The Structure of PEG-Modified Poly(Ethylene Imines) Influences Biodistribution and Pharmacokinetics of Their Complexes with NF-κB Decoy in Mice

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Purpose. To study the relationship between structure of poly(ethylene imine-co-ethylene glycol), PEI-PEG, copolymers and physicochemical properties as well as in vivo behavior of their complexes with NF-κB decoy.

Methods. A variety of copolymers of PEG grafted onto PEI as well as PEI grafted onto PEG were synthesized and their complexes with a double stranded 20mer oligonucleotide were examined regarding size, surface charge, biodistribution and pharmacokinetics.

Results. Polyplexes of copolymers were smaller compared to polyplexes formed by non-PEGylated PEI 25 kDa (58 – 334 nm vs. 437 nm for a nitrogen/phosphate ratio of 3.5 and 85 – 308 nm vs. 408 nm for N/P 6.0) and showed reduced zeta potential (–2.5 – 6.4 mV vs. 14.5 mV for N/P 6.0). IV injection into mice revealed liver (35–76 % of injected dose), kidney (3 – 22 %) and spleen (2 – 16 %) to be the main target organs for all injected complexes. Complexes formed by copolymers with few PEG blocks of higher molecular weight (5 kDa and 20 kDa) grafted onto PEI 25 kDa did not show different blood levels from PEI 25 kDa. In contrast, a copolymer with more short PEG blocks (550 Da) grafted onto PEI showed elevated blood levels with an increase in AUC of 62 %.

Conclusions. A sufficiently high density of PEG molecules is necessary to effectively prevent opsonization and thereby rapid clearance from blood stream.

KEY WORDS: gene delivery, polyethylenimine, poly(ethylene glycol), DNA, body distribution, pharmacokinetic behavior.

INTRODUCTION

The transport of DNA to target tissue remains a major challenge for *in vivo* gene therapy. Viruses have achieved high efficiency in delivering nucleic acids to host cells and are the most effective vectors available today. Drawbacks such as serious immune or toxic reactions have stimulated intensive research into non-viral gene delivery systems among which lipofection using cationic lipids and polyfection employing cationic polymers seem to hold promise. Poly(ethylene imine) (PEI), a branched polyamine, has been used as non-viral vector under *in vitro* (1) and *in vivo* (2) conditions, due to the hypothetical "proton sponge" mechanism (3).

Avoidance of rapid clearance from circulation seems to be an important prerequisite for systemic administration of non-viral gene delivery systems. Cationic complexes of polymers and DNA, so called polyplexes, are subject to opsonization (4) and thereby enhanced uptake by the mononuclear phagocytic system (MPS) is reducing their half-life in circulation. On the other hand, an excess of positive charges is needed to form small toroid-like complexes with DNA (5).

Several hydrophilic macromolecules such as poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA), poly(N-vinylpyrrolidone) (PVP) or poly(ethylene glycol) (PEG), have been used to prolong the circulation time of drugs or particles in blood (6).

Two different approaches have been investigated to modify the surface of polyplexes. One of them is the formation of complexes of DNA and cationic polymers, which are subsequently coupled with hydrophilic non-ionic polymers. This approach offers the advantage that the hydrophilic non-ionic polymer does not interfere with complex formation, but is less practicable. Systems following this method are pLL/DNA complexes coupled with PHPMA (7) or PEI/DNA complexes coupled with PEG (8).

Another approach is the synthesis of block or graft copolymers of cationic and hydrophilic non-ionic polymers, which are then used to form polyplexes with DNA. Oupicky (9) described the supramolecular structure of such complexes to consist of a hydrophobic core formed by DNA neutralized by polycation blocks, surrounded by a shell of hydrophilic non-ionic polymers. Copolymers of this type include poly-(L-lysine) (PLL) grafted with PEG (10–12), dextran or PHPMA (12) as well as PEI grafted with PEG (13,14). However, these polyplexes have mainly been examined in terms of physicochemical properties and *in vitro* transfection efficiency but not regarding their *in vivo* distribution and pharmacokinetics. Furthermore, none of these studies has investigated the influence of PEG block length and degree of substitution, which seems crucial to optimize such systems.

We therefore synthesized a variety of copolymers consisting of PEI and PEG with PEI core and PEG shell or branched PEG core and PEI shell to study structure/function-relationship. Copolymers with PEI core and PEG shell have a PEI 25 kDa core that has been most widely used for gene delivery. By systematic variation of PEG block length and degree of substitution we got a row of copolymers ranging from a AB - block type copolymer, $PEI(PEG)_1$, up to a copolymer with 50 PEG blocks per PEI core, $PEI(PEG)_{50}$. The weight ratio of PEG to PEI could be kept almost constant. The idea underlying the second copolymer type with reverse configuration was to create a structure similar to histones with a neutral core and charges on the surface to create very dense complexes with DNA. Such a copolymer type has not been studied for gene therapy before.

