Synthesis, Characterization, and Biocompatibility of Polyethylenimine-*graft*-poly(ethylene glycol) Block Copolymers

Holger Petersen, Petra M. Fechner, Dagmar Fischer, and Thomas Kissel*

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

Received November 26, 2001

ABSTRACT: Two series of block copolymers were prepared by grafting linear poly(ethylene glycol) (PEG) onto branched polyethylenimine (PEI). In the first series, the PEI (25 000) was grafted with varied numbers of PEG blocks (5000). The second series was composed of copolymers all containing an equal amount of PEG (50%) and PEI (50%), but with PEG of different molecular weights (MW: 550−20 000). In a two-step synthesis, both the activation of monomethyl-PEGs and the coupling reactions of the PEGs with PEI were performed with hexamethylene diisocyanate (HMDI), leading to water-soluble copolymers with hydrolytically stable urethane and urea bonds. The molecular structure of the resulting copolymers was evaluated spectroscopically (NMR, FTIR). Thermal and calorimetric analysis (TGA, DSC) as well as size exclusion chromatography (SEC) verified the successful formation of the copolymers. MW was determined by static light scattering in combination with SEC. With respect to their application as gene transfer agents, the biocompatibility of the copolymers was studied using an in vitro cytotoxicity assay (lactate dehydrogenase assay) and blood compatibility tests (hemolysis and erythrocyte aggregation). It was found that PEG reduced the toxicity of PEI and prevented hemolysis as well as the aggregation of erythrocytes. The extent of the positive influence of PEG on the biocompatibility of the copolymers was found to be dependent upon both the number of PEG blocks and the structure of the block copolymers.

Introduction

Block and graft copolymers containing cationic polyelectrolytes and nonionic hydrophilic polymers gained significant interest as nonviral gene delivery systems.[1,2] The task of the cationic block is to condense nucleic acids, whereas the nonionic, hydrophilic block is thought to increase the solubility of the interpolyelectrolyte complex and to stabilize the complex against opsonization. As polycations, mainly poly(L-lysine)[3,4] (PLL) and polyethylenimine[5] (PEI) have been used. Apart from PEI, other aliphatic polyamines (e.g., polyspermine[6]) were also investigated. Furthermore, some cationically modified methacrylic polymers, such as poly(trimethylammonioethyl methacrylate chloride)[7] and poly(2-(*N*,*N*'-dimethylamino)ethyl methacrylate),[8] have also found interest as the cationic component of a copolymeric gene delivery vehicle. As nonionic hydrophilic polymers, poly(ethylene glycol)[9] (PEG), poly[*N*-(2-hydroxypropyl) methacrylamide][10] (HPMA), and polysaccharides such as dextran[11] and cyclodextrin[12] were studied. Despite the large numbers of publications about the use of these copolymers for gene delivery, a systematic investigation of the influence of the nonionic hydrophilic blocks on the condensation process of a polycation with nucleic acids has not been properly performed yet. To perform such a systematic study, a comparable set of several copolymers should be available. Furthermore, to study the influence of the nonionic component of the copolymers, the polycation should remain the same within this sample set. We chose a branched PEI with a molecular weight (MW) of 25 000 as polycation since it is one of the most efficient cationic polymers with regard to transfection activity.[13] Linear PEGs served as nonionic polymers in our study. In a first series, we varied the number of PEG blocks which were all of MW 5000, as presented in Scheme 1A. The second series was produced with PEGs of different MW. However, the composition of the copolymer remained constant, composing approximately 50% PEI and 50% PEG (see Scheme 1B).

Several routes of synthesis for PEI-*g*-PEG copolymers have been described in the literature. In most cases, well-defined homopolymer blocks were linked together. Some of the authors[5,14,15] employed commercially available activated PEGs which could be directly used for the coupling reaction with PEI. The groups that performed the activation of PEG in their own laboratories[9,16,17] used a rather time-consuming synthesis route with at least three steps. Furthermore, their activated PEGs had to be separated from polymeric side products, which again was quite time-consuming and complicated the isolation of the product. We did not want to be limited to products of commercial suppliers. Therefore, we developed a new synthetic route[18] with only two steps, no side products, and complete conversion of both reaction steps. This simplified the isolation of the copolymers. In the following report, we described this facile method of synthesis, the characterization of the copolymers, and the influence of PEG on the cytotoxicity of PEI.

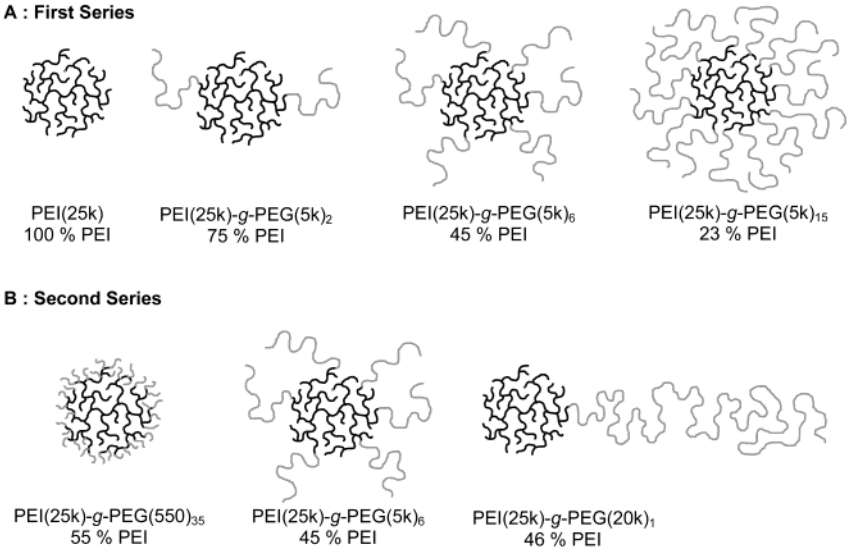
Experimental Section

Materials. Branched polyethylenimine (PEI) with MW of 25 000 (Polymin water free 99%) was a gift from BASF. PEI of MW 1200 (99%) was purchased from Polysciences. PEG-monomethyl ether (mPEG) 550 and mPEG 5000 were obtained from Aldrich, and monoamino-PEG-monomethyl ether (mPEG-NH_2) 20 000 was from Rapp Polymere, Tuebingen, Germany. Hexamethylene diisocyanate (HMDI) (≥99%) was from Fluka. Chloroform (Riedel-de Haen, ≥99%) was treated with HMDI for 4 h at 60 °C and distilled to remove any traces of water and ethanol. Dichloromethane (≥99%) and diethyl ether (≥99,5%), both from Merck, Darmstadt, Germany, were dis-

* Corresponding author: Tel +49-6421-282-5881; Fax +49-6421-282-7016; e-mail kissel@mailer.uni-marburg.de.

