The crystalline and filtration were repeated 3 times. The changes of the structure over time were measured by ^{1}H NMR.

2.8. Cytotoxicity

In vitro cytotoxicity tests of the copolymers were performed by MTT-assays. A549 cells (human non-small-cell lung carcinoma; ATCC: CCL-185, purchased from LG Promochem, Wesel, Germany) were seeded in 96-well microtiter plates (8,000 cells/well) 24 h prior to application of increasing concentrations of the polymer solutions (0.001, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/ml in cell culture media). Cells were incubated in DMEM high glucose (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (Cytogen, Sinn, Germany) in humidified atmosphere with 5% CO2 at 37 °C. After an initial incubation of 24 hours, polymer solutions were added to the cells, which were incubated for another 24 hours. The concentrations of all hy-PEI-g-PCL-b-mPEGs used are expressed in mg/ml of their PEI-content to ensure comparability to unmodified hy-PEI25k. After the incubation period, the polymer solutions were 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 hours at 37 °C in humidified atmosphere, before the un-reacted dye was removed and 200 μl 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 and 690 nm. The relative cell viability (%) was set relative to control wells containing only cell culture medium and calculated by [A] test/[A] control × 100. The IC_{50} was calculated as polymer concentration which inhibits growth of 50% of cells relative to nontreated control cells. The results of optical density measurements were fitted logistically by the Levenberg–Marquardt methods of least-squares minimization for nonlinear equation under the default conditions using Origin 7.0 (OriginLab software, Northampton, USA) by the following equation:

$$Y = Y_0 + (Y_m - Y_0)/(1 + (C/C_0))$$

where C_0 is the IC_{50} dose, Y is the optical density in a well containing a particular polymer/extract of concentration C. Y_0 and Y_m are the optical density corresponding to 0% viability and 100% viability, respectively. Data are presented as a mean of independent four experiments (±SD). The statistical analysis of the IC50 values was performed with Origin 7.0 as well, using unpaired Student's *t*-tests. Probability values $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were marked with asterisks.

2.9. Hemolytic activity

The hemolytic activity of polyplexes was investigated as reported earlier [23]. Briefly, human erythrocytes were isolated from fresh citrated blood by centrifugation at 850×g. Red blood cells were washed in PBS until the supernatant was clear and colorless. Erythrocytes were diluted with PBS to 5×10^8 cells/ml. Polyplexes were prepared at N/P 10 in a total volume of 160 μl (DNA concentration 0.1 μg/μl). 1% of Triton X-100 in PBS (100% lysis, pH 7.4) and pure PBS buffer (0% lysis, pH 7.4) were used as controls. Aliquots (50 μl) of polyplex solutions were mixed with 50 μl erythrocyte suspension. Samples were incubated for 30 min at 37 °C under constant shaking. After centrifugation at 850×g, supernatant was analyzed for hemoglobin release at 541 nm. Samples were prepared in triplicates.

For quantification of hemoglobin, a UV spectrum of a 1:1 dilution of Triton X-100 lysed erythrocytes was recorded showing maxima at 541 nm and 576 nm (Ultrospec 3000, GE Healthcare, Munich, Germany). Totally lysed erythrocytes were subsequently diluted with HEPES-buffered saline (HBS) to give a standard curve. Experiments were performed in triplicates, and results are given as mean value ± standard deviation.

2.10. Polyplexes formation and the measurements of size and zeta-potential

5% of glucose solution was filtered freshly through 0.20 μm pore sized filters (Nalgene® syringe filter, Sigma–Aldrich, Taufkirchen, Germany) before use. 10 μl of stock copolymer solution (1 mg/ml based on hy-PEI25k) was diluted by 5% of glucose to a final volume of 50 μl in microcentrifuge tubes. The volume of a 2 mg/ml DNA stock solution (V_{DNA}) required for a special N/P ratio was calculated as follows:

$$V_{DNA} = (C_{copolymer} \times 10\ \mu l \times 330) \Big/ (C_{DNA} \times 43 \times N/P) \qquad (1)$$

Where Ccopolymer is the concentration of the stock copolymer solution based on hy-PEI25k, and C_{DNA} is the concentration of the stock DNA solution. The certain amount of DNA stock solution (V_{DNA}, calculated before) was also diluted by 5% of glucose to a final volume of 50 μl in microcenrifuge tubes. The 50 μl DNA aliquots were added to the microcentrifuge tubes containing the 50 μl diluted copolymer aliquots and mixed by pippetting 15 times before the mixture was incubated for 30 minutes at room temperature for complex and equilibrium formation.

The size and zeta potential of the polyplexes were monitored by Malvern Zetasizer 3000HS (Marvern Instrument, Worcestershire, UK). The viscosity (0.88 mPa.s) and the refractive index (1.33) of distillied water at 25 °C were used for data analysis. The measurement angle was 173° backscatter. Polyplexes were incubated for 30 min at room temperature before the polyplex size was measured in a low volume cuvette (100 μl) firstly, and then zeta-potential measurements were performed on the samples prepared by diluting 100 μl of polyplexes solution by additional 600 μl of 5% glucose solution to a final DNA concentration of 11 ng/μl and volume of 700 μl. The samples were carried out in the standard clear capillary electrophoresis cell at 25 °C. Three measurements were performed on each sample. Each measurement of size

consists of 15 runs of 10sec each. Each measurement of zeta-potential consists of 15–100 runs, which was set to be automatic. Measurements were analyzed by CONTIN algorithm. Values given are means of three separate experiments with three measurements ± standard deviation.

