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The Impact of Molecular Weight and PEG Chain Length on the Systemic Pharmacokinetics of PEGylated Poly L-Lysine Dendrimers

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Abstract: The impact of PEGylation on the pharmacokinetics and biodistribution of ^{3}H-labeled poly L-lysine dendrimers has been investigated after intravenous administration to rats. The volumes of distribution, clearance and consequently the plasma half-lives of the PEGylated dendrimers were markedly dependent on the total molecular weight of the PEGylated dendrimer, but were not specifically affected by the PEG chain length alone. In general, the larger dendrimer constructs (i.e. >30 kDa) had reduced volumes of distribution, were poorly renally cleared and exhibited extended elimination half-lives ($t_{1/2}$ 1–3 days) when compared to the smaller dendrimers (i.e. <20 kDa) which were rapidly cleared from the plasma principally into the urine ($t_{1/2}$ 1–10 h). At later time points the larger dendrimers concentrated in the organs of the reticuloendothelial system (liver and spleen); however, the absolute extent of accumulation was low. Size exclusion chromatography of plasma and urine samples revealed that the PEGylated dendrimers were considerably more resistant to biodegradation in vivo than the underivatized poly L-lysine dendrimer cores. The results suggest that the size of PEGylated poly L-lysine dendrimer complexes can be manipulated to optimally dictate their pharmacokinetics, biodegradation and bioresorption behavior.

Keywords: Polyethylene glycol; dendrimer; poly L-lysine; pharmacokinetics; biodistribution

Introduction

The practical utility of drug candidates may be limited by unfavorable systemic pharmacokinetic properties such as high renal or hepatic clearance, short plasma half-lives, and limited distribution to sites of action. A number of colloidal and polymeric drug-carrier systems have been proposed and investigated in an attempt to prolong circulating half-lives,[1,2] improve cellular targeting[2,3] and reduce systemic toxicities.[4–7] The surface characteristics and size of these delivery systems

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typically dictate their pharmacokinetic behavior; however, the widespread application of macromolecular systems as parenteral delivery vehicles has historically been limited by rapid uptake by the phagocytic cells of the reticuloendothelial system (RES), resulting in reduced plasma half-lives and decreased systemic exposure.[8–12]

To circumvent this rapid plasma clearance, several groups have shown that modification with polyethylene glycol (PEG) may reduce the recognition of drug carriers by opsonic factors, decrease particle uptake by the RES and increase plasma circulation times. PEGylated delivery systems have been used to clinical advantage in the treatment of tumors and inflammation, since long circulating carriers inevitably accumulate at sites where the vascular architecture is compromised and capillary permeability is increased (the enhanced permeation and retention (EPR) effect).[13–15] PEGylation has also been utilized to enhance the utility of therapeutic proteins, since covalent attachment of PEG chains reduces immunogenicity, inhibits recognition by protease enzymes, and enhances protein stability and solubility.[16] However, increasing the PEG molecular weight above a critical threshold may result in reduced efficacy, presumably reflecting a reduction in receptor binding affinity.[17] While some colloidal systems have been approved for clinical use and many others are in phase I or II clinical trials,[18–20] the more widespread application of PEGylated colloidal drug delivery systems has, at least in part, been limited by their complexity and potential physical and chemical instability, issues which inevitably increase the complexity of the product approval process.

Dendritic polymer (dendrimer) based drug delivery systems provide a number of possible advantages over similar linear polymeric materials (reviewed by Qiu and Bae[21]) and have the potential to provide the drug targeting and pharmacokinetic advantages of typical colloidal or macromolecular delivery systems, but in the form of well-defined, chemically stable molecular entities with great flexibility in structure and surface functionality.[22] Dendrimers are characterized by a defined globular structure that is composed of a central core surrounded by a number of concentric polymeric layers. The stepwise synthesis of dendrimers was first described by Tomalia and colleagues in the early 1980s,[23] and the size, surface charge and solubility of these molecules can be precisely controlled by alteration of the size and functionality of both the core and surface repeating units. A number of studies have examined the potential utility of, for example, poly amidoamine (PAMAM) and polyester bowtie dendrimers, as vehicles for improved drug solubility, reduced systemic toxicity and enhanced drug delivery to tumor tissue.[24,25]

Most recently, interest in polyester or poly L-lysine (PLL) core dendrimers has increased due to the potential advantages of delivery systems based on biodegradable and (in the case of poly L-lysine) bioresorbable polymers.[24,26] Modified PLL-based dendrimers also provide useful antibiotic and antiviral properties in their own right.[27,28] We have recently described the systemic pharmacokinetic behavior of cationic PLL-based dendrimers possessing either 16 and 32 surface amine

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groups[29] and a series of anionic arylsulphonic and succinylated poly lysine dendrimers.[30] These studies described the influence of dendrimer size and charge on pharmacokinetic behavior, and provided evidence of the in vivo biodegradation and bioresorption of the PLL dendrimer cores. However, in all cases, the time scale of plasma exposure was relatively short and dendrimers were rapidly degraded or cleared by the cells of the RES. This highlights a key issue regarding the choice of core structure for a dendrimer vector, such that while biodegradation of the core is important in facilitating dendrimer clearance following liberation of drug, the core needs to be sufficiently stable to facilitate the extended circulation of the complex and therefore participation in the EPR effect. The current investigation describes approaches to modify these PLL dendrimer cores with PEG units in order to reduce the rate of in vivo biodegradation, decrease renal and RES clearance and enhance plasma exposure.

The influence of PEGylation on the pharmacokinetics of a series of related polyester bow-tie dendrimers has been described previously;[26] however, extrapolation of these data to the likely impact of changes to core size and PEG chain length on the pharmacokinetics of PLL dendrimers is not possible since the composition of the core and the shape of the dendrimer are likely to markedly affect both metabolic and dispositional characteristics. A further study by Okuda and colleagues[31] has made some preliminary observations of the influence of surface PEGylation on the pharmacokinetic behavior of PLL dendrimers; however, this study examined only a single generation six (G6) poly L-lysine core and did not evaluate in detail the influence of dendrimer core size, PEG chain length or the overall molecular mass of the PEGylated dendrimers on dendrimer pharmacokinetic profiles. Importantly, the impact of surface PEGylation on metabolism of the poly lysine core was also not evaluated. Finally the dendrimer structures evaluated comprised a statistical mixture of systems with differing degrees of surface PEGylation, yielding dendrimer mixtures with a high degree of polydispersity.

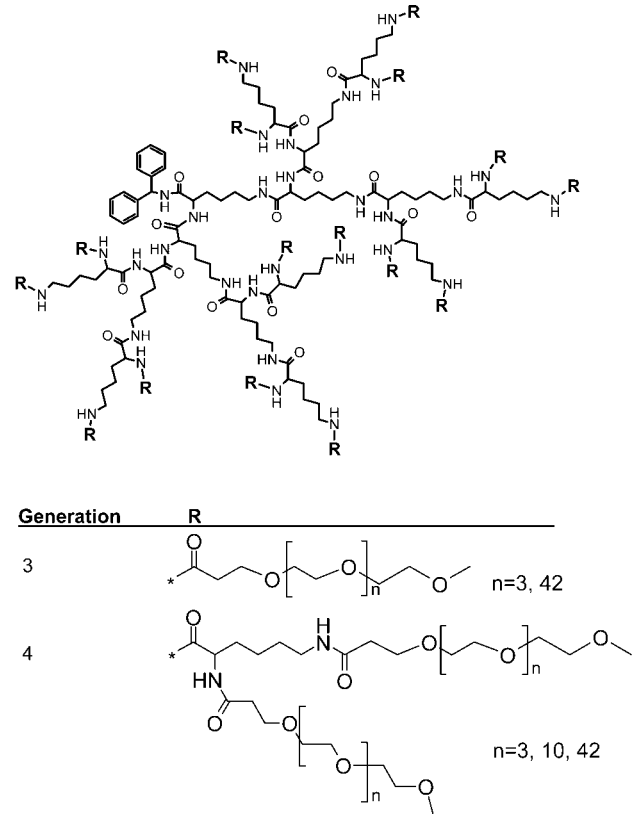

Figure 1. Structure of generation 3 and 4 PEGylated poly L-lysine dendrimers. N refers to repeating units of PEO to give the final PEG chains of MW 200, 570 or 2000 Da. ^{3}H radiolabel was located on the surface lysine residues. * indicates where the R group is attached to the dendrimer core.

In the current study therefore, we describe a synthetic approach that ensures a high degree of monodispersity of PEGylated material. This method has been utilized to generate dendrimers with ^{3}H-labeled generation 3 (16 surface lysine groups) and generation 4 (32 surface lysine groups) cores that are fully capped with PEG chains of varying molecular weight (MW 200, 570 and 2000) as illustrated in Figure 1. The pharmacokinetics and biodistribution profiles of these systems have subsequently been evaluated after intravenous administration in rats. Urinary and fecal excretion profiles have also been quantified, and the ^{3}H-labeled species present in plasma and urine investigated to provide an indication of the impact of PEGylation on biodegradation.

Methods and Materials

Materials. Chemicals were purchased from Aldrich and were used without further purification. Unlabeled lysine for synthesis was purchased from Bachem (Bunbendorf, Switzerland). Soluene-350 and Starscint were purchased from Packard Biosciences (Meriden, CT). Heparin (10,000 U/mL) was obtained from Faulding (SA, Australia). Saline was from Baxter Healthcare (NSW, Australia). Isopropyl alcohol was

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AR grade and was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). (L)-(4,5-^{3}H)-Lysine (1 mCi/mL) was purchased from MP Biomedicals (Irvine, CA). Polyethylene glycol (PEG, 200, 570 Da) for synthesis was purchased from Quanta Biodesign (Powell, OH), while PEG, 2000 Da was obtained from Nektar Therapeutics (Huntsville, AL). All other solvents were HPLC grade and were used without any further purification.