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ABBREVIATIONS: AUC, area under curve; NHS, N-hydroxysuccinimide ; PEG, poly(ethylene glycol); PEI, poly(ethylene imine); PHPMA, poly[N-(2-hydroxypropyl)methacrylamide]; PLL, poly-(L-lysine); PVP, poly(N-vinylpyrrolidone).

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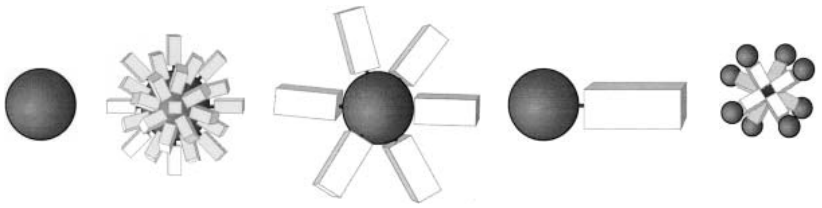
Table I. Characterization of PEI and Copolymers of PEI and PEG Investigated in This Study. PEI Content, Molecular Weight of PEI and PEG Blocks and Total Molecular Weight as Well as Symbolic Structure Are Given

Compound	PEI	$PEI(PEG)_{50}$	$PEI(PEG)_6$	$PEI(PEG)_1$	$PEG(PEI)_8$
PEI content	100%	41%	45%	46%	30%
Mw PEI	25000	25000	25000	25000	800
Mw PEG	n.a.	550	5000	20000	10000
Mw	25000	61000	56000	54000	16000



Note: Spheres symbolize PEI blocks and cuboids represent PEG blocks. The volume of spheres and cuboids is proportional to the molecular weight of the polymer blocks. All copolymers have a very similar weight ratio of PEG to PEI.

Table I gives an overview of the applied polymers. Complexes at different charge ratios were examined regarding their physicochemical characteristics and injected to mice to study their biodistribution and pharmacokinetics. Results were compared to PEI alone.

MATERIALS AND METHODS

Polymer Synthesis

Branched PEI 25kDa (Aldrich, Taufkirchen, Germany) was the starting material for the synthesis of the copolymers $PEI(PEG)_{50}$, $PEI(PEG)_6$ and $PEI(PEG)_1$. PEG-monomethylether (550 Da) and PEG-monomethylether (5 kDa) from Aldrich and monoamino-PEG-monomethylether (20 kDa) from Rapp Polymere (Tübingen, Germany) were dissolved in anhydrous chloroform (200 g/L) and activated for the reaction with the amino groups of PEI with an 10 – 100 fold excess of hexamethylene diisocyanate (HMDI, Fluka, Deisenhofen, Germany) (60°C, 24 h). Unreacted HMDI was carefully removed by repetitive extraction with light petrol. The activated PEGs were reacted with PEI at concentrations of 10 g/L to give the copolymers $PEI(PEG)_{50}$, $PEI(PEG)_6$ and $PEI(PEG)_1$. Reactions were carried out in anhydrous chloroform at 60°C for 24 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 vacuum. Similarly, branched PEI 800 Da (Aldrich) was linked to an 8 arm branched PEG (10 kDa) from Shearwater Polymers (Huntsville, USA) to form the copolymer $PEG(PEI)_8$ via reaction with HMDI under conditions as described earlier. Polymers were characterized by ^1H and ^{13}C-NMR spectroscopy that verified the structure of the copolymers and allowed calculation of the content of ethylene imine and ethylene glycol units in the copolymer. Size exclusion chromatography proved the absence of unreacted PEG and PEI homopolymers. Thus, no further purification step was necessary. Further details of the polymer synthesis and characterization are reported in (15) and will be published elsewhere.

Preparation of DNA-Polymer-Complexes

The double-stranded decoy used was a 20-mer DNA oligo containing the NF-κB cis element (5′-CCTTGAAGGGATTTCCCTCC-3′) and its complement (MWG-Biotech AG). Complexes of DNA and polymer were prepared by adjusting concentration of polymer solutions (in 10mM Hepes buffer pH 7.4 with 150mM NaCl) with 5% glucose and subsequent addition of these solutions to solutions of NF-κB decoy in 5% glucose to yield complexes of the required N/P ratio and polymer amount at pH 7.4. For example, for complexes of PEI and oligo with a dose of 1 μg PEI and N/P 6.0, 79.0 μL of labeled polymer with a concentration of 36.4 μg/ml as obtained after purification were diluted with 36.0 μL of 5% glucose. This solution was then added to a solution of 3.71 μg DNA in 115 μL 5% glucose. Thereby, 80 μL contained 1 μg PEI. Mixtures were vortexed immediately and incubated for 10 min at room temperature. For the copolymers, dose was related to PEI, not total copolymer. DNA concentrations of complex solutions prepared for animal experiments can be calculated from the indicated dose of injected polymer, the injected volume of 80 μL and the N/P ratios. (DNA: 330 Da/phosphate, PEI: 43.1 Da/nitrogen). DNA concentrations of complex solutions prepared for physico-chemical characterization are given below.

