Stabilized Nanocarriers for Plasmids Based Upon Cross-linked Poly(ethylene imine)

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Received August 11, 2006; Revised Manuscript Received September 18, 2006

Stabilized PEI/DNA polyplexes were generated by cross-linking PEI with biodegradable disulfide bonds. The reaction conversion of different PEIs with the amine reactive cross-linker dithiobis(succinimidyl propionate) (DSP) was investigated, and the molecular weight of the reaction products was identified. Light scattering and microelectrophoresis were employed to assess size and zeta potential of the resulting polyplexes. Polyplex morphology and mechanic stability were investigated using atomic force microscopy. Finally, albumin and erythrocyte interactions and stability against polyanions and high ionic strength were checked. Polyplexes of PEI and DNA were prepared by two different formulation methods, either using pre-cross-linked polymers or by cross-linking polyplexes after complexation. Only the latter method yielded small (100−300 nm) polyplexes with a positive zeta potential when HMW PEI was used, whereas cross-linked LMW PEI resulted in polyplexes with increased size (>1000 nm) and zeta potentials down to −20 mV. In addition, only cross-linking after polyplex formation was able to enhance resistance against polyanion exchange and high ionic strength. AFM images revealed no changes in the morphology of cross-linked HWM PEI polyplexes, and indentation force measurements using AFM significantly increased mechanical stability of cross-linked HMW PEI polyplexes. These polyplexes also displayed significantly reduced interactions with major blood components like albumin and erythrocytes. The resulting biocompatible particles offer a means of combining enhanced polyplex stability with redox-triggered activation for in vivo application.

Introduction

Systemic delivery of therapeutic genes remains a major objective for human gene therapy. While locoregional administration of nucleic acids has shown some promise,[1] intravenous gene delivery is required to reach more disseminated targets, such as metastatic cancer cells. Systemic applications of nanoscale delivery systems, such as positively charged polymeric gene transfer vectors (polyplexes), liposomes, or micelles, are currently limited by their rapid clearance from the bloodstream following intravenous injection, with only 10−20% of the injected dose remaining in circulation after 10 min, and only 2% after 30 min.[2−6] Usually, these nanocomplexes are quickly cleared by first-pass organs of the reticuloendothelial system, such as the liver or spleen,[6−8] or in the lung capillaries.[4,6] The positive charge of polyplexes may lead to interactions with cellular blood components[9] and plasma proteins,[4] causing aggregate formation. In addition, polyplexes of cationic polymers and DNA may undergo exchange reactions with endogenous polyanions, such as albumin, thereby releasing DNA.[10,11] Thus, for efficient systemic application, polyplexes should bear only a low positive surface charge.[12,13]

Hydrophilic polymers, such as poly(ethylene glycol) (PEG) or poly[*N*-(2-hydroxypropyl) methacrylamide] (PHPMA), have been used to shield surface charges and reduce interactions of the polyplexes with blood components or endothelia, thus resulting in prolonged circulation times.[5,7,14−16] Increased blood concentrations of about 30% versus 6% after 30 min for PEGylated PEI/transferrin polyplexes and a 7-fold higher area under the curve for PEGylated polyplexes have been reported.[9,14] However, even for PEGylated polyplexes, the disruption of the polyplexes remains a challenge, as this leads to DNA degradation after systemic application. Double labeling of the DNA and the polymers showed differences in blood level profiles and organ distribution profiles after 2 and 12 h, likely due to separation of the polyplexes and degradation of the DNA.[5,17] Therefore, in addition to steric shielding, it is necessary to further stabilize the polyplexes by enhancing their resistance against dissociation.[18]

Surface cross-linking of polyplexes has emerged as a tool to address these critical stability issues and has been investigated for different polymers in vitro and in vivo (e.g., PLL and PEI).[19,20] Surface coating of polyplexes with PHPMA resulted in enhanced resistance against disruption by polyanions,[20] indicating minimal extracellular release of the DNA by polyelectrolyte exchange reactions, which play an essential role in the process of DNA delivery.[21] However, these constructs suffered from low surface charges of about zero or even negative values due to the shielding of the copolymers, which impaired efficient cell interaction[22] and likely caused them to be subject to phagocytosis via the polyanion receptors of macrophages.[23] Thus, a balance between charge shielding and positive surface charge seems to be a key factor for the stabilization of polyplexes for systemic application. Such a balance may improve target cell interactions while reducing unwanted side effects.

Our aim was to design a vector system for intravenous administration, which is stabilized by the introduction of bioreversible disulfide cross-links. Disulfide cross-linking is thought to facilitate intracellular release of the DNA, because glutathione-mediated reduction will occur predominantly in the intracellular milieu.[22,24−26] Poly(ethylene imine) (PEI) was used because it is one of the most successful polycationic carrier systems.[27−29] Cross-links were introduced to the primary amine

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10.1021/bm060788z CCC: $33.50 © 2006 American Chemical Society
Published on Web 11/14/2006

functions of PEI using a low molecular weight reagent, dithiobis(succinimidyl propionate) (DSP). This cross-linking method yields neutral amide bonds, which reduces the cationic charge of the PEI and shows favorable transfection efficiencies as compared to charge preserving reagents.[24] Besides the use of cross-linked PEI as absorbent in wastewater treatment or chromatography,[30,31] cross-linking has been employed to improve DNA complexation with low molecular weight PEI.[32,33] In this work, we evaluate the potential of two PEIs with different molecular weights in forming polyplexes with plasmid DNA prepared with two different formation strategies. Polyplexes formed with cross-linked PEI as well as surface cross-linked PEI/DNA polyplexes are compared in terms of their biophysical properties, stability, and cytocompatibility.

Experimental Section

Materials. *Polymers.* Poly(ethylene imine) (25 kDa, HMW (high molecular weight) PEI and 5 kDa, LMW (low molecular weight) PEI, as specified by the manufacturer) were gifts from BASF, Ludwigshafen, Germany. *DNA.* The plasmid pGL3-CMV encoding the firefly luciferase gene was amplified in JM-109 competent cells and purified using a commercial kit (Qiagen, Hilden, Germany). Salmon testes DNA (Sigma, Taufkirchen, Germany) was used for light scattering experiments. Dithiobis(succinimidyl propionate) (DSP, Lomant's reagent), dry dimethylsulfoxide (DMSO, HPLC grade), dithiobis(2-nitrobenzoic acid) (Ellman's reagent), and fluorescamine were purchased from Sigma, Taufkirchen, Germany. All other reagents used were of analytical grade. Pure water (0.22 μm filtered, 0.055 S/cm, USF Seral, Seradest BETA 25 and Serapur DELTA UV/UF) was used to prepare analytical solutions and buffers.

Polymer Cross-linking. HMW PEI and LMW PEI stock solutions (1 g/L in pure water, pH 7.5) were diluted in either low ionic strength buffer (5% glucose/25 mM Hepes, pH 7.5) or high ionic strength buffer (150 mM sodium chloride, pH 7.5). Stock solutions of water-insoluble DSP were prepared by dissolving DSP in water-free DMSO. The primary amines of PEI were cross-linked with 0.01 M dithiobis(succinimidyl propionate) (DSP) in DMSO. The solutions were mixed by vigorous pipetting and allowed to incubate for 30 min at room temperature. All degrees of cross-linking are reported as molar ratios between DSP and PEI amines assuming that HMW PEI (25 kDa) contains 580 amines per molecule and LMW PEI (5 kDa) contains 126 amines per molecule. Calculation of primary amines was based on ^{13}C NMR data obtained by a recently reported method.[34]

Characterization of Cross-linked Polymers. *Solubility Testing.* The solubility of cross-linked polymers was tested in different media. HMW PEI and LMW PEI were diluted to 1 and 0.1 mg/mL and cross-linked by the addition of 0.01 M DSP in DMSO. The solutions were mixed by vigorous pipetting and allowed to stand for 30 min at room temperature. The solutions were rated visually for any turbidity and precipitates.