Synthesis and Characterization of Tritium Labeled Dendrimers. The preparation of defined tritium labeled dendrimer cores from L-lysine has been described in previous papers,[29,30] and the supplementary data to support these methods is available in the Supporting Information. In these dendrimer cores, the γ, δ tritiated L-lysine branching unit is attached via an amide bond to the outermost layer of dendrimers to give radiolabeled generation 3 (G3) and generation 4 (G4) dendrimers.

To support the description of the PEGylated dendrimers in the current publication, the following nomenclature has been adopted, where the more complete nomenclature (left-hand column) has been utilized in the detailed synthetic methods described in the Supporting Information (and previously for the synthesis of poly L-lysine dendrimers[1]), and the abbreviated nomenclature (right-hand column) has been utilized in the body of the text to enhance readability.

BHALys [Lys]$_4$ [^{3}H-Lys]$_8$ [PEG$_{200}$]$_{16}$	$\equiv$ Lys$_8$(PEG$_{200}$)$_{16}$
BHALys [Lys]$_8$ [^{3}H-Lys]$_{16}$ [PEG$_{200}$]$_{32}$	$\equiv$ Lys$_{16}$(PEG$_{200}$)$_{32}$
BHALys [Lys]$_8$ [^{3}H-Lys]$_{16}$ [PEG$_{570}$]$_{32}$	$\equiv$ Lys$_{16}$(PEG$_{570}$)$_{32}$
BHALys [Lys]$_4$ [^{3}H-Lys]$_8$ [PEG$_{2000}$]$_{16}$	$\equiv$ Lys$_8$(PEG$_{2000}$)$_{16}$
BHALys [Lys]$_8$ [^{3}H-Lys]$_{16}$ [PEG$_{2000}$]$_{32}$	$\equiv$ Lys$_{16}$(PEG$_{2000}$)$_{32}$

In Lys$_X$(PEG 200, 570, 2000)$_Y$ X refers to number of outermost lysine groups and Y refers to number of surface functional groups, where the bifunctional nature of lysine dictated that $Y = 2X$. The PEGylated functional groups utilized varied with molecular weight of 200 Da, 570 Da and 2 kDa. The radiolabel was located on the outermost lysine layer for each dendrimer (i.e. PEG was attached to the labeled lysine).

Detailed synthetic methods are available in the Supporting Information. To summarize, PEGylated dendrimers required multiple links of amide bonds to ensure complete coverage of the surface amine groups of the dendrimers. For the PEG$_{200}$ and PEG$_{2000}$ dendrimers (Schemes 1 and 2) multiple amide formations were achieved with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium-hexafluorophosphate (pyBop) in the presence of Hunig's base (DIPEA), and in some cases activated esters of PEGylated material were exploited. The pyBop coupling conditions involved adding an excess of pyBop on a "per amine" basis to a solution of the uncapped dendrimer as the TFA salt in dimethylformamide (DMF), followed by the addition of excess DIPEA and PEG group. The formation of multiple amide bonds to L-lysine dendrimers from the benzhydryl core has been described in more detail elsewhere.[30] The PEGylated (PEG$_{200}$ and PEG$_{2000}$) dendrimers were then purified by ultrafiltration on a Pall-Gellman stirred cell or minimate (with a 5 or 10 kDa cutoff) or by size exclusion chromatography on an LH20 Sephadex column as described in the Supporting Information. All fractions were analyzed by reverse phase LCMS (ESI +ve) and UV (diode array). The UV and ESI active fractions were combined and lyophilized to give the PEGylated dendrimer for pharmacokinetic analysis.

The preparation of Lys$_{16}$(PEG$_{570}$)$_{32}$ was achieved via an alternative method (Scheme 2). An excess of the activated *N*-hydroxy succinimide PEG ester on a "per amine" basis was added to a solution of the uncapped dendrimer as the TFA salt in a mixture of DMF and an excess triethylamine. The product was purified by preparatory HPLC on an Xterra Prep RP18 column, and the UV active fractions were combined and freeze-dried to give the PEGylated dendrimer.

The PEGylated dendrimers were characterized by ^{1}H NMR spectroscopy and HPLC/MS. Characteristic PEGylated peaks for all compounds were present in the ^{1}H NMR. Also, the integrals between the dendrimer structure and the various PEG functional groups provided a ratio of dendrimer to capping as a way of validating complete coverage. Using size exclusion chromatography with postcolumn fraction collection and scintillation counting of eluting tritium, we have also estimated polydispersity values for each PEGylated dendrimer and these are shown in the Supporting Information. Detailed analysis of the PEGylated dendrimers are also given in the Supporting Information. These analytical techniques certified the purity and identity for each targeted compound. HPLC/(ESI)MS data for the purified PEGylated dendrimers presented multiply charged ions which were deconvoluted to provide molecular weight identity for all targets.

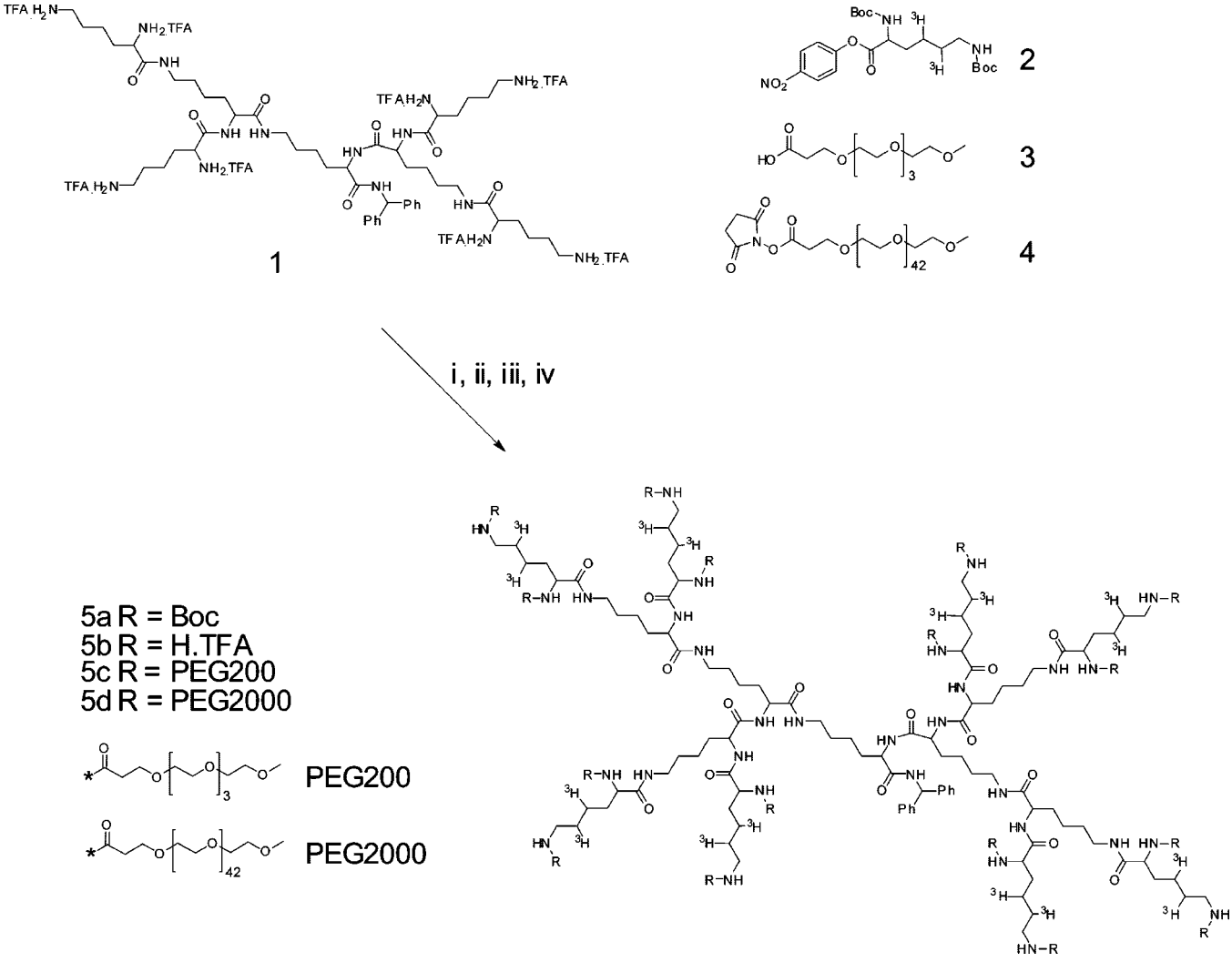
NMR spectra were recorded in D_2O on a Bruker (Bruker Daltronics Inc., NSW, Australia) 300 UltraShield 300 MHz NMR instrument. HPLC/(ESI)MS were conducted on a Waters (Millipore Corporation, Milford, MA) 2795 with 2996 diode array detector (DAD) coupled to a Waters ZQ4000 with ESI probe, inlet flow split to give approximately 50 μL/min to the MS. All dendrimers were diluted in PBS and frozen at -20 °C until used.

The specific radioactivity of the dendrimers was determined by dilution of a known mass of dendrimer (in triplicate) in 1 mL of Starscint, followed by scintillation counting using a Packard Tri-Carb 2000CA liquid scintillation analyzer (Meriden, CT). The specific activities of the dendrimers examined are listed in Table 1.

