EFFECT OF POLY(ETHYLENE IMINE) MOLECULAR WEIGHT AND PEGYLATION ON ORGAN DISTRIBUTION AND PHARMACOKINETICS OF POLYPLEXES WITH OLIGODEOXYNUCLEOTIDES IN MICE

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ABSTRACT:

The in vivo body distribution and the pharmacokinetics of a 20mer double-stranded nuclear factor X b decoy oligodeoxynucleotide (ODN) complexed with 25-kDa poly(ethylene imine) (PEI), low molecular weight 2.7-kDa PEI, and PEGylated PEI [bPEI(25k)-g-PEG(550)gal] after intravenous injection were studied in BALB/c mice using a double-labeling technique to follow simultaneously the distribution of both complex components. The polymers were radioactively labeled with 125I by Bolton-Hunter reagent and the decoys with /H9253/H9260 double-stranded nuclear factor X b DNA with /H9253/H9260-32P. In contrast to the behavior of free ODN, the complexes were mainly distributed into liver and spleen. Whereas the organ concentrations of 125I remained very high over 12 h, the 32P values of ODN decreased in a time-dependent manner, likely due to separation of the complexes and degradation of the DNA. Although PEGylated PEI demonstrated a slower 125I-uptake into the RES organs compared with 25-kDa PEI due to the shielding effect of PEG [poly(ethylene glycol)], it was not able to better stabilize the complexes in the circulation or protect DNA from degradation.

Gene therapy using nonviral vectors promises considerable advances in the treatment of many severe genetic and acquired diseases due to their safety and low immunogenicity profiles. However, the in vivo application of gene drug delivery systems is still a great challenge (Dash et al., 1999; Merdan et al., 2002). Rapid elimination from the circulation, interactions with biological fluids and extracellular matrix, nonspecific accumulation in organs, and rapid degradation of the nucleic acids were reported as drawbacks for successful systemic gene delivery. Particularly, the delivery of gene carriers to disseminated and widespread disease targets or metastatic nodules can only be achieved by systemic administration and have to overcome these obstacles.

Many reports about the use of the polycation poly(ethylene imine) (PEI) as a nonviral gene delivery system for in vivo applications were published during the last few years (Boussif et al., 1995; Lemkine and Demeneix, 2001). Due to its high ability to protect DNA and RNA against degradation as well as the intrinsic endosomolytic activity based on the buffering capacity, PEI gained considerable attention (Behr, 1997). Copolymers of PEI and hydrophilic poly(ethylene glycol) (PEG) have been designed to provide a stealth-like shield around polymer/DNA complexes, reducing nonspecific interactions, inhibiting activation of the reticuloendothelial system, and prolonging the half-life of the complexes in the blood (Ogris et al., 1999; Nguyen et al., 2000; Ochietti et al., 2002). The influence of copolymer structure regarding PEG block length and the degree of substitution on in vivo behavior was shown previously (Kunath et al., 2002).

To characterize body distribution and pharmacokinetics of polymer/DNA complexes in vivo, different techniques were described. Many reports focused on quantification of gene expression as the last step of the transfection process using marker genes such as luciferase and green fluorescent protein, but in studies that were mostly limited to specific organs or cell types (Oh et al., 2001). Others followed the body distribution of DNA that was radioactively labeled with 125I, 123I, and 35S (Liu et al., 1997; Dash et al., 1999) or fluorescently labeled with a fluorescent dye such as rhodamine-PEI.

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ABBREVIATIONS: PEI, poly(ethylene imine); AUC, area under the curve; LMW, low molecular weight; NF-xB nuclear factor X b; ODN, oligodeoxynucleotide; PEG, poly(ethylene glycol); T4 PNK, T4 polynucleotide kinase; TCA, trichloroacetic acid; PEI(PEG)350, bPEI(25k)-g-PEG(550)gal; N/P ratio, total nitrogen of the polymer per DNA phosphate ratio; %ID, percentage of injected dose; ANOVA, analysis of variance; RES, reticuloendothelial system.
labeled with fluorescein isothiocyanate and rhodamine (Yockman et al., 2003). To deal with the limited stability of some of these label bindings, these techniques were used especially for short-term studies. Southern blots (Kircheis et al., 2001) and polymerase chain reaction (Oh et al., 2001) were used to characterize the quantity as well as the stability of DNA in organs. However, all these reports investigated mainly the distribution of DNA and neglected the distribution fate of the polymers in vivo. Information about organ accumulation, excretion, and half-life time of the polymers is important with regard to repeated, long-term, and depot applications. Previously, we reported the body distribution and pharmacokinetics of 125I-labeled 25-kDa PEI and its PEGylated derivatives and found a relationship between polymer structure and in vivo behavior (Kunath et al., 2002). However, to explain some of our observations, simultaneous determination of DNA and polymer distribution would be necessary.