Determination of Primary Amine Content. Samples were prepared as described above. The colorimetric assay was performed according to the methods of Read et al.[35] Briefly, 15 μL samples of polymer solution were added to 190 μL of 0.1 M borate buffer at pH 8.0 in 96-well plates. 75 μL of freshly prepared 0.01% acetonic fluorescamine solution was added, mixed vigorously, and incubated for 10 min. Fluorescence was measured with a Perkin-Elmer LS 50B luminescence spectrometer equipped with a well plate reader at $\lambda_{Ex} = 392$ nm, $\lambda_{Em} = 480$ nm, and slit width 4 nm. Measurements were carried out in quadruplicate, and the concentration of the primary amines of the cross-linked polymers was calculated using a standard curve of PEI 5 kDa and PEI 25 kDa standards, respectively. The primary amine content of these standards was independently determined by ^{13}C NMR measurements.[34] The results are expressed in mmol/L primary amines, and results are given as the mean of quadruplicate experiments ± standard deviation.

Determination of Thiol Content. Thiol content was determined according to a method reported in the literature.[36] Briefly, 100 μL of the sample solution was added to 150 μL of 0.1 M phosphate buffer at pH 8.0 in 96-well plates. The color was developed by adding 50 μL per well of a solution containing 0.5 mg/mL dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB) diluted in phosphate buffer (pH 8.0) and incubated for 15 min at room temperature. Absorbance values were obtained with a microplate reader (TitertekPlus MS 212, ICN, Germany) at 405 nm, and thiol content was calculated using a standard curve of cysteine and expressed as mmol/L free thiol. Results are given as the mean of quadruplicate experiments ± standard deviation.

Determination of Disulfide Content. A method reported in the literature was modified for microplate assays.[37] The reagent 2-nitro-5-thiosulfobenzoate (NTSB) was prepared by air oxidation of a solution of 100 mg of dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB) in 10 mL of 0.1 M Na_2SO_3 at 37 °C, yielding a yellowish stock solution of NTSB that was stored at −20 °C. The working solution was prepared by diluting the stock solution 1:100 with 50 mM glycine, 100 mM Na_2SO_3, 3 mM EDTA buffer at pH 9.5. 190 μL of the reagent working solution was added to 10 μL of sample per well and incubated in the dark for 30 min at room temperature. Absorbance values were measured in a microplate reader (TitertekPlus MS 212, ICN, Germany) at 405 nm. The disulfide content was calculated using a DSP standard curve and expressed as mmol/L disulfides. Results are given as the mean of quadruplicate experiments ± standard deviation.

Size Exclusion Chromatography in Combination with Multiple Angle Laser Light Scattering (SEC-MALLS). The SEC setup consisted of an HPLC pump L-6000 from Merck-Hitachi, Darmstadt, Germany, and a Merck-Hitachi autosampler AS-200A. 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, DAWN EOS, GaAs Laser 690 nm, 30 mW, K5 cell). The SEC columns Hema 40 (precolumn) and Novema 3000 were from Polymer Standard Service (Mainz, Germany).

Cross-linked polymers were prepared by reacting 10 mL of a 0.1 g/L PEI 25 kDa solution in 0.1 M borate buffer at pH 7.5 with increasing amounts of 0.01 M DSP dithiobis(succinimidyl)propionate in DMSO for 30 min. After 30 min, the reaction solution was purified by ultrafiltration (Amicon filter membranes, 10 kDa Mw cutoff) with 0.5% formic acid. The eluent was prepared with pure water and degassed with a four-channel online vacuum degasser (DDG-75, Duratec, Reilingen, Germany). Sample size was 100 μL with a 40 μL loop volume for each run. A flow rate of 1 mL/min was applied. MWs were calculated with Astra 4.73 for Windows Software.

Formation of Polymer−DNA Polyplexes and Cross-linking. Luciferase reporter gene plasmids (pGL3) and the appropriate amounts of uncross-linked or cross-linked PEI were dissolved separately in either low ionic strength buffer (5% glucose/25 mM Hepes at pH 7.5) or high ionic strength buffer (150 mM sodium chloride at pH 7.5), mixed by vigorous pipetting, and incubated for 10 min to allow polyplex formation. Polyplex cross-linking was achieved by adding the necessary amount of 0.01 M DSP in DMSO for the desired molar ratios of DSP/PEI amines to the preformed polyplexes. The solutions were mixed by vigorous pipetting and incubated for 30 min. Polyplexes were prepared at a concentration of 2 μg/100 μL plasmid and the appropriate amount of polymer to yield an N/P (nitrogen to phosphate) ratio of 7.

Physicochemical Characterization of Polymer−DNA Polyplexes. *Determination of Polyplex Size and Zeta Potential.* The hydrodynamic diameters as well as the zeta potentials of freshly prepared polyplexes were measured using a Zetasizer Nano-ZS from Malvern Instruments (Herrenberg, Germany) equipped with a 4 mW He−Ne laser at a wavelength of 633 nm at 25 °C. Scattered light was detected at a 173° backward scattering angle with automatic measurement position and automatic laser attenuation. The viscosity and refractive index of pure water at 25 °C were used for data analysis. Hydrodynamic diameters and zeta potential were measured in folded capillary cells after 1:3 dilution with the appropriate buffer and were calculated using DTS

software v4.10. Reference measurements with Malvern size and zeta potential standards were run routinely to check for correct instrument operation. Values are given as the mean of three measurements of 10 runs each.

Atomic Force Microscopy. The polyplexes were prepared as described above and diluted in pure water. 10 μL of the polyplex containing solution was directly transferred onto a prewashed glass slide. Afterward, polyplexes were allowed to immobilize on the glass slides under laminar airflow overnight. Finally, the glass slides were washed with pure water to remove buffer residues and dried with air. AFM experiments were performed using a vibration-damped NanoWizard instrument (JPK instruments, Berlin, Germany). Commercial pyramidal tips (Micromash, Estonia) attached to I-type cantilevers with a length of about 230 μm, a resonance frequency of about 160 kHz, and a nominal force constant of 40 N/m were used. Pictures were recorded in intermittent contact mode at a scan speed of approximately 1 Hz to avoid damage of the sample surface. The acquired pictures had a resolution of 512 $\times$ 512 pixels.

Nanoindentation experiments were carried out with V-type shaped contact mode cantilevers (Micromash, Estonia). The cone angle of the tips was smaller than 10°, and the cantilever had a typical length of 290 μm and a force constant of about 0.03 N/m. The deflection of the cantilever was recorded while the cantilever was extended toward the sample, indicating the force exerted on the tip of the AFM. The setup was calibrated on a precleaned glass slide as an incompressible surface. To determine sensitivity, the slope of the retracted part of the obtained spectroscopy curve was taken. Calibration of the spring constant of the installed cantilever was carried out using a thermal noise method.[38] The height of the polyplexes was measured to ensure that the experiment was performed in the center of the polyplex and the indentation of each polyplex did not exceed 10% of polyplex height. Force scans were performed with an extension speed of about 5 nm per second. During each experiment, 256 data pairs, deflection of the cantilever, and extension of the cantilever holder were recorded. Values are reported as the mean $\pm$ standard deviation of 35−50 particles, each measured twice.

