molecular pharmaceutics

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Impact of Surface Derivatization of Poly-L-lysine Dendrimers with Anionic Arylsulfonate or Succinate Groups on Intravenous Pharmacokinetics and Disposition

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Received April 26, 2007; Revised Manuscript Received August 21, 2007; Accepted September 8, 2007

Abstract: Tritium-labeled poly-L-lysine dendrimers displaying 8 or 16 surface lysines have been capped with benzene sulfonate (BS), benzene disulfonate (BDS), or succinate (Succ) groups, and the intravenous pharmacokinetics and disposition profiles of the resulting dendrimers ($Lys_8(BS)_{16}$, $Lys_{16}(BS)_{32}$, $Lys_{16}(BDS)_{32}$, $Lys_{16}(Succ)_{32}$) have been evaluated. $Lys_{16}(Succ)_{32}$ was rapidly removed from the plasma primarily via renal elimination. $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$ were opsonized, resulting in more prolonged plasma elimination kinetics and increased uptake by the liver. Data obtained at higher doses suggested some evidence of nonlinear pharmacokinetics. $Lys_8(BS)_{16}$ had reduced affinity for plasma proteins and was cleared more rapidly than the larger $Lys_{16}(BS)_{32}$ or $Lys_{16}(BDS)_{32}$ dendrimers. $Lys_8(BS)_{16}$ and $Lys_{16}(BS)_{32}$ were metabolized in vivo, resulting in the production of a low molecular weight species (possibly the cleavage product $Lys(BS)_2$) that was extensively renally eliminated and accounted for almost all of the radioactivity recovered in urine (~20–45% of administered ^3H). In contrast, only 3–5% of the administered ^3H was recovered in the urine of rats administered $Lys_{16}(BDS)_{32}$, suggesting increased resistance to in vivo degradation. The plasma clearance, distribution, and metabolic profiles of lysine dendrimers are therefore significantly influenced by the structure and charge of the capping groups. In particular, larger arylsulfonate-capped lysine dendrimers are rapidly opsonized and initially cleared from the plasma by the reticuloendothelial organs. The degree of metabolism is subsequently dictated by the nature of the surface capping group with BDS surfaces seemingly more resistant to breakdown. In contrast, smaller arylsulfonate-capped dendrimers are less readily opsonized and phagocytozed but are metabolically labile, and succinate-capped dendrimers are rapidly eliminated by the kidneys.

Keywords: Pharmacokinetics; biodistribution; dendrimer; poly-L-lysine; arylsulfonate

Introduction

The molecular diversity and structural flexibility of dendritic polymers or dendrimers provide potential for these systems to constitute both a new class of drugs or vaccines (with therapeutic activity in their own right) and a potentially novel class of drug delivery system.[1–4]

Certain dendritic systems, however, and in particular some cationic dendrimers, may be both hemolytic and cytotoxic,[5,6]

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(1) Chaves, F.; Calvo, J. C.; Carvajal, C.; Rivera, Z.; Ramirez, L.; Pinto, M.; Trujillo, M.; Guzman, F.; Patarroyo, M. E. Synthesis, Isolation and Characterization of Plasmodium Falciparum Antigenic Tetrabranched Peptide Dendrimers Obtained by Thiazolidine Linkages. *J. Pept. Res.* **2001**, *58*, 307–316.

(2) Ihre, H. R.; Padilla De Jesus, O. L.; Szoka, F. C., Jr.; Frechet, J. M. J. Polyester Dendritic Systems for Drug Delivery Applications: Design, Synthesis, and Characterization. *Bioconjugate Chem.* **2002**, *13*, 443–452.

10.1021/mp070047s CCC: $37.00
Published on Web 10/23/2007

although it is also apparent that the degree of toxicity may be reduced by functionalizing the surface of the dendrimer with nonionic groups such as poly(ethylene glycol).[6] Surface functionalization of polylysine-based dendrimers with phenyldicarboxylate and naphthylsulfonate groups to provide for an anionic surface at physiological pH has also been shown to significantly reduce cytotoxic and hemolytic effects while retaining antiviral activity.[3,7–9] Pharmacokinetically, the elimination half-lives of anionic or PEGylated PAMAM dendrimers are typically longer than those of their cationic counterparts; however, the paucity of data in the literature describing the pharmacokinetics of different dendrimer systems makes definitive conclusion difficult.[9]

Most of the early studies describing dendrimers and dendrimer pharmacokinetics have focused almost exclusively on PAMAM dendrimers. In recent years, however, increasing interest in the development of biocompatible dendrimers comprising biodegradable intramolecular linkages has led to the development of several lysine-based dendrimers. Poly-L-lysine dendrimers have both antibiotic and antiviral activity,[10,11] and one naphthyl disulfonate-modified lysine-based dendrimer is currently in human clinical trials as a vaginal gel to prevent the transmission of HIV and other sexually transmitted diseases.[3]