10.1021/ma012060a CCC: $22.00 © 2002 American Chemical Society
Published on Web 07/23/2002

Scheme 1. Schematic Representation of the Two Copolymer Series Used in This Study[a]



[a] The black component of the structure represents the cationic branched PEI and the gray component the nonionic linear PEG blocks. It should be remarked that the structures are reduced to two dimensions. Thus, the density of the PEG shield in (3D) reality is smaller than it appears in this scheme. PEI(25K)-*g*-PEG(5K)$_6$ is a member of both series.

tilled before use. Light petrol (≥99%, 40−60 °C) was from Riedel-de Haen.

Block Copolymer Synthesis. *Activation of mPEG 550*. In a 50 mL flask fitted with a reflux condenser and an oil bubbler, 4.00 g of mPEG 550 was dissolved in 10 mL of CH_2Cl_2. 10 mL of HMDI was added, and the mixture was heated under reflux for 8 h. The polymer was precipitated in 250 mL of petrol. The oily precipitate was washed with a further 100 mL of petrol, redissolved in 20 mL of CH_2Cl_2, and precipitated in 250 mL of petrol again. This reprecipitation and washing steps were repeated four times before the polymer was isolated. Residues of solvents were removed at reduced pressure. 0.77 g of a colorless and viscous liquid was obtained with a 15% yield. ^{1}H NMR ($CDCl_3$): δ = 0.79 (m, OCN(CH$_2$)$_3$C*H*$_2$−, 2H), 0.84 (m, OCN(CH$_2$)$_2$C*H*$_2$−, 2H), 0.96 (m, OCN(CH$_2$)$_4$C*H*$_2$−, 2H), 1.07 (m, OCNCH$_2$C*H*$_2$−, 2H), 2.59 (t, OCN(CH$_2$)$_5$C*H*$_2$−, 2H), 2.77 (t, OCNC*H*$_2$−, 2H), 2.82 (s, −OC*H*$_3$, 3H), 3.00 (m, −NHC(O)OCH$_2$C*H*$_2$O−, 2H), 3.10 (s, −OC*H*$_2$C*H*$_2$O−, 60H), 3.63 (t, −NHC(O)OC*H*$_2$−, 2H) ppm. ^{13}C NMR ($CDCl_3$): δ = 13.9 (OCN(CH$_2$)$_3$*C*H$_2$−), 25.4 (OCN(CH$_2$)$_2$*C*H$_2$−), 29.1 (OCN(CH$_2$)$_4$*C*H$_2$−), 30.4 (OCNCH$_2$*C*H$_2$−), 39.9 (OCN(CH$_2$)$_5$*C*H$_2$−), 42.1 (OCN*C*H$_2$−), 58.1 (O*C*H$_3$), 62.9 (−NHC(O)O*C*H$_2$−), 68.8 (−NHC(O)OCH$_2$*C*H$_2$O−) 69.8 (−O*C*H$_2$*C*H$_2$O−), 71.1 (−*C*H$_2$OCH$_3$), 121.3 (−N*C*O), 155.8 ppm (−NH*C*(O)O−). IR (KBr): $\tilde{\nu}$ = 3450 (w, H_2O), 3338 (m, N−H amide ν), 2867 (s, C−H ν), 2274 (s, N=C=O ν_{as}), 1724 (s, C=O urethane, amide I ν), 1559 (s, N−H urethane, amide II δ), 1456 (m, −CH$_2$− δ), 1351 (m), 1251 (m), 1136 (s, C−O−C ether ν_{as}), 952 (w), 852 (w), 778 (w), 585 cm^{-1} (w).

Activation of mPEG 5000. Using the same procedure as described above, 15.23 g of mPEG 5000 was dissolved in 15 mL of $CHCl_3$ in a 100 mL flask and treated with 60 mL of HMDI for 24 h under reflux. The polymer was precipitated in 750 mL of petrol. The light yellow precipitate was washed three times with 400 mL of petrol, redissolved in 20 mL of $CHCl_3$, and precipitated in 500 mL of petrol again. Reprecipitation was repeated 10 times before the polymer was isolated and dried in vacuo. 8.33 g of a white solid was obtained with a 55% yield.

Activation of mPEG 20 000. Using the same procedure as described above, 1.50 g of mPEG-NH$_2$ 20 000 was dissolved in 20 mL of $CHCl_3$ in a 50 mL flask and treated with 5 mL of HMDI for 72 h under reflux. The polymer was precipitated in 250 mL of petrol. The white precipitate was washed with 250 mL of petrol, redissolved in 10 mL of $CHCl_3$, and precipitated in 250 mL of petrol again. Reprecipitation was repeated six times before the polymer was isolated and dried in vacuo. 1.38 g of a white solid was obtained with a 92% yield.

Synthesis of PEI(25K)-g-PEG(550)$_{35}$. 0.80 g of PEI 25 000 was dissolved in 80 mL of $CHCl_3$, and 0.77 g of activated PEG 550 was dissolved in 100 mL of CH_2Cl_2 in a 250 mL flask. The PEI solution was added dropwise to the PEG solution, and the flask was fitted with a reflux condenser and an oil bubbler. The mixture was heated under reflux for 12 h. The clear light yellow solution was concentrated to 30 mL volume, and the polymer was precipitated in 500 mL of diethyl ether. The precipitate was dried in vacuo. 1.15 g of a yellowish powder was obtained with a 73% yield.

As described above, the following polymers were synthesized:

Synthesis of PEI(25K)-g-PEG(5K)$_2$: 2.19 g of PEI 25 000 in 150 mL of $CHCl_3$; 0.73 g of activated PEG 5000 in 50 mL of $CHCl_3$. Reaction time: 24 h. Yield: 2.03 g (69%); yellowish waxy solid.

Synthesis of PEI(25K)-g-PEG(5K)$_6$: 1.10 g of PEI 25 000 in 100 mL of $CHCl_3$; 1.10 g of activated PEG 5000 in 100 mL of $CHCl_3$. Reaction time: 24 h. Yield: 1.72 g (78%); yellowish waxy solid.

Synthesis of PEI(25K)-g-PEG(5K)$_{15}$: 0.54 g of PEI 25 000 in 60 mL of $CHCl_3$; 1.62 g of activated PEG 5000 in 140 mL of $CHCl_3$. Reaction time: 24 h. Yield: 1.95 g (90%); white powder.

Synthesis of PEI(25K)-g-PEG(20K)$_1$: 1.00 g of PEI 25 000 in 100 mL of $CHCl_3$; 1.00 g of activated PEG 20 000 in 100 mL of $CHCl_3$. Reaction time: 72 h. Yield: 1.54 g (77%); fluffy, sticky white solid.