2.11. In vitro transfection

Human lung epithelial cell line (A549) cells, passages 8–15, were seeded at a density of 15,000 cells/well in 48-well cell culture plates (Nunc, Wiesbaden, Germany) 24 h prior to all experiments. 50 μl of polyplexes solution prepared as the description above, containing 1 μg pDNA, was added to 450 μl fresh culture medium + 10% FCS in each well and incubated for 4 h. The medium was exchanged and cells were harvested after 44 h. Transfection activity was measured according to the protocol provided by Promega (Madison, WI, USA). Cells were lysed in 100 μl cell culture lysis buffer for 15 min. Luciferase activity was quantified by injection of 50 μl luciferase assay buffer, containing 10 mM luciferin, to 20 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). Data were expressed in ng luciferase per mg protein (±SD). All experiments performed in triplicate were representative of three independent experiments.

3. Results and discussion

3.1. Synthesis of copolymer hy-PEI-g-PCL-b-mPEG

The synthesis of hy-PEI-g-PCL-b-mPEG by the conjugation of succinimidyl carbonated mPEG–PCL (mPEG–PCL–NHS) with hy-PEI was reported earlier [21]. These copolymers demonstrated promising properties as gene delivery systems. However, the synthesis required four steps and the purification of pre-products and products proved to be complicated. Therefore, we attempted to shorten the synthesis and to simplify the purification methods as shown in Scheme 1.

As a new coupling strategy we selected the conjugation of acrylated mPEG–PCL to PEI according to a Michael addition under mild conditions [24]. In the acrylation step, an excess of acryloyl chloride was used to ensure complete conversion of all terminal hydroxyl groups of mPEG–PCL. The only byproduct, triethylamine hydrochloride, was readily removed by filtration. The last addition reaction could be easily followed using ^{1}H NMR spectroscopy as the signals for acrylated groups disappeared during the coupling reaction. Further purification of hy-PEI-g-PCL-b-mPEG was unnecessary but dialysis could be employed if needed.

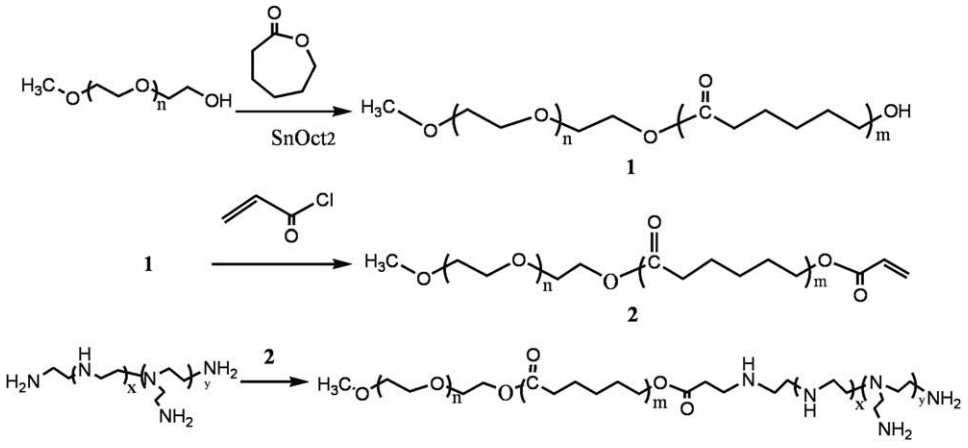
The structures of hy-PEI-g-PCL-b-mPEG and the intermediates were verified using ^{1}H NMR and ^{13}C NMR spectroscopy as shown in Fig. 1. In all three ^{1}H NMR spectra, the resonance of mPEG and PCL blocks were observed. The assignment of the signals from mPEG, PCL and PEI is displayed in Fig. 1. In Fig. 1B, the resonance signals at 5.7–6.5 ppm belong to –CH═CH_2 groups of acrylated mPEG–PCL. The signals disappeared in Fig. 1C of hy-PEI-g-PCL-b-mPEG. This indicates that no unreacted acrylated mPEG–PCL remained in the reaction mixture. Fig. 1D depicts the ^{13}C NMR spectrum of hy-PEI-g-PCL-b-mPEG in deutero-chloroform. The resonance signal at 173.4 ppm originated from the carbon of ester bonds. FTIR was also used to confirm the generation of hy-PEI-g-PCL-b-mPEG from the absorption of amino groups and ester bonds (1735–1732 cm^{-1}). Higher grafted density displayed higher absorption of hydrogen bonds between amino groups and ester bonds (3286 cm^{-1}) (supporting information Fig. S1). These assignments indicated the successful synthesis of hy-PEI-g-PCL-b-mPEG copolymer.

The molecular weight of PCL blocks and the grafting density of mPEG–PCL on hy-PEI were calculated from the internal values of peaks at 4.03 ppm (–OCH_2– CH_2– CH_2– CH_2– CH_2CO–, PCL), 3.37 ppm (CH_3O–, mPEG) and 2.3–2.9 ppm (–CH_2CH_2N–, PEI), based on the known molecular weight of mPEG and PEI [21]. The molecular weights of copolymers measured by GPC, as summarized in Table 1, were mostly in agreement with those calculated from ^{1}H NMR, especially for copolymers with lower grafted density. Furthermore, the molecular weight distributions of all the copolymers were narrow and mono-modal, suggesting no or negligible homopolymer residues in the copolymer. The structural characterization suggested that the new synthesis method was feasible and reproducible.

Notably, all the copolymers were soluble in chloroform but did not dissolve in other organic solvents or water after drying from organic solvents. The copolymers were soluble in water only after displacing chloroform by methanol, and then by water. This solubility pattern is probably due to the strong inter-molecular or intra-molecular hydrogen bonds.

3.2. The degradation of hy-PEI-g-PCL-b-mPEG under in vitro conditions

It is well known that ester bonds are hydrolyzed as polyester gets into contact with water. Following the scheme of bulk degradation, after water uptake, ester bonds are cleaved and the molecular weight decreases until water-soluble oligomers are



Scheme 1. Synthesis outline of hy-PEI-g-PCL-b-mPEG.