Animals. Rats (male, Sprague–Dawley, 270–350 g) were supplied by animal services at Monash University, Australia. Rats were maintained on a 12 h light/dark cycle and were fed standard rodent chow prior to surgery. Food was withheld after surgery and for 8 h after administration of the iv dose, but water was available at all times. Food was provided at all other times. All animal experiments were approved by the Victorian College of Pharmacy Animal Ethics Committee, Monash University (Melbourne, Victoria).

Intravenous Plasma Pharmacokinetics. Rats were cannulated via the right jugular vein and carotid artery under

Scheme 1. Synthetic Scheme for Generation 3 Pegylated Dendrimers: $Lys_8(PEG_{200})_{16}$ and $Lys_8(PEG_{2000})_{16}$[a]

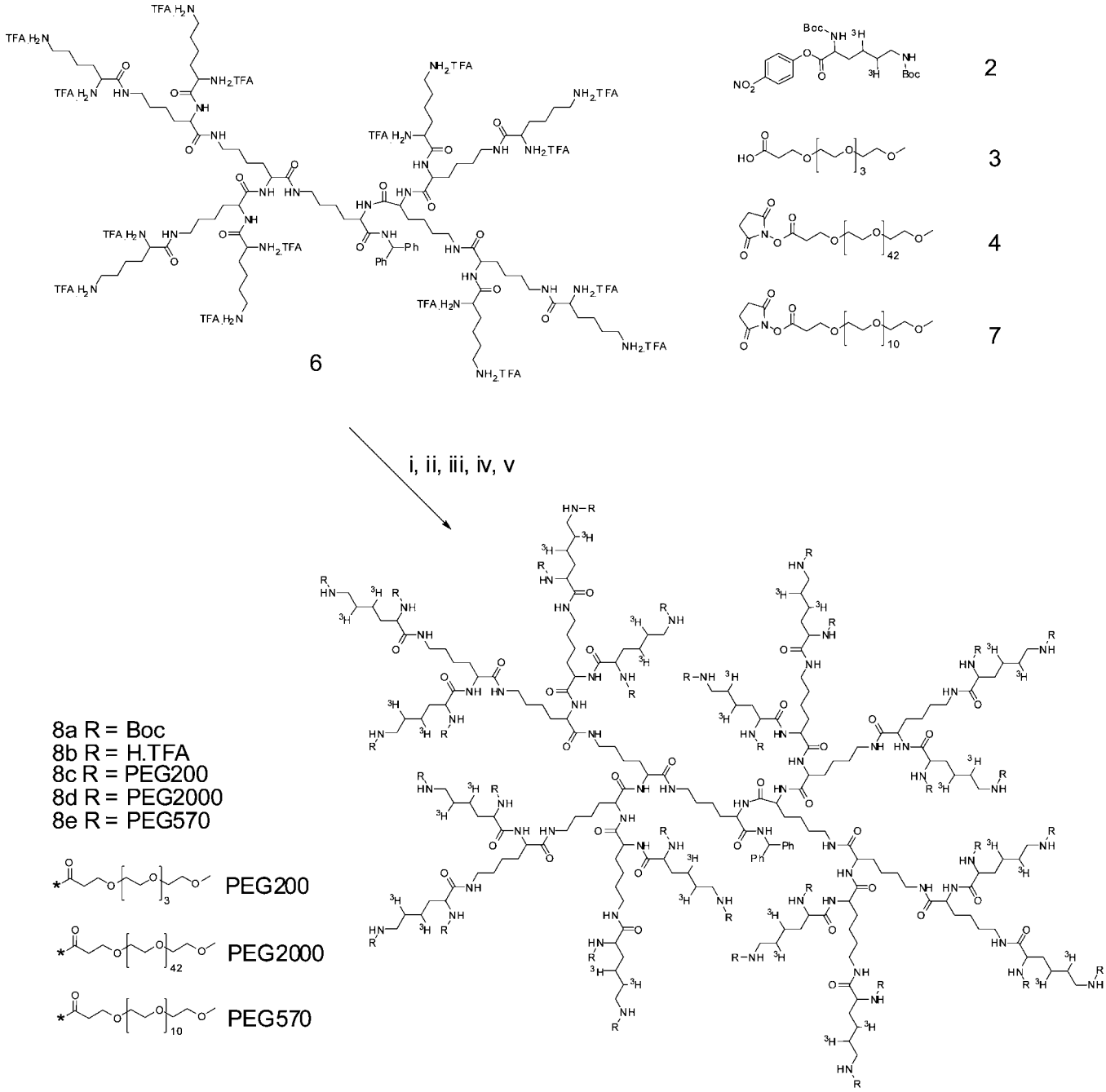


[a] Structure **1** represents a generation 2 dendrimer with primary amines as the TFA salts on the outer layer. This was treated with ^{3}H-Lysine-Boc protected **2** to form the radiolabeled generation 3 **5a**, reaction i. The labeled, protected generation 3 dendrimer **5a** was deprotected under acidic conditions (reaction ii) to give the multiple amines as the TFA salt **5b**. The PEGylated compounds **3** and **4** were coupled to the deprotected generation 3 dendrimer **5b** via pyBop coupling conditions (reactions iii, iv) to give the generation 3 PEGylated 200 and 2000 dendrimers **5c** and **5d**.

isoflurane anesthesia as described previously.[29] After surgery, rats were transferred to metabolism cages (which allowed collection of urine and feces), and were allowed to recover overnight prior to administration of 1 mL of dendrimer solution (in 50 mM PBS, pH 7.4) via the jugular vein to provide a final dose of 5 mg/kg (total radioactivity dosed was 0.2 to 3.3 μCi). Prior to dosing, a blank blood sample (150 μL) was collected via the carotid artery into a heparinized (10 U) Eppendorf tube. Animals were dosed with each dendrimer as a bolus over 1.5 min. Dendrimer remaining in the cannula was then flushed through the cannula with a further 200 μL of heparinized saline (2 U heparin/mL) over a further 30 s, after which a zero time point sample (200 μL, $t = 0$) was collected via the carotid artery cannula to facilitate estimation of C_p° and V_c. Further blood samples (150 μL) were collected at appropriate time points and similarly stored in heparinized Eppendorf tubes. Blood samples were collected for varying periods of time in order that terminal plasma elimination kinetics could be estimated; specifically the last blood sample was taken at 30 h for $Lys_8(PEG_{200})_{16}$, $Lys_{16}(PEG_{200})_{32}$ and $Lys_{16}(PEG_{570})_{32}$, at 120 h for $Lys_8(PEG_{2000})_{16}$ and at 168 h for $Lys_{16}(PEG_{2000})_{32}$. Sample times for PEG_{200} and PEG_{570} dendrimers were 0, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 1440 and 1800 min. Sample times for $Lys_8(PEG_{2000})_{16}$ were identical to those above, with additional samples taken at 2880, 3240, 4320, 4680 and 5760 min. In the case of $Lys_{16}(PEG_{2000})_{32}$ additional samples were also taken at 7200, 8640 and 10080 min. Whole blood samples were centrifuged (3500$\times$ g) for 5 min to isolate plasma. Plasma samples (100 μL) were then mixed with 1 mL of Starscint in 6 mL scintillation vials and counted for ^{3}H-content as described above. Values that were below the validated limit of quantification (LOQ) were not reported.

Excretion of Radiolabel in Urine and Feces and Biodistribution Studies. Urine and feces were collected and analyzed for ^{3}H-content as described previously.[29]

Scheme 2. Synthetic Scheme for Generation 4 Pegylated Dendrimers: $Lys_{16}(PEG_{200})_{32}$, $Lys_{16}(PEG_{570})_{32}$, and $Lys_{16}(PEG_{2000})_{32}$[a]



[a] The ^3H-labeled generation 4 dendrimer as the protected and deprotected **8a** and **8b** was synthesized by the same method as described in Scheme 1 (reaction i, ii). Compounds **3** and **4** were coupled to the deprotected generation 4 dendrimer **8b** under pyBop coupling conditions (reactions iii, iv) to give the generation 4 PEGylated 200 and 2000 dendrimers **8d** and **8e** respectively. However, the generation 4 PEGylated 570 dendrimer **8e** was prepared by treating **8b** with the activated PEG ester **7** under basic conditions (reaction v).

Feces collected from each rat over the sampling period were pooled before determination of the percentage of tritium radiolabel recovered in total excreted feces. A detailed description of the assay for measuring tritium in feces is described in Boyd et al.[29]

For the biodistribution studies, rats were euthanized by intravenous infusion of 1 mL of sodium pentobarbital (Lethabarb, 60 mg of pentobarbitone sodium/mL) immediately following collection of the final blood sample. Major organs (liver, kidneys, spleen, pancreas, heart, lungs, brain) were removed, weighed and homogenized in 5–10 mL Milli-Q water using a Waring miniblender (Extech Equipment Pty. Ltd., Boronia, Australia) for 5 × 10 s intervals. The homogenates were processed and the ^3H-content of the homogenates was determined as described previously. [29]

Separation of Radiolabeled Species in Plasma and Urine by SEC. To determine whether PEGylated PLL dendrimers remained intact after intravenous administration

Table 1. Dendrimer Properties

	$Lys_8(PEG_{200})_{16}$	$Lys_{16}(PEG_{200})_{32}$	$Lys_{16}(PEG_{570})_{32}$	$Lys_8(PEG_{2000})_{16}$	$Lys_{16}(PEG_{2000})_{32}$
MW (kDa)	6	11.1	22.4	34.1	68
^3H activity (μCi/mg)	0.134 ± 0.007	2.195 ± 0.022	1.012 ± 0.028	0.623 ± 0.019	0.469 ± 0.022

or were metabolized in vivo, size exclusion chromatography (SEC) was employed to separate the radiolabeled species present in plasma and urine.