Photon Correlation Spectroscopy

Hydrodynamic diameters of the DNA/polymer complexes were determined by photon correlation spectroscopy. To have conditions comparable to the *in vivo* application, complexes of unlabeled polymer and DNA were prepared as described earlier in poly(ethylene) tubes with a final DNA concentration of 16 μg/mL. After 10 min incubation time, 0.5 mL of the complexes were diluted with 0.5 mL of a 1:1 mixture of 5 % glucose solution and 10mM Hepes buffer with 150mM NaCl. Measurements were performed on a Zetasizer 3000 HS from Malvern Instruments, Herrenberg, Germany (10 mW HeNe laser, 633 nm). Scattering light was detected at 90° angle through a 400 micron pin hole at a temperature of 25°C. For data analysis, the viscosity (0.88 mPa*s) and the refractive index (1.33) of distilled water at 25°C were used. The instrument was routinely calibrated using Standard Reference latex particles (AZ 55 Electrophoresis Standard Kit, Malvern Instruments). Measurements were analyzed by CONTIN algorithm. Values given are the means of 3 runs ± standard deviation. One-way ANOVA with Dunnett's post-

test was performed using GraphPad InStat v3.05 to find significant differences between copolymers and PEI. Differences were considered significant if $P \leq 0.05$.

Zeta Potential Measurements

Samples were prepared as for the photon correlation spectroscopy, but 0.5 mL complex solution (16 μg/mL DNA) was diluted with 1.5 mL of the of the Hepes/NaCl mixture. Zeta-potential measurements were carried out in the standard capillary electrophoresis cell of the Zetasizer 3000 HS from Malvern Instruments at position 17.0 at 25°C. Sampling time was set to automatic. Average values were calculated with the data of 3 runs. Statistics were performed as described earlier.

Radioactive Labeling of PEI

PEI was labeled employing N-succinimidyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionate (Amersham Pharmacia Biotech, Freiburg, Germany) by the method of Bolton and Hunter (16). Therefore, polymers were dissolved in 0.1 M borate buffer pH 8.5 to a concentration of 1 mg PEI/mL (for application of 0.2 and 1 μg polymer) or 10 mg PEI/mL (for application of 10 μg polymer) and Bolton Hunter reagent was dissolved in DMSO to a concentration of 22.5 μCi/μL. 60 μL of polymer solution were reacted with 20 μL of Bolton Hunter reagent solution and reaction was carried out for 60 min at room temperature. Purification from low molecular weight products and buffer exchange were performed on a Sephadex G-25 column (PD10, Pharmacia) with a 10 mM Hepes buffer with 150 mM NaCl, pH 7.4. Fractions of 0.8 mL each were collected for process monitoring by determination of radioactivity and sample collection.

Reproducibility of labeling and purification were tested by cold labeling with N-succinimidyl-3-(4-hydroxyphenyl) propionate (SHPP, Pierce, Rockford, USA) in triplicate under the same conditions as above. PEI concentration in peak fractions (fraction 4 and 5) was determined by complex formation with copper (17) in triplicate. Shortly, to 100 μl of polymer solution 100 μl of Copper (II) acetate (0.02 M) were added on a 96 well plate and absorption at 630 nm was read on a Dynatech MR5000 plate reader (Dynatech, Denkendorf, Germany). A standard curve was recorded with samples of the same polymer in known concentrations on the same plate. Polymer concentration was assumed identical for SHPP labeled and Bolton Hunter labeled polymers after reaction and purification under exactly the same conditions.

Organ Distribution and Pharmacokinetic Behavior

All animal experiments followed the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985) and were approved by external review committee for laboratory animal care. Male balb/c mice with a body weight of approximately 25 g were anaesthetized by injection of Ketamine (Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and Xylazine (Rompun, Bayer AG, Leverkusen, Germany). Complexes were injected as a bolus of 80 μL through the jugular vein. Blood samples were obtained through a catheter in the common carotid artery and urine was sampled by flushing the bladder with sodium chloride solution through a 2-way catheter. After 120 min mice were sacrificed by decapitation and organs (cortex, liver, kidneys, heart, lungs, spleen) were sampled and weighed.

Radioactivity of all samples was measured on a 1277 Gammamaster (Perkin Elmer Wallac, Freiburg, Germany). Measurements of complex solutions were used to determine the injected dose of radioactivity. Polymer concentrations in the samples were then calculated as percent of injected dose (%ID), %ID/mL or %ID/g, respectively. Unpaired *t*-test was performed using GraphPad InStat v3.05 to compare blood levels of different copolymers at corresponding time points. Differences were considered significant if two-tail $P \leq 0.05$.

Non-Linear Curve Fitting

Concentration time curves were fitted to a two compartmental model with the Software Kinetica 1.1 from Simed. The model used was $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ and the weighting applied was $1/(c_{calc})^2$. For all complexes, each concentration time curve was fitted individually as well as the mean of the concentrations for each time point. Pharmacokinetic parameters given are the mean of the parameters calculated from individual fits. In addition, α and β values were transformed to half-life periods. Plots shown in the results section are fits for the mean concentrations with the standard deviation for each time point shown.