Synthesis of PEI(1200)-g-PEG(550)$_2$: 1.53 g of PEI 1200 in 80 mL of $CHCl_3$; 1.83 g of activated PEG 550 in 80 mL of $CHCl_3$. Reaction time: 12 h. Yield: 3.19 g (95%); yellowish, highly viscous oil. ^{1}H NMR ($CDCl_3$): δ = 0.77 (s, −NHC(O)NH(CH$_2$)$_{2and3}$C*H*$_2$−, 4H), 0.93 (s, −NHC(O)NH(CH$_2$)$_{1and4}$ C*H*$_2$−, 4H), 1.96−2.28 (m, −NC*H*$_2$C*H*$_2$N-, 43H), 2.54 (s, OCN(CH$_2$)$_{0and5}$ C*H*$_2$−, 4H), 2.79 (s, −OC*H*$_3$, 3H), 2.98 (m, −NHC(O)OCH$_2$C*H*$_2$O−, 2H), 3.07 (s, −OC*H*$_2$C*H*$_2$O−, 62H) 3.60 (s, −NHC(O)OC*H*$_2$−, 2H) ppm. ^{13}C NMR ($CDCl_3$): δ = 13.5 (−NHC(O)NH(CH$_2$)$_3$*C*H$_2$−), 25.1 (−NHC(O)NH(CH$_2$)$_2$*C*H$_2$−), 28.5 (−NHC(O)NH(CH$_2$)$_4$*C*H$_2$−), 29.0 (−NHC(O)NHCH$_2$*C*H$_2$−), 38.3 (H$_2$N*C*H$_2$CH$_2$N⟨), 39.3 (−NHC(O)NH(CH$_2$)$_5$*C*H$_2$−), 39.5 (−NHC(O)NHC*H*$_2$−), 40.1 (H$_2$NCH$_2$*C*H$_2$NH−), 46.2 (−NH*C*H$_2$CH$_2$N⟨), 48.0 (−NHCH$_2$*C*H$_2$NH−), 50.9 (−HN*C*H$_2$CH$_2$NH$_2$), 51.6 (⟩N*C*H$_2$CH$_2$N⟨), 53.3 (⟩N*C*H$_2$CH$_2$NH−), 56.0 (⟩N*C*H$_2$CH$_2$NH$_2$), 57.5 (−O*C*H$_3$), 62.1 (−NHC(O)O*C*H$_2$−), 68.1 (−NHC(O)OCH$_2$*C*H$_2$O−) 69.1 (−O*C*H$_2$*C*H$_2$O−), 70.5 (−*C*H$_2$OCH$_3$), 155.3 (−NH*C*(O)O−), 158.1 ppm (−NH*C*(O)NH−). IR (KBr): $\tilde{\nu}$ = 3294 (m, N−H amine ν), 2921 (s, C−H ν), 1714 (s, C=O urethane, amide I ν), 1620 (m, C=O urea ν), 1541 (s, N−H

urethane, amide II δ), 1460 (m, $-CH_2-$ δ), 1351 (m), 1253 (m), 1106 (s, C−O−C ether ν_{as}), 953 (w), 851 (w), 752 (w), 578 cm^{-1} (w).

Techniques. *Size Exclusion Chromatography in Combination with Multiple Angle Laser Light Scattering (SEC-MALLS).* The MW and molecular weight distribution (MWD) of all homopolymers and copolymers were determined using SEC in combination with MALLS. The SEC consisted of a HPLC pump L-6000 (Merck-Hitachi, Darmstadt, Germany), a Merck-Hitachi autosampler AS-200A, and a Merck column thermostat T-6300 (25 °C). Polymers were detected by a differential refractive index (RI) detector RI-71 from Merck and an 18 angle laser light scattering detector from Wyatt Technologies (DAWN EOS, GaAs laser 690 nm, 30 mW, K5 cell). The SEC column, Suprema Max 3000, was from Polymer Standard Service, Mainz, Germany. 1% formic acid (Riedel-de Haen, 98−100%) was used as eluent. The eluent was prepared with pure reagent water (0.22 μm, 0.055 μS/cm, USF Seral, Seradest BETA 25 and Serapur DELTA UV/UF), filtered through a 0.2 μm sterile filter, and degassed with a four-channel online vacuum degasser DDG-75 from Duratec, Reilingen, Germany. Additionally, an inline filter (0.45 μm, stainless steel, Rheodyne, Cotati, CA) was inserted between the columns and the MALLS detector. A flow rate of 0.5 mL/min was applied. Polymer concentrations were in the range 2−30 g/L, and 20 μL was injected for each run.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR and ^{13}C NMR spectra were recorded in D_2O (Merck) (except PEI(1200)-*g*-PEG(550)$_2$ in $CDCl_3$, Aldrich) on a GX 400 D spectrometer from JEOL at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR spectra, respectively. Spectra were evaluated with the NMR data processing program MestRe-C Version 1.5.1. Integration of the signals in ^{1}H NMR spectra for $-CH_2-CH_2-O-$ and for $-CH_2-CH_2-ND-$ yielded the composition of the copolymers. Indices in the nomenclature of the copolymers in this paper are calculated from this integration and with the MW provided by the suppliers.

Fourier Transformed Infrared Spectroscopy (FTIR). FTIR spectroscopy was conducted on a FT-IR 510P spectrometer from Nicolet with PC/IR v. 3.20 software using KBr (Uvasol, Merck) disks.

Thermogravimetric Analysis (TGA). TGA was performed on a thermogravimetric analyzer TGA 7 with a thermal analysis controller TAC 7/DX from Perkin-Elmer using an approximately 10 mg polymer sample. The scanning rate was 20 K/min, and thermograms were recorded within the temperature range 25−700 °C. Analysis was performed under a nitrogen gas atmosphere in platinum crucibles.

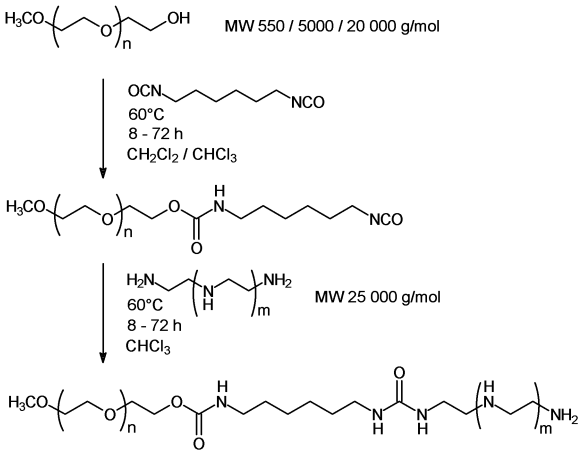
Differential Scanning Calorimetry (DSC). DSC measurements were conducted on a Toledo DSC 821e from Mettler with approximately 10 mg polymer. Scans were run in nitrogen at a heating and cooling rate of 10 K/min (temperature range −100 to 120 °C). Glass transition (T_g) and melting temperature (T_m) are derived from the second heating curve.

Lactate Dehydrogenase (LDH) Assay. In vitro cytotoxicity of the polymers was evaluated by a LDH assay with 3T3 mouse fibroblasts. 500 000 cells per well (12 well plate) were seeded in 2 mL of Dulbecco's modified eagle medium (DMEM) and cultivated for 24 h. The cells were washed with 2 mL of phosphate buffered saline (PBS) (with Ca^{2+}/Mg^{2+}, pH 7.4) and treated with 1.8 mL of fresh PBS and 200 μL of a diluted polymer solution. As a reference, both PBS (negative, 0%) and 0.1% (w/w) Triton X-100 solution (ICN, Eschwege, Germany) in PBS (positive, 100%) were used. After 4 h, 100 μL samples were withdrawn, and the LDH content of a 30 μL sample was measured using a commercial test kit (DQ 1340-K, Sigma) which allows the photometric determination of the reduction of nicotinamid adenine dinucleotide (NAD) in the presence of lactate and LDH at 340 nm. The percent value of LDH release indicates the amount of released LDH compared to total LDH contained in the intact cells. Experiments were performed in triplicate.