Therefore, we took the next step and investigated in the present study the time-dependent organ distribution and pharmacokinetics of double-labeled complexes based on 125I-PEI gene delivery systems with a 20mer double-stranded 32P-labeled NF-xB decoy oligodeoxynucleotide over a period of up to 12 h. To the best of our knowledge, only one report has been published about a double-labeling technique using fluorescent markers to determine the biodistribution of lipopolymer/plasmid complexes after intratracheal injection (Yockman et al., 2003). In contrast to many reports (Mann and Dzau, 2000) that described the administration of stabilized phosphorothioate decoys, we used nonmodified phosphodiester decoys. Two PEI homopolymers of different molecular weights were chosen to investigate the influence of molecular size on in vivo behavior: the commercially available 25-kDa PEI widely used for in vivo applications and the low molecular weight (LMW)-PEI of 2.7 kDa synthesized in our laboratory. The LMW-PEI gained specific attention due to lower cytotoxicity as well as higher transfection efficiencies in vitro compared with commercial products (Fischer et al., 1999; Kunath et al., 2003). To examine the effect of PEGylation on in vivo distribution, the block copolymer bPEI(25k)-g-PEG(550) [shortened form, PEI(PEG)30], consisting of 25-kDa PEI grafted with 50 chains per molecule of a linear 550-Da PEG, was included in the study, which showed a prolonged circulation time in the blood compared with the nonmodified 25-kDa PEI (Kunath et al., 2002).

Materials and Methods

Polymers and Oligonucleotides. Branched 25-kDa PEI was purchased from BASF AG (Ludwigshafen, Germany). The LMW-PEI (2.7-kDa) was synthesized by acid-catalyzed ring-opening polymerization of aziridine in aqueous solution, purified, and characterized as previously described (von Harpe et al., 2000). The synthesis and characterization of the block copolymer PEI(PEG)30 was reported by Kunath et al. (2002). Briefly, poly(ethylene glycol)-monomethylether (550-Da; Rapp Polymere, Tübingen, Germany) was reacted after activation with hexamethylene diisocyanate (Fluka, Deisenhofen, Germany) with 25-kDa PEI in anhydrous chloroform at 60 °C for 5 min, followed by annealing by a stepwise cooling down to ambient temperature.

Radioactive Labeling of Polymers and Oligodeoxynucleotides. The polymers were labeled using N-succinimidyl-3-(4-hydroxy-3-125I)iodophenyl)propionate (PerkinElmer Life and Analytical Sciences, Boston, MA) by the method of Bolton and Hunter as published elsewhere (Bolton and Hunter, 1973; Kunath et al., 2002). Briefly, 60 μl of polymer solution (1 mg/ml) were reacted for 60 min in 0.1 M borate buffer, pH 8.5, with 100 μCi of Bolton-Hunter reagent dissolved in dried dimethyl sulfoxide. The product was purified on a Sephadex G-25 column (PD10; Amersham Biosciences Inc., Piscataway, NJ) with 10 mM HEPES buffer containing 150 mM NaCl, pH 7.4. For collection of the product, 800-μl fractions were sampled, and the radioactivity was measured in the gamma counter (Cobra II Auto Gamma; PerkinElmer Life and Analytical Sciences).