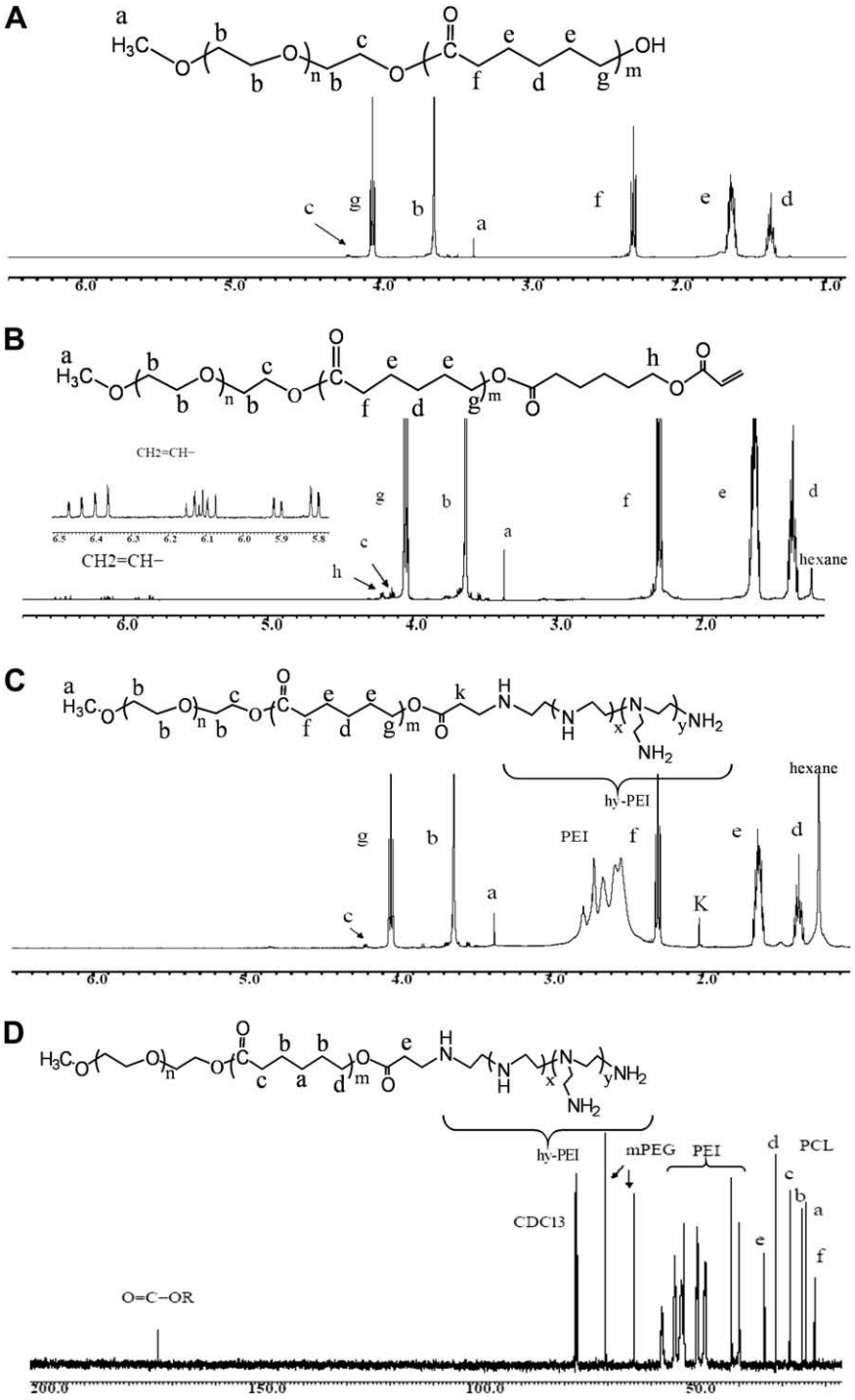


Fig. 1. ^{1}H NMR spectra of mPEG–PCL–OH (A), the arrow points to the position of c; mPEG–PCL-acrylate (B), the arrows point to the positions of c and h, respectively, and the insert content is the close-up of CH_2═CH–; and hy-PEI-g-PCL-b-mPEG (C), the arrow points to the position of c; and ^{13}C NMR spectrum of hy-PEI-g-PCL-b-mPEG (D), the arrows point to the positions of mPEG. Deuterated chloroform is the solvent.

formed whereupon mass-loss of water insoluble polymers is observed [25]. In the case of hy-PEI-g-PCL-b-mPEG this pattern becomes more complicated as these copolymers are water-soluble and only the reduction of the copolymer molecular weight can be observed.

Introducing hydrophilic segments into polyesters can accelerate the degradation due to the improved hydration. The degradation of polyesters can also be accelerated by amino groups [26]. In principle one could expect that hy-PEI-g-PCL-b-mPEG would be cleaved in PCL chains to form mPEG–PCL_x and $PCL_{(n-x)}$–PEI in the first step and after complete hydrolysis of the PCL segments caprolactic acid and mPEG would be generated.

Comparing ^{1}H NMR spectra of hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$ before and after 3 days degradation in water, a broad signal at 8.2 ppm appeared. This signal comes from amide groups or the amino groups linked with carboxylic groups by hydrogen

Table 1
The molecular weight and molecular weight distribution of copolymers.