While polylysine dendrimers show potential as possible drug candidates or drug delivery systems, relatively few studies have addressed the fundamental pharmacokinetic properties of these systems, and little is known of the impact of, for example, changes to molecular weight, surface charge, and surface functionality on pharmacokinetic parameters such as clearance, volume of distribution, and half-life. With this in mind we have recently investigated the in vivo pharmacokinetics and biodistribution of "uncapped" poly-L-lysine dendrimers following intravenous dosing in rats. In these studies cationic Generation 3 and 4 ^{3}H-lysine dendrimers with either 16 surface amine groups or 32 surface amine groups, respectively, were rapidly cleared from plasma and appeared to be broken down to the constituent lysine monomers before intercalation into protein resynthetic pathways.[12]

To complement these studies, the current investigation has sought to investigate the effect of functionalizing the surface of cationic poly-L-lysine dendrimers with anionic arylsulfonate and succinate groups on dendrimer pharmacokinetics. To this end, anionic lysine dendrimers with either Lys_8 or Lys_{16} cores and 4-benzene sulfonate (BS), 3,5-benzene disulfonate (BDS), or succinic acid (Succ) surface groups (Figure 1, Table 1) have been synthesized and their in vivo pharmacokinetics and biodistribution profiles evaluated in rats. The data indicate that anionic dendrimers display longer plasma half-lives when compared with their cationic counterparts and that the relative proclivity with respect to renal or reticuloendothelial clearance is determined by the size and charge of the surface functional groups.

Methods

Materials. Chemicals were purchased from Aldrich and were used without further purification. Unlabeled lysine was purchased from Bachem (Bunbendorf, Switzerland) while (L)-(4,5-^{3}H)-lysine was purchased from MP Biomedicals (Irvine, CA). Soluene-350 tissue solubilizer, Starscint scintillation cocktail, and 6 and 20 mL scintillation vials were obtained from Packard Biosciences (Meriden, CT). Heparin (10 000 U/mL) was from Faulding (SA, Australia). Saline for injection was obtained in 100 mL polyethylene bags from Baxter Healthcare (NSW, Australia). Acetonitrile was HPLC grade and was obtained from Ajax Finechem (Auckland, New Zealand). All buffer reagents were AR grade.

Synthesis and Characterization of Tritium-Labeled Dendrimers. Detailed methods for the preparation of the anionic dendrimers described in this article are given in the

(3) McCarthy, T. D.; Karellas, P.; Henderson, S. A.; Giannis, M.; O'Keefe, D. F.; Heery, G.; Paull, J. R. A.; Matthews, B. R.; Holan, G. Dendrimers as Drugs: Discovery and Preclinical and Clinical Development of Dendrimer-Based Microbicides for HIV and STI Prevention. *Mol. Pharmaceutics* **2005**, *2*, 312–318.

(4) Lee, C.; Gillies, E.; Fox, M.; Guillaudeu, S.; Frechet, L.; Dy, E.; Szoka, F. A. Single Dose of Doxorubicin-Functionalized Bow-Tie Dendrimer Cures Mice Bearing C-26 Colon Carcinomas. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 16679–16654.

(5) Fischer, D.; Li, Y. X.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. In Vitro Cytotoxicity Testing of Polycations: Influence of Polymer Structure on Cell Viability and Hemolysis. *Biomaterials* **2003**, *24*, 1121–1131.

(6) Jevprasesphant, R.; Penny, J.; Jalal, R.; Attwood, D.; McKeown, N. B.; D'Emanuele, A. The Influence of Surface Modification on the Cytotoxicity of Pamam Dendrimers. *Int. J. Pharm.* **2003**, *252*, 263–266.

(7) Witvrouw, M.; Fikkert, V.; Pluymers, W.; Matthews, B.; Mardel, K.; Schols, D. R. J.; Debyser, Z.; De Clercq, E.; Holan, G.; Pannecouque, C. Polyanionic (Ie, Polysulphonate) Dendrimers Can Inhibit the Replication of Human Immunodeficiency Virus by Interfering with Virus Adsorption and Later Steps (Reverse Transcriptase/Integrase) in the Virus Replicative Cycle. *Mol. Pharmacol.* **2000**, *58*, 1100–1108.