Stability Testing. Polyanion-mediated dissociation of polyplexes was studied using agarose gel electrophoresis as recently described.[39] Polyplex solutions were incubated for 10 min with increasing amounts of heparin. 20 μL aliquots were loaded onto a 1% agarose gel containing EtBr. Gels were run for 60 min at 70 V, and then scanned with a Biometra gel analyzing system.

Albumin-Induced Aggregation. Polyplexes were prepared at a plasmid concentration of 15 μg/mL and cross-linked as described. After the addition of 2 μL of a 40 mg/mL solution of bovine serum albumin (Behring, Germany), polyplexes were allowed to incubate for 10 min. The optical density of the polyplex solution was measured with a Shimadzu UV-160 UV/vis spectrometer at 400 nm. Results are given as the mean of triplicate experiments $\pm$ standard deviation.

Polyplex Stability against High Ionic Strength. The intensity of scattered light of the polyplex solutions was assessed as kilocounts per second (kcps) with a fixed pinhole (200 μm) on an Autosizer Lo-C from Malvern (Herrenberg, Germany, 90° angle, 10 mW HeNe laser, 633 nm). Polyplexes were prepared according to the procedure for size measurements using herring testes DNA in 150 mM sodium chloride. Dissociation of the polyplexes was achieved by adding aliquots of 5 M sodium chloride solution. After each addition, polyplexes were incubated for 5 min, and the scattered light intensity was measured as kcps. Results are given as the mean of triplicate experiments $\pm$ standard deviation.

Hemocompatibility Testing. Hemolytic effects were investigated as reported earlier.[40] Briefly, fresh blood from healthy human volunteers collected in EDTA containing tubes was centrifuged at 4 °C for 3 min at 3000 rpm and washed several times with phosphate buffered saline (PBS) at pH 7.4 until the supernatant was clear and colorless. 150 μL of a 2.5% (v/v) suspension of the erythrocytes was mixed with 15 μL of the polymer or the polyplex solution, respectively, prepared in 5% glucose/25 mM Hepes buffer in microcentrifuge tubes. After an incubation time of 60 min at 37 °C, the blood cells were removed by centrifugation and the supernatant was transferred to 96-well plates. The supernatant was spectroscopically investigated at 570 nm with a TitertekPlus MT 212 plate reader (ICN, Germany). Pure glucose buffer and a 1% Triton X-100 solution in water were used as negative and positive controls, respectively. Hemolysis is reported as percent ($OD_{Triton} - OD_{sample}/OD_{Triton} - OD_{buffer}$)*100%. Results are given as the mean of triplicate experiments $\pm$ standard deviation.

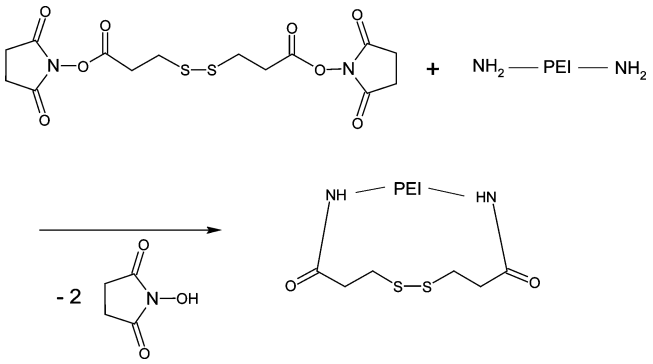


Figure 1. Reaction scheme for the conversion of PEI primary amines with dithiobis(succinimidyl propionate) (DSP).

Statistics. Experiments were performed at least in triplicate. Significance between the mean values was calculated using one-way ANOVA analysis using Origin 7.0 software (OriginLab Corp., Northampton, USA). Probability values <0.05 were regarded to be significant.

Results and Discussion

Stabilization of polyplexes using cross-linking strategies could be a promising way to overcome dissociation of DNA and polycations in the bloodstream after intravenous administration.[18,41] Polyplexes containing PEI have been the subject of numerous investigations.[27,28] Two strategies to stabilize PEI/DNA polyplexes from a low (5 kDa) as well as a high (25 kDa) molecular weight PEI were investigated here. First, PEI was cross-linked with the low molecular weight cross-linker DSP and subsequently complexed to DNA. Second, preformed PEI/DNA polyplexes were cross-linked with DSP to achieve surface stabilization.

Synthesis and Characterization of Cross-linked Polymers. Because cross-linked low molecular PEI has recently been used for polyplex formation, we attempted to investigated the cross-linking reaction in more detail.[32,42] The reaction of HMW PEI and LMW PEI with the cross-linking reagent DSP was characterized by determining the amounts of primary amines of PEI and disulfide groups that were modified by the cross-linking reagent (Figure 1). DSP is a homobifunctional *N*-hydroxysuccinimide (NHS) ester-based electrophilic cross-linking reagent, containing an 8-atom spacer with a length of 12 Å.[43] The amide bond formation mediated by DSP results in the elimination of a positive charge. DSP reacts with primary amines to form stable covalent bonds, and its centrally located disulfide linkage is potentially cleavable after conjugation using common reducing agents. A fluorescamine-based assay was used to determine the amount of primary amines in the polymers. This analysis revealed an initial decrease in primary amines after the addition of DSP, reaching an end point after 15−30 min (data not shown), suggesting successful conversion. Thus, a reaction time of 30 min was chosen for all further cross-linking experiments.

The residual primary amine content after reaction of the polymers with DSP decreased with increasing amounts of DSP.

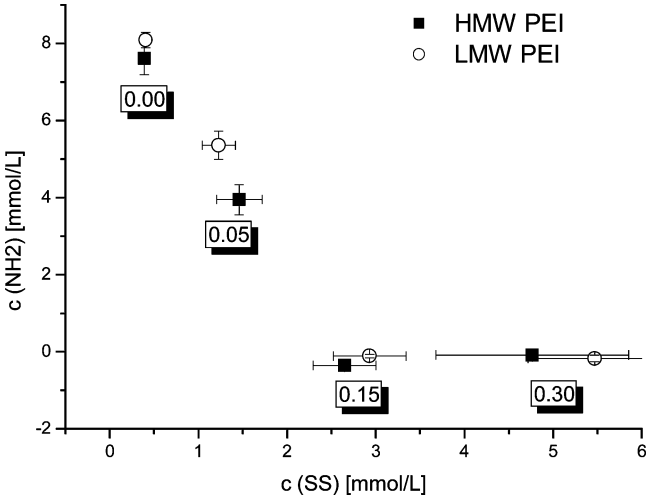


Figure 2. Loss of primary amines and introduction of disulfides due to the reaction of LMW PEI and HMW PEI with DSP (numbers in boxes indicate molar ratio of DSP to amines, $n = 4$).

