Structurally Modified Polyethylenimines and their Interpolyelectrolyte Complexes with DNA as Non-Viral Gene Delivery Systems

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Structurally Modified Polyethylenimines and their Interpolyelectrolyte Complexes with DNA as Non-Viral Gene Delivery Systems

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Die vorliegende Arbeit

entstand auf Anregung und unter der Leitung von

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„Ich wette mit ihnen:

Es ist viel schwieriger Viren zu zähmen,

als Polymere intelligenter zu machen.“

T. Kissel

Arbeitskreis-Seminar, 4. August 1998

Claudia

und meinen Eltern

in Liebe und Dankbarkeit

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Preface

General Introduction, Aims and Organization of this Thesis

General Introduction

It was the outcome of research efforts in the last decade that genes as molecular information units can be considered as "prodrugs" generating specific proteins with therapeutic potential. The completion of sequencing the human genome by the international Human Genome Project and Celera Genome Corporation [1] will very likely accelerate the development of new therapeutic genes. Therefore, gene therapy is likely to become more and more important for the treatment of a wide variety of diseases on a molecular basis.

However, there is a need for the development of a parenteral dosage form for this new class of prodrugs that allows a save and reliable application of the gene for the human patient. Furthermore, in the field of somatic gene therapy it often is crucial to treat exclusively a special tissue or organ. Thus, targeting issues are also important. Therefore, gene delivery has become an important field in pharmaceutics.

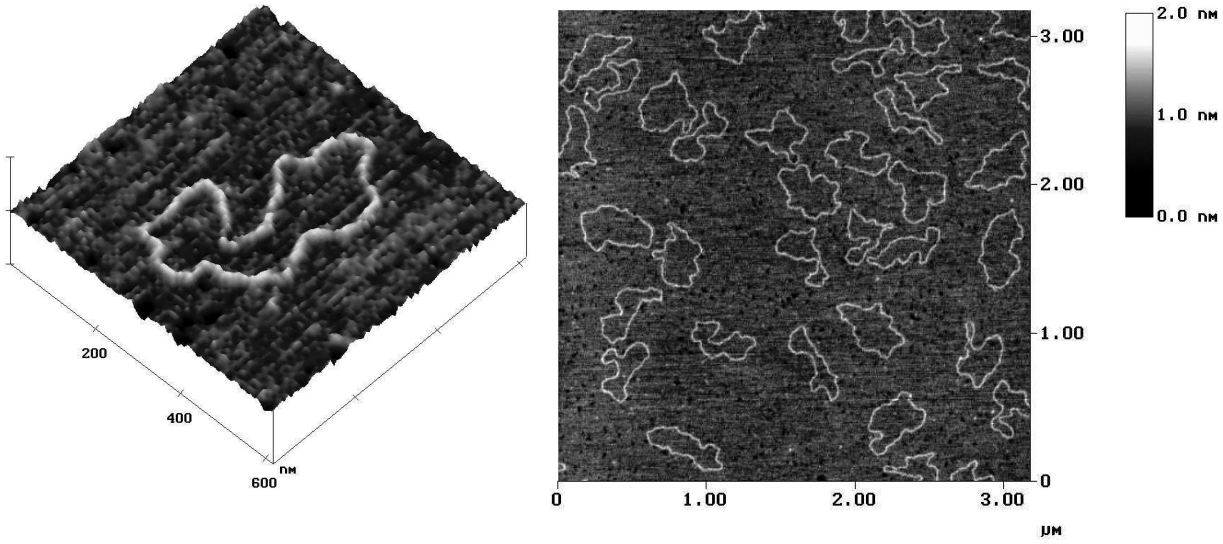

Figure 1: A typical prodrug of the 21st century? AFM images of a plasmid DNA (in this case: pBR322, 4363 bp). [reprinted with kind permission of A. L. Martin, University of Nottingham].

A dosage form for genes has to meet at least the following three requirements. First, a therapeutic gene is often incorporated into an plasmid. These plasmids exhibit a ring-form of the double-helix of DNA and are about 500 nm in size (Figure 1). Since this size is too large for a sufficient blood circulation and for the transport through the diverse cellular barriers [2], the DNA has to be packaged into a smaller unit. A second crucial feature is, that the DNA needs to be protected against enzymatic attacks. The open ring form of the plasmid is easily degraded by DNase [3,4]. The packaged form should compact the DNA so that enzymes do

no longer have access to the plasmid. Third, the dosage form should allow a chemical modification with special home devices or targeting molecules to address the targeted tissue.

Viruses have a natural ability to deliver genes and have, therefore, been used for gene delivery by many groups [5]. They might be understood as a natural capsule-like dosage form for the gene. Although viral gene transfer has been shown to be highly efficient in transfection, current viral based therapies might have inherent problems including immune and toxic reactions as well as the potential for viral recombination [6]. Beyond these safety concerns, the disadvantages of viruses are their limited capacity for DNA (limit for adenovirus: 7.5 kb [7], limit for adeno-associated virus: 2.5 kb [8] or 4.5 kb[9]) and the difficulties in chemical modifications for targeting reasons.

As a consequence, non-viral gene delivery vehicles have been developed as an alternative to viruses that are less expensive, easier and safer to synthesize, and can be stored for relatively long periods of time without special conditions. It was found that almost every cationic species such as inorganic cations (e.g. $Co(NH_3)_6^{3+}$ [10]) or biogenic oligoamines (e.g. spermidine^{3+}, spermine^{4+} [11]) are able to form complexes with the negatively charged DNA and a vigorous search for new cationic species with a possible potential for gene delivery is still ongoing. For instance, even inorganic materials such as anionic clays with cationic brucite-like layers have recently been applied for gene transfer [12]. However, most prominent species for a non-viral gene therapy are cationic liposomes [13] and cationic polymers [14].

Cationic polymers seem to play a key role in the development of gene transfer agents due to their extraordinarily good potential to condense DNA [15]. The polycation-induced DNA condensation is generally assumed to be an entropy-driven process [16]. The complexation of a high MW polycation with DNA is thermodynamically favored over the complexation with low MW cations since the change of entropy during complex formation is much higher if the new counterion of the DNA is large releasing a large number of low MW counterions. As a consequence, recent development of liposome-based gene delivery uses polycations to condense the DNA. In a second step, this polymer/DNA complex is encapsulated by a liposome formulation leading to a so-called liposome entrapped polycation condensed DNA [17]. A similar strategy was to incorporate the polymer/DNA complexes in biodegradable nano- and microspheres for a controlled release of the complex [18]. It should also stressed here that even viruses use “polycations” for DNA condensation. Viral proteins with a large amount of basic and, therefore, cationically charged amino acids act as DNA condensing agents [19].

To summarize, the polycation-induced DNA condensation is one of the key features for all types of gene delivery. To gain a better understanding of the mechanism of gene transfer it is well worth to concentrate the research efforts in the field on the polycation/DNA complexes since efficient gene transfer can not occur without polycations.

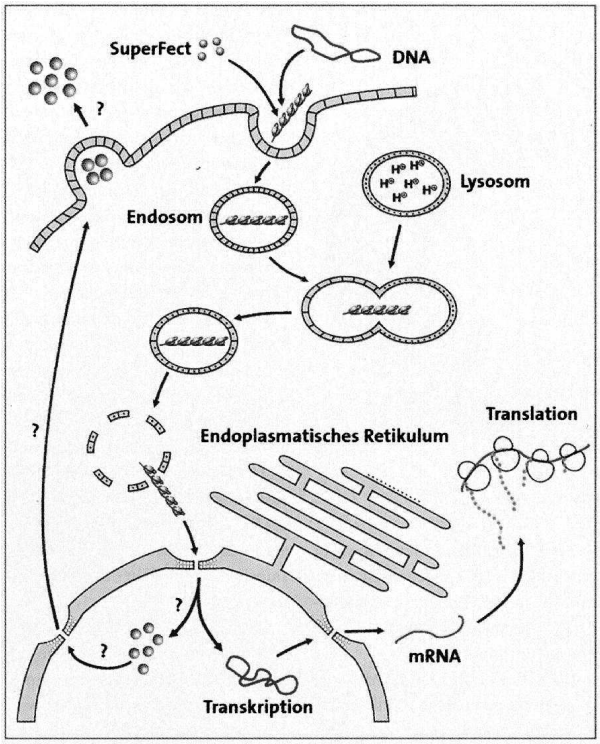

Figure 2: Gene delivery and gene expression. SuperFect™ is the brand name of fractured PAMAM dendrimers. The figure was adapted from M. Weber (*Nachrichten aus der Chemie*, **2000**, *48*, 18-23.).

Synthetic polycations used for gene delivery have been reviewed in [20] and [21]. Whereas for most of these polycations DNA complexation was observed, only very few have shown activity leading to gene expression. The most effective polymeric gene transfer vectors presently known are fractured polyamidoamine dendrimers (PAMAM) [22] and polyethylenimines (PEI) [23]. These two polymers share the feature of a hyperbranched structure. As a result, the density (polymer mass / hydrodynamic volume) is much higher for these polymers in comparison with linear polymers. As a consequence, the cationic charge density of this hyperbranched polycations is also much higher than the charge density of linear polycations with the same primary molecular structure. Moreover, a crucial aspect for their efficiency seems to be the fact that both polycations are not fully protonated at physiological pH. Therefore, they possess a buffer capacity. Their high potential to deliver genes is likely to be linked to their unique endosome-disruptive mechanism also designated as proton-sponge-effect [24]. After endocytosis of the polycation/DNA complexes, PEI and PAMAM dendrimers will capture protons in the endosomal compartment when the pH drops on its route to the lysosomal stage. It is hypothesized that swelling and disruption of the

endosomes finally occur due to water entry as a consequence of the net increase in ion concentration and expansion of the PEI or dendrimer by internal charge repulsion. This allows the application of these polycations without additional endosome-disruptive agents as it is necessary for many other linear polycations such as PLL [25]. The hypothetical pathway of the polycation/DNA complex is shown in Figure 2. It should be emphasized that many steps of this gene delivery process are still under investigation.

In our group we focus on the use of PEI and its derivatives for gene delivery. Inspite of the many advantages of PEI over the other polycations, it still shares two disadvantages that have been observed for all polycations. First, the solubility of a polycation/DNA complex is very poor at neutral net charge and strong aggregation occurs leading to very large particles which precipitate both under *in vitro* and *in vivo* conditions [26]. Second, only high molecular weight PEI showed sufficient gene transfection. Low molecular weight PEI were almost inactive in gene transfection experiments [27]. On the other hand low MW PEI is much less cytotoxic than high MW PEI and would be, therefore, more suitable for medical application [28]. To overcome the first drawback, the polycation was grafted with PEG by several groups which enhanced the solubility of the complex significantly [for a review: 29]. However, in some cases the PEGylation led to poor DNA condensation [30,31] and consequently to reduced transfection activities [32,33]. Other groups reported conflicting results and found an enhanced transfection activity for the PEGylated polycations [34]. The second problem, the poor transfection activity of low MW PEI has not been addressed in depth. Enhance transfection activity of low ME PEI by adding an endosome-disruptive agent [35] was reported recently. Third and finally, PEI is not biodegradable. For a long term gene therapy biodegradability is a prerequisite to avoid accumulation of the polycation in the body. So far, few attempts have been reported to generate biodegradable polycations, e. g. the degradation of PAMAM polymers has been investigated [36]. Only scant information exists in the literature about biodegradable polycations for gene delivery [37,38] in general and no hydrolytically degradable PEI derivative have been reported to our knowledge.

Aims of this thesis

The research described in this thesis was aimed at identifying how structural modifications of the polycation, PEI, affect the condensation of nucleic acids and the biological activity of the PEI/DNA complexes.

The key modification of PEI is grafting the polycationic with PEG. Since a systematic study of the PEGylation of PEI has not been reported yet and conflicting results about the gene delivery potential of PEGylated polycations have been described in the literature it was one aim of this thesis to address this task and to supply a set of data which allows to understand the influence of PEG on the polymer-induced DNA condensation. This study should allow a more rational design of a PEGylated polycation as delivery vehicle for gene transfer.

Further, a second task was to understand the poor transfection activity of low MW PEI and to overcome this drawback by an appropriate strategy.

Third, a biodegradable PEI should be developed and the degradation mechanism and profile should be investigated.

Organization of this thesis

In **Chapter 1** the synthesis and characterisation of PEGylated PEIs is reported. Here a new strategy to couple PEG and PEI blocks using diisocyantes is described. A set of copolymers were obtained which were thoroughly characterized and the biocompatibility of these copolymers was evaluated.

In **Chapter 2** the polymer/DNA complexes of the copolymers from Chapter 1 are described. Here, the influence of PEG on the PEI-induced DNA condensation and the biological activity of these complexes is reported.

Chapter 3 describes another synthesis route for the formation of PEG-*block*-PEI copolymers: the macrostopper-route. This new synthesis concept involving the ring-opening polymerization of ethylenimine is introduced, and it is demonstrated how this synthesis lead exclusively to diblock copolymers. In this chapter it is also shown that these diblock copolymers result in enhanced DNA condensation compared to conventionally PEGylated PEIs.

In **Chapter 4** the poor DNA condensation potential of low molecular weight (MW) PEI is reported. It is also demonstrated how the DNA condensation is significantly enhanced when star-shaped PEG-*block*-PEI copolymers are formed composed of the low MW PEIs and star-PEGs.

Chapter 5 describes the synthesis of a biodegradable PEI derivative. The polymers is physicochemically characterized and its hydrolytic degradation is reported as a function of

time and pH. Further, cytotoxicity and transfection activity of the degradable polycation is shown in *in vitro* experiments.

All the synthesized polymers described in this thesis had to be characterized. Therefore, in **Chapter 6** the determination of the molecular weight and the degree of branching of PEI and its derivatives by size exclusion chromatography in combination with light scattering analysis is described.

Finally, in the **Conclusions** the results of this thesis are summarized and put into perspective.

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Chapter 1

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

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(Accepted by Macromolecules)

[a] LDH assay, erythrocyte aggregation, hemolysis assay

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 (5,000). 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.

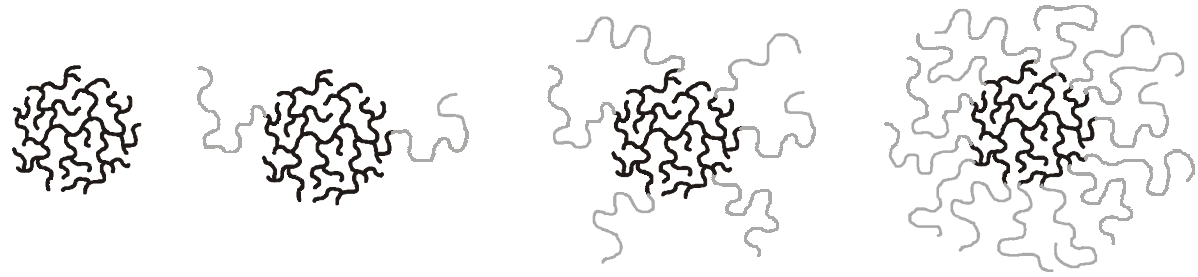
Introduction

Block and graft copolymers containing cationic polyelectrolytes and non-ionic hydrophilic polymers gained significant interest as non-viral gene delivery systems.[1,2] The task of the cationic block is to condense nucleic acids, whereas the non-ionic, 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 non-ionic hydrophilic polymers, poly(ethylene glycol)[9] (PEG), poly[N-(2-hydroxypropyl) methacrylamide][10] (HPMA) and polysaccharides as dextran[11] and cyclodextrin[12] were studied. Despite of the large numbers of publications about the use of these copolymers for gene delivery, a systematic investigation of the influence of the non-ionic hydrophilic blocks on the condensation process of a polycation with nucleic acids has not been properly performed yet. In order to perform such a systematic study a comparable set of several copolymers should be available. Furthermore, to study the influence of the non-ionic 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 (PEG) served as non-ionic polymers in our study. In a first series, we varied the number of PEG blocks which were all of MW 5,000, as presented in Scheme 1 (A). The second series was produced with PEGs of different MW. However, the composition of the copolymer remained constant, composing of approximately 50 % PEI and 50 % PEG (see Scheme 1 (B)).

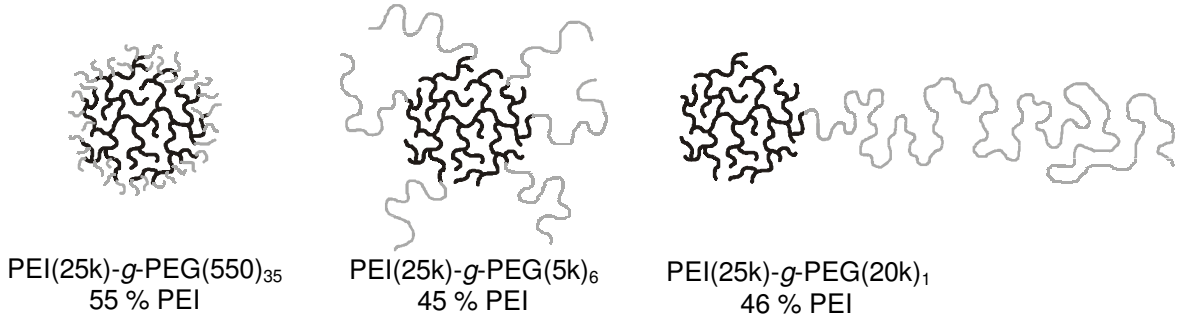
Several routes of synthesis for PEI-g-PEG copolymers have been described in the literature. In most cases, well-defined homopolymer blocks were linked together. Some of the authors[5,14,15] employed commercially available activated PEGs which could be directly used for the coupling reaction with PEI. The groups which 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, as well as the influence of PEG on the cytotoxicity of PEI.

A : First Series



B : Second Series



Scheme 1: Schematic representation of the two copolymer series used in this study. The black component of the structure represents the cationic branched PEI and the grey component the non-ionic 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.

Experimental Section

Materials.

Branched polyethylenimine (PEI) with MW of 25,000 (Polymin^{TM} water free 99%) was a gift from BASF. PEI of MW 1,200 (99%) was purchased from Polysciences. PEG-monomethylether (mPEG) 550 and mPEG 5,000 were obtained from Aldrich and monoamino-PEG-monomethylether (mPEG-NH_2) 20,000 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 distilled 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 reflux condenser and oil bubbler 4.00 g of mPEG 550 were dissolved in 10 mL CH_2Cl_2. 10 mL of HMDI were added and the mixture was heated under reflux for 8 h. The polymer was precipitated in 250 mL petrol. The oily precipitate was washed with further 100 mL petrol, redissolved in 20 mL CH_2Cl_2 and precipitated in 250 mL 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 were obtained with a 15 % yield. ^{1}H-NMR ($CDCl_3$): δ = 0.79 (m, OCN(CH_2)$_3$CH_2-, 2H), 0.84 (m, OCN(CH_2)$_2$CH_2-, 2H), 0.96 (m, OCN(CH_2)$_4$CH_2-, 2H), 1.07 (m, OCNCH_2CH_2-, 2H), 2.59 (t, OCN(CH_2)$_5$CH_2-, 2H), 2.77 (t, OCNCH_2-, 2H), 2.82 (s, -OCH_3, 3H), 3.00 (m, -NHC(O)OCH_2CH_2O-, 2H), 3.10 (s, -OCH_2CH_2O-, 60H), 3.63 (t, -NHC(O)OCH_2-, 2H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 13.9 (OCN(CH_2)$_3$CH_2-), 25.4 (OCN(CH_2)$_2$CH_2-), 29.1 (OCN(CH_2)$_4$CH_2-), 30.4 (OCNCH_2CH_2-), 39.9 (OCN(CH_2)$_5$CH_2-), 42.1 (OCNCH_2-), 58.1 (OCH_3), 62.9 (-NHC(O)OCH_2-), 68.8 (-NHC(O)OCH_2CH_2O-) 69.8 (-OCH_2CH_2O-), 71.1 (-CH_2OCH_3), 121.3 (-NCO), 155.8 (-NHC(O)O-) ppm. 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 (w) cm^{-1}.

Activation of mPEG 5,000. Using the same procedure as described above, 15.23 g of mPEG 5,000 were dissolved in 15 mL $CHCl_3$ in a 100 mL flask and treated with 60 mL HMDI for 24 h under reflux. The polymer was precipitated in 750 mL petrol. The light yellow precipitate was washed three times with 400 mL petrol, redissolved in 20 mL $CHCl_3$ and precipitated in 500 mL petrol again. Reprecipitation was repeated 10 times before the polymer was isolated and dried *in vacuo*. 8.33 g of a white solid were 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 were dissolved in 20 mL $CHCl_3$ in a 50 mL flask and treated with 5 mL HMDI for 72 h under reflux. The polymer was precipitated in 250 mL petrol. The white precipitate was washed with 250 mL petrol, redissolved in 10 mL $CHCl_3$ and precipitated in 250 mL petrol again. Reprecipitation was repeated 6 times before the polymer was isolated and dried *in vacuo*. 1.38 g of a white solid were obtained with a 92 % yield.

Synthesis of PEI(25k)-g-PEG(550)$_{35}$. 0.80 g of PEI 25,000 were dissolved in 80 mL $CHCl_3$ and 0.77 g of activated PEG 550 were dissolved in 100 mL 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 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 PEI 25,000 in 150 mL $CHCl_3$; 0.73 g activated PEG 5,000 in 50 mL $CHCl_3$. Reaction time: 24 h. Yield: 2.03 g (69 %); yellowish waxy solid.

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

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

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

Synthesis of PEI(1200)-g-PEG(550)$_2$: 1.53 g PEI 1,200 in 80 mL $CHCl_3$; 1.83 g activated PEG 550 in 80 mL $CHCl_3$. 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)$_{2\ and\ 3}$CH_2-, 4H), 0.93 (s, -NHC(O)NH(CH_2)$_{1\ and\ 4}$CH_2-, 4H), 1.96 – 2.28 (m, -NCH_2CH_2N-, 43H), 2.54 (s, OCN(CH_2)$_{0\ and\ 5}$CH_2-, 4H), 2.79 (s, -OCH_3, 3H), 2.98 (m, -NHC(O)OCH_2CH_2O-, 2H), 3.07 (s, -OCH_2CH_2O-, 62H) 3.60 (s, -NHC(O)OCH_2-, 2H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 13.5 (-NHC(O)NH(CH_2)$_3$CH_2-), 25.1 (-NHC(O)NH(CH_2)$_2$CH_2-), 28.5 (-NHC(O)NH(CH_2)$_4$CH_2-), 29.0 (-NHC(O)NHCH_2CH_2-), 38.3 (H_2NCH_2CH_2N<), 39.3 (-NHC(O)NH(CH_2)$_5$CH_2-), 39.5 (-NHC(O)NHCH_2-), 40.1 (H_2NCH_2CH_2NH-), 46.2 (-NHCH_2CH_2N<), 48.0 (-NHCH_2CH_2NH-), 50.9 (-HNCH_2CH_2NH2), 51.6 (>NCH_2CH_2N<), 53.3 (>NCH_2CH_2NH-), 56.0 (>NCH_2CH_2NH_2), 57.5 (-OCH_3), 62.1 (-NHC(O)OCH_2-), 68.1 (-NHC(O)OCH_2CH_2O-) 69.1 (-OCH_2CH_2O-), 70.5 (-CH_2OCH_3), 155.3 (-NHC(O)O-), 158.1 (-NHC(O)NH-) ppm. 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 (w) cm^{-1}.

Techniques.

Size Exclusion Chromatography in combination with Multiple Angle Laser Light Scattering (SEC-MALLS). 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 degased with a 4 channel online vacuum degaser DDG-75 from Duratec, Reilingen, Germany. Additionally, an inline filter (0.45 µm, stainless steel, Rheodyne, Cotati, CA, USA) 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 of 2 – 30 g/l and 20 µL were 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 approximately 10 mg polymer sample. Scanning rate was 20 K/min and thermograms were recorded within temperature range of 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 – 120 °C). Glass transition (T_g) and melting temperature (T_m) are derived from the 2nd heating curve.

Lactate dehydrogenase (LDH) assay. *In vitro* cytotoxicity of the polymers was evaluated by LDH assay with 3T3 mouse fibroblasts. 500,000 cells per well (12 well plate) were seeded in 2 mL Dulbecco's modified eagle medium (DMEM) and cultivated for 24 h. The cells were washed with 2 mL phosphate buffered saline (PBS) (with Ca^{2+}/Mg^{2+}, pH 7.4) and treated with 1.8 mL 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.

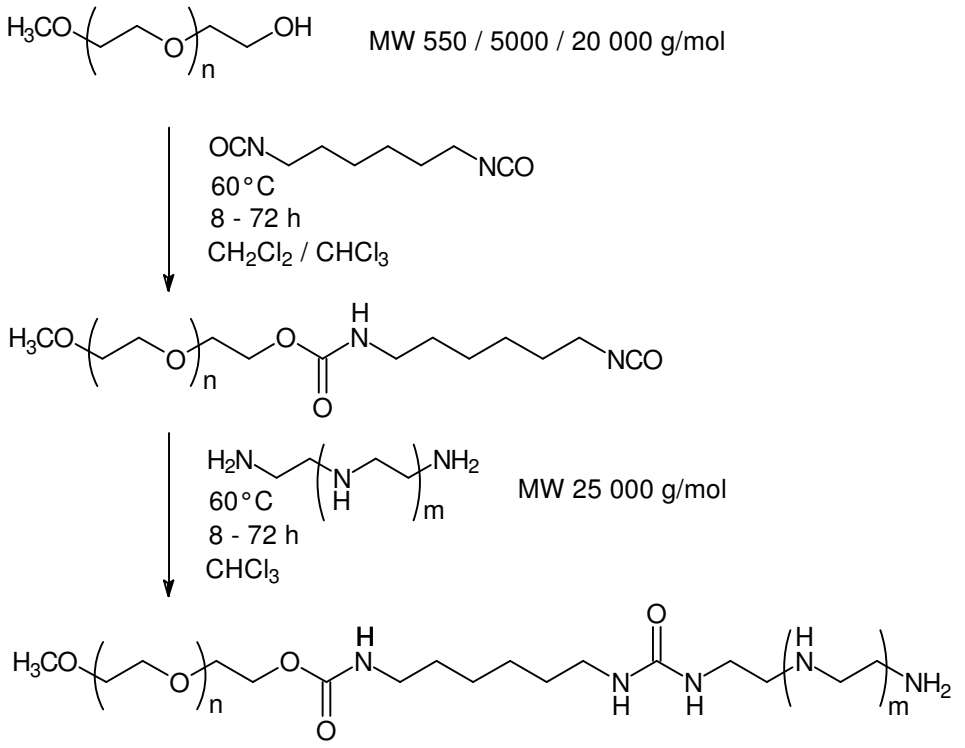
Hemolysis tests. Fresh blood from 6 month old rats (Fischer 344 rats, Institute for Pharmacology and Toxicology, University of Marburg), collected in heparinized-tubes, was centrifuged at 700 g at 4 °C for 10 min and washed several times with PBS until the supernatant was colorless. 500 µL of a 2.5 % (v/v) suspension of erythrocytes were mixed with 500 µL 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 were 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 times magnification. Experiments were performed in triplicate.

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 towards 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 towards 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, due to 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 and long durability.



Scheme 2: Synthetic route of PEI(25k)-*g*-PEG copolymers. The monomethylether-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.

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 crosslinking 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 5,000 and 20,000). Details about synthesis are provided in Table 1.

Table 1: Synthesis of the Block Copolymers PEI(25k)-*g*-PEG

Copolymer	activation of mPEGx with HMDI						synthesis of block copolymers			
	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.

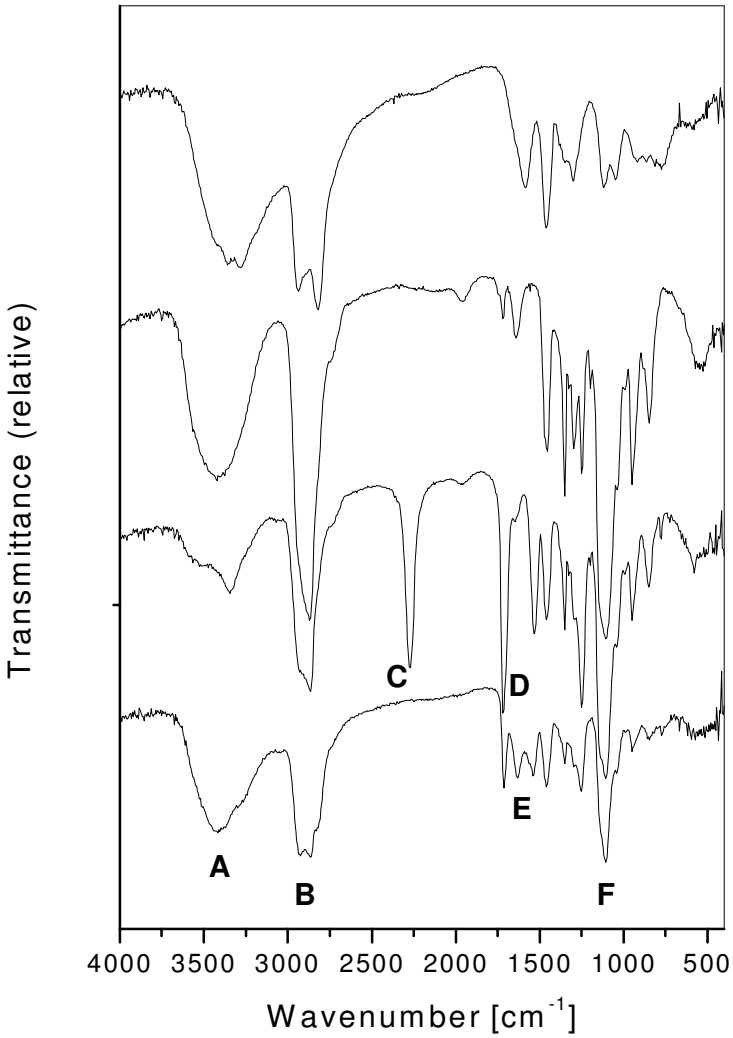


Figure 1: FT-IR spectra (from top to bottom) of PEI 1,200, PEG 550, activated PEG 720 and the block copolymer PEI(1200)-*g*-PEG(550)$_2$. Characteristic absorptions: (A) N-H amines stretching 3300 cm^{-1}, (B) C-H stretching 2921 cm^{-1}, (C) O=C=N isocyanate stretching 2274 cm^{-1}, (D) C=O urethane stretching 1724 cm^{-1}, (E) C=O urea stretching 1620 cm^{-1}, (F) C-O ether stretching 1106 cm^{-1}.

For the activation of PEG 550 and 5,000 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 5,000 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 towards isocyanates in comparison with the hydroxy group. In this case, an even longer reaction time was necessary for successful coupling. Due to 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, 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 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 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 maximum degradation at about 360 °C. Low MW PEGs 550 and 5,000 degraded at lower temperatures (270 – 280 °C), whereas high molecular weight PEG 20,000 showed maximum degradation at a higher temperature (about 390 °C). In the case of the copolymers, a characteristic two step degradation was observed as exemplarily shown for PEI(25k)-*g*-PEG(5k)$_6$ in Figure 2.

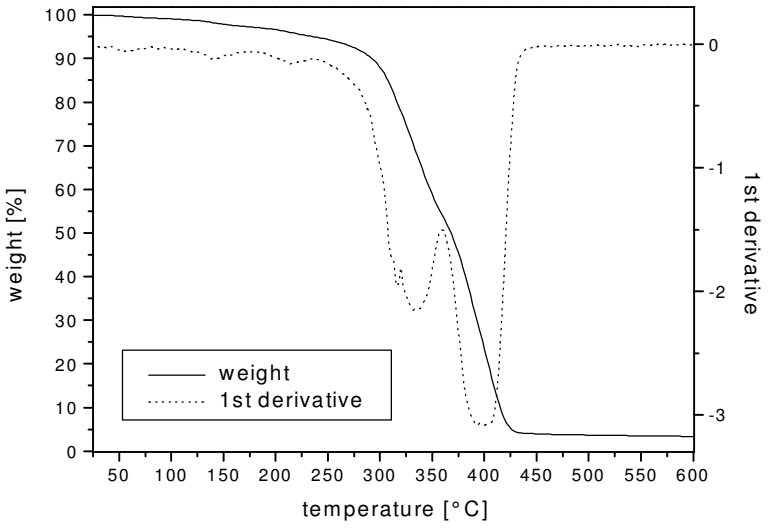


Figure 2: The two-step degradation of the PEI(25k)-*g*-PEG(5k)$_6$ copolymer as monitored by TGA. The first degradation step is derived mainly from the PEG blocks, the second one from the PEI block.

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 can not 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 can not 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 5,000 (63 °C). T_m increased only slightly with MW higher than 5000. PEI, instead, exhibited only one glass transition step at -52 °C. Due to 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 a stronger interaction between both blocks and therefore a loss of mobility (physical crosslinking). 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.

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 condition, 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 of 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 5,000, which did not react with PEI.

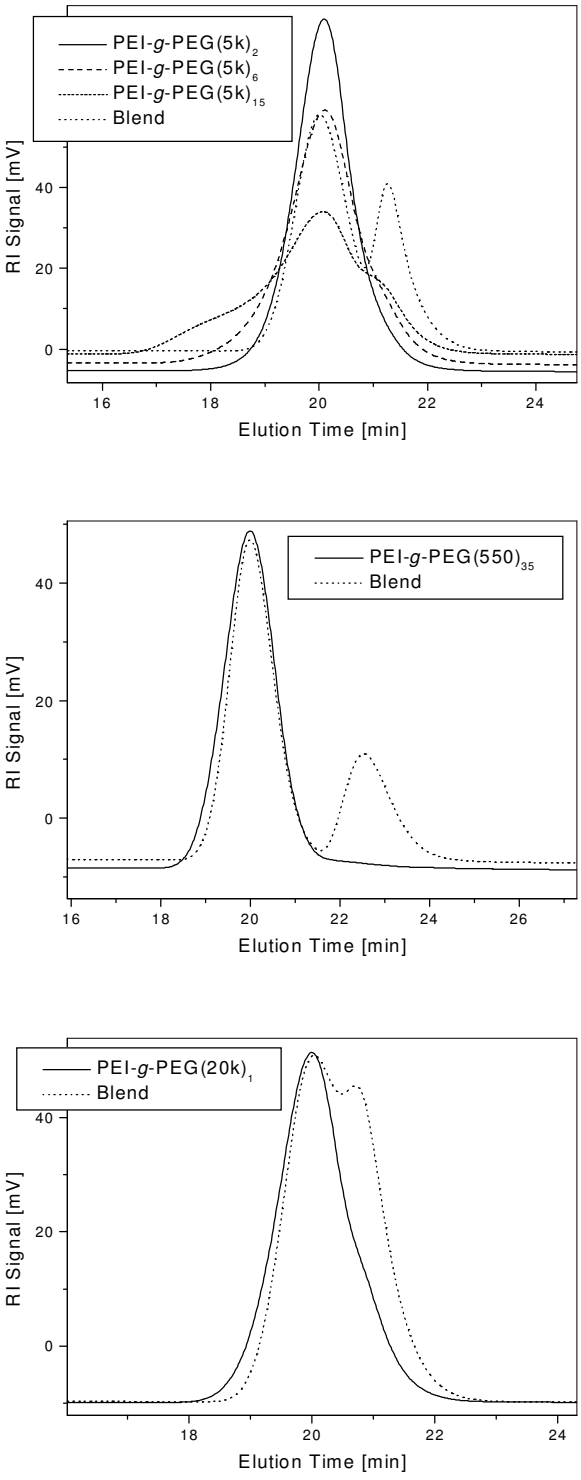


Figure 3: SEC eluograms of copolymers and blends in 1 % formic acid. The signals shown were detected by a refractive index detector.

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 5,000 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, due to 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 of 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 supplement. The refractive index increments dn/dc of the copolymers were found to be between the values for the homopolymers: the higher the PEI content, the higher dn/dc. 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. Polydispersity index (Mw/Mn) 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 were 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 toxic PEI(25k)-*g*-PEG(5k)$_{15}$. This copolymer showed, however, only 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 5,000 blocks significantly reduced the 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.

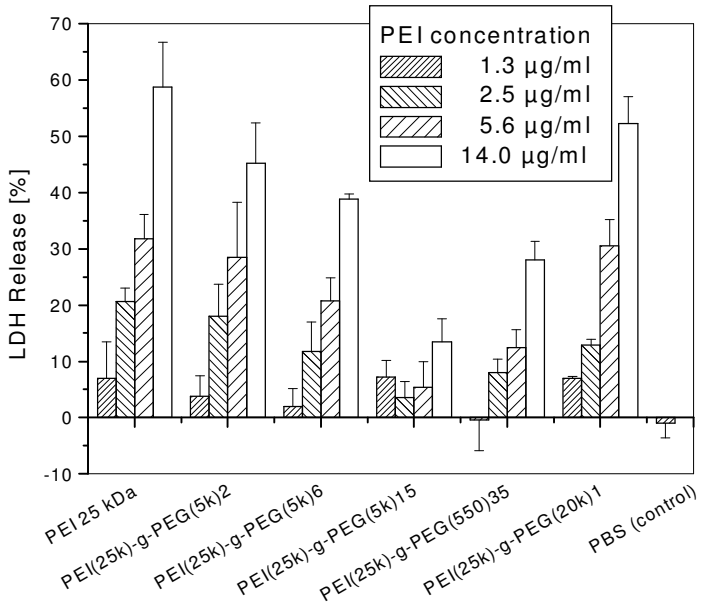


Figure 4: LDH assay of homopolymer PEI 25,000 and copolymers after an incubation period of 4 h at different concentrations of PEI.

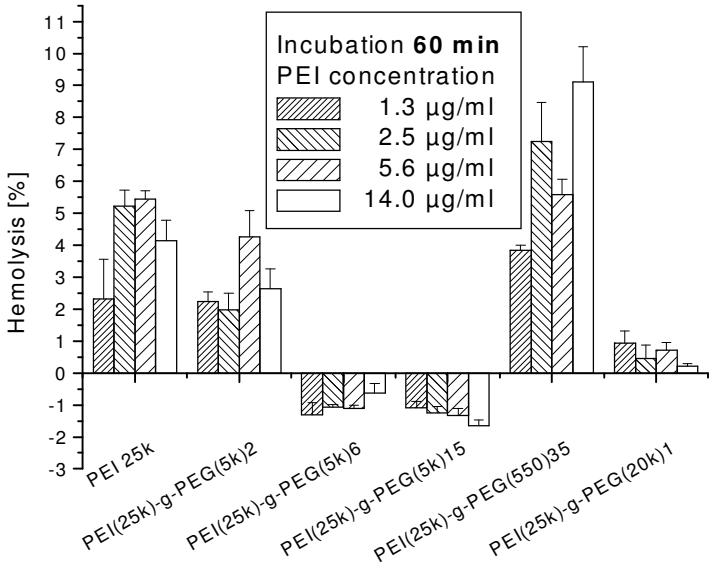
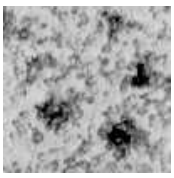
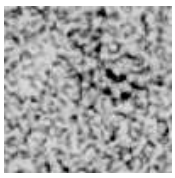
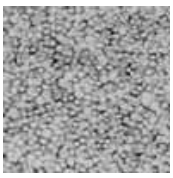
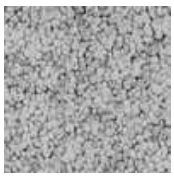
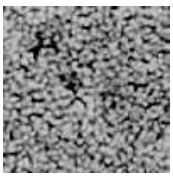
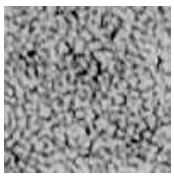


Figure 5: Hemolysis of homopolymer PEI 25,000 and PEI-*g*-PEG copolymers after an incubation period of 60 min.

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 supplement) 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 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.

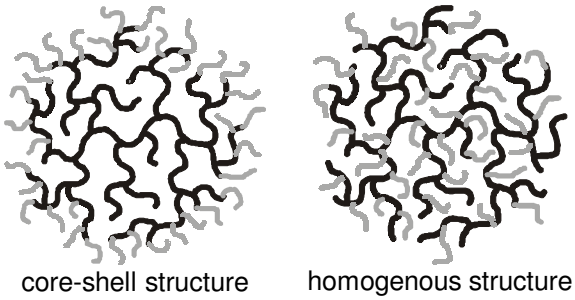
Table 2: Semiquantitative results of the erythrocyte aggregation induced by homopolymer PEI and copolymers PEI-*g*-PEG at different PEI concentrations. Classification: - no aggregation, + weak aggregation, ++ medium aggregation. +++ strong aggregation, ++++ very strong aggregation. Images were taken by phase contrast light microscopy at a PEI concentration of 5.6 µg/mL.

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						

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 5,000 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 of 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, as well as 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 5,000 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, not only react 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 homogenously distributed throughout the entire PEI macromolecule (Scheme 3: right structure).



Scheme 3: The two possible copolymer structures of PEI(25k)-*g*-PEG(550)$_{35}$. The results reported in this paper suggest that the PEG (grey) 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 non-viral 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. Due to 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. Due to 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.

Acknowledgments

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.

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Supplement of Chapter 1

Table 1: Physical Data of Homopolymers PEI, PEG and Block Copolymers PEI(25k)-*g*-PEG

Polymer	PEI[a] [%]	MW_{calc}[b] [g/mol]	dn/dc[c] [ml/g]	M_n[d] [g/mol]	M_w[d] [g/mol]	M_w/M_n	T_{degr}[e] [°C]	Copolymer T_g[f] [°C]	Copolymer T_m[g] [°C]	Blend T_g[f] [°C]	Blend T_m[g] [°C]
PEI(25k)-g-PEG(5k)$_{15}$	23	102550	0.154	117 300	297 400	2.53	373 382	-18[h]	60	-	65
PEI(25k)-g-PEG(5k)$_6$	45	61000	0.209	95 410	229 300	2.40	329 388	-29	60	-47	62
PEI(25k)-g-PEG(5k)$_2$	75	35340	0.260	33 250	75 850	2.28	322 379	-39	55	-46	63
PEI 25 kDa	100	25 000	0.328	23 480	31 030	1.32	357	-52	-	-52	-
PEI(25k)-g-PEG(550)$_{35}$	55	50200	0.203	53 980	168 200	3.12	352	35	-	-46	18
PEI(25k)-g-PEG(20k)$_1$	46	45000	0.230	27 430	63 180	2.30	333 413	-28	63	-48	65
PEG 550 Da	0	550	0.128	555	556	1.00	274	-	16	-	16
PEG 5 kDa	0	5 000	0.141	5 169	5 171	1.00	284	-	63	-	63
PEG 20 kDa	0	20 000	0.143	13 890	14 080	1.01	392	-	65	-	65

[a] PEI content in copolymer as determined by ^{1}H NMR spectroscopy.
[b] As calculated with MW data given by supplier.
[c] As determined by differential refractometer in 1% formic acid and at 690 nm wavelength.
[d] As determined by GPC-MALLS.
[e] Temperature of maximum degradation as determined by TGA. Two numbers indicate a two step degradation process.
[f] Glass transition temperature as determined by DSC.
[g] Melting temperature as determined by DSC.
[h] Weak glass transition step, only observed at the first heating-up curve.

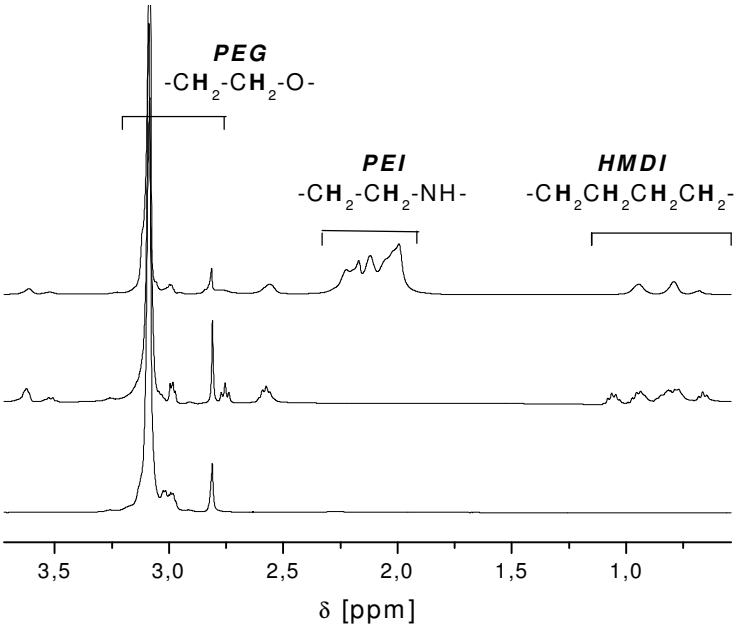

Figure 1: ^{1}H-NMR spectra in $CDCl_3$ of (from bottom to top) mPEG 550, activated PEG and the copolymer bPEI(1200)-g-lPEG(550)$_2$. A detailed assignment of the signals is given in the experimental section.