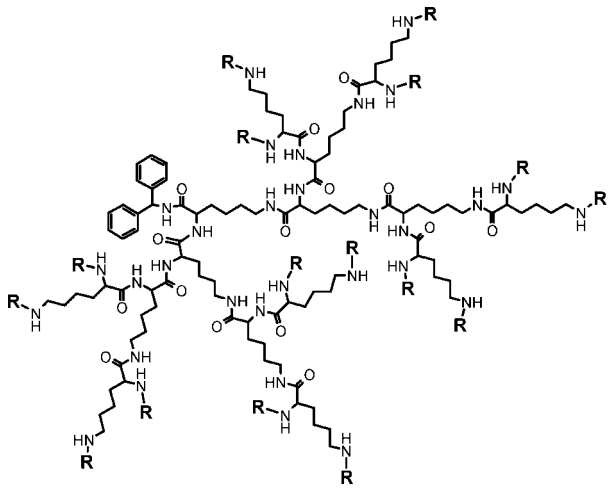
(8) Matthews, B. R.; Holan, G. Antiviral Dendrimers. US Patent 6190650, 2001.

(9) Malik, N.; Wiwattanapatapee, R.; Klopsch, R.; Lorenz, K.; Frey, H.; Weener, J. W.; Meijer, E. W.; Paulus, W.; Duncan, R. Dendrimers: Relationship Between Structure and Biocompatibility in Vitro, and Preliminary Studies on the Biodistribution of I-125-Labelled Polyamidoamine Dendrimers in Vivo. *J. Controlled Release* **2000**, *65*, 133–148.

(10) Bourne, N.; Stanberry, L. R.; Kern, E. R.; Holan, G.; Matthews, B.; Bernstein, D. I. Dendrimers, a New Class of Candidate Topical Microbicides with Activity against Herpes Simplex Virus Infection. *Antimicrob. Agents Chemother.* **2000**, *44*, 2471–2474.

(11) Janiszewska, J.; Swieton, J.; Lipkowski, A. W.; Urbanczyk-Lipkowska, Z. Low Molecular Mass Peptide Dendrimers That Express Antimicrobial Properties. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3711–3713.

(12) Boyd, B. J.; Kaminskas, L. M.; Karellas, P.; Krippner, G.; Lessene, R.; Porter, C. J. H. Cationic Poly-L-Lysine Dendrimers: Pharmacokinetics, Biodistribution and Evidence for Metabolism and Bioresorption after Intravenous Administration in Rats. *Mol. Pharmaceutics* **2006**, *3*, 614–627.



Dendrimer	R
$Lys_8(NH_2)_{16}$	—H
$Lys_{16}(NH_2)_{32}$	—Lys
$Lys_8(BS)_{16}$	—Ph(4)-SO_3Na
$Lys_{16}(BS)_{32}$	-Lys-Ph(4)-SO_3Na
$Lys_{16}(BDS)_{32}$	—Lys-Ph(3,5)-(SO_3Na)$_2$
$Lys_{16}(Succ)_{32}$	—CO-CH_2CH_2-COOH

Figure 1. Chemical structures of the cationic ^{3}H-lysine dendrimer cores and their anionic arylsulfonate or succinate substituted counterparts. Benzene-4-sulfonate, benzene-3,5-disulfonate, and succinic acid functional groups were attached to the ϵ-amino groups of terminal lysine molecules. Tritium is located at C2 and C3 on the outermost lysine layer in each case.

Table 1. Selected Properties of the Dendrimers Used in This Study[a]

dendrimer	number of anionic groups	molecular weight by ESI-MS	specific activity (μCi/mg, mean $\pm$ s.d., $n = 3$)
$Lys_8(BS)_{16}$	16	5404	0.109 ± 0.001
$Lys_{16}(BS)_{32}$	32	10053	0.176 ± 0.006
$Lys_{16}(BDS)_{32}$	64	14020	0.098 ± 0.001
$Lys_{16}(Succ)_{32}$	32	7360	2.765 ± 0.017

[a] The molecular mass and number of anionic groups for each dendrimer were confirmed by LC/MS.

Supporting Information. The tritium radiolabel was incorporated using a lysine moiety that contained tritium labels at the γ- and δ-positions, and in all cases the tritium-labeled lysine was used in the outermost lysine layer of the dendrimer.