RESULTS

Size of the Complexes

Size of complexes was determined in a 1:1 mixture of 5 % glucose solution and 10 mM Hepes buffer with 150 mM NaCl to measure complexes in the same medium in which they were injected into mice. Photon correlation spectroscopy demonstrated a decrease in size of the polyplexes with a change of N/P from 3.5 to 6.0 (Fig. 1). The excess of positive charges leads to a more condensed structure of the complexes. All copolymers showed a decrease in complex size compared to PEI alone, which was found significant for PEI(PEG)$_1$ and PEG(PEI)$_8$ at N/P 3.5 and for all copolymers at N/P 6.0. Obviously PEG prevents aggregation of the complexes and thereby leads to smaller sizes. Within the series of PEGylated PEIs, an influence of PEG block length could be seen in the way that the copolymers with fewer PEG blocks

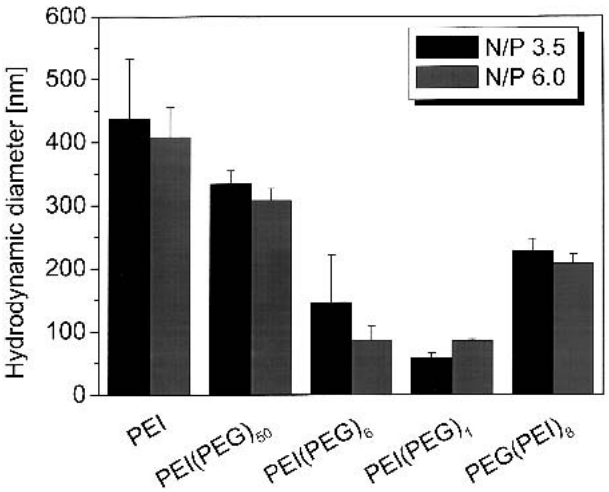


Fig. 1. Size of polyplexes formed with NF-κB decoy at two different nitrogen/phosphate (N/P) ratios as determined by photon correlation spectroscopy. Values given are the means of three runs ± standard deviation. All copolymers formed complexes with reduced size compared to PEI at both N/P ratios tested.

formed smaller complexes than copolymers with numerous shorter blocks. It should be taken into account that the viscosity of this mixture is slightly higher than that of water and therefore leads to slower movement of the complexes. This might lead to a calculation of sizes which are slightly too high because the calculations are based on the viscosity of water.

Zeta Potential

Results of zeta potential measurements are shown in Fig. 2. It can clearly be seen that all tested polymers and copolymers formed complexes with a slightly negative surface charge at the lower nitrogen/phosphate (N/P) ratio of 3.5. No significant difference could be seen between the different copolymers and PEI. Since only every fourth to fifth nitrogen of PEI is protonated at physiological pH there is still a slight excess of negative charge. Therefore surface charge is dominated by DNA and not by PEI or PEG, respectively. At the N/P ratio of 6.0 all copolymers showed a significantly reduced zeta potential compared to PEI. At this N/P ratio there is an excess of positive charges from PEI over negative charges from DNA. Obviously, at least part of the PEG blocks of the copolymers orientate towards the surface and thereby shield the positive charge.

Labeling of the Polymers

Bolton Hunter reagent containing radioactive ^{125}I was coupled to free amino groups of the polymers by aminolysis of the reactive N-hydroxysuccinimide (NHS) ester by the method of Bolton and Hunter (16). By γ-counting of the collected fractions we demonstrated that the Bolton Hunter reagent was coupled to PEI. Labeled polymer and free hydrolyzed label could be separated effectively by gel filtration (data not shown).

Organ Distribution

Organ values for liver, kidney, spleen, lungs and urine for all tested complexes are shown in Table II. Evaluation of organ distribution revealed that the liver was the main target organ for all polyplexes (54 ± 13% of injected dose), followed by kidney (9 ± 7 %ID) and spleen (6 ± 5 %ID). All other organs including the lungs contained less than 2 %ID after 120 min. Mean renal excretion as examined by radioactivity in the collected urine and bladder flush liquid was 3 ± 2%. Almost no radioactivity was found around the injection site (not shown).

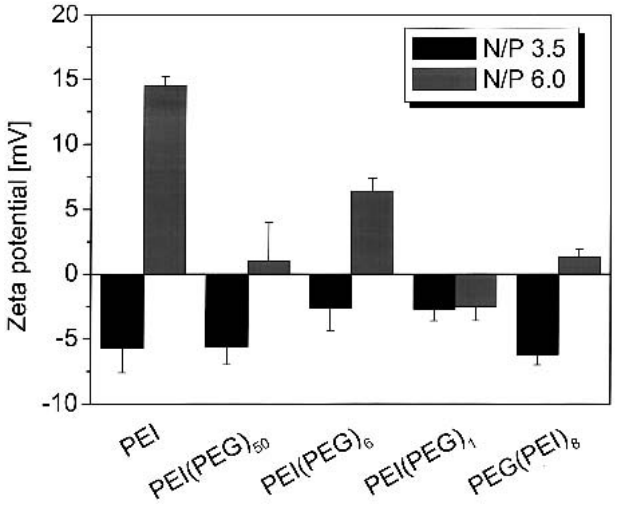


Fig. 2. Surface charge measured as zeta potential. Values are the means of three runs ± standard deviation. Zeta potential was slightly negative at N/P 3.5 with no significant differences between PEI-PEG copolymers and PEI. At N/P 6.0 all copolymers showed reduced zeta potential compared to PEI.