The oligonucleotides were radioactively labeled by a 5'-end-labeling technique using T4 polynucleotide kinase (T4 PNK; Fisher Scientific, Houston, TX) and [γ-32P]ATP (PerkinElmer Life and Analytical Sciences) according to the manufacturer’s protocol. Briefly, 10 pmol of oligodeoxynucleotide were mixed with 20 pmol of [γ-32P]ATP and 6 U of T4 PNK reaction buffer, and incubated at 37 °C for 1 h. The reaction was terminated at 65 °C for 5 min. Unincorporated nucleotides were separated from the 32P-labeled product by centrifugation on a Probe Quant G-50 micro spin column (Amersham Biosciences Inc.) at 800g for 2 min. The purity of the product was controlled by precipitation of the oligodeoxynucleotides with trichloroacetic acid (TCA). Ice-cold TCA (500 μl; 20% v/v) was added to a mixture of 1 μl of sample and 10 μl of sheared salmon sperm DNA (10 mg/ml; Brinkmann Instruments, Westbury, NY), vortexed, and incubated on ice for 10 min. After 5 min of centrifugation (4300g, 4 °C), the supernatant was collected and the pellet was diluted in 500 μl of 1 N NaOH. For counting in the Beckman LS 6000SC Liquid Scintillation System (Beckman Coulter, Fullerton, CA), samples were diluted in 4 ml of ScintiSafe Econo 2 (Fisher Scientific). The TCA-precipitable fraction of radioactivity was calculated as % (precipitable fraction) = cpm pellet × 100/(cpm pellet + cpm supernatant). Only batches with precipitation >95% were used for the experiments.

Preparation of the Polymer/DNA Complexes. The desired amounts of decoy and polymer were each diluted in 5% glucose and vortexed. After 10 min at room temperature, the polymer solution was added to the DNA solution and the resulting mixture was vortexed again. The complexes were allowed to equilibrate 10 min at room temperature before use. The complex composition was characterized by total nitrogen (N) of the polymer per DNA phosphate (P) and expressed as N/P ratio. All complexes were prepared at a N/P ratio of 6 as described earlier (Kunath et al., 2002). Nonlabeled ODN was mixed with 32P-ODN to reach a final ratio of 125I (dpm)/32P (dpm) of 3:1.

Organ Distribution and Pharmacokinetics. Male BALB/c mice (approximate weight 20–25 g) were obtained from Charles River Laboratories Inc. (Wilmington, MA). The animal experiments were approved by the local Animal Care Committee and followed the National Institutes of Health guidelines.

Ketamine (Ketaset; Fort Dodge Animal Health, Ford Dodge, IA) and xylazine (Rompun; Bayer Corp., Shawnee, KS) were used to anesthetize the mice at an initial dose of 100 mg/kg and 4 mg/kg i.m., respectively. The left jugular vein was exposed at the level of the clavicle for intravenous bolus injection, and 80 μl of the complex solution corresponding to 1 μg of poly(ethylene imine) per mg. For the copolymer, dose was related to PEI, not to total copolymer. For organ sampling, animals were sacrificed by decapitation 15 min, 2 h, and 12 h after injection, and organs (fat, liver, spleen, kidney, heart, lung, cortex), blood, and urine were collected and weighed.

For pharmacokinetic studies, a PE-10 catheter was placed in a retrograde direction into the right common carotid artery. Arterial blood samples were drawn after nine different time points from 20 s to 120 min, and the sample volume (30 μl) was replaced immediately with physiologic saline containing 10 U/ml heparin (Heparin sodium, USP, Elkins-Sinn Inc., Cherry Hill, NJ, USA). After the last sampling time (120 min), mice were decapitated and organs and blood were removed for analysis. Plasma was obtained by centrifugation of blood at 2000g and 4 °C for 10 min. The TCA-precipitation of 5 μl of each blood and plasma sample was performed according to the radioactivity isolated organs, 5 μl of blood and 5 μl of plasma were each dissolved in 1 ml of Soluene (PerkinElmer Life and Analytical Sciences) and diluted with 15 ml of Hionic-Fluo (PerkinElmer Life and Analytical Sciences). Measurements were run on the LS 6000SC Liquid Scintillation System. Cross-channel corrections and corrections for decay were performed. The applicability and reproducibility of the technique were confirmed by measuring complexes of 32P-ODN and 125I-polymer over a wide range of concentrations and N/P ratios (data not shown).