The dendrimer nomenclature utilized in this synthetic section and in the Supporting Information has been described previously.[12] However, to simplify identification of the dendrimers in the text, the following shortened nomenclature has been used elsewhere in the paper:

$$\text{BHALys[Lys]}_{16}\ [\text{COCH}_2\text{CH}_2(\text{CO}_2\text{Na})]_{32} \equiv \text{Lys}_{16}(\text{Succ})_{32}$$

$$\text{BHALys[Lys]}_{8}\ [\text{CO-4-Ph}(\text{SO}_3\text{Na})]_{16} \equiv \text{Lys}_{8}(\text{BS})_{16}$$

$$\text{BHALys[Lys]}_{16}\ [\text{CO-4-Ph}(\text{SO}_3\text{Na})]_{32} \equiv \text{Lys}_{16}(\text{BS})_{32}$$

$$\text{BHALys[Lys]}_{16}\ [\text{CO-3,5-Ph}(\text{SO}_3\text{Na})]_{32} \equiv \text{Lys}_{16}(\text{BDS})_{32}$$

where $Lys_X(Succ, BS, BDS)_Y$ refers to X number of outermost lysine groups and Y number of surface functional groups, Succ is the succinate surface functionality, BS is the 4-benzene sulfonate surface functionality, and BDS is the 3,5-benzene disulfonate surface functionality.

The preparation of the aryl sulfonic acid dendrimers made use of commercially available carboxylic acid precursors (4-sulfobenzoic acid, 3,5-disulfobenzoic acid, and succinic anhydride; see Supporting Information) as their sodium forms and used the potent peptide coupling agent benzotriazole-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate (pyBop) to ensure the highest efficiency in the formation of the multiple amide bonds required to cap all of the surface amine groups of the dendrimers.

In a typical procedure, solid pyBop in excess on a "per amine" basis was added to a solution of the uncapped dendrimer as the TFA salt in DMSO and/or DMF. The preparation of lysine dendrimers from the benzhydryl amide protected lysine core has been described in detail elsewhere.[12] To this was added a "per amine" excess of the monosodium salt of 4-sulfobenzoic acid as a solution in a mixture of DMSO and a large excess of Hunig's base to ensure a reaction pH of ≥ 9. The reaction was carried out at room temperature and generally allowed to proceed for 24 h, although in-process controls making use of capillary electrophoresis provided evidence that the reaction was essentially complete in less than 15 min.

The contents of the reaction were then poured into a 10-fold volume of water and filtered through a 0.45 μm filter. The dendrimer solution was then concentrated to approximately one quarter of the quench volume by tangential flow filtration using a Pall-Gellman Minimate with a 1 kDa cutoff or by ultrafiltration on a Pall-Gellman stirred cell with a 5 kDa cutoff. The retentate was washed with a 10-fold (vs retentate) volume of deionized water. The presence of small molecule impurities in the retentate was monitored by CE and NMR. Once the filtration process had removed all detectable traces of contaminants from the retentate, the anionic dendrimer was recovered by lyophilization of the retentate. In most cases ^{1}H NMR analysis of the lyophylate indicated incomplete ion exchange of sodium for the protonated Hunig's base, and in such cases a final ion exchange of the anionic dendrimer was carried out using a column of Dowex ion exchange resin 69F (Dow Water Solutions, Sydney, Australia), equilibrated using sodium chloride and washed well with deionized water prior to use. The eluant

containing the anionic dendrimer was again lyophilized to provide material for animal studies.

The anionic dendrimers were characterized by ^{1}H NMR, capillary electrophoresis, and HPLC/MS. Each of these techniques provided evidence to support the identity and purity of the target material, and the combined analytical evidence, summarized in Table 1 (and in the Supporting Information), supported the proposed structures of the target materials. In particular, the integrals of the ^{1}H NMR spectra provided evidence that the ratio of the capping group to elements of the dendrimer structure as a gross property of the material was appropriate for complete capping, the use of CE provided evidence that the dendrimeric material was a single component free of small molecule capping agents, and the HPLC/MS using an ion pairing eluent to facilitate ionization of the polyanionic dendrimers as the protonated form provided molecular weight identity of the target material and confirmed the absence of significant amounts of undercapped dendrimeric components. The raw data are provided in the Supporting Information.

For the preparation of the succinlylated dendrimer BHALys[Lys]$_{16}$[COCH$_2$CH$_2$(CO$_2$Na)]$_{32}$, succinic anhydride at 2 equiv per amine was added as a solid to a solution of the amine-terminated dendrimer BHALys[Lys]$_{16}$[NH$_2$]$_{32}$ as its TFA salt in a mixture of DMF and excess Hunig's base. The succinylated dendrimer was isolated after an overnight reaction in a manner similar to that described for the aryl sulfonic dendrimers, with the exception that a solution of sodium bicarbonate, not sodium carbonate, was used to wash the retentate.