On a weight basis, spleen showed the highest concentration of radioactivity after 120 min (69 ± 51 %ID/g), followed by liver (52 ± 14 %ID/g) and kidney (24 ± 16 %ID/g) whereas lungs showed a much lower concentration of only 5 ± 3 %ID per gram.

When the two tested N/P ratios are compared, an increased liver accumulation for the complexes with the higher N/P ratio was found for PEI. Such an increase was not found for $PEI(PEG)_{50}$ and $PEG(PEI)_8$ where the surface charge does only marginally increase. A variation of dose from 0.2 over 1 to 10 μg of PEI did not significantly alter organ distribution.

A comparison of the different polymers for a dose of 1 μg (related to PEI) and N/P 6.0 is shown in Fig. 3. PEI showed the highest accumulation in spleen and therefore reduced liver accumulation. The three PEGylated PEIs showed a shift from spleen to liver compared to PEI. Within the series of PEGylated PEIs, this effect becomes less pronounced when the block length of PEI is increased and the block number decreased. Kidney accumulation drops with increasing PEG block length. The "PEIylated" PEG showed a strong increase in kidney accumulation and a strong decrease in entrapment by the spleen compared to PEI.

Pharmacokinetic Behavior of the Complexes

Concentration-time curves for whole blood could be fitted to a biexponential disposition equation by non-linear curve fitting with Simed Kinetica for all polymer/DNA complexes. A first phase of fast disposition and a second phase of slower elimination were found for all complexes studied (Fig. 4).

Comparisons of two different N/P ratios at a dose of 10 μg revealed no striking differences as can be seen from the pharmacokinetic parameters shown in Table III.

Comparison of three different doses as for PEI and $PEI(PEG)_{50}$ at an N/P ratio of 6 showed that lower doses had increased the area under the curve. Smaller half-time periods in the distribution phase were found for lower concentrations (Table III).

A comparison of complexes of DNA and the different polymers at an N/P ratio of 6 and a dose of 1 μg PEI per animal revealed major differences between the different structures (Fig. 4). The copolymer with PEG core and PEI shell, $PEG(PEI)_8$, showed clearly reduced blood levels compared to PEI. The copolymer with the highest number of PEG blocks grafted onto PEI, $PEI(PEG)_{50}$, showed a clearly increased area under the curve (+ 63%) compared to PEI with an elevated beta phase. The blood levels were significantly different from PEI for all time points from 30 to 120 min. An increased AUC compared to PEI was also found for the other doses examined. The two other PEGylated PEIs, $PEI(PEG)_6$ and $PEI(PEG)_1$, did not show changes in blood levels compared to PEI.

Table II. Organ Distribution of Polyplexes of NF-κB Decoy and PEI or Copolymers of PEI and PEG 120 Minutes after Injection into the Jugular Vein

Polymer	N/P	Polymer dose [μg]	Liver	Kidney	Spleen	Lungs	Urine
PEI	3.5	10	34.8 ± 8.5	2.7 ± 0.3	11.6 ± 2.8	1.95 ± 0.18	6.1 ± 1.9
	6.0	10	76.3 ± 16.9	4.7 ± 1.4	7.7 ± 1.2	0.47 ± 0.11	2.9 ± 0.9
	6.0	1	46.0 ± 6.9	4.2 ± 0.3	15.7 ± 5.5	0.78 ± 0.16	1.7 ± 0.4
	6.0	0.2	65.3 ± 12.6	5.0 ± 1.9	13.2 ± 3.0	0.64 ± 0.18	1.4 ± 0.6
$PEI(PEG)_{50}$	3.5	10	55.5 ± 10.5	10.6 ± 1.6	4.0 ± 0.9	0.58 ± 0.20	0.7 ± 1.0
	6.0	10	39.6 ± 9.0	12.5 ± 0.8	4.2 ± 1.4	1.68 ± 0.80	1.3 ± 0.1
	6.0	1	68.9 ± 2.6	8.6 ± 0.3	3.6 ± 0.6	0.57 ± 0.14	1.6 ± 0.1
	6.0	0.2	61.7 ± 2.2	6.9 ± 0.9	3.6 ± 0.8	0.33 ± 0.16	1.6 ± 0.4
$PEI(PEG)_6$	6.0	1	64.4 ± 5.4	5.1 ± 1.0	5.5 ± 2.0	0.99 ± 0.26	2.7 ± 0.4
$PEI(PEG)_1$	6.0	1	56.7 ± 7.9	3.6 ± 0.2	6.1 ± 1.0	1.04 ± 0.13	0.5 ± 0.1
$PEG(PEI)_8$	3.5	10	39.1 ± 1.8	22.2 ± 1.3	1.6 ± 0.1	0.37 ± 0.06	5.9 ± 1.3
	6.0	10	41.2 ± 0.2	20.8 ± 3.3	1.5 ± 0.2	0.32 ± 0.01	5.0 ± 1.3
	6.0	1	52.6 ± 4.3	15.6 ± 1.6	1.8 ± 0.4	0.57 ± 0.15	3.5 ± 0.3

Note: Values shown are percent of injected dose (%ID) two hours after injection and represent the means ± standard deviation of three animals. Liver was the main target organ for all injected polyplexes under all conditions tested, followed by kidney and spleen.