Calculations. The organ radioactivity was expressed as percentage of injected dose (%ID/organ and %ID/g) and the blood and plasma radioactivity as %ID/ml. The 15-min values of organ distributions were corrected using the...
complexes decreased clearance of the $^{32}$P-ODN from the circulation of blood cells. PEI(PEG)$_{50}$ showed similar results to those for blood, with the exception of $V_t$ for PEI(PEG)$_{50}$ samples. The declines in the concentration-time data were fitted to a biexponential disposition function: $C(t) = Ae^{-at} + Be^{-bt}$, with the WinNonlin Standard 2.1 software (Pharsight, Mountain View, CA). Iterative reweighting with $1/(C_{calc})^2$ was applied, with $C_{calc}$ corresponding to the estimated concentration. Diagnostic plots and statistical significance between the different polymers. In contrast, the concentration-time curves for the PEI derivatives were fully inspected, and alternative models (monoexponential and triexponential equations) could be rejected. Statistical comparisons were performed with GraphPad InStat 3.0a for Macintosh (GraphPad Software Inc., San Diego, CA; www.graphpad.com), using the $t$ test (two groups) or one-way ANOVA (multiple groups) followed by the Tukey-Kramer multiple comparisons test.

**Results**

**Pharmacokinetics of Free ODN and ODN/Polymer Complexes.**

The plasma concentration time course of the free ODN is shown in Fig. 1A and serves as reference for the kinetics of the ODN complexes with the PEI derivatives. These data represent TCA-precipitable $^{32}$P-ODN only. The plasma profile fitted by a biexponential model revealed a fast disposition phase and a second phase of slower elimination. Already after 10 min, less than 50% of the total plasma $^{32}$P activity was TCA-precipitable, and after 20 min, less than 25% of the ODN remained intact. The kinetics in whole blood and plasma following i.v. bolus injection of dual labeled polyplexes are shown in Fig. 2. It depicts the concentrations of the intact (TCA-precipitable) fractions of the PEI derivatives and of the ODN from 0 to 2 h. Table 1 shows the corresponding pharmacokinetic parameters of DNA and injected complexes obtained by fitting the data to a biexponential disposition equation.

Comparison of blood concentration-time curves of $^{32}$P and $^{125}$I for the individual polymers revealed that these tracers have similar initial concentration values $C_{(0)}$ (Fig. 2, A, C, and E). According to the initial volumes of distribution, $V_{(0)}$ (Table 1), which correspond well to the total blood volume in mice (Diehl et al., 2001), the polyplexes apparently distributed in the blood space after administration. For blood samples, no significant differences occurred either between the two tracers or between the different polymers (one-way ANOVA of all $V_{(0)}$ values, based on blood samples). Measurement of the plasma samples yielded results similar to those for blood, with the exception of the 25-kDa PEI. Polyplexes formed with the 2.7-kDa PEI and the PEI(PEG)$_{50}$ showed $V_{(0)}$ values for both $^{32}$P and $^{125}$I compatible with an initial distribution in plasma, with little binding to blood cells (i.e., the value of plasma $V_{(0)}$ was about half the corresponding value in blood; see Table 1). In contrast, the $V_{(0)}$ values in blood and plasma for $^{32}$P and $^{125}$I were discordant after administration of polyplexes made with 25-kDa PEI; the $V_{(0)}$ values of $^{32}$P-ODN in blood ($1.93 \pm 0.32$ ml) and plasma ($1.07 \pm 0.09$ ml) were compatible with distribution in plasma, whereas the $V_{(0)}$ values of $^{125}$I-25-kDa PEI in blood (2.25 $\pm$ 0.64 ml) and plasma (3.77 $\pm$ 1.47 ml) indicated significant binding to blood cells.

When compared with the administration of free ODN, all polymer complexes decreased clearance of the $^{32}$P-ODN from the circulation (see Figs. 1 and 2, B, D, and F). The corresponding pharmacokinetic parameters obtained by fitting the data to a biexponential disposition equation are given in Table 1. Noticeably, the declines in the concentration-time curves for the two tracers were not coincident. This indicates that disintegration of polyplexes began within the first few minutes after i.v. injection.