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 Corp., Milford, MA) 2795 with 2996 diode array detector (DAD) coupled to a Waters ZQ4000 with ESI probe, inlet flow split to give ~50 μL/min to the MS. Capillary electrophoresis was conducted on a Beckman P/ACE MDQ with diode array detector; capillary was underivatized fused silica, 75 μm i.d. × 50 cm to detector. The position of the UV window was 40 cm from sample loading at 15 kV normal polarity The running buffer was 80 mM borate pH unadjusted (~9.1). Samples of 0.1–0.5 mg/mL were loaded under hydrodynamic pressure: 0.5 psi for 5 s. All dendrimers were diluted in PBS and frozen at −20 °C until used.

Activity Determinations and Scintillation Counting. The specific activity of the dendrimers was determined in triplicate by dilution of stock solutions containing a known mass of dendrimer into phosphate buffered saline (PBS). An aliquot was subsequently added to 1 mL of Starscint and scintillation counted on a Packard Tri-Carb 2000CA liquid scintillation analyzer. The average of triplicate specific activity determinations was used for all subsequent calculations, and these data are listed in Table 1.

Animals. Male Sprague-Dawley rats (270–350 g) were used in these experiments and were supplied by Monash University Animal Services. Rats were provided with water at all times. Food was withheld after surgery and until 8 h after administration of the IV dose. All protocols involving animal experimentation were approved by the Victorian College of Pharmacy Animal Ethics Committee, Monash University, Parkville, VIC, Australia.

Intravenous Pharmacokinetic Studies. On the day prior to dendrimer administration, rats had cannulas (0.96 × 0.58 mm polyethylene tubing, Paton Scientific, Victor Harbour, Australia) inserted into the jugular vein and carotid artery, under isoflurane anaesthesia as described previously.[12] Animals were allowed to recover overnight prior to dosing. After surgery and during experimentation rats were housed in metabolic cages to permit separate collection of urine and feces and the cannulas attached to a swivel/leash assembly to facilitate drug administration and blood collection. Dendrimers were dissolved in 1 mL of sterile saline and administered a dose of 1, 5, or 25 mg/kg by intravenous injection over 2 min as described previously.[12] Whole blood (0.15 mL) was obtained from the carotid artery cannula immediately after complete infusion of the dose ($t = 0$) and at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 1440, and 1800 min. Blood samples were placed in heparinized (10 U) tubes and centrifuged (3500g) for 5 min. Plasma samples (50–100 μL) were then mixed with 1 mL of Starscint before scintillation counting.

Distribution of Dendrimers in Urine, Feces, and Major Organs. Urine and feces were collected and assayed for tritium as described previously.[12] On completion of the pharmacokinetic studies, rats were euthanized with 1 mL of Lethabarb (sodium pentobarbital, 60 mg/mL) 30 h after dosing. Major organs (liver, kidney, lung, heart, pancreas, spleen, brain) were removed, weighed, and homogenized in 5–10 mL of Milli-Q water using a Waring miniblender (Extech Equipment Pty. Ltd., Boronia, Australia) as reported previously.[12] The distribution of the arylsulfonic dendrimers at an earlier time point (the time at which ~90% of dendrimer had been cleared from plasma) was also investigated by IV dosing $n = 1$ rat at 5 mg/kg, euthanizing the animals 4 h later for Lys$_{16}$(BS)$_{32}$ and Lys$_{16}$(BDS)$_{32}$ or 1 h later for Lys$_8$(BS)$_{16}$ and removing liver, kidneys, and spleen for distribution studies as above. Tissue homogenate was stored at −20 °C prior to analysis. The radioactivity present in each tissue was calculated as % dose present per gram of tissue and per whole organ based on the wet weight of each organ after sacrifice.

Size Exclusion Chromatography. We have previously shown that uncapped poly-L-lysine dendrimers are rapidly metabolized after intravenous dosing.[12] As such, size exclusion chromatography was employed in this study to investigate the species present in plasma and urine samples obtained from rats dosed with anionic dendrimers. An analytical size exclusion column (Superdex 75 HR 10/30, GE Healthcare, NSW, Australia) coupled to a Waters 590 HPLC pump (Millipore Corp., Milford, MA) was used to generate plasma and urine separations. Because of the low specific activity of the dendrimers used in this study, 150–200 μL aliquots of plasma or urine were typically injected onto

the column without further dilution. Samples were eluted with 10% acetonitrile/50 mM PBS pH 7.0 at 0.5 mL/min, and aliquots were collected at 1 min (0.5 mL) intervals using a Gilson FC10 fraction collector (John Morris Scientific Pty. Ltd.). Fractions eluting from the column were then mixed with Starscint (3 mL) and analyzed by liquid scintillation to determine the radioactivity in each fraction. The retention times of each dendrimer were characterized by injection of intact parent ^{3}H dendrimers diluted in PBS. Dendrimers were also incubated in fresh plasma or 20 mg/mL bovine serum albumin (BSA, Sigma Chemical Co.) for 1 h, and the dendrimer–plasma and dendrimer–BSA mixtures were analyzed by SEC to provide an indication of whether physical interaction of dendrimer with plasma components may lead to the production of opsonized high-MW species. The void volume for the column was 15 min as determined by injection of Blue dextran 2000 (Pharmacia, Uppsala, Sweden).