Hemolysis Tests. Fresh blood from 6 month old rats (Fischer 344 rats, Institute for Pharmacology and Toxicology, University of Marburg), collected in heparinized tubes, was centrifuged at 700*g* at 4 °C for 10 min and washed several times with PBS until the supernatant was colorless. 500 μL of a 2.5% (v/v) suspension of erythrocytes was mixed with 500 μL of polymer solution in Eppendorf cups. After incubation of 2 and 60 min at 37 °C (shaking water bath), the blood cells were removed by centrifugation, and the supernatants were investigated spectroscopically at 540 nm for the release of hemoglobin. As a reference, PBS (negative = 0%) and 0.2% Triton X-100 solution (positive = 100%) were used. Experiments were performed in triplicate.

Aggregation of Erythrocytes. To study polymer-induced aggregation of erythrocytes, rat blood (see hemolysis test) was applied. Ringer's solution (with addition of sodium citrate, pH 7.4) was used to prevent coagulation. The blood was washed several times with Ringer's solution until the supernatant was colorless. Finally, the erythrocytes were diluted with Ringer's solution 1:50. 200 μL of this cell suspension was treated with 100 μL of polymer solution in 24 well plates (Nunc). After incubation for 2 h at 37 °C pictures were taken of the cells with a Contax RTS 2 camera from AGFA (ASA 100) fitted to a reverse phase contrast microscopy (Nikon TMS) at 40× magnification. Experiments were performed in triplicate.

Scheme 2. Synthetic Route of PEI(25K)-*g*-PEG Copolymers[a]



[a] The monomethyl ether-PEG 20 000 has an amino terminal group instead of the hydroxy terminal group as shown in this scheme. To simplify, the PEI is drawn as linear polymer and not as a branched macromolecule, as it exists in reality.

Results and Discussion

Copolymer Synthesis. Copolymer synthesis was achieved by coupling linear PEG blocks to a branched PEI macromolecule. The synthesis route is shown in Scheme 2. In a first step the mPEG was activated. The single hydroxy terminal group of mPEG had to be transformed into a functionality, which is reactive toward amino groups of PEI. Since we were performing a reaction, in which only the terminal groups of the polymers are involved, we were looking for a coupling reagent with very reactive functional groups toward both the hydroxy group of mPEG and the amino groups of PEI. Isocyanates meet these requirements. Using a diisocyanate, in our case HMDI, we had one functional group for conversion of the hydroxy group of PEG and the other one for the coupling reaction with PEI. There are at least two advantages of isocyanates for this application. First, because of the nature of an addition reaction, no side products are formed during the reaction, thus allowing a facile isolation of the product. Second, both bonds, the urea and the urethane bond, are very stable against hydrolytic cleavage. This provides aqueous solutions of the copolymers, which may potentially be part of a gene delivery kit, a high stability,

Table 1. Synthesis of the Block Copolymers PEI(25K)-*g*-PEG

	activation of mPEGX with HMDI						synthesis of block copolymers			
copolymer	mPEGX [g/mol]	end group X	solvent	t_R [h]	excess of HMDI [times]	yield [%]	PEI in feed [%]	PEI found[a] [%]	time of reaction [h]	yield [%]
PEI(25K)-*g*-PEG(550)$_{35}$	550	-OH	CH_2Cl_2	8	8	15	57	55	12	73
PEI(25K)-*g*-PEG(5K)$_2$							75	75		69
PEI(25K)-*g*-PEG(5K)$_6$	5000	-OH	$CHCl_3$	24	100	55	50	45	24	78
PEI(25K)-*g*-PEG(5K)$_{15}$							25	23		90
PEI(25K)-*g*-PEG(20K)$_1$	20000	-NH_2	$CHCl_3$	72	400	92	50	46	72	77

[a] As determined by ^{1}H NMR spectroscopy.

and long durability. However, two critical points need to be considered. First, a possible side reaction during the activation step could lead to the conversion of both isocyanate groups, thus forming a PEG with a doubled MW. This problem was overcome by using an excess of HMDI. The large excess also had the advantage of improving the conversion of the activation reaction. However, this raised a second problem; the excessive HMDI had to be removed before the coupling reaction with PEI could be conducted. Even a small amount of residual HMDI would result in cross-linking of the PEI macromolecules and in loss of solubility. Therefore, HMDI was carefully removed by repetitive extraction with petrol (for PEG 550) or with repetitive reprecipitation in petrol from chloroform (for PEG 5000 and 20 000). Details about synthesis are provided in Table 1. For the activation of PEG 550 and 5000 the monohydroxy derivative was used for synthesis. The reaction could be run under mild conditions and with short reaction times. However, a longer reaction time was necessary for a complete conversion of the terminal groups of the middle sized PEG 5000 in comparison with the small PEG 550. Since the terminal group/repeating unit ratio was even less favorable for the high MW PEG 20 000, we chose the monoamino derivative of the PEG, because of the higher reactivity of this group toward isocyanates in comparison with the hydroxy group. In this case, an even longer reaction time was necessary for successful coupling. Because of their limited stability, activated PEGs were immediately used for the coupling reaction with PEI. To verify the presence of an activated intermediate, the copolymer PEI(1200)-*g*-PEG(550)$_2$ was synthesized, and as an example for all other activated PEGs and copolymers the FT-IR spectra of the intermediate and the resulting block copolymer are shown in Figure 1. Since both homopolymers are of low MW, signals of linker and terminal groups can clearly be seen in the FT-IR as well as in the NMR spectra.

Spectroscopic Characterization of the Copolymers. The two-step reaction could be easily monitored by spectroscopic methods. The activation of the PEG with HMDI, resulting in the urethane bond, could be followed by ^{13}C NMR. Two carbonyl signals, one for the urethane carbonyl at 156 ppm and the other for the free isocyanate group at 121 ppm, verified successful activation. Even more characteristic is the IR spectrum (Figure 1) which showed a very strong absorption for the isocyanate at 2274 cm^{-1} and one for the urethane at 1724 cm^{-1}. After linking the activated PEG with PEI, the urea carbonyl C gave a broad signal in the NMR spectrum at 158 ppm. This signal is broader than that of the urethane carbonyl signal, due to the many different amino groups in the branched PEI. The IR spectrum showed an absorption at 1620 cm^{-1} for the urea bond. The ^{1}H NMR signals for both the PEG protons and PEI protons appeared at different chemical