Arterial blood was collected for SEC analysis immediately after infusion of the $Lys_{16}(PEG_{200})_{32}$ dose (t_0) and at 10, 20, 30, 45 and 60 min postdose. Blood samples for SEC were collected at t_0 and at 8 and 24 h postdose for $Lys_{16}(PEG_{570})_{32}$. For $Lys_8(PEG_{2000})_{16}$ and $Lys_{16}(PEG_{2000})_{32}$ SEC analysis of plasma was conducted on samples collected at t_0 and at t = 48 h. Plasma was obtained by centrifugation as described above. For $Lys_{16}(PEG_{200})_{32}$ and $Lys_{16}(PEG_{570})_{32}$ the short sampling times were necessary to allow collection of plasma with sufficient radiolabel for SEC, while 48 h was deemed an appropriate sampling time for examination of the in vivo stability of the larger long circulating PEGylated poly L-lysine dendrimers. In addition, samples of urine were analyzed by SEC over periods of 0–8 h for $Lys_{16}(PEG_{200})_{32}$ and 8–24 h for $Lys_{16}(PEG_{570})_{32}$. Urine from these time periods was analyzed in detail by SEC since these time points contained sufficient radiolabel to obtain meaningful data to be generated during the elimination phase of the plasma concentration−time curves. Radiolabel in urine samples collected over the elimination phase of the concentration−time profiles for the Lys_{2000} dendrimers was not high enough to obtain meaningful SEC. Plasma and urine samples were stored at −20 °C before analysis.

Prior to analysis, plasma and urine samples were diluted 1:1 in mobile phase (50 mM PBS + 0.3 M NaCl, pH 3.5), and sample aliquots (100–200 μL) subsequently injected onto a Superdex 75 SEC column (Amersham Biosciences, NJ), and eluted with mobile phase at 0.5 mL/min using a Waters 590 pump (Millipore Corporation, Milford, MA). Fractions were collected every 1 min using a Gilson FC10 fraction collector (John Morris Scientific Pty. Ltd.) and mixed with 3 mL of Starscint in 6 mL scintillation vials prior to scintillation counting for ^3H-content as described above.

Calculation of Pharmacokinetic Parameters. The amount of radiolabel in each plasma sample was converted to ng dendrimer equivalents using the specific activity of the ^3H-labeled dendrimer. Plasma concentrations have been expressed as ng equivalents/mL; however, this should be viewed with the caveat that this approach assumes that the ^3H-content is still associated with intact dendrimer.

The terminal elimination rate constants (k) were obtained by regression analysis of the individual postdistributive plasma concentration vs time profiles. Half-lives ($t_{½}$) were determined from ln 2/k. The area under the plasma concentration vs time profiles ($AUC^{0-\infty}$) were calculated using the linear trapezoidal method. The extrapolated area ($AUC^{tlast-\infty}$) was determined by division of the last measurable plasma concentration (C_{last}) by k. The initial distribution volume (V_c) was calculated by dividing the administered dose by the concentration in plasma at t = 0 ($C_p°$). Plasma clearance (Cl) was calculated by dose/$AUC^{0-\infty}$. Renal Cl (Cl_r) was subsequently determined by multiplying Cl by the fraction excreted unchanged in urine (F_e), and nonrenal clearance (Cl_{nr}) was estimated by Cl − Cl_r.

Results

Plasma Pharmacokinetics. The plasma concentration vs time profiles (expressed as ng equivalents of dendrimer per mL of plasma) for the PEGylated poly L-lysine dendrimers are shown in Figure 2, panel A, and the pharmacokinetic parameters calculated from the plasma concentration versus time profiles are provided in Table 2. The plasma concentration−time profiles were almost identical for the smaller dendrimers ($(Lys_8(PEG_{200})_{16}$ and $Lys_{16}(PEG_{200})_{32}$) and were substantially different from those of the larger dendrimers.

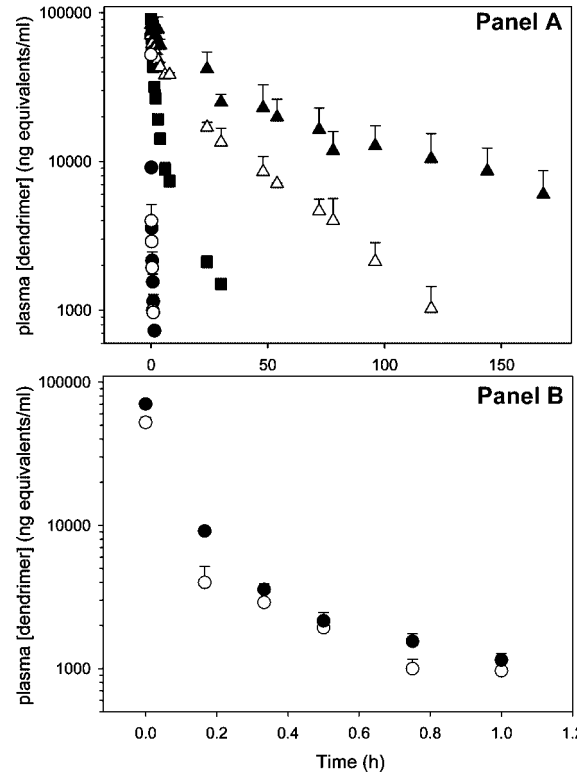


Figure 2. Plasma concentration−time profiles for PEGylated poly L-lysine dendrimers after iv administration at a dose of 5 mg/kg to rats. Dendrimers were $Lys_8(PEG_{200})_{16}$ (○), $Lys_{16}(PEG_{200})_{32}$ (●), $Lys_{16}(PEG_{570})_{32}$ (■), $Lys_8(PEG_{2000})_{16}$ (▽) and $Lys_{16}(PEG_{2000})_{32}$ (▼). Panel A shows the plasma pharmacokinetics for all dendrimers over 1–7 days. Panel B shows the plasma pharmacokinetics of the PEG_{200} dendrimers over a reduced time of 1 h. Values are mean ± SD (n = 3–5 rats).

Table 2. Pharmacokinetic Parameters after Intravenous Administration of PEGylated Poly L-Lysine Dendrimers to Rats at 5 mg/kg[a]

	$Lys_8(PEG_{200})_{16}$	$Lys_{16}(PEG_{200})_{32}$	$Lys_{16}(PEG_{570})_{32}$	$Lys_8(PEG_{2000})_{16}$	$Lys_{16}(PEG_{2000})_{32}$
C_p° (μg/mL)	57.4 ± 4.5	70.4 ± 3.3	90.8 ± 5.3	70.8 ± 2.5	83.2 ± 15.3
k (h^{-1})	1.21 ± 0.14	0.99 ± 0.12	0.073 ± 0.003	0.029 ± 0.003	0.0093 ± 0.0013
$t_{½}$ (h)	0.6 ± 0.1	0.7 ± 0.1	9.5 ± 0.3	23.9 ± 2.1	75.4 ± 9.3
V_c (mL)	24.7 ± 1.1	18.6 ± 0.9	14.8 ± 0.5	18.0 ± 0.4	19.1 ± 3.1
Cl (mL/h)	194 ± 5.4	115 ± 4.9	5.2 ± 0.8	0.9 ± 0.1	0.4 ± 0.1
Cl_r (mL/h)	160 ± 9.2	92.6 ± 17.9	2.07 ± 0.41	0.24 ± 0.03	0.01 ± 0.01
Cl_{nr} (mL/h)	34.9 ± 13.1	22.3 ± 14.7	2.73 ± 0.34	0.68 ± 0.11	0.37 ± 0.09

[a] Values are reported as mean ± SD; n = 3–5.

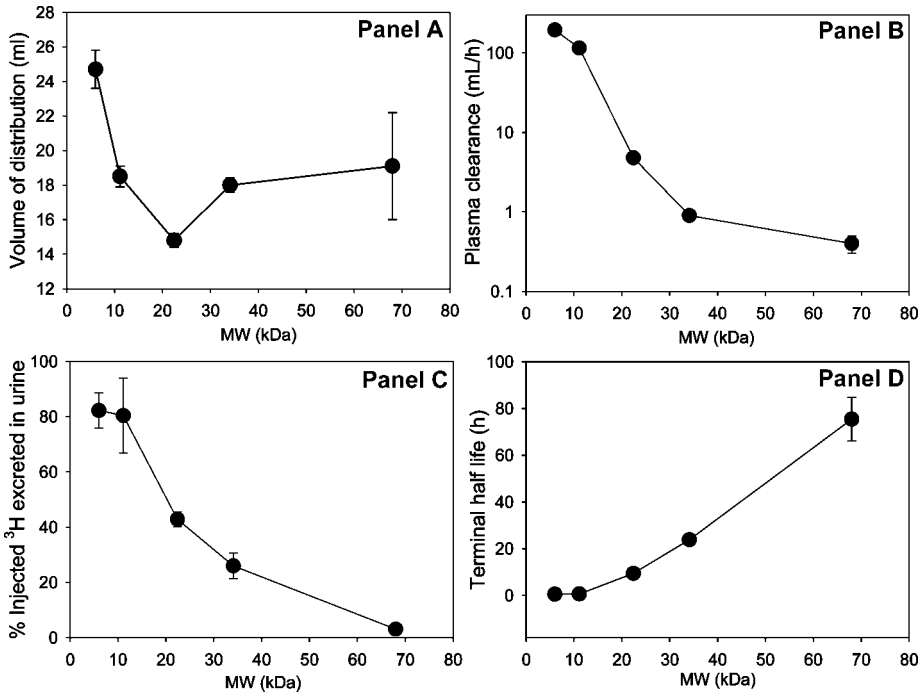


Figure 3. Correlations between plasma pharmacokinetic parameters and dendrimer molecular weight (MW). Panel A: MW vs initial distribution volume (V_c) in plasma. Panel B: MW vs plasma clearance of dendrimer. Panel C: MW vs the proportion (%) of injected ^3H excreted in urine over the sampling period. Panel D: MW vs terminal plasma half-life. Values are mean ± SD in all cases (n = 3–5 rats).