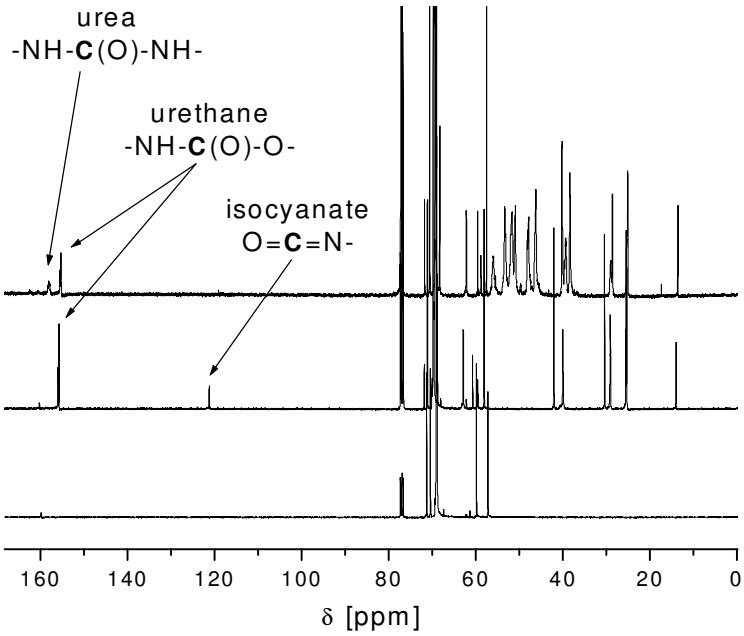

Figure 2: ^{13}C-NMR spectra in $CDCl_3$ of (from bottom to top) mPEG 550, activated PEG and the copolymer bPEI(1200)-g-lPEG(550)$_2$. A detailed assignment of the signals is given in the experimental section.

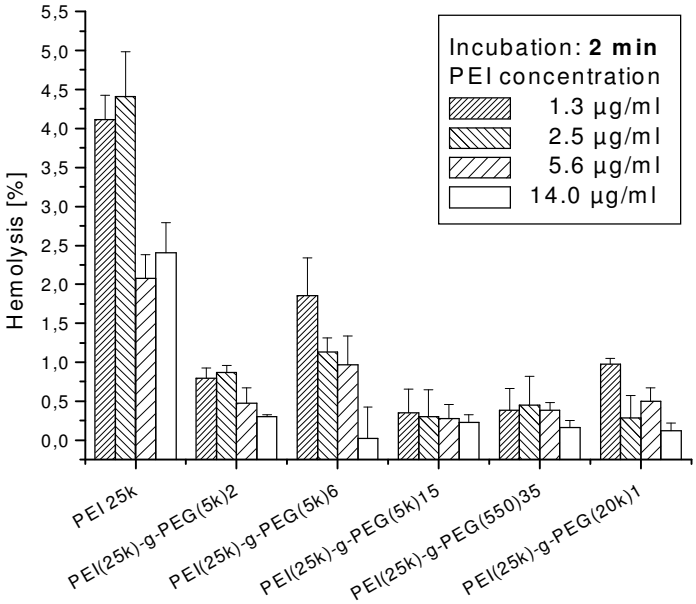

Figure 3: Hemolysis of homopolymer PEI 25,000 and PEI-*g*-PEG copolymers after incubation of 2 min.

Chapter 2

Polyethylenimine-*graft*-Poly(ethylene glycol) Copolymers: Influence of Copolymer Block Structure on DNA Complexation and Biological Activities as Gene Delivery System

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(Submitted to Bioconjugate Chemistry)

[a] Agarose retardation assay, LDH assay, erythrocyte aggregation, hemolysis assay
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ABSTRACT

For two series of polyethylenimine-*graft*-poly(ethylene glycol) (PEI-*g*-PEG) block copolymers the influence of copolymer structure on DNA complexation were investigated and physicochemical properties of these complexes were compared with the results of blood compatibility, cytotoxicity and transfection activity assays. In the first series PEI (25 kDa) was grafted to different degrees of substitution with PEG (5 kDa) and in the second series the molecular weight (MW) of PEG was varied (550 Da – 20 kDa). Using atomic force microscopy we found that the copolymer block structure strongly influenced the DNA complex size and morphology: PEG 5 kDa significantly reduced the diameter of the spherical complexes from 142 ± 59 nm to 61 ± 28 nm. With increasing degree of PEG grafting complexation of DNA was impeded and complexes lost their spherical shape. Copolymers with PEG 20 kDa yielded small, compact complexes with DNA (51 ± 23 nm) whereas copolymers with PEG 550 Da resulted in large and diffuse structures (130 ± 60 nm). Zeta-potential of complexes was reduced with increasing degree of PEG grafting if MW ≥ 5 kDa. PEG 550 Da did not shield positive charges of PEI sufficiently leading to hemolysis and erythrocyte aggregation. Cytotoxicity (LDH assay) was independent on MW of PEG but affected by the degree of PEG substitution: all copolymers with more than 6 PEG blocks formed DNA complexes of low toxicity. Finally, transfection efficiency of the complexes was studied. The combination of large particles, low toxicity and high positive surface charge as in the case of copolymers with many PEG 550 Da blocks proved to be most efficient for *in vitro* gene transfer. To conclude, the degree of PEGylation and the MW of PEG were found to strongly influence DNA condensation of PEI and therefore also affect the biological activity of the PEI-*g*-PEG/DNA complexes. These results provide a basis for the rational design of block copolymer gene delivery systems.

INTRODUCTION

Surface-modification of drug delivery systems with poly(ethylene glycol) (PEG) has met with increasing interest to enhance biocompatibility (*1*), increase systemic circulation times (*2*) and alter their biodistribution (*3*). For instance, nanoparticles (*4*), liposomes (*5*), and also adenoviruses (*6*) have been "PEGylated" mainly to obtain long-circulating particulate delivery systems, based on the so-called "stealth" effect. Recently, PEGylation methodology has been reviewed (*7*). PEGylation of polycations for gene delivery has also been realized leading to block or graft copolymers as summarized in (*8*,*9*). Among other polycations, poly(L-lysine) (PLL) has been modified by PEGylation (*10*,*11*,*12*) most extensively with the result of reduced complement activation and an increase of DNA complex stability. However, PLL requires endosome-disruptive reagents such as chloroquine (*13*) to attain sufficient transgene expression. Compared to PLL, polyethylenimine (PEI) has successfully been used for gene delivery both under *in vitro* (*14*,*15*) and *in vivo* (*16*,*17*) conditions. PEI offers a high positive charge density and exhibits a strong proton buffer capacity over a broad pH range (*18*). The latter characteristic is advantageous for PEI since the proton-sponge effect (*19*) allows sufficient gene transfer without endosome-disruptive reagents.

PEGylated PEIs have been studied by several authors as potential gene delivery systems. For instance, PEI 25 kDa was grafted with 3 and 10 PEG 3400 Da blocks carrying a terminal galactose ligand for hepatocyte targeting (*20*). Kabanov and coworkers modified PEI 25 kDa with 37 and 61 PEG 5 kDa blocks and PEI 2 kDa with two blocks of PEG 8 kDa and one block of PEG 10 kDa (*21*, *22*) to study the influence of PEG on oligonucleotide and plasmid DNA complexation. Instead, Choi et al. grafted PEI 25 kDa with 40 and 133 short blocks of PEG 550 Da (*23*) and applied this copolymers for *in vitro* gene transfer. While all these groups grafted the PEG onto PEI first and formed the DNA complexes in a subsequent step, Wagner and coworkers first condensed DNA with PEI 800 kDa and subsequently grafted the complex with more than 1600 blocks of PEG 5 kDa (*24*, *25*).

Nevertheless, PEGylation of PEI has not been studied varying both the degree of substitution and the MW of PEG under similar experimental conditions to our knowledge. A retrospective comparison of literature data obtained with PEI of different MW and different experimental conditions is notoriously difficult. Therefore, our investigations were aimed at providing a PEI-*g*-PEG block copolymer sample set in which PEI is kept constant but the extent of PEGylation *and* the MW of the PEG is varied. Here, we use two series of PEI-*g*-PEG block copolymers in an effort to elucidate the influence of copolymer block structure on DNA condensation (copolymer structure/complex property - relationship), and in a second

step to compare the physicochemical properties of the complexes with their biological activity revealed in cell culture experiments (complex property/biological activity - relationship).

MATERIALS AND METHODS

Materials

Branched PEI (bPEI) with MW of 25 kDa (Polymin^{TM} water free, 99 %) was a gift of BASF, Ludwigshafen, Germany. PEG-monomethylether (mPEG) 550 Da and mPEG 5 kDa were obtained from Aldrich, Milwaukee, WI, USA. Monoamino-PEG-monomethylether (mPEG-NH_2) 20 kDa was purchased from Rapp Polymere, Tübingen, Germany. All polymers were thoroughly dried *in vacuo* before use. HMDI (≥ 99 %) was from Fluka, Deisenhofen, Germany. Chloroform (Riedel-de Haën, Seelze, Germany, ≥ 99 %) was treated with HMDI for 4 h at 60 °C and distilled to remove any traces of water and ethanol. Diethylether (≥ 99.5 %) from Merck, Darmstadt, Germany was distilled before use. Light petrol (≥ 99 %, 40 – 60 °C) was from Riedel-de Haën.

Three different types of DNA were used in this work. DNA from herring testes (Type XIV, 0.3 – 6.6 MDa, 400 – 10000 bp) and plasmid DNA pBR322 (2.9 MDa, 4363 bp) were from Sigma, Steinheim, Germany. Plasmid pGL3 control, luciferase reporter vector (3.5 MDa, 5256 bp) was from Promega, Heidelberg, Germany, and was amplified with a competent E. coli strain JM 109 (Promega) according to a protocol from QIAGEN, Hilden, Germany (*26*).

Block Copolymer Synthesis

Synthesis and characterization of the block copolymers has been described in detail elsewhere (*27*, *28*). Briefly, PEG dissolved in anhydrous chloroform (c = 200 g/L) was activated for the reaction with the amino groups of PEI with an 10 – 100 fold excess of hexamethylene diisocyanate (HMDI) at 60 °C for 8 - 72 h depending on the MW of PEG (higher MW required longer reaction times). Unreacted HMDI was carefully removed by repetitive extraction with light petrol. Activated PEGs were reacted with PEI at concentrations of 10 g/L in anhydrous chloroform at 60 °C for 8 - 72 h. The reaction solutions were concentrated to 100 g/L by evaporation of the solvent and dropped into a 20 fold larger volume of diethyl ether to obtain the copolymer by precipitation. Finally, the products were dried *in vacuo*. Polymers were characterized by ^{1}H and ^{13}C-NMR spectroscopy which verified the structure of the copolymers and allowed calculation of the composition of ethylenimine and ethylene glycol units in the copolymer. Size exclusion chromatography demonstrated absence of unreacted PEG and PEI homopolymers. Thus, no further purification steps were necessary.

Formation of the Copolymer-DNA Complexes

All complexes of DNA and polymer were prepared freshly before use. Polymer solutions were added to the DNA solutions in equal volumes, mixed by vortexing and incubated for 10 min before use unless otherwise stated. DNA stock solutions were diluted to a concentration of 20 µg/ml with 10 mM NaCl at pH 7.4 (AFM experiments) or to 40 µg/ml (all other experiments) with 150 mM NaCl at pH 7.4. The polymer stock solutions were diluted with the same medium as above to the appropriate concentration depending on the required polycation-nitrogen/polyanion-phosphorous ratio (N/P ratio) and filtered through 0.22 µm. DNA stock solutions were 2 mg/ml pGL3 plasmid in 10 mM TRIS HCl (pH 8) and 1 mM EDTA, 100 µg/ml pBR322 plasmid in pure reagent water and 1.11 mg/ml herring testes DNA in 10 mM TRIS HCl (pH 7.4). Homopolymer and copolymer stock solutions were 0.5 mg PEI/ml (AFM experiments) and 0.9 mg PEI/ml (all other experiments) so that all polymer solutions contained the same PEI concentration.

Physicochemical Characterization of Copolymer-DNA Complexes.

Atomic Force Microscopy (AFM). AFM images were conducted on a Dimension™3000 Scanning Probe Microscope from Digital Instruments (Santa Barbara, CA) with a Nanoscope IIIa controller. The solution of polymer-DNA complex was prepared as described above and incubated for 5 minutes before being deposition onto freshly cleaved mica and imaged under 10 mM NaCl solution. Immobilization was achieved using electrostatic forces between the opposite charges of the mica and polymer-DNA complexes. All imaging was carried out in the tapping mode at a scan speed of approximately 2 Hz with 512x512 pixel data acquisition. V-shaped cantilevers with a pyramidal tip of silicon nitride were used (Park Scientific, CA). The experiments were repeated twice and the observed structures were found to be reproducible. To determine the mean complex size from these images, the diameter at about half-height of 30 complexes were measured.

Dynamic Light Scattering (DLS). Complex size measurements were carried out with a Zetasizer 3000 HS from Malvern Instruments, Worcs., UK at 25 °C. (10 mW HeNe laser, 633 nm). Scattering light was detected at 90° angle through a 400 µm pinhole. Measurements yielding hydrodynamic diameters (Z average mean) and polydispersity indices (PDI) were performed with count rates of about 100 - 400 kCps in form of 10 runs of 60 min duration each and analyzed in the CONTIN mode. For data analysis, the viscosity (0.8905 mPas) and refractive index (1.333) of pure water at 25 °C were used. The instrument was calibrated with Nanosphere™ Size Standards (polymer microspheres in water, 220 ± 6 nm) from Duke Scientific, Palo Alto, CA.

Laser Doppler Anemometry. ζ-potential (zeta-potential) measurements of the complexes were carried out in the standard capillary electrophoresis cell of the Zetasizer 3000 HS from Malvern Instruments at position 17.0 and at 25 °C. Measurement duration was set to automatic. Average values of the ζ-potential were calculated with the data from 8 runs. The instrument was calibrated with DTS5050 Electrophoresis Standards from Malvern Instruments.

Agarose Gel Retardation Assay. 40 µl aliquots of the complex solution were mixed with 4 µl loading buffer [glycerol (85%) and TAE (40 mM Tris/HCl, 1 % acetic acid, 1 mM EDTA, pH 7.4) in equal parts] and loaded onto an ethidium-bromide (EtBr) containing 1% agarose gel. Electrophoresis (Blue Marine 200, Serva, Heidelberg, Germany) was carried out at a voltage of 80 V (LKB Bromma 2197 Power Supply, Pharmacia, Freiburg, Germany) for 2 h in TAE running buffer solution. UV light (254 nm) detection was conducted with a Video Copy Processor P67E from Mitsubishi, Japan.

Biological Characterization of PEI-PEG-Copolymer-DNA Complexes.

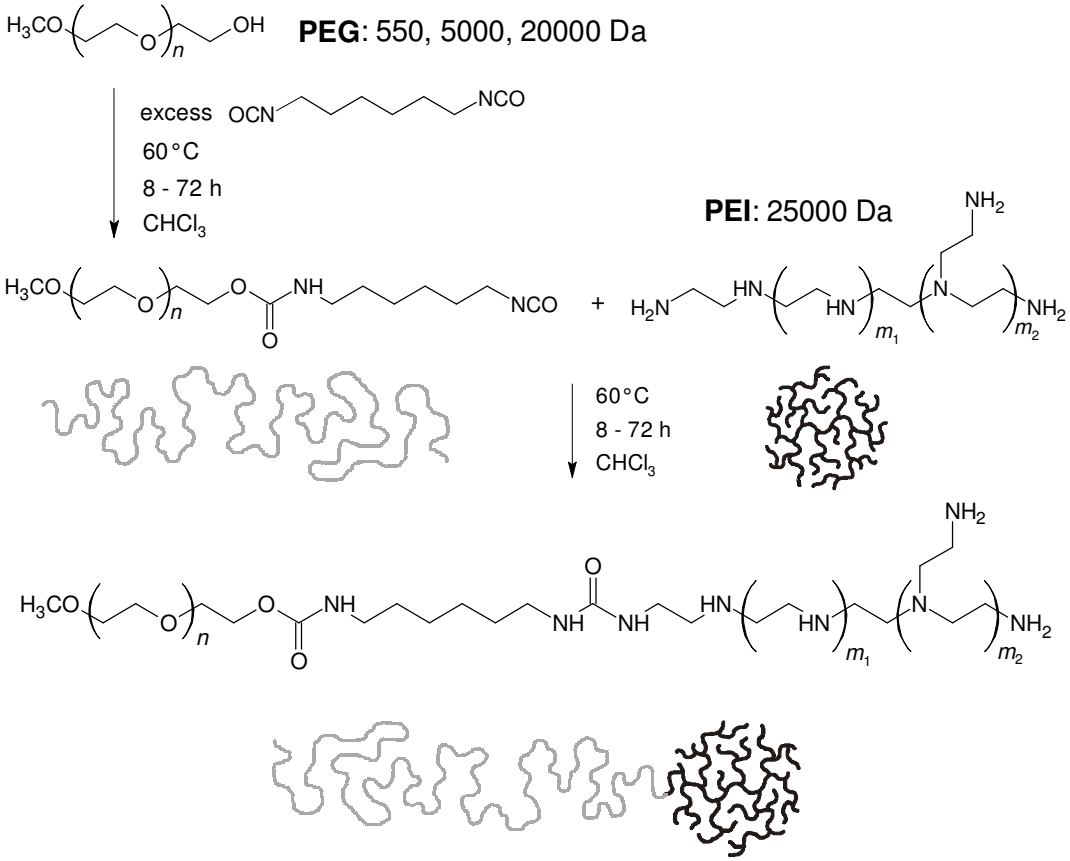
Hemolysis Assay. For hemolysis fresh blood from 6 month old rats (Fischer 344 rats, Institute for Pharmacology and Toxicology, University of Marburg) collected in heparinized-tubes was centrifuged at 700 g at 4 °C for 10 min and washed several times with PBS until the supernatant was colorless. 500 µL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

Aggregation of Erythrocytes. To study polymer/DNA complex 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 as described above. Finally, the erythrocytes were diluted with Ringer's solution 1:50. 200 µL of this cell suspension were treated with 100 µL of polymer solution in 24 well plates (Nunc). After incubation of 2 h at 37 °C pictures were taken of the cells with a Contax RTS 2 camera from AGFA (ASA 100)

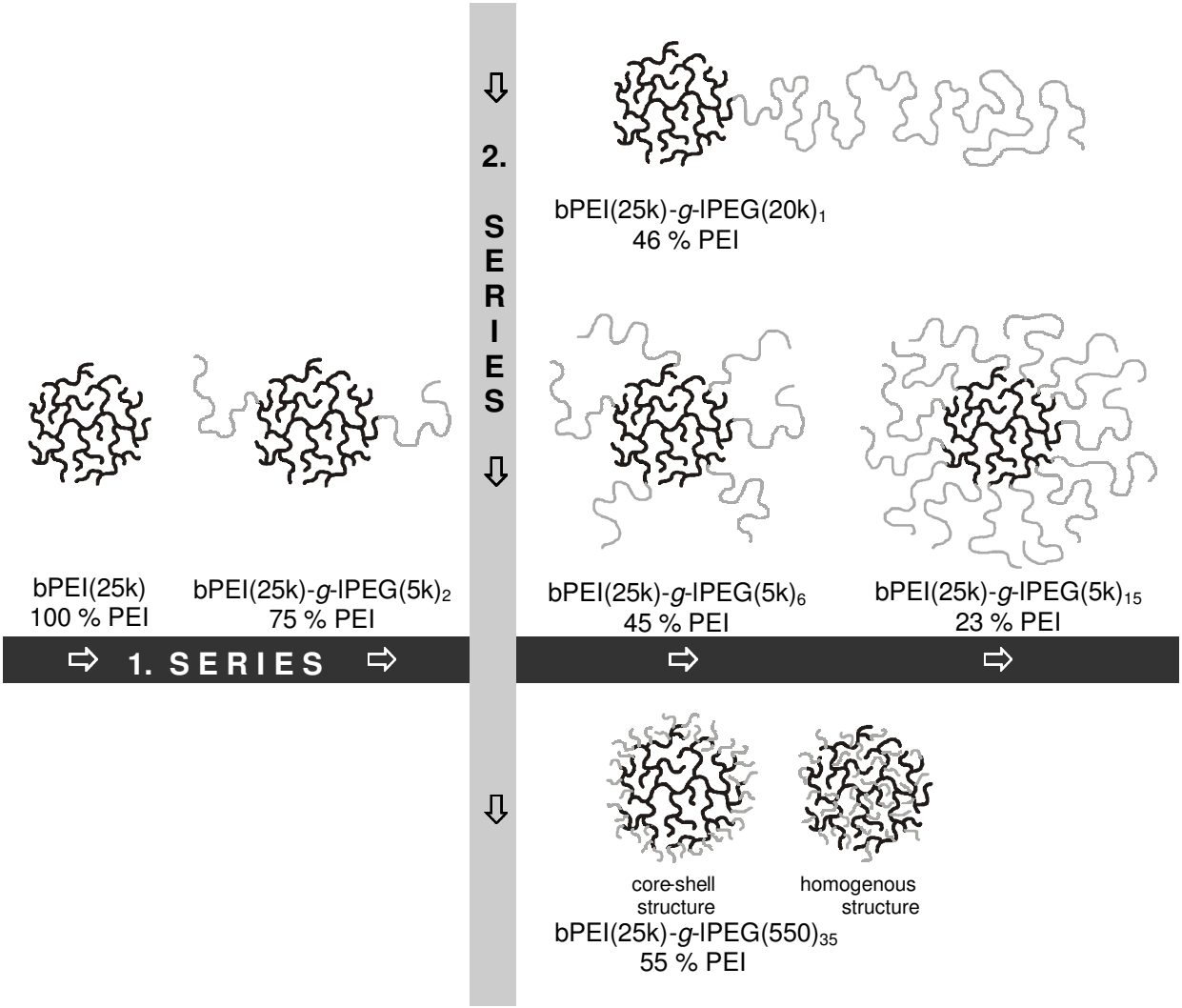
fitted to a reverse phase contrast microscopy (Nikon TMS) at 40 times magnification. Experiments were performed in triplicate.

Lactate dehydrogenase (LDH) assay. *In vitro* cytotoxicity of the complexes with herring testes DNA was evaluated using an LDH assay with 3T3 mouse fibroblasts (500,000 cells/well, 4 h incubation). As reference PBS (negative, 0 %) and 0.1 % (w/w) Triton X-100 solution (ICN, Eschwege, Germany) in PBS (positive, 100 %) were applied. The LDH-content was measured photometrically (340 nm) using a commercial test kit (DQ 1340-K, Sigma) as described in more detail earlier (*29*). Experiments were performed in triplicate.

Transfection Experiments. Transfection activity of the copolymer complexes was studied on 3T3 mouse fibroblasts (NIH 3T3, Swiss mouse embryo, ATCC, Rockville, Maryland) using a Luciferase assay kit from Promega. Measurements of relative light units (RLU) were conducted on a Sirius Luminometer from Berthold, Pforzheim, Germany. Protein quantification was performed with freshly prepared bicinchonic acid (BCA) reagent (Pierce, Rockford, IL) and was carried out with an ELISA plate reader from Dynatech MR 5000, Denkendorf, Germany at 570 nm. Experiments were performed in triplicate as described in more detail in (*30*).



Scheme 1: Synthesis of bPEI(25k)-*g*-lPEG copolymers. The PEG has a linear, the PEI a hyperbranched structure.



Scheme 2: Schematic of the bPEI(25k)-*g*-lPEG copolymer sample set. The black part of the structure represents the cationic branched PEI and the grey part the non-ionic 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. The copolymer bPEI(25k)-*g*-lPEG(550)$_{35}$ has two possible structures. However, PEG is more likely homogenously distributed within the PEI than concentrated at the outer sphere of the PEI (core-shell structure).

RESULTS AND DISCUSSION

Two series of block copolymers were prepared by grafting linear PEG onto branched PEI using a straightforward synthesis route (Scheme 1). Diisocyanate HMDI was used as linker generating hydrolytically stable urethane bonds between PEG and linker and urea bonds between PEI and linker. In the first series (Scheme 2: horizontal) the PEI (25 kDa) was grafted with different degrees of substitution using PEG blocks all of MW 5 kDa in order to

study the quantitative effect of PEG blocks. As a result, the polycationic domain of the copolymers was increasingly surrounded by PEG blocks in this series.

The second series (Scheme 2: vertical) was arranged with copolymers all containing equal weight % of PEG and PEI (about 50 % : 50 %) but with PEGs of different molecular weights (MW 550 – 20,000 Da). Thus, we varied the copolymer structure in such a way that on one extreme PEG is homogeneously distributed in short blocks within the PEI domain, whereas in the other extreme a clear separation of the PEG and the PEI domain is realized. In other words, the copolymer structure is varied from multi -arm-grafting to a diblock structure.

For these graft (*g*) copolymers we chose following nomenclature: bPEI(25k)-*g*-lPEG(x)$_n$ where b and l denote a branched or linear structure. The numbers in brackets (25k or x, where x = 550, 5k or 20k) represent the MW of the PEI or the PEG block and the index n is the average number of PEG blocks per one PEI macromolecule. The index n was calculated from integration of the ^{1}H-NMR signals and with the MW values of the homopolymers given by the suppliers.

Size and morphology of the complexes. To study the influence of copolymer structure on size and shape of the complexes formed with plasmid DNA, we performed AFM experiments in aqueous solution. While transmission electron microscopy (TEM) has been frequently used to characterize DNA/polycation complexes (*20,21,22*) the advantage of AFM stems from the direct observation of these complexes in aqueous solution. Samples for TEM are visualized *in vacuo* and need to be pretreated and stained, e.g. with zinc uranyl acetate. Therefore, dimensions and structure of DNA/polycation complexes determined in aqueous media using AFM and by TEM could be different. In this study, physiological salt concentrations (150 mM NaCl) as used for the biological experiments were not feasible technically due to formation of salt crystals during the AFM imaging process. Therefore, a low salt concentration of 10 mM NaCl and pH 7.4 was selected. Since immobilization was based on electrostatic forces between the negative charge of mica substrate and the complex, the latter had to exhibit a distinct positive net charge. Therefore, we chose the N/P ratio 9 for this study (compare with ζ-potential measurements below).

The homopolymer PEI showed images of spherical and compact complexes of 142 ± 59 nm diameter (Figure 1, A and Figure 2). The first copolymer series (images A, B, C and D) demonstrated that PEG 5 kDa significantly reduced the diameter of the complexes from 142 nm to about 60 nm but with increasing number of PEG blocks DNA condensation was affected and complexes lost their spherical shape. Whereas the copolymer containing 2 PEG blocks gave spherical complexes with diameters of 77 ± 36 nm, bPEI(25k)-*g*-lPEG(5k)$_6$

showed a mixture of spherical and rod-like particles (diameter 61 ± 28 nm). When PEI was grafted with 15 PEG blocks complexes of ill-defined shape were found (diameter 60 ± 46 nm). Furthermore, some of the complexes exhibited stringy structures which can be attributed to poorly condensed DNA.

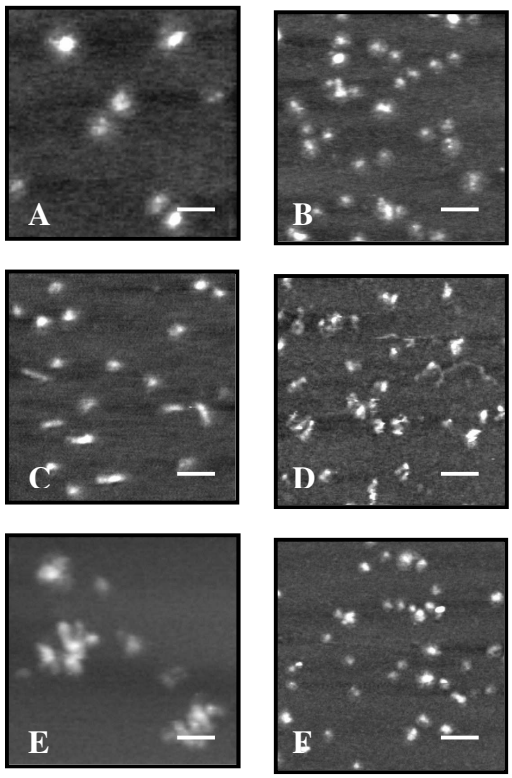


Figure 1: AFM images of polymer/plasmid DNA complexes at N/P ratio 9: Image A shows PEI 25 kDa homopolymer DNA condensates. Images B, C and D show DNA-polymer condensates produced with plasmid DNA and bPEI(25k)-*g*-lPEG(5k)$_n$ where n = 2, 6 and 15 respectively. Images E and F show DNA condensates produced with bPEI(25k)-*g*-lPEG(550)$_{35}$ and bPEI(25k)-*g*-lPEG(20k)$_1$ respectively. Scale bars are equivalent to 250nm. The color-encoded height scale extends 15 nm.

Complex size was also determined by dynamic light scattering (DLS) in the same medium as used for AFM (10 mM NaCl, pH 7.4). Hydrodynamic diameters are shown in Figure 2 in comparison with the results of the AFM experiments.

Differences in complex size were much smaller compared to the AFM results and no specific trend could be observed for the first series. The trend found by AFM that PEG 5 kDa reduces the size of the complex was observed by DLS only for bPEI(25k)-*g*-lPEG(5k)$_2$. Their complexes (88.7 ± 2.2 nm) were on average slightly smaller than complexes formed by the homopolymer PEI (94.5 ± 1.1 nm). However, copolymers with 6 and 15 PEG blocks formed complexes with slightly larger average diameters of about 105 nm probably due to the rod-like shape of some of the complexes as observed in AFM.

In the second series another trend is observed in AFM (Figure 1, Images E, C and F): Complexes gradually lose their compact and spherical shape with decreasing MW of PEG. Copolymers with one long PEG 20 kDa formed small (51 ± 23 nm) and clearly spherical complexes, whereas copolymers with many short PEG 550 Da gave rather large complexes

(131 ± 60 nm) which were fluffy or diffuse and almost shapeless. For this series, DLS measurements revealed the same trend: For bPEI(25k)-*g*-lPEG(20k)$_1$ a small complex diameter of 68.7 ± 4.3 nm was found, whereas in the case of bPEI(25k)-*g*-lPEG(550)$_{35}$ larger diameters (110.4 ± 12.0 nm) were determined.

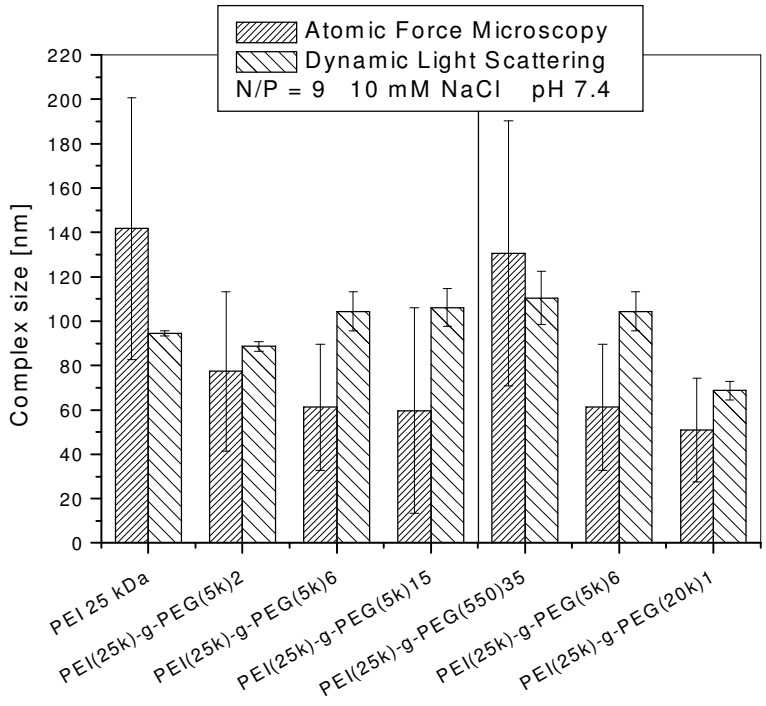


Figure 2: DNA-polymer complex size as determined by AFM (n = 30) and DLS (n = 10) at N/P ratio 9 in 10 mM NaCl. The error bars indicate the standard deviation (SD). In the case of the AFM results the SD reflects the size distribution.

The results of both the AFM experiments and the DLS measurements demonstrated that DNA complexation with PEI-*g*-PEG is strongly dependent on the block copolymer structure. In the case of PLL-*g*-PEG copolymers a negative influence of PEG and other hydrophilic polymers on the condensation of DNA was observed (*11,31*). Similar disadvantageous effects were anticipated for PEI; therefore PEGylation of the PEI/DNA complex was carried out as a second step after condensation (*25*). This procedure may, however, be impractical in a clinical setting. Here, we demonstrated that PEG does not necessarily interfere with the condensation process of DNA. Attaching only one or two PEG blocks to PEI can even enhance DNA condensation forming compact and spherical complexes with smaller sizes compared to homopolymer PEI complexes.

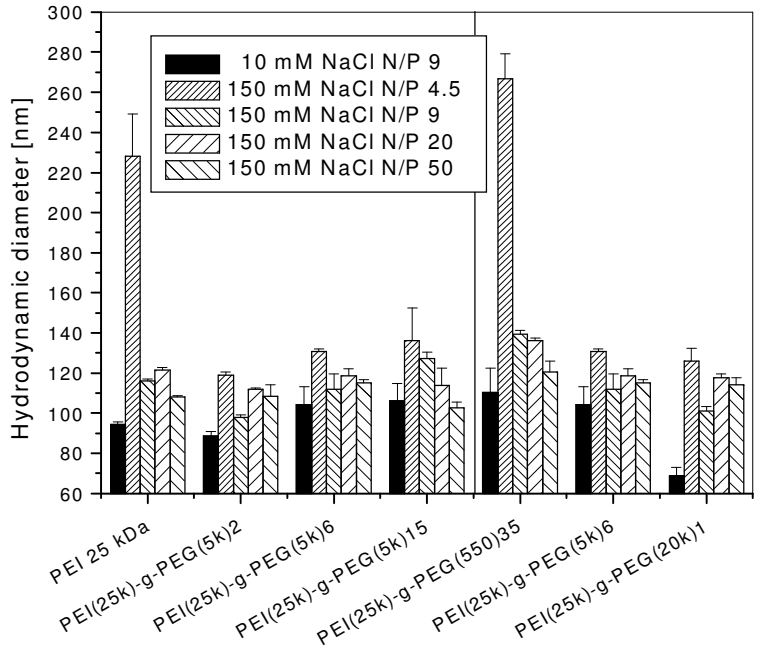


Figure 3: Hydrodynamic diameters of complexes assembled by plasmid DNA with bPEI 25 kDa and bPEI-*g*-lPEG copolymers at different ionic strength and different N/P ratios as determined by dynamic light scattering.

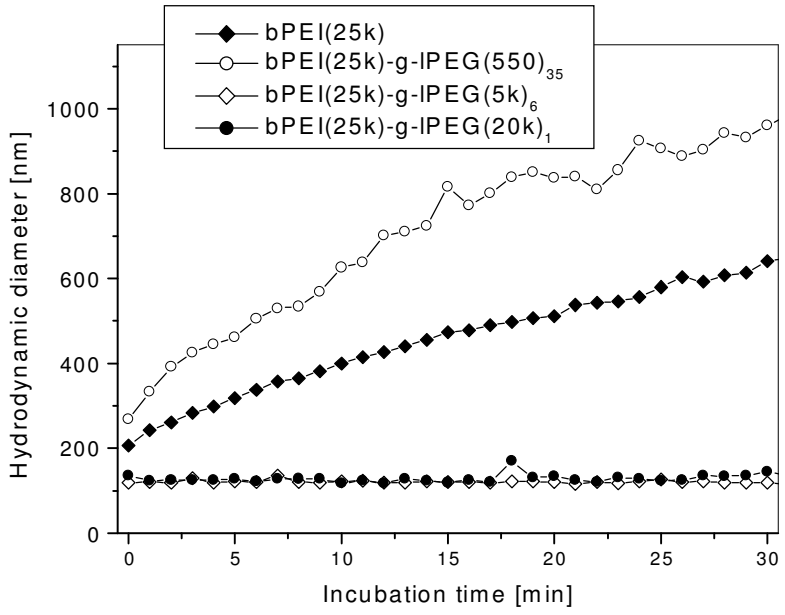


Figure 4: Stabilization study of the complexes assembled by plasmid DNA with bPEI 25 kDa and bPEI-*g*-lPEG at N/P 4.5 and 150 mM NaCl. Complex size (diameter) as function of incubation time as determined by dynamic light scattering.

Using DLS, complexes were also studied at high ionic strength (150 mM NaCl, pH 7.4) and at different N/P ratios (Figure 3). Under these (more physiological) conditions all polymers formed larger complexes as compared to low salt concentration. However, with increasing N/P ratio the complex size was reduced again. Two exceptional polymers were found: At low N/P ratio (4.5) PEI and bPEI(25k)-*g*-lPEG(550)$_{35}$ did not give stable complexes. Over a period of 30 min complex size increased from 200 nm to 600 nm and from 270 nm to 900 nm, respectively (Figure 4). In contrast, all copolymers with PEG of 5 kDa and 20 kDa formed stable complexes at N/P 4.5 (Copolymers bPEI(25k)-*g*-lPEG(5k)$_n$ where n is 2 or 15 are not shown in Figure 4). A stabilizing effect of PEG against aggregation of PEI/DNA complexes has been noted previously (*21,25*). Short PEG blocks seem to provide little stabilization and MW of PEG ≥ 5 KDa is necessary to obtain this effect. At higher N/P ratios (≥ 9) all homo- and copolymers formed stable complexes which are probably stabilized electrostatically under these conditions by a larger excess of polycations present.

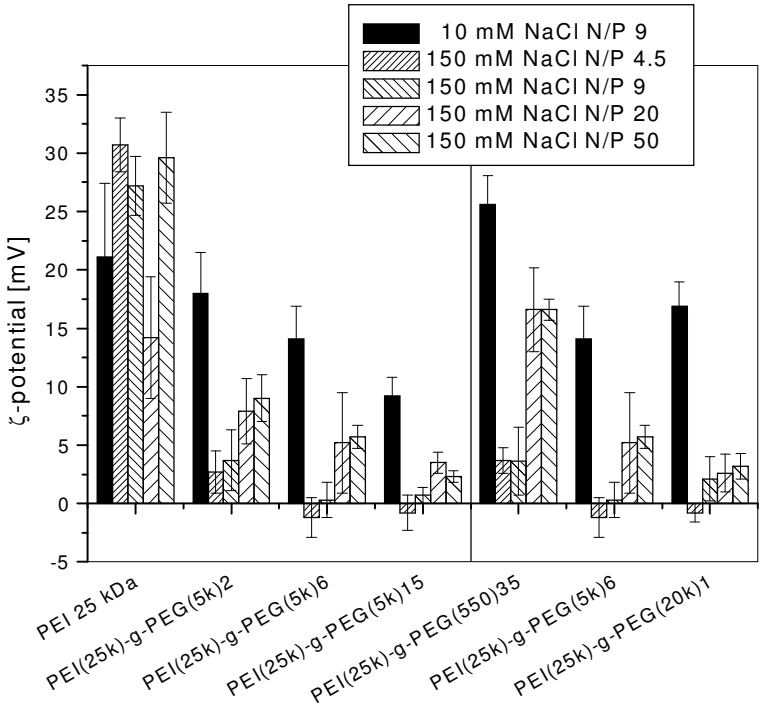


Figure 5: ζ-Potential of plasmid DNA complexes with PEI 25 kDa and bPEI-*g*-lPEG block copolymers at different ionic strength and at different N/P ratios as determined by laser Doppler anemometry.

Surface charge of the complexes. To estimate the surface charge of the complexes we measured the ζ-potential of the complexes by laser Doppler anemometry (LDA). The results

are shown in Figure 5. In the first series of bPEI(25k)-*g*-lPEG(5k)$_n$ copolymers ζ-potential is reduced with increasing number n of PEG blocks. This is true for different salt concentrations and different N/P ratios. Thus, PEG 5 kDa blocks seem to orientate towards the surface of the complex and shield its core with the cationic net charge. In the case of the copolymers the ζ-potential increases with increasing N/P ratios. The ζ-potential did not increase further from N/P 20 to 50 since free copolymer was present at N/P 50. In the case of homopolymer PEI, even at low N/P ratio 4.5 ζ-potential is highly positive and is found in the range of +25 to +35 mV. Similar values have been reported by others (*24,25,32*). A ζ-potential of +35 mV seems to be the maximum surface charge of the PEI-DNA complexes. PEI was reported to possess a ζ-potential of +37 mV (*33*).

In the second series with the copolymers containing about 50 % PEG, ζ-potential is reduced with increasing MW of PEG. Therefore, PEG 5 kDa and especially PEG 20 kDa are able to shield the charged core of the complex. The high ζ-potential of complexes formed with bPEI(25k)-*g*-lPEG(550)$_{35}$ points to the poor shielding properties. As we have previously shown using differential scanning calorimetry (*28*) bPEI(25k)-*g*-lPEG(550)$_{35}$ probably did not form a simple core-shell structure in which the PEG blocks surrounded the positively charged PEI core. During the synthesis the short PEG blocks might penetrate into the branched PEI structure and to react also with the inner amino groups. This would yield a copolymer where PEG is more homogeneously distributed over the PEI macromolecule as schematically shown in Scheme 2. This structure could explain the high ζ-potential of the complex.

DNA Complexation and Condensation. Finally, to study DNA complexation and condensation as a function of N/P ratio agarose gel retardation assays were performed. The gels are shown in Figure 6. No difference was found for the copolymers from the first series with PEG 5kDa: Complete retardation of DNA (indicating complete DNA complexation) was achieved at N/P = 2.0 - 2.2 whereas for complete exclusion of EtBr (indicating complete DNA condensation) a further excess of the polycation was needed (N/P = 4.5, data not shown). In the case of homopolymer PEI DNA was complexed completely at N/P = 1.6 and condensed at 3.0. Thus, grafting PEI with PEG 5 kDa slightly hindered the interaction with DNA. When PEI was grafted with short PEG blocks as in the case of copolymer bPEI(25k)-*g*-lPEG(550)$_{35}$ the complexation was hindered slightly (N/P = 2.0) but condensation was realized at the same N/P ratio (N/P = 3.0). Here again we found that short PEG blocks behaved differently than longer PEG chains: PEG 550 Da did not stabilize the complex against aggregation, it did not reduce the positive surface charge and here, it did not affect DNA condensation. However, the most interesting result from the agarose gel retardation

assay is the enhanced DNA condensation observed for copolymer bPEI(25k)-*g*-lPEG(20k)$_1$ already at N/P = 2.6. This is in line with the results from AFM images where small and compact particles were found. The excellent DNA condensation might be attributed to the special copolymer structure. With only one PEG block on average this is a diblock-like copolymer and there might be a PEI domain and a PEG domain clearly separated from each other. Thus, the DNA seems to interact with the PEI domain without interference from PEG. Furthermore, some authors reported the DNA condensation ability of PEG (*34*,*35*). Therefore, PEG might additionally participate in the condensation process in synergistic manner.

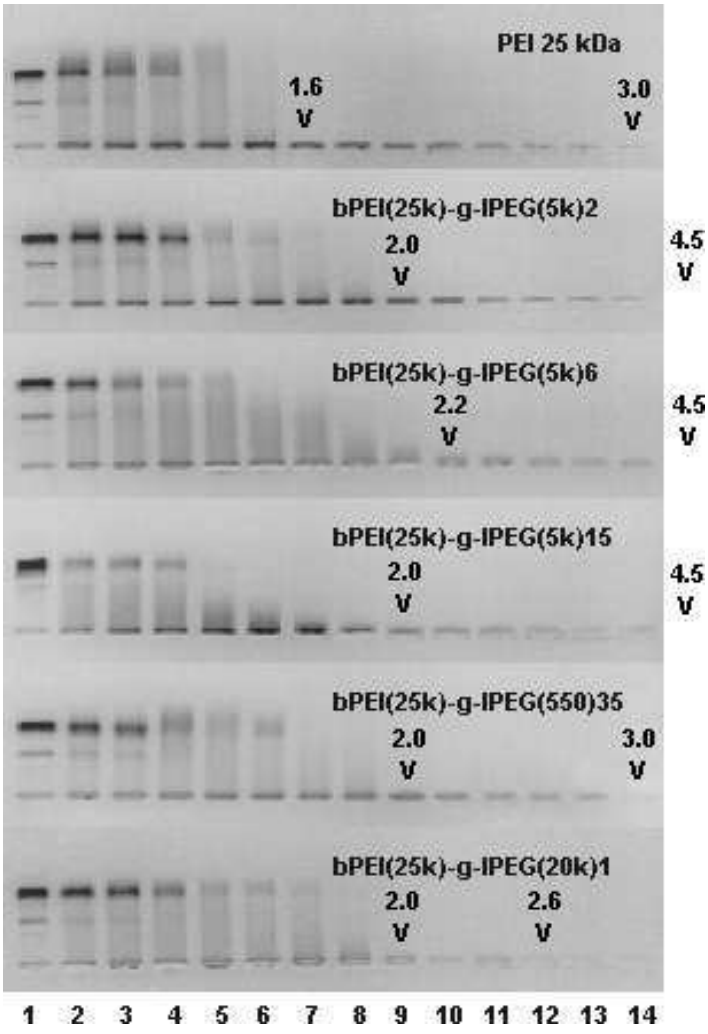


Figure 6: Agarose gel electrophoresis of the complexes of plasmid DNA with bPEI 25 kDa and bPEI-*g*-lPEG block copolymers. Lane 1: N/P = 0 (plasmid only); lanes 2-14: N/P 0.6 - 3.0 with increments of 0.2. N/P ratios of completed DNA retardation (left mark) and of completed ethidium bromide exclusion (right mark) sign the appropriate lanes. N/P ratio 4.5 was determined with another gel experiment (data not shown).