Copolymers	Feed ratio (wt%)		Composition[a] (wt%)		Mn[a]	Mn[b]	Mw[b]	Mw/Mn[b]
	PEG	PCL	PEG	PCL				
Hy-PEI25k-g-(PCL800-b-mPEG2k)$_1$	67	33	71	29	27,800	36,440	44,610	1.224
Hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$	77	23	78	22	27,570	32,270	48,220	1.494
Hy-PEI25k-g-(PCL342-b-mPEG2k)$_1$	80	20	85	15	27,340	31,040	50,760	1.635
Hy-PEI25k-g-(PCL342-b-mPEG2k)$_8$	80	20	85	15	48,730	36,710	61,400	1.673
Hy-PEI25k-g-(PCL342-b-mPEG2k)$_1$	83	17	93	7	30,340	38,130	54,290	1.424
Hy-PEI25k-g-(PCL342-b-mPEG2k)$_8$	83	17	93	7	67,760	41,690	68,580	1.645

[a] Calculated from NMR.
[b] Detected from GPC.

bonds. A broad resonance signal between 3.0–3.3 ppm also appeared, which was assigned to the hydrogen bonds between amino groups and hydroxyl groups at the terminal of mPEG. A smaller signal at 1.53 ppm appeared as well (Fig. 2A). In order to destroy the hydrogen bonds between amino groups and carboxylic groups or hydroxyl groups and confirm the degradation and the formation of amide bonds, deuterated hydrochloric acid (20% DCl in D_2O) and deuterated water were added to the methanol-d_4 solution of degradation products. The signal at 8.2 ppm moved to 8.1 ppm and became a single small peak, which comes from amide groups, the signals between 3.0–3.3 ppm disappeared, while the signal at 1.53 ppm still existed. It indicated that the degradation occurred and some amide bonds were generated. In order to confirm this degradation rout further, 0.1 M of NaOH was added to degradation medium and heated at 60 °C for 10 min, then incubated at room temperature for 3 h. The small signal at 8.1 ppm existed as in the acid environment. The signals comes from CH_2 of PCL at 1.63 ppm and 1.38 ppm shifted to 1.53 ppm and 1.3 ppm, while the signals being assigned to CH_2 of PCL segments connecting caprolactic acid units and CH_2 of mPEG connected PCL segments at 4.03 ppm and 4.2 ppm disappeared completely.

Esters are hydrolyzed in aqueous solution at base as well as at acid conditions and should be more stable at neutral pH. Amides are less susceptible to hydrolysis but can be degraded at base conditions [28]. In case of the possible generated amides bonds by aminolysis, the degradation studies of hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$ were also performed in PBS buffer (0.1 M, pH7.4), disodium hydrogen phosphate–potassium dihydrogen phosphate buffer (0.1 M, pH 5.2) and borate buffer (0.1 M, pH 9.0) at 37 °C to investigate if various buffers can hamper the aminolysis or catalyse the hydrolysis of amides. Fig. 2B exhibited that the signals of PCL segments were obscure in base degradation product, and they are clearer in pH7.4 buffer than in pH5.2 buffer. This suggested that hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$ degraded faster in a basic environment than in acidic buffers, and the degradation in the buffer of pH 7.4 showed the slowest degradation. This can be explained by the accelerated cleavage of the ester bonds under base and acid catalysis. The three ^{1}H NMR spectra also exhibited the signal of amide bonds at 8.2 ppm (Fig. 2B). Degradation in pH 7.4 buffer displayed the least signal possibly due to the slowest hydrolysis. The amides signal did not disappear even in pH 9.0 buffer for 3 days. This strongly proved the degradation of PCL and the generation of amides, which were assumed to be generated by the aminolysis of ester bonds. As reported by Lim et al. [27], amines can react with ester bonds in the copolymers of polyesters bearing amines during degradation. Petersen et al. [28] has also reported the aminolysis between ester bonds and amines during degradation. The aminolysis would possibly lead to too rapid degradation and undefined molecules to be able to delivery the gene efficiently before the degradation. A very rapid degradation might be only tolerable in the case of in vitro transfection experiment since only a few hours are required to yield a maximum transfection in vitro. With respect to in vivo experiments the genes have to be protected for more hours than in vitro due to the longer circulation time of polyplexes in blood system. The generation of amides of the copolymers might be more appropriate for an in vivo gene delivery since the amides shows a higher stability as compared to ester bonds [28]. On the other hand the aminolysis of ester bonds reported so far was mainly happened in poly (lactic acid-co-glycolic acid) (PLGA) rather than PCL. As we know the degradation of PLGA was very quick due to its amorphous structure [29]. Usually the half-life of PLGA was much less than PCL [30]. At the same time the shielding effects of mPEG chains can partially hurdle or slow the aminolysis. Thereby the degradation and aminolysis of the ester bonds of PCL might be slower than those of PLGA. In our studies, Fig. 2B(5), there is only very indefinite amides but obvious PCL signal even incubation in pH 7.4 buffer about 3 days. Additionally, the amide bonds were believed to be degradable in physiological conditions. Accordingly the copolymers, hy-PEI-g-PCL-mPEGs, would be finally degraded into hy-PEI, caprolactic acid and mPEG.

Interestingly, the solubility of copolymer also changed during degradation. Before and after degradation for 10 days, all copolymers dissolved both in water-miscible solvents as well as in chloroform, whereas, copolymers with longer PCL segments did not dissolve in chloroform after more than 45 days of degradation. The solubility did not change after degradation in water for the copolymers with very short PCL segments (e.g. 342 and 570 Da). The reason for this solubility behavior might come from the increased hydrogen bonds after copolymer degradation.