For clarity, the plasma concentration versus time profiles for the smaller dendrimers have been replotted on a more informative scale in Figure 2, panel B. Calculation of dendrimer pharmacokinetic parameters based on total plasma radioactivity requires the assumption that the dendrimers remain intact (and therefore that the radiolabel remains associated with the dendrimer) through the sampling period. The degree to which label was liberated was investigated by size exclusion chromatography and is reported in detail in the following section; however, it is apparent that in large part liberation of radiolabel from the dendrimers did not occur. The assumption has therefore been made that for the majority of the period of plasma exposure the primary species present in plasma was the parent (unchanged) dendrimer and therefore that the pharmacokinetic parameters calculated represent a reasonably accurate reflection of the true pharmacokinetic profile.

The initial extravascular distribution of the dendrimers (V_c) was minimal regardless of PEG length, dendrimer generation or molecular weight. In all cases V_c approximated plasma volume (approximately 18 mL) (Table 2 and Figure 3, panel A), although the value obtained for V_c after administration of $Lys_8(PEG_{200})_{16}$ was moderately higher at 24.7 ± 1.1 mL, which may indicate slightly greater initial distribution of the smaller dendrimer (approximately 6 kDa).

Postdistributive volumes of distribution have not been reported as their estimation is highly dependent on accurate estimates of terminal plasma concentrations, which was complicated in the current study by the presence of trace quantities of long circulating impurities in the smaller dendrimers (see section entitled "Analysis of Plasma and Urine Radioactivity Profiles by Size Exclusion Chromatography"). The high molecular weights and low initial distribution volumes of the dendrimers suggest, however, that the postdistributive volumes of distribution are also likely to be low.

Table 3. Percent of Injected ^{3}H Excreted in Urine Fractions Collected over 8–24 h Time Periods[a]

urine fraction (h)	$Lys_8(PEG_{200})_{16}$	$Lys_{16}(PEG_{200})_{32}$	$Lys_{16}(PEG_{570})_{32}$	$Lys_8(PEG_{2000})_{16}$	$Lys_{16}(PEG_{2000})_{32}$
0–8	69.0 ± 17.7	51.2 ± 44.4	27.4 ± 3.4	4.0 ± 0.5	
8–24	11.5 ± 11.0	28.2 ± 30.7	13.5 ± 0.8	2.6 ± 0.9	0.6 ± 0.3[b]
24–30/24–48[c]	1.7 ± 6.4	1.0 ± 0.6	2.2 ± 0.6	5.0 ± 1.1	0.6 ± 0.9
48–72				5.5 ± 0.7	0.3 ± 0.1
72–96				5.6 ± 1.6	0.3 ± 0.1
96–120				3.4 ± 2.4	0.6 ± 0.8
120–144					0.4 ± 0.2
144–168					0.3 ± 0.2
total	82.2 ± 6.4	80.4 ± 13.5	42.9 ± 2.7	26.0 ± 4.6	3.1 ± 1.9

[a] Urine fractions indicate the time period after dosing that the % excreted ^{3}H was determined. Values are mean ± SD (n = 3–5). [b] Urine collection period 0–24 h. [c] Urine collection period 24–48 h for $Lys_8(PEG_{2000})_{16}$ and $Lys_{16}(PEG_{2000})_{32}$.

The total plasma clearance of the PEGylated dendrimers was markedly dependent on molecular weight and decreased from approximately 100–200 mL/h for the smallest PEG_{200} dendrimers to <1 mL/h for the larger PEG_{2000} dendrimers (Table 2 and Figure 3, panel B). In a reflection of the changes to clearance, the plasma concentration of the smallest dendrimer ($Lys_8(PEG_{200})_{16}$) had dropped below quantifiable limits after only 1 h, whereas the plasma concentrations of the largest dendrimer ($Lys_{16}(PEG_{2000})_{32}$) were maintained at relatively high levels for greater than 7 days postdosing.

The proportion of the administered radioactivity that was recovered in the urine is tabulated in Table 3, and the calculated values for Cl_r and Cl_{nr} are reported in Table 2. Figure 3, panel C, also illustrates the relationship between the proportion of administered ^{3}H that was recovered in urine and the molecular weight of the dendrimers. A correlation was evident between molecular weight and % injected ^{3}H excreted in urine, and while approximately 80% of the ^{3}H dose of the smaller PEG_{200} dendrimers (MW 6–11.1 kDa) was eliminated in urine over 30 h, less than 2% of the larger $Lys_{16}(PEG_{2000})_{32}$ (MW 68 kDa) was recovered in the urine over the same time period. For the larger dendrimers (i.e. those that were not appreciably renally cleared), proportionately larger values for Cl_{nr} were seen when compared with Cl_r (Table 2), suggesting that nonrenal mechanisms were the predominant route of dendrimer plasma clearance. The reduction in plasma clearance of the larger dendrimers in combination with relatively limited changes to volume of distribution also resulted in a significant increase in terminal half-life with increases in molecular weight of the dendrimer −PEG complex (Figure 3, panel D).

Radiolabel was only detected in pooled feces after administration of $Lys_{16}(PEG_{570})_{32}$ and $Lys_{16}(PEG_{200})_{32}$ with the ^{3}H-content reflecting only 2.3 ± 2.1% and 1.7 ± 1.4% of the injected dose respectively.

Analysis of Plasma and Urine Radioactivity Profiles by Size Exclusion Chromatography (SEC). To provide an indication of the stability of the PEGylated dendrimers in plasma and urine, size exclusion chromatography was used to elucidate the species present in selected plasma and urine samples. The retention times of the PEGylated dendrimers when applied to the column (Superdex 75) as a simple solution in phosphate buffered saline (i.e., the vehicle in which they were administered) were 23, 20, 18 and 17 min for $Lys_{16}(PEG_{200})_{32}$, $Lys_{16}(PEG_{570})_{32}$, $Lys_8(PEG_{2000})_{16}$ and $Lys_{16}(PEG_{2000})_{32}$ respectively. The retention times for unchanged dendrimer are indicated by the arrows in Figures 4−6.

Due to the low specific activity and rapid renal elimination and plasma clearance of the $Lys_8(PEG_{200})_{16}$ dendrimer, further investigation of metabolic fate was not possible. The higher specific activity of $Lys_{16}(PEG_{200})_{32}$, however, permitted an exploratory evaluation of the species present in plasma over the 60 min postdose period in which plasma concentrations were quantifiable. These exploratory studies suggested the presence of a higher MW product (that eluted 2–3 min earlier by SEC than the intact dendrimer) that became quantitatively more significant at later time points. To investigate further, plasma samples were collected from two additional animals at time points of 0, 10, 20, 30, 45 and 60 min postdose, and analyzed by SEC to determine the relative contribution of the high MW product to total plasma radiolabel at each time point.

Figure 4, panel A, shows that, immediately after the completion of the IV infusion of $Lys_{16}(PEG_{200})_{32}$, intact dendrimer was the major species present in plasma, although the presence of a very small quantity of the high molecular weight species was apparent at an elution time of 21 min (relative peak height 3%). Size exclusion chromatography of plasma samples at later time points (SEC profiles shown for t = 20 min in Figure 4, panel B, and t = 60 min in panel C) revealed the increasing relative presence of the higher molecular weight species as the total plasma radioactivity levels declined. However, the relatively small quantities of lower molecular weight species in the chromatograms, and in particular lysine (which elutes at 42 min), suggested that significant metabolism of the dendrimer did not occur, and was not responsible for the higher molecular weight product (previous studies have described the liberation of lysine from PLL dendrimers and subsequent appearance in plasma of radiolabeled high molecular weight species, presumably reflecting reincorporation of liberated lysine into protein resynthesis pathways[30]). Incubation of $Lys_{16}(PEG_{200})_{32}$ in fresh plasma for 5 h also failed to generate the high molecular weight material suggesting that this species was not formed by interaction of the dendrimers with plasma components