Evaluation of the AUC of intact $^{32}$P-ODN in either blood or plasma following administration of the ODN as polyplexes revealed no statistical significance between the different polymers. In contrast, the pharmacokinetic behavior of the $^{125}$I-labeled polymers themselves differed significantly: compared with the non-PEGylated parent compound, 25-kDa PEI, the PEI(PEG)$_{50}$ derivative displayed a 3.66-fold higher blood AUC$_{0\rightarrow120}$ ($p < 0.05$). The differences were even more pronounced in the plasma samples: the plasma AUC$_{0\rightarrow120}$ was 5.5-fold
higher for PEI(PEG)50 (p < 0.01) and 5.1-fold higher for 2.7-kDa PEI than for 25-kDa PEI (p < 0.05). This apparently results from differences in binding to blood cellular elements among the polymers. In fact, PEI(PEG)50 and 2.7-kDa PEI had 2- to 3-fold higher concentrations and AUCs in plasma versus blood, indicating lower binding to blood cells (Fig. 2; Table 1). In contrast, the concentrations and AUC for 25-kDa PEI were similar in blood and plasma. Therefore, about 50% of 25-kDa PEI appeared to be bound by blood cells. The partial disintegration of polyplexes and binding of the free 25-kDa PEI to blood cells were rapid events, as evident from the discrepancy in C(o) and V(o) between 32P-ODN and 125I-PEI in plasma (Table 1; Fig. 2B).

Organ Distribution. Figures 3 to 7 illustrate the organ distributions of the tracers after 15 min, 2 h, or 12 h. To facilitate comparisons, all data are expressed in units of percentage injected dose per gram (%ID/g) and percentage injected dose per organ (%ID/organ). The distribution after administration of free 32P-ODN was not studied at
the later time points because blood concentrations had already declined by 15 min to about 1% of the initial values (Fig. 1).

As shown in Fig. 1B, 15 min after dose, the $^{32}$P radioactivity of noncomplexed ODN was found primarily within kidney (10.40 ± 1.21% ID/organ) and liver (8.44 ± 0.28% ID/organ), and very low levels (<0.5% ID/organ) in all other organs tested. On a weight basis (%ID/g), kidney (39.82 ± 4.39%) and urine (54.17 ± 14.66%) showed the highest amount of radioactivity, suggesting a rapid excretion of the $^{32}$P-ODN by the renal pathway (Fig. 1C).

Administration of PEI(PEG)$_{50}$/ODN complexes at N/P ratio 6 resulted in a $^{32}$P-body distribution profile, which was very similar to the distribution of free ODN after 15 min with regard to the pattern of organ accumulation (Fig. 3, A and B). Kidney (6.79 ± 1.16% ID/organ) and liver (6.64 ± 0.73% ID/organ) showed the highest con-