Identification of High Molecular Weight Species Formed in Plasma. High molecular weight species identified in the plasma of rats administered arylsulfonic dendrimers were thought to be dendrimer–protein complexes. This hypothesis was tested by employing a protein precipitation procedure based on chloroform/phenol/isoamyl alcohol DNA purification methods.[13] Briefly, 200 μL of dendrimer-spiked plasma was mixed with 200 μL of concentrated ammonium hydroxide, 200 μL of ethanol, and 600 μL of phenol/chloroform/isoamyl alcohol (25/24/1 w/v). After vortexing, the mixture was centrifuged at 10 000*g* for 3 min to pellet precipitated protein. The aqueous layer was removed, and the organic layer was washed twice with water (2 × 400 μL). The combined aqueous layers (containing liberated dendrimer) were dried overnight at 50 °C, reconstituted in 200 μL of water, and analyzed by size exclusion chromatography as above.

Characterization of the Urinary Metabolite of Benzene Sulfonate Dendrimers. It was anticipated that the most likely identity of the metabolite observed in the urine sample of rats administered $Lys_8(BS)_{16}$ and $Lys_{16}(BS)_{32}$ was radiolabeled lysine covalently linked to two BS surface capping groups (i.e., $Lys(BS)_2$). On the basis of this assumption, ^{3}H-$Lys(BS)_2$ was synthesized, and its chromatographic and biopharmaceutical characteristics were compared to that of the urinary metabolite. Details of the procedures used are described in the Supporting Information.

Calculation of Pharmacokinetic Parameters. The quantity of radiolabel in each plasma sample was converted to nanogram dendrimer equivalents using the specific activity of the tritiated dendrimer and plasma concentrations expressed as ng equiv/mL. It is apparent that this approach assumes that the ^{3}H quantified is entirely associated with intact dendrimer and should therefore be viewed with the caveat that in some instances this is not the case. In the latter circumstances the pharmacokinetic parameters obtained should be viewed as estimates.

Terminal elimination rate constants (k) were obtained by linear regression of the individual postdistributive plasma concentration vs time profiles. Half-lives ($t_{1/2}$) 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. Less than 15% of the total AUC for each dendrimer was contained in the extrapolated area. The initial distribution volume (V_c) was calculated by dividing the administered dose by the concentration in plasma at the end of the 2 min infusion ($t = 0$, C_p^0). Since C_p^0 was taken at the end of the 2 min infusion (and not after immediate bolus), this may underestimate C_p^0 (and therefore overestimate V_c) but in this instance was assumed to represent a reasonable estimate since the infusion period (2 min) was short. Postdistributive steady-state volumes of distribution (V_{Dss}) were determined by calculation of the area under the first moment plasma concentration vs time profiles (AUMC) using the trapezoidal method and by the equation V_{Dss} = (dose/AUC) × (AUMC/AUC). Plasma clearance (Cl) was calculated by the dose/$AUC^{0-\infty}$.

Results

Plasma Pharmacokinetics of Anionic Poly-L-lysine Dendrimers. The concentration of tritium in plasma declined rapidly following administration of 5 mg/kg $Lys_{16}(Succ)_{32}$ with an elimination half-life of 12.5 ± 6.0 min (Figure 2A). Plasma concentrations were less than 1% of C_p^0 30 min after administration, and clearance was 521 mL/h (Table 2). The initial distribution volume (V_c) of the succinate dendrimer was 49.5 ± 11.8 mL, suggesting some rapid extravascular distribution or binding to nonplasma related components. Despite the similarity in size, the concentration of tritium in plasma declined considerably more slowly following administration of $Lys_8(BS)_{16}$ with a terminal half-life of ∼1 h and clearance of 21.4 mL/h (Figure 2A, Table 2). The V_c was also lower than that of the succinate dendrimer and approximated the plasma volume although slightly greater distribution occurred at later times (V_{Dss}).