3.3. Cytotoxicity and hemolysis

To evaluate the cytotoxicity of hy-PEI-g-PCL-b-mPEG polymers in A549 cells an MTT-assay was performed. We selected pure polymers instead of DNA polyplexes to measure the cytotoxicity. This was done to simulate a worse case scenario and obtain larger sensitivity results. It has reported that the cytotoxicity was reduced when polymers complexing with DNA [9]. However, results of a lactate dehydrogenase (LDH) assay demonstrated that the cytotoxicity of polyplexes was closed related to the cytotoxicity of the free polymers [10]. It was found that the cytotoxicity profile of hy-PEI-g-PCL-b-mPEG copolymers was dependent on PCL-segment length, PEG-chain length, and graft density. As illustrated in Fig. 3A, the cytotoxicity of the copolymers displayed the concentration dependent. Hy-PEI25k reduced the cell viability dramatically at concentration of 25 μg/ml (~9% viability), while all hy-PEI-g-PCL-b-mPEG copolymers displayed less toxicity on cells than hy-PEI25k. Considering the IC_{50} values, increasing the PCL-segment length, as it is the case in hy-PEI25k-g-(PCL342-b-mPEG2k)$_1$ < hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$ < hy-PEI25k-g-(PCL800-b-mPEG2k)$_1$, lead to decreased cytotoxicities with IC_{50} values of 0.134 mg/ml < 0.178 mg/ml < 0.187 mg/ml respectively (Fig. 3B). Qiu et al also reported that the cytotoxicity decreased

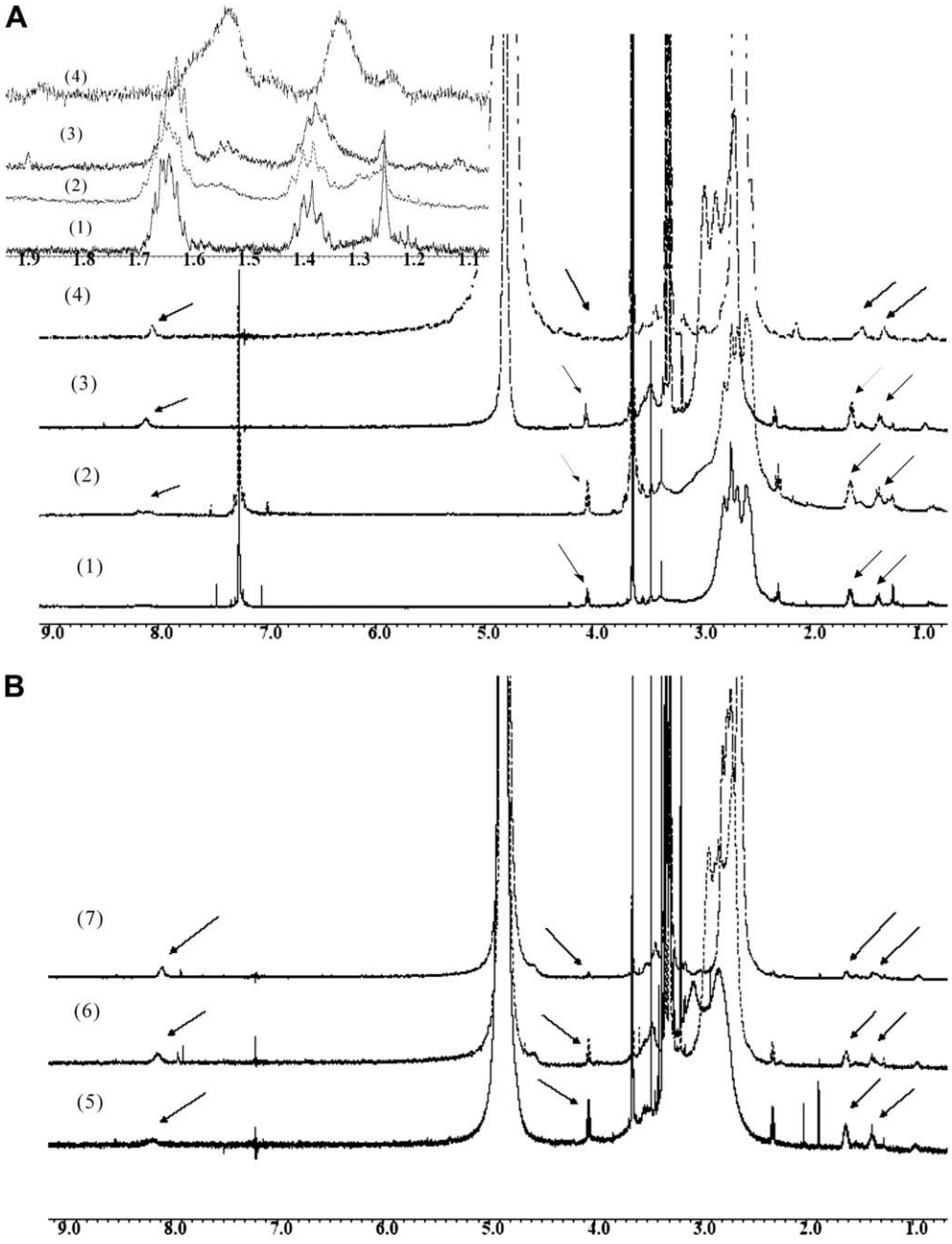


Fig. 2. ^{1}H NMR spectra comparison of hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$ before [0 day, A (1)] and after degradation of 3 days A (2) in water (Deuterated chloroform as the solvent); A (3) was using 20% DCl/D_2O/methanol-d_4 (1/1/7.5, v/v/v) as the solvent; A (4) was using 0.1 M NaOH/methanol-d_4 (1/3, v/v, heating) as solvent; the insert part is the close-up of PCL segments (–$COCH_2CH_2CH_2CH_2CH_2O$– and –$COCH_2CH_2CH_2CH_2CH_2O$–) respectively. Degradation of 3 days in various buffers (methanol-d_4 was the solvents): B (5) was degradation in pH 7.4 buffer; B (6) was degradation in pH 5.2 buffer; B (7) was degradation in pH 9.0 buffer. The arrows point to the positions of PCL segments. The arrows points to the signals of PCL segments and amides (at 8.2 ppm) respectively.

as increasing hydrophobic PCL segment length for PEI–PCL copolymers [31]. Longer PEG-chain lengths and higher graft densities can also achieve the same effect. The cytotoxicty of cationic polymers was thought to be a result of membrane damaging effects [10]. The toxic sites of copolymers were the amino groups of PEI. They were shielded by the grafted neutral mPEG–PCL segments. Therefore, the reduction of the cytotoxicty was expected to be a function of higher graft density as well as higher molecular weight of PCL segments and mPEG chains due to the higher flexibility of mPEG–PCL chains [32].