Hemocompatibility of the complexes. The blood-compatibility of PEI-*g*-PEG/DNA complexes was screened using a hemolysis and an aggregation of erythrocytes under *in vitro* conditions. These assays are a prerequisite for the intravenous administration of PEI-*g*-PEG/DNA complexes in animals or humans.

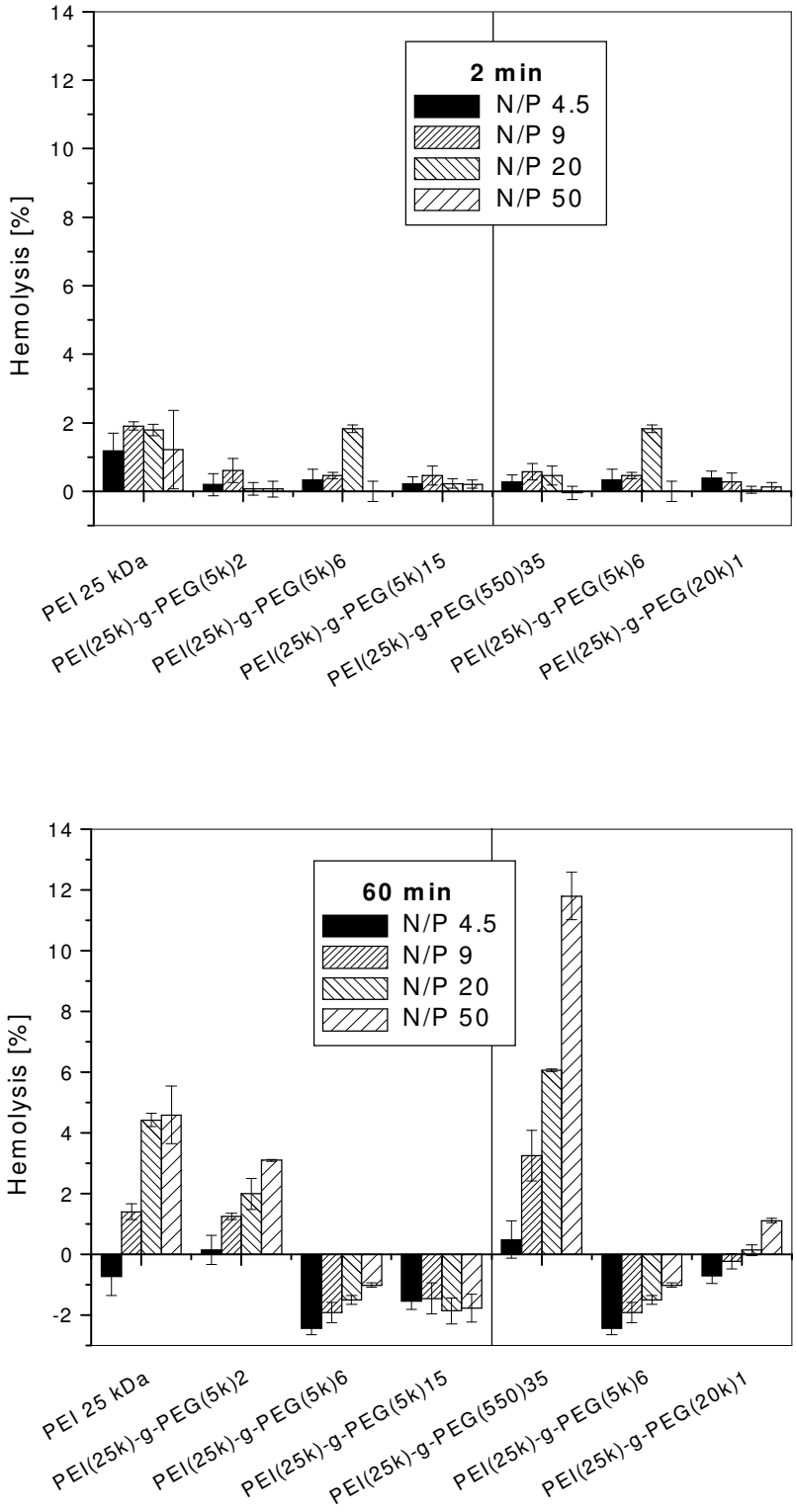
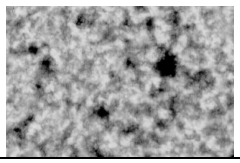
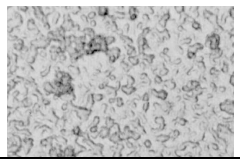
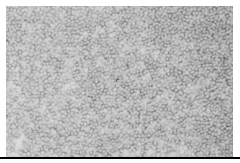
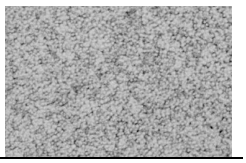
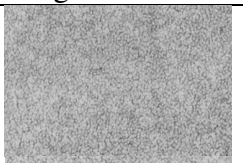


Figure 7: Hemolysis of bPEI 25 kDa and bPEI-*g*-lPEG copolymer complexes with herring testes DNA at different N/P ratios after incubation of 2 min (top) and 60 min (bottom).

The results of the hemolysis assay are shown in Figure 7. In a short term experiment (incubation time with erythrocytes: 2 min) the acute phase of an intravenous administration is reflected. No hemolysis was observed after short incubation time for any of the PEI-g-PEG preparations. Complexes with PEI 25 kDa showed a slight hemolytic activity (1-2 %). The long term experiment (60 min incubation) still showed only negligible hemolysis for the complexes of PEI and most copolymers. PEI, bPEI(25k)-*g*-lPEG(5k)$_2$ and bPEI(25k)-*g*-lPEG(550)$_{35}$ caused more hemolysis (up to 5 %) after 60 min in comparison with the results after 2 min incubation. Only bPEI(25k)-*g*-lPEG(550)$_{35}$ exhibited a remarkable value of 12 % at very high N/P ratios which is still tolerable since substances are classified as non-hemolytic when hemolysis remains < 15 % (*36*).

Table 1: Semiquantitative results of the erythrocyte aggregation induced by complexes formed with herring testes DNA and bPEI(25k)-*g*-lPEG copolymers at different N/P ratios. Classification: - no aggregation + weak aggregation ++ medium aggregation +++ strong aggregation ++++ very strong aggregation. Images were taken by phase contrast light microscopy and are exemplarily shown for N/P 20.

N/P ratio	PEI 25 kDa	bPEI(25k)-*g*-lPEG(5k)$_2$	bPEI(25k)-*g*-lPEG(5k)$_6$	bPEI(25k)-*g*-lPEG(5k)$_{15}$
4.5	+	-	-	-
9	++	++	-	-
20	+++	+++	-	-
50	++++	++++	+	-
20				
N/P ratio	bPEI(25k)-*g*-lPEG(550)$_{35}$	bPEI(25k)-*g*-lPEG(5k)$_6$	bPEI(25k)-*g*-lPEG(20k)$_1$	Controls: DNA Ringer´s solution
4.5	-	-	-	
9	+	-	-	
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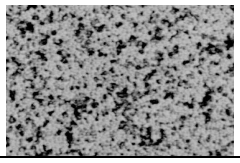
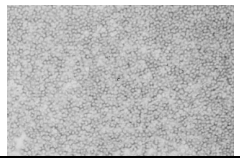
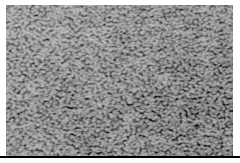
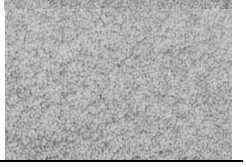
50	+++	+	++	
20				

The erythrocyte aggregation assay allowed only semiquantitative estimations about the hemocompatibility of the complexes. Results of this study are given in Table 1. Exemplarily, microscopic images of the erythrocytes are shown in this table for N/P ratio 20. Ringer's solution and free DNA did not show any aggregation (control experiment). In case of the

complexes, aggregation tendency was found as a function of N/P ratio: The larger the excess of polycation in the complex, the stronger the aggregation. Complexes of PEI, bPEI(25k)-*g*-lPEG(5k)$_2$ and bPEI(25k)-*g*-lPEG(550)$_{35}$ showed strong to very strong aggregation of the red blood cells. Since aggregation is often caused by electrostatic interactions between the anionic cell membranes and a cationic object, the surface charge of the complexes was an important key to interpret these results. When the extent of aggregation is plotted against the ζ-potential (Figure 8) a threshold at a ζ-potential of about 6 mV is observed. Below this value no or only weak aggregation was observed, whereas above this ζ-potential strong aggregation occurred. In all cases where PEGylation reduced ζ-potential of the complexes below 6 mV no erythrocyte aggregation was observed. This was achieved by grafting 6 PEG 5 kDa blocks or one 20 kDa block. Since the three polymers showing strong aggregation also induced a relatively strong hemolytic activity it is likely that high ζ-potentials also affect hemolysis.

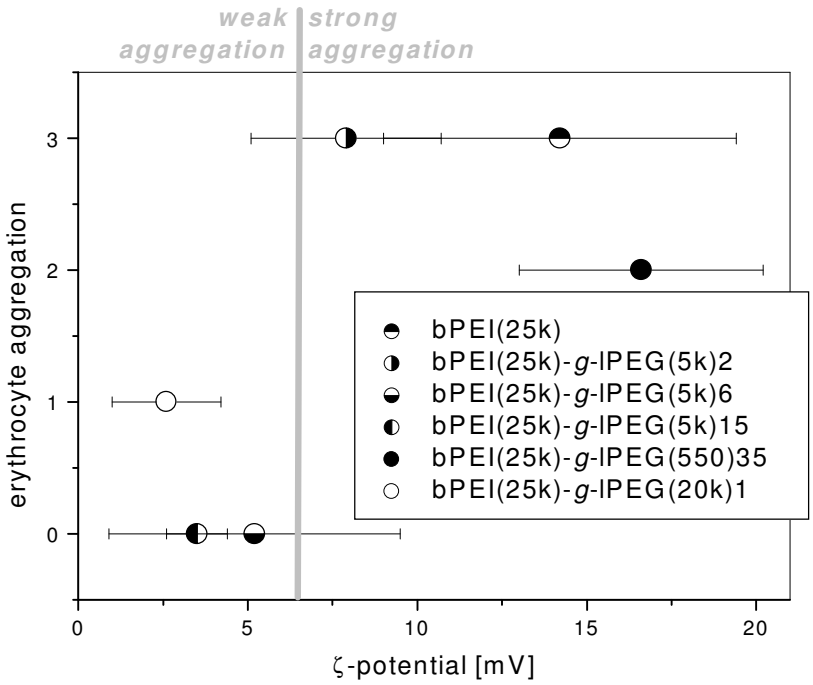


Figure 8: Plot of polymer/DNA complex induced erythrocyte aggregation (N/P 20) against ζ-potential of the complexes (N/P 20, 150 mM NaCl). Erythrocyte aggregation is given semiquantitatively. 0: no aggregation, 1: weak aggregation, 2: strong aggregation, 3: very strong aggregation.

Cytotoxicity of the complexes. The cytotoxicity of the complexes was studied using the LDH assay which provides information on the destructive interaction of the complexes with cell membranes. It was found that with increasing N/P ratio the cytotoxicity of the complexes increased (Figure 9). In the first series with the bPEI(25k)-*g*-lPEG(5k)$_n$ copolymers toxicity is

reduced with increasing number n of PEG blocks. Another trend is observed for the second copolymer series: Toxicity is reduced with decreasing MW of PEG. Consequently, there are two copolymers which formed complexes with a low toxicity profile: bPEI(25k)-*g*-lPEG(550)$_{35}$ and bPEI(25k)-*g*-lPEG(5k)$_{15}$. Since their LDH release is < 10 % DNA complexes with these copolymers can be regarded as relatively non-toxic (*37*). These two copolymers have two features: First, they both form complexes of ill-defined and more diffuse complex shape. Severe cell membrane damage is often observed when many charges are locally concentrated on the cell membrane (*37*). In contrast, the charges of a diffuse complex are more distributed over a wider area of the cell membrane in comparison with compact complexes. Second, both copolymers are characterized by a high degree of PEG substitution (n = 15 and 35, respectively). The toxic sites of the copolymers are the amino groups of PEI. If they are substituted with PEG, the basic amino groups are transformed into neutral urea bonds (according to our synthesis route). Thus, the reduction of the toxicity of PEI is expected to be a function of degree of PEG substitution. Indeed, a plot of % LDH release against the degree of PEGylation (Figure 10) clearly shows this function which seems to be independent on the MW of the PEG.

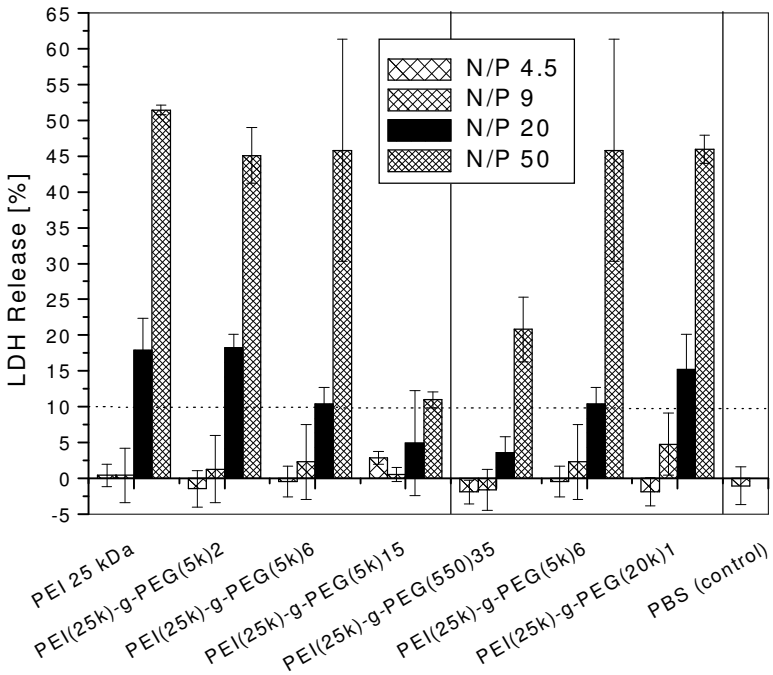


Figure 9: LDH-Assay of bPEI 25 kDa and bPEI-*g*-lPEG copolymer complexes with herring testes DNA at different N/P ratios after an incubation of 4 h. The experiment was performed with 3T3 mouse fibroblast cell line. A release of less than 10 % can be understand as non-toxic.

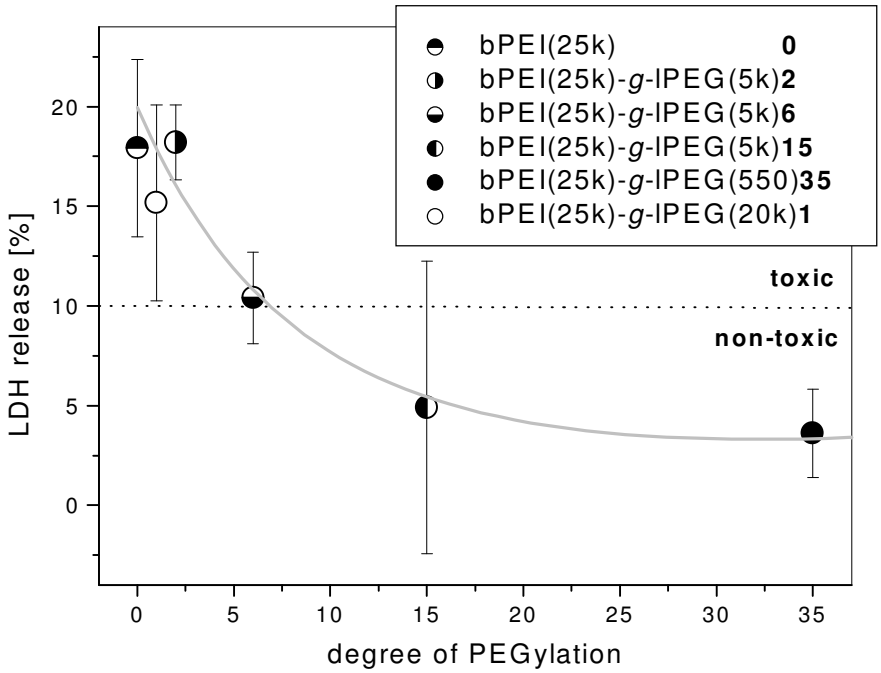


Figure 10: Correlation of degree of PEG grafting (average number of PEG blocks per one PEI molecule) with cytotoxicity of the polymer/DNA complexes as determined by LDH assay (LDH release at N/P = 20).

Transfection efficiency of the complexes. Finally, the transfection activity of the complexes was studied using the same cell line as in the cytotoxicity assay. The transfection efficiency expressed by amount of luciferase per amount protein was measured at three different N/P ratios for each polymer. As a consequence of the results from preliminary experiments, not every N/P ratio was repeatedly measured for all polymers. Due to the high toxicity of the homopolymer PEI, all cells died at N/P = 50 and a transfection experiment was not feasible. No perceptible transfection could be observed at N/P = 4.5. Therefore, the experiments were run only at higher N/P ratios. The results are presented in Figure 11.

In spite of PEGylation all copolymers showed transfection activity at least as high as homopolymer PEI. Furthermore, at higher N/P ratios the copolymers turned out to be even more efficient than PEI. This is quite remarkable since most other groups found a significant decrease in transfection activity for the PEG modified PEIs (*20 ,21, 23*). All polymers showed higher transfection activities with increasing N/P ratios. In the first series with bPEI(25k)-*g*-lPEG(5k)$_n$ copolymers transfection efficiency decreases with increasing number of PEG blocks. Complexes of copolymer with n = 2 were even more efficient in gene transfer than complexes of homopolymer PEI. In case of the second series, copolymer bPEI(25k)-*g*-lPEG(20k)$_1$ gave similar results as the other 50 % PEG copolymer bPEI(25k)-*g*-lPEG(5k)$_6$.

Remarkable results were found for bPEI(25k)-*g*-lPEG(550)$_{35}$ even with a high N/P ratio of 50.

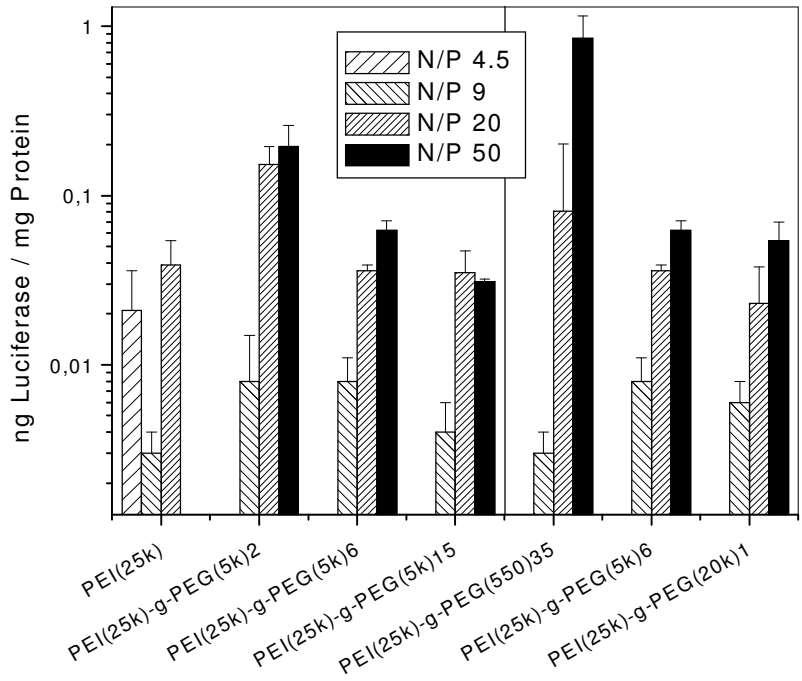


Figure 11: Transfection efficiency of polymer/plasmid DNA complexes with bPEI 25 kDa and bPEI-*g*-lPEG block copolymers at different N/P ratios. The experiment was performed with 3T3 mouse fibroblast cell line.

The complexity of the gene transfer process prevents the correlation of transfection data with a single physicochemical or biological parameter. For instance, ζ-potential or complex size alone can not explain why the two polymers with high ζ-potential and large complex size (PEI and bPEI(25k)-*g*-lPEG(550)$_{35}$) transfected differently, the first very poorly, the latter very strongly. Also, the cytotoxicity alone did not give a reasonable relationship with transfection activity. Here again, the two polymers with high LDH release (PEI and bPEI(25k)-*g*-lPEG(5k)$_2$) transfected differently, the first poorly, the latter strongly. Furthermore, the two copolymers of low cytotoxicity (bPEI(25k)-*g*-lPEG(5k)$_{15}$ and bPEI(25k)-*g*-lPEG(550)$_{35}$) also showed quite different transfection activities. Therefore, the results of the transfection experiments could be explained only by a combination of physicochemical and biological parameters. Cytotoxicity and ζ-potential turned out to be the key factors for the explanation of the results of the gene transfer experiments. Whereas a strong cytotoxicity was unwanted, a high ζ-potential is generally assumed to be advantageous for *in vitro* gene delivery since it enhances the adhesion of the cationic complex with the

negatively charged cell membrane (*38*). In Figure 12, the cytotoxicity in means of LDH release is plotted against ζ-potential and the transfection efficiency is represented semiquantitatively by the size of the polymer symbols.

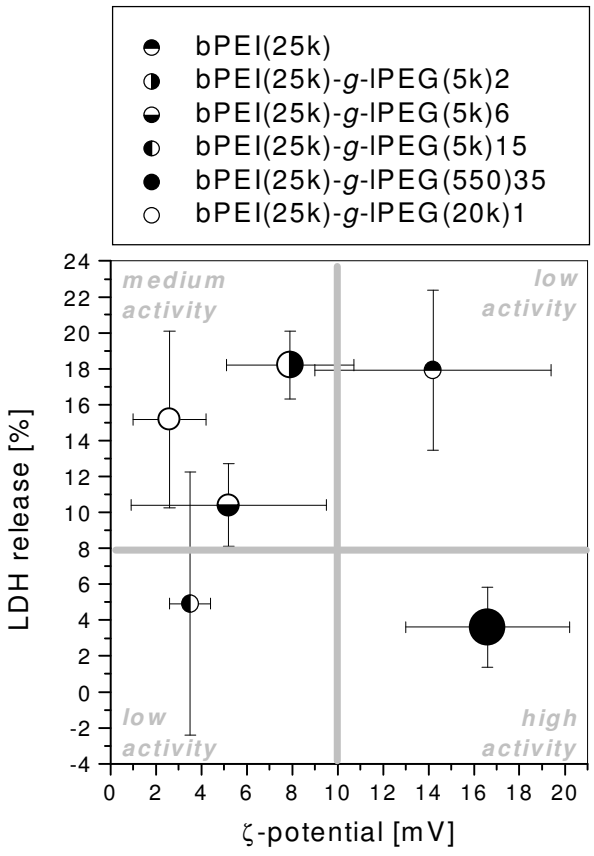


Figure 12: Transfection efficiency in correlation to ζ-potential (N/P 20, 150 mM NaCl) and cytotoxicity as determined by LDH assay (N/P 20) on 3T3 fibroblast. The size of the dot indicates semiquantitatively the transfection efficiency (the larger the symbol, the higher transfection activity).

The six polymer/DNA complexes could be divided into four groups of different transfection activities. First, in the case of low toxicity *and* of low ζ-potential the complexes showed only poor gene expression. These complexes are probably too inert due to the thoroughly shielding PEG blocks and do not interact with the cells. Second, when the ζ-potential was relatively low but the LDH release was high medium transfection activities were observed for the appropriate complexes. Due to the low ζ-potential the concentration of cell-adherent complexes might remain low and therefore, the cells survived the treatment of these toxic complexes. Third, the combination of high ζ-potential and high LDH release yielded poor transfection possibly due to a strong interaction of the highly toxic complexes with the cells which did not survive. Forth and finally, complexes with high ζ-potential but with low cytotoxicity showed high transfection activities and seemed to be most efficient as *in vitro* gene transfer vectors. Here, highly positive surface charge is not accompanied by toxic side

effects. The representative copolymer of this group is bPEI(25k)-*g*-lPEG(550)$_{35}$. Also another explanation for the good transfection activity of this copolymer can be put forward. It has been reported for *in vitro* gene delivery that large complexes more easily reach the cells due to sedimentation (*39*). Thus, the large complexes formed by bPEI(25k)-*g*-lPEG(550)$_{35}$ could show higher sedimentation.

CONCLUSIONS

By variation of the degree of substitution and the MW of PEG we have obtained a set of PEI-*g*-PEG copolymers which represent a broad range of different block copolymer structures. We have demonstrated that copolymer structure has a strong influence on the shape of the polycation/DNA complex and its surface charge. Copolymers with many short PEG blocks formed large and diffuse complexes of high positive surface charge. Copolymers with only a few but long PEG blocks self-assembled to small and compact condensates of low surface charge. Copolymers with many long PEG blocks generated complexes of ill-defined shape and of almost no surface charge. Furthermore, the physicochemical parameters have a decisive impact on the interaction of the complexes with cells. A high positive ζ-potential of the complexes resulted in a strong erythrocyte aggregation and hemolysis. However, cytotoxicity monitored with fibroblasts was a function of the degree of PEGylation independent of the MW of the PEG. Sufficient *in vitro* gene expression was realized with the copolymer that formed large complexes with a high positive surface charge and a low toxicity profile, as it was found for the copolymer with many short PEG 550 Da blocks.

ACKNOWLEDGMENTS

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Chapter 3

The Macrostopper-Route: A New Synthesis Concept Leading Exclusively to Diblock Copolymers with Enhanced DNA Condensation Potential

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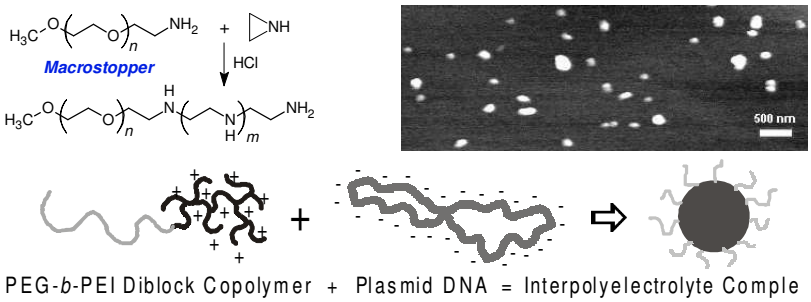


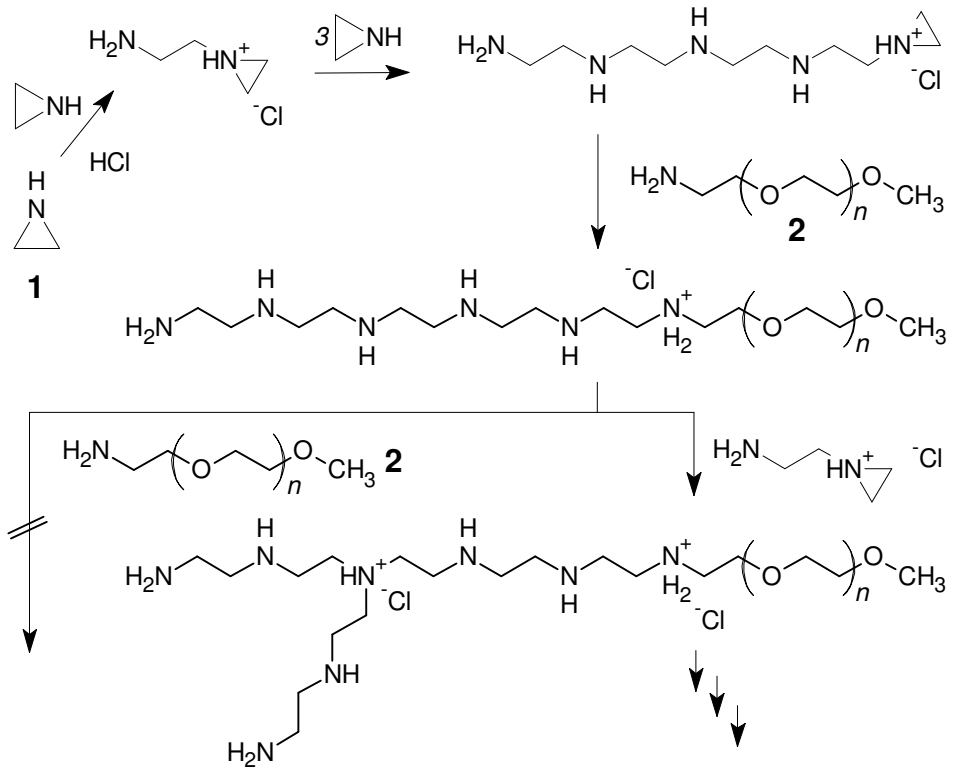
ABSTRACT

A new synthesis concept has exclusively led to diblock copolymers of branched polyethylenimine (PEI) and poly(ethylene glycol) (PEG). Using monoamino-PEGs as "macrostoppers" for the termination of the ethylenimine polymerization avoids inherently the formation of PEIs that are multiply grafted with PEG blocks. This concept works perfectly for PEGs with MW of 5000 while with higher MW increasing populations of free homopolymers were obtained as side products. The diblock copolymers self-assembled with plasmid DNA to very compact and spherical complexes with diameters of approximately 100 nm. The single PEG block of these copolymers was able to generate stable and soluble complexes even under critical conditions such as neutral net charge and high ionic strength.

Interpolyelectrolyte complexes (IPEC) of DNA and synthetic polycations have been proposed as an promising alternative to viral gene delivery systems.[1] However, the lack of stability of those complexes has lead to the development of block copolymers composed of one cationic block for DNA condensation grafted with several non-ionic hydrophilic blocks to enhance the complex solubility and shielding them against non-specific interactions with biological components.[2] Prominent homopolymers for such copolymers are the cationic polyethylenimine (PEI) which is an effective gene transfection agent,[3] and poly(ethylene glycol) (PEG) a well known shielding moiety.[4] Unfortunately direct PEGylation of polycations such as PEI can diminish the DNA condensation properties.[5] To overcome this problem we synthesized pure diblock copolymers and discovered that PEI-induced DNA condensation is not hampered when the polycation is grafted with only one PEG.

PEI is a hyperbranched polycation with many terminal amino groups. The synthesis of a pure diblock copolymer composed of branched PEI and linear PEG is not feasible via coupling of the homopolymers since in this case side products with more than one PEG per PEI macromolecule would also result.[6] The use of a PEG-macroinitiator for the ring-opening-polymerization (ROP) of ethylenimine (EI) would also result in side products with higher degrees of PEGylation.[7] The only possibility to avoid multiple PEG grafting of PEI is to terminate the polymerization of EI by a PEG macromolecule. According to this function we propose the term “macrostopper”.

The ROP of EI **1**, initiated by hydrochloric acid, was performed in aqueous solution containing the monomethyl-monoamino-PEG **2** as macrostopper (Scheme 1).[8] It is well known that low molecular weight (MW) amines terminate the polymerization reaction of EI.[9] Thus, we expected the same termination with high MW amines, such as monoamino-PEG. The nature of the EI polymerization mechanism ensures formation of diblock copolymers, exclusively. Once the propagating PEI chain is terminated by the PEG, the resulting copolymer can no longer react with a second PEG **2**. Only coupling of further oligoethylenimine with a reactive, alkylated three-membered ring to the PEI block is possible, leading to the formation of the hyperbranched structure of PEI. We performed this polymerization using a monoamino-PEG with MW of 5000 (for which we determined a MW of 7370 by light scattering (LS), Figure 1). The resulting copolymer exhibited a number average MW (M_n) of 11870 which is in line with the theoretically expected MW of 11780 since the M_n of PEI in a control experiment without macrostopper was found to be 4410.



Scheme 1: Synthesis of the copolymers via macrostopper-route.

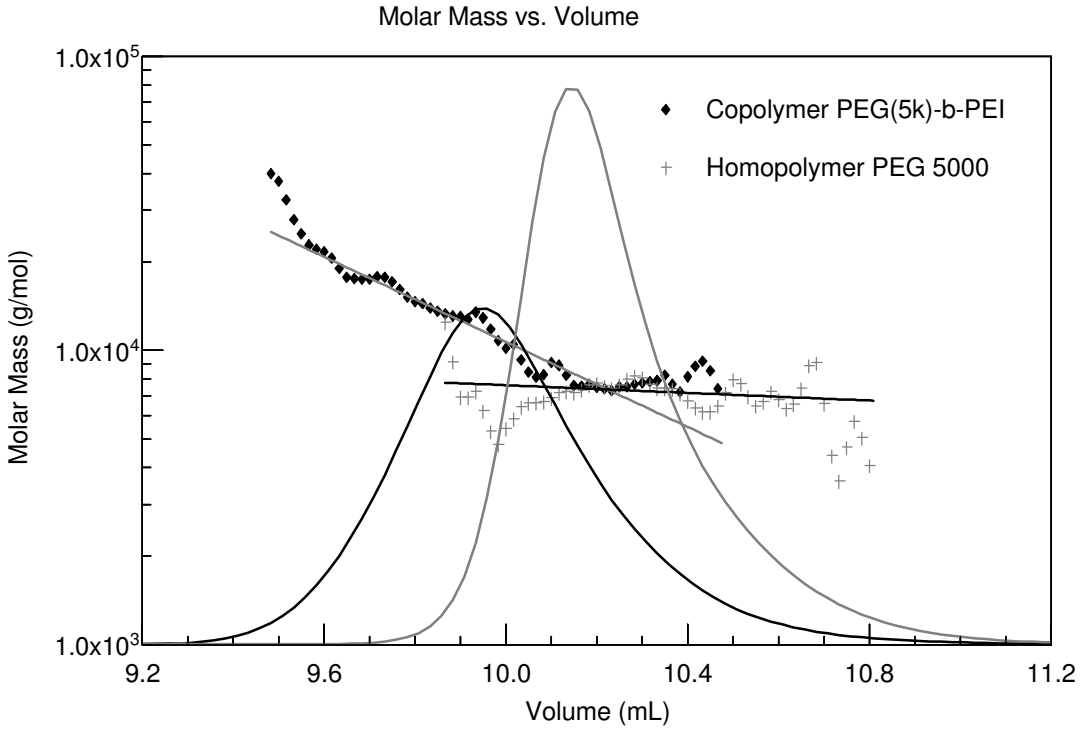


Figure 1: SEC-eluograms in 1% formic acid as detected by a refractive index detector with MWs of the appropriate polymer fractions as determined by a multiple-angle-laser-LS detector.

The formation of the copolymers was also confirmed by ^{13}C-NMR. The signals of the homopolymer end group PEG-O-*C*H_2-*C*H_2-NH_2 at 71.4 and 40.8 ppm were shifted to 70 ppm (overlapped by the PEG signal) and to 46 ppm (overlapped by one of the PEI signals) for the copolymer. All other signals appeared at the same chemical shift since we produced a copolymer without a linker group that would cause further shifts of signals. The absence of any ester or amide bonds for the linkage between the two polymer blocks yielded a copolymer, which is extremely stable against hydrolysis. A ^{13}C-NNE-NMR experiment that allowed the integration of PEI's ethylene carbon signals by avoiding the nuclear Overhauser effect (NOE) demonstrated the unique degree of branching of the PEI block. While for commercially available PEIs the ratio of primary/secondary/ternary amino groups was found to be approximately 1/1/1,[10] the PEI block in this copolymer exhibited a more linear structure (ratio 1/2/1).[11] This lower degree of branching could be advantageous since linear PEI exhibited a higher activity in gene transfection experiments *in vitro*.[12]

While the macrostopper-route perfectly functioned for PEG with MW of 5000, the feasibility of this route for higher PEG-MWs seems to be limited. The ROP of EI in the presence of PEGs with MWs of 10000 and 20000 led to the formation polymers with M_n of 8000 and a weight average MW (M_w) of 11000. With a MW > 10000 the terminal amino groups of PEGs seem to be no longer sufficiently accessible. This led to the formation of a blend consisting of unreacted PEG, PEI and copolymer. With increasing MW of the macrostopper the amount of free homopolymer increased.

IPEC were prepared upon mixing aqueous solutions of PEG-*b*-PEI and plasmid DNA (c = 40 µg/mL), in equal volumes in 10 mM NaCl, at pH 7.4 and at a PEI-amines/DNA-phosphate (N/P) ratio of 9. These self-assembling complexes were imaged by atomic force microscopy (AFM) under 10 mM NaCl solution. Uniformly spherical complexes with diameters of about 100 nm were observed for the PEG(5k)-*b*-PEI copolymer (Figure 2 C). The compact appearance of the complexes formed with this diblock copolymer support the impression of very efficient DNA condensation. This is in contrast to multi-grafted PEGylated PEIs where poorly condensed DNA was observed (Figure 2 A and B). PEG(10k)-*b*-PEI and PEG(20k)-*b*-PEI also formed compact spherical complexes of about 100 nm diameter. These results were confirmed by dynamic LS (DLS) analysis demonstrating an enhanced DNA condensation of the diblock copolymers compared with the homopolymer PEI. Significantly, smaller hydrodynamic diameters of about 100 ± 15 nm were found for all three copolymers, in comparison with complexes of the homopolymer PEI (325 ± 38 nm). The surface charge of the IPECs was estimated by ζ-potential measurements. The ζ-potential of the complexes was

reduced from 24 ± 3 mV (no PEG) to 19 ± 3 mV by the PEG 5000 blocks and to 13 ± 4 mV by PEG 10000. The stability and solubility of the complexes under critical conditions (neutral net charge and high ionic strength) was studied by DLS. Whereas PEI formed complexes aggregating to particles with diameters up to 800 nm within 20 min, the copolymers yielded stable complexes. To summarize, our results have shown that the macrostopper concept led to diblock copolymers that enhance DNA condensation in comparison with homopolymer PEI and multi-PEGylated PEIs. Therefore, these PEG-*b*-PEI diblock copolymers are promising candidates for gene delivery and their potential to transfect genes *in vitro* is currently investigated in our laboratories.

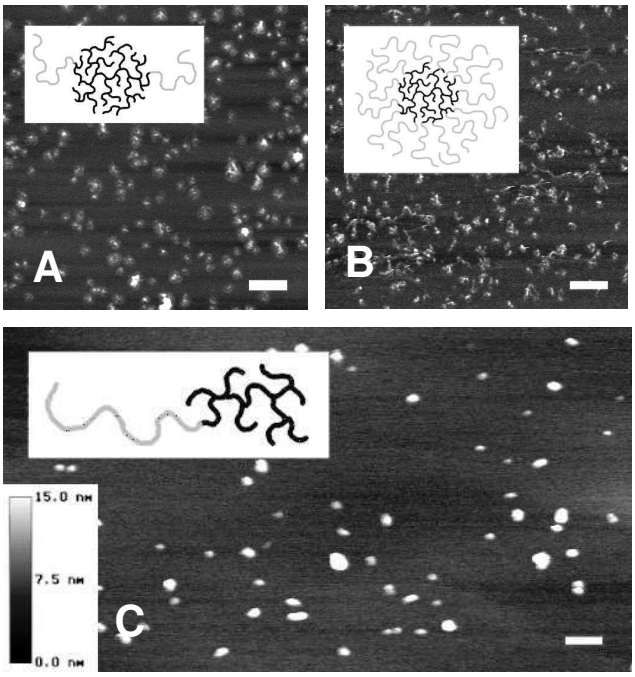


Figure 2: AFM images of copolymer/pDNA complexes at N/P = 9, 10 mM NaCl, pH 7.4. PEI 25000 grafted with two (A) and 15 (B) PEG 5000 blocks in average. MonoPEGylated PEI: PEG(5k)-*b*-PEI (C). The scale bar is equivalent to 500 nm.

Acknowledgment. We thank Klaus Kunath and Thomas Merdan for plasmid preparation and gratefully acknowledge support of the Deutsche Forschungsgemeinschaft (DFG). A.L.M. acknowledges the Royal Pharmaceutical Society of Great Britain for funding.

Supporting Information: Scheme of the macroinitiator-route, ^{13}C-NMR spectra, MW data, elemental (C,H,N) analysis data, SEC eluograms, DLS/ζ-potential measurement data and AFM images (PDF). This material is given in the supplement of Chapter 3.

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(6) The reaction of PEG blocks onto PEI in a stoichiometric 1:1 ratio is to a large extent a random process, which should lead to significant populations of PEI without any PEG blocks and with two or even more PEG blocks.

(7) Two PEG blocks that initiated the ROP of EI are able to react with each other during the propagation steps. The reactive site (the alkylated three-membered ring) of the one growing copolymer might react with the amines of PEI of the other copolymer.

(8) 1 ml of EI and 0.83 g of the monoamino-PEG were dissolved in a 25 ml flask containing 10 ml water. The reaction was initiated using 50 µl of a 37 % hydrochloric acid and stirred for 4 days at 25 °C. Finally, the solution was heated up to 60 °C for 24 h. The solvent was removed under reduced pressure. The polymer was dissolved in ethanol, precipitated in diethyl ether, and the isolated polymer was dried *in vacuo*. PEG(5k)-*b*-PEI: 0.74 g, 40 % yield. PEG(10k)-*b*-PEI: 0.82 g, 45 % yield. PEG(20k)-*b*-PEI: 0,92 g, 50 % yield.

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(11) The exact ratio of 1°/2°/3° amines of PEG(5k)-*b*-PEI was determined as 25 % / 54 % / 21 %.

(12) Bragonzi A., Boletta A., Biffi A., Muggia A., Sersale G., Cheng S. H., Bordignon C., Assael B. M. *Gene Ther.* **1999**, *6*, 1995-2004.

Supplement of Chapter 3

Table 1: Molecular weights of homopolymers and copolymers.

Polymer	PEI content[a] [%]	MW [g/mol] calcd.	M_n [g/mol] found[b]	M_w [g/mol] found[b]	M_w/M_n	dn/dc[c] [mL/g]
mPEG-NH_2	0	5 000[d]	7 370	7 370	1.00	0.13
mPEG-NH_2	0	10 000[d]	11 970	12 060	1.01	0.13
mPEG-NH_2	0	20 000[d]	20 200	20 640	1.02	0.13
PEI	100		4 410	5 800	1.32	0.26
PEG(5k)-*b*-PEI	32[e]	11 780	11 870	15 270	1.29	0.17
PEG(10k)-*b*-PEI	39	16 380	7 970	11 310	1.42	0.18
PEG(20k)-*b*-PEI	30	24 610	8 420	11 470	1.36	0.17

[a] As determined via integration of ^{1}H-NMR signals.
[b] As determined by SEC-MALLS in 1% formic acid.
[c] PEG: according to J. Brandrup, E. H. Immergut, Polymer Handbook, 3rd ed., Interscience Wiley, New York, 1989. PEI: measured by RI detector. Copolymers: calculated from PEI content.
[d] Molecular weight data provided by the supplier.
[e] Integration of the ^{13}C-NNE signals resulted in a 28 % PEI content.

Table 2: Elemental analysis of the polymers.

Polymers	Analysis[b] C calcd	C found	H calcd	H found	N calcd	N found
PEI	55.78	52.10	11.70	10.90	32.52	28.05
PEG(5k)-*b*-PEI	54.92	53.90	9.95	9.49	10.25	10.18
PEG(10k)-*b*-PEI	55.01	52.95	9.13	9.22	12.58	9.92
PEG(20k)-*b*-PEI	54.90	53.46	9.90	9.49	9.58	10.52

[a] Calculated for $(C_2H_5N)_n$, $C_{200}H_{439}N_{32}O_{67}$, $C_{200}H_{439}N_{39}O_{61}$ and $C_{200}H_{430}N_{29}O_{70}$, respectively.