LMW PEI and HMW PEI did not differ in their reaction behavior with DSP, which can be attributed to similar degrees of branching and content of primary amines.[34] For cross-linking degrees (molar ratio of DSP/amines) of 0.15 and above, no residual primary amines were detected anymore (see Figure 2). Because the cross-linker is bifunctional, a molar ratio of 0.15 corresponds to a molar ratio of 0.30 in amine reactive NHS groups. Recent reports showed that about 1/3 of all amines present in commercially available PEI were primary amines.[34] This points to a more than 90% reaction conversion of the primary amines in the polymers after reaction with DSP at a cross-link degree of 0.15. A further increase of the DSP/amines ratio did not show further reduction of the primary amines content, suggestive of full conversion. Thus, one can assume that at least 90% of the primary amines of both LMW PEI and HMW PEI are susceptible to reaction with DSP. Because the length of the cross-linker is about 12 Å, this indicates the close vicinity of the primary amines due to the flexibility of the polymer chains. A more rigid polymer structure is obtained following the cross-linking reaction due to suppressed chain flexibility of the branched polymer structure.[44]

A recently reported method was used to determine the disulfide content of the cross-linked polymer samples.[37] The thiol content caused by a possible disulfide reduction was checked beforehand. The freshly prepared cross-linking reagent contained only a negligible amount of free thiols, $1.61 \pm 1.1\%$. Figure 2 shows the primary amine content as a function of disulfide modification for both cross-linked PEIs. The primary amine content of both LMW PEI and HMW PEI was observed to decrease in a linear manner with increasing disulfide and, consequently, DSP concentrations. A molar ratio of DSP to amines of 0.15 is considered to be sufficient to obtain the maximum possible cross-link density in branched PEIs of either high or low molecular weight. These measurements allow the adjustment of the desired cross-linking degrees based on the determination of reactive groups instead of by the feeding ratio alone.[32,33]

Solubility of Cross-linked Polymers. The cross-linked polymers were investigated for their solubility in a broad range of aqueous buffer systems to optimize polyplex formation. Table 1 shows the results for two polymer concentrations reacted in different buffers at pH 7.5. As precipitation would impair polyplex preparation, tests were performed at a high cross-link degree of 0.30 combined with relatively highly concentrated polymer solutions to investigate conditions that might cause precipitation. Low ionic strength media were found to be favorable in terms of preventing precipitation of the cross-linked polymers. High ionic strength sodium chloride solutions also showed no precipitation, but high ionic strength phosphate and borate ion containing buffers led to precipitation, especially for the higher concentrated polymer solutions. Thus, low ionic strength 5% glucose/25 mM Hepes buffer and sodium chloride, both at pH 7.5, were chosen as media for complexation with DNA in further experiments.

Table 1. Influence of the Buffer on the Reaction of LMW PEI and HMW PEI with DSP (Cross-link Degree 0.30)[a]

	LMW PEI		HMW PEI	
buffer	0.1 g/L	1 g/L	0.1 g/L	1 g/L
ultrapure water	s	s	s	s
5% glucose/25 mM Hepes	s	s	s	s
150 mM sodium chloride	s	s	s	s
100 mM borate buffer	t	p	t	p
100 mM phosphate buffer	t	p	t	p
DMSO	s	s	s	s

[a] "s" indicates solubility, "t" is for turbid solutions, and "p" represents precipitates.

Molecular Weight of Cross-linked Polymers. The cross-linking of polycations was recently reported to enhance the transfection efficiency of low molecular weight polycations by creating higher molecular weight conjugates due to intermolecular cross-linking.[32,33] LMW PEI and HMW PEI solutions were found to react differently with DSP because the absolute amount of primary amines per molecule is different for the LMW and HMW PEIs used in this study (about 10 times higher per molecule for HMW PEI). The molecular weight of cross-linked PEIs was determined by size exclusion chromatography combined with multi-angle laser light scattering (Table 2). The reaction of LMW PEI with DSP resulted in generally higher molecular weight products. Increased weight-average molecular weights were found, from an initial value of about 3.7 kDa to more than 6 kDa at a cross-link degree of 0.15, suggesting intermolecular cross-linking. A similar rise in the molecular weight has been recently reported for a cross-linked mixture of 423 Da and 2 kDa,[33] and 800 Da PEI resulted in 10−30-fold higher molecular weight after cross-linking.[32] Interestingly, in contrast to LMW PEI, HMW PEI derivatives retained their original molecular weight after cross-linking, which was found to be in the range of 45 kDa for all cross-link degrees up to a DSP/amines molar ratio of 0.15, which is in line with recently reported values.[45,46] In fact, the chromatograms of the size exclusion chromatography revealed even higher elution volumes for the higher cross-link degrees with steady molecular weight, indicating a more dense and globular structure upon cross-link formation (data not shown).[44] The LMW PEI eluted earlier with increasing cross-link degrees, which is in agreement with the M_w results obtained by MALLS. Comparing these data, one can assume a molecular weight-dependent reaction behavior, leading from large aggregates of intermolecular cross-linked low molecular weight PEI species to intramolecular cross-linked polymer chains if higher molecular weight PEI is used.

Characterization of Polyplexes. So far, either constructs generated from cross-linked low molecular weight polymers or cross-linked polyplexes have been investigated separately.[22,42,47] Here, we compare the two different strategies to assess differences in their polyplex formation. Polyplexes between LMW PEI as well as HMW PEI with plasmid DNA were prepared using either cross-linked polymers or polyplexes

Table 2. Molecular Weights of Cross-linked Polymers As Determined by Size Exclusion Chromatography Combined with Multiple Angle Laser Light Scattering

	LMW PEI			HMW PEI		
cross-link degree	M_w [Da]	M_n [Da]	PDI	M_w [Da]	M_n [Da]	PDI
0.00	$3.9 \times 10^{+3}$	$3.7 \times 10^{+3}$	1.1	$4.7 \times 10^{+4}$	$3.2 \times 10^{+4}$	1.5
0.05	$5.3 \times 10^{+3}$	$4.5 \times 10^{+3}$	1.2	$4.8 \times 10^{+4}$	$4.1 \times 10^{+4}$	1.2
0.15	$6.2 \times 10^{+3}$	$5.7 \times 10^{+3}$	1.1	$4.3 \times 10^{+4}$	$4.2 \times 10^{+4}$	1.0