Hemolysis was measured by testing the induced lysis of erythrocytes as a model system. It can serve as a marker for membrane activity and a prediction of potential side effects for polycationic vectors in intravenous administration [30]. Hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$ was chosen to test hemolytic effects due to the almost lowest IC_{50} among all copolymers. Since the polyplexes were acquired with respect to the transfection, we measured the hemolysis of the polyplexes directly. Triton-X 100 in PBS buffer (0.15 M, pH 7.4) was used as positive control while pure PBS buffer (0.15 M, pH 7.4) was selected as the negative control. The result was related to these controls. Fig. 4 illustrates the percentage of hemolysis. Erythrocytes treated with hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$/DNA polyplexes showed almost as little hemolysis as the blank cells and considerably lower hemolysis compared to hy-

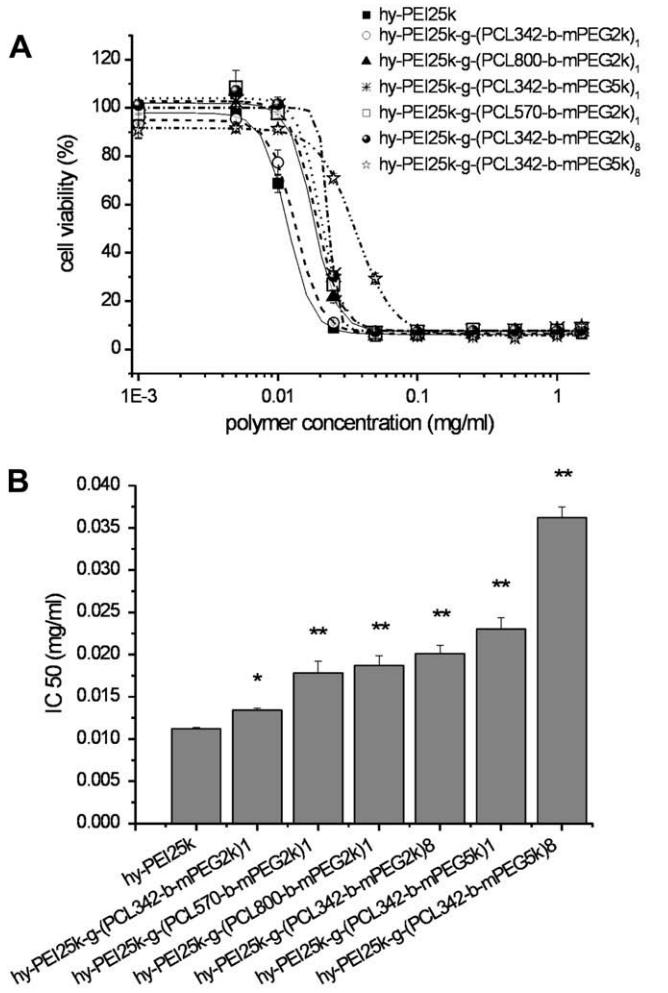


Fig. 3. Cell viability of hy-PEI-g-PCL-b-mPEG copolymers on A549 cells (A), IC_{50} values of hy-PEI-g-PCL-b-mPEG copolymers compared to hy-PEI25 kDa (B) ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

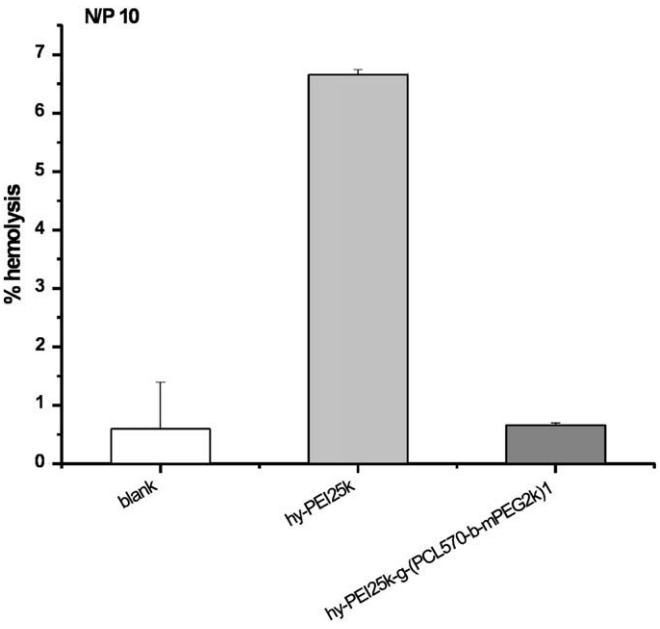


Fig. 4. Percentages of hemolysis for the polyplexes of hy-PEI25k and hy-PEI25k-g-$(PCL570\text{-}b\text{-}mPEG2k)_1$ at the same concentration; the N/P ratio is 10. Experiments were performed in triplicates, and results are given as mean value ± standard deviation.

PEI25k/DNA complexes. The hemolytic effect of polyplexes is known to increase with increasing N/P ratio since the amount of free polymer increases. As the polycations interact with cells and cause cell lysis or agglutination, an increase of their concentration coactively causes higher hemolysis. At N/P 7, which was also used for transfection, hemolysis is expected to be slightly lower than at N/P 10.