Scheme 1: Macroinitiator-route. A possible synthesis-route to produce PEG-*b*-PEI copolymers is to modify a monomethyl-PEG **1** with tosyl chloride **2**. In this way, an alkylating agent is obtained, which is able to initiate the ring-opening-polymerization (ROP) of ethylenimine **3** (EI). Two PEG blocks that initiated the ROP of EI are able to react with each other during the propagation steps. The reactive site (the alkylated three-membered ring) of the one growing copolymer might react with the amines of PEI of the other copolymer.

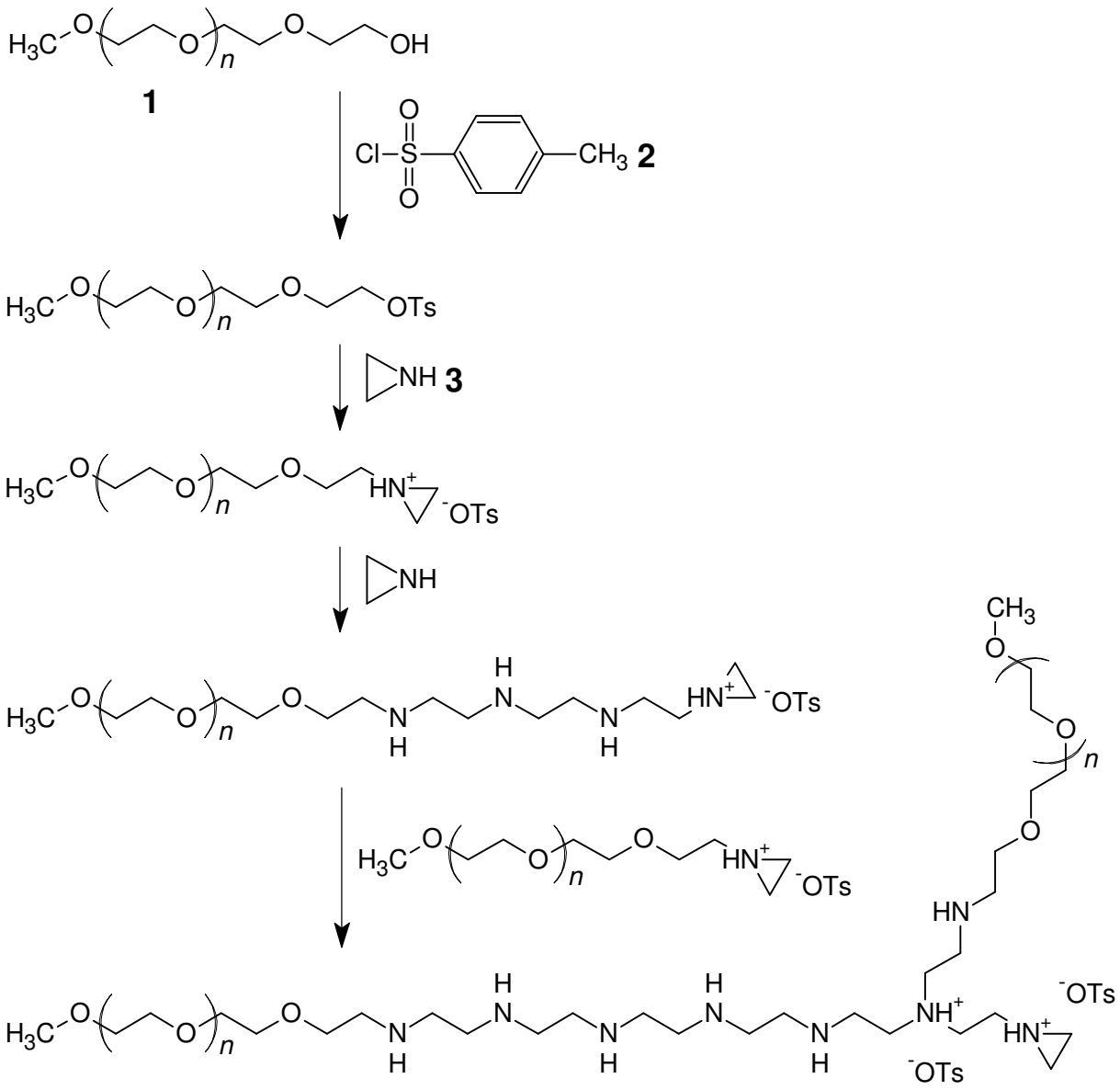


Figure 1: ^{13}C-NMR spectra of homopolymer PEG-NH_2 5000 (**A**) and copolymer PEG(5k)-*b*-PEI (**B**) in D_2O at 50 °C, recorded on a Eclipse+ 500 from Joel, 125 MHz.

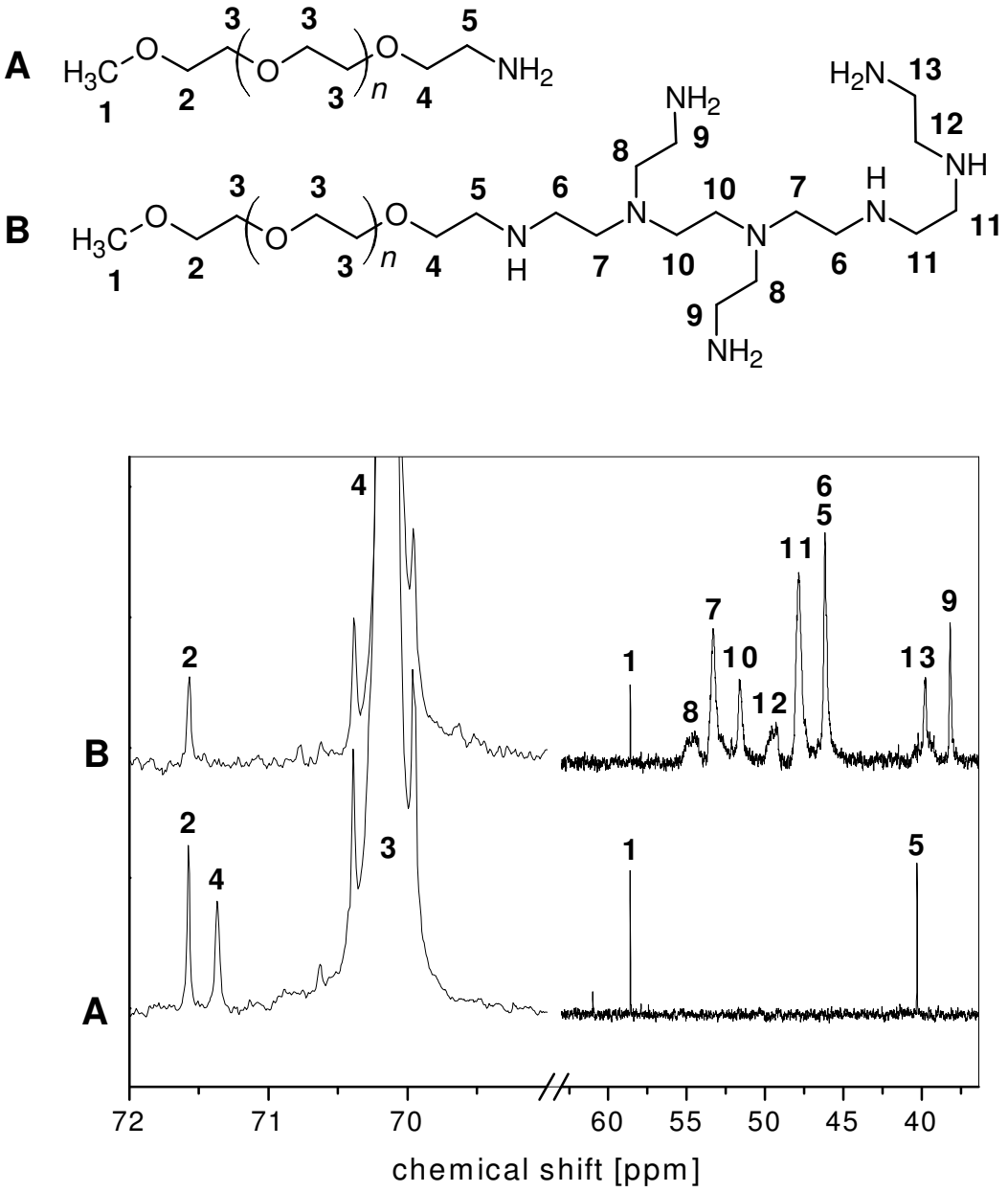
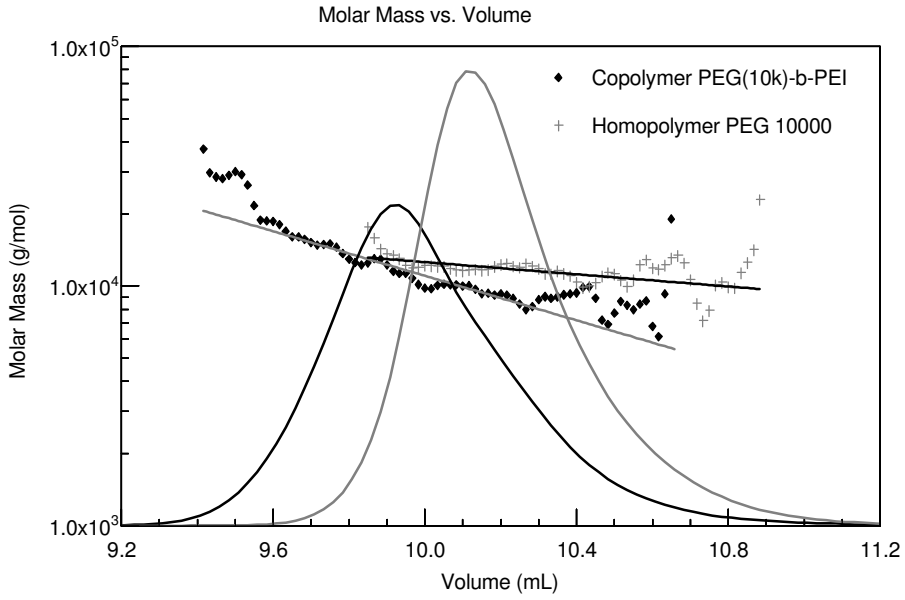


Figure 2: SEC-eluograms of PEG(10k)-*b*-PEI (top) and PEG(20k)-*b*-PEI (bottom) in 1% formic acid detected by refractive index detector RI-71 from Merck (Darmstadt, Germany) with MWs of the appropriate polymer fractions as determined by multiple-angle-laser-light-scattering detector from Wyatt Technologies (DAWN®EOS™, Santa Barbara, CA). Column: Suprema Max 3000 from Polymer Standard Service (Mainz, Germany). Flow rate: 1 ml/min. Temperature: 35 °C. MWs were determined via Zimm-plots.



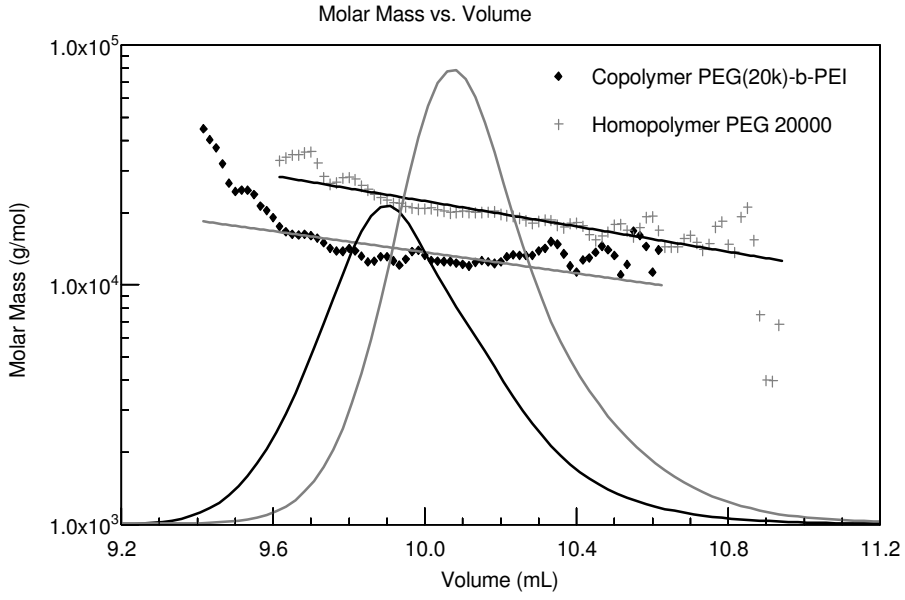


Figure 3: Complex size (Z-average) of the copolymer/pDNA complexes as determined by dynamic light scattering at 25 °C, 90°, and ζ-potential as determined by laser-Doppler-anemometry (Zetasizer 3000 HS from Malvern Instruments, Worcs., UK). The columns represent the mean and SD of 10 measurements. Plasmid: pGL3, 5256 bp, Promega (Heidelberg, Germany).

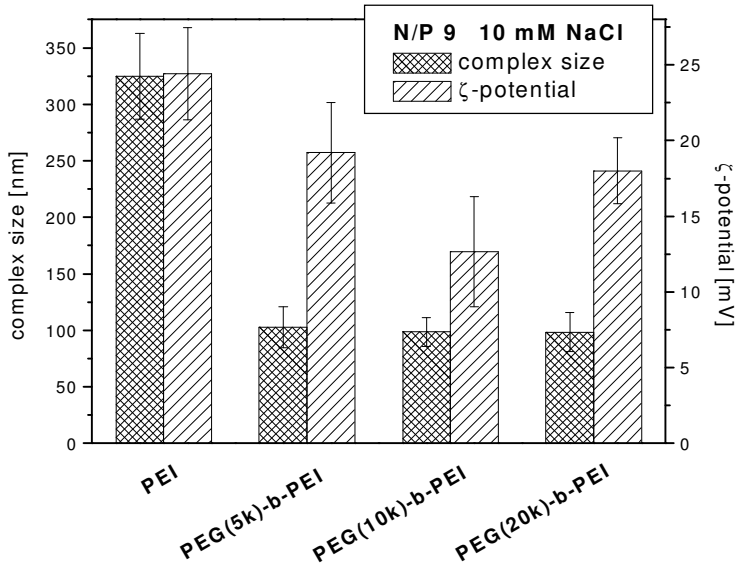


Figure 4: Complex size (Z-average) of the polymer/pDNA complexes as a function of incubation time as determined by dynamic light scattering in 150 mM NaCl and at N/P ratio 4.5 (25 °C, 90°, pH 7.4, Zetasizer 3000 HS from Malvern Instruments, Worcs., UK). Plasmid: pGL3, 5256 bp, Promega (Heidelberg, Germany). The blend exhibited the same composition of PEI and PEG as the copolymer PEG(5k)-*b*-PEI.

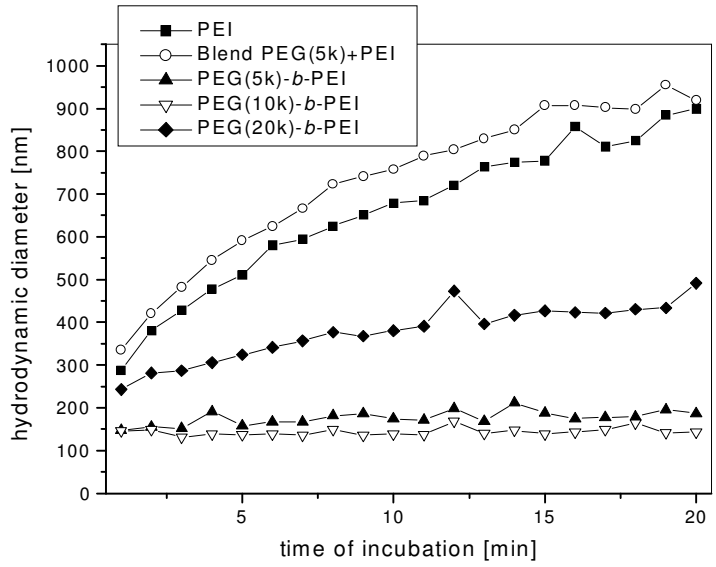
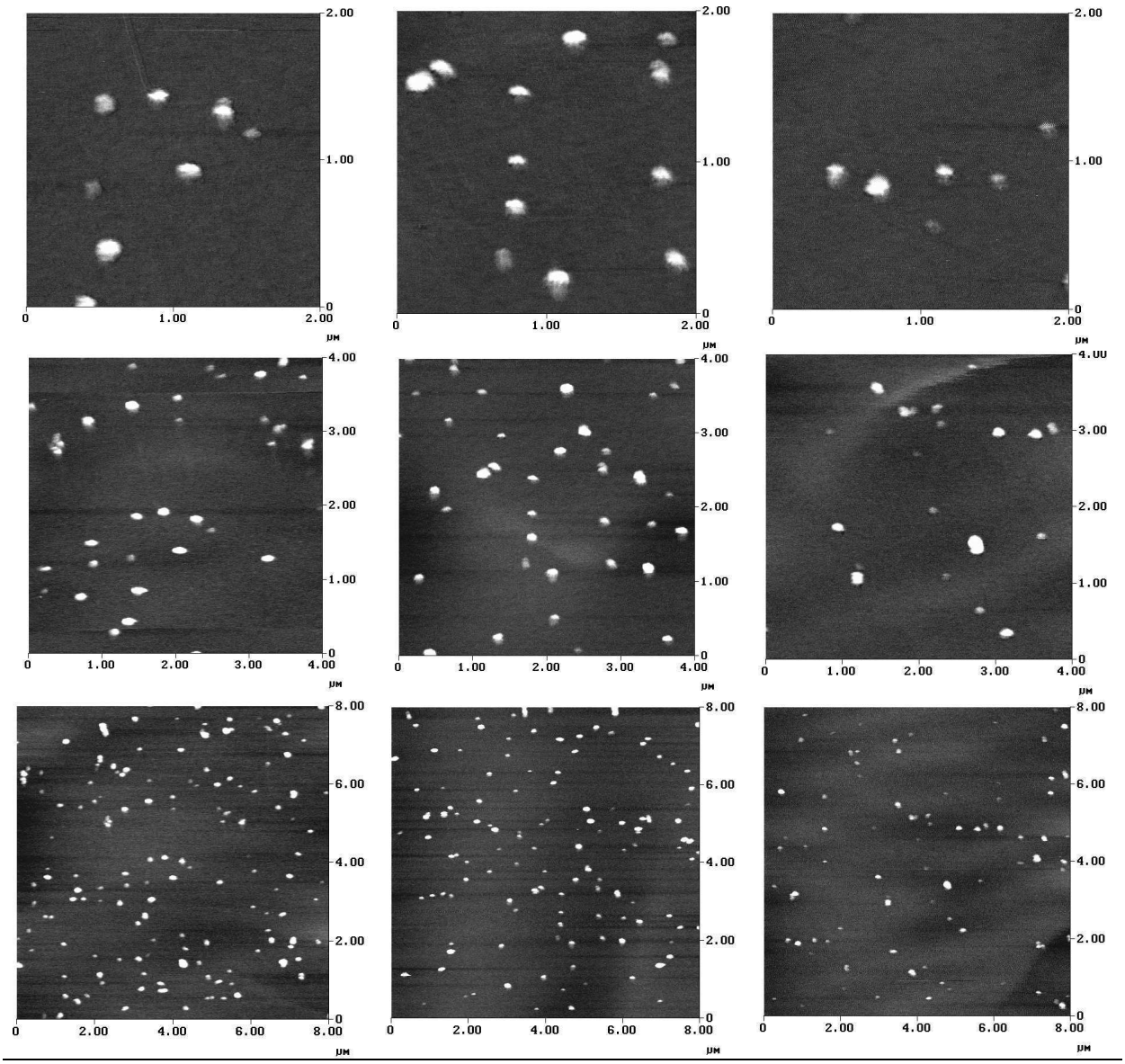


Figure 5: AFM-images of copolymer/pDNA complexes as imaged under 10 mM NaCl on a mica substrate. Images were conducted on a Dimension™3000 Scanning Probe Microscope from Digital Instruments (Santa Barbara, CA) with a Nanoscope IIIa controller. N/P ratio: 9. Plasmid: pBR322, 4363 bp, Sigma (St. Louis, MO).



Chapter 4

Star-shaped Poly(ethylene glycol)-*block*-Polyethylenimine Copolymers Enhance DNA Condensation of Low Molecular Weight Polyethylenimines

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ABSTRACT

Star-shaped poly(ethylene glycol)-*block*-polyethylenimine [*star*-(PEG-*b*-PEI)] significantly enhance plasmid DNA condensation of low molecular weight (MW) PEIs. The star-block copolymers were prepared via a facile synthesis route using hexamethylene diisocyanate as linker between PEG and PEI blocks. NMR and FT-IR spectroscopy confirmed the structures of intermediately activated PEG and final products. Furthermore, the copolymers were characterized by size exclusion chromatography, static light scattering and viscosimetry. Their MW (M_w: 19 - 26 kDa) were similar to high MW PEI (25 kDa). Thermoanalytical investigations (TGA, DSC) were also performed and verified successful copolymer synthesis. DNA condensation with the low MW PEI (800 Da and 2000 Da) and their 4- and 8-star-block copolymers was studied using atomic force microscopy, dynamic light scattering, zeta-potential measurements and ethidium bromide (EtBr) exclusion assay. It was found that low MW PEIs formed huge aggregates (500 nm – 2 µm) in which DNA is only loosely condensed. By contrast, the star-block copolymers yielded small (80 - 110 nm), spherical and compact complexes that were stable against aggregation even at high ionic strength and charge neutrality. Furthermore, as revealed in the EtBr exclusion assay these star-block copolymers exhibited a DNA condensation potential as high as high MW PEI. Since these *star*-(PEG-*block*-PEI) copolymers are composed of relatively non-toxic low MW PEI and biocompatible PEG their potential as gene delivery agents merits further investigations.

INTRODUCTION

Condensation of DNA is the first and very important step in gene delivery. In the condensed form the DNA is protected against digestion by enzymes and is packaged into a compact and small unit which enables trafficking through the diverse barriers towards the nucleus of the target cell where the gene can be expressed. In the case of viral gene transfer, DNA is condensed by viral proteins containing a large number of basic and therefore positively charged amino acids in their primary structure.[1] Also other cationic species such as cationic lipid formulations[2] or cationic polymers[3] have been studies as DNA condensation agents in the context of non-viral gene delivery. Polyethylenimine (PEI) is one of the most frequently used polycations for this application.[4] PEI can be synthesized with different degrees of branching and molecular weights (MW). With regard to the degree of branching, three different types of PEI have been proposed as gene transfer vehicles: Highly branched[5], moderately branched[6] and linear PEI[7].

The MW of commercially available PEIs range from 423 Da to 1 MDa. Several authors reported that the size of the PEI has a strong influence on the efficiency of gene transfer with regard to transfection activity[8,9] and cytotoxicity.[10] Generally, branched PEI can be categorized into three groups with respect to the MW. Low MW PEIs (LMW PEI $\leq$ 2000 Da) proved to be non-toxic but displayed very poor transfection activity. By contrast, high MW PEIs (HMW PEI $\geq$ 25 kDa) showed high transgene expression but also significant cytotoxicity. In the intermediate MW range from > 2000 Da to < 25 kDa the PEIs are of a medium to low cytotoxicity and also a medium activity in transfection experiments.[8,11] Due to their relatively low cytotoxicity higher doses of the polycation could be tolerated in the gene transfer experiments which resulted in high gene expression.[12] Although this is known for several years the reason for the poor transfection activity of the low MW PEIs still remained unclear. Nguyen et al. reported that DNA complexes formed with PEI 2000 Da were not stable and precipitated rendering the determination of complex size and the gene expression activity in transfection experiments[13] impossible. Others performed transfection experiments but did not supply additional physicochemical information about their complexes which might be helpful to explain the poor results of their gene transfer experiments.[8,9] In this paper we focus on the physicochemical aspects of DNA condensation of LMW PEI and determined the morphology, size and ζ-potential of the complexes using atomic force microscopy (AFM), dynamic light scattering (DLS) and laser Doppler anemometry (LDA), respectively. Furthermore, we studied the DNA condensation potential using an ethidium bromide (EtBr)

exclusion assay. With these physicochemical results we hope to offer an explanation for the poor transfection activity of LMW PEI.

Despite of its poor transfection activity, LMW PEI is of general interest as gene delivery agents due to the cytotoxicity profile. Since size of PEI seems to matter both for cytotoxicity as well as transfection,[8] our hypothesis was to assemble LMW PEI chemically in a new way to increase the MW. Further, we hypothesized that the internal charges of HMW PEI may not critically contribute to DNA condensation. Thus, our idea was to create a core-shell structure where the core consists of a hydrophilic, inert polymer to which a shell of LMW PEI molecules is attached. Using this strategy we hoped to obtain a large macromolecular unit in which only the external shell contains cationic charges. These units should attain the size of a HMW PEI but consist only of the non-toxic LMW PEI. Here, we report a facile synthesis route for these macromolecules and how experimental data on DNA condensation.

MATERIALS AND METHODS

Materials.

4-*star*-PEG (15 kDa) and 8-*star*-PEG (10 kDa) were obtained from Shearwater Polymers (Huntsville, AL). They are designated as 4 and 8 arm branched PEG or diglycerol and hexaglycerol polyethyleneglycol ether by the supplier. Branched PEI with MW of 800 Da (water free, 99 %) and 2000 Da (50 % aqueous solution) were purchased from Aldrich (Milwaukee, WI). In case of PEI 2 kDa water was removed by lyophilization. Furthermore, this PEI was dissolved in dichloromethane (≥ 99 %, Merck, Darmstadt, Germany) and treated with Na_2SO_4 until the solution became clear. The dried solution was filtered and PEI was isolated by evaporation of solvent. Finally PEI was dried *in vacuo* (1 mbar) at 40 °C for 48 h before use. Hexamethylene diisocyanate (HMDI, ≥ 99 %) was purchased from Fluka (Buchs, Switzerland). Chloroform (Riedel-de Haën, Seelze, Germany, ≥ 99 %) was treated with HMDI for 4 h at 60 °C and distilled to remove any traces of water and ethanol. Diethyl ether (≥ 99.5 %, Merck) and light petrol (≥ 99 %, 40 - 60 °C, Riedel-de Haën) were distilled before use.

Three different DNA samples were used in this study: Plasmid DNA pBR322 (2.9 MDa, 4363 bp) were from Sigma (St. Louis, MO) and the luciferase reporter vector plasmid pGL3 control, (3.5 MDa, 5256 bp) was from Promega (Heidelberg, Germany) and was amplified with a competent E. coli strain JM 109 (Promega) according to a protocol from QIAGEN (Hilden, Germany).[14] Salmon testes DNA (type III) was purchased from Sigma.

Block Copolymer Synthesis.

Activation of 4- and 8-star PEG. In a 100 mL flask fitted with a reflux condenser and an oil bubbler 3.8 g of HMDI (22.5 mmol, 80 eq.) were dissolved in 10 mL $CHCl_3$. 2 g of 8-*star*-PEG (0.28 mmol, 1 eq.) were dissolved in 20 mL $CHCl_3$ and slowly added to the HMDI solution under stirring. The mixture was heated under reflux for 4 h. Stirring was continued for 12 h at ambient temperature. The solution was concentrated to a volume of 10 mL and the polymer was precipitated in 100 mL petrol ether. The precipitate was washed 3 times with 50 mL petrol ether before the polymer was isolated by solvent evaporation under reduced pressure. 1.38 g of an orange waxy polymer were obtained in 58 % yield. In the same way, 4-

star-PEG (2 g, 0.13 mmol, 1 eq.) was activated with HMDI (2.1 g, 12.5 mmol, 96 eq.). In this case, a white solid polymer was isolated in quantitative yield.

Synthesis of 8-star-*[PEG(10k)*-b-*PEI(800)]*. 3 g of PEI 800 Da (5 mmol, 25 eq.) were dissolved in 20 mL $CHCl_3$. In a 100 mL flask fitted with a reflux condenser and an oil bubbler 2.27 g of activated 8-*star*-PEG (0.2 mmol, 1 eq.) were dissolved in 30 mL $CHCl_3$ and the PEI solution was slowly added to the PEG solution under stirring. The mixture was heated under reflux for 12 h. The clear light yellow solution was poured into 500 mL diethyl ether. After 12 h a yellowish oil precipitated from the white-turbid supernatant. Again, the precipitate was dissolved in 40 mL ethanol and poured into 500 mL diethyl ether again. The precipitated polymer was isolated by decantation. Residues of solvent were removed under reduced pressure. 1.65 g of a yellowish and waxy polymer was obtained in 46 % yield. Similar as described above, following polymers were synthesized:

Synthesis of 4-star-*[PEG(15k)*-b-*PEI(800)]*. 2 g PEI 800 Da (3.3 mmol, 26 eq.) in 20 mL $CHCl_3$, 2.04 g activated 4-*star*-PEG (0.13 mmol, 1 eq.) in 30 mL $CHCl_3$. Yield: 2.5 g (quantitative) of a yellowish solid but sticky polymer.

Synthesis of 8-star-*[PEG(10k)*-b-*PEI(2k)]*. 1 g PEI 2 kDa (0.5 mmol, 16 eq.) in 50 mL CH_2Cl_2, 0.41 g activated 8-*star*-PEG (0.03 mmol, 1 eq.) in 150 mL CH_2Cl_2. Reaction time 72 h. The polymer was solved in water and precipitated in THF. Yield: 0.74 g (90%) of a yellowish solid polymer.

Polymer Characterization

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H-NMR and ^{13}C-NMR spectra were recorded in D_2O (Merck) at 35 °C or $CDCl_3$ (Merck, containing TMS as reference) at 25 °C on a Eclipse+ 500 spectrometer from Jeol, Tokyo, Japan, at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR spectra, respectively. Spectra were evaluated with the NMR data processing program MestRe-C Version 2.2.

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 (for IR spectroscopy, Spektranal®, Riedel-de Haën) disks. The waxy and oily samples were smeared on the KBr disk.

Thermogravimetric Analysis (TGA). TGA was performed on a Thermogravimetric Analyzer TGA 7 with a Thermal Analysis Controller TAC 7/DX from Perkin-Elmer with about 10 mg polymer sample. Scanning rate was 20 K/min and curves were recorded within temperature range of 25 – 500 °C. Analysis was done under 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 sample. Scans were run in nitrogen at a heating and cooling rate of 10 K/min (temperature range from -100 to +60 °C). Data in Table 3 are derived from 2nd heating curve.

Capillary Viscosimetry. The polymers were characterized by capillary viscosimetry (Ubbelohde, Schott, type No. 50101/0a) at 25.0 °C in 0.5 M $NaNO_3$. Polymer concentration was 0.5 g/dL. Inherent viscosity (η_{inh}) was calculated by following equation: $\eta_{inh} = \ln (t/t_0) / c$, where t is the flow time of the polymer solution, t_0 the flow time of the solvent and c the polymer concentration.

Size Exclusion Chromatography in combination with Multiple Angle Laser Light Scattering (SEC-MALLS). The SEC setup consisted of a HPLC Pump L-6000 from Merck-Hitachi, Darmstadt, Germany, a Merck-Hitachi autosampler AS-200A and a Merck column thermostat T-6300. Polymers were detected by an differential refractive index (RI) detector RI-71 from Merck and an 18 angle laser light scattering detector from Wyatt Technologies, Santa Barbara, CA, USA (DAWN® EOS™, GaAs Laser 690 nm, 30 mW, K5 cell). SEC columns Novema 100 (for PEIs), Novema 3000 (for copolymers) and Suprema Max 3000 (for PEGs) were from Polymer Standard Service (Mainz, Germany). As eluent 1% formic acid

(Riedel-de Haën, 98-100%) was used. 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 0.2 µm sterile filter and degassed with a 4 channel online vacuum degaser DDG-75 from Duratec (Reilingen, Germany). Additionally, an inline filter (0.45 µm, stainless steel, Rheodyne, Cotati, CA) was set between columns and MALLS detector. A flow rate of 1 ml/min was applied. Polymer concentration was 5 g/l and 20 µl were injected for each run. MWs were calculated by Astra for Windows Software, version 4.73.04, using Zimm plots.

Formation of the Polymer-DNA Complexes

All complexes of DNA and polymer were prepared freshly before use. Polymer solutions were added to the DNA solutions in equal volumes, mixed by vortexing and incubated for 10 min before analysis. DNA stock solutions were diluted to a concentration of 20 µg/mL with 10 mM NaCl at pH 7.4 (for AFM) or to 40 µg/mL (for DLS and LDA) with 150 mM NaCl at pH 7.4. The polymer stock solutions were diluted with the same medium as above to the appropriate concentration depending on the required polycation-nitrogen/polyanion-phosphorous ratio (N/P ratio) and filtered through 0.22 µm. DNA stock solutions were 2 mg/mL pGL3 plasmid in 10 mM TRIS HCl (pH 8) and 1 mM EDTA, 100 µg/mL pBR322 plasmid in pure reagent water. Homopolymer and copolymer stock solutions were 0.5 mg PEI/mL (AFM) and 0.9 mg PEI/mL (DLS/LDA).

Characterization of Copolymer-DNA Complexes.

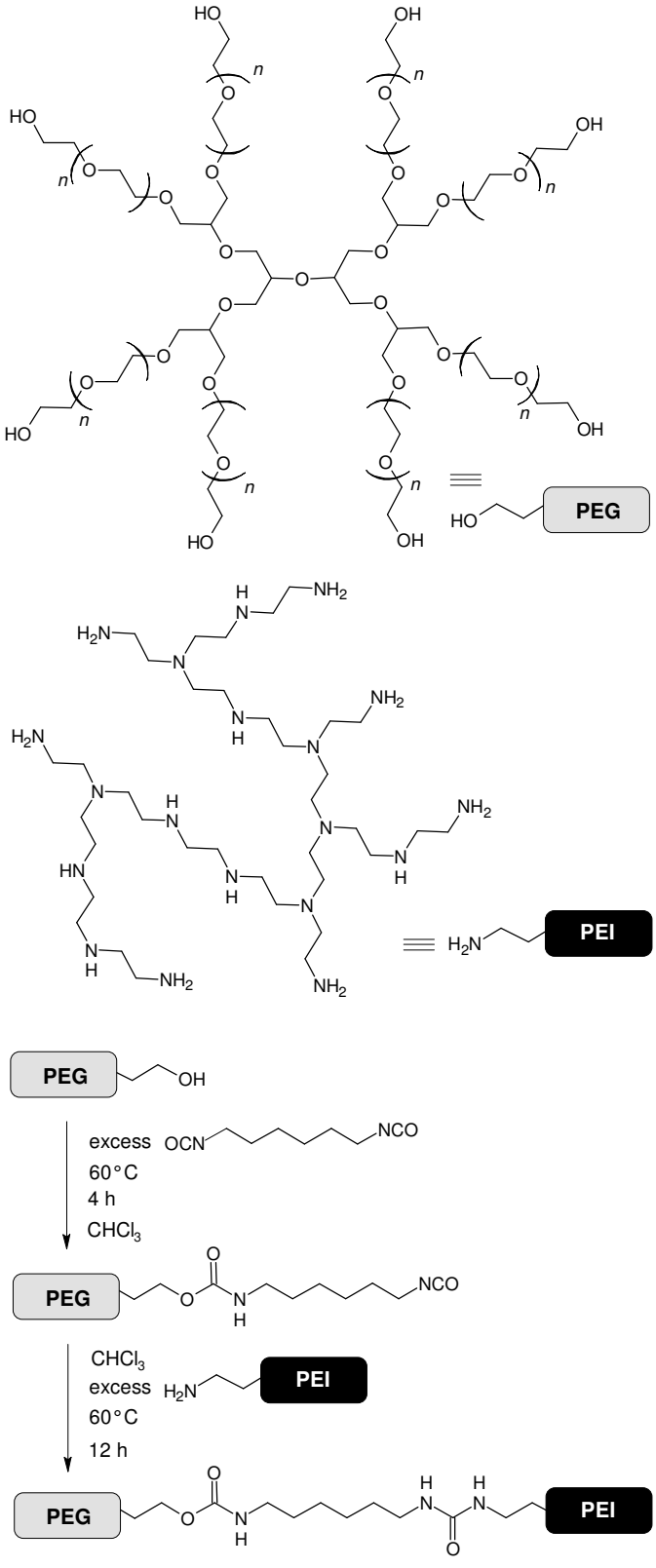
Atomic Force Microscopy (AFM). AFM images were conducted on a Dimension™3000 Scanning Probe Microscope from Digital Instruments (Santa Barbara, CA) with a Nanoscope IIIa controller. The solution of polymer-DNA complex was prepared as described above and incubated for 5 minutes before being deposited onto freshly cleaved mica and imaged under 10 mM NaCl solution. Immobilisation was achieved using electrostatic forces between the opposed charges of the mica and polymer-DNA complexes. Imaging was carried out in the tapping mode at a scan speed of approximately 2 Hz with 512x512 pixel data acquisition. V-shaped cantilevers with a pyramidal tip of silicon nitride (Park Scientific, CA) were used. The experiments were repeated and the structures were found to be reproducible.

Dynamic Light Scattering (DLS). Complex size measurements were carried out with a Zetasizer 3000 HS from Malvern Instruments (Worcs., UK) at 25 °C. (10 mW HeNe laser, 633 nm). Scattering light was detected at 90° angle through a 400 micron pinhole. Measurements yielding hydrodynamic diameters (Z average mean) and polydispersity indices (PDI) were performed with count rates of about 100 - 400 kCps in form of 10 runs of 60 min duration each and analyzed in the CONTIN mode. For data analysis, the viscosity (0.8905 mPas) and refractive index (1.333) of pure water at 25 °C were used. The instrument was calibrated with Nanosphere™ Size Standards (polymer microspheres in water, 220 nm +/- 6 nm) from Duke Scientific, Palo Alto, CA.

Laser Doppler Anemometry (LDA). ζ-potential (zeta-potential) measurements of the complexes were carried out in the standard capillary electrophoresis cell of the Zetasizer 3000 HS from Malvern Instruments at position 17.0 and at 25 °C. Measurement duration was set to automatic. Average values of the ζ-potential were calculated with the data from 10 measurements. The instrument was calibrated with DTS5050 Electrophoresis Standards from Malvern Instruments.

DNA condensation assay. DNA condensation was measured by fluorescence of complex solutions upon addition of ethidium bromide (EtBr). The assay was performed in 96 well plates in quadruplicate. 80 µL of an 100 µg/mL aqueous salmon testes DNA solution were placed per well. 50 µL of 60 mM Tris HCl buffer (pH 7.4) were added. Finally, additional water was given to yield a final volume of 300 µL per well. The appropriate volumes of 0.05 mg PEI/mL polymer solutions were added to yield N/P ratios from 0.25 to 10. After 10 minutes incubation time 20 µL of an 100 µg/mL aqueous EtBr solution were added. The plate was shaken and analyzed on a Perkin Elmer LS 50 B fluorescence plate reader with excitation

at 518 nm and a 15 nm slit, a 515 nm emission filter and emission wavelength of 605 nm with a 20 nm slit. Results are given as relative fluorescence values where 1 is the fluorescence without polycation and 0 is the remaining fluorescence of non-intercalating EtBr.



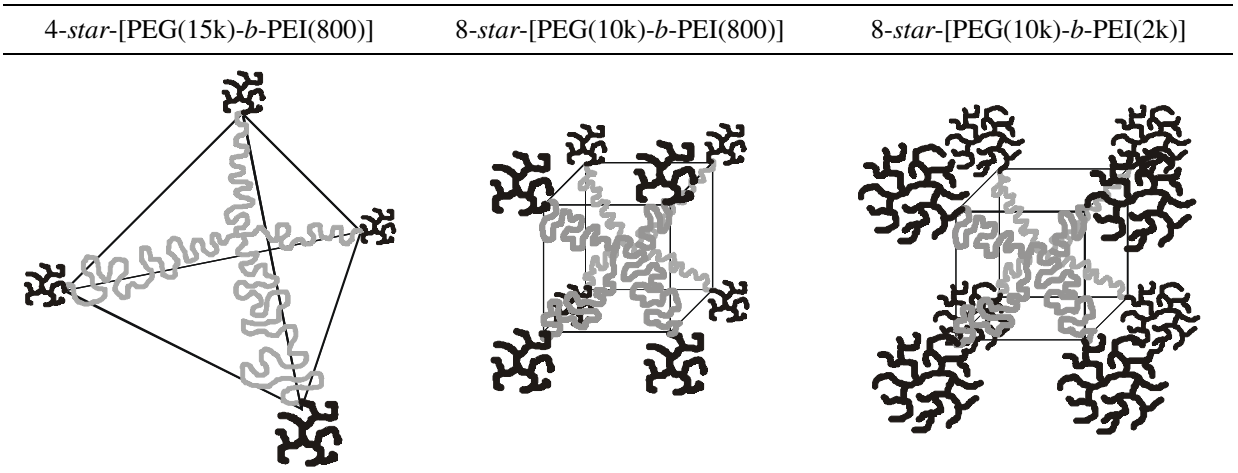
Scheme 1: Synthesis of the *star*-copolymers and schematics of the structures of 8-*star*-PEG and branched PEI.

RESULTS AND DISCUSSION

Copolymer Synthesis

Our aim was to synthesize a copolymer with a core-shell structure. The shell should be made from cationic LMW PEIs and the core should be non-charged and chemically as well as biologically inert. As core we selected multi-star poly(ethylene glycol)s (PEG). 4-*Star*- and 8-*star*-PEGs were applied for our synthesis. These branched PEGs have a multiglycerol ether unit in their center which is grafted with linear PEG branches. In the case of 4-*star*-PEG it is a diglycerol ether unit, in the case of 8-*star*-PEG a hexaglycerol ether unit (Scheme 1). Every PEG branch has one hydroxy end group. For the shell we used the low MW PEIs 800 Da and 2000 Da. These polycations are hyperbranched and contain a mixture of primary, secondary and ternary amino groups. We have previously shown that the ratio between primary, secondary and ternary amino groups is 1 : 1 : 1 for both the PEI 800 Da and PEI 2000 Da.[6] To attain a core-shell structure every single PEG branch should be grafted with one PEI macromolecule which seems to be difficult to achieve in synthesis without cross-linking because both, the PEG and the PEI are multivalent polymers with regard to their respective amino- and hydroxy-end groups. We solved this problem using a large excess of one of the reactants and subsequent removal of the non-reacted excess. The two-step synthesis route is presented in Scheme 1. In a first step, PEG was activated for the coupling reaction with PEI. This activation step was performed with hexamethylene diisocyanate (HMDI). A diluted PEG solution was added slowly to a concentrated HMDI solution. The large excess (80fold) of the diisocyanate prevented that both isocyanate groups would react with two hydroxy end groups of PEG and that two PEG molecules would be linked together. Additionally, the large excess of HMDI allowed a high conversion of the reaction. The non-reacted HMDI was easily removed by precipitation of the polymer in petrol ether in which HMDI is soluble. Further, to ensure that all residues of HMDI were removed repetitive extraction was performed. In the second step a diluted solution of the activated PEG was slowly added to a concentrated solution of PEI. Again, the large excess of PEI prevented that one PEI molecule would react with several isocyanate end groups of the same PEG or of an different PEG molecule. The non-coupled PEI was removed by precipitation of the copolymer in diethyl ether since LMW-PEI did not or only very slowly precipitate in ether whereas the PEG-PEI copolymer precipitated quickly.

Via this straightforward synthesis we produced a sample set of three *star-block copolymer*s (Scheme 2): PEI 800 Da was grafted onto a 4-*star*-PEG of MW 15 kDa and an 8-*star*-PEG of MW 10 kDa. The copolymers are designated as 4-*star*-[PEG(15k)-*b*-PEI(800)] and 8-*star*-[PEG(10k)-*b*-PEI(800)], respectively, where *b* denotes the abbreviation for *block*. Additionally, the 8-*star*-PEG was also coupled with PEI 2000 Da yielding copolymer 8-*star*-[PEG(10k)-*b*-PEI(2k)]. We expect that these copolymers form a core-shell structure in solution due to repulsive forces of the positively charged PEI molecules in the shell structure. The PEI molecules were thought to minimize the electrostatic potential of the copolymer by a maximum separation from each other.



Scheme 2: Schematic presentation of the *star*-copolymer sample set. The *star*-PEGs are drawn in grey. PEI blocks are represented by the black structures.