In comparison to the plasma profiles obtained for the $Lys_{16}(Succ)_{32}$ and $Lys_8(BS)_{16}$ dendrimers, plasma tritium concentrations declined more slowly again for the larger arylsulfonate dendrimers ($Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$) following 5 mg/kg intravenous administration (Figure 2A). These profiles also showed some degree of convexity when plotted as log–linear profiles, suggesting potential nonlinearity with respect to elimination and/or distribution processes for these larger and more negatively charged dendrimers. Increasing the size and surface charge of the benzene sulfonate dendrimer (i.e., from $Lys_8(BS)_{16}$ to $Lys_{16}(BS)_{32}$) resulted in a reduction in clearance and steady-state volume of distribution (Table 2); however, the terminal half-lives were relatively unchanged. The initial distribution volumes (V_c) of the arylsulfonic dendrimers were low in all cases and

(13) Hsia, M.; Kreamer, B. Induction of Unscheduled DNA Synthesis in Suspensions of Rat Hepatocytes by an Environmental Toxicant 3,3′,4,4′-Tetrachloroazobenzene. *Cancer Lett.* **1979**, *6*, 207–212.

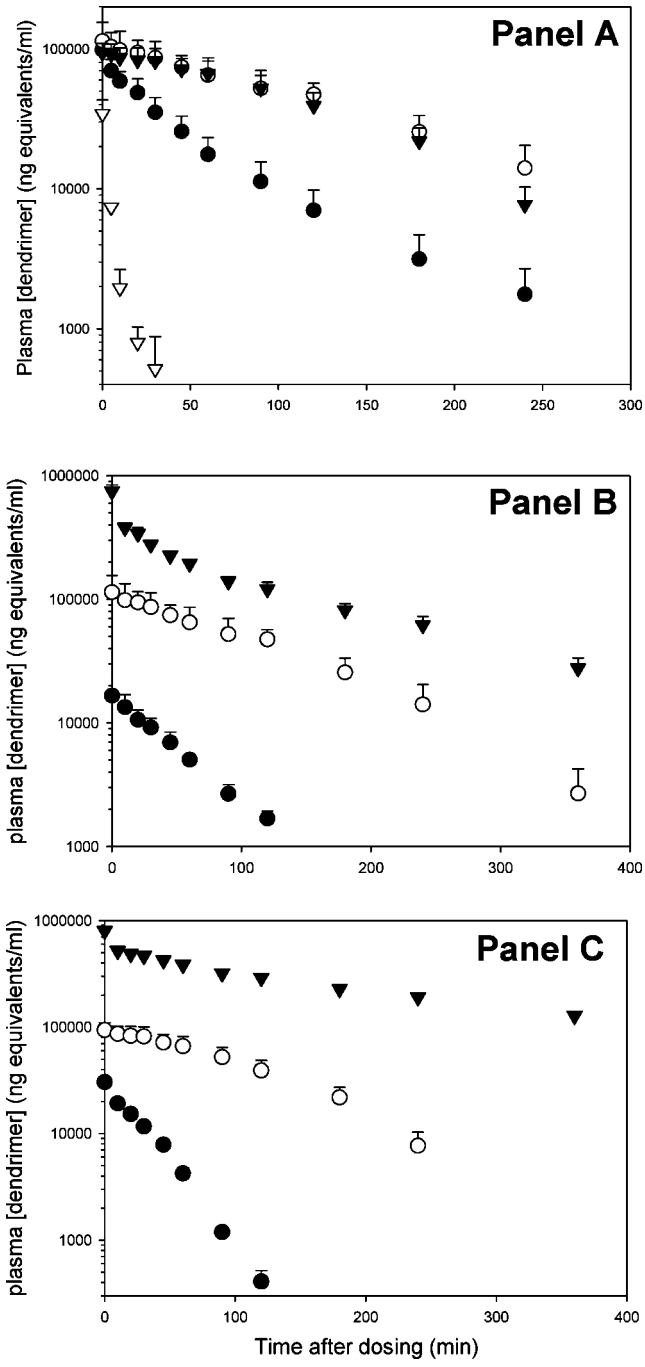


Figure 2. Dose normalized plasma concentrations of anionic ^{3}H-dendrimers following intravenous administration of dendrimers to rats. Panel A: plasma concentrations following administration of 5 mg/kg anionic dendrimers. Closed circles denote $Lys_8(BS)_{16}$, open circles $Lys_{16}(BS)_{32}$, closed triangles $Lys_{16}(BDS)_{32}$, and open triangles $Lys_{16}(Succ)_{32}$. Panel B: plasma concentrations of ^{3}H-$Lys_{16}(BS)_{32}$ following intravenous administration of 1 (closed circles), 5 (open circles), and 25 mg/kg (closed triangles) to rats. Panel C: plasma concentrations of ^{3}H-$Lys_{16}(BDS)_{32}$ following intravenous administration of 1 (closed circles), 5 (open circles), and 25 mg/kg (closed triangles) to rats. Values are mean $\pm$s.d., $n = 3$–4.

similar to the plasma volume (Table 2), suggesting little distribution out of the vasculature. Interestingly, the pharmacokinetic profiles and parameters of the $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$ dendrimers at the 5 mg/kg dose were similar, suggesting that the additional size and charge associated with the benzene disulfonate vs benzene sulfonate capping groups had relatively little impact on the kinetics of distribution and elimination, although as described in the subsequent section the mechanism of distribution and clearance of the two dendrimers did vary.