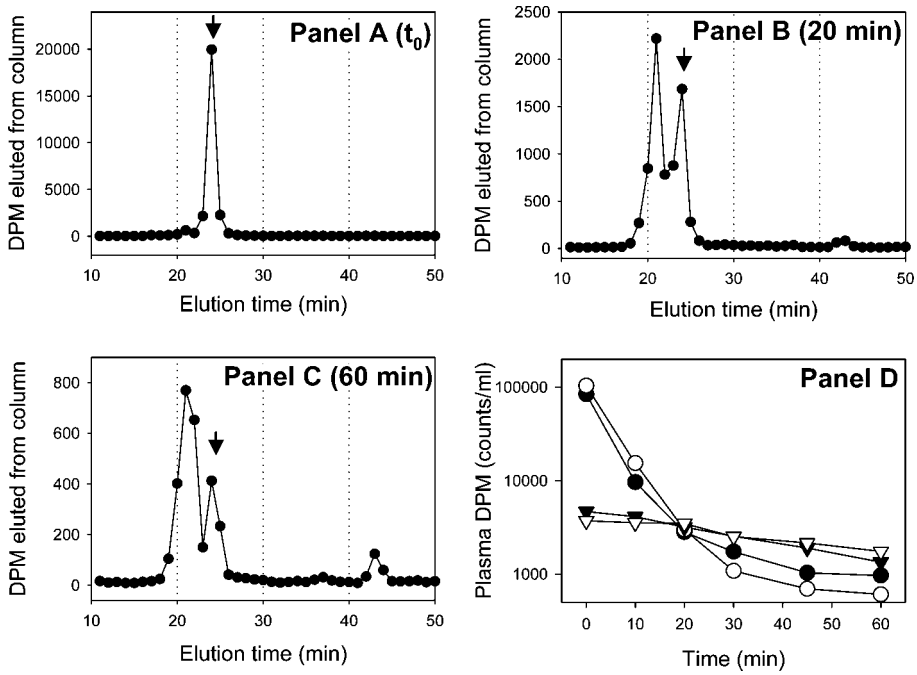


Figure 4. Size exclusion profiles of plasma from a rat (rat 2) administered $Lys_{16}(PEG_{200})_{32}$, separated using a Superdex 75 SEC column. Panel A (t_0 plasma), panel B (20 min plasma) and panel C (60 min plasma). The elution time of the intact dendrimer is indicated by arrows. Panel D shows the deconvoluted plasma radioactivity−time data where plasma radioactivity attributed to intact dendrimer and to a high MW product from two rats administered 5 mg/kg $Lys_{16}(PEG_{200})_{32}$ is indicated. The high MW product represents <5% of the ^3H content in stock samples of the dendrimer. DPM attributed to each species per mL of plasma were calculated from the area under each peak eluting from a Superdex 75 SEC column at 0.5 mL/min. Fractions eluting from the column were collected at 1 min intervals, mixed with Starscint in a 6 mL scintillation vial and scintillation counted for ^3H. Symbols represent rat 1 intact dendrimer (●), rat 2 intact dendrimer (○), rat 1 high MW product (▼), rat 2 high MW product (▽).

as has previously been reported for PLL dendrimers with anionic surface capping groups.[31] In contrast, closer inspection of the SEC profiles of the dendrimer stock solution (when applied to the column in PBS) revealed the presence of small quantities of a higher molecular weight species (approximately 3–5% of the total radiolabel). The high MW product therefore likely represents a contaminant that was present in the dosing solution at low levels (<5%), but was cleared from plasma much more slowly than the intact dendrimer and as such became apparent once most (approximately 90%) of the dendrimer had been cleared from plasma.

The deconvoluted plasma radioactivity profiles for intact dendrimer and the contaminant are presented in Figure 4, panel D. The pharmacokinetic parameters for $Lys_{16}(PEG_{200})_{32}$ were recalculated from the deconvoluted data and compared to the parameters calculated in the same animals using total plasma activity in Table 4. In general, the pharmacokinetic parameters calculated using the deconvoluted data and total plasma activity were similar, although a slight decrease (approximately 20%) in plasma clearance was apparent (presumably reflecting the slower clearance of the high MW contaminant).

SEC evaluation of plasma samples obtained after intravenous infusion of $Lys_{16}(PEG_{570})_{32}$ suggested that at time periods up to at least 8 h postdose, the principal species that could be quantified in plasma was intact dendrimer (data not shown)). At 8 and 24 h postdose, however, a shoulder peak that eluted ahead of the intact dendrimer was apparent (Figure 5, panels A and B respectively). Importantly, while the shoulder peak became more obvious at longer time points (due to elimination of intact dendrimer), the absolute peak area associated with the higher molecular weight species again decreased from $t = 8$ h to $t = 24$ h, suggesting that in an analogous fashion to the $Lys_{16}(PEG_{200})_{32}$ dendrimer, the higher molecular weight species was not formed from the administered material but reflected a high molecular weight contaminant that was present in very low quantities in the injected material, and slowly cleared thereafter. At 24 h post dose, when the high molecular weight product became most apparent, the majority of the dendrimer had been eliminated and the total concentration of radiolabeled material present in plasma was only 2.5% of that at time zero (2.11 ± 0.22 μg/mL vs 90.8 ± 5.3 μg/ml dendrimer equivalents). As the shoulder peak was responsible for only approximately 30% of the total activity in that sample, it seems likely that the original level of contaminant was of the order of 1–2%, although the higher molecular weight species could not be identified in stock solutions of the dendrimer (presumably reflecting its presence at extremely low levels). The small amounts of this high molecular weight contaminant are therefore unlikely to greatly affect the pharmacokinetic parameters calculated for the $Lys_{16}(PEG_{570})_{32}$ dendrimer

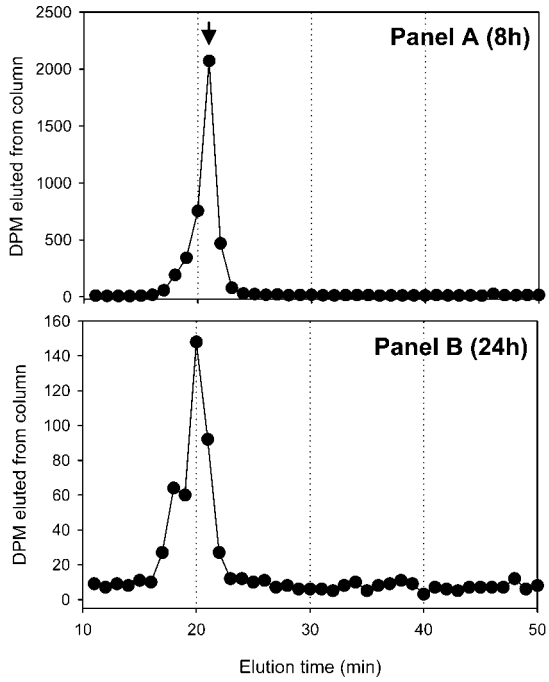


Figure 5. Size exclusion profiles of plasma from rats administered $Lys_{16}(PEG_{570})_{32}$, separated using a Superdex 75 SEC column. Panel A (8 h plasma) and panel B (24 h plasma). The elution time for the intact dendrimer applied to the column as a solution in PBS is indicated by the arrow.

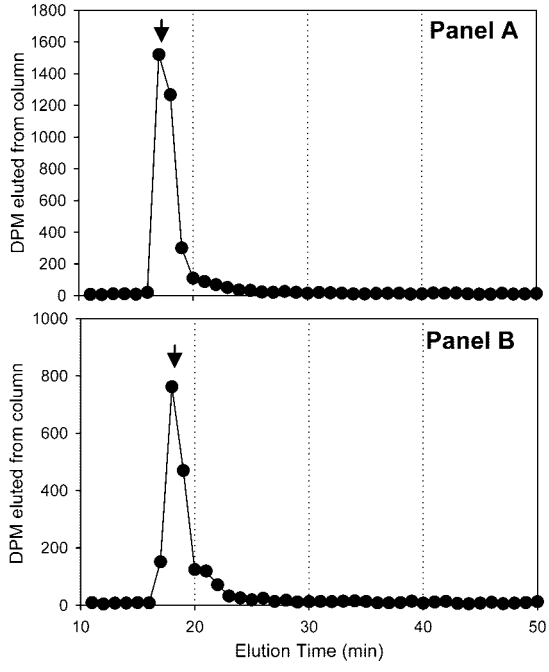


Figure 6. Size exclusion profiles of plasma from rats administered PEG_{2000} dendrimers, separated using a Superdex 75 SEC column. Panel A: 48 h $Lys_8(PEG_{2000})_{16}$. Panel B: 48 h $Lys_{16}(PEG_{2000})_{32}$. The elution times for the intact dendrimers applied to the column as a solution in PBS are indicated by the arrows.

reported in Table 2. Similarly to the $Lys_{16}(PEG_{200})_{32}$ dendrimer, incubation of $Lys_{16}(PEG_{570})_{32}$ species in fresh plasma for 5 h did not result in the generation of the high molecular weight material.

Table 4. Pharmacokinetic Parameters Obtained after Intravenous Administration of $Lys_{16}(PEG_{200})_{32}$ to two Individual Rats at 5 mg/kg[a]

	rat 1		rat 2	
	plasma ^3H	^3H-dendrimer	plasma ^3H	^3H-dendrimer
$C_p°$ (μg/mL)	88.6	83.8	107	103
k (h^{-1})	1.32	1.18	1.24	1.16
$t_{½}$ (h)	0.5	0.6	0.6	0.6
V_c (ml)	16.9	17.0	14.9	14.7
Cl (mL/h)	95.1	118	86.1	105

[a] Pharmacokinetic parameters were generated via total plasma ^3H and specifically for ^3H-dendrimer alone using SEC to separate intact dendrimer and the high MW contaminant.

In contrast to the chromatograms observed for the two smaller dendrimers, Figure 6, panels A and B, illustrates that for the two larger PEG_{2000} dendrimers there was no evidence of a higher MW contaminant, even after 48 h. The absence of the higher molecular weight contaminant was confirmed by injecting the same plasma samples onto a Superdex 200 column designed to better separate higher molecular weight materials (data not shown). In addition, no evidence of lower MW ^3H-labeled species (indicative of e.g. lysine liberation) was evident in either sample.

SEC analysis was also conducted on urine collected after administration of $Lys_{16}(PEG_{200})_{32}$ (pooled urine from the 0–8 h collection point) and $Lys_{16}(PEG_{570})_{32}$ (pooled urine from the 8–24 h time point). Only intact dendrimer was observed by SEC in these urine samples (data not shown). Since only intact dendrimer was observed in the plasma of animals administered the larger PEG_{2000} dendrimers, and the extent of renal elimination of these dendrimers was low, SEC analysis of urine was not conducted.