### Table 1

<table>
<thead>
<tr>
<th>Polymer Sample Type</th>
<th>Label</th>
<th>A</th>
<th>B</th>
<th>Initial Volume ($V_0$)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood $^{125}$I</td>
<td>PEI(PEG)$_{50}$</td>
<td>19.7 ± 3.2</td>
<td>19.6 ± 3.0</td>
<td>2.60 ± 0.21</td>
<td>1559 ± 187</td>
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<tr>
<td>Blood $^{32}$P</td>
<td>PEI(PEG)$_{50}$</td>
<td>30.4 ± 4.6</td>
<td>2.49 ± 0.65</td>
<td>278 ± 129</td>
<td>3.27 ± 0.50</td>
</tr>
<tr>
<td>Plasm $^{125}$I</td>
<td>PEI(PEG)$_{50}$</td>
<td>51.6 ± 2.3</td>
<td>25.8 ± 6.3</td>
<td>86.1 ± 17.7</td>
<td>N.D.</td>
</tr>
<tr>
<td>Plasm $^{32}$P</td>
<td>PEI(PEG)$_{50}$</td>
<td>55.5 ± 8.6</td>
<td>3.37 ± 0.83</td>
<td>75.6 ± 16.7</td>
<td>N.D.</td>
</tr>
<tr>
<td>Blood $^{125}$I</td>
<td>25-kDa PEI</td>
<td>57.1 ± 21.7</td>
<td>2.86 ± 0.74</td>
<td>81.6 ± 25.6</td>
<td>2.25 ± 0.64</td>
</tr>
<tr>
<td>Blood $^{32}$P</td>
<td>25-kDa PEI</td>
<td>54.8 ± 9.6</td>
<td>1.90 ± 0.31</td>
<td>267 ± 130</td>
<td>1.93 ± 0.32</td>
</tr>
<tr>
<td>Plasm $^{125}$I</td>
<td>25-kDa PEI</td>
<td>29.4 ± 13.3</td>
<td>2.78 ± 1.96</td>
<td>50.7 ± 21.7</td>
<td>N.D.</td>
</tr>
<tr>
<td>Plasm $^{32}$P</td>
<td>25-kDa PEI</td>
<td>91.6 ± 7.5</td>
<td>2.46 ± 0.51</td>
<td>90.0 ± 27.6</td>
<td>N.D.</td>
</tr>
<tr>
<td>Blood $^{125}$I</td>
<td>2.7-kDa PEI</td>
<td>30.0 ± 1.5</td>
<td>1.47 ± 1.89</td>
<td>83.6 ± 20.3</td>
<td>2.48 ± 0.21</td>
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<td>Blood $^{32}$P</td>
<td>2.7-kDa PEI</td>
<td>36.3 ± 4.4</td>
<td>2.42 ± 0.83</td>
<td>735 ± 411</td>
<td>2.79 ± 0.45</td>
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<td>Plasm $^{125}$I</td>
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<td>53.8 ± 8.4</td>
<td>5.35 ± 2.63</td>
<td>94.7 ± 32.5</td>
<td>N.D.</td>
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<td>Plasm $^{32}$P</td>
<td>2.7-kDa PEI</td>
<td>63.8 ± 15.9</td>
<td>3.09 ± 1.54</td>
<td>113 ± 11</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

**Fig. 3.** Organ distribution of free ODN and polyplexes formed by ODN and 25-kDa PEI, 2.7-kDa PEI, and PEI(PEG)$_{50}$ at a dose of 1 μg of poly(ethylene imine) and a N/P ratio 6, 15 min after injection. The distribution profile of the $^{32}$P values (A and B) was compared with that of the $^{125}$I values (C and D). Values are given as the mean ± S.E. of four animals as percentage of injected dose per gram (A and C) and per organ (B and D).
Fig. 4. Organ distribution of polycation/ODN complexes (N/P 6) given as mean ± S.E. (n = 4) of the percentage of injected dose per gram (A and C) and organ (B and D) 2 h postinjection (1 μg of PEI/mouse) with comparison of 32P levels (A and B) and 125I levels (C and D) of 25-kDa PEI, 2.7-kDa, and PEI(PEG)50.

Fig. 5. Comparison of organ distribution of 32P values (A and B) and 125I values (C and D) of ODN/polymer complexes with N/P ratio 6 and 1 μg of PEI/mouse, 12 h after intravenous administration. Values are percentage of injected dose per gram (A and C) and organ (B and D) given as the mean ± S.E. of four animals.
To further analyze the organ distribution of both tracers, the organ concentrations of $^{32}$P and $^{125}$I labels were plotted relative to each other (Figs. 6 and 7). All organs should fall on a straight line with a slope of 1, provided the polyplexes are stable and taken up intact by tissues. This scenario applies to the 15-min sampling time after administration of polyplexes based on 2.7-kDa and 25-kDa PEI (Fig. 6A). However, the polyplexes based on PEI(PEG)$_{50}$ display a distinct pattern (Fig. 6B). The two tracers accumulated in the organs to varying degrees, indicating that part of the $^{32}$P-ODN and $^{125}$I-PEI(PEG)$_{50}$ must have been released from the complexes.

At the later sampling times (2 h and 12 h), significant differences between the $^{32}$P and $^{125}$I tracers in terms of organ accumulation were obvious for all three types of complex. These differences are best illustrated in Fig. 7, which highlights the longer retention of the $^{125}$I label in the organs compared with $^{32}$P. The side by side comparison of the polymers (Figs. 3, 4, and 5) shows that after 2 h, an increase of $^{125}$I activity was observed for all three in liver and kidney, with a simultaneous decrease in the spleen, lung, and heart. By 2 h, the distribution of the PEI(PEG)$_{50}$ approached the distribution pattern of the homopolymers (Fig. 4, C and D), and this was also the case after 12 h (Fig. 5, C and D).