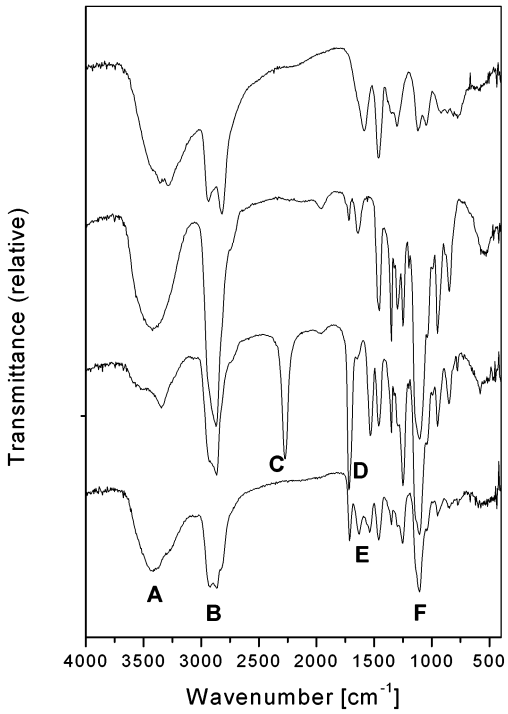


Figure 1. FT-IR spectra (from top to bottom) of PEI 1200, PEG 550, activated PEG 720, and the block copolymer PEI-(1200)-*g*-PEG(550)$_2$. Characteristic absorptions: (A) N−H amines stretching 3300 cm^{-1}, (B) C−H stretching 2921 cm^{-1}, (C) O═C═N isocyanate stretching 2274 cm^{-1}, (D) C═O urethane stretching 1724 cm^{-1}, (E) C═O urea stretching 1620 cm^{-1}, (F) C−O ether stretching 1106 cm^{-1}.

shifts, and the ratio of ethylene glycol/ethylenimine repeating units was calculated via integration. The signals for the methylene protons of the HMDI linker did not interfere with the polymer peaks and, therefore, did not disturb the calculation. The calculated content of PEG and PEI in the copolymer was in reasonable agreement with the homopolymer feed of the coupling reaction (Table 1). This calculation was also the basis for the nomenclature of the copolymers PEI(25K)-*g*-PEG(x)$_n$, where x is the MW of PEG (given by the supplier) and the index n represents the average number of PEG blocks per one PEI macromolecules. Note that in the case of PEI(25K)-*g*-PEG(20K)$_1$ the index was 1.4, which was rounded off to the integer 1.

Thermoanalytical Characterization of the Copolymers. Data derived from TGA and DSC are provided in the supplement and in Figure 2. A MW dependent degradation behavior was observed for the homopolymers. PEI 25 000 showed a maximum degradation at about 360 °C. Low MW PEGs 550 and 5000 degraded at lower temperatures (270−280 °C), whereas high molecular weight PEG 20 000 showed a maximum degradation at a higher temperature (about 390 °C). In the case of the copolymers, a characteristic two-step

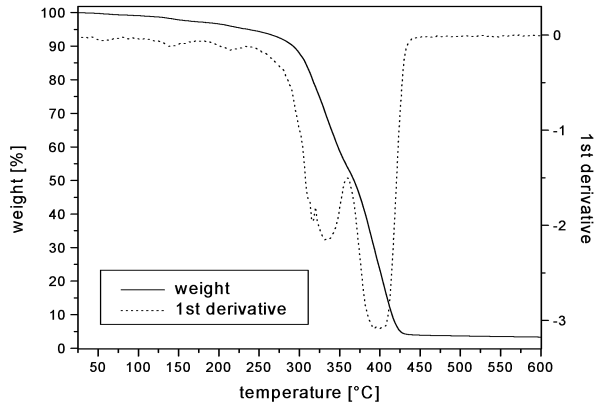


Figure 2. The two-step degradation of the PEI(25K)-*g*-PEG-$(5K)_6$ copolymer as monitored by TGA. The first degradation step is derived mainly from the PEG blocks and the second one from the PEI block.

degradation was observed as exemplarily shown for PEI(25K)-*g*-$PEG(5K)_6$ in Figure 2. In most cases, the PEG is degraded first at temperatures of about 330 °C. A second degradation step at 380 °C can be attributed to PEI degradation. Since both steps are close to each other and because of the relative high heating rate (20 K/min), it cannot be assumed that all of the PEG is degraded when the first amount of PEI begins to degrade. Therefore, the content of EG and EI in the copolymer cannot be derived from the TGA. This is especially true for PEI(25K)-*g*-$PEG(550)_{35}$, where degradation occurs over a broad temperature range and only one step can be seen. Here, the first derivative of the degradation curve indicates a maximum degradation at 350 °C. In the case of PEI(25K)-*g*-$PEG(20K)_1$, the first degradation step (333 °C) must be attributed to PEI and the second step to PEG (413 °C), using the data of the homopolymers as a reference.

DSC measurements showed an endothermic signal for PEG at its melting point, but no glass transition step. Again, a MW dependency was observed: The higher the MW of PEG, the higher T_m. The greatest change was observed from MW 550 (16 °C) to 5000 (63 °C). T_m increased only slightly with MW higher than 5000. PEI, instead, exhibited only one glass transition step at −52 °C. Because of its highly branched structure, it is completely amorphous. Blends were prepared by solvent evaporation of a solution with both homopolymers in the same constitution of the appropriate copolymer. DSC measurements of all combinations of PEI and PEG showed that the blend exhibited both the T_g of PEI as well as the T_m of PEG. Therefore, the two homopolymers do not interact with each other and do not form a thermodynamically stable mixture when they are blended. In contrast, the copolymers clearly showed a shift of T_g to higher values in comparison to the T_g of homopolymer PEI. The increased T_g of the copolymer might be due to H bonding between the protons of PEI and the free electrons of the PEG oxygen atoms, resulting in a stronger interaction between both blocks and therefore a loss of mobility (physical cross-linking). The shift of T_g to higher values is dependent on the amount of PEG. T_g increases from −53 °C for the PEI homopolymer over −39 °C (PEI(25K)-*g*-$PEG(5K)_2$) and −29 °C (PEI(25K)-*g*-$PEG(5K)_6$) to −18 °C (PEI(25K)-*g*-$PEG(5K)_{15}$) with increasing numbers of PEGs blocks. A remarkable shift of T_g is observed for the copolymer with many short PEG blocks: +35 °C for PEI(25K)-*g*-$PEG(550)_{35}$. In this case, the PEG is well mixed within the PEI and, therefore, can interact very well with the polyamine, thus resulting in a clear shift of the T_g.