The results obtained by the MTT assay and RBC hemolysis assay strongly indicate that the introduction of the PCL and the PEG chain decreases the cytotoxicity and hemolytic activity of the hy-PEI-g-PCL-b-mPEG polymers compared to unmodified hy-PEI25k as expected. As reported by Petersen et al [33], cytotoxicity is decreased with an increasing number of PEG blocks and increasing degree of PEGylation. Furthermore the introduction of a hydrophobic moiety, such as cholesterol, to hy-PEI can also lead to less interaction with erythrocyte membranes and decreased cytotoxicity [17]. The advantageous combination of PCL and PEG chains in hy-PEI-g-PCL-b-mPEG polymers provides shielding from the positive charges of hy-PEI thus causing less cytotoxicity and hemolysis of erythrocytes.

3.4. Characterizations and DNA transfection efficiency of Polyplexes

To investigate the application of the hy-PEI-g-PCL-b-mPEG copolymers in the DNA transfection a panel of copolymers has been investigated. These hy-PEI-g-PCL-b-mPEG copolymers consist of a hy-PEI25k but differ in PCL segments length, mPEG-chain length, and the graft densities. The sizes and zeta-potentials of the polyplexes depicted in Fig. 5A and 5B. The polyplex size was largest, within the copolymers investigated in this study, when the molecular weight of the hydrophobic PCL segments was 800 Da (hy-PEI25k-g-$(PCl800\text{-}mPEG2k)_1$) (Fig. 5A). This is consistent with the report [31]. A positive surface charge of polyplexes has been believed to be necessary for binding to anionic cell surfaces, which consequently facilitates cell uptake [10]. All polyplexes presented net positive charge and the charge was larger when the graft density was one, and mPEG was 2k Dalton long as well as molecular weight of PCL segments was 800 Da (Fig. 5B). This can be explained by the PEGylation effects [32] and the larger hydrophobic core bulk with longer PCL segments.

The stability of polyplexes over time was measured by the size changes of polyplexes with incubation time (Fig. 5C). It showed the slightly decrease of size within first 30 min. This might be the equilibration process of complexes. Then, no significant size change was observed for all polyplexes within 60 min. Thereby the polyplexes was considered to be stable. As depicted in Fig. 6, hy-PEI25k-g-$(PCL342\text{-}b\text{-}mPEG2k)_1$ and hy-PEI25k-g-$(PCL570\text{-}b\text{-}mPEG2k)_1$ display the highest transfection efficiency. With a luciferase expression of around 35 ng luciferase/mg protein, an up to 6-fold higher transfection efficiency compared to unmodified hy-PEI25k could be achieved. hy-PEI25k-g-$(PCL342\text{-}b\text{-}mPEG5k)_8$ and hy-PEI25k-g-$(PCL342\text{-}b\text{-}mPEG2k)_8$ exhibited lower transfection efficiency. All polymers, except for the latter, showed higher transfection efficiency upon increasing the N/P ratio, which can be understood as an effect of better condensation, smaller sizes and better protection of DNA. Increasing either the PCL segment lengths or the PEG chain lengths, or both, lowered the transfection efficiency to the level of hy-PEI25k. There was no obvious correlation of size and transfection efficiency among this panel of copolymers that all formed particles of more or less the same size. Therefore stability of the polyplexes might play a role as well as membrane interaction. We assumed that a very short hydrophobic PCL segments length (i.e. 342 and 570 Da) in combination with a hydrophilic mPEG chain length of 2 kDa