To more closely examine the linearity of the pharmacokinetics of the Lys_{16} arylsulfonate dendrimers, rats were administered additional IV doses of 1 and 25 mg/kg of $Lys_{16}(BS)_{32}$ (Figure 2B) and $Lys_{16}(BDS)_{32}$ (Figure 2C). For both dendrimers, plasma clearance was considerably greater following administration of the lower 1 mg/kg dose. Terminal plasma half-lives similarly decreased (Table 2). Following administration of the higher 25 mg/kg dose, clearance decreased for $Lys_{16}(BDS)_{32}$ but did not change for $Lys_{16}(BS)_{32}$. Terminal half-lives increased to approximately 2 and 3 h for the $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$ dendrimers, respectively, after administration of the 25 mg/kg dose. No substantial changes in distribution volume were observed following administration of the different doses of either dendrimer.

Excretion of Dendrimers in Urine and Feces. The succinate derivatized dendrimer was rapidly eliminated into the urine within the first 8 h after dosing, and by 30 h postdose 63.7 $\pm$ 9.0% of the injected radiolabel had been recovered in pooled urine (Table 3). ^{3}H derived from $Lys_8(BS)_{16}$ and $Lys_{16}(BS)_{32}$ was also recovered in significant (albeit lower) quantities in 30 h urine (25.4 $\pm$ 8.2% and 30.4 $\pm$ 6.8% of the injected tritium, respectively) (Table 3). In contrast to the succinate dendrimer, however, urinary recovery of tritium after administration of $Lys_8(BS)_{16}$ and $Lys_{16}(BS)_{32}$ was delayed, and the majority of the eliminated radiolabel recovered in urine was recovered in the 8–24 h postdose period (Table 3) and was not associated with intact dendrimer (metabolic profile shown by SEC in the following section). Increasing the expected number of anionic charges on the Lys_{16} dendrimer from 32 ($Lys_{16}(BS)_{32}$) to 64 ($Lys_{16}(BDS)_{32}$) and consequently increasing molecular weight from 10 to 14 kDa resulted in a 10-fold decrease in the proportion of radiolabel eliminated in urine over 30 h (from 30.4 $\pm$ 6.8% to 3.0 $\pm$ 4.1% of administered dose, respectively) (Table 3).

The dose dependence of urinary elimination of ^{3}H from the Lys_{16} arylsulfonic dendrimers is shown in Figure 3. The extent of urinary elimination was low and largely unchanged with increasing dose (from 1 to 25 mg/kg) for the $Lys_{16}(BDS)_{32}$ dendrimer, whereas increasing the dose of $Lys_{16}(BS)_{32}$ resulted in a reduction in the radioactive fraction eliminated into the urine from ~45% of the injected dose to 20% on increasing the dose from 1 to 25 mg/kg.

No tritium was detected in feces for any of the three arylsulfonic dendrimers, although the LOQ for the assay was high (up to 24% of administered dose, data not shown). The

Table 2. Plasma Pharmacokinetic Parameters for Anionic Dendrimers Following Intravenous Administration at Doses 1–25 mg/kg to Rats (Mean ± s.d., $n = 3$)

dendrimer	dose (mg/kg)	terminal $t_{1/2}$ (h)	total AUC (μg/(mL h))	V_c (mL)	V_{Dss} (mL)	Cl (mL/h)
$Lys_{16}(Succ)_{32}$	5	0.2 ± 0.1	3.2 ± 0.7	49.5 ± 11.8	42.3 ± 6.9	521 ± 97
$Lys_8(BS)_{16}$	5	0.9 ± 0.2	65 ± 18	13.9 ± 2.7	20.2 ± 0.8	21.4 ± 4.1
$Lys_{16}(BS)_{32}$	1	0.6 ± 0.0	14.4 ± 2.4	14.8 ± 1.3	14.7 ± 3.1	17.3 ± 3.2
	5	0.9 ± 0.2	219 ± 46	12.8 ± 3.6	10.7 ± 2.9	6.4 ± 1.6
	25	2.0 ± 0.1	795 ± 101	10.5 ± 2.0	20