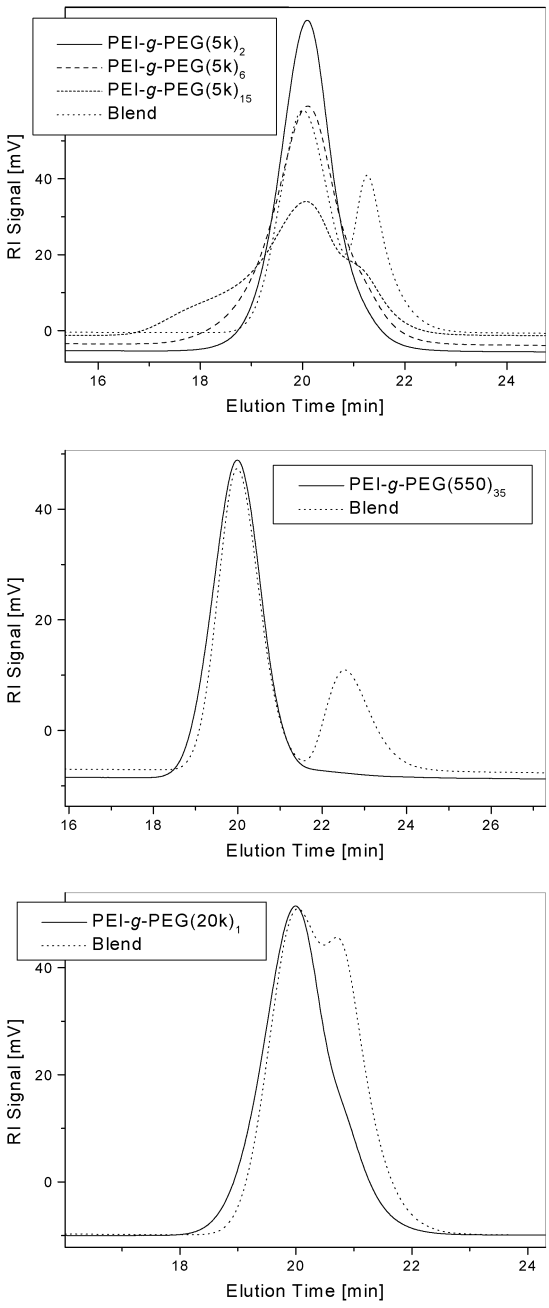


Figure 3. SEC eluograms of copolymers and blends in 1% formic acid. The signals shown were detected by a refractive index detector.

Molecular Weight Determination of the Polymers. The SEC eluograms (Figure 3) verified the successful coupling of the PEG blocks onto the PEI. Under the chosen conditions, blends of the homopolymers could be separated, even in the case of the similar molecular weight polymers PEG 20 000 and PEI 25 000. The acidic eluent caused a maximum expansion of the polycation, PEI, and allowed sufficient separation on the column. Additionally, since the column material itself was cationic, polycations were eluated earlier from the column, due to repulsive electrostatic forces than neutral polymers, such as PEG. In contrast to the blend, the copolymers exhibited only one signal, which had shifted to earlier elution time, thus indicating a higher molecular weight. It should be emphasized that, in the

case of the copolymers, PEG partly shielded the cationic charges of PEI so that the copolymers were retarded by the gel more efficiently in comparison to the homopolymer, PEI. As a consequence, the shift to an earlier elution time for the copolymers remained small despite the significant changes in MW as determined by MALLS. In two cases only asymmetrical signals were obtained. The copolymer PEI(25K)-*g*-PEG(5K)$_{15}$ gave a signal which showed an asymmetrical expansion at a later elution time. This can be explained by an amount of free PEG 5000, which did not react with PEI. The amount of the free PEG could be calculated by the integrals of the ^{1}H NMR signals (HO−CH_2−CH$_2$−O−, free PEG, 3.47 ppm and −NH−C(O)−O−CH_2−CH$_2$−O−, coupled PEG, 3.60 ppm) and was found to be 14%. It seems to be a sterical problem to graft 15 PEG blocks of MW 5000 onto a PEI 25 000. Hence, it must be taken into account that the nomenclature of PEI(25K)-*g*-PEG-(5K)$_{15}$ is not quite correct and that the number of PEG blocks is probably less than 15. However, because of its lack of toxicity, very low immunogenicity, and antigenicity,[19] the amount of free PEG is not problematic for the gene delivery application of the copolymers and can be tolerated. The copolymer PEI(25K)-*g*-PEG(20K)$_1$ also showed an asymmetrical expansion of its signal, which again may be explained by a small amount of residual PEG. In this case, the problem was probably due to the disadvantageous terminal group/repeating units ratio, which resulted in an insufficient activation step with HMDI, despite the stronger reaction conditions. The asymmetry of the signal can hardly be detected, suggesting that the amount of free and residual PEG is very low in this copolymer and can be neglected. The MW was determined by static light scattering, and the data are listed in the Supporting Information. The refractive index increments d*n*/d*c* of the copolymers were found to be between the values for the homopolymers: the higher the PEI content, the higher d*n*/d*c*. M_n is close to the calculated MW of the polymers. The measured M_w was always too high, except for the PEGs. This can be explained by the formation of few large aggregates generating intense light scattering signals. The polydispersity index (M_w/M_n) was about 2.5 for the copolymers and 1.3 for PEI. The PEGs showed very low polydispersities because the column could not separate these samples sufficiently, and the light scattering detector analyzed the poorly fractionated polymer.

Cytotoxicity. In vitro cytotoxicity of the copolymers was studied in the 3T3 mouse fibroblasts cell line in a PEI concentration range that represented the conditions of in vitro transfection experiments. The LDH assay provides information about the destructive impact of the polymers on the cell membranes. In a reference experiment without polymer (only PBS) it was ascertained that the cells are stable for the duration of the LDH experiment. The results are shown in Figure 4. A reasonable concentration dependency was found for all samples. With increasing polymer concentration the toxicity increased. Linear regression gave correlation coefficients (r) of about 0.95 ($r^2 = 0.90$) for all polymers except for the low-toxicity PEI(25K)-*g*-PEG(5K)$_{15}$. This copolymer showed, however, only a poor correlation was found ($r = 0.66$, $r^2 = 0.44$). In the series of PEI(25K)-*g*-PEG(5K)$_n$ copolymers, toxicity was reduced as the number n of PEG blocks increased. Whereas from $n =$ 0 to 2 the decrease was not significant, PEGylation with 6 and 15 PEG 5000 blocks significantly reduced the

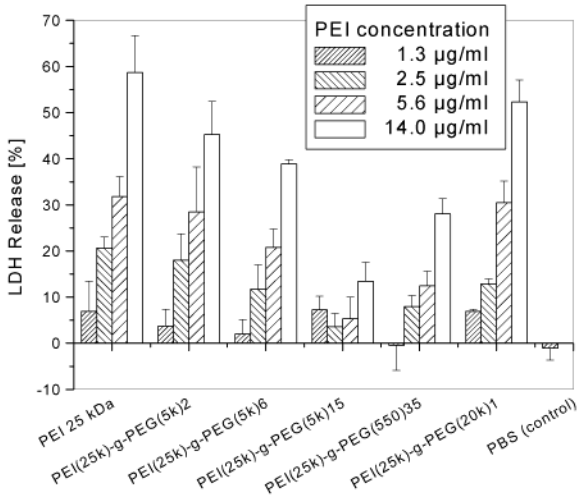


Figure 4. LDH assay of homopolymer PEI 25 000 and copolymers after an incubation period of 4 h at different concentrations of PEI.