Biodistribution of PEGylated Dendrimers. At the completion of blood sampling (30 to 168 h postdose, depending on the dendrimer administered) the major organs were collected and analyzed for ^3H to determine the biodistribution pattern of the PEGylated dendrimers. The % of injected ^3H recovered in each organ at sacrifice is shown in Figure 7, panel A, and the same data, after normalizing for the mass of the organ (% injected radiolabel per gram of tissue), is shown in panel B. The total amount of administered tritium recovered in blood, selected tissues, urine and feces at the end of the study was 82, 82, 50, 34 and 26% for $Lys_8(PEG_{200})_{16}$, $Lys_{16}(PEG_{200})_{32}$, $Lys_{16}(PEG_{570})_{32}$, $Lys_8(PEG_{2000})_{16}$ and $Lys_{16}(PEG_{2000})_{32}$ respectively, suggesting that for the long circulating PEG_{570} and PEG_{2000} dendrimers, considerable quantities of administered radiolabel were retained in the nonsampled tissues and carcass.

There was no quantifiable ^3H present in any of the organs that were removed 30 h after iv administration of the smallest ($Lys_8(PEG_{200})_{16}$) dendrimer. However, increasing quantities of ^3H were recovered in the selected organs at sacrifice as the dendrimer size was increased, such that after 7 days a total of approximately 11% of injected ^3H was recovered

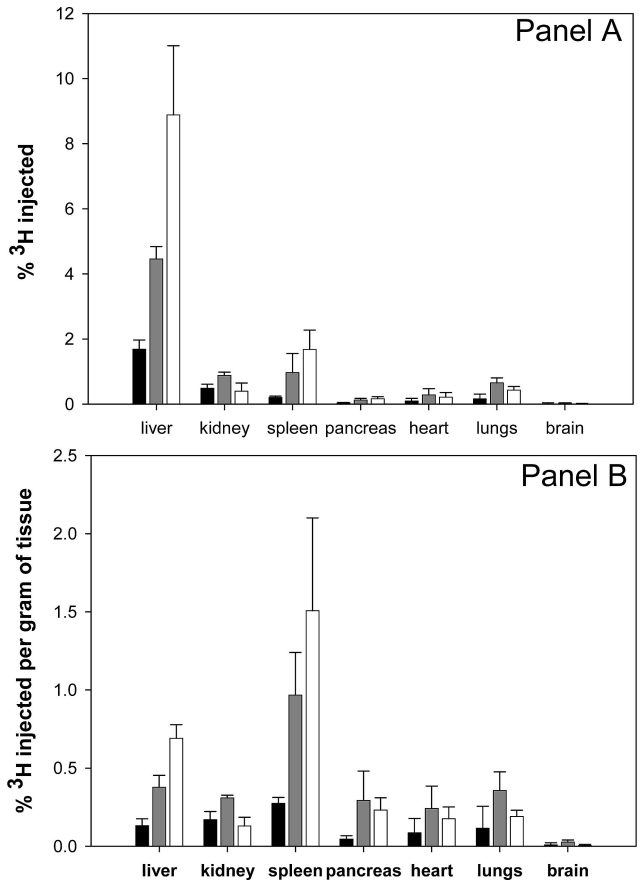


Figure 7. Distribution of residual ^{3}H to major organs at sacrifice after intravenous administration of PEGylated ^{3}H-dendrimers (5 mg/kg) to rats. Rats administered $Lys_{16}(PEG_{570})_{32}$, $Lys_8(PEG_{2000})_{32}$ and $Lys_{16}(PEG_{2000})_{32}$ were sacrificed after 30 h, 5 days and 7 days respectively. Panel A represents the % of injected radiolabel retained in each organ. Panel B provides data normalized for tissue mass (mean ± SD, $n = 3$). Black bars represent data for $Lys_{16}(PEG_{570})_{32}$ dosed rats, gray bars depict data for $Lys_8(PEG_{2000})_{32}$ dosed rats and white bars represent the remaining radiolabel for $Lys_{16}(PEG_{2000})_{32}$ dosed rats. Values are shown as mean ± SD ($n = 3$–5).

from the pooled organs after administration of the largest dendrimer, $Lys_{16}(PEG_{2000})_{32}$.

The majority of the ^{3}H recovered in organs after administration of the PEG_{570} and PEG_{2000} dendrimers was associated with the liver; however, as a proportion of organ weight, the ^{3}H associated with $Lys_{16}(PEG_{570})_{32}$ was more evenly distributed between the liver, spleen and kidneys, with smaller amounts detected in the pancreas, heart, lungs and brain. In contrast, per gram of tissue, most of the ^{3}H recovered after administration of the PEG_{2000} dendrimers was associated with the spleen. In the case of the $Lys_8(PEG_{2000})_{16}$ dendrimer, an approximately equal concentration of ^{3}H was recovered in the kidneys, liver, pancreas, heart and lungs, whereas for $Lys_{16}(PEG_{2000})_{32}$ ^{3}H recovered was primarily concentrated in the liver and spleen, with relatively smaller amounts recovered in the other organs.

To determine whether uptake of the $Lys_{16}(PEG_{2000})_{32}$ dendrimer into the liver and spleen only occurred over extended time periods or whether this occurred at earlier time points, the biodistribution pattern of the dendrimer was also determined 24 h postdose (when the ^{3}H levels in the plasma had declined by only approximately 50%). Approximately 16% of injected ^{3}H was recovered in major organs at 24 h (in comparison to 11% recovered at 48 h). However, the plasma concentrations of dendrimer were also much higher at 24 h postdose. When the organ deposition data were normalized for the radioactivity that reflected organ blood volumes (using published data for organ blood content[32–36]), the total recovered activity fell to 8% (normalization for blood-related radioactivity was not required for organs removed at 168 h since the plasma levels of activity were approximately 10-fold lower than they were at 24 h and did not significantly alter the calculated biodistribution for each organ). Thus, only approximately 0.2–0.3% of the injected ^{3}H was present per gram of tissue in the liver, kidney, pancreas and heart (blood-corrected and uncorrected organ distribution data for $Lys_{16}(PEG_{2000})_{32}$ at 24 h is shown in the Supporting Information). Higher accumulation of radiolabel was evident in the spleen and lungs (approximately 0.8 and 0.4% injected ^{3}H per gram of tissue respectively); however, the extent of radiolabel uptake into the liver and spleen 24 h after dosing was still only approximately half of that observed at 7 days, suggesting gradual accumulation over time rather than initial clearance into the liver followed by removal or breakdown. Less than 0.1% injected ^{3}H was found per gram of brain tissue.

Discussion

PEGylation has been shown to shield proteins and colloids from proteolysis, and opsonization, to attenuate phagocytosis and immune cell stimulation, and consequently to increase the circulating half-life of conjugated (PEGylated) molecules and carriers. To this point, however, examination of the effect of PEGylation on the in vivo disposition of dendrimers has mostly been limited to studies describing PAMAM dendrimers (and in particular systems designed for magnetic reso-

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nance imaging (MRI) applications[37–40]), polyethylene-"bow-tie" hybrid dendrimers[24,26] and a single recent description of a G6 PEGylated PLL dendrimer.[31] The present study was therefore conducted to investigate whether the circulatory half-lives and tissue distribution profiles of a series of PLL dendrimers could be altered by surface modification with various molecular weight PEGs after iv administration, and to evaluate the impact of PEGylation on renal and nonrenal clearance processes. Examination of the nature of the species present in plasma and urine by size exclusion chromatography has also allowed evaluation of the impact of PEGylation on in vivo degradation of PLL dendrimers.

In all cases, the pharmacokinetic profiles of the PEGylated dendrimers were significantly different from the equivalent "uncapped" cationic PLL dendrimers[29] and anionic arylsulfonate and succinate-capped dendrimers.[30] In general, terminal half-lives were significantly lengthened and plasma clearance dramatically reduced by PEGylation (Figure 2). For example, even after conjugation with relatively short chain length PEG (200 Da) the plasma profiles of the G3 and G4 dendrimers were altered such that initial half-lives were increased from 3 to 4 min to 30–40 min respectively when compared with the fully uncapped cationic dendrimers. Furthermore, unlike the uncapped cationic dendrimers, the PEG_{200} capped dendrimers were rapidly eliminated into the urine (Table 3) and little or no evidence of biodegradation or uptake into RES organs was apparent (Figure 7). It appears likely therefore that, for the smaller dendrimers, PEGylation "caps" the cationic charge inherent in poly lysine systems and therefore prevents electrostatic interaction with cell surfaces and also reduces proteolysis. The relatively low molecular weight of these species, however, is not sufficient to prevent renal excretion and as such, rapid elimination of intact dendrimer into the urine occurs. Indeed the renal clearance of both the PEG_{200} PEGylated dendrimers (100–200 mL/h) approached GFR for male Sprague–Dawley rats (approximately 220 mL/h),[41] suggesting relatively unhindered filtration into the urine. Limited uptake into the RES was also seen, suggesting that even PEGylation with relatively small PEG chains was sufficient to inhibit the opsonization and phagocytosis seen previously for similar anionic dendrimers.[30]

In contrast, as the molecular weight of the PEG−dendrimer complex increased, the proportion of the dose eliminated into the urine (fe) decreased (Figure 3, panel D) such that renal elimination was essentially zero for the 68 kDa Lys_{16}-$(PEG_{2000})_{32}$ dendrimer (renal clearance <0.1 mL/h; fe = 0.03). Indeed for this high molecular weight dendrimer, although total plasma clearance was extremely low, renal clearance was attenuated sufficiently that nonrenal mechanisms were the major contributors to clearance. In contrast to previous reports describing the elimination pathways of biocompatible PEG hybrid polyester dendrimers,[26] little dendrimer was recovered in pooled feces, suggesting limited intestinal secretion, and dendrimers appeared to eventually concentrate in the organs of the RES, albeit over extended timescales.