DISCUSSION

In this report we describe investigation of poly(ethylene imine) (PEI) as well as copolymers of PEI and PEG with different structures regarding their ability to form long circulating complexes with DNA.

Several studies on polyplexes of DNA and poly(ethylene glycol)-poly(ethylene imine) graft copolymers with (14,18,19) or without (13,20) targeting moieties have been undertaken. However, none of these studies has systematically examined the influence of PEG block length and number as well as PEI-PEG orientation (PEI or PEG core with PEG or PEI shell) on complex formation. Only little is known about the pharmacokinetic behavior of PEI or PEI-PEG copolymers.

All copolymers formed relatively small complexes with oligomeric DNA even in the presence of ions which is know to lead to larger complexes by aggregation (21). To correlate complex sizes with *in vivo* behavior, it seemed to be important to measure complexes under the same conditions under which they were prepared for injection into mice. To obtain a good separation from unreacted label, the presence of salts was necessary. The formation of small complexes by copolymers of PEI and PEG was questioned by Ogris and co-workers (8) who therefore formed complexes of DNA and PEI first and subsequently PEGylated them. These complexes showed highly decreased interactions with plasma proteins and erythrocytes and extended circulation in blood, but this approach is inconvenient for clinical applications. In fact, our copolymers even lead to reduced complex sizes compared to PEI, probably caused by reduced aggregation. The hydrophilic nonionic PEG molecules prevent charge-charge interactions of the cationic PEI chains and counter ions. This effect is more pronounced for PEG blocks of higher molecular weight as we found smaller complex sizes with increasing PEG block Mw for the PEGylated PEIs. We think that those PEGylated PEIs are especially well qualified for oligonucleotide transfer. Because of the small size of oligos, they can interact with single PEI cores. In contrast, plasmids with several thousand base pairs have to interact with several copolymer molecules at once and thereby not all PEG blocks will be arranged towards complex surface.

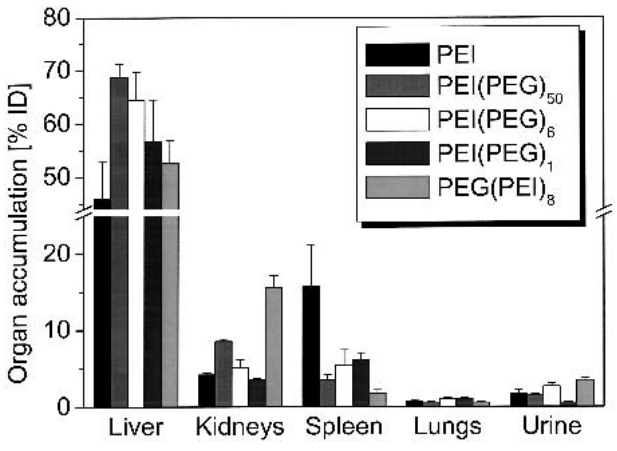


Fig. 3. Organ distribution of polyplexes formed by PEI or PEI-PEG copolymers and NF-κB in comparison with unmodified PEI at a dose of 1 μg PEI and N/P ratio of 6 two hours after injection. Values are percent of injected dose given as the mean of three animals. Error bars represent standard deviation. PEI-PEG copolymers showed increased liver and decreased spleen accumulation compared to PEI.

Remarkably, the "PEIylated" PEG also formed small complexes. Obviously the connection of 8 short PEI molecules by 8-arm PEG to a larger histone-like structure with cationic charges on the outer shell of the copolymer molecule and a neutral core leads to molecules that can effectively form small polyplexes. We hypothesize that in the complexes formed PEG blocks orientate towards the surface as indicated by the reduced zeta potential while PEI and DNA are located in the inner part of the complexes. Due to the flexibility of the molecule, PEG and PEI domains might form within the molecule and the PEG domains might orientate towards the surface of the complexes.

One disadvantage of size determination by photon correlation spectroscopy is that it only gives average diameters and polydispersities, but does not give information on the shape of particles or whether complexes form distributed networks. We have therefore employed atomic force microscopy and results, which corresponded very well to the PCS data, will be published elsewhere.