Copolymer Analysis

The successful conversion of this two-step synthesis can be monitored by spectroscopic methods. The conversion of the hydroxy end groups of the 8-*star*-PEG by HMDI into the urethane bond can be seen in the ^{1}H-NMR spectrum (Figure 1) where the signal (no. 5) of the terminal methylene protons was shifted from 3.69 to 4.38 ppm (no. 5') [compare with assignment in literature[15]]. The activated PEG showed characteristic absorptions for the isocyanate (stretching, 2274 cm^{-1}) and urethane bond (stretching, 1724 cm^{-1}) in the FT-IR spectrum (Figure 2) and the appropriate signals in the ^{13}C-NMR spectrum (121.3 and 155.9 ppm, Figure 1). After coupling with PEI the signals for isocyanate vanished and new signals appeared for the urea bond: stretching absorption at 1620 cm^{-1} in FT-IR and a signal at 158.2 ppm in ^{13}C-NMR. The urea signal (13') is broader than the urethane signal (6) due to the large variety of structurally different amino groups of the branched PEI.

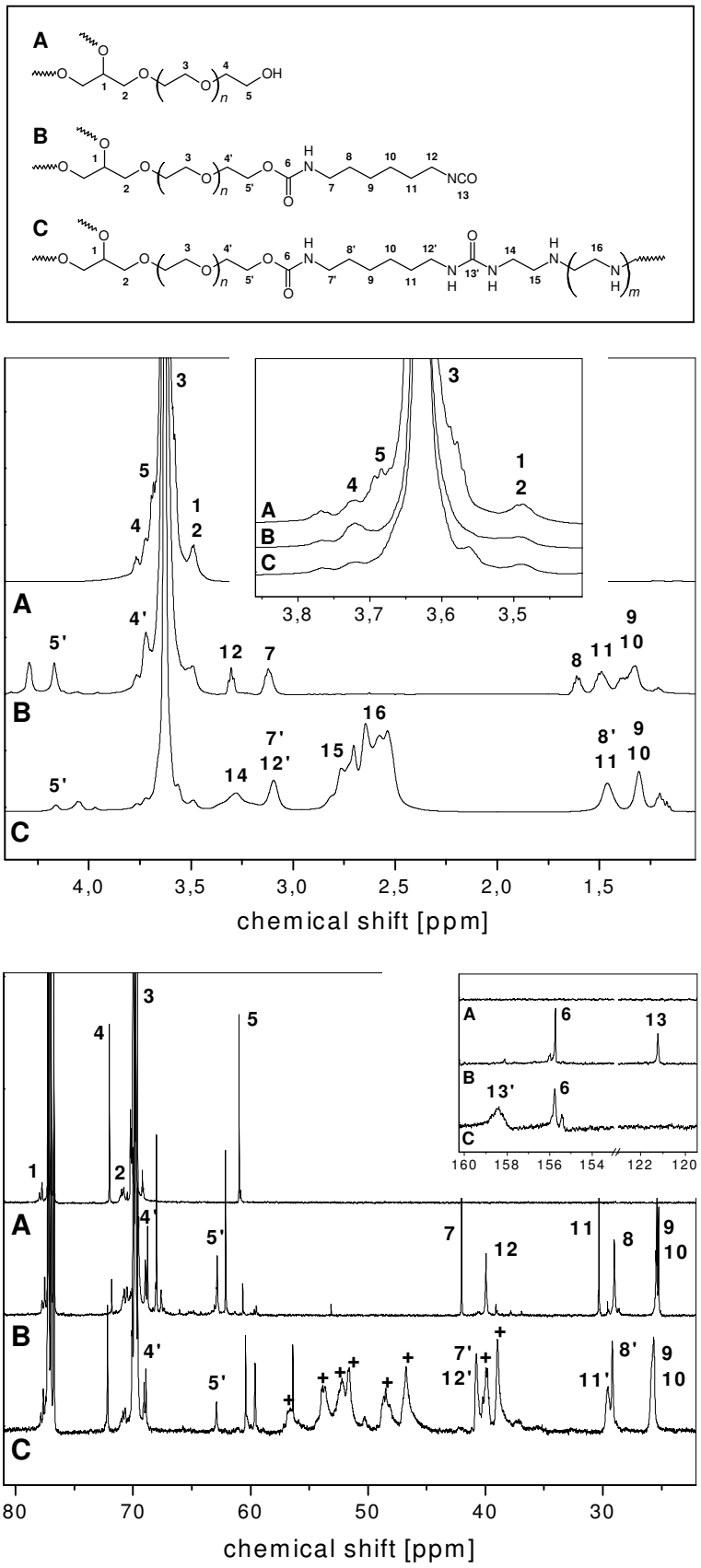


Figure 1: ^{1}H-NMR (top) and ^{13}C-NMR (bottom) spectra in $CDCl_3$ of 8-*star*-PEG, activated PEG and copolymer 8-*star*-[PEG(10k)-*b*-PEI(800)]. Signals of the branched PEI are marked with "+".

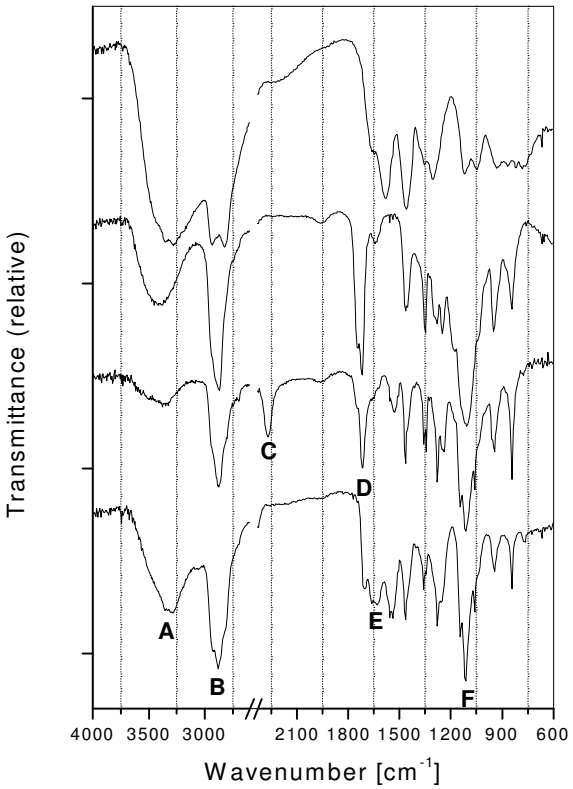


Figure 2: FT-IR spectra (from top to bottom) of PEI 800 Da, 8-*star*-PEG, activated PEG and copolymer 8-*star*-[PEG(10k)-*b*-PEI(800)]. Characteristic absorptions: (A) N-H amines stretching 3300 cm^{-1}, (B) C-H stretching 2921 cm^{-1}, (C) O=C=N isocyanate stretching 2274 cm^{-1}, (D) C=O urethane stretching 1724 cm^{-1}, (E) C=O urea stretching 1620 cm^{-1}, (F) C-O ether stretching 1106 cm^{-1}.

MWs of homopolymers and copolymers were determined by a combination of SEC and static light scattering. The eluograms are exemplarily given for 8-*star*-[PEG(10k)-*b*-PEI(800)] and the appropriate homopolymers (Figure 3).

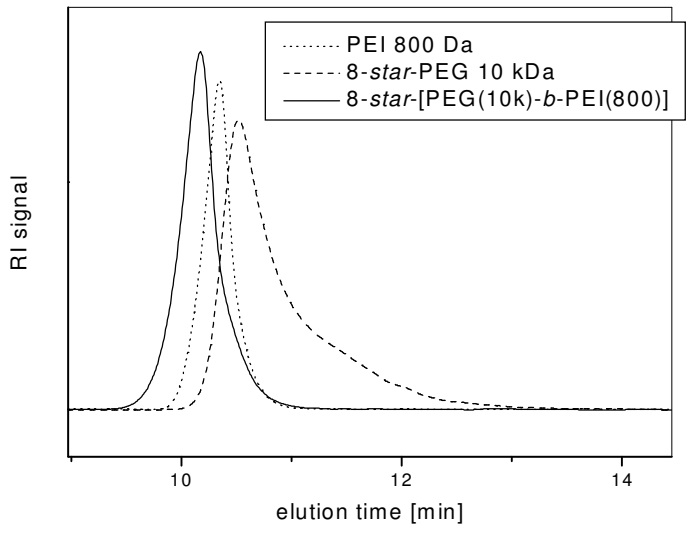


Figure 3: SEC eluograms of 8-*star*-[PEG(10k)-*b*-PEI(800)] and the appropriate homopolymers. The signals were detected by an differential refractive index detector (1 % formic acid, 1 ml/min, 35 °C, column: PSS Suprema Max 3000).

It should be emphasized that the column material was cationic. Consequently, cationic polymers were retained in the caves of the hydrogel matrix not as efficient as non-charged polymer samples. This explains why LMW- PEI (800 Da) which is probably fully protonated under the acidic conditions (eluent: 1% formic acid) was eluted earlier from the column than the PEG with higher MW (10 kDa). Number and weight average MWs (M_n, M_w) as well as MW distributions (M_w/M_n) of all polymers are given in Table 1.

Table 1: Molecular weights and inherent viscosity of homopolymers and copolymers.

Polymer	MW [g/mol] calculated	M_n [g/mol] found[a]	M_n [g/mol]	M_w [g/mol] found[b]	M_w/M_n	dn/dc[c] [mL/g]	η_{inh}[d] [dL/g]
PEI	800[e]		660	746	1.13	0.263	0.0504
PEI	2 000[e]		1 953	2 127	1.01	0.272	0.0726
PEI	25 000[e]		16 810	44 670	2.66	0.272	0.1354
4-*star*-PEG	15 000[e]	10 831	8 696	9 871	1.14	0.135	0.2188
8-*star*-PEG	10 000[e]	10 138	6 287	11 260	1.61	0.089	0.0794
4-*star*-[PEG-*b*-PEI(800)]	19 012		16 800	19 020	1.13	0.175	0.1646
8-*star*-[PEG-*b*-PEI(800)]	18 014		22 650	25 120	1.11	0.148	0.1202
8-*star*-[PEG-*b*-PEI(2k)]	27 267		18 660	26 410	1.42	0.131	0.1109

[a] As determined by end group analysis using ^{1}H-NMR (signals 3 and 5 in Figure 1).
[b] As determined by SEC-MALLS in 1% formic acid.
[c] As determined by differential refractometer in 1% formic acid.
[d] As determined by capillary viscosimetry in 0.5 M $NaNO_3$ at polymer concentration of 0.5 g/mL and 25.0 °C.
[c] Molecular weight given by supplier.

The determined MWs are in good agreement with the stoichiometrically expected data. The M_w of the star-blocks copolymers were in the range of 19 – 26 kDa and therefore, are of comparable size with the high MW PEI 25 kDa. The inherent viscosities of both, the homopolymers and copolymers are also listed in Table 1. Here again, comparable data were obtained for the star-block copolymers (η_{inh} = 0.11 - 0.16 dL/g) and for high MW PEI (η_{inh} = 0.135 dL/g). Thus, not only the absolute MWs of the copolymers are similar to the high MW PEI but also the hydrodynamic properties. Furthermore, composition of the copolymers was determined by integration of the appropriate signals in ^{1}H-NMR spectra and showed good agreement with the calculated values (Table 2) that again documents the feasibility of the proposed synthetic route. Elemental analysis (Table 2) gave results which were roughly in line with the calculated data. In the case of copolymer 8-*star*-[PEG(10k)-*b*-PEI(800)] found and calculated data were in good agreement. For 4-*star*-[PEG(15k)-*b*-PEI(800)] more nitrogen was found than expected. Slightly more PEI was also found by NMR. Adsorption of unmodified PEI during the isolation procedure could be a possible reason for this finding.

This unwanted effect is most likely for the 4-*star*-PEG because it was the PEG that precipitated fastest due to its higher MW compared with the 8-*star*-PEG. In the case of 8-*star*-[PEG(10k)-*b*-PEI(2k)] less nitrogen was found than calculated which might be due to residues of water in the sample which can not be easily removed. Indeed, it was this copolymer for which water/THF was used for precipitation.

Table 2: Composition of the *star*-copolymers as determined by ^{1}H-NMR spectroscopy and elemental analysis.

star-copolymers	composition of copolymers % PEG : % HMDI : % PEI		elemental analysis[b]					
			C		H		N	
	calculated	found[a]	calcd	found	calcd	found	calcd	found
4-*star*-[PEG-*b*-PEI(800)]	79 : 4 : 17	79 : 3 : 18	54.39	52.03	9.48	9.05	6.34	8.91
8-*star*-[PEG-*b*-PEI(800)]	56 : 8 : 36	56 : 9 : 35	54.14	53.01	9.85	9.50	13.45	12.22
8-*star*-[PEG-*b*-PEI(2k)]	37 : 5 : 58	38 : 3 : 59	55.41	42.30	10.62	8.78	19.06	16.63

[a] As determined by integration of ^{1}H-NMR signals of measurement conducted in D_2O (PEG signals 1-5, HMDI signals 8' and 11, PEI signals 15 and 16 in Figure 1).
[b] Calculated for $C_{861}H_{1788}N_{86}O_{354}$, $C_{812}H_{1760}N_{173}O_{254}$ and $C_{1258}H_{2874}N_{371}O_{254}$, respectively.

Table 3: Thermoanalytical data of the homopolymers and the copolymer 8-*star*-[PEG(10k)-*b*-PEI(800)] determined by DSC measurements (2nd heating curve, 10 K/min, N_2).

Polymer	T_g [°C]	T_m [°C]	ΔH [J/g]
PEI 800 Da	-59.5	-	-
8-*star*-PEG 10 kDa	-54.3	39.3	89.9
Blend	-38.3	41.0	39.9
Copolymer	-30.4	38.2	40.4

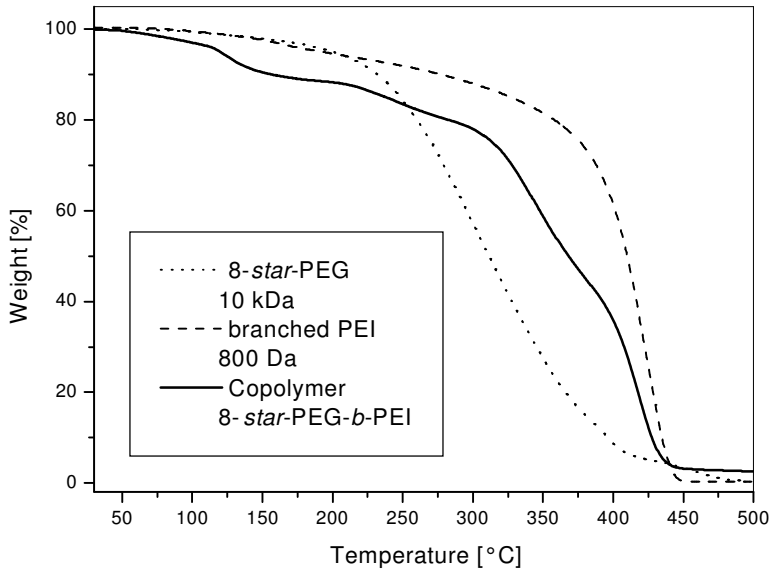


Figure 4: TGA of the homopolymers and the copolymer 8-*star*-[PEG(10k)-*b*-PEI(800)].

Finally, the copolymers were characterized thermoanalytically. Exemplarily, the results are presented for copolymer 8-*star*-[PEG(10k)-*b*-PEI(800)] in Figure 4 and Table 3. The thermogravimetric profiles of this copolymer in comparison with its homopolymers is shown in Figure 4. Four steps could be observed. The first mass loss step was due to solvent residues of the sample. At temperature higher than 200 °C the 8-*star*-PEG began to degrade and caused a second degradation step. However, strong thermal degradation was monitored above 300 °C where also the PEI began to degrade (third step). Maximum degradation for PEI was observed at 400 °C, when the copolymer showed the forth step in its degradation curve. The mass loss of the second and the third step attributed mainly to the PEG is about 50 % and that of the forth step which can be derived from PEI about 35 %. This again is in reasonable agreement with the composition determined by NMR (PEG 56 %, PEI 35 %). The results of the DSC measurements are given in Table 3. PEI 800 Da and 8-*star*-PEG seemed to form thermodynamically stable mixtures since the glass transition temperature of the blend of these homopolymers (T_g - 38.3 °C) is significantly higher than T_g of the homopolymers themselves (T_g - 59.5 °C and - 54.3 °C). The increase of the T_g can be explained by non-covalent H-bonds between the O of PEG and the H of the amino groups of PEI (>N-H···O<). In the case of copolymer 8-*star*-[PEG(10k)-*b*-PEI(800)] the increase of T_g is even higher (-30.4 °C).

To summarize, the facile synthesis route presented herein, yielded indeed to the expected multi-star-block copolymers in good quality. These copolymers are composed of only LMW PEI but have the molecular size of a HMW PEI.

DNA Condensation

Polymer induced DNA condensation was performed by adding the polycation solutions to the DNA solutions in equal volumes. The DNA concentration was kept constant. The concentration of the polycation was adjusted to the desired N/P ratio (ratio amino-nitrogen of the polycation/phosphate of the DNA). We have employed four methods to probe the interaction of the polycations with DNA. These methods include imaging by atomic force microscopy (AFM), particle size measurements by dynamic light scattering (DLS), ζ-potential measurements by laser Doppler anemometry (LDA) and an ethidium bromide (EtBr) exclusion assay.

We studied the morphology of the complexes formed with our polycations by AFM. The AFM images were taken in aqueous solution to reflect the conditions of gene delivery and to avoid artifacts due to staining or drying procedures. Due to interference of the scanning process by salt formation the buffer used for AFM experiments had to be changed to low

ionic strength. A salt concentration of 10 mM NaCl turned out to be ideal for these experiments which were performed at an N/P ratio of 9 and at pH 7.4. The AFM images of the complexes are shown in Figure 5. In the case of the high MW PEI (25 kDa) small and compact particles were obtained with diameters of about 100 nm. In contrast, the low molecular weight PEIs (800 Da and 2 kDa) formed huge aggregates with the DNA of diameters up to 2 µm. Areas with these large objects which also exhibited a large height (up to 300 nm) were difficult to scan. Therefore, the quality of the AFM images was poorer compared to the images with objects all in the nanometer scale. However, the results are obvious: HMW PEI induced formation of small complexes with diameters of 80 – 100 nm and heights of about 10 - 20 nm, whereas LMW PEI formed huge aggregates with diameters between 500 nm – 2 µm and heights of up to 300 nm.

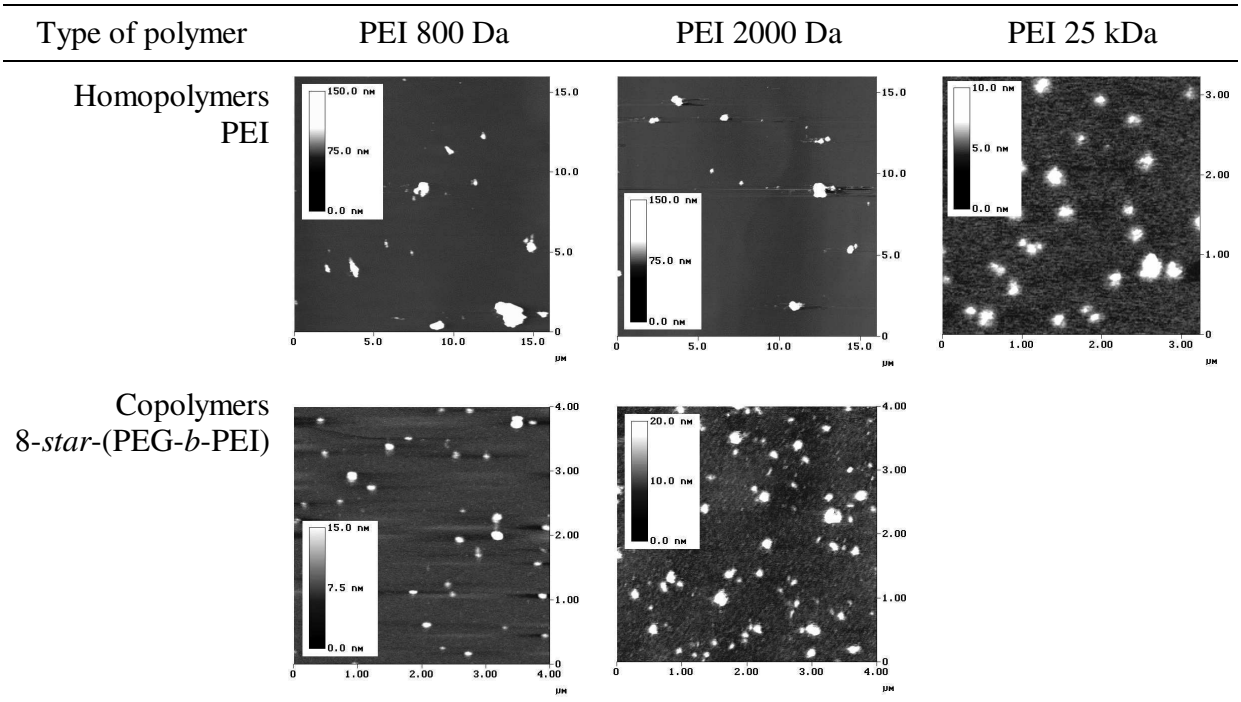


Figure 5: AFM images of the plasmid DNA complexes formed with homopolymer PEI and copolymers 8-*star*-[PEG(10k)-*block*-PEI].

Unlike LMW PEIs, their 8-*star*-(PEG-*b*-PEI) copolymers formed significantly smaller complexes. For copolymer 8-*star*-[PEG(10k)-*b*-PEI(800)] complexes with diameters of about 100 nm and heights of about 10 nm were found, whereas for 8-*star*-[PEG(10k)-*b*-PEI(2k)] the complexes seemed to be slightly larger on average due to some particles of about 200 nm size and about 25 nm height.

The results of the AFM experiments could be confirmed by DLS measurements of the complexes (Figure 6). Under the same conditions as for the AFM experiments (10 mM NaCl,

N/P 9) small particles with 90 nm diameter were found for HMW PEI (25 kDa). In contrast, LMW PEIs yielded particles of about 700 nm for PEI 800 Da and 450 nm for PEI 2000 Da. All star-block copolymers, however, formed complexes with small diameters. Whereas the copolymers based on PEI 800 Da produced complexes of 80 nm in size, the copolymer 8-*star*-[PEG(10k)-*b*-PEI(2k)] showed a slightly larger complex size (105 nm) which is in line with the observations by AFM. DLS experiments were also performed under physiological conditions (150 mM NaCl) but did not show significant differences compared with the measurements at low ionic strength.

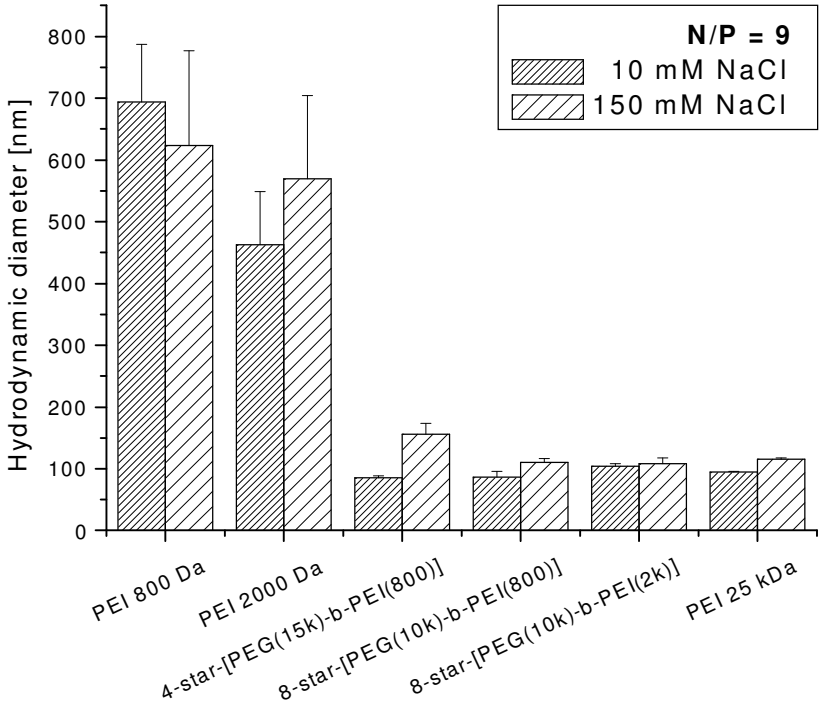


Figure 6: Complex size of PEI homopolymers and *star*-(PEG-*b*-PEI) copolymers as determined by DLS at N/P ratio 9 and at different salt concentrations. The bars represent the mean z-average of 10 measurements within 10 min.

Furthermore, no significant differences in complex size were observed when the N/P ratio was varied (Figure 7). There was only one exception: The HMW PEI showed at N/P ratio 5 larger complexes with diameters of 220 nm compared with higher N/P ratios. This effect can be explained by instabilities of the complexes. As a result, the complex size increased from 200 nm to about 500 nm during the DLS measurement as shown in Figure 8. Instabilities at N/P 5 were also found in the case of LMW PEI as shown in Figure 8 for PEI 800 Da. In contrast, all star-block copolymers formed stable complexes. Their complex size

remained small and constant for more than 20 min as demonstrated for 8-*star*-[PEG(10k)-*b*-PEI(800)] in Figure 8. At higher N/P ratios (≥ 9) both the *star*-(PEG-*b*-PEI) copolymers and the high MW PEI formed complexes that were stable at low and at high ionic strength. Only complexes of LMW PEI were not stable even at extremely high excess of the polycations (N/P 50).

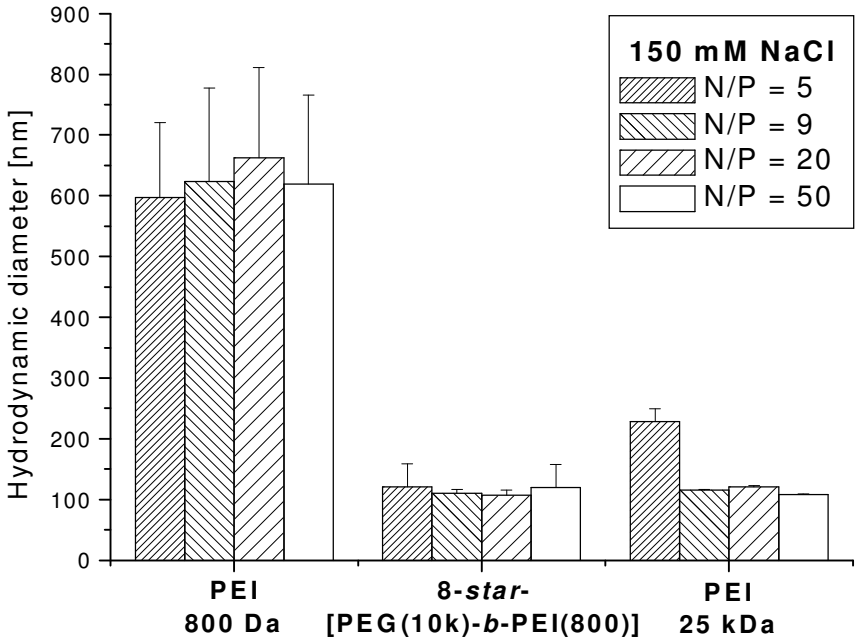


Figure 7: Complex size of PEI homopolymers and 8-*star*-[PEG(10k)-*b*-PEI(800)] copolymer as determined by DLS at 150 mM NaCl and at different N/P ratios. The bars represent the mean z-average of 10 measurements within 10 min.

To estimate the surface charge of the polymer/DNA complexes we measured the ζ-potential. The results are shown in Figure 9. High ζ-potentials of more than +20 mV were measured for HMW PEI both at low and at high ionic strength. In contrast, only small ζ-potentials were determined for the star-block copolymer. At low ionic strength and moderate excess of the polycation (N/P = 9) the ζ-potential was weakly positive, while at high ionic strength it was weakly negative. Even a large excess of the polycation (N/P = 50) did not increase the ζ-potential sufficiently. An only weak ζ-potential was also observed for LMW PEI (800 Da) at 10 mM NaCl. However, at high salt concentration the ζ-potential was strongly negative. Even with a large excess of polycation the negative ζ-potential could be only reduced to a moderately negative value.

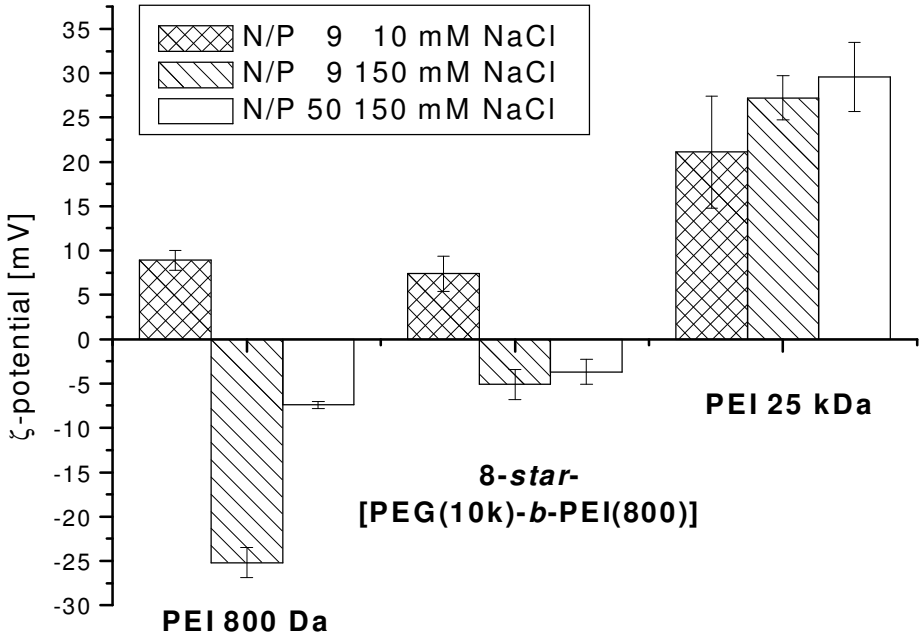


Figure 9: ζ-Potential of PEI 800 Da and 25 kDa homopolymers and 8-*star*-[PEG(10k)-*b*-PEI(800)] as determined by laser Doppler anemometry at different N/P ratios and at different salt concentrations.

As a forth method to study the condensation of DNA we applied an ethidium bromide (EtBr) exclusion assay. EtBr produces strong fluorescence when intercalating with DNA but only very weak fluorescence when it is free in solution. If the DNA is condensed by a polycation EtBr can not intercalate with DNA anymore. In Figure 10 the relative fluorescence is presented as a function of the N/P ratio. It was found that DNA condensation is more sufficient in the case of the star-block copolymers compared to LMW PEI. Within the group of LMW PEIs, PEI 2000 Da showed a slightly better DNA condensation than PEI 800 Da. For the latter fluorescence could be reduced only to the half even at high excess of polycation. Similarly, the star-block copolymer derived from PEI 2 kDa also exhibited a slightly stronger DNA condensation compared to the star-block copolymers of PEI 800 Da. Surprisingly, no significant differences were observed for the 4- and 8-star-block copolymers of PEI 800. Only a very weak advantage of the 8-star- over the 4-star-block copolymer was measured.

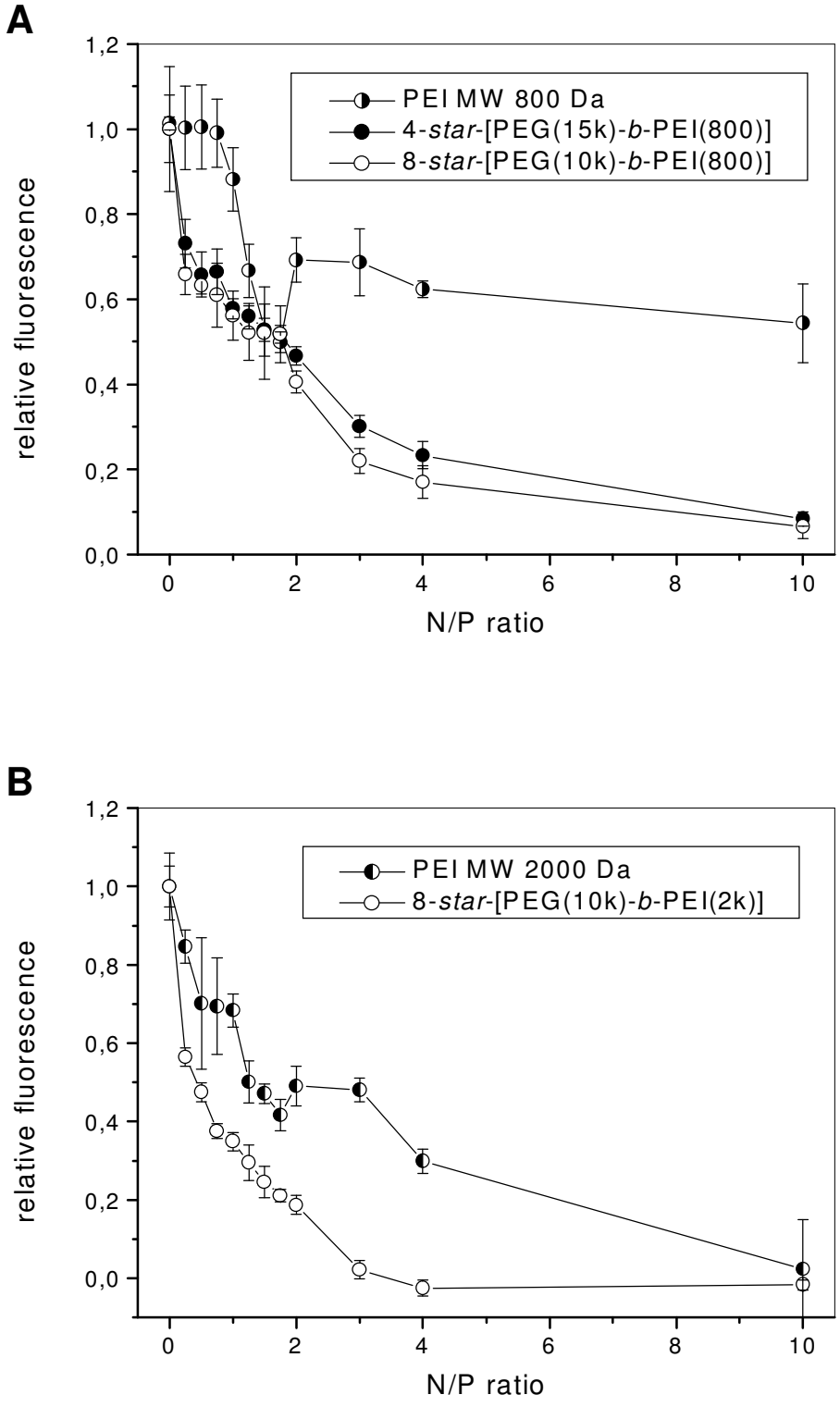


Figure 10: Condensation potential of the homopolymers PEI 800 Da (A) and PEI 2 kDa (B) and their *star*-copolymers as determined by EtBr-exclusion assay.

We concluded from these results that LMW PEI poorly condensed DNA and these polymer/DNA complexes aggregated to large units. Furthermore, large aggregates were often unintentionally torn apart during the scanning process of AFM which gives rise to the conclusion that the aggregates are not strongly associated but are only loosely packed. Additionally, in the EtBr exclusion assay we found that LMW PEI did not condensed DNA sufficiently so that EtBr still can intercalate with the DNA. This again is in line with the strongly negative ζ-potential of these aggregates, which probably arose from DNA molecules which were not complexed by LMW PEI. All star-block copolymers showed a strong condensation potential which is comparable with the DNA condensation of HMW PEI (25 kDa, data not shown). In this case the small complexes observed with AFM seem to be very compact which is in line with an sufficient EtBr exclusion at low N/P ratios. Hence, it can be expected that the *star-block copolymer*s very sufficiently protect the DNA against enzymatic attack.

The phenomenon of the huge particles formed with LMW PEI is explained by aggregation. As observed in DLS measurements the diameter of the complexes increased dramatically within the first 20 min of incubation. LMW PEI might form small complexes initially. Nevertheless, after already one minute the complexes aggregated to particles of more than 300 nm diameter and aggregation continued for several minutes. Aggregation was very likely due to the low ζ-potential of LMW PEI complexes. The weakly repulsive electrostatic forces were easily overcome by attractive forces especially in the presence of salt resulting in aggregation (DLVO theory). In contrast, complexes of HMW PEI (25 kDa) exhibited a high ζ-potential (+35 mV) and thus were stable against aggregation. The low ζ-potential of LMW-PEI can be explained by the finding that small multivalent cations or cationic oligomers condensed DNA, but bind reversibly[16] and therefore could not associate in a complex with charge ratios above 1:1. Thus, strongly positive ζ-potentials are not achieved, not even at very high oligoamines excess as shown for pentalysine and spermine.[17] Cationic polymers, however, possess high binding affinity to DNA[18] and therefore can associate with DNA in excess of 1:1 charge ratio, Hence strongly positive ζ-potentials can be observed that stabilize the complex against aggregation and guarantee small complexes.

As indicated earlier, PEIs of MW of 2 kDa or less were found to be inefficient as gene transfer reagents even at high N/P ratios, although they are able to form complexes with DNA.[19,20] For PEI 800 Da gene expression was immeasurable below an N/P ratio of 18 and at this N/P ratio only a negligible gene expression was found.[21] It has been speculated that a possible reason for the poor transfection activity is the dissociation of the complex during

dilution[22] as it was found for interpolymer systems with relatively few salt bonds.[23] However, experimental evidence for this explanation has not been provided yet. Our results suggest three possible reasons for the poor transfection efficiency of low MW PEI. First, the huge size of the aggregates is probably too large for efficient cellular uptake.[24] For *in vitro* experiments, Ogris et al. have found that smaller (40 nm) PEI(25 and 800 kDa)/DNA complexes, prepared at low salt concentrations, have lower transfection efficiencies than larger (> 1000 nm) complexes made from the same components at physiological salt levels.[25] Others reported that larger complexes were favored for *in vitro* transfection experiments due to faster sedimentation onto the cell layer.[26] Consequently, larger sizes of LMW PEI aggregates do not lead necessarily to poor transfection efficiency under in vitro conditions.

A second explanation for poor transfection efficiencies could be the low the ζ-potential of the complexes formed with LMW PEI since it is generally believed that a high surface charge enhance the interaction with the negatively charged cell membranes.[27] A third aspect, could be insufficient DNA condensation with LMW PEI, since it is crucial that DNA is sufficiently protected against DNases in the endo/lysosomal compartment. This explanation supported by observations that PEI < 2 kDa were not efficient in gene transfer, unless endosomolytic agents such as replication-defective adenoviruses were added to the DNA complexes.[20, 28] These endosomolytic agents reduce the time of the complex present in the lysosome. Thus, the enzymatic stress of the DNA is decisively reduced which becomes an important feature when DNA is not well condensed by the polycation.

The formation of large aggregates were not only observed for LMW PEI. Plank et al.[29] investigated a series of oligocationic peptides in the MW range from 900 to 1800 Da and found that within minutes after mixing in the presence of salt, DNA complex sizes reached 500 and 1000 nm. Furthermore, these complexes continued to aggregate until they precipitated. Interestingly, they also found that the branched peptides bound DNA with affinities that increased with the number of cationic groups. This is in line with our finding that PEI 2000 Da formed smaller complexes and quenched EtBr more effectively than PEI 800 Da.

Whereas we found that with increasing MW of PEI the size of the DNA complex was reduced, somewhat contradictory results seemed to be found for poly(L-lysine) (PLL).[30] In this case, the smallest PLL formed also the smallest complexes and with increasing MW of PLL the complex size increased. This contradiction can be resolved when the MWs of the samples are taken in consideration. We investigated the complex size of PEI 800 Da, 2 kDa and 25 kDa, whereas Wolfert et al.[30] studied the plasmid DNA complexes of 4 different PLL

with MWs of 4, 24, 54 and 225 kDa. For the 24 kDa PLL they found complex diameters in the range of 80 – 120 nm which is in agreement with our results for PEI 25 kDa. Apparently, the nature of the polycation has not a strong influence on the plasmid DNA-complex size. The decisive feature is the MW of the polycation. It seems that in the low MW range from 800 Da – 4000 Da the complex size is reduced with increasing MW probably due to a decreased aggregation tendency with increasing MW. However, in the high MW range (24 kDa – 225 kDa) the complex size increased again with increasing MW. Here, no aggregation was observed and the size of the polycation reflects the size of the complex. As a result, there seems to be a size minimum somewhere in the range between 2 and 24 kDa for plasmid DNA complexes.

Therefore, all these findings suggest that the condensation potential of LMW PEI has to be enhanced to improve the potential for gene delivery. We found that this can be realized by linking some of these low MW cationic polymers together to a larger unit with approximately the same MW as high MW PEI (25 kDa). Interestingly, we found for these polymers form complexes of the same size as PEI 25 kDa and bind DNA as strong as PEI 25 kDa. Therefore, it is reasonable to assume that for DNA condensation only the external shell of cationic charges is important and that it does not matter if the core is composed of a neutral or charged polymer.

However, the core-shell structure must not be considered as a rigid arrangement of the star-block copolymers but as a relatively flexible system. As indicated earlier, the core-shell formation of the polymer blocks is likely to be realized in solution due to repulsion of the cationic PEI macromolecules. However, in the presence of polyanionic DNA the cationic star-block copolymers might lose their core-shell structure. Since the ζ-potential of the complexes formed with the copolymers was about zero and the complexes were remarkably stable, we hypothesize that in the outer sphere of the complex the multi-star PEG block orientates towards the surface leaving the conjugated PEI macromolecules in interaction with DNA. In this way the PEG shielded the positive charges and stabilized the complex against aggregation. The block structure in the internal sphere of the complexes remains unknown and requires further investigations.

CONCLUSIONS

In summary, LMW PEI formed large (> 500 nm) aggregates upon mixing with plasmid DNA. Within these aggregates, DNA was only loosely packaged so that DNA is not

sufficiently protected. Insufficient condensation seems to explain the poor transfection activity which was found for LMW PEI. The condensation potential of LMW PEI could be significantly enhanced by coupling with multi-*star*-branched PEG. In this way copolymers with core-shell structures were obtained that had a similar MW as high MW PEI and formed small and compact complexes of about 100 nm upon mixing with plasmid DNA similar to HMW PEI. In comparison with HMW PEI these star-block copolymers contain less cationic units and their DNA complexes are more stabile. Therefore, these *star*-(PEG-*b*-PEI) copolymers could be promising candidates for non-viral gene delivery. Their biocompatibility and transfection activity is currently under investigation.

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Chapter 5

Poly(ethylenimine-*co*-L-lactamide-*co*-succinamide): A Biodegradable Polyethylenimine Derivative with an Advantageous pH-Dependent Hydrolytic Degradation for Gene Delivery

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[a] Transfection experiment
[b] MTT assay

ABSTRACT

A biodegradable gene transfer vector has been synthesized by linking several low molecular weight (MW) polyethylenimine (PEI, 1200 Da) blocks using an oligo(L-lactic acid-*co*-succinic acid) (OLSA, 1000 Da). The resulting copolymer P(EI-*co*-LSA) (8 kDa) is soluble in water and degrades via base catalyzed hydrolytic cleavage of amide bonds. With regard to its application as a gene transfer agent, the polymer showed an interesting pH dependency of degradation. At pH 5, when DNases are highly active, the degradation proceeds at a slower rate than at a physiological pH of 7.4. PEI and P(EI-*co*-LSA) spontaneously formed complexes with plasmid DNA. Whereas the complexes formed with PEI were not stable and aggregated, forming particles of up to 1 µm hydrodynamic diameter, P(EI-*co*-LSA) formed complexes, which were about 150 nm in size and of narrow size distribution. The latter complexes were stable, due to their high surface charge (ζ-potential + 18 mV). Similar to low MW PEI, the copolymer exhibited a low toxicity profile. At the same time, the copolymer showed a significant enhancement of transfection activity in comparison to the low MW PEI. This makes P(EI-*co*-LSA) a promising candidate for long-term gene therapy where biocompatibility and biodegradability become increasingly important.

INTRODUCTION

Over the past decade charged macromolecules have found considerable interest as drug carrier systems. Whereas anionic polymers have often been reported to adsorb proteins (*1*), polycations (*2*) have shown promise as interpolyelectrolyte complexes containing nucleic acids in the field of non-viral gene transfer. Nevertheless, several requirements have to be fulfilled by these polymers used for *in vivo* gene delivery. One of the most important demands on macromolecular drug carriers is that they must not accumulate in the human body. The elimination of the polycation as a function of time is very important. Almost all types of polycations have already been studied as gene delivery vehicles (for a review see *3*). It is has been reported that cationic macromolecules and their drug complexes accumulate mostly in the liver or kidney (*4*). Furthermore, it is well known that hydrophilic low molecular weight (MW) substances can be eliminated by the kidney, when their MW is lower than 30 kDa. Therefore, high MW polymers must be degraded or metabolized before they can be eliminated. In the case of natural polymers this metabolic process can be performed by enzymes. However, synthetic polymers are often resistant to enzymatic degradation. Thus, their application as a component of a drug delivery system is limited, especially in the case of chronic or multiple administrations. Whereas in specific indications, such as a terminal cancer, a higher dose of polycations might be tolerable, special care must be taken when treating young patients with inherited genetic disorders. In this case, accumulation of polycations over years might cause severe organ damage.