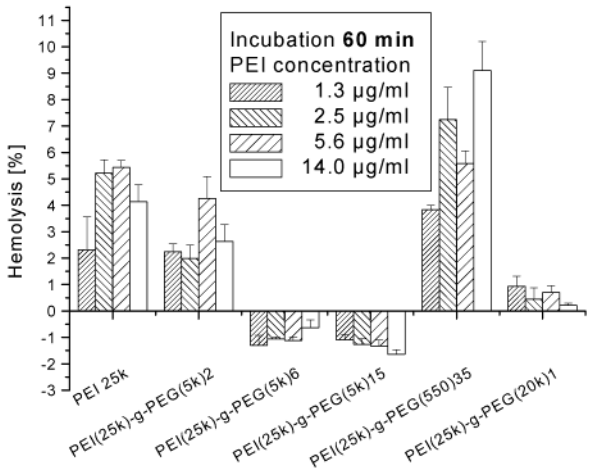


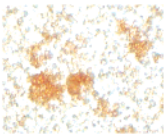
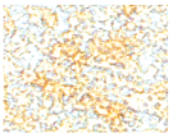
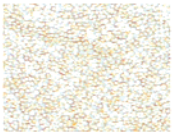
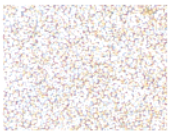
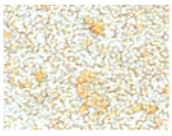
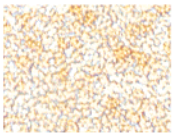
Figure 5. Hemolysis of homopolymer PEI 25 000 and PEI-*g*-PEG copolymers after an incubation period of 60 min.

toxicity of PEI at a high polymer concentration ($c = 14$ μg/mL). Another trend was found for the second series. An increase in the MW of PEG from PEG 550 to PEG 20 000 increased the cytotoxicity significantly. While the copolymer PEI(25K)-*g*-PEG(550)$_{35}$ showed a relative low toxicity, PEI(25K)-*g*-PEG(20K)$_1$ exhibited a toxicity comparable to that of PEI and PEI(25K)-*g*-PEG(5K)$_2$. This suggests that excessive membrane destruction always occurs when the cationic domain is accessible, as in the case of no or only a few PEG blocks. When the cationic bock of the copolymer is shielded by many PEG blocks, as in the case of PEI(25K)-*g*-PEG(550)$_{35}$ and PEI(25K)-*g*-PEG(5K)$_{15}$, the copolymer does not exhibit cell membrane toxicity.

Blood Compatibility. The interaction of the polymers with erythrocytes was studied using both a hemolysis and aggregation assay, which were performed with red blood cells of rats.

The results of the hemolysis assay are shown in Figure 5. In a short-term experiment (incubation 2 min, data shown in the Supporting Information), the acute stress of an intravenous administration was simulated. No significant hemolysis (<2%) was observed for all copolymers during this short incubation time. Only PEI 25 000 showed a slight hemolytic activity (2−4.5%). The long-term experiment (60 min incubation) also showed

Table 2. Semiquantitative Results of the Erythrocyte Aggregation Induced by Homopolymer PEI and Copolymers PEI-*g*-PEG at Different PEI Concentrations[a]

PEI conc. [μg/mL]	PEI 25,000	PEI(25k)-*g*-PEG(5k)$_2$	PEI(25k)-*g*-PEG(5k)$_6$	PEI(25k)-*g*-PEG(5k)$_{15}$	PEI(25k)-*g*-PEG(550)$_{35}$	PEI(25k)-*g*-PEG(20k)$_1$
1.3	+	-	-	-	+	-
2.5	++	++	-	-	+	++
5.6	+++	+++	+	-	++	++
14.0	++++	+++	+	-	+++	++
5.6						

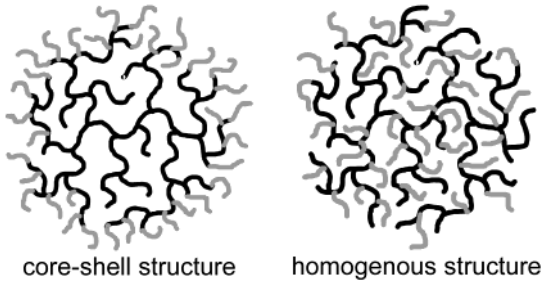
[a] Classification: −, no aggregation; +, weak aggregation; ++, medium aggregation; +++, strong aggregation; ++++, very strong aggregation. Images were taken by phase contrast light microscopy at a PEI concentration of 5.6 μg/mL.

a negligible rate of hemolysis (<6%) for PEI and most copolymers. Only PEI(25K)-*g*-PEG(550)$_{35}$ exhibited a higher value (9.1 ±1.1%) at high concentrations.

The erythrocyte aggregation assay allowed only semiquantitative estimations about the blood compatibility of the polymers. Results of this study are given in Table 2. Exemplary microscopic images of the erythrocytes are shown in this table for the polymer concentration, 5.6 μg/mL. Once again, the aggregation activity was concentration-dependent. PEI and PEI(25K)-*g*-PEG(5K)$_2$ showed a very strong aggregation of the red blood cells. More than two PEG blocks were necessary to prevent aggregation, as shown for the copolymers with 6 and 15 PEG 5000 blocks. The copolymers PEI(25K)-*g*-PEG-(20K)$_1$ and PEI(25K)-*g*-PEG(550)$_{35}$ exhibited a medium strong aggregation. The latter copolymer showed a strong aggregation at high concentrations, which is consistent with the results of the hemolysis assay (long-term experiment). Aggregation is caused by electrostatic interactions between the anionic cell membranes and the cationic polymer. Therefore, when the cationic block of the copolymer is sufficiently shielded by the PEG blocks, aggregation is prevented. In the case of the diblock copolymer, such as PEI(25K)-*g*-PEG(20K)$_1$, the cationic block still seems to be accessible, despite 54% PEG. The single PEG block is not able to shield the entire cationic PEI block.

The many extraordinary results for PEI(25K)-*g*-PEG-(550)$_{35}$, such as the extreme shift of the T_g in the DSC experiment, the single, but broad, degradation step in TGA, and the strong hemolysis and aggregation activity, might be explained by the synthesis of the copolymers. PEI is a highly branched and, therefore, very dense and compact polymer. The high density is verified by viscosimetry. The Mark−Houwink parameter, α, is very small for branched PEI (α = 0.26[20]). The activated PEG 5000 and 20 000 probably cannot penetrate into the PEI macromolecule during the coupling reaction. Thus, they have to react with amino groups at the outer sphere. This resulted in a clear core−shell structure, in which the PEG blocks shield the cationic PEI domain. In contrast, the PEG 550 might be small enough to penetrate into the PEI macromolecule and can, therefore, react not only with outer-sphere amino groups but also with inner amino groups. Thus, in this case, a pure core−shell structure (Scheme 3: left structure) might be unlikely. The PEG is probably homogeneously distributed throughout the entire PEI macromolecule (Scheme 3: right structure).

Scheme 3. Two Possible Copolymer Structures of PEI(25K)-*g*-PEG(550)$_{35}$[a]



[a] The results reported in this paper suggest that the PEG (gray) is more likely homogenously distributed within the PEI (black) than concentrated at the outer sphere of the PEI (core−shell structure).