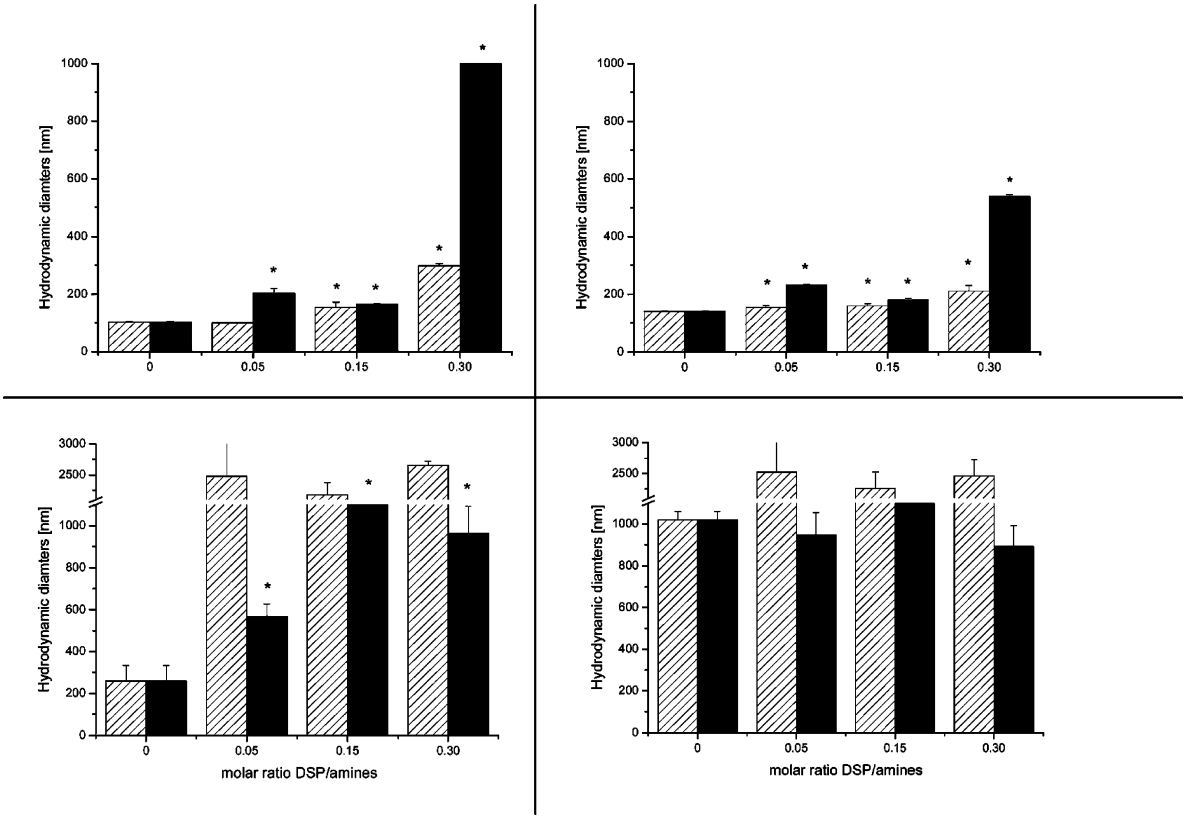


Figure 3. Hydrodynamic diameters of HMW PEI (upper) and LMW PEI (lower) polyplexes. Left: 5% glucose/25 mM Hepes buffer (pH 7.5). Right: 150 mM sodium chloride (pH 7.5). Striped bars indicate polyplexes cross-linked after polyplex formation; black bars indicate polyplex formation using pre-cross-linked polymers. Cross-link degrees are reported as the molar ratio of DSP/amines (* = significant differences from unmodified polyplexes ($p < 0.05$, $n = 3$)).

between plasmid DNA and unmodified polymers, which were cross-linked after formation. All polyplexes were prepared at an N/P ratio of 7, which is commonly used for transfection experiments,[45] and tested in low as well as high ionic strength medium.

Polyplex Size and Zeta Potential. The hydrodynamic diameters (Figure 3) and the zeta potential values (Figure 4) are shown for both HMW PEI/plasmid and LMW PEI/plasmid polyplexes. Polyplexes prepared with pre-cross-linked HMW-PEI showed hydrodynamic diameters up to 500−1000 nm for the highest cross-link ratio of 0.30. In contrast, the size of HMW PEI polyplexes cross-linked after polyplex formation increased only slightly with increasing cross-link degree, ranging from 102 ± 3 nm in 5% glucose/25 mM Hepes buffer and 140 ± 2 nm in 150 mM sodium chloride to 200−300 nm for a cross-link degree of 0.30. The sizes of polyplexes prepared with pre-cross-linked LMW PEI were larger than those of polyplexes cross-linked after formation, independent of the medium used for preparation. Pre-cross-linked LMW PEI resulted in polyplex sizes of up to 1000 nm, which is much higher than polyplex diameters of previously reported cross-linked 2 and 1.8 kDa PEIs.[33,42] Presumably, the reduction of cationic amine groups led to decreased complexation ability. LMW PEI resulted in generally larger hydrodynamic diameters than HMW PEI for both uncross-linked and cross-linked forms, reflecting a lower overall complexation efficiency due to its lower molecular weight.[33,48] Interestingly, while HMW PEI polyplexes could be successfully surface cross-linked with only negligible size increase, LMW PEI polyplexes became much larger when reacted with DSP after formation. Presumably, a looser structure is obtained due to the loss in complexation efficiency, which is not compensated by stabilizing surface cross-links as was the case for the HMW PEI complexes.[24]

The surface charge of HMW PEI polyplexes was only moderately affected by the cross-linking when prepared in 150 mM sodium chloride at pH 7.5, retaining a comparably high zeta potential of $>+25$ mV. A slight decrease was seen for polyplexes formed with pre-cross-linked polymers, where the surface charge decreased to +20−25 mV at high cross-link degrees. By contrast, in low ionic strength medium, high cross-link degrees led to a decrease of the surface charge for polyplexes formed via both preparation methods, yielding lowered zeta potentials of about +10−20 mV. Zeta potentials of LMW PEI polyplexes prepared according to either method decreased with increasing cross-link degrees. The surface charge was found to be in the range of +6−16 mV in 150 mM sodium chloride at pH 7.5 and, in contrast, decreased to negative values in glucose buffer, presumably indicating a dissociation of the