Therefore, one aim of further development in the field of polymeric gene delivery should be the development of biodegradable polycations. Surprisingly, only scant information about water-soluble, biodegradable polymers for gene transfer is available in the literature. For example, Lim et al. (*5*) reported a biodegradable aliphatic polyester poly[α-(4-aminobutyl)-L-glycolic acid)] (PAGA), which initially degraded very rapidly within the first 100 min and then gradually degraded to its monomers within 6 months. The polymer showed approximately a 2-fold higher transfection efficiency than its structural amide analogue poly(L-lysine). Putnam et al. (*6*) synthesized another amino group bearing aliphatic polyester poly(4-hydroxy-L-proline ester), which were of low toxicity. However, results of degradation studies or transfection experiments have not been reported as of yet. Apart from their biodegradability, these polymers need to be optimized with respect to transfection efficiency.

Our strategy was to use polycations well known for their high transfection activity and render this polymer degradable. We selected polyethylenimine (PEI) since it has already been

widely and successfully used for gene delivery both under *in vitro* (*7*, *8*) and *in vivo* (*9*, *10*) conditions.

It has been reported (*11*, *12*) that PEI with high MW (≥ 25 kDa) is characterized by a high transfection activity, but unfortunately by a high cytotoxicity, as well. In contrast, low MW PEI (≤ 2 kDa) shows almost no transfection, but seems to be less toxic (*13*). Our concept was to combine the good transfection efficiency of high MW PEI and the low toxicity of low MW PEI by linking several low MW PEIs with an appropriate spacer to form a biodegradable, high MW polycation.

All of the published biodegradable polycationic gene transfer systems exhibited ester bonds as degradable linkages. Here, we report the first synthesis of a biodegradable PEI derivative and its degradation mechanism based on the hydrolytic cleavage of amide bonds. This novel polycation exhibited a degradation profile with a favorable pH dependency.

Finally, it should be noted that Gosselin et al. (*14*) very recently published a concept, which is similar to ours. They coupled several PEIs with a MW of 800 Da together. In contrast to our work, they used a linker chemistry involving disulfide bonds so that the resulting polymer is expected to be degraded via intracellular reduction and not by hydrolysis.

MATERIALS AND METHODS

Polymer Synthesis

Synthesis of the oligo(L-lactic acid-co-succinic acid) (OLSA) **3**. The oligomer was prepared by polycondensation of 40.51 g L(+) lactic acid **1** (Fluka, 90 % in water, 0.405 mol, 20 eq) in the presence of 2.39 g succinic acid **2** (Fluka, ≥ 99.5 %, 20 mmol, 1 eq). The reaction was carried out *in vacuo* (20 mbar) at 160 °C for 4 h and resulted in a clear, colorless and highly viscous material. The amount of condensed water (11 g, 0,386 mol condensation product, 19 eq) corresponds to a conversion of the polycondensation of 90 %, which is related to the formation of decamers. Thus, the MW of OLSA is approximately 1000 Da. Determination of the MW by end group analysis from ^{1}H-NMR spectra is not possible due to very similar chemical shifts of the methyl-protons and the methine-protons adjacent to an acid or ester group. ^{1}H-NMR ($CDCl_3$): δ = 5.11 (m, O-C*H*, 1H), 4.33 (m, HO-C*H*, terminal, 0.2 H), 2.64 (m, -C(O)CH_2CH_2C(O)-, 0.2 H), 1.51 (m, -CH_3, 3 H), 1.41 (m, -CH_3, terminal, 0.6 H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 174.9 (*C*=O, lactic acid (LA) terminal), 171.5 (*C*=O, succinic acid (SA)), 169.5 (*C*=O, LA), 68.9 (*C*H, LA), 66.6 (*C*H, LA terminal), 28.5 (*C*H_2*C*H_2, SA), 20.2 (*C*H_3, LA terminal), 16.5 (*C*H_3, LA) ppm.

Synthesis of Poly(ethylenimine-co-L-lactamide-co-succinamide) [P(EI-co-LSA)] **6**. 2.34 g of PEI **4** (1200 Da, Polyscience, 99%, 1.95 mmol, 1.2 eq) and 1.66 g of OLSA **3** (1000 Da, 1.66 mmol, 1 eq) were dissolved in 25 mL absolute ethanol. After 1 h, the solvent was removed under reduced pressure at 40 °C and the product was isolated as a clear, colorless and highly viscous material in quantitative yield. This isolated product is referred to as intermediate **5** in this report and was treated at 160 °C for 2 h *in vacuo* (1 mbar). The resulting polymer was dissolved in ethanol and precipitated in diethyl ether. Solvent residues were removed *in vacuo*. The orange-red colored polymer was isolated with a yield of 81 %.

Polymer Characterization

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H-NMR and ^{13}C-NMR spectra were recorded in d_6-DMSO (Merck) at 35 °C or $CDCl_3$ (Merck) at an ambient temperature on a Eclipse+ 500 spectrometer from Jeol, Tokyo, Japan, at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR spectra, respectively. Spectra were evaluated with the NMR data processing program MestRe-C Version 2.2.

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 (for IR spectroscopy, Uvasol®, Merck) disks. The waxy and oily samples were smeared on the KBr disk.

Capillary Viscosimetry. The polymers were characterized by capillary viscosimetry (Ubbelohde, Schott, type No. 50101/0a) at 25.0 °C in filtered water (0.22 µm, 0,055 µS/cm). The polymer concentration was 0.5 g/dL. Inherent viscosity (η_{inh}) was calculated using the equation: $\eta_{inh} = \ln (t/t_0) / c$, where t is the flow time of the polymer solution, t_0 the flow time of the solvent and c the polymer concentration. The η_{inh} listed in Table 1 and Figure 4A is the mean of five measurements.

Degradation Study. Degradation was studied with the polymer solution used for viscosimetry, incubated for several days in a slowly rotating heating block (Rotatherm, Liebisch, Bielefeld, Germany) at 37.2 °C. Solutions of P(EI-*co*-LSA) at a concentration of 0.5 g/dL exhibited a pH of 9. Solutions with a pH of 7 and 5 were prepared by adding the appropriate amount of HCl. To compare the degradation process at different pH values, relative η_{inh} values were plotted in Figure 8 A. Absolute MW determination of these samples were carried out using SEC-MALLS.

Size Exclusion Chromatography in combination with Multiple Angle Laser Light Scattering (SEC-MALLS). The SEC setup consisted of a HPLC Pump L-6000 from Merck-Hitachi, Darmstadt, Germany, a Merck-Hitachi autosampler AS-200A and a Merck column thermostat T-6300. 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, Santa Barbara, CA, USA (DAWN® EOS™, GaAs Laser 690 nm, 30 mW, K5 cell). SEC column Novema 3000 was from Polymer Standard Service, Mainz, Germany. As an eluent, 1% formic acid (Riedel-de Haen, 98-100%) was used. 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 0.2 µm sterile filter and degassed with a 4 channel online vacuum degasser DDG-75 from Duratec, Reilingen, Germany. Additionally, an inline filter (0.45 µm, stainless steel, Rheodyne, Cotati, CA, USA) was set between the columns and MALLS detector. A flow rate of 1 mL/min was applied. Polymer concentration was 5 g/l and 20 µl were injected for each run. MWs were calculated by Astra for Windows Software, version 4.73.04, using Zimm plots. We have evaluated our SEC-MALLS with an commercially available PEI 25 kDa (Aldrich) and found following standard deviations (n = 5). dn/dc: 0.64 %. Mn: 4.16 %. Mw: 1.67 %. Mw/Mn: 3.48 %. Similar errors can be expected for the PEI and the PEI derivatives used in this study.

Formation of the Polymer-DNA Complexes

All complexes of DNA and polymer were prepared freshly before use. Plasmid DNA pGL3 control (luciferase reporter vector, 3.5 MDa, 5256 bp) was from Promega, Heidelberg, Germany and was amplified with a competent E. coli strain JM 109 (Promega) according to a protocol from QIAGEN, Hilden, Germany (*15*). Plasmid DNA was used for all experiments except for the MTT assay, in which DNA from herring testes (Type XIV, Sigma, Steinheim, Germany, 0.3 – 6.6 MDa, 400 – 10000 bp) was used.

The polymer solutions were added to DNA solutions in equal volumes, mixed by vortexing and incubated for 1 min (physicochemical experiments) or 10 min (biological experiments) before use. DNA stock solutions were diluted to a concentration of 40 µg/mL with 150 mM

NaCl at pH 7.4. The polymer stock solutions were also diluted with 150 mM NaCl (pH 7.4) to the appropriate concentration depending on the required polycation-nitrogen/polyanion-phosphorous ratio (N/P ratio) and filtered through 0.22 µm pore sized filters. DNA stock solutions were composed of 1 mg/mL pGL3 plasmid in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl) (pH 8) and 1 mM ethylenediaminetetraacetic acid (EDTA). Polymer stock solutions exhibited a concentration of 0.9 mg/mL. Polymer concentration was based on the PEI content in the copolymer only, the lactic acid and succinic acid is ignored in this calculation. Thus, all polymer stock solutions contain the same polycation concentration for a better comparability of the results.

Physicochemical Characterization of Copolymer-DNA Complexes.

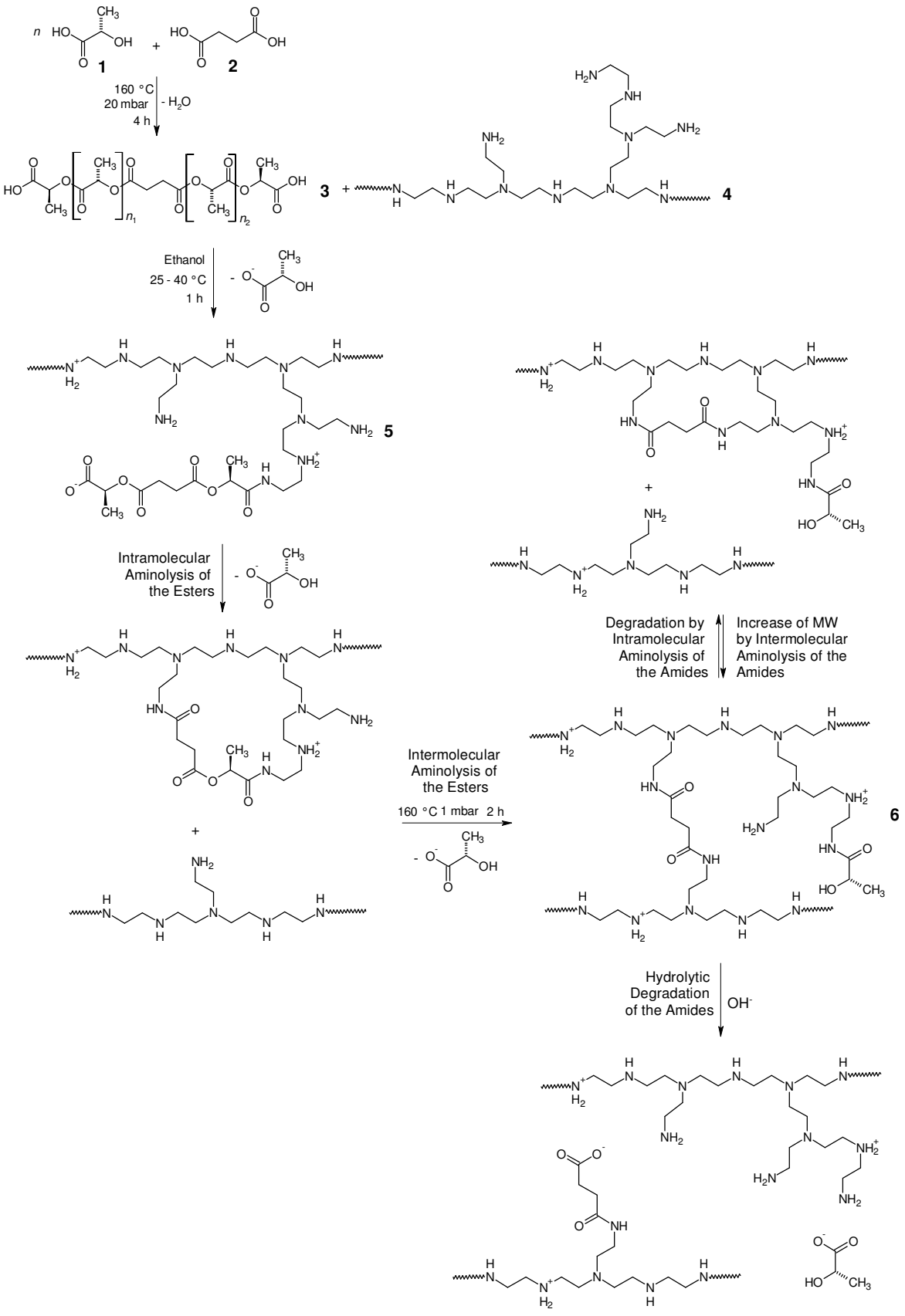
Dynamic Light Scattering (DLS). Complex size measurements were carried out with a Zetasizer 3000 HS from Malvern Instruments, Worcs., UK at 25 °C. (10 mW HeNe laser, 633 nm). Scattered light was detected at a 90° angle through a 400 micron pinhole. Measurements yielding hydrodynamic diameters (Z average mean) and polydispersity indices (PDI) were performed with count rates of about 100 - 400 kCps in form of 10 runs of 60 min duration each and analyzed in the CONTIN mode. The viscosity (0.8905 mPas) and refractive index (1.333) of pure water at 25 °C were used for data analysis. The instrument was calibrated with Nanosphere^{TM} Size Standards (polymer microspheres in water, 220 nm +/- 6 nm) from Duke Scientific, Palo Alto, CA.

Laser Doppler Anemometry. ζ-potential (zeta-potential) measurements of the complexes were carried out in the standard capillary electrophoresis cell of the Zetasizer 3000 HS from Malvern Instruments at position 17.0 and at 25 °C. Measurement duration was set to automatic. Average values of the zeta-potential were calculated with the data from 8 runs. The instrument was calibrated with DTS5050 Electrophoresis Standards from Malvern Instruments.

Biological Characterization of the Polymers and the Polymer-DNA Complexes.

MTT Cytotoxicity Assay. *In vitro* cytotoxicity was studied on L929 mouse fibroblasts by MTT assay according to the method of Mosmann (*16*). The cells were incubated with the polymers for 3, 12 and 24 h (8000 cells/well, 96-well microtiter plates) as described in detail earlier (*17*). Each point in Figure 10 represents the mean +/- SD of seven determinations.

Transfection Experiments. Transfection activity of the polymers was also studied on L929 mouse fibroblasts using a Luciferase Kit from Promega. Measurements of relative light units (RLU) were conducted on a Sirius Luminometer from Berthold, Pforzheim, Germany. Protein quantification was performed with freshly prepared bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL, USA) and was carried out with an ELISA plate reader from Dynatech MR 5000, Denkendorf, Germany, at 570 nm. Experiments were performed in triplicates as described in more detail in a previous report (*18*).



Scheme 1: Synthesis route and degradation mechanism of P(EI-*co*-LSA) **6**.

RESULTS AND DISCUSSION

Synthesis and Characterization of P(EI-*co*-LSA). In order to link several macromolecular PEI units together we chose the simple bifunctional succinic acid, which had to be activated for the reaction with the amino groups of PEI. It is well known that primary amines react with many esters just upon mixing and sometimes even at room temperature to yield amides (*19*). Therefore, an easy way to activate succinic acid for the reaction with PEI was to transform the acid into an ester. We used the α-hydroxy acid L-lactic acid as an alcohol. The use of this alcohol for esterification had at least two advantages: On the one hand, this alcohol allows the formation of a polyester, which incorporates the succinic unit. As a consequence, there are not only two ester bonds per linker which can react with PEI, but a large number of ester bonds instead. This should improve the conversion of the coupling reaction. On the other hand, it does not matter if not all of the ester bonds of this polyester react with PEI. In this case, the linker will contain amide and ester bonds, so that hydrolytic degradation is guaranteed in every case.

In a first step, we synthesized the polyester to activate the succinic acid (Scheme 1). Polycondensation of succinic acid and L-lactic acid were carried out in bulk. As a result, the conversion of this polycondensation reaction was 90 %, which implied that a decamer (10mer) with a MW of about 1000 Da was formed. Because of the low degree of polymerization the polyester will be referred to as oligo(L-lactic acid-*co*-succinic acid) (OLSA) in this article. Due to the incorporated succinic unit, the oligomer did not crystallize, but remained in its glassy state.

PEI and OLSA are highly viscous substances and cannot be mixed easily in bulk. Therefore, in preparation for the next reaction step a blend of these homopolymers was prepared by dissolving the two components in ethanol and removing the solvent again under reduced pressure. During this process the first reactions took place. The ^{13}C-NMR (175 ppm, signal 2 in Figure 2) and the FTIR (1653 cm^{-1}, signal E in Figure 3) spectra verified the formation of amide bonds. Signals of the ester groups (169 ppm, 1761 cm^{-1}, signal D_1 in Figure 3) disappeared. Only in the FTIR spectrum a weak signal of the ester bond remained. Due to H-bonding between N-H of PEI and the C=O of OLSA, the ester signal had shifted to 1733 cm^{-1} (signal D_2 in Figure 3). In the ^{1}H-NMR spectrum a new signal (6 in Figure 1) at 3.05 ppm appeared and was assigned to methylene protons of the PEI, which are neighbored to an amide bond. Hence, aminolysis of the ester bonds yielded an intermediate of the structure **5** shown in Scheme 1.

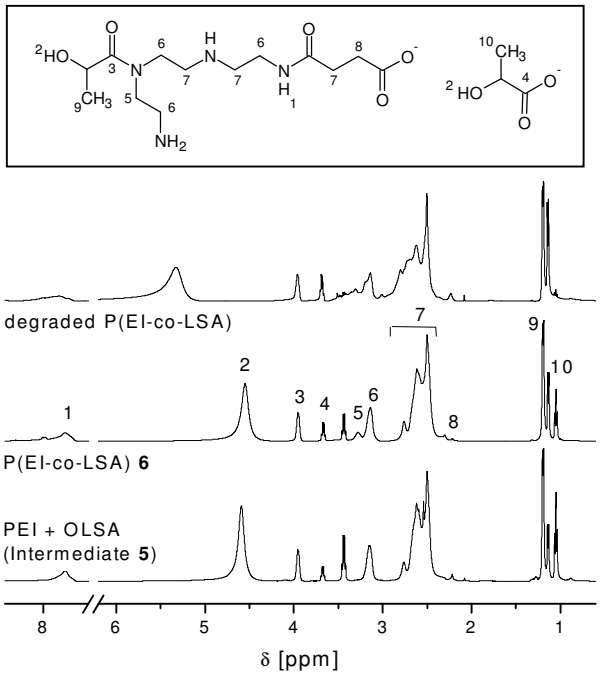


Figure 1: ^{1}H-NMR spectra (500 MHz, d_6-DMSO) of polymers **5**, **6** and the degraded P(EI-*co*-LSA) **6'** after 500 days. The structure shown in this figure is only a part of the macromolecules. Signals at δ = 1.05 and 3.45 ppm are derived from residues of ethanol in the product.

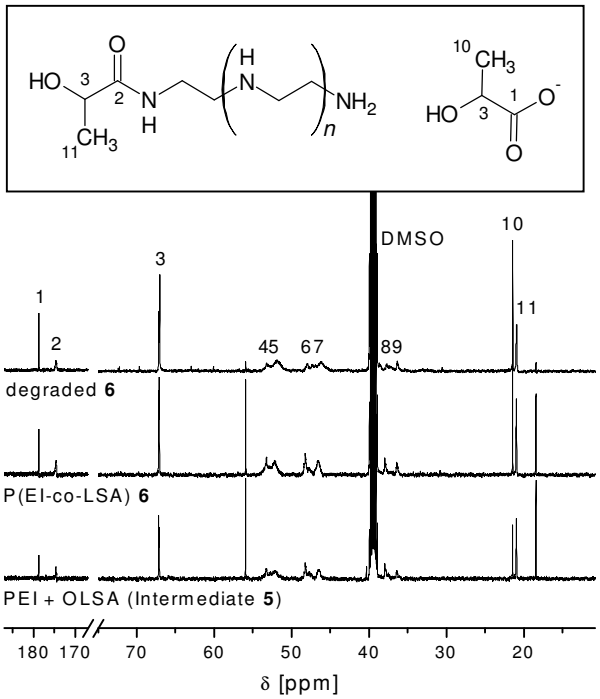


Figure 2: ^{13}C-NMR spectra (125 MHz, d_6-DMSO) of polymers **5**, **6** and the degraded P(EI-*co*-LSA) **6'** after 500 days. The structure shown in this figure is only a part of the macromolecules. Signals at δ = 18.9 and 56.5 ppm are derived from residues of ethanol in the product. Signals 4 – 9 are derived from the methylene carbons of PEI.

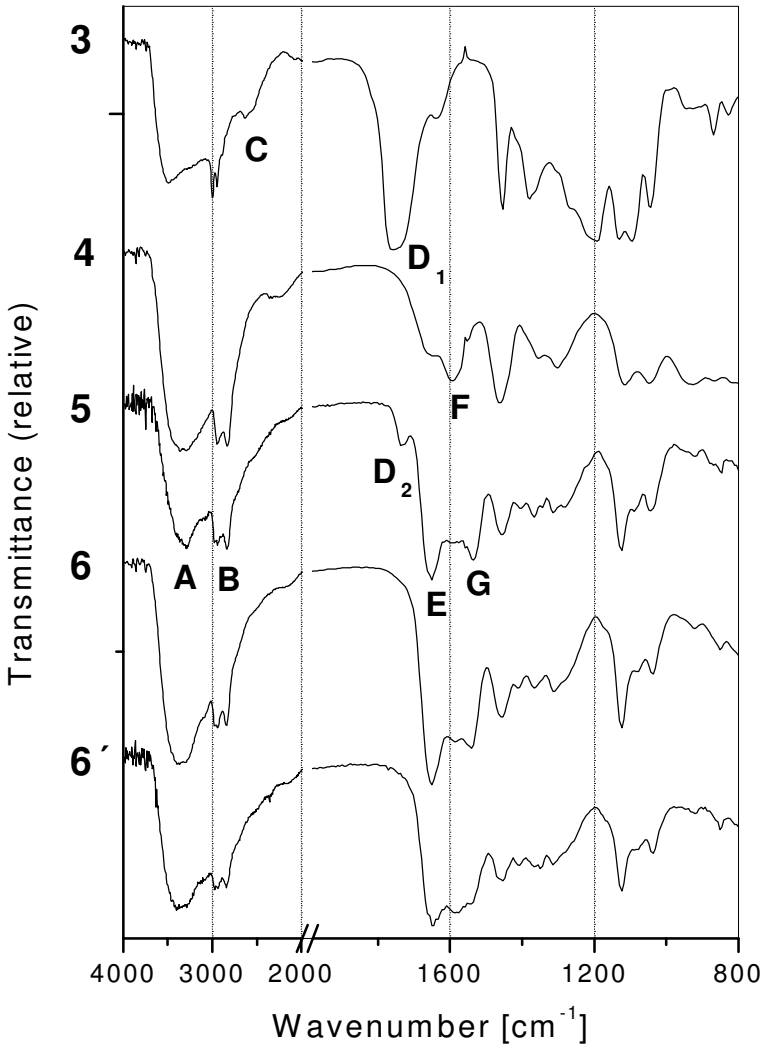


Figure 3: FTIR spectra (from top to bottom, KBr): Oligo(lactic acid-*co*-succinic acid) (OLSA) **3**, PEI 1200 Da **4**, intermediate **5**, copolymer P(EI-*co*-LSA) **6**, degraded copolymer **6'**. Characteristic stretching absorptions: (A) N-H amines 3300 cm^{-1}, (B) C-H 2921 cm^{-1}, (C) COOH 2634 cm^{-1}, (D_1) C=O ester 1761 cm^{-1}, (D_2) C=O···H-N ester, (E) C=O amide I 1653 cm^{-1}. Bending absorptions: (F) N-H amines 1594 cm^{-1}, (G) N-H amide II 1541 cm^{-1}.

Only a slight MW increase occured at this step. While viscosimetry does not indicate any increase of MW, SEC-MALLS revealed an MW (M_w) increase from 1116 to 2853 (Table 1). This suggests that OLSA most likely reacted with PEI only to modify and graft the polycation, but did not link different PEI macromolecules together. In this case, a theoretical MW of about 2000 Da is expected for **5** which is in good agreement with M_n = 1795 Da found by SEC-MALLS. Since the reactants were in diluted solution the aminolysis will most likely occur *intra*molecularly. This also explains why the inherent viscosity did not increase, but instead slightly decreased (from 0.050 to 0.042 dL/g, Table 1).

Table 1: Synthesis and degradation of P(EI-*co*-LSA): Molecular weight M_n, M_w and molecular weight distribution M_w/M_n of the polymers **4**, **5** and **6** as determined by SEC-MALLS. The refractive index increment dn/dc was determined by a refractive index detector and the inherent viscosity η_{inh} by capillary viscosimetry. Degradation was studied at pH 9, 7 and 5.

Polymer sample		Degradation time [d]	dn/dc [mL/g]	M_n [g/mol]	M_w [g/mol]	M_w/M_n	η_{inh} [dL/g]
PEI 1200 Da	**4**	-	0.359	713	1116	1.57	0.050
Intermediate	**5**	-	0.199	1795	2853	1.59	0.042
				pH 9			
Copolymer P(EI-*co*-LSA)	**6**	0	0.195	3284	7906	2.41	0.096
		3	-	-	-	-	0.086
		7	0.219	2775	6115	2.20	0.072
		21	0.217	2360	4455	1.89	0.061
		46	0.219	2097	3449	1.65	-
		61	0.217	2112	3112	1.47	-
	6'	500	0.221	1593	1789	1.12	0.053
				pH 7			
		0	-	-	-	-	0.1421
		3	-	-	-	-	0.1326
		7	0.219	3412	7017	2.06	0.1217
		28	0.235	2595	5124	1.97	0.1057
		43	0.224	2478	4181	1.69	-
				pH 5			
		0	-	-	-	-	0.1242
		3	-	-	-	-	0.1247
		7	0.218	3473	7563	2.18	0.1206
		28	0.234	3114	6463	2.08	0.1016
		43	0.219	3241	6360	1.96	-

Despite the grafting of new blocks onto PEI, the density of the macromolecule remained constant or was even enlarged by the intramolecular linkages. Since density and viscosity are antiproportional to each other (Einstein's viscosity law: $\eta_{sp}/c = 2.5/\rho$, where η_{sp} is the specific viscosimetry [$\eta_{sp} = (t - t_0)/t_0$]) and ρ is the equivalent coil density) the viscosity of the polymer solution is expected to decrease in this case even if MW increases.

In a second reaction step the intermediate **5** was allowed to react at high temperatures (160 °C) and at maximum concentration (in bulk). Under these conditions, the aminolysis of the ester bonds was completed (no remaining ester absorptions in FTIR for **6**) and two additional processes began: Aminolysis of amides (amine-amide interchange reaction) and transamidification (amide-amide interchange reaction) (*20*). The latter reaction most likely

played only a minor role, while the predominant reaction was the aminolysis of the amides (*21*). Aliphatic primary amines and, under forced reaction conditions secondary amines as well, can react with the amides (*22*, *23*, *24*). Hence, a new signal (Signal 5 in Figure 1) appears in the ^{1}H-NMR spectrum at 3.3 ppm. This signal was attributed to methylene protons of PEI adjacent to an N,N-disubstituted amide, which was formed by the reaction of a secondary amine with an N-monosubstituted amide. The formation of N,N-disubstituted amides was also verified by the decreased intensity of the N-H amide II absorption in the FTIR spectrum (Signal G in Figure 3) from **5** to **6**. While during the first step most reactions probably occured *intra*molecularly, due to the relatively high dilution, this step favors *inter*molecular reactions, because of the extremely high polymer concentration in bulk. These rearrangements towards intermolecular linkages are responsible for the large MW increase up to 8000 observed by SEC-MALLS. At the same time, inherent viscosity increased from 0.05 dL/g to almost 0.1 dL/g by a factor of two.

Finally, the solubility behavior of oligomers and polymers are compatible with the proposed reaction scheme. OLSA is soluble in organic solvents, such as ethanol, chloroform and dimethylsulfoxid (DMSO), but insoluble in water. In contrast, the intermediate **5** and the resulting polymer **6** are insoluble in chloroform, but soluble in water, ethanol and DMSO. This also is a measure for the progress of the reaction between PEI and OLSA at ambient temperature after as little as 1 h.

We are aware that copolymer synthesis can be further optimized. Especially, the optimum ratio between lactic acid and succinic acid in the oligomer OLSA has still to be determined. With less lactic acid in OLSA, more amino groups of the PEI will remain unsubstituted, which will probably affect the toxicity of the polymer and the DNA complexation. Copolymers with higher MW can be achieved when the ratio OLSA to PEI is increased. However, this ratio is probably limited. A larger amount of succinic acid linker could lead to cross-linking and would result in loss of solubility of the polymeric network. This optimization process is currently being carried out in our laboratories.

Polymer-DNA Complexes. PEI 1200 Da **4** and copolymer P(EI-*co*-LSA) **6** spontaneously formed complexes with plasmid DNA upon mixture of the solutions. These complexes were studied by DLS, which determined the hydrodynamic diameters and the polydispersity indices (PDI). The results are shown in Figures 4 and 5.

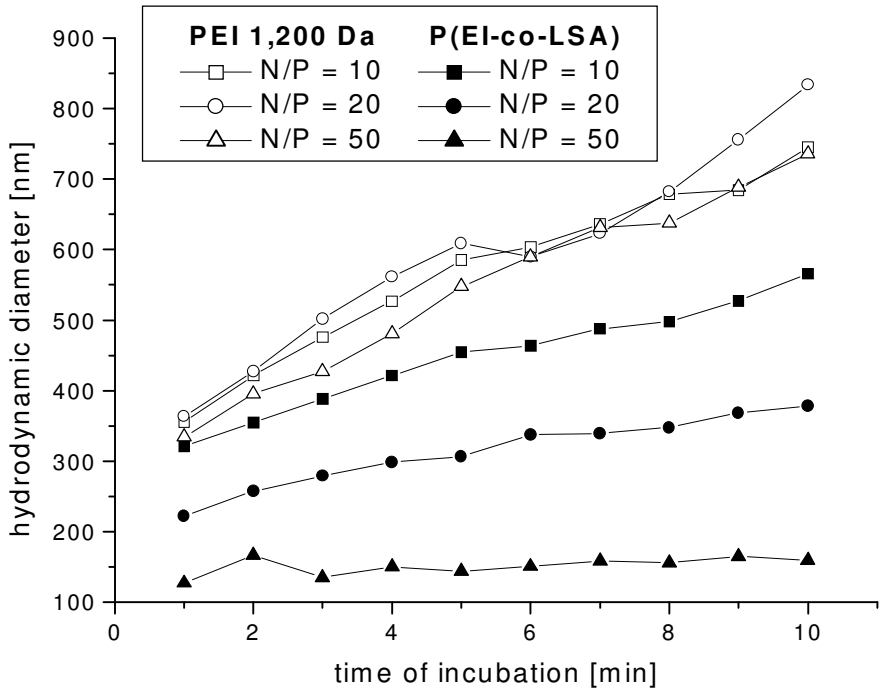


Figure 4: Hydrodynamic diameter of the polymer-DNA complexes as determined by dynamic light scattering at different N/P ratios in 150 mM NaCl at 25 °C.

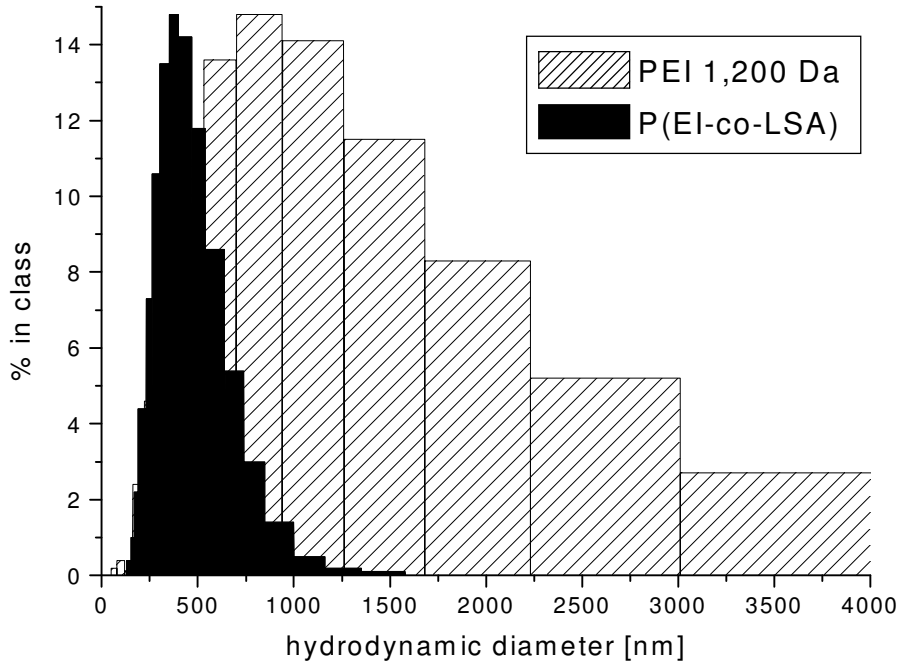


Figure 5: Size distributions of polymer-DNA complexes at N/P = 20 and after 10 min incubation as determined by dynamic light scattering in 150 mM NaCl at 25 °C.

The low MW PEI generated complexes of 350 nm in size with a PDI of 0.15 indicating a narrow complex size distribution. However, within 10 min the complexes aggregated to particle diameters of more than 700 nm. Furthermore, the PDI also increased significantly up to 0.5 implying an extreme broad size distribution. Thus, DNA complexes formed with low MW PEI were prone to aggregation. Surprisingly, a decisive enhancement of DNA condensation was observed in the case of copolymer P(EI-*co*-LSA), where the tendency towards aggregation was significantly reduced. In this case, size and stability of the complexes were strongly dependent on the N/P ratio. At N/P 10 complexes were not stable and aggregated from 320 nm to 570 nm within 10 min. Even at N/P 20 complexes were not quite stable, although the aggregation process (from 220 nm to 380 nm) was reduced significantly. However, stable complexes were obtained with N/P 50. In this case, no aggregation was observed at all. Complexes were found to be very small in size (150 nm). Also, the size distribution of the P(EI-*co*-LSA) complexes was considerably narrower (PDI < 0.1) in comparison to the PEI 1200 Da complexes. The large difference in complex size distribution between the homopolymer (PDI = 0.601) and copolymer (PDI = 0.077) is exemplarily presented in Figure 8 for N/P 20 after 10 min incubation.

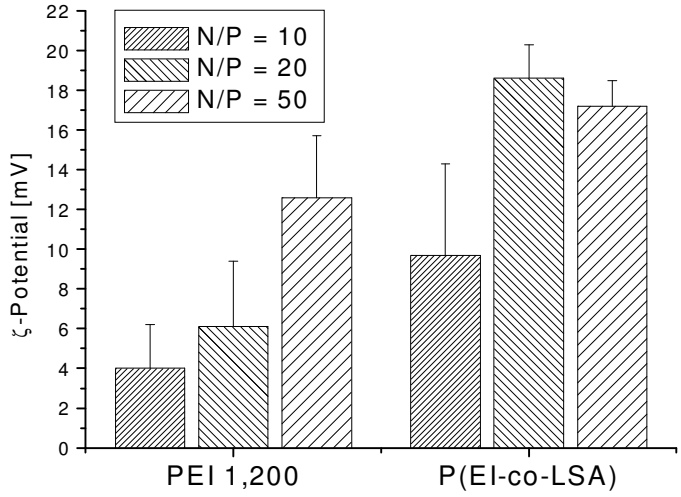


Figure 6: Zeta-potential of the complexes formed with plasmid DNA and PEI 1200 Da and copolymer P(EI-*co*-LSA) as determined by laser Doppler anemometry at different N/P ratios in 150 mM NaCl at 25 °C.

These results could be explained by the differences in ζ-potential of the complexes (Figure 6). Complexes formed with PEI 1200 Da were only of moderate ζ-potential (4 – 13 mV), which apparently was not sufficient to electrostatically stabilize the complexes against

aggregation. In contrast, the stable complexes of P(EI-*co*-LSA) exhibited highly positive ζ-potentials of about 18 mV for N/P 20 and 50.

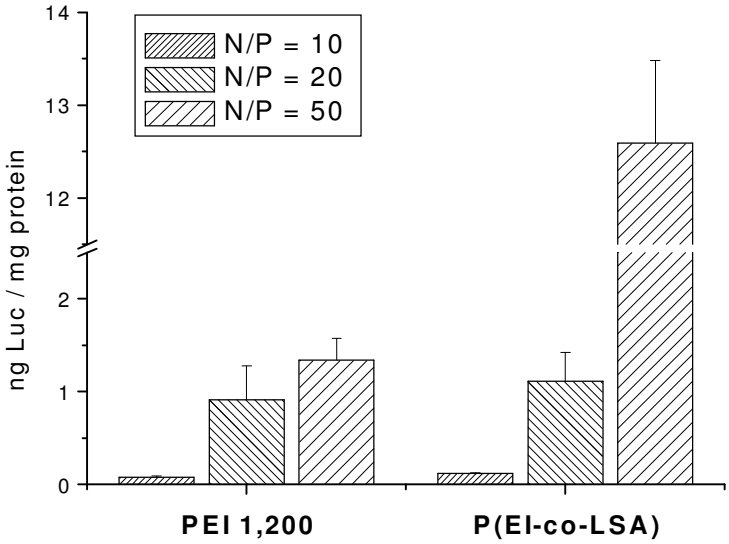


Figure 7: Transfection efficiency of PEI 1200 Da and copolymer P(EI-*co*-LSA) on L929 mouse fibroblasts at different N/P ratios.

Transfection experiments. Transfection experiments were performed with L929 mouse fibroblasts, as opposed to 3T3 cells, which are more widely used in transfection studies. However, we used the L929 cells in order to provide results which could be correlated with the cytotoxicity data raised from the MTT assay for which this cell line is recommended (see below). In previous experiments using PEI and its derivatives we found that the transfection activities with L929 cells are similar to the results with 3T3 fibroblasts (data not shown). Results of the transfection experiments are shown in Figure 7. At N/P ratios 10 and 20 no significant differences in transfection activity could be observed between the homopolymer **4** and the copolymer **6**. However, at N/P ratio 50 a 10fold higher reporter gene expression was found for copolymer P(EI-*co*-LSA) as compared to the homopolymer PEI. This high transfection activity can be attributed to the highly positive surface charge of the complexes (ζ-potential +18 mV) which favors a strong interaction with the negatively charge cell membrane, as well as to the small complex size (150 nm) which allows an easier cellular uptake by endocytosis.

For an efficient gene delivery with polymeric transfer agents it seems to be important that the polycation has a MW > 2000 Da. It has already been reported that low MW PEIs (≤ 2000 Da) do not transfect efficiently and sometimes not at all (*11, 12*). Herein, we give an

explanation for this poor transfection activity: The instability of the complexes, due to the relatively low ζ-potential, resulted in the formation of aggregates, which might be too large (> 800 nm) for a non-specific uptake into the cells. Apart from increasing the MW of the polycation by optimized polymerization methods, we demonstrate that improved transfection activity can also be achieved by linking low MW polycation units together. These conjugates of higher MW form stable and small complexes with DNA and, therefore, exhibit higher transfection efficiencies.

Degradation of P(EI-*co*-LSA). Biodegradable polymers, especially those for implants or other medical devices, have, in the past, been based on aliphatic polyesters, such as poly(lactic acid), poly(glycolic acid) or poly(ε-caprolactone). Creating a degradable polycation with cleavable ester bonds is difficult, since almost all polycations are based on amines, which can easily react with esters. This also is the reason why the amino group bearing polyester, PAGA, reported by Lim et al. (*5*) degraded very quickly. The initial MW is reduced to 1/3 after only 100 min. The authors explained the rapid degradation as being a result of self-degradation of the polymer's own ester linkages by the ε-amino groups. Generally, ester bonds are easily cleaved if the polymer and the degradation products are water-soluble. Thus, a polycation linked with ester bonds would possibly degrade too fast and is not able to delivery the therapeutic gene efficiently before degradation. A very rapid degradation might be just tolerable in the case of cell culture experiments since only a few hours are required to yield a maximum transfection of the cells. In the case of *in vivo* gene delivery, however, the gene has to be protected for several hours or more, due to longer circulation times of the complexes in the blood system. A degradable polymeric network based on amides might be more appropriate for an *in vivo* gene delivery since it shows a higher stability as compared to a system with ester bonds, however, is still degradable, as we will show here.

The degradation pattern of our copolymer was studied in aqueous solutions at pH 5, 7 and 9 at 37 °C. The results of the viscosity measurements (Figure 8 A) and MW determination by SEC-MALLS (Figure 8 B and 9, Table 1) demonstrated that P(EI-*co*-LSA) had degraded within months and that degradation was accelerated with increasing pH. This finding is compatible with a degradation process which is base catalyzed by the amino-groups of PEI. Table 1 displays the absolute MW during the degradation process. At pH 9 the copolymer **6** is degraded from M_w 8000 to the MW of the starting material **5** (M_w 3000) within 2 month. At the same time, the MWD is reduced to the original distribution of the PEI homopolymer. This is reasonable because the degradable copolymer P(EI-*co*-LSA) is cleaved into parts of the

non-degradable PEI macromolecules with their narrower distribution. While the copolymer **6** is degraded after 1½ month to a M_w of 3449 Da at pH 9, it is degraded to M_w = 4181 Da at pH 7 and M_w = 6360 Da at pH 5, respectively. Hence, at neutral and physiological pH the copolymer still degrades relatively fast, whereas under acidic conditions (pH 5) the degradation process is remarkably inhibited. We think that this pH dependency is quite favorable for a biodegradable gene delivery system, since it should protect the DNA against enzymatic degradation, especially at the pH at which DNAses are extremely active, as is true for the acidic conditions found in lysosomes (pH 5).

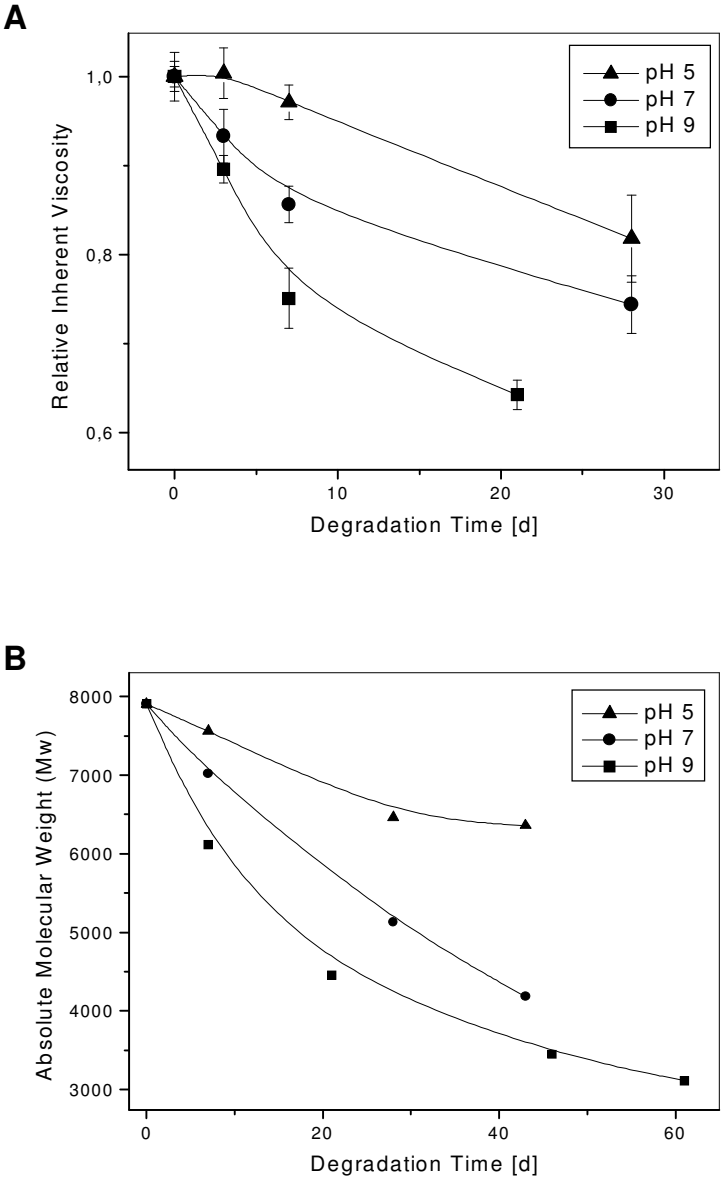


Figure 8: Degradation experiments with P(EI-*co*-LSA) **6** in aqueous solution at 37.2 °C and pH 5, 7 and 9. (A) Change of inherent viscosity during the degradation process as determined by an Ubbelohde viscosimeter. Relative values are presented in this plot. (B) Absolute MW (M_w) during degradation process as determined by SEC-MALLS.