**Discussion**

The transcription factor NF-κB is involved in gene regulation during different cellular processes (Mann and Dzau, 2000). It is a central mediator of immune and stress responses, can exert pro- and antiapoptotic effects, and plays a key role in cell proliferation and inflammatory diseases. Classical cell types underlying the regulation of NF-κB are monocytes/macrophages, endothelial cells, smooth muscle cells, and T-lymphocytes. Therefore, the inhibition of the NF-κB activation would be a promising strategy for the treatment of diseases such as atherosclerosis, restenosis, arthritis, chronic colon disease, bacterial infections, and the metastasis of tumors.

In the present study, we investigated the influence of molecular weight and PEGylation of poly(ethylene imine) on the time-dependent organ distribution and pharmacokinetics of complexes with a 20mer double-stranded phosphodiester DNA NF-κB decoy oligodeoxyribonucleotide. No signs of toxicity or anaphylactic responses were observed under the described experimental conditions and the administered dose (equivalent to approximately 40 μg/kg poly(ethylene imine)).

In contrast to previous studies, we used a double-labeling technique to follow the distribution of both complex components simultaneously. The $^{125}$I-labeling of the polymers was chosen according to our previous studies (Kunath et al., 2002). In addition, a $^{32}$P-label was attached to the oligonucleotide using an enzymatic 5'-end-labeling technique. In pilot experiments with free ODN, we did not observe significant differences in the pharmacokinetic behavior of 5'-$^{32}$P-labeled ODN and $^3$H-ODN labeled at multiple internal thymidine bases (data not shown). In either case, metabolism of the free ODN after i.v. injection was rapid, which was apparent from the decline in the fraction of TCA-precipitable tracer in plasma samples. $^{32}$P-labeling has also recently been utilized in a pharmacokinetic study of polycation/plasmid-DNA complexes in mice (Upicky et al., 2002). These authors based their pharmacokinetic evaluation on total radioactivity. The measurement of intact versus total tracer using TCA precipitation, as performed in the present experiments for the plasma samples, provides additional information about the in vivo stability of the polyplexes in the circulation. We hypothesized that if there is a stabilizing and protective effect of the polymers on DNA, the tracer should also show enhanced stability in the circulation in vivo.

In accordance with studies reporting plasma half-lives of degradation of approximately 5 min for intravenously injected unmodified...
phosphodiester ODNs (Akhtar and Agrawal, 1997), our noncomplexed 20mer NF-κB decoy ODN was almost completely degraded after 15 min circulation in the blood as shown by TCA precipitation. Given the recently reported instability of free decoy ODN against nucleases present in the blood (Fischer et al., 2001), the rapid disappearance of ODN from the circulation may, rather, be due to metabolic degradation than to extensive uptake into tissues. Consistent with previous reports on oligonucleotide biodistribution (Akhtar and Agrawal, 1997; Takakura et al., 2001), we consider the hepatic and renal pathways as the major routes of excretion, since most of the label was found in liver, kidney, and urine already after 15 min. Miyao et al. (1995) and Sawai et al. (1996) described that single-stranded ODNs undergo glomerular filtration, but they are also subject to tubular reabsorption via a scavenger receptor-like mechanism and to

Fig. 7. Correlations of the 125I- and 32P-organ concentrations of decoy complexes with 2.7-kDa LMW-PEI (A and B), 25-kDa PEI (C and D), and PEI(PEG) 50 after 2 h (A, C, and E) and 12 h (B, D, and F), given as percentage of injected dose per gram.
uptake from the capillary side. Liver seems to play a role, not only due to the phagocytic activity. Also, scavenger receptors were reported to recognize a wide range of polyamines, since preadministration of polyamines was found to reduce the liver uptake of naked DNA (Takakura et al., 2001).