Two hours after injection, all complexes showed highest accumulation in the liver, followed by kidney and spleen. Nakane and colleagues (22) have proposed that cationic macro-

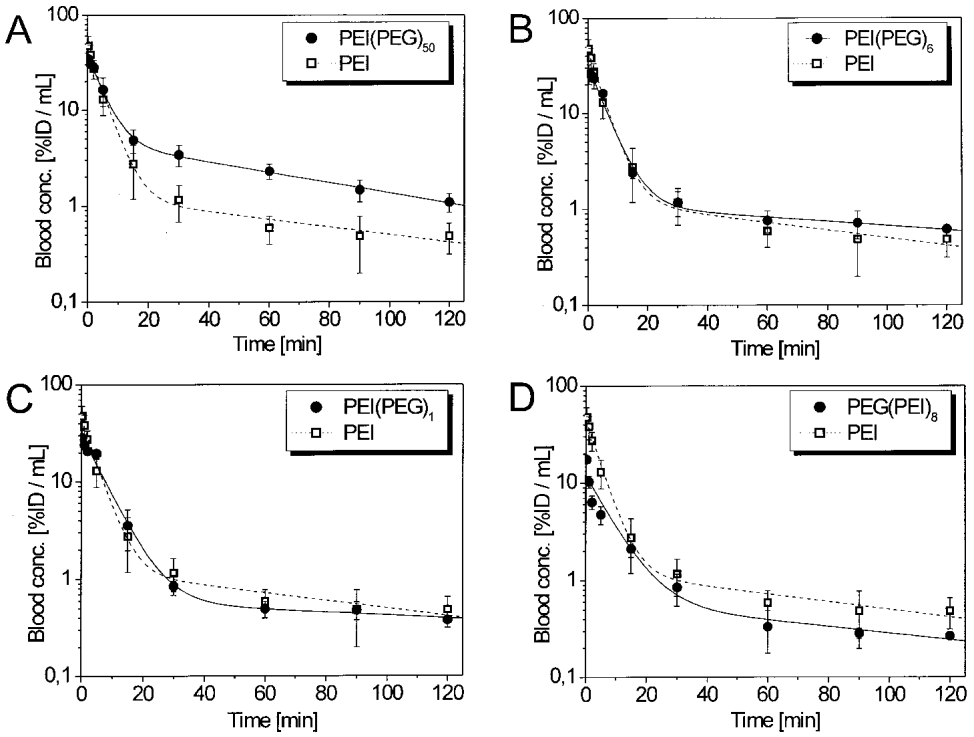


Fig. 4. Blood concentration time profiles of polyplexes formed by different copolymers of PEI and PEG in comparison to unmodified PEI at a dose of 1 μg PEI and N/P ratio of 6. Plots show mean concentration of three animals for each time point with error bars indicating standard deviation. Line graphs are fits through the mean concentrations for each time point employing a biexponential disposition equation. (A) $PEI(PEG)_{50}$ showed elevated blood levels. (B) $PEI(PEG)_6$ and (C) $PEI(PEG)_1$ did not cause changes compared to PEI. (D) $PEG(PEI)_8$ showed reduced blood concentrations.

molecules are taken up by the liver through adsorption to the surface of liver parenchymal and non-parenchymal cells in the space of Disse. However, fenestrae in liver sinusoids are believed to be smaller than the size of the injected complexes and it is questionable if they are able to freely diffuse through them. More probably, opsonization of the polyplexes as found by Plank and co-workers (4) leads to rapid clearance by the mononuclear phagocytic (MPS) system. Uptake by MPS would be in agreement with the observed liver and spleen accumulation.

PEGylation of PEI is expected to decrease opsonization of copolymer/DNA complexes in a similar fashion as for PEGylated stealth liposomes (for review see (23)). In fact, we found decreased spleen accumulation for all copolymers but liver accumulation was increased compared to PEI. This might be caused by the differing sizes of the complexes. Litzinger and co-workers (24) found that smaller liposomes showed a shift towards the liver when compared to larger ones. However, we examined organ distribution two hours after injection when the injected dose is almost completely

Table III. Pharmacokinetic Parameters of Injected Polyplexes

Polymer	N/P	Polymer dose [μg]	AUC [%ID/mL^{-1}*min]	A [%ID/mL]	$t_{1/2,alpha}$ [min]	B [%ID/mL]	$t_{1/2,beta}$ [min]
PEI	3.5	10	288 ± 79	27.9 ± 0.1	3.0 ± 0.9	2.1 ± 0.2	55 ± 10
	6.0	10	223 ± 8	25.8 ± 7.1	3.3 ± 0.5	1.1 ± 0.2	67 ± 20
	6.0	1	334 ± 121	44.7 ± 8.7	3.0 ± 0.5	1.3 ± 0.5	78 ± 13
	6.0	0.2	640 ± 217	40.0 ± 7.4	6.5 ± 0.3	2.0 ± 1.3	111 ± 57
$PEI(PEG)_{50}$	3.5	10	624 ± 301	15.9 ± 6.7	1.6 ± 0.2	2.1 ± 0.7	184 ± 34
	6.0	10	278 ± 65	15.4 ± 0.3	1.5 ± 0.2	2.7 ± 0.3	62 ± 12
	6.0	1	542 ± 32	34.5 ± 5.7	3.0 ± 0.2	4.9 ± 2.0	60 ± 18
	6.0	0.2	704 ± 91	34.5 ± 5.5	6.5 ± 1.0	5.5 ± 0.9	48 ± 12
$PEI(PEG)_6$	6.0	1	457 ± 8	31.0 ± 6.3	3.7 ± 0.4	1.1 ± 0.4	214 ± 83
$PEI(PEG)_1$	6.0	1	419 ± 199	30.9 ± 4.3	4.5 ± 0.7	0.7 ± 0.1	247 ± 206
$PEG(PEI)_8$	3.5	10	197 ± 23	18.6 ± 1.4	1.5 ± 0.1	1.7 ± 0.4	67 ± 25
	6.0	10	199 ± 96	13.5 ± 2.0	2.0 ± 0.9	1.7 ± 0.4	73 ± 61
	6.0	1	129 ± 26	15.2 ± 2.5	1.8 ± 1.5	2.8 ± 1.7	28 ± 13