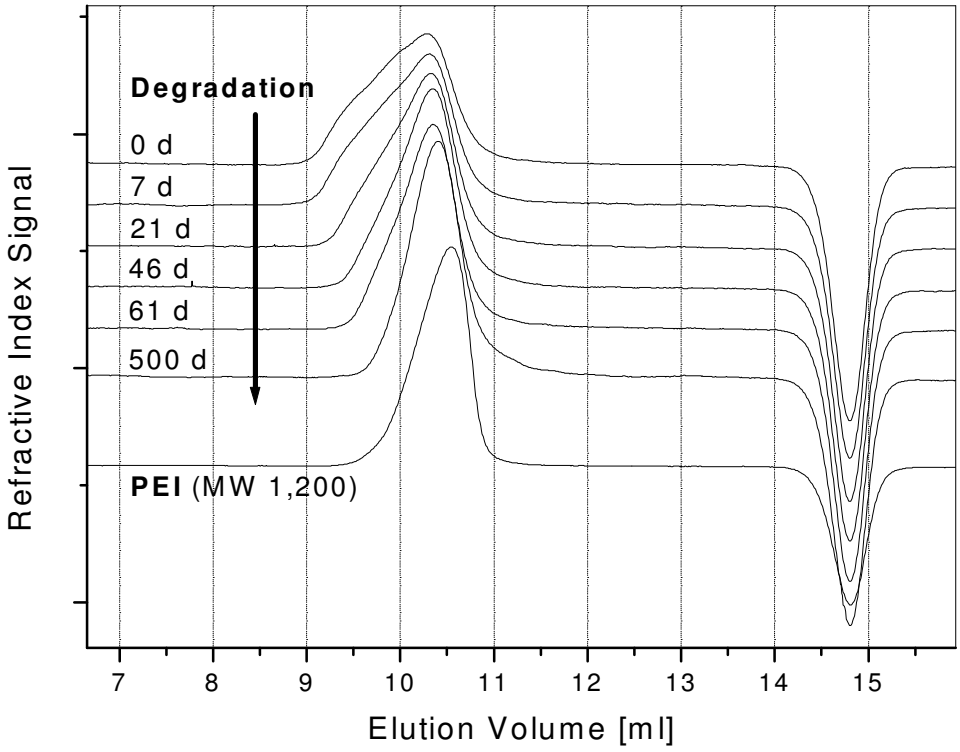


Figure 9: Degradation of P(EI-*co*-LSA): SEC eluograms of PEI 1200 Da **4** and its biodegradable derivative P(EI-*co*-LSA) **6** as detected by a refractive index detector. The degradation study was performed at pH 9 and 37.2 °C.

Two possible degradation mechanisms have to be discussed (see Scheme 1). On the one hand, since the degradation process is performed in diluted solution, *intra*molecular aminolysis might cleave the *inter*molecular linkages, which would results in a decrease of MW. Still, it remains questionable if the aminolysis of amides really occurs under these mild conditions. On the other hand, the large number of primary amino groups in the vicinity of the amide bonds might facilitate this reaction. The second possible degradation process is the hydrolytic cleavage of the amides. It is generally assumed that the amide bond is rather stable against hydrolysis under mild conditions and that hydrolysis of amides only occurs at extreme pH and temperatures. Nevertheless, three points should be taken into account. First, in order to significantly decrease the MW of polyamides only a few amide bonds have to be cleaved. Second, the basic environment of the amides in our copolymer caused by the amino groups of PEI probably accelerates the hydrolytic cleavage. Third, several authors (e. g. *25*, *26*) reported the hydrolysis of polyamides under mild conditions and demonstrated that polyamide degradation can be observed after a few days. For instance, it has been reported that even water-*in*soluble aliphatic polyamides made from D- and L-tartaric acids and 1,6-

hexamethylene diamine showed a weight loss between 10-30% and decays in the MW of nearly 50% after 2 months upon incubation in water under physiological conditions (pH 7.3, 37 °C) (*25*). In contrast, our P(EI-co-LSA) and all its degradation products are water-soluble. Thus, in our case the contact of the polymer with water is optimized which favors the amide hydrolysis.

The spectroscopic data support the theory of hydrolytic cleavage of amide bonds. A comparison of the NMR spectra of intermediate **5**, the copolymer **6** and the degraded copolymer **6'** (see Figure 1 and 2) showed an increase in the intensities of free lactic acid (^{1}H: signals 4 and 10, ^{13}C: signals 1 and 10) and a decrease in the intensities of polymer bound lactamide (^{1}H: signals 3 and 9, ^{13}C: signals 2 and 11) is clearly observed. During the reaction from **5** to **6**, aminolysis of the remaining ester bonds led to the release of additional lactic acid. The degradation process from **6** to **6'** released even more lactic acid, which is only possible by hydrolytic cleavage. A decrease of amide bonds by hydrolysis was also detectable by FTIR (Figure 3). Compared to the spectrum of **6**, the intensity of the carbonyl amide I absorption (Signal E) was decreased in the spectrum of **6'**, while the intensity of the amino N-H bending absorption (Signal G) had increased, thus, indicating an increasing number of free amino groups.

Consequently, if our copolymer is degradable two other polycations known for their application as gene delivery vehicles should also degrade via hydrolytic cleavage of its amide bonds since they also exhibit neighboring amido bonds and amino groups: poly(L-lysine) (PLL) (*27*) and polyamidoamine dendrimers (PAMAM) (*28*). In the case of polylysine, Oppermann et al. (*26*) states that almost nothing is known about the degradation behavior of this poly(amino acid). However, Kunioka et al. (*29*) reported that the similar polyamide poly(ε-lysine), had slightly degraded at its amide bonds (2 % weight loss after 17 h at 40 °C) in water. In the case of PAMAM dendrimers, it was found that a short heat treatment of aqueous polymer solutions caused hydrolysis of some of the amide bonds, which surprisingly resulted in an even better transfection activity (*30*). Ranucci et al. (*31*) studied the hydrolysis of PAMAMs under physiological conditions and found that, after incubation for 24 h, polymer intrinsic viscosity was decreased by approximately 50 %. Interestingly, they also pointed out that hydrolytic degradation progressed much more slowly at pH 5.5 which is in good agreement with our pH-rate profile for polyamide hydrolysis.

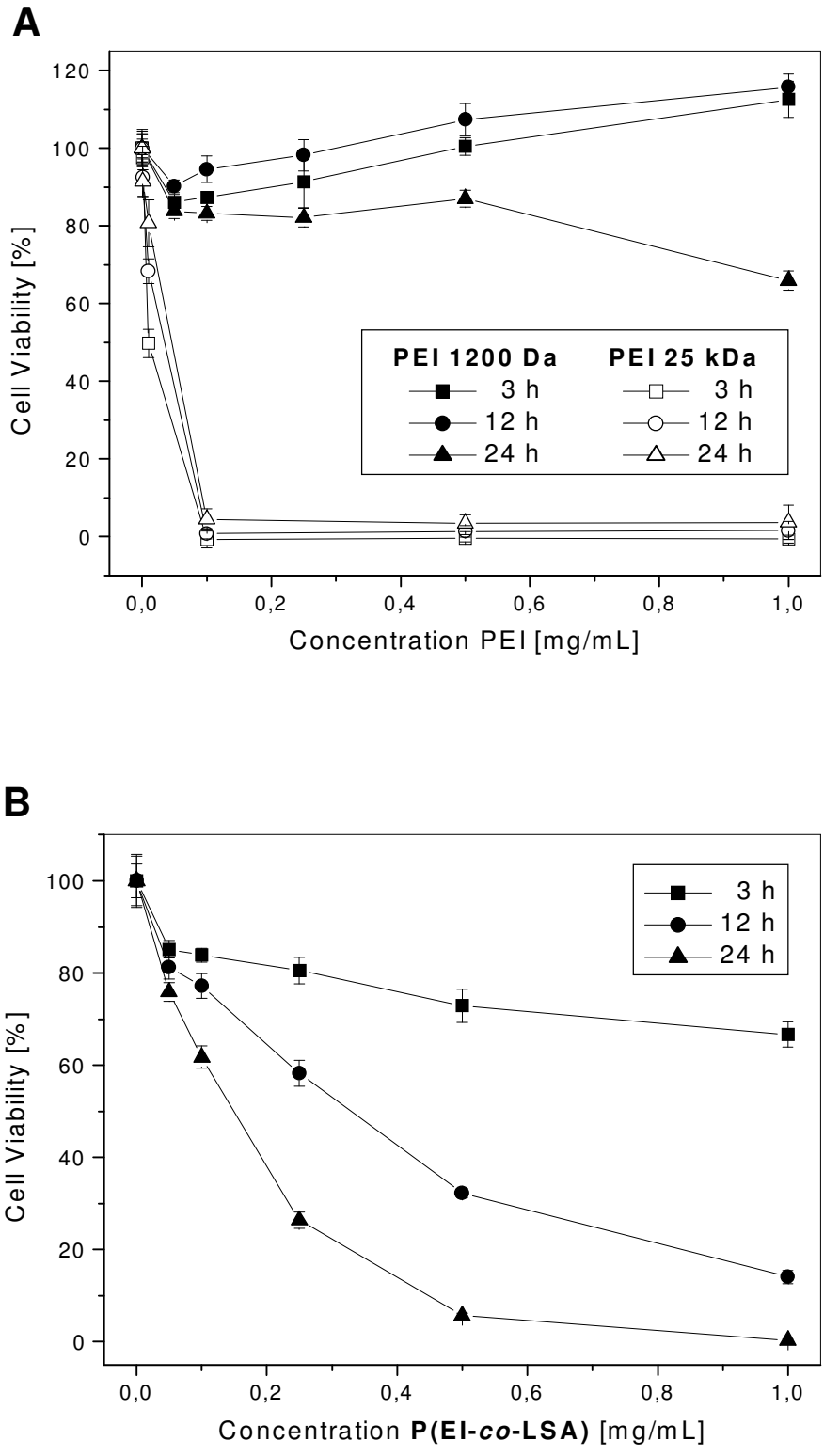


Figure 10: Dose- and time-dependent effects of PEI **4** 1200 Da in comparison to PEI 25 kDa (A) and copolymer **6** P(EI-*co*-LSA) (B) on L929 mouse fibroblasts determined by MTT-assay. Each point represents the mean +/- SD of seven determinations.

Cytotoxicity of the Polymers. The cytotoxicity of the copolymer **6** was studied using the same cell line as in the transfection experiments. As recommended by many standard institutions, L929 mouse fibroblasts were used as cell line to test the cytotoxicity of the polymers (*32*, *33*). PEI **4** was also studied as a reference (Figure 10 A) and was found to be non-toxic up to a concentration of 0.5 mg/mL for 24 h. Only a slight decrease of cell viability (70 %) was found at 1 mg/mL after a 24 h incubation time. IC_{50} values (> 1.0 mg/mL) could not be determined in this case. The copolymer **6** (Figure 10 B) also showed only a slight decrease in cell viability (67%) after 3 h incubation (IC_{50} > 1.0 mg/mL). At 12 h and 24 h incubation time, an increase in toxicity with increasing polymer concentrations was observed (IC_{50}: 0.34 and 0.16 mg/mL, respectively). However, in comparison to commercially available high MW PEI, such as PEI 800 kDa from Fluka or PEI 25 kDa from Aldrich [IC_{50} approximately 0.01 mg/mL, see Figure 10 A and (*13*)], the copolymer **6** was still of relative low toxicity. Its toxicity is comparable with the low MW PEI that is described in the literature as a highly effective gene transfer reagent with a low toxicity profile (*17*). Thus, the results of this cytotoxicity assay indicate a good biocompatibility of the biodegradable copolymer. A positive contribution towards the low toxicity profile of the copolymer might have the lactamide units with their terminal hydroxy groups which are generally assumed to have a positive influence on the biocompatibility of drug carrier systems (*34*). Furthermore, biocompatibility should even increase when degradation proceeds. With progressive degradation, copolymer **6** is expected to show a decrease in cytotoxicity, since the degradation products are non-toxic low MW PEI and lactic acid, whose acidity is neutralized by the basic PEI. Finally, it should be emphasized that we measured cytotoxicity of the free polymers. When applied as a gene transfer vehicle, the polymer is complexed with DNA. These complexes are generally less toxic compared to the free polymer (*35*, *36*). Therefore, the results of our cytotoxicity study represent the maximum toxic effect of the polymers.

CONCLUSIONS

A new type of copolymer has been prepared by linking several low MW PEI macromolecules using an oligomer of lactic acid and succinic acid. The resulting copolymer of high MW spontaneously formed complexes with plasmid DNA. Compared to low MW PEI the complexes were much smaller in size and more stable against aggregation which resulted in a 10fold higher transfection activity of the copolymer. However, the decisive advantage is the biodegradability of this new copolymer which is based on hydrolytic cleavage of amide

bonds. It was found that degradation was strongly dependant on pH. Keeping in mind the fact that DNAses are highly active under acidic conditions, as seen in lysosomes (pH 5), biodegradable gene transfer agents should be stable under acidic conditions in order to sufficiently protect the therapeutic gene. On the other hand, the polycation should be degradable under physiological conditions (pH 7.4) where the DNA should be released from the system. Our copolymer fulfils these demands and degrades within a reasonable time period that is long enough to secure gene transfer, but as short as necessary to prevent accumulation in the human body. Additionally, a low toxicity profile was observed for the new copolymer, which indicates its good biocompatibility.

These results demonstrate that the copolymer, P(EI-*co*-LSA), formed by a new synthesis method is a promising candidate for *in vivo* gene therapy applications. Further development will focus on the modification of the synthesis to improve the MW of the copolymer. It is expected that the transfection efficiency can be even enhanced by this process.

ACKNOWLEDGMENTS

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Chapter 6

Characterization of Low Molecular Weight and Hyperbranched Polyethylenimines using Size Exclusion Chromatography Combined with Static and Dynamic Light Scattering

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(in preparation)

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[b] Viscosity measurements, solution polymerization

ABSTRACT

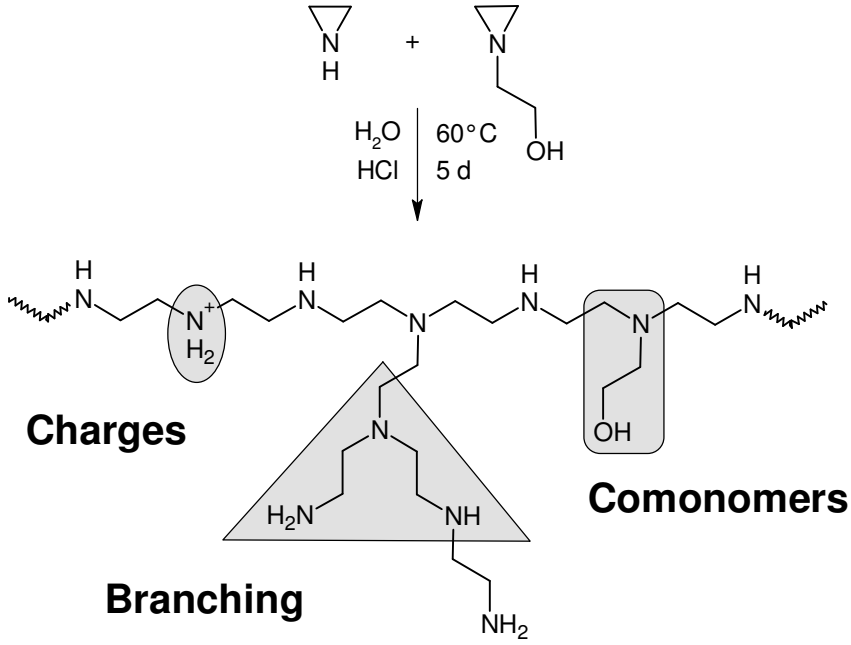
Polyethylenimines (PEI), poly(N-hydroxyethyl-ethylenimine)s (PHEEI), and their copolymers were characterized by size exclusion chromatography (SEC) in combination with static and dynamic light scattering (SLS/DLS). Commercially available and synthetically obtained PEIs were investigated. Synthesis of the PEIs were performed both in solution and bulk. Solution polymerisation of EI yielded polymers with molecular weights (MW) up to 7000 Da. Higher MW (27000 Da) were achieved by bulk polymerisation. PEIs were fractionated by SEC and analysed by a multi angle laser LS (MALLS) and a differential refractive index (DRI) detector using vacuum degassed 1 % formic acid of high purity as eluent. SEC columns consisting of hydrophilic non-charged hydroxyethyl methacrylate gel-matrices were used. These columns did not show any absorptive effects with the cationic analyte and did not shed any particulate matter. Further, the non-charged nature of the columns allowed a good fractionation of the polymer. The MALLS detector with its enhanced optical system was able to accurately determine the MW of PEIs as low as MW < 1000 Da. The hydrodynamic radii (R_h) of the PEIs were determined by quasi-elastic LS (QELS = DLS). The results of the MW and R_h determination demonstrated a lower degree of branching of the synthesized PEIs compared to the commercially available PEIs. Using results of viscosimetry measurements and the absolute MW data, the Mark-Houwink parameter a and K were determined. The a value of the synthesized PEIs (a = 0.295 ± 0.015) was significantly higher compared to commercially available PEIs (a = 0.186 ± 0.028). This finding supports the results about the differences in degree of branching. The combination of SEC with SLS and DLS detectors has been shown to be a powerful tool for the determination of the molecular parameters of very difficult polymeric analytes, such as hyperbranched and highly charged copolymers of low MW.

1. Introduction

Polyethylenimines (PEI) have a long history as additives in the paper industry [1,2] and for waste water treatment [3]. In recent years PEI has been gradually substituted by other polycations such as poly(vinyl amine) in many fields of industrial application [4]. More recently, PEI has experienced a "renaissance" as a speciality chemical in the field of drug delivery, when Behr and coworkers first introduced this hyperbranched polycation as non-viral gene delivery system [5]. The exceptionally high potential of PEI to transfect cells both *in vitro* and *in vivo* was explained by its ability to escape from endosomal compartments presumably by a proton-sponge effect [6].

PEI is commercially available in different isomeric forms, linear and branched, and with a wide variety of different molecular weights (MW), 423 – 1 Mio. Da. Since it is not clear, whether the MW or the degree of branching is a critical feature for its potential as gene transfer agent, it is of importance to determine both parameters accurately. Relative methods for the determination of the MW, such as viscosimetry or size exclusion chromatography (SEC), can be excluded since the degree of branching affects the hydrodynamic diameters of the macromolecules and would result in incorrect data. As a consequence, absolute methods, such as osmometry or light scattering (LS), need to be used to determine the absolute MWs. Once the MWs of the PEIs are determined, the degree of branching can be estimated when the absolute MW data are compared with the results of the relative methods. Absolute methods to determine the degree of branching of PEI are based on special NMR techniques, such as a ^{13}C-NNE-NMR experiment, in which the spectrum is recorded using inverse gated decoupling pulse sequences, allow the exact evaluation of the ratio of the primary, secondary and tertiary amino groups [7]. This NMR method is independent of the MW of the polymer and yields reliable data even when MW determinations are affected with errors.

Generally, polymer characterization is problematic when the analyte is a polyelectrolyte or in the case of branching or in the case of copolymers [8]. In the field of polymeric drug delivery systems one has to deal very often with polymers that combine all these three difficulties of polymer characterization in one macromolecule. For instance, in our case we wanted to characterize a hyperbranched, positively charged copolymer (Scheme 1). Another complication would be if the analyte is only of low molecular weight. In that case, it is for instance difficult to find an appropriate membrane for osmometry or the intensity of the scattered light is often too low to allow a reliable evaluation of the data.



Scheme 1: Schematic of the polycation synthesis. The three problematic aspects of polymer characterization are marked in the structure.

Here, we describe the use of static and dynamic light scattering (SLS and DLS) in combination with SEC to determine the MW and MW distribution (polydispersity, PD) of commercially available PEIs and PEIs that have been synthesized in our own laboratories. We will also demonstrate that with the enhanced optical systems of modern LS instruments we were able to determine even low MW polymers quite accurately. These absolute MW results were used to determine the Mark-Houwink parameters that verified the results about the degree of branching that we found in ^{13}C-NNE-NMR experiments and reported earlier [7]. Further verification of the different degree of branching was achieved by an DLS experiment.

2. Experimental

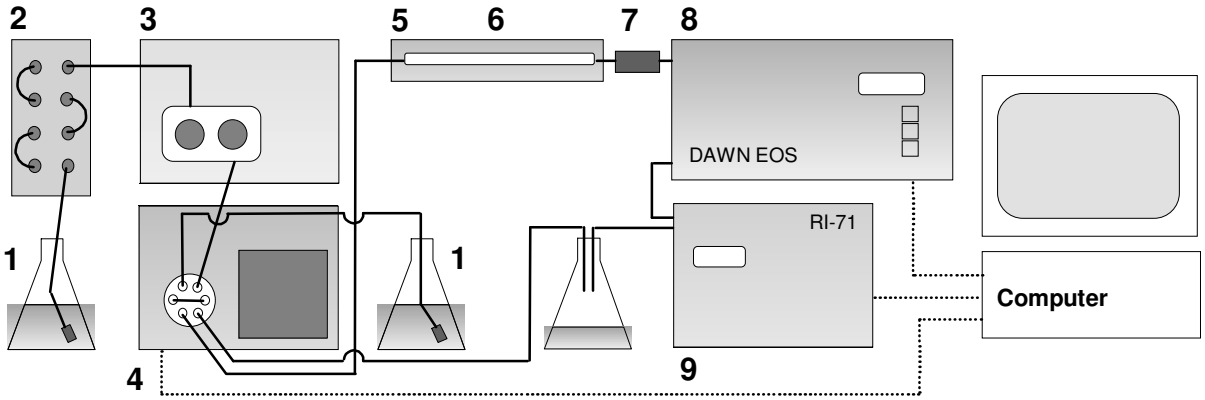
2.1 Commercially available PEIs

The supplier of the commercially available PEIs and of the other polymers used in this study are given in the Tables 1 and 2. A large number of PEIs were a gift of the BASF, Ludwigshafen, Germany. Other PEIs were obtained form Aldrich, Milwaukee, WI, USA, from Polysciences, Warrington, PA, USA, and from Fluka, Buchs, Switzerland. The Poly(diallyldimethylammonium cloride)s (PDADMAC) were a kind gift of W. Jaeger at the Fraunhofer Institute of Applied Polymer Science in Teltow, Germany, and of H. Dautzenberg at the Max-Planck-Institute (MPI) of Kolloid and Surface Sciences in Potsdam, Germany. The poly(amino acids) poly(L-lysine) and poly(L-histidine) were obtained from Sigma, St. Louis, MO, USA. The poly(amido amine) (PAMAM) dendrimer was purchased form Aldrich. The pullulan and dextran standards were from Polymer Standards Service, Mainz, Germany. The α-cyclodextrin was obtained from Schwarz Pharma, Monheim, Germany.

2.2 Synthesized PEIs

Solution-polymerization of Ethylenimine (EI) and N-Hydroxyethyl-ethylenimine (HEEI). The acid catalyzed ring-opening-polymerization (ROP) of EI in water was described in [9]. The solution-polymerization of HEEI will be published elsewhere [10].

Bulk-polymerization of EI and HEEI. Freshly destilled ethylenimine (2 ml, 1.664 g, 38.63 mmol) was placed under nitrogen atmosphere in a dry 25 ml flask fitted with a magnetic stirring bar. The monomer bulk was cooled to -70 °C, and the polymerization was initiated by adding 100 µl of a 32 % hydrochloride acid (116 mg, 1.02 mmol) under stirring. The mixture was allowed to warm up slowly to room temperature, and stirring was continued for 5 days. Finally, the substance was heated up to 100 °C for 6 h. The resulting highly viscous, slightly yellowish polymer was obtained in quantitative yield. In the same way, HEEI was polymerized in bulk and a viscous, yellowish polymer was obtained.



1	Eluent (with filter): 1 % Formic acid (Riedel-de Haën, purest, 98-100%), purest water (0,22 µm, 0,055 µS/cm), USF Seral, Ransbach, Germany, Seradest BETA 25 + Serapur DELTA UV/UF
2	Online vacuum degasser DDG-75 (4 channels), Duratec Analysentechnik, Reilingen, Germany
3	HPLC-pump L-6000, Merck-Hitachi, Darmstadt, Germany (flow rate: 1 ml/min)
4	Autosampler AS-2000A, Merck-Hitachi (20 µl sample loop, 60 µl injection volume)
5	Column thermostat T-6300, Merck, Darmstadt, Germany (35°C)
6	SEC columns Novema 30 and 3000, (8 x 300 mm, 10 µm), Polymer Standards Service, Mainz, Germany
7	Inline filter, Rheodyne, Cotati, CA, USA (stainless steel, 0.45 µm)
8	18 angle laser light scattering detector, Wyatt Technology, Santa Barbara, CA, USA, DAWN® EOS™ (Enhanced Optical System), GaAs laser, 690 nm, 30 mW (vertical polarisized light), beam diameter: ($1.0/e^2$) 0,2 mm, K5 cell (angles between 25° and 163°)
9	Differential Refractometer RI-71, Merck, Darmstadt, Germany

Figure 1: SEC-MALLS set-up for polycations in aqueous solution.

2.3 Size exclusion chromatography in combination with multiple angle laser light scattering (SEC-MALLS)

The SEC set-up is shown in Figure 1. In the attached table of this figure, all the experimental details are given. The concentration of the polymer solution were between 5 mg/ml and 20 mg/ml. The LS detector was calibrated using a polystyrene standard in toluene. The calibration constant of the RI detector was determined with a pullulan standard (MW 47900 Da) in 1 % formic acid. Data were processed using the ASTRA for Windows software (Version 4.73.04). Zimm plots [11] were used for the calculation of the MW.

2.4 SEC-MALLS in combination with quasi-elastic light scattering (QELS)

For a DLS (= QELS) experiment, the WyattQELS™ digital correlator from Wyatt Technologies, Santa Barbara, CA, USA, containing a single photon-counting avalanche photodiode and an optical fibre that was connected to the read head of the MALLS detector at the 111° position. The data were processed by the ASTRA software. The viscosimetry (0.8905 mPas) and the refractive index (1.333) of pure water were used for the calculation of the hydrodynamic radii (R_h).

2.5 Capillary viscosimetry

The inherent viscosimetry of the polycations was determined with a capillary viscosimeter in 0.5 M $NaNO_3$ at 25 °C. Details are described in [7].

2.6 ^{13}C-NNE-NMR.spectroscopy

The ^{13}C-NMR spectra were recorded on a LA 500 Eclipse+ Delta FT spectrometer from Joel, Tokyo, Japan, with 100 mg polymer in D_2O (Merck, Darmstadt, Germany) at 125 MHz (8000 scans, 30 °C). To avoid any influence of the Nuclear Overhauser effect (NOE) the spectra were recorded using inverse gated decoupling pulse sequences. The ratio of primary/secondary/tertiary amino groups of the PEIs was calculated from these NMR spetra as described in detail in [7].

3. Results and discussion

3.1 Polymer synthesis

The ring-opening polymerizaiton (ROP) of ethylenimine (EI) initiated by hydrochloric acid (HCl) could be performed in solution and in bulk. The solution-polymerization yielded polymers with MW up to 7 000 Da. Higher MW PEIs (27 000 Da) were achieved when the ROP was performed in bulk. Since the ROP of EI is exotherm and the HCl reacts violently with the monomer the polymerization had to be initiated at low temperature (-70 °C). Higher MW polymers with higher monomer concentrations were also reported for other hyperbranched systems [12]. Schlüter et al. explained this finding by hindered propagation steps in diluted monomer solutions caused by a high concentration gradient the monomers are

exposed to when the monomer diffuses to the reactive end of the propagating polymer chain [13]. At high monomer concentration (bulk = highest monomer concentration) there is no concentration gradient, and therefore the reaction of further monomers to the propagating polymer chain is kinetically facilitated.

3.2 Polymer characterization by SEC-MALLS

Measurement principle. The combination of SEC and SLS allows the determination of both the MW and the MW distribution of the polymer sample. The polymer is fractionated by the SEC column and the single fractions are detected by a differential refractive index (DRI) detector (concentration dependent) and by an multi angle laser LS detector (MALLS) (molar mass dependent). Therefore, the weight average MW (M_w) is determined of the single polymer fractions and the number average MW (M_n) and the MW distribution (M_w/M_n) is calculated based on these absolutely determined M_w. The refractive index increment (dn/dc), which is used for the calculation of MW from the LS intensities, is determined by the RI detector.

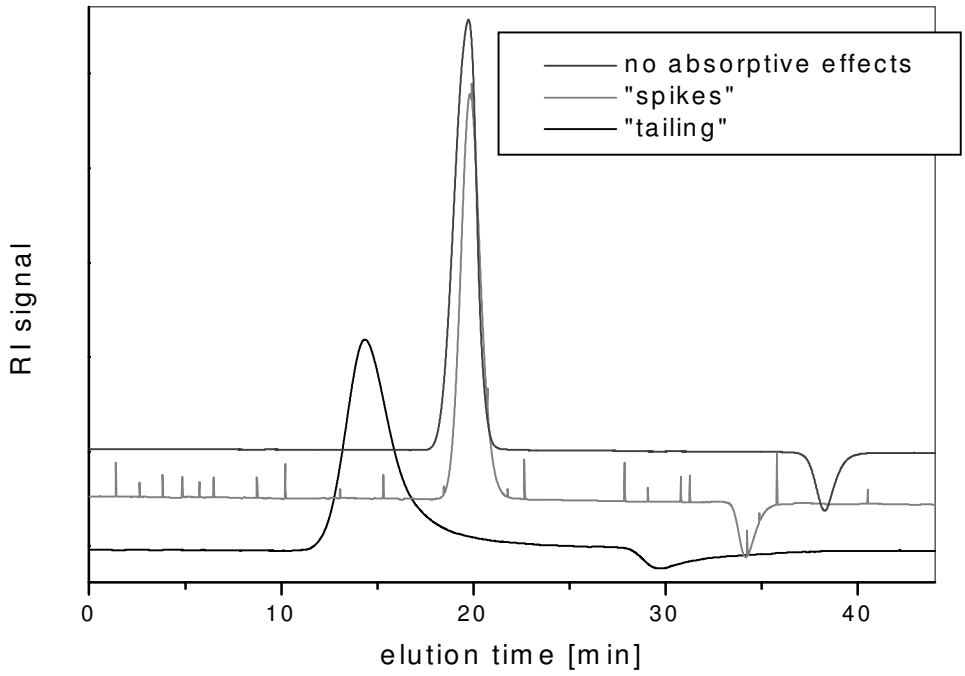


Figure 2: Technical problems with aqueous SEC. From bottom to top: (1) PEI 25 kDa was fractionated on a ordinary column for aqueous solutions (PSS Suprema linear). Absorptive effects can be seen. After the polymer peak the signal does not return to the baseline immediately. (2) The same polymer on a cationic column (Suprema Max 3000). The RI detector shows repeatedly sharp signals, “spikes”. (3) The same polymer on the same column with use of degassed eluent did not show absorptive effects or spikes.

SEC columns for SEC-MALLS. The dn/dc is evaluated by the RI detector under the assumption of 100 % polymer mass recovery. This implies that the SEC column does not absorb any amounts of the polymer. Since most stationary phases for aqueous SEC bear a negative charges [14], substrate-macromolecule interactions have been specially problematic for polycations leading to strong and often irreversible adsorption. Additionally, the very high affinity of PEI for many surfaces [15] makes it extremely difficult to apply PEI for chromatoghraphic analysis. These absorptive effects can often be seen in the eluograms as "tailing" as it is demonstrated in Figure 2 for a PEI 25 kDa on a ordinary hydroxyethyl methacrylate (HEMA) column for aqueous solutions. This absorptive effects were the reason for many unsuccessful SEC experiments of polycations as for instance reported recently by Lukkari et al. [16]. For a long time, absorption of the polycation to the column was minimized by using cationic column materials [17].

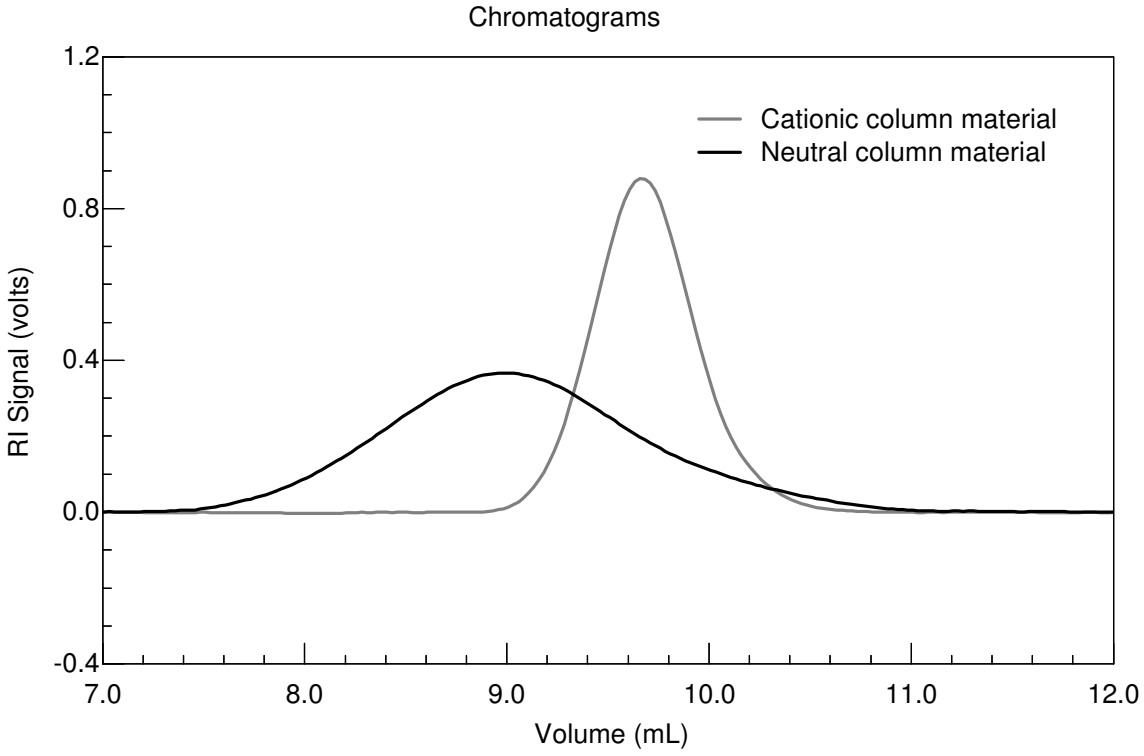


Figure 3: The potential of different columns to separate a polycation. PEI 25 kDa (Lupasol WF) was less fractionated on a cationic column (PSS Suprema Max 3000) compared to a neutral column (PSS Novema 3000).

However, this strategy creates a disadvantage. Since both the column and the analyte are cationic the polymer is strongly repelled by the column material and the cavities of the column matrix are not accessible for the polymer [18]. This results in only poor separation as demonstrated in Figure 3. However, the combination of SEC and LS only gives reliable data for the polydispersity if the SEC column is able to sufficiently separate the polymer sample.

As a consequence, neutral column materials without any charged groups are thought to be more appropriate for polymer analysis by SEC-MALLS.

Furthermore, the SEC column must not show particle shedding. Column particles that are released from the column matrix would cause a high and turbulent LS background signal that makes it difficult to determine low MW macromolecules (Figure 4). Furthermore, the released particle easily aggregate with polycations such as PEI and induced a very strong LS signal. This makes it impossible to achieve correct MW data.

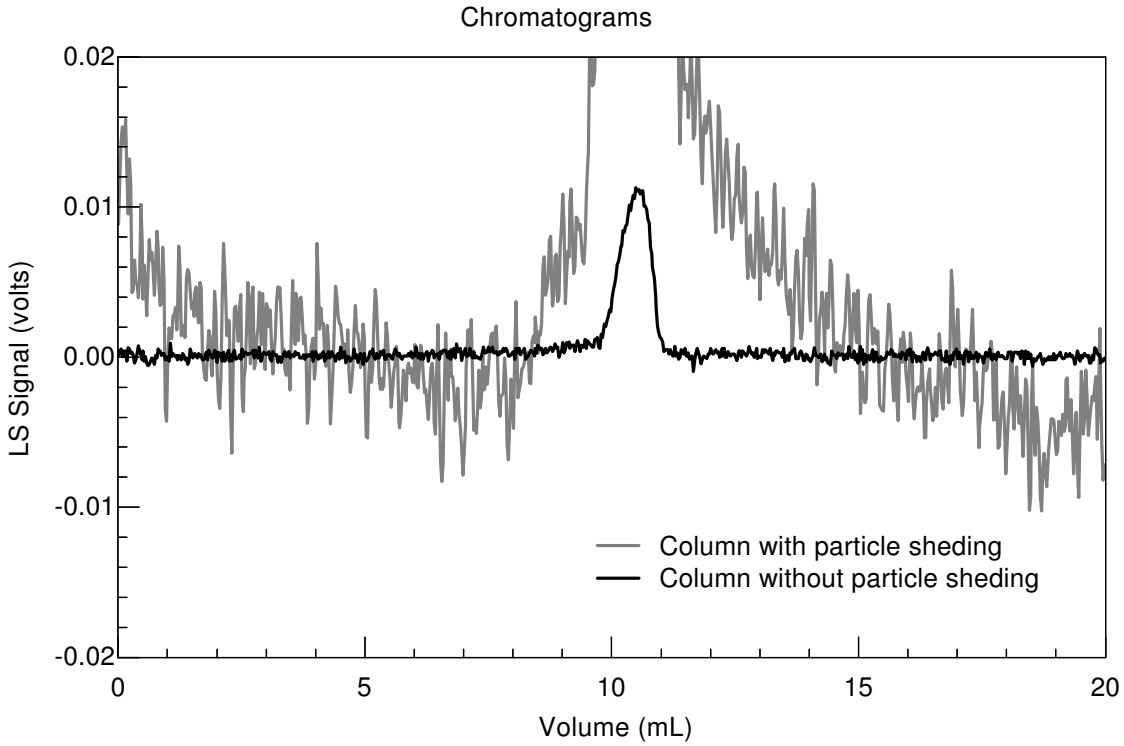


Figure 4: Particle shedding from a column. A low MW PEI (800, Aldrich) was used as analyte. The grey curve shows the turbulent baseline and the huge LS signal (maximum of peak at 1.4 volts !) induced by aggregates of the PEI with the shed particles (Column: PSS Suprema Max 100). The black curve demonstrate the very calm baseline and the small signal for the low MW polymer (Column: PSS Novema 30).

We have identified a column that perfectly meets all these requirements and that is suitable for the characterisation of polycations in aqueous solutions. This SEC column is based on a neutral hydroxyethylene methacrylate (HEMA) matrix that has been additionally hydophilisized by further hydroxy groups [19]. The column did not shed particles, thus the baseline of the scattered light was very smooth and allowed the evaluation even of the very smalls signals of the low MW PEIs. Further, the column did not show any absorptive effects with PEI samples. The baseline is reached again very quickly (no “tailing”) and the signal is symmetrical. In an experiment injecting a PEI with and without the column, the integration of

the curves with the column resulted in less than 10 % smaller values compared to the experiment without a column.

Eluents for SEC-MALLS. Another important feature using a LS detector in the SEC set-up is the quality of the eluent. The eluent has not only be free of any dust particles but also free of gas that might be dissolved in the eluent. Whereas for organic solvent a relative short treatment with ultrasound is sufficient to remove gas, aqueous solutions have to be degassed *in vacuo*. This degassing procedure is important to avoid "spikes" in the eluogram detected by the RI detector (Figure 2). Dust and impurities are easily taken up by aqueous solutions due to the high polarity of the water molecules. This makes LS experiments difficult to perform in water. However, this problem is solved by the combination of SEC and MALLS. The column ensures that the dust is retained by the column matrix releasing only the polymer.

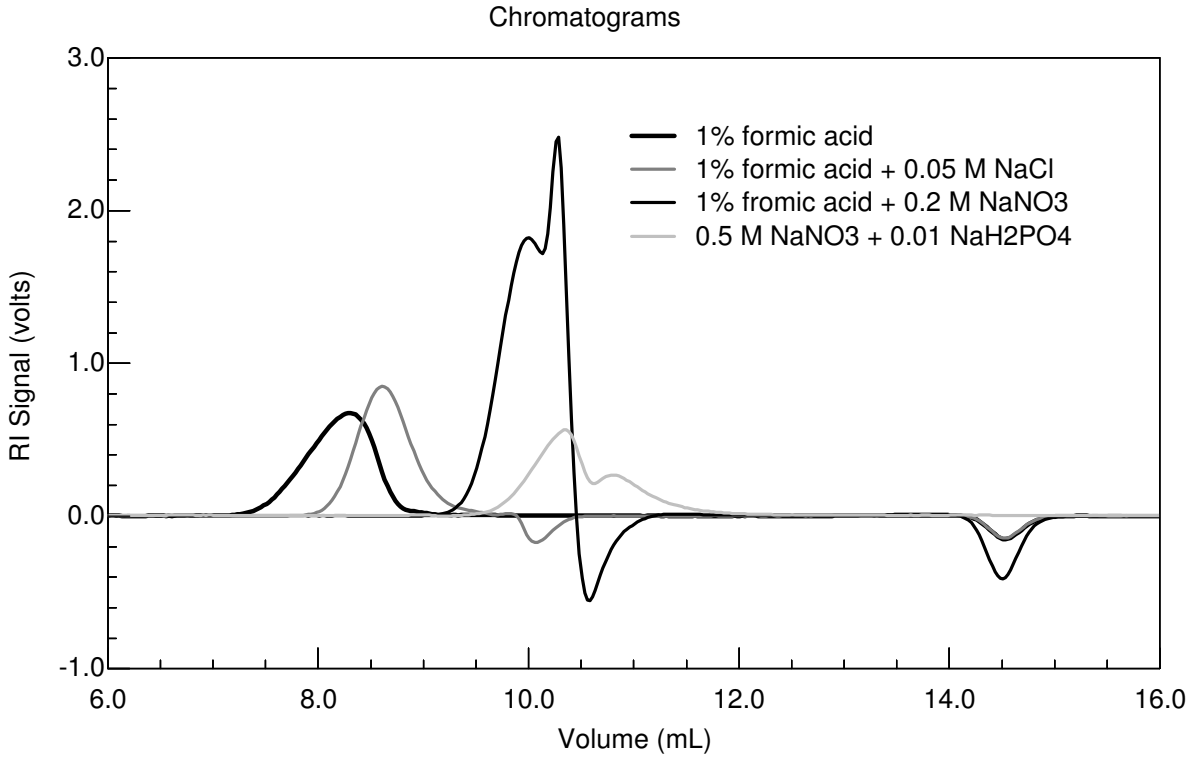


Figure 5: PEI 800 Da (Lupasol FG) on a PSS Novema 30 column in different eluents.

Different aqueous eluents were studied. The best results were obtained with 1 % formic acid. At that concentration the pH is about 1, but this low pH was tolerated by the columns used in this study. Addition of salts as it is necessary for relative methods to avoid the "polyelectrolyte effect" [8] was not necessary here since the LS detector is molar mass dependend. In this way interfering "salt exclusion peaks" could be avoided, which made it possible to determine even low MW polycations which are normally overlapped with the salt

peak. This is demonstrated in Figure 5. The overlapping of the "salt peaks" with the polymer peak made it impossible to determine the MW accurately without knowing the dn/dc. The polymer peak is increasingly shifted to larger elution volumes with increasing salt concentration.

Aggregates. Polycations, and especially the hyperbranched systems, tend to aggregate once they have been in the dried state [13]. Dissolution of the dried polycation in water revealed these aggregates. As an example, the eluograms of a PEI sample is shown in Figure 6. Next to the low MW polymer (2 kDa), aggregates could be detected, which were of about 100 kDa. It is a decisive advantage of the SEC-MALLS combination that aggregates can be easily recognized in the eluograms and the appropriate part of the LS signals can be excluded for the calculation of the MW by the LS data. A simple LS experiment would yield incorrect data due to the strong scattered light caused by the aggregates.