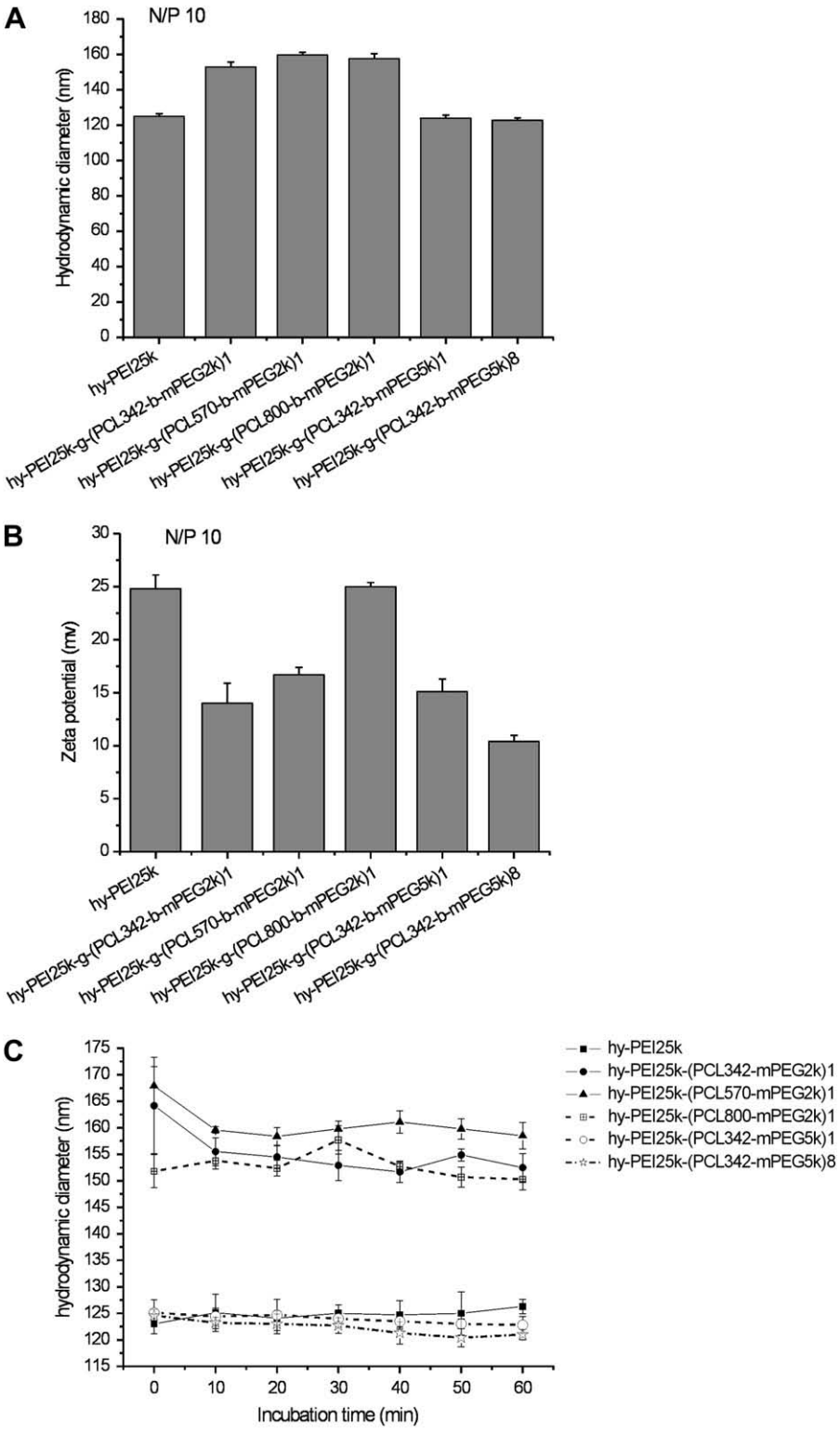


Fig. 5. Sizes (A) and zeta-potentials (B) of the pCMV–Luc/polymers polyplexes in 5% of glucose solution after incubating at room temperature for 30 min; the size changes of pCMV–Luc/copolymer polyplexes in 5% of glucose solution over time at room temperature (C); the N/P ratio in all diagrams is 10. The data presented here as the means of three independent experiments with three measurements of each sample.

caused higher membrane interactions followed by improved cellular uptake and luciferase expression. This is in accordance with the findings by Kurisawa et al [34]. They could increase the transfection efficiency of 2-(dimethylamino)ethyl methacrylate-polymers by incorporating hydrophobic units. Qui et al have also reported that introducing suitable length of hydrophobic PCL segments in hy-PEI increased the transfection efficiency [30]. The very low transfection efficiency of higher grafted copolymer might

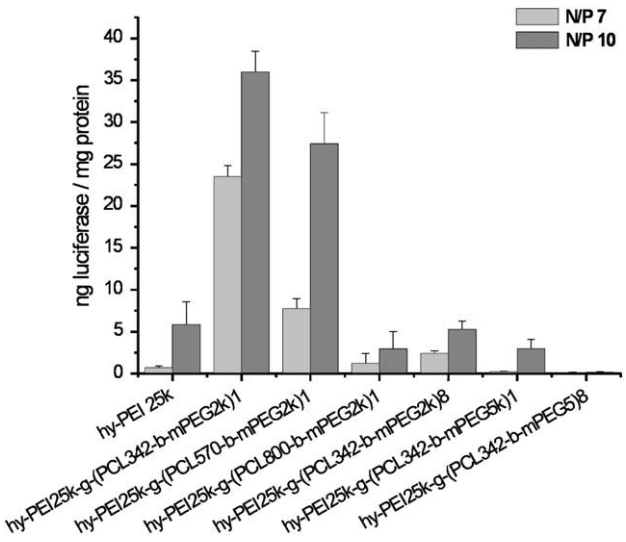


Fig. 6. Transfection efficiency of hy-PEI-g-PCL-b-mPEG copolymers in comparison to hy-PEI25 kDa in A549 cells at N/P ratios of 7 and 10.

be explained by the higher shielding effects and the lower mobility of the grafted chains [35].

4. Conclusion

The copolymers of hy-PEI-g-PCL-b-mPEG were synthesized by the conjugation of acrylated mPEG–PCL with hy-PEI based on the Michael addition reaction. The copolymer structures were characterized and confirmed by NMR and FTIR spectroscopy. The copolymers hy-PEI-g-PCL-b-mPEGs proved to be similar with the structures previously synthesized by the conjugation of succinimidyl carbonate and hy-PEI except for the linker. Degradation studies of the copolymers were performed in water and buffers with varied pH values. ^{1}H NMR spectra of degraded copolymers proved the degradation and suggested that some amide bonds were possibly generated due to the aminolysis of the ester bonds. The application in DNA delivery studies displayed a strong effect of PCL and mPEG chain lengths and graft density on cytotoxicity and transfection efficiency of these copolymers in A549 cells as expected. Lower cytotoxicity was observed for all copolymers compared to hy-PEI25k. Longer mPEG chains, longer PCL segments and higher graft densities led to lower cytotoxicity. Erythrocytes treated with hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$/DNA complexes displayed lower hemolysis compared to hy-PEI25k/DNA complexes. Increasing either PCL length or PEG length or both decreased the transfection efficiency to the level of hyper-branched PEI25k. Higher graft density of mPEG–PCL on hy-PEI25k also decreased bioactivity. The copolymers hy-PEI25k-g-(PCL342-mPEG2k)$_1$ and hy-PEI25k-g-(PCL570-b-mPEG2k)$_1$ demonstrated 6-fold higher transfection efficiencies compared to hy-PEI25k. This is most likely due to the aggregation of polyplexes on cell membranes, thereby increasing cell membrane interaction and uptake [31]. However no significant correlation was showed between the size of polyplexes and transfection efficiencies.

The copolymers hy-PEI-g-PCL-b-mPEG, after all, showed a promising feature for effective gene delivery, but the reason of suitable length of hydrophobic PCL enhancing transfection efficiency has not been clear. So far even no sufficient literatures reported the effect mechanism. More details about the correlation between molecular structure or assemble architecture and copolymer properties or bioactivities, the relationship of polymer structure and the degradation behaviors are under investigation in our laboratory. The application of these copolymers in siRNA transfection is also in studying. Additionally in this report only copolymers with short PCL segments were studied. It is still very interesting what will happen when increasing further the molecular weight of PCL segments in this structure.

Acknowledgements

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.polymer.2009.06.043.

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