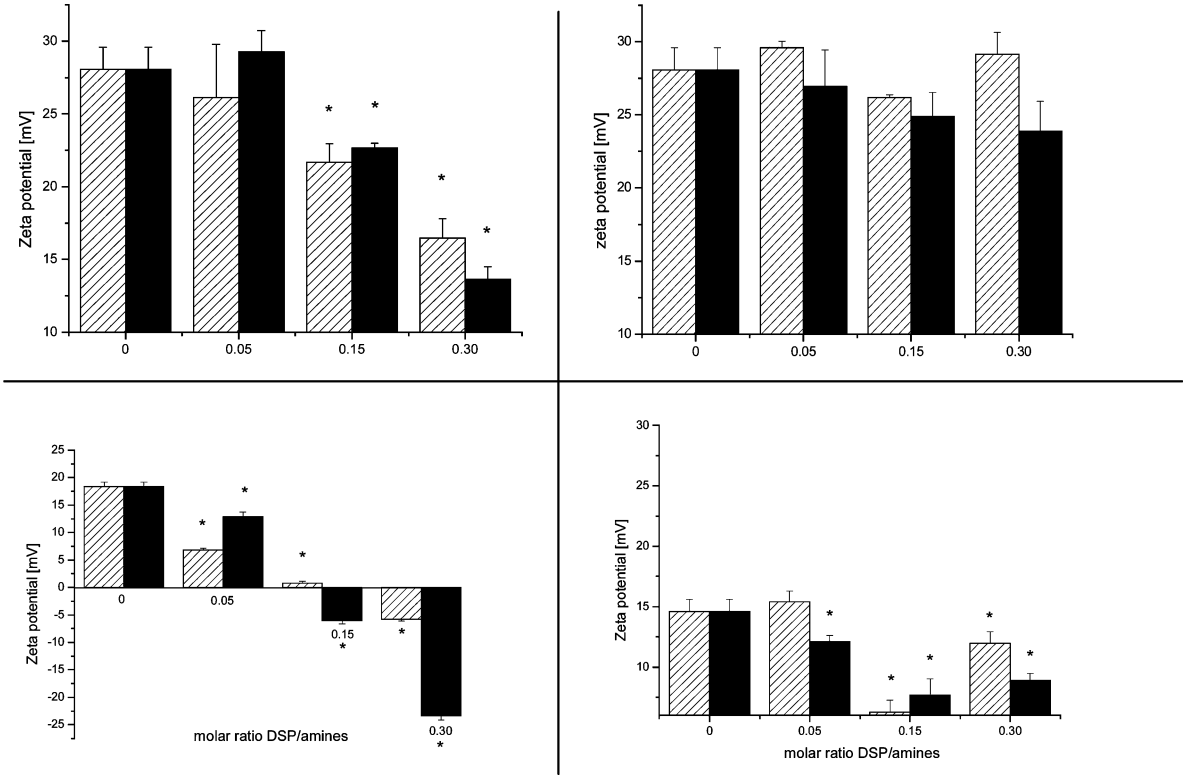


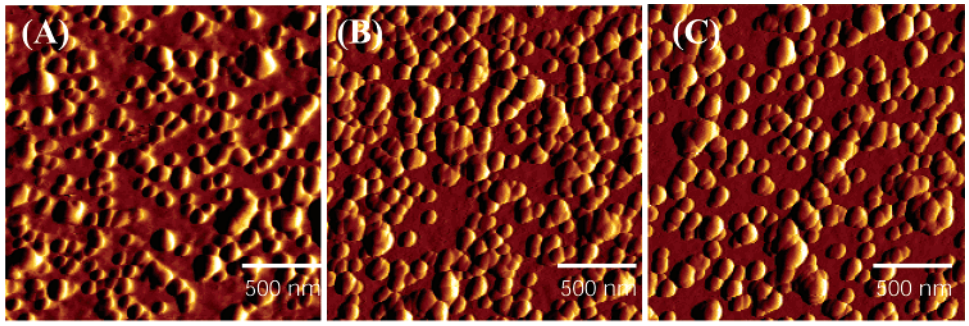
Figure 4. Surface charge of HMW PEI (upper) and LMW PEI (lower) polyplexes. Left: 5% glucose/25 mM Hepes buffer (pH 7.5). Right: 150 mM sodium chloride (pH 7.5). Striped bars indicate polyplexes cross-linked after polyplex formation; black bars indicate polyplex formation using pre-cross-linked polymers. Cross-link degrees are reported as molar ratio DSP/amines (* = significant differences from unmodified polyplexes ($p < 0.05$, $n = 3$)).

polyplexes. The comparably low surface charges could also cause aggregation and, thereby, explain the resulting high hydrodynamic diameters of the cross-linked LMW PEI polyplexes.

Taken together, LMW PEI seemed to be unfavorable for the formation of cross-linked polyplexes using DSP, independent of the preparation method, because hydrodynamic diameters exceeded 500 nm. HMW PEI polyplexes cross-linked after formation displayed only a slight increase in their size. For efficient endocytosis and transfection, polyplexes must be small and compact,[49] and the cross-linked HMW PEI polyplexes fulfill these criteria. The low influence of cross-linking upon the polyplex sizes of the HMW PEI polyplexes can be attributed to its superior complexation efficiency as compared to the LMW PEI. This leads to more stable polyplexes and, thus, preferential surface cross-linking with only minor dislocation of the DNA. A net loss of cationic charges by cross-linking can be observed for both types of polymers. The formation of charge-lowering amide bonds instead of cross-linking with charge-preserving cross-linkers was recently reported to enhance the transfection efficiency for PEG-PLL/plasmid polyplexes, presumably by facilitating DNA escape from the carrier.[17,24] Non-charge consuming cross-linkers, such as dimethyl-dithiobis(propione imidate) (DTBP) or ethylene glycol-bis(succinimidyl succinate) (ESS), have only been reported for cross-linking LMW PEIs to higher molecular weight conjugates, but not for surface stabilization, as no stabilization effect was intended.[32,42] However, according to recent reports, the cationic charge of the polyplexes plays a major role in their cellular uptake. Thus, a delicate balance between charge loss and charge preservation is necessary. The surface charge of cross-linked LMW PEI polyplexes was greatly reduced and even yielded negative values in glucose buffer, rendering the cell surface interaction problematic and indicating polyplex dissociation and release of free DNA.[50] By adjusting the cross-link degree with HMW PEI polyplexes, however, this balance can be retained with a positive surface charge of about at least +10 mV.

AFM Measurements. AFM imaging of HMW PEI polyplexes cross-linked after formation was carried out to support the size measurements by DLS. Images shown in Figure 5 illustrate that non-cross-linked HMW PEI polyplexes prepared at an N/P ratio of 7 formed defined, globular polyplexes with plasmid DNA (A), in good agreement with earlier reports.[17,40] Polyplexes cross-linked with increasing cross-linking degrees (B, C) did not show any differences in their morphology as compared to uncross-linked polyplexes, supporting the results from DLS measurements. Some larger aggregates were observed in all three cases. The introduction of the cross-links did not seem to alter the DNA complexation properties of HMW PEI, as the polyplexes were of similar shape for all cross-link degrees and no free DNA was observed. Size measurements using AFM confirmed the results from DLS for cross-linked HMW PEI polyplexes. For cross-link degrees of 0.05 and 0.15, the size measured with AFM was similar to that of uncross-linked polyplexes (Figure 5).

AFM has been considered as an appropriate technique to investigate mechanical properties of soft materials, such as polymer films, at a submicrometer scale.[51−53] A linear relationship was reported between the resistance to indentation and the amount of cross-links for some rubber-like polymers.[54] The elastic moduli measured with AFM are comparable to those determined with macroscopic tensile tests.[52] However, macroscopic tests are not suitable in this study, as the tested structures are in the range of only 100 nm. The advantage of AFM measurements is the capability to perform local measurements with such a high lateral resolution, allowing one to focus on small structures like nanoparticles or, as in this study, polyplexes. To our knowledge, no one has previously tried to



Crosslink degree	Complex Size AFM [nm]	Indentation force [nN/µm]
0.00	85 +/- 16	392.0
0.05	82 +/- 15	608.7
0.15	83 +/- 15	-

Figure 5. HMW PEI complexes cross-linked with increasing amounts of DSP (molar ratio DSP/amines): (A) 0.00, (B) 0.05, and (C) 0.15, visualized by atomic force microscopy in amplitude mode. Complexes were prepared at N/P 7 in 5% glucose/25 mM Hepes buffer (pH 7.5). Images display defined complexes with no morphological differences at different cross-link degrees. No free DNA could be observed in any image. Some larger aggregates are visible in all formulations, independent of the cross-link degree. Size (n = 40) and indentation force measurements (n = 35−50) are based on AFM images.

determine the mechanical stability of particulate polyplexes, besides testing polyelectrolyte multilayer films in the micrometer range.[55,56] Cross-linked HMW PEI polyplexes were tested for their mechanical stiffness in terms of their indentation resistance by measuring the force needed to indent the polyplex surface at constant indentation depth using atomic force spectroscopy. Higher forces needed to indent a specific depth directly indicate higher stiffness of the polyplex. Uncross-linked polyplexes showed a deflection force of about 30.9 ± 7.4 nN/μm, corresponding to an indentation force of 392.0 nN/μm (n = 35). The mechanical behavior of cross-linked HMW PEI polyplexes was tested at a cross-link degree of 0.05. A significant increase in the force exerted on the tip was found (35.8 ± 6.5 nN/μm, which corresponds to an indentation force of 608.7 nN/μ, n = 50), suggesting that the network of cross-links stabilizes the shape of the polyplexes and thereby increases the stiffness of the polyplexes. Presumably, the cross-linking not only provides higher polyplex stability in a functional sense by minimizing polyplex dissociation, but it also enhances polyplex mechanical stability, which is a prerequisite for resistance against shear stress in the blood stream. The results of the nanoindentation experiments reveal a difference in the indentation resistance between polyplexes that did not contain any cross-links and polyplexes that had been stabilized. This suggests that AFM nanoindentation experiments may be an appropriate method to measure the mechanical properties of polyplexes. The impact of the mechanical properties of polyelectrolyte polyplexes will be investigated further.

Stability Testing of Polyplexes. The objective of this study was to investigate whether polyplexes of LMW PEI and HMW PEI could be efficiently stabilized with a low molecular weight cross-linker to improve their in vivo applicability. Polyplexes prepared according to both preparation methods were investigated in terms of complexation efficiency, resistance against polyanion exchange reactions, and albumin-induced aggregation. Cross-linked HMW PEI polyplexes were additionally investigated by laser light scattering to examine the role of ionic strength on polyplex stability.