Complexation of the ODNs with polymers induced a higher and prolonged organ retention depending on the structure of the polymers. However, the main difference between the decoy-complexes of the homopolymer PEIs and the PEGylated PEI was in the stability of the complexes. Whereas after 15 min the distribution profiles of the $^{125}$I-homopolymers and the $^{125}$P-decoys correlated well with regard to percentage of injected dose in the organs, for the PEI(PEG)$_{50}$ major differences were obvious. Already 15 min after injection, the organ distribution of $^{32}$P-ODN complexed with the PEGylated PEI was comparable to that of noncomplexed ODN. PEGylated PEI did not mediate higher retention of $^{32}$P in the organs, as detected for the homopolymers. The degradation of the $^{32}$P-ODN was also shown in the TCA precipitation experiments where decoys complexed with PEI(PEG)$_{50}$ showed the lowest precipitable fraction of all polymers tested 15 min after intravenous administration. The pharmacokinetics followed the same trend. The lower stability of the polyplexes with PEGylated PEI could be due to the fact that the electrostatic interactions between polymer and DNA were inhibited by the PEG moieties. Weaker binding between the complex components may result in less compact structures and a less efficient protection against nucleases (Merdan, 2003). Similar observations were made by Mullenn et al. (2000) using poly-l-lysine derivatives. Compared with nonmodified polymers, substitution of poly-l-lysine ε-amino sites with PEG or hydrocarbon chains resulted in faster degradation of polyplexes, even at higher N/P ratios.

With regard to the pharmacokinetic behavior of the polymers, all of them showed the highest accumulation in the liver, followed by spleen, already after 15 min. Because liver and spleen represent the main organs of the RES and fenestrations of their endothelia are smaller than the size of the injected particles, a rapid opsonization of the polyplexes and subsequent clearance by phagocytic macrophages of the MPS seemed to occur. This is indirectly confirmed by the results of PEGylated PEI, whose hydrophobic and noncharged PEG shell is expected to decrease opsonization and therefore RES processing. Whereas the $^{125}$I levels of PEI(PEG)$_{50}$ after 15 min in liver and spleen were only about 50% of those observed for the 25-kDa PEI, they increased after 2 h to values comparable with those of the homopolymers. This indicates a slower uptake rate of the PEGylated polymer into the RES organs. Simultaneously, a higher amount of copolymer was circulating in blood.

The values of $^{125}$I for 25-kDa and 2.7-kDa PEI in liver and spleen were comparable with previously reported data, with a sampling time of 2 h (Kunath et al., 2003). The present study provides information on the change of organ distribution over time and on both complex components for these homopolymers. The equal distribution of $^{32}$P and $^{125}$I at the early sampling time (15 min) can be interpreted as evidence for organ uptake of intact complexes, indicating stability during the distribution phase. However, the discordant concentration time curves for $^{32}$P and $^{125}$I in blood and plasma, also argue in favor of an early disintegration of a fraction of the complexes. Although disintegration was evident for both homopolymers and for the PEI(PEG)$_{50}$, 25-kDa PEI seemed to be least affected. On the other hand, only when the ODN complexes with 25-kDa PEI were administered did the polymer seem to bind significantly to blood cells. Taken together, our findings reinforce the importance of in vivo pharmacokinetic experiments in the overall efforts to optimize non-viral vector systems. Simultaneous measurement of biodistribution of both components of the polyplexes gives valuable insight into the stability of polyplexes in vivo. The different molecular weights of the two investigated homopolymers resulted only in minor differences in organ distribution. PEGylation of PEI was intended as a steric barrier against opsonization, activation of the reticuloendothelial system, and rapid clearance from blood after intravenous injection. However, the effect was apparently limited to the pharmacokinetics and distribution of the polymer itself. Due to a weaker binding of the ODN, complex instabilities led to a rapid elimination of ODN formulated with PEGylated PEI from the body. Whereas the copolymers seem to be promising nonviral carriers for local administration, with regard to systemic applications, further modifications of the PEG-shell to stabilize the system and to increase the interaction with DNA are necessary. Additionally, higher doses of complexes will be investigated since it is conceivable that there is a saturable mechanism of complex destabilization that can be overcome or delayed with higher concentrations.

References


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