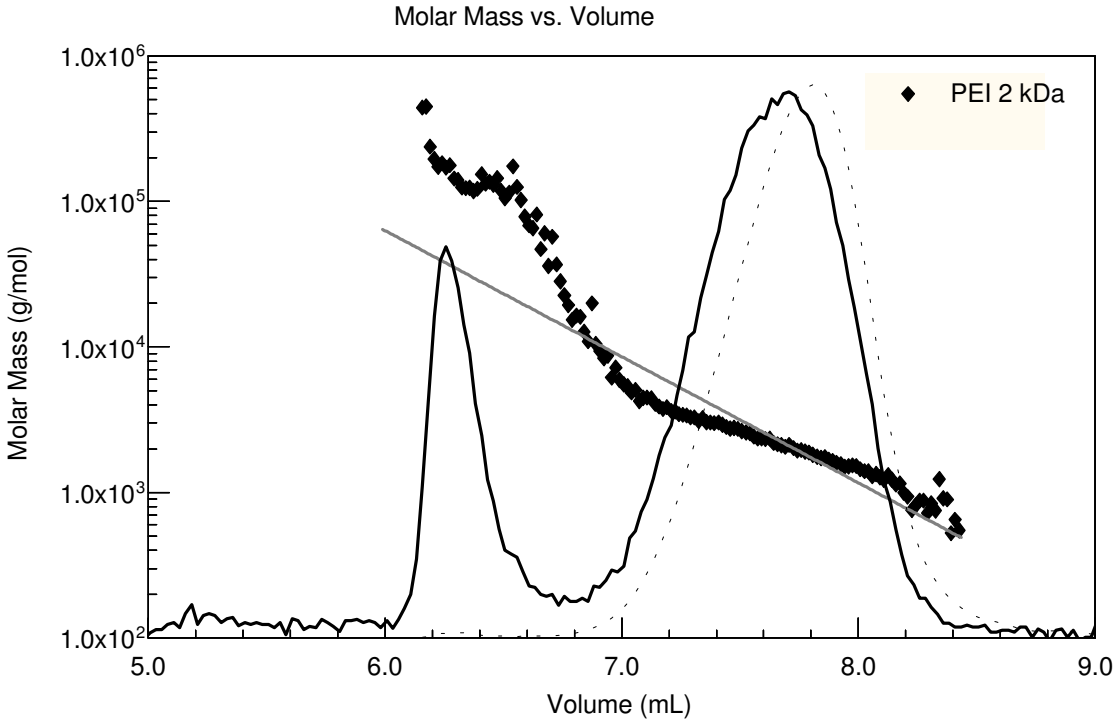


Figure 6: Aggregates PEI 2000 Da, Lupasol PR 8515 (Lot: 95-1079). LS Signal (bold line), RI signal (dashed line). Note that the concentration of the aggregates is very small (small RI signal), though a strong LS signal is obtained.

Table 1: MW data of the different polycations and non-charged hydrophilic polymers (standards) as determined by SEC-MALLS in 1 % formic acid. SEC columns: PSS Novema 3000.

Polymer samples	Supplier	Data of supplier MW M_n	M_w	Data of SEC-MALLS dn/dc [ml/g]	M_n	M_w	PD
PAMAM dendrimer, generation 3	Aldrich	6 909		0.263	6 803	10 780	1.58
PDADMAC	MPI	5 000		0.165	7 918	13 740	1.74
PDADMAC	MPI	30 000	45 000	0.187	29 200	41 580	1.42
Poly(L-lysine) HCl	Sigma	16 800	21 000	0.182	8 952	11 520	1.29
Poly(L-histidine) HCl	Sigma		14 000	0.183	9 635	11 140	1.16
Dextran	PSS	504		0.142	548	558	1.02
Pullulan	PSS	5 400	5 900	0.140	6 688	6 699	1.00
Pullulan	PSS	10 700	11 800	0.140	12 120	12 120	1.00
Pullulan	PSS	21 300	22 800	0.142	21 790	21 790	1.00
α-Cyclodextrin	Schwarz	973		0.133	1 076	1 087	1.01

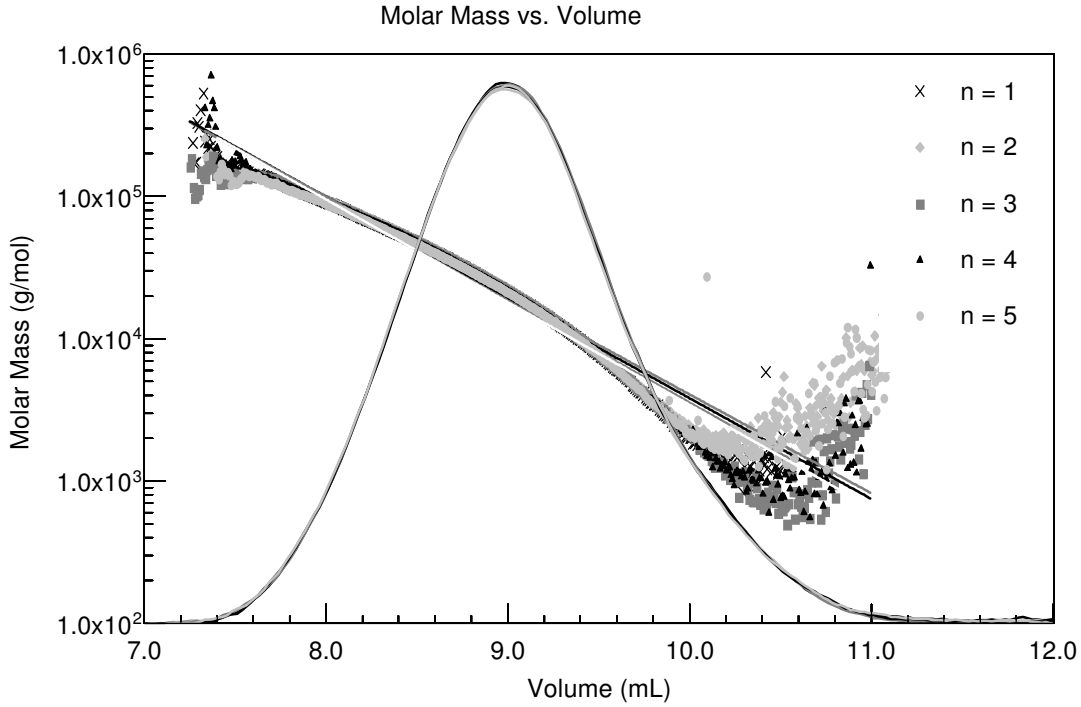


Figure 7: Reproduction of the eluograms and the MW as determined by SEC-MALLS. The PEI 25 kDa (Lupasol WF) was injected 5 times onto the column PSS Novema 3000.

Validation of the SEC-MALLS. The accuracy of our SEC-MALLS analysis was checked by a PEI sample that has been injected 5 times (Figure 7). The eluograms could be very well reproduced. The standard deviations (SD) of the MWs were found to be less than 5 %. Diverse non-charged hydrophilic polymer standards were measured. The results are listed in Table 1 demonstrating that our system works properly and yield reliable and accurate MW

data even for low MW samples below 1000 Da. Also, the MW of diverse polycations, such as hyperbranched polycations, such as PAMAM dendrimers or linear polycations, such as PDADMACs or poly(amino acids) could be accurately determined. Small errors might be attributed to the fact that the laser diode (690 nm) of the LS detector has a different wavelength than the RI detector (tungsten lamp). However, the error due to the different wavelength is expected to be less than 10 %. Further contributions to errors might be due to inaccurate weighing of the sample or due to a small absorption of the polymer on the column. It should be also mentioned that the data of the supplier can be incorrect. However, despite of all this critical points the MW determined by our SEC-MALLS are in good agreement with the data of the suppliers.

Table 2: MW data of the commercially available PEIs as determined by SEC-MALLS in 1 % formic acid. SEC columns: Novema 30 for MW ≤ 2000 and Novema 3000 for MW ≥ 5000. Inherent viscosity as determined by capillary viscosimetry in 0.5 M $NaNO_3$ at a polymer concentration of 0.5 g/dL and at a temperature of 25.0 °C.

Polymer sample	Supplier	Data of supplier MW		Data of SEC-MALLS				Viscosi-metry
		M_n	M_w	dn/dc [ml/g]	M_n	M_w	PD	η_{inh} [dL/g]
PEI linear	Aldrich	423	-	0.278	360	363	1.01	
Lupasol FG	BASF	600	800	0.256	679	780	1.15	
PEI	Aldrich	600	800	0.263	674	752	1.12	0.057
PEI	Polysciences	1 200		0.273	1 474	1 533	1.04	0.063
Lupasol G20	BASF	1 200	1 300	0.276	1 082	1 200	1.11	
Lupasol PR8515	BASF	1 800	2 000	0.260	1 965	2 184	1.11	
PEI	Aldrich	1 800	2 000	0.272	1 953	2 127	1.09	0.068
Lupasol G100	BASF		5 000	0.280	4 523	5 027	1.11	
PEI	Polysciences	10 000		0.260	17 690	23 070	1.30	0.092
Lupasol WF	BASF	10 000	25 000	0.272	16 810	44 670	2.66	
PEI	Aldrich	10 000	25 000	0.253	15 010	41 780	2.78	0.132
EI Polymer	Fluka	600 000 – 1 000 000		0.255	215 400	493 900	2.29	0.252

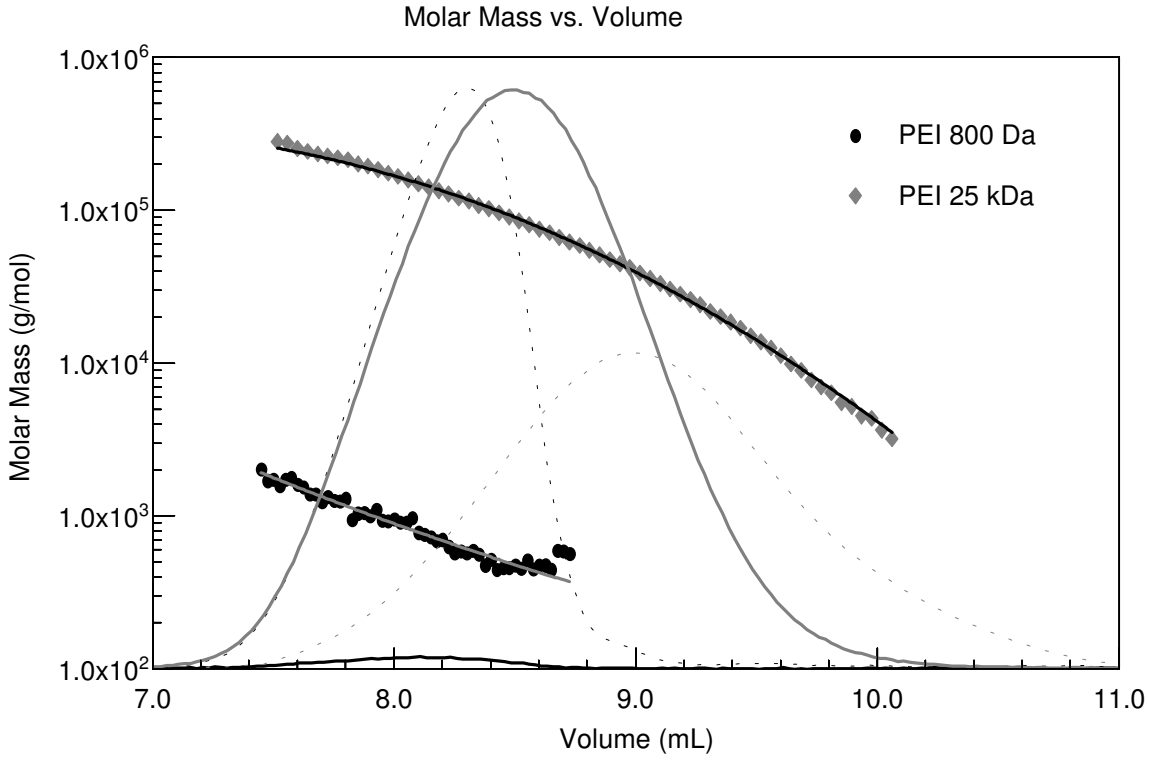
3.3 MW determination of commercially available PEIs

Various commercially available PEIs were characterized. The results are listed in Table 2. The MW are in reasonable agreement with the data of the supplier or producer, respectively. Only in three cases differences were found. The PEIs 10'000 and 25'000 were found to be of higher MW and the PEI 600'000-1'000'000 were found to be of lower MW. However, the results we found are still of the same order of magnitude and the data of the supplier are not necessarily based on absolute MW determinations. The dn/dc was found to be

relatively high with values of about 0.28 ml/g. These high dn/dc values are common for polycations and are quite advantageous since the LS signal is strong even at relatively low concentration and for low MW polymers.

The high dn/dc and the enhanced optical system (EOS®) of the LS detector allowed even the analysis of low MW PEI such as PEI 423, which is actually only an oligomer (degree of polymerization = 10). The LS signal of a low MW PEIs is shown in Figure 8 in comparison to the signal of a high MW PEI.

Figure 8: RI (dotted lines) and LS (bold lines) signals of a high MW PEI (25 kDa, Lupasol WF) and a low MW PEI (800 Da, Lupasol FG). Column PSS Novema 30 for PEI 800 Da, PSS Novema 3000 for 25 kDa. Both polymers were of the same concentration (5 mg/ml), thus the area under the curve is equal. However, the LS signal shows huge differences.



3.4 MW determination of the synthesized PEIs

The MW of the PEIs that were synthesized in our laboratories are listed in Table 3. The maximum MW which can be achieved by polymerization in solution is about 7000 Da. The dependency of the MW on the reaction conditions have been discussed in detail in [7]. Herein, we additionally characterized the PEI that was synthesized in bulk and for which we found a significantly higher MW (M_w 27 000 Da) compared to the PEI prepared by solution-polymerization. A similar result was found for the PHEEI homopolymers. The solution-

polymerization yielded a polymer with MW 4300 Da, whereas in bulk a MW of 6800 Da was achieved. The dependency of dn/dc on the composition of the polymer could be demonstrated by the series of P(EI-*co*-HEEI) copolymers. The dn/dc values correlated quite well with copolymer composition (Figure 9).

Table 3: MW data of the synthesized PEIs as determined by SEC-MALLS in 1 % formic acid. SEC columns: Novema 30 for MW ≤ 2000 and Novema 3000 for MW ≥ 5000. Inherent viscosity as determined by capillary viscosimetry in 0.5 M $NaNO_3$ at a polymer concentration of 0.5 g/dL and at a temperature of 25.0 °C.

Polymer sample	Synthesis	Data of SEC-MALLS				Viscosi-metry
		dn/dc [ml/g]	M_n	M_w	PD	η_{inh} [dL/g]
PEI	solution	0.309	6 345	7 251	1.14	0.130
PEI	solution	0.309	4 293	4 860	1.13	0.113
PEI	solution	0.294	2 922	3 594	1.23	0.104
PEI	solution	0.315	3 079	3 601	1.17	0.102
PEI	solution	0.306	3 001	3 479	1.16	0.100
PEI	solution	0.304	4 159	4 744	1.14	0.110
PEI	solution	0.307	3 404	3 979	1.17	0.100
PEI	bulk	0.283	16 420	27 280	1.66	0.183
PEI	solution (100 : 0)	0.249	3 648	5 041	1.38	
P(EI-*co*-HEEI)	solution (76 : 24)	0.221	2 648	4 082	1.54	
P(EI-*co*-HEEI)	solution (61 : 39)	0.202	2 857	4 227	1.48	
P(EI-*co*-HEEI)	solution (44 : 56)	0.185	2 698	3 918	1.45	
P(EI-*co*-HEEI)	solution (27 : 73)	0.180	2 456	3 746	1.53	
P(EI-*co*-HEEI)	solution (7 : 93)	0.172	2 126	3 417	1.61	
PHEEI	solution (0 : 100)	0.162	2 866	4 327	1.51	
PHEEI	bulk	0.165	3 940	6 838	1.74	

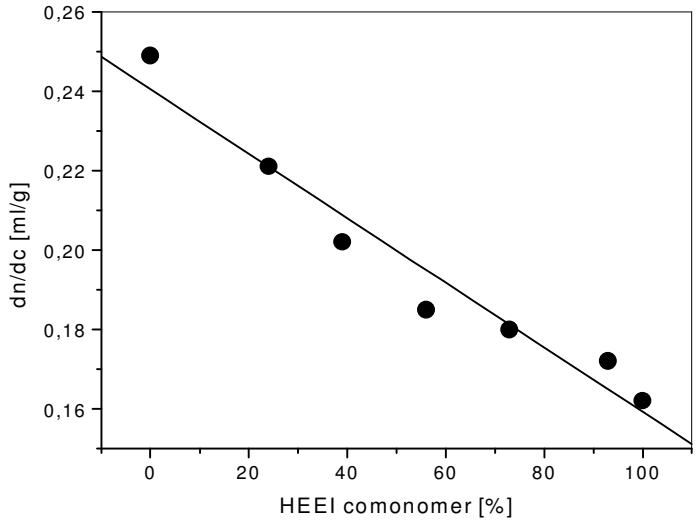


Figure 9: Refractive index increment (dn/dc) as a function of the comonomer composition. (Linear regression: R = -0.97, SD = 0.0075)

As reported earlier [7], a more linear structure was found for the synthesized PEI compared to the commercially available ones. As shown by the basic reseach of Zimm and Stockmayer a linear polymer is larger in size than a branched one [20]. As a consequence, the fractions of the synthesized PEI exhibited a higher hydrodynamic volume compared to a commercially available PEI with the same MW. Therefore, the fractions of the synthesized PEI were eluted earlier from the SEC column, compared to the commercially available PEIs of the same MW. This is demonstrated in Figure 10.

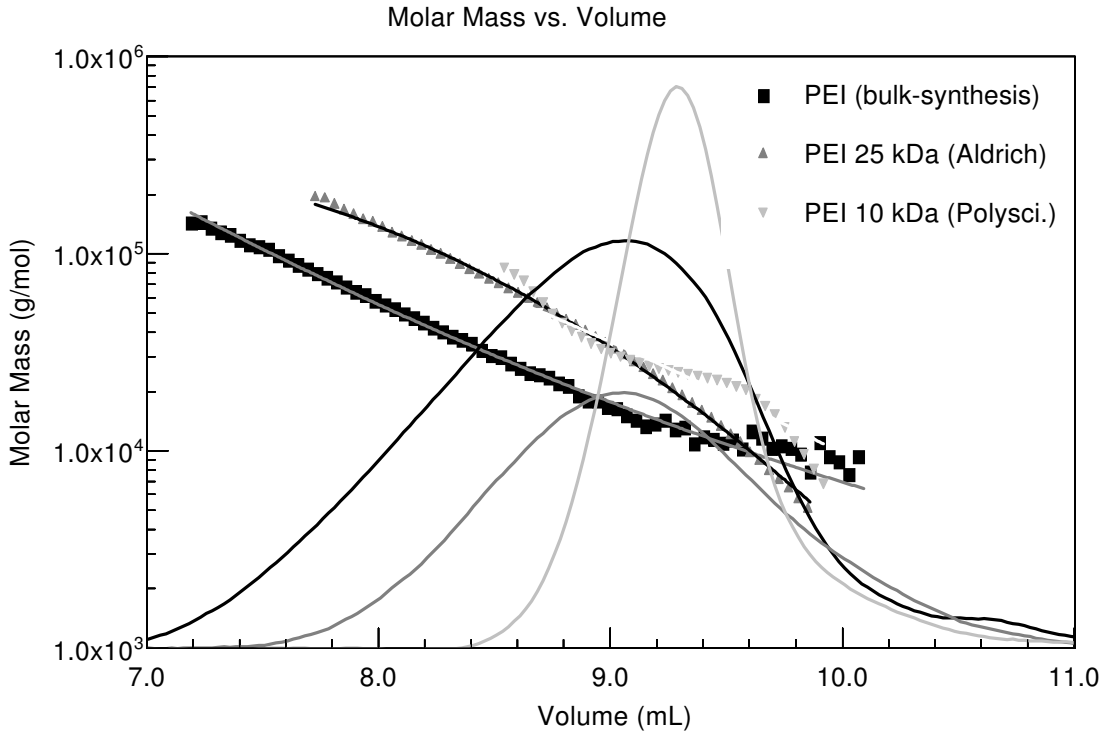


Figure 10: PEIs of different degree of branching. The branched PEI synthesized by bulk-polymerization exhibited a more linear structure compared to the commercially available PEIs. Therefore, fractions of the same absolute MW were of different R_h, and were eluted after different times. Column: PSS Novema 3000.

3.5 Viscosimetry

The polycations were also characterized by capillary viscosimetry. The results have been described in [7]. Since the absolute MWs of both the commercially and our own PEIs have been determined, these data can be set in relation to the viscosity data. In Figure 11 the inherent viscosity is plotted against the Mw. A linear regression of the data yielded the slope of the line which is equivalent to the Mark-Houwink parameter a. In the case of the commercially available PEIs we found the Mark-Houwink parameter a = 0.186 ± 0.028 (K = 1.619 ml/g). Hosteler et al. reported for commercially PEIs in 0.1 M NaCl an even smaller

parameter for a = 0.14 (K = 2.32 ml/g) [21]. In contrast, our own PEIs exhibited a relative high parameter a = 0.295 ± 0.015 (K = 0.909 ml/g). This suggests that our PEIs are more linear than the commercial PEIs.

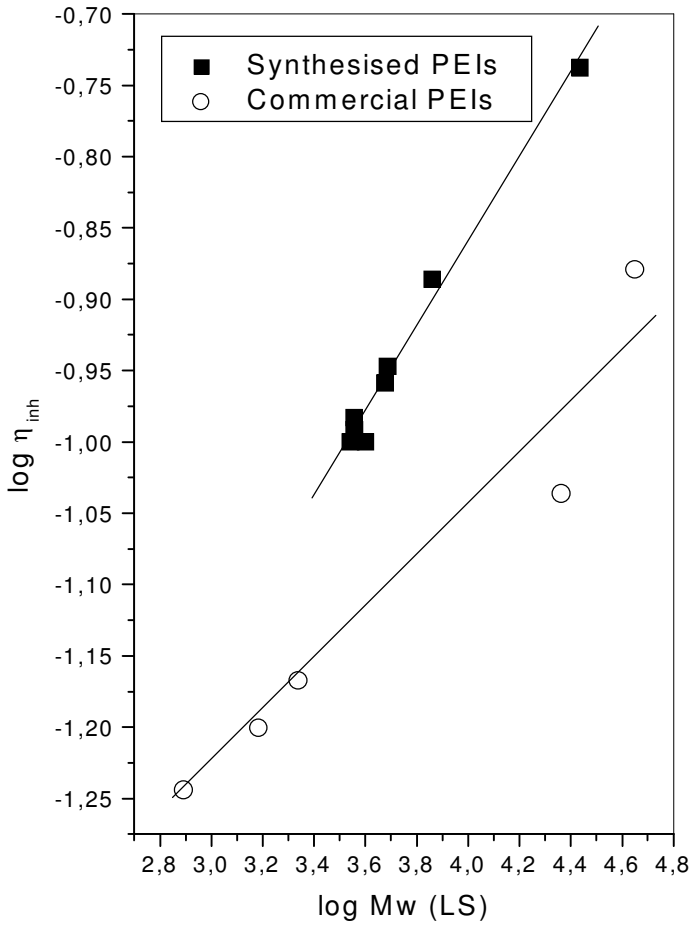


Figure 11: Mark-Houwink Plot of the synthesized and commercially available PEIs: The inherent viscosity (dL/g) is doubled logarithmically plotted against the absolute MW (g/mol) as determined by MALLS.

3.6 SEC-MALLS-QELS

A further support for the finding that our PEIs are of a different degree of branching was given by the DLS measurements which allowed the determination of the hydrodynamic radii (R_h) of each fraction of the polymer sample. The PEI 10 kDa (Polysciences) and the PEI synthesized by bulk polymerization were of similar MW and were, therefore, applied in this comparative study. The results are shown in Figure 12, in which the R_h are plotted against log M_w. Whereas the commercial available PEI exhibited a R_h of about 3 nm for the fraction of M_w = 30 kDa, a R_h of about 5 nm were found for the 30 kDa-fraction of the more linear synthesized PEI. Further information about the degree of branching was achieved by the slope of the curve indicating again the higher degree of branching of the commercial PEIs (3.84 ± 0.70) over the synthesized polycations (7.57 ± 0.76). In a doubled-logarithmic R_h-M_w-plot,

the slope of the synthesized PEI was found to be 0.52 ± 0.03, whereas the slope of the commercially available PEI was 0.38 ± 0.04. The slope in this doubled logarithmic plot represents the scaling exponent ν that was found to be about 0.6 for fully-flexible linear polymers [22]. The lower value of ν found for PEI is due to the branched structure.

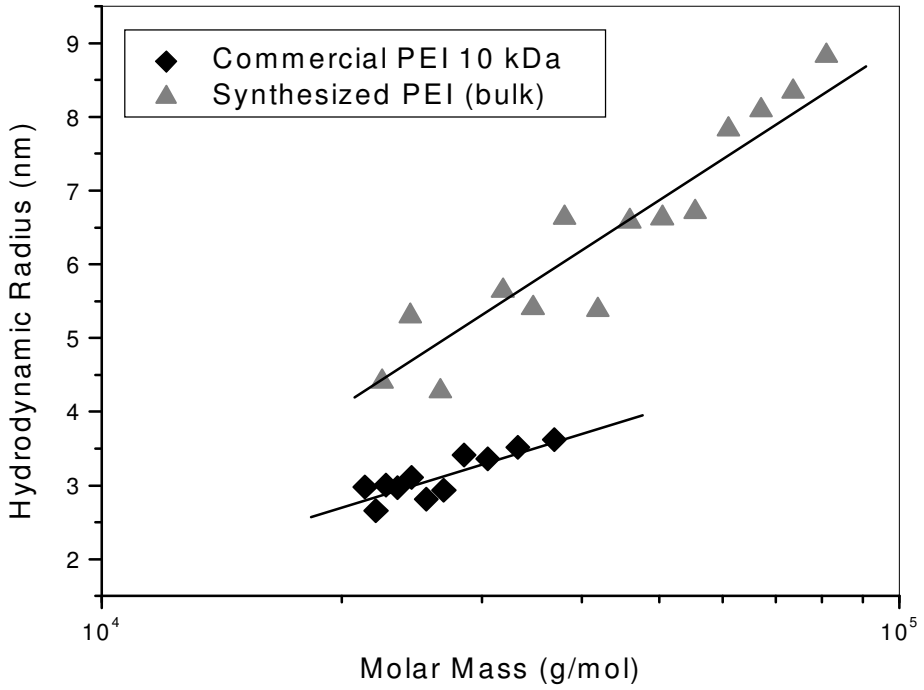


Figure 12: Dynamic LS (= Quasi-elastic LS) in combination with SEC-MALLS. Synthesized PEI via bulk-polymerization. Commercially available PEI 10 kDa (Polysciences). Column: PSS Novema 3000.

3.7 Degree of branching as determined by ^{13}C-NNE-NMR

The structural difference between the commercial and our own PEIs has also been supported by the ^{13}C-NNE-NMR experiment. As described in [7], the ratio between the primary, secondary and tertiary amino groups was found to be 1 : 1 : 1 in the case of the commercial products. In contrast, our PEIs exhibited a more linear structure which was concluded from the found ratio 1:2:1. This ratio of the PEI prepared by solution polymerization was also found in the case of the bulk polymerisation of EI (27.2 % : 51.6 % : 21.2 %).

Our results of the degree of branching point to the more qualitative nature of the data in the literature, where the ratio of the amines of the commercially available PEIs was reported to be roughly 30 % : 40 % : 30 % [23] or 1 : 2 : 1 [24]. Simple ^{13}C-NMR and ^{15}N-NMR techniques were used to determine the degree of branching [25]. Quantitative evaluation of these NMR data is problematic because the signal intensities are influenced by NOEs which are certainly different for the C and N atoms of primary, secondary and tertiary amines.

Differences in polymer synthesis were the reason for the different degree of branching we observed for our synthesized PEIs in comparison with the commercially available PEIs. It has been reported that the reaction conditions of the polymerization of EI have a strong influence on the degree of branching of the resulting PEI [26]. Frey et al. emphasized in a article about hyperbranched polymers [27] that in an ideal random polymerisation of an AB_2 monomer (ethylenimine can be understand as such a monomer), the ratio of dendritic (in the case of PEI: primary amines), linear (secondary amines) and terminal (tertiary amines) is expected to be 1 : 2 : 1. This is the ratio we found for our PEIs made in the laboratory in a batch-synthesis. However, under condition of slow-monomer-addition it has been theoretically predicted that the probability of the formation of linear units is only half of that in a common bulk polymerization, leading to an abundance of dendritic, linear and terminal units of 1 : 1 : 1 [28,29]. Experimental results confirmed this prediction [30]. This is also the ratio we found for the commercially available PEIs produced in an industrial scale.

4. Conclusion

The combination of SEC with static and dynamic LS did not only allow to determine accurately and absolutely the MW and the MW distribution of the polymers but also to the determine the R_h of the analyte, as well as to estimate the degree of branching of the polymer. To be able to perform LS experiments in combination with SEC the column has to be of high quality. First, it must neither absorb any amount of the polymer nor shed any particles from the gel matrix. Second, it must efficiently fractionate the polymer sample by allowing the diffusion of the analyte into the cavities of the column. In the case of the highly charged, hyperbranched polycation, PEI, with its very high absorption tendency towards many surfaces a non-charged, additionally hydrophilisized HEMA column were found to met all these criteria. Furthermore, the enhanced optical system of modern LS detectors allowed also the accurate MW determination of oligomers.

Acknowledgements

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Conclusion

SUMMARY

A variety of different block copolymers containing polyethylenimine (PEI) and poly(ethylene glycol) (PEG) blocks, as well as biodegradable PEI derivatives were synthesized, and their potential as gene delivery vehicles were investigated with regard to DNA condensation, transfection efficiency and biocompatibility.

In a first study, two series of PEI-*graft*-PEG copolymers were synthesized. PEI 25 kDa was grafted with different numbers of PEG 5 kDa blocks and PEI 25 kDa was grafted with PEGs of different molecular weight (MW, 550 Da – 20 kDa). The homopolymer blocks were coupled using hexamethylene diisocyanate (HMDI) as linker which led to hydrolytically stable urethane and urea bonds. The copolymer block structure was found to have a strong impact on the DNA condensation. With an increasing degree of PEGylation PEI-induced DNA condensation was increasingly hindered and the complexes lost their spherical shape as demonstrated by atomic force microscopy. When PEI was grafted with only one large PEG 20 kDa block small and compact polycation/DNA complexes were obtained with a size of about 50 nm. Grafting PEI with many short PEG 550 Da blocks yielded complexes of large and diffuse shape (130 nm). The ζ-potential of the complexes was reduced by PEGylation only when PEG with a MW $\geq$ 5 kDa was used. With increasing degree of PEGylation the ζ-potential was reduced to charge neutrality. The cytotoxicity of the copolymers and their DNA complexes was found to be dependent only on the degree of PEGylation and was independent of the MW of the PEG. The complexes could be considered as non-toxic when the degree of PEGylation of the copolymers was $\geq$ 15. The copolymer/DNA complexes did not show significant hemolytic activity. However, erythrocyte aggregation was observed for the complexes with a ζ-potential > 7 mV. The transfection efficiency of the copolymers were found to be dependend on the combination of the cytotoxicity and the ζ-potential of the complexes. The combination of high ζ-potential and low cytotoxicity was found to be most effective for *in vitro* gene delivery.

Since this first study revealed that with increasing degree of PEGylation DNA condensation is impaired, a synthesis route was developed that exclusively led to PEG-block-PEI diblock copolymers where the degree of PEGylation did not exceed one. The new synthesis concept involved the ring-opening-polymerization of ethylenimine in the presence of a so called “macrostopper”, a PEG with a primary amine as end group allowing to

terminate the polymerization reaction. Compact spherical and stable polymer/DNA complexes of about 100 nm size were obtained with these diblock copolymers.

With low MW (≤ 2 kDa) PEI DNA complex formation was achieved but these complexes aggregated to larger particles (> 500 nm). Since the ζ-potential of this complexes remained negative even at high polycation excess and ethidium bromide (EtBr) showed intercalation with DNA, we concluded that in these complexes DNA were poorly condensed. These findings explain the poor transfection efficiency which was found for low MW polycations.

The DNA condensation potential of low MW PEIs was significantly enhanced when they were coupled to a larger unit by multi-star-PEGs using HMDI as linker. The resulting 4- and 8-*star*-(PEG-*block*-PEI) copolymers yielded small (100 nm) and stable polymer/DNA complexes. EtBr could no longer intercalate into the DNA within these complexes, thus, the DNA was properly condensed by these star-block copolymers.

Biodegradable PEIs was synthesized by connecting several low MW PEIs to larger units using oligo(L-lactic acid-*co*-succinic acid)s. As a result, the PEI macromolecules were conjugated by succinamide units to the copolymer poly(ethylenimine-*co*-L-lactamide-*co*-succinamid) [P(EI-*co*-LSA)]. The hydrolytic degradation of these amide bonds was found to be pH dependent. At pH 5 degradation proceeded very slowly, whereas at pH 7 degradation was accelerated. An even stronger degradation was observed at pH 9 where the half life of MW decay was ca. one month. This pH-denpendency is advantageous for gene delivery systems, since in the lysosome the DNases are highly active under the acidic conditions of pH 5. Here, the protection of the DNA should be guaranteed by the polycation, whereas at physiological pH (7.4) the rapid degradation is desirable. This biodegradable PEI derivative was found to be of low cytotoxicity and will degrade into the non-toxic low MW cleavage products. In comparison with the low MW PEI 1200 Da the P(EI-*co*-LSA) exhibited a tenfold stronger transfection efficiency.

The characterization of the synthetic polycations, mainly PEI and its derivatives, was successfully performed by size exclusion chromatography in combination with static and dynamic laser light scattering. Using these methods the MW, the polydispersity and the hydrodynamic radii of the polycations was determined. The degree of branching of the hyperbranched PEIs was measured using ^{13}C-NNE-NMR spectroscopy. Further, estimations of the degree of branching were also achieved by the evaluation of viscosity data in combination with the absolute data of the static light scattering experiments (Mark-Houwink parameters). As a result, the structure of synthesized PEIs were found to be of a more linear

structure compared to the commercially available PEIs. Consequently, higher hydrodynamic radii were measured for the more linear PEIs (5 nm) in comparison to the more branched PEI of the same MW (3 nm).

PERSPECTIVES

So far the PEI/DNA complexes described in this thesis have been characterized physicochemically and have been biologically studied in *in vitro* cell culture assays only. Consequently, as a next step the potential of these complexes for gene delivery need to be evaluated in animal models. However, for an *in vivo* application further modifications of the PEGylated PEIs need to be performed. First, a target molecule has to be fitted at the end of the PEG blocks to be able to address a specific tissue or organ. Second, the results of the *in vitro* experiments suggest that grafting a combination of different PEG blocks onto a single PEI macromolecules might be recommendable for *in vivo*. In order to reduce the toxicity the PEI should be grafted with many short PEG blocks. On the other hand, this PEI should additionally be grafted with one or two longer PEG block to stabilize the complex against aggregation and to yield a sufficient shielding of the complex. The target molecule should be connected to the ends of these longer PEG blocks to allow a targeting gene delivery. Further, we recommend for *in vivo* applications not to use toxic high MW PEIs for the PEGylation but rather to build up polycations with several non-toxic low MW PEIs to a larger unit with a biodegradable linker as described in this thesis. In conclusion, a more rational design of new *in vivo* gene delivery systems became feasible using the different approaches of physicochemical and *in vitro* cell culture experiments described in this PhD thesis.

Appendices

Zusammenfassung

Eine Vielzahl verschiedener Blockcopolymere bestehend aus Polyethylenimin (PEI) und Polyethylenglykol (PEG), sowie bioabbaubare PEI-Derivate wurden synthetisiert. Ihr Potential als Gentransport-Vehikel in Bezug auf DNA-Kondensation, Transfektionseffizienz und Biokompatibilität wurde untersucht.

In einer ersten Studie wurden zwei Serien von PEI-*graft*-PEG-Copolymeren synthetisiert. Zum einen wurde PEI 25 kDa mit unterschiedlicher Anzahl von PEG 5 kDa-Blöcken gepfropft. Zum anderen wurde PEI 25 kDa mit PEG-Blöcken mit unterschiedlichen Molekulargewichten (MG, 550 Da – 20 kDa) gepfropft. Die Homopolymer-Blöcke wurden mittels Hexamethylendiisocyanat (HMDI) als Brückenglied aneinander gekoppelt, was zu hydrolysestabilen Urethan- und Harnstoffbindungen führte. Die Blockcopolymer-Struktur hatte hohen Einfluß auf die DNA-Kondensation. Mit zunehmendem Grad der PEGylierung wurde die PEI-vermittelte DNA-Kondensation zunehmend gestört und die Komplexe verloren ihre sphärische Form wie mittels Rasterkraftmikroskopie gezeigt werden konnte. Wenn das PEI mit nur einem PEG 20 kDa-Block gepfropft wurde, wurden kleine und kompakte Komplexe erhalten (ca. 50 nm). In dem Fall, daß PEI mit vielen kurzen PEG 550 Da-Blöcken gepfropft wurde, wurden Komplexe von großer und diffuser Gestalt erhalten (ca. 130 nm). Das ζ-Potential der Komplexe wurde durch die PEGylierung nur reduziert, wenn das PEG ein MG von $\geq$ 5 kDa aufweist. Mit zunehmendem Grad der PEGylierung wurde das ζ-Potential bis zur Ladungsneutralität reduziert. Die Zytotoxizität der Copolymere und ihrer DNA-Komplexe war abhängig vom Grad der PEGylierung aber unabhängig vom MG der PEG-Blöcke. Die Komplexe konnten als nicht-toxisch betrachtet werden, wenn der PEGylierungsgrad der Copolymere $\geq$ 15 war. Die Copolymer/DNA-Komplexe zeigten keine nennenswerte hämolytische Aktivität. Dagegen wurde Erythrozyten-Aggregation für Komplexe mit einem ζ-Potential > 7 mV festgestellt. Die Transfektionseffizienz war von der Kombination aus Zytotoxizität und ζ-Potential der Komplexe abhängig. Die Kombination von hohem ζ-Potential mit niedriger Zytotoxizität erwies sich als am effektivsten für den *in vitro*-Gentransfer.

Da die erste Studie zeigte, daß mit zunehmenden PEGylierungsgrad die DNA-Kondensation gestört wurde, wurde eine Syntheseroute entwickelt, die ausschließlich zu PEG-*block*-PEI Diblockcopolymeren führte und die eine Mehrfach-PEGylierung ausschloß. Das neue Synthesekonzept beinhaltete die ringöffnende Polymerisation von Ethylenimin in Gegenwart von einem sogenannten Makroregler, ein PEG mit einer endständigen Aminogruppe, die die Polymerisationsreaktion stoppen konnte. Kompakte, sphärische und stabile

Polymer/DNA-Komplexe von etwa 100 nm Größe wurden mit diesen Diblockcopolymeren erhalten.

Niedermolekulares PEI (MG $\leq$ 2 kDa) war zwar in der Lage DNA zu komplexieren, aber die Komplexe aggregierten zu großen Partikeln (> 500 nm). Da das ζ-Potential dieser Komplexe selbst bei hohem Polykation-Überschuß negativ blieb und Ethidiumbromid (EtBr) immer noch in der Lage war, mit der DNA zu interkalieren, konnte daraus geschlossen werden, daß in diesen Komplexen die DNA nur schlecht kondensiert wurde. Diese Befunde konnten eine Erklärung für die schlechte Transfektionseffizienz liefern, die für niedermolekulare Polykationen gefunden wurde.

Das DNA-Kondensationspotential von niedermolekularem PEI konnte entscheidend verbessert werden, wenn letztere zu größeren Einheiten durch sternförmige PEGs mit HMDI als Brückenglieder zusammengesetzt wurden. Die resultierenden 4- und 8-*star*-(PEG-*block*-PEI) Copolymere ergaben kleine (100 nm) und stabile Polymer/DNA-Komplexe. EtBr konnte nicht mehr mit der DNA interkalieren, folglich wurde die DNA durch die sternförmigen Copolymere effektiv kondensiert.

Ein bioabbaubares PEI wurde durch das Verknüpfen mehrerer niedermolekularen PEIs mittels eines Oligo(L-Milchsäure-*co*-Bernsteinsäure) zu größeren Einheiten synthetisiert. Die PEI-Makromoleküle wurden dabei durch Bernsteinsäureamid-Einheiten zu dem Copolymer Poly(ethylenimin-*co*-L-Milchsäureamid-*co*-Bernsteinsäureamid) [P(EI-*co*-MBA)] verknüpft. Der hydrolytische Abbau dieser Amidbindungen war pH-abhängig. Bei einem pH-Wert von 5 vollzog sich der Abbau nur sehr langsam, während bei pH 7 der Abbau beschleunigt wurde. Ein noch schnellerer Abbau wurde bei pH 9 beobachtet, bei dem sich nach einem Monat das MG des Polymers um die Hälfte reduzierte. Die pH-Abhängigkeit ist für einen Gentransfer-Vektor vorteilhaft, da im Lysosom unter den relativ stark sauren Bedingungen die DNasen hoch aktiv sind. Unter solchen Bedingungen sollte der Schutz der DNA durch das Polykation garantiert sein, während unter physiologischen Bedingungen (pH 7,4) der schnelle Abbau erwünscht ist. Das bioabbaubare PEI war von niedriger Zytotoxizität und wird zu nichttoxischen Spaltprodukten degradieren. Im Vergleich zu dem niedermolekularen PEI (1200 Da) zeigte das P(EI-*co*-MBA) eine 10fach höhere Transfektionseffizienz.

Die Charakterisierung der synthetisierten Polykationen, hauptsächlich PEI und seine Derivate, konnte erfolgreich über Größenausschlußchromatographie in Verbindung mit statischer und dynamischer Lichtstreuung durchgeführt werden. Auf diese Weise konnte das MG, die Polydispersität und der hydrodynamische Radius der Polykationen bestimmt werden. Der Verzweigungsgrad der hochverzweigten PEIs wurde über ^{13}C-NNE-NMR-Spektroskopie be-

stimmt. Ferner erlaubte die Auswertung der Viskositätsdaten in Zusammenhang mit den absolut bestimmten MG-Daten aus der statischen Lichtstreuung eine Abschätzung des Verzweigungsgrades (Mark-Houwink-Parameter). Dabei wurde eine stärker lineare Struktur bei den selbst dargestellten PEIs im Vergleich zu den kommerziell erhältlichen PEIs gefunden. Folglich wurde mit der dynamischen Lichtstreuung größere hydrodynamische Radien (5 nm) für das stärker lineare PEI gemessen als für das stärker verzweigte PEI von gleichem MG (3 nm).

AUSBLICK

Die in dieser Arbeit beschriebenen PEI/DNA-Komplexe wurden bisher physiko-chemisch charakterisiert und biologisch nur *in vitro*-Zellkultur-Versuchen untersucht. Konsequenterweise müßte nun das Potential der Komplexe für den Gentransfer *in vivo* studiert werden. Für eine *in vivo*-Applikation müßten aber noch weitere Modifikationen an den PEGylierten PEIs vorgenommen werden. Erstens müßte ein Zielmolekül an das freie Ende der PEG-Blöcke gekoppelt werden, damit spezifisch ein gewünschtes Gewebe oder Organ als Adressat bestimmt werden kann. Zweitens, die Ergebnisse der *in vitro*-Versuche legen eine Kombination aus verschiedenen PEG-Blöcken an ein und demselben PEI nahe. So sollte das PEI mit vielen kurzen PEG-Blöcken gepfropft werden, um die Toxizität herabzusetzen. Desweiteren sollte dieses PEI mit einem längeren PEG-Block gepfropft werden, um den Komplex zu stabilisieren, vor Aggregation zu schützen und ausreichend abzuschirmen. Das Zielmolekül sollte an das Ende dieser längeren PEG-Blöcke gekoppelt werden, um einen gezielten Gentransfer zu erlauben. Außerdem wäre es für eine *in vivo*-Applikation empfehlenswert, nicht auf toxische hochmolekulare PEIs zurückzugreifen, sondern die PEGylierung an mehreren nicht-toxischen niedermolekularen PEIs vorzunehmen, die - wie in dieser Arbeit beschrieben - zu einer größeren polykationischen Einheit über bioabbaubare Brückenglieder zusammengesetzt werden könnten. Zusammenfassend läßt sich festhalten: Ein rationales Design eines vielversprechenden *in vivo*-Gentransfer-Vehikels sollte durch die Anwendung der verschiedenen Resultate der hier beschriebenen physiko-chemischen und in vitro-Zellkultur-Versuche möglich geworden sein.

Curriculum Vitae

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