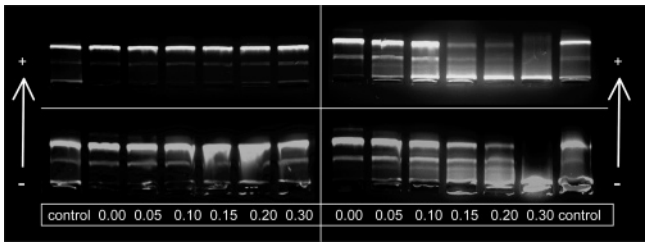


Figure 6. Stability of polyplexes against polyanion exchange. HMW PEI (upper pictures) and LMW PEI (lower pictures) polyplexes challenged with 1 I.U. heparin per 1 μg of plasmid. Left: Polyplexes prepared with pre-cross-linked polymer. Right: Polyplexes cross-linked after complex formation. The numbers in each lane represent the molar ratio of DSP/amines.

Stability against Heparin Exchange. Resistance of polyplexes to polyanionic-mediated dissociation is a method used to study the impact of negatively charged compounds in the blood, such as albumin.[11] A high amount of heparin (1 I.U. per 1 μg plasmid), which is able to release DNA quantitatively from unstabilized HMW PEI polyplexes at N/P 7, was used to challenge the polyplexes and investigate highly stabilized polyplexes.[39]

The use of pre-cross-linked polymers resulted in no retention of the DNA for both tested molecular weights, and the DNA was markedly shifted in the electric field (Figure 6, left-hand side). The loss of charged amine functions and a more rigid polymer structure after the cross-link reaction may be responsible for this significantly reduced complexation efficiency, which resulted in polyplex dissociation for both LMW and HMW PEI. The results from both size and zeta potential measurements support these assumptions, because pre-cross-linked polymers resulted in generally larger hydrodynamic diameters with decreased surface charge, which indicate a lower compaction ability. The overall lower complexation ability of LMW PEI[45] was not affected by the intermolecular cross-linking, suggesting that for pre-cross-linked polymers, a higher molecular weight could not compensate for the loss of charges and flexibility. In contrast, both HMW PEI (Figure 6, upper

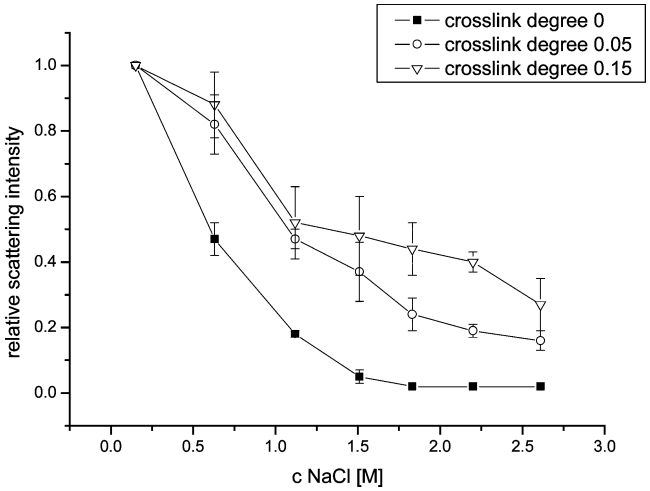


Figure 7. Stability of surface cross-linked HMW PEI polyplexes against high ionic strength. Polyplexes were challenged with increasing amounts of sodium chloride, and polyplex dissociation was investigated by light scattering ($n = 3$).

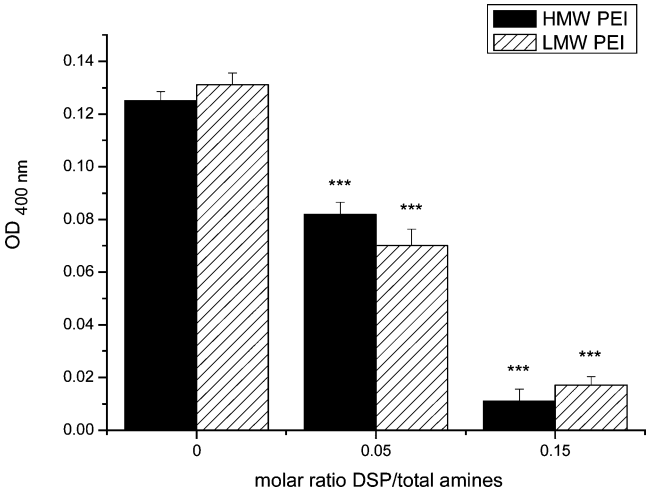


Figure 8. Turbidimetric measurement of albumin-induced aggregation of complexes cross-linked after complexation. Optical density was measured after 10 min incubation with 0.2 mg/mL bovine serum albumin (*** $p < 0.001$, $n = 3$).

pictures) and LMW PEI (Figure 6, lower pictures) polyplexes cross-linked after polyplex formation showed increased stability after exposure to heparin, suggestive of a successful surface cross-linking of polyplexes. The reduction of the electrophoretic shift of the DNA is related to the degree of cross-linking. Heparin fully displaced DNA from non-stabilized polyplexes. Complete retention of the plasmids was achieved for HMW PEI and LMW PEI at molar ratios of DSP to amines of 0.15 and 0.20, respectively. Treating the polyplexes with less DSP resulted in some retention as well, with only limited stabilization. Even though both polymers showed protection of the DNA against dislocation by heparin, only HMW PEI at the same time retained the polyplex size. Thus, cross-linking of HMW PEI polyplexes after formation seems to be the most favorable strategy to efficiently protect plasmid DNA against polyanionic exchange reactions.

Stability in High Ionic Strength Medium. The formation of polyplexes between PEI and DNA is an electrostatic process, which is mainly driven by entropic forces arising from the exchange of sodium ions associated with DNA, thereby shifting the equilibrium toward polyplexes and releasing low molecular weight salt. Sufficiently increasing the concentration of the counter ions is known to shift the equilibrium.[22] Light scattering was used to study nanocomplex dissociation, because particulate polyplexes exhibited increased light scattering intensity as compared to dissociated polyplexes.[22,57] For example, PLL/plasmid DNA polyplexes (N/P 2) have been reported to dissociate at a sodium chloride concentration of 1.1 M by measuring the reduction in the intensity of scattered light.[22] For these reasons, the stability of surface cross-linked HMW PEI polyplexes was investigated in high salt concentrations. Enhanced stability due to cross-linking HMW PEI polyplexes was studied by increasing the concentration of sodium chloride and measuring the resulting intensity of scattered light at a detection angle of 90°. An initial sodium chloride concentration of 150 mM was used to form the polyplexes, and the scattered light intensity was calculated relative to this starting value. A decrease in the relative scattering intensity, corresponding to a dissociation of the polyplexes, was observed for uncross-linked HMW PEI polyplexes immediately after the addition of sodium chloride, leading to a relative scattering intensity of <0.1 at a sodium chloride concentration of 1.5 M (Figure 7). Further addition of sodium chloride did not decrease the relative scattering intensity anymore, suggesting complete dissociation of the polyplexes at 1.5 M. Reaction of the HMW PEI polyplexes with DSP at a molar ratio of 0.05 and 0.15 resulted in an increase in the relative scattered light intensity as compared to the unstabilized polyplexes. Cross-linking of the polyplexes seemed to increase their resistance against salt-induced dissociation. This can be attributed to surface cross-linking, probably in combination with an apparent increase of the polymer molecular weight inside the polyplexes.[22] The higher residual intensity of the cross-linked polyplexes points to an effective cross-linking of polyplexes yielding stable systems even at high salt concentrations of more than 2.5 M.