The data therefore suggest that for completely PEGylated (i.e. 100% conjugation) dendrimers, even conjugation with relatively small molecular weight PEG chains is sufficient to preclude dendrimer clearance via electrostatic interaction, phagocytosis and proteolysis and clearance in large part reflects the ease of renal filtration. In this regard, the molecular weight of the total dendrimer−PEG complex (rather than the molecular weight of the dendrimer or PEG chain individually) dictated the extent of renal filtration, with significant increases in plasma retention times for systems with molecular weights in excess of 30 kDa. This finding agrees with the generally accepted view that molecules of molecular weight <20 kDa are well filtered by the kidneys, whereas molecules >70 kDa are sterically excluded from glomerular filtration as a means of preventing the loss of plasma proteins via renal filtration, although this is also influenced by polymeric architecture.[42–44]

The trends in PEGylated PLL dendrimer pharmacokinetics described here are consistent with the results of others who have investigated the influence of PEGylation on, for example, the circulation times of proteins[16,44,45] and various dendrimer systems.[26,31,46] Thus, Knauf et al.[44] reported that the renal clearance and plasma half-lives of PEG-conjugated recombinant interleukin-2 (IL-2) were directly related to the overall molecular weight of the

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protein complex, where increasing the number of PEG_{4000} molecules bound to IL-2 resulted in decreasing rates of plasma clearance. In addition, and in keeping with the PEGylated PLL dendrimer data reported in the current study, the authors also suggested that the length of PEG chains attached to IL-2 had relatively little influence on plasma clearance, and rather that the overall molecular weight of the complex was a stronger determinant of plasma clearance.

PEGylated dendrimers have been used previously to enhance the utility of dendrimer−gadolinimum (Gd) complexes as MRI contrast agents. In the first of these studies Margerum and co-workers examined the effect of increasing the molecular size of PAMAM-based dendrimers via increasing the dendrimer generation and by PEGylation.[47] In all cases the dendrimers were also surface modified with the macrocycle 1-(4-isothiocyanatobenzyl)amido-4,7,10-triacetic acid-tetraazacyclododecane (DO3A-bz-NCS) to provide a chelating agent for Gd. The authors reported a reduction in plasma clearance with increasing molecular weight of non-PEGylated dendrimers, and in general described an increase in blood half-life for PEGylated rather than non-PEGylated dendrimers. In contrast to the data reported here, however, no clear molecular weight dependence in either blood clearance or organ accumulation was evident for the PEGylated systems. In addition, while the largest PEGylated dendrimer examined was of similar molecular dimensions (69.3 kDa) to the 68 kDa $Lys_{16}(PEG_{2000})_{32}$ dendrimer examined here, the elimination half-life reported was significantly lower (20.3 vs 75.4 h) (although the pharmacokinetic analysis employed by Margerum et al. differed slightly from the methods employed here). Kobayashi et al.[46] subsequently examined the impact of PEGylation on the pharmacokinetics and organ distribution profile of similar generation 4 PAMAM dendrimers surface modified with the Gd chelating agent 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriamine-pentaacetic acid (1B4M). In these studies the authors evaluated both a non-PEGylated control G4 dendrimer (G4D-$(1B4M\text{-}Gd)_{64}$ (57 kDa)) and dendrimers modified with either one (PEG_1-G4D-$(1B4M\text{-}Gd)_{63}$ (77 kDa)) or two (PEG_2-G4D-$(1B4M\text{-}Gd)_{63}$ (97 kDa) 20 kDa PEG chains. In keeping with expectation PEGylation resulted in enhanced plasma retention and decreased plasma clearance, although in contrast to the current study, increased PEGylation and therefore molecular weight resulted in increased (rather than decreased) renal excretion.

In the most recently reported examination of the impact of PEG on dendrimer pharmacokinetics, Gillies et al.[26] examined a series of "bow-tie" polyester dendrimers, which differ from the PLL systems described here in that they consist of two covalently attached and orthogonally functionalized polyester dendrons. Under these circumstances one dendron (i.e. one "side" of the bow-tie) may be selectively deprotected to facilitate conjugation with PEG. The other dendron was left unfunctionalized in the study described, but provides attachment points for subsequent drug and or targeting moiety conjugation. The systems examined were based on a G3 polyester dendron, covalently linked to a second G1−G3 dendron functionalized with 5, 10 and 20 kDa PEG, where G1, G2 and G3 allowed for two, four and eight conjugation points respectively. This provided a range of hydrophilic dendrimers with molecular weights of 21.8 kDa to ~160 kDa. Consistent with the PEGylated PLL dendrimers described here, PEG hybridization, in general, led to a significant increase in plasma half-life, although the degree of enhancement was more dependent on the dendrimer generation (and therefore numbers of conjugated PEG molecules). Thus the G3 systems with eight conjugated PEG chains of molecular weight 5 to 20 kDa were retained in the circulation for very extended periods and elimination half-lives ranged from 31 to 50 h. Similarly elimination half-lives for the 10 and 20 kDa PEG conjugated G2 systems were also extended (26 and 25 h respectively), although the smaller 5 kDa conjugate was more readily renally eliminated, in keeping with a molecular weight (23 kDa) below the nominal cut off point for renal filtration. The "bow-tie" PEG dendrimers reported by Gillies make an interesting point of comparison with the PEG−PLL systems described here, since at each generation only half the conjugation points are available when compared to the fully PEGylated systems reported here. Thus G3 PLL dendrimers provide 16 available amino groups and therefore 16 conjugated PEG chains, in comparison to the 8 points of attachment in the polyester systems. Comparison of the elimination kinetics of systems with similar overall molecular weights across the two studies (for example $Lys_8(PEG_{2000})_{16}$ (34.1 kDa, $t_{1/2}$ = 23.9 h) and $G3_{polyester}(PEG_{5000})_8$ (44.7 kDa, $t_{1/2}$ = 31 h)) suggests that the orientation of the hydrophilic chains made relatively little difference to the overall elimination kinetics for the G3 systems. However, the elimination half-life for the $Lys_{16}(PEG_{2000})_{32}$ dendrimer (68 kDa, $t_{1/2}$ = 75.4 h) was longer than the much larger $G3_{polyester}(PEG_{20,000})_8$ dendrimer (~160 kDa, $t_{1/2}$ = 50 h) suggesting that for the very large dendrimer systems the greater degree of flexibility in the bow-tie dendrimers may lead to slightly higher clearance.

In contrast to previous studies, the current investigation has also examined plasma and urine samples by SEC to provide an indication of the in vivo stability of the PEGylated dendrimers. It is apparent that in comparison to the uncapped PLL dendrimers described previously, PEGylation markedly enhances plasma stability. For the

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fully PEGylated systems described here, conjugation of both G3 and G4 dendrimers with 2000 Da PEG appeared to essentially eliminate degradation, and no evidence of smaller radiolabeled fragments, or larger reintegration products, were evident by SEC. Similarly, even for systems conjugated with lower molecular weight PEG (200 Da), the majority of the dose was eliminated in the urine and SEC of urine suggests that the dendrimer was eliminated intact. Close examination of plasma samples obtained from animals administered $Lys_{16}(PEG_{200})_{32}$ and $Lys_{16}(PEG_{570})_{32}$ revealed the presence of a higher molecular weight species; however, this was most likely a byproduct of dendrimer synthesis that was present in relatively low quantities (<5%) and possibly represents either a dendrimer multimer or a higher molecular weight impurity derived from molecular weight variability in the PEG reactant. While this product became apparent in later plasma samples (due to slower clearance when compared with the intact dendrimer), it did not greatly affect the calculated pharmacokinetic parameters. The predominance of the high MW product in later plasma samples did, however, highlight that, even for byproducts that are present in dosing solutions at very low concentration, where clearance of the byproduct is much lower than that of the parent species, persistence in the plasma at later time points may complicate estimation of terminal half-lives and clearance, particularly for long circulating PEGylated dendrimers such as those examined here.

Conclusion

The current study has shown that PEGylation significantly alters the pharmacokinetics of poly L-lysine dendrimers, leading to enhanced retention in the plasma, reduced clearance and significantly increased plasma half-lives. The decrease in plasma clearance when compared to the uncapped cationic PLL dendrimer likely reflects (a) capping the cationic charge associated with poly lysine and therefore prevention of electrostatic dendrimer interactions with cell surfaces, (b) a reduction in proteolysis, (c) reduced opsonization and phagocytosis by the RES and (d) a reduction in renal filtration with increases in molecular weight. Dendrimers conjugated with higher molecular weight PEG, and which displayed the longest plasma half-lives, were eventually distributed to the liver and spleen, while dendrimers with short elimination half-lives were rapidly eliminated via the urine. No clear evidence of dendrimer metabolism was seen with any of the dendrimers, suggesting that PEGylation also shields the core from hydrolytic breakdown. These results suggest that PEGylation may be used as an appropriate means to alter the pharmacokinetics of poly L-lysine dendrimers to achieve increased plasma residence times and may find application in the generation of drug delivery systems amenable to concentration at sites of e.g. inflammation or tumor growth via the enhanced permeability and retention effect.

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Supporting Information Available: Detailed descriptions of synthetic procedures for the dendrimers described here and supporting mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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