Note: Values were calculated by fitting individual concentration time curves to a biexponential disposition model with the Software Kinetica 1.1 from Simed. Values are the means of three animals ± standard deviation. Increased area under the curve was found for $PEI(PEG)_{50}$ compared to PEI under all conditions tested.

cleared from blood stream. Therefore, we cannot speculate about the rate of organ uptake so that a slower uptake by the liver for the copolymer/DNA complexes compared to PEI/DNA is possible. Upcoming studies with determination of organ distribution after a variety of time intervals are expected to yield further information of the time dependence of body distribution.

It is also important to note that lung accumulation is relatively low for all injected complexes although the lungs have been found the main organ of gene expression after systemic injection of PEI/plasmid complexes (25). One possible explanation for this somewhat surprising observation is that biodistribution of the complexes and gene expression do not necessarily coincide. Redistribution from lungs to liver and spleen as has been observed for liposomes (26) might also happen to the polyplexes in our study. Again, organ examination at different time points is necessary to verify this hypothesis. Furthermore, different tissues show different levels of response to the same amounts of DNA (27). This means that the high gene expression found in the lungs by Bragonzi (25) is not necessarily due to high accumulation of DNA or polyplexes, respectively. In addition, polyplexes with oligos are known to behave differently from polyplexes formed with plasmids (28) in terms of physico-chemical properties and biologic activity. Therefore, it can not be assumed that polyplexes with plasmids and with oligos show the same biodistribution. Third, these studies have examined biodistribution of the DNA contained in polyplexes while we have labeled the polymers and examined their biodistribution. Double labeling of polymer and DNA would be necessary to explore the stability and distribution of polyplexes under *in vivo* conditions.

The blood levels show the expected bi-exponential disposition curve. A dose dependency of AUC was found in the way that increased doses lead to decreased AUCs with smaller half-life times in the distribution phase. A possible explanation for this unexpected behavior could be increased complement activation or aggregation with entrapment in capillary beds for the higher doses, caused by the higher concentration of the injected solutions.

An increase in blood levels was found for $PEI(PEG)_{50}$. This is most certainly caused by the shielding effect of PEG, which reduces opsonization and therefore leads to a slower MPS uptake. However, the prolongation in circulation seems to depend on the exact composition of the copolymers and no significant effect was found for the two other copolymers of the $PEI(PEG)_x$ type. Grafting of many short PEG blocks obviously leads to a stronger shielding of the complexes than fewer larger blocks when the weight ratio of PEG to PEI is kept constant. We assume that this is due to a more dense distribution of PEG chains on the complex surface.

For polymers grafted on solid surfaces two regimen have been distinguished (29) designated mushroom and brush regimen for low and high grafting densities (30). Only a brush regimen offers effective shielding since in a mushroom regimen only a part of the surface is covered by PEG. Interestingly, these results are contrary to complex size measurements. Probably a very dense order of PEG is needed for the avoidance of interaction with blood components and MPS, while a sufficiently high PEG block length might be necessary to avoid interaction of copolymer molecules with each other and thereby aggregation. Maybe the coupling of numerous PEG blocks with molecular weight between 550 and 5000 could lead to a $PEI(PEG)_x$ copolymer that offers both reduced aggregation and more prolonged circulation. Synthesis and evaluation of a larger variety of copolymers with a series of copolymers with same PEG block length but different amount of blocks per PEI core as well as a row with the same amount of PEG blocks per PEI core but with different block length should allow a more detailed analysis of the factors responsible for complex size, zeta potential, body distribution and blood levels.

In summary, we have shown that PEG to PEI orientation, PEG block length and substitution degree determine physico-chemical properties as well as *in vivo* behavior of PEI-PEG block copolymers. All tested copolymers lead to reduced complex sizes with DNA compared to PEI. Within the copolymers of the $PEG(PEI)_x$ type, the one with the highest substitution degree showed a prolonged circulation. PEI-PEG copolymers are therefore promising candidates for *in vivo* oligonucleotide delivery. Investigations on complement activation and time dependence of organ distribution might help to elucidate the exact mechanisms of polyplex elimination from the blood stream as well as to clarify the importance of PEG molecular weight and substitution degree. Thereby further optimization of our copolymers should be possible.

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