In this study we investigated the biocompatibility of the free polymers as a worst case scenario. In the transfection experiments these cationic polymers are complexed with DNA which will alter their cytotoxicity. However, the biocompatibility of the free polymers is interesting for two reasons: First, not all of the cationic macromolecules participate in complex formation with DNA.[21] This is especially true when a higher excess of the polycation is used for in vitro gene transfer experiments to yield positive net charges of the complexes. Second, one of the last steps of the complex gene delivery process is assumed to be complex dissociation to release the DNA from the complex.[22] This step has not been thoroughly studied so far, but it is likely that during complex dissociation at least part of the polycations will also be set free.

In comparison to the homopolymer PEI, the PEG−PEI copolymers generally showed an improved cytotoxicity profile under in vitro conditions and are, therefore, promising candidates for further in vivo studies as potential nonviral gene delivery systems.

Conclusions

We have shown an elegant two-step route to synthesize PEI-*g*-PEG block copolymers using HMDI. In the first reaction step, the activation of PEG, an excessive application of HMDI, prevented the formation of side products and helped to reach complete conversion of the terminal hydroxy group of PEG. In this manner, the activated PEG exhibits a highly reactive isocyanate terminal group for the coupling reaction with PEI, the second step. Because of the high reactivity of the

isocyanate, all of the PEG blocks reacted with PEI and the resulting copolymer could be easily isolated without any time-consuming separation and purification steps. DSC and SEC verified the formation of copolymers and excluded the existence of blends. ^{13}C NMR and FTIR spectroscopy verified the molecular structure. Finally, via ^{1}H NMR analysis the exact constitution of the copolymers could be calculated and was in reasonable agreement with the homopolymer feed used in the synthesis. Because of the urethane and urea bonds, the copolymers are very stable against hydrolysis and aqueous solutions can be stored for a long time.

To summerize, we got a set of fully characterized copolymers, which have enalbed us to study the influence of PEG on the condensation of DNA with PEI and on the transfection ability of these complexes. This investigations have already been performed in our laboratories and will be published very soon elsewhere.

Acknowledgment. We acknowledge the Deutsche Forschungsgemeinschaft (DFG) for generous support. We also thank D. Braga (Department of Chemistry, University of Marburg) for DSC and TGA measurements and C. Culmsee (Inst. of Pharmacology, University of Marburg) for the rat blood used in this study.

Supporting Information Available: Table of physical data of PEI, PEG, and PEI(25K)-*g*-PEG and figure showing hemolysis of PEI 25 000 and PEI-*g*-PEG. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Lemieux, P.; Vinogradov, S. V.; Gebhart, C. L.; Guerin, N.; Paradis, G.; Nguyen, H.-K.; Ochietti, B.; Suzdaltseva, Y. G.; Bartakova, E. V.; Bronich, T. K.; St-Pierre, Y.; Alakhov, V. Yu.; Kabanov, A. V. *J. Drug Targeting* **2000**, *8*, 91−105.

(2) Kabanov, A. V.; Kabanov, V. A. *Adv. Drug Delivery Rev.* **1998**, *30*, 49−60.

(3) Toncheva, V.; Wolfert, M. A.; Dash, P. R.; Oupicky, D.; Ulbrich, K.; Seymour, L. W.; Schacht, E. H. *Biochim. Biophys. Acta* **1998**, *1380*, 354−368.

(4) Choi, Y. H.; Lui, F.; Kim, J.-S.; Choi, Y. K.; Park, J. S.; Kim, S. W. *J. Controlled Release* **1998**, *54*, 39−48.

(5) Ngyugen, H.-K.; Lemieux, P.; Vinogradov, S. V.; Gebhart, C. L.; Guerin, N.; Paradis, G.; Bronich, T. K.; Alakhov, V. Y.; Kabanov, A. V. *Gene Ther.* **2000**, *7*, 126−138.

(6) Kabanov, A. V.; Vinogradov, S. V.; Suzdaltseva, Y. G.; Alakhov, V. Y. *Bioconjugate Chem.* **1995**, *6*, 639−643.

(7) Read, M. L.; Toncheva, V.; Schacht, E. H.; Ulbrich, K.; Seymour, L. W. *Eur. J. Pharmacol. Sci.* **2000**, *10*, 169−177.

(8) Kataoka, K.; Harada, A.; Wakebayashi, D.; Nagasaki, Y. *Macromolecules* **1999**, *32*, 6892−6894.

(9) Vinogradov, S. V.; Bronich, T. K.; Kabanov, A. V. *Bioconjugate Chem.* **1998**, *9*, 805−812.

(10) Oupicky, D.; Konak, C.; Dash, P. R.; Seymour, L. W.; Ulbrich, K. *Bioconjugate Chem.* **1999**, *10*, 764−772.

(11) Maruyama, A.; Katoh, M.; Ishihara, T.; Akaike, T. *Bioconjugate Chem.* **1997**, *8*, 3−6.

(12) Gonzalez, H.; Hwang, S. J.; Davis, M. E. *Bioconjugate Chem.* **1999**, *10*, 1068−1074.

(13) Remy, J.-S.; Abdallah, B.; Zanta, M. A.; Boussif, O.; Behr, J.-P.; Demeneix, B. *Adv. Drug Delivery Rev.* **1998**, *30*, 85−95.

(14) Erbacher, P.; Bettinger, T.; Belguise-Valladier, P.; Zou, S.; Coll, J.-L.; Behr, J.-P.; Remy, J.-S. *J. Gene Med.* **1999**, *1*, 210−222.

(15) Ogris, M.; Brunner, S.; Schüller, S.; Kircheis, R.; Wagner, E. *Gene Ther.* **1999**, *6*, 595−605.

(16) Bronstein, L. M.; Sidorov, S. N.; Gourkova, A. Y.; Valetsky, P. M.; Hartmann, J.; Breulmann, M.; Cölfen, H.; Antonietti, M. *Inorg. Chim. Acta* **1998**, *280*, 348−354.

(17) Choi, J. H.; Choi, J. S.; Suh, H.; Park, J. S. *Bull. Korean Chem. Soc.* **2001**, *22*, 46−52.

(18) Kissel, T.; Petersen, H.; Fischer, D.; Kunath, K.; von Harpe, A. DE 199 33 024 A1 (1999), patent pending WO 01/05875 A1/EP 00/06214 (2000).

(19) Dreborg, S.; Akerblom, E. B. *Crit. Rev. Ther. Drug Carrier Syst.* **1990**, *6*, 315−365.

(20) Harpe, A. v.; Petersen, H.; Li, Y.; Kissel, T. *J. Controlled Release* **2000**, *69*, 309−322.

(21) Godbey, W. T.; Wu, K. K.; Hirasaki, G. J.; Mikos, A. G. *Gene Ther.* **1999**, *6*, 1380−1388.

(22) De Smedt, S. C.; Demeester, J.; Hennink, W. E. *Pharm. Res.* **2000**, *17*, 113−126.

MA012060A