Interactions of Stabilized Polyplexes with Blood Components. Interactions with blood components are likely to occur after injection of polyplexes into the blood stream.[9,10] Therefore, polyplexes were incubated under in vitro conditions with two major components of the blood, albumin and erythrocytes.

Binding of albumin is known to lead to aggregation of positively charged colloidal particles.[14] Turbidimetric measurements were performed to assess the extent of aggregation. Whereas uncross-linked PEI polyplexes showed severe aggregation after incubation with albumin, the introduction of cross-links after polyplex formation was found to significantly reduce the aggregation tendency (Figure 8). In fact, at a cross-link degree of 0.15, the stabilized polyplexes were completely stable against aggregation. No differences could be found between cross-linked HMW PEI and LMW PEI polyplexes in their interactions with albumin. It is likely that the reduction of the surface charge renders the polyplexes less sensitive to aggregation. Similarly, the reduced aggregation may be caused by less cross-bridging of polycationic particles with each other or with albumin, due to the surface cross-linking.[58]

The interaction of the polymers with erythrocytes was studied using a hemolysis assay, which was performed with freshly purified human red blood cells. 5% glucose/25 mM Hepes buffer (pH 7.5) and 1% Triton X-100 were used to define the 0% and 100% values, respectively. The results of the hemolysis assay are shown in Figure 9. In general, HMW PEI polymers and polyplexes showed higher hemolysis as compared to LMW PEI. A hemolysis of 11% was found for the uncross-linked HMW PEI control, which is in good agreement with earlier reports.[59] After cross-linking of the pure polymers, an initial increase in hemolysis could be observed, reaching more than 25% for

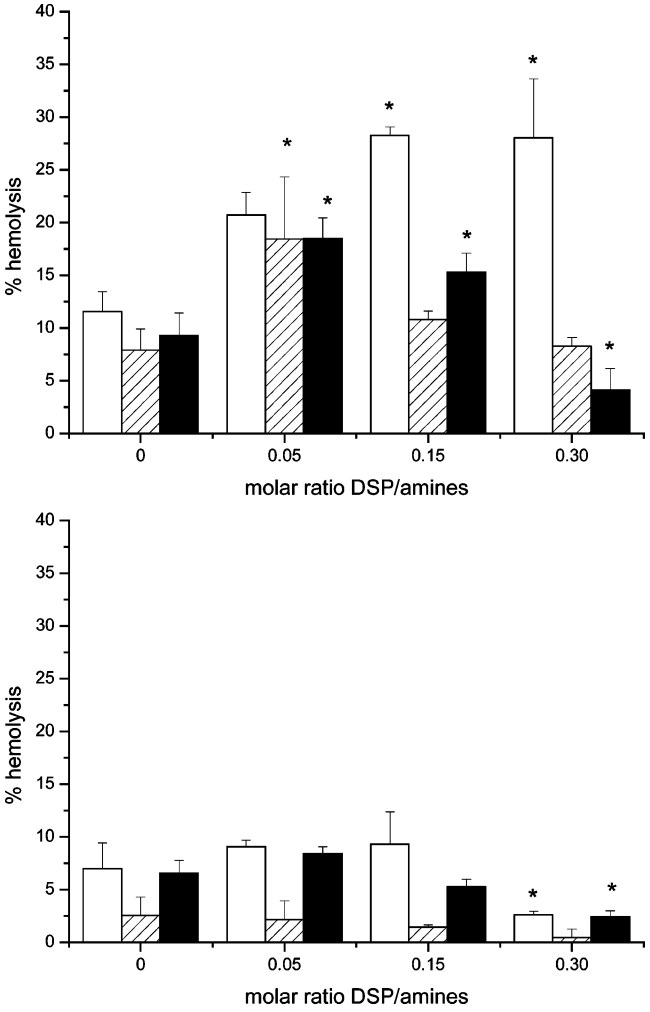
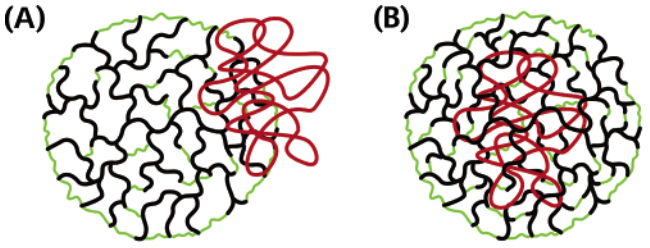


Figure 9. Hemolysis of pure pre-cross-linked polymers (white bars), polyplexes cross-linked after formation (striped bars), and polyplexes prepared with pre-cross-linked polymers (black bars) after an incubation time of 60 min at 37 °C. Upper picture: HMW PEI. Lower picture: LMW PEI (* = significant differences from the value of the corresponding molar ratio of 0, $p < 0.05$, $n = 3$).

Scheme 1. Proposed Differences in Complex Formation of Pre-cross-linked Polymers or Cross-linking of Already-Formed Complexes[a]



[a] The DNA compaction behavior of pre-cross-linked polymers decreases presumably due to their lowered cationic charge and chain flexibility (A). Introduction of cross-links after formation results in increased polyplex stability (B). Color scheme: black, branched PEI; red, plasmid DNA; green, cross-links.

HMW PEI and about 10% for LMW PEI. The initial increase was followed by decreased hemolysis at higher cross-link degrees, presumably caused by the reduction of the cationic charges, which are known to account for the hemolytic activity of cationic polymers.[60] The hemolysis for both preparation methods was comparable for HMW PEI polyplexes. In contrast, LMW PEI polyplexes were significantly less hemolytic if prepared by cross-linking preformed polyplexes. A higher amount of free polymer in formulations with pre-cross-linked LMW PEI, as indicated by size and stability tests, may account for this observation. Substances are classified as hemolytic if the hemolytic activity is higher than 15%.[61] All stabilized polyplexes prepared with LMW PEI and surface cross-linked HMW PEI polyplexes met this requirement, except at a cross-link degree of 0.05. Pure cross-linked HMW PEI, however, reached a hemolytic activity of up to 30% at high cross-link ratios, which decreased after complexation with DNA. Even if a certain hemolytic activity of gene transfer vectors has been claimed to be favorable due to destabilization of biological membranes,[62] low hemolytic activity is critical for systemic administration.

Conclusion

One important prerequisite for systemic gene delivery of nonviral vectors is their stability in the bloodstream. Surface cross-linked PEI/plasmid polyplexes were found to show interesting properties for systemic plasmid delivery. The reaction of DSP with PEI can be easily controlled, allowing one to adjust desired degrees of disulfide cross-linking. Our study points to a remarkable influence of the molecular weight of the polymer as well as the cross-linking procedure. Pre-cross-linked PEIs showed decreased plasmid compaction and stabilization properties, suggesting incomplete caging of the DNA (Scheme 1). High molecular weight PEI in combination with surface cross-linking resulted in polyplexes with enhanced stability against dissociation by polyanions and ionic strength of the medium. In addition, the mechanical properties were improved, as shown by AFM indentation studies. Such polyplexes exhibited hydrodynamic diameters and zeta potential values compatible with endocytic cellular uptake mechanisms. The resulting vector systems showed improved biocompatibility in terms of albumin and erythrocyte interactions.

Further characterization of the polyplexes in terms of their triggered activation by the cells and in vivo properties is currently under investigation. We believe that this is an important step toward the goal of designing stable vectors for intravenous plasmid delivery.

Acknowledgment. We thank Dr. Erik Rytting for careful revision of this manuscript.

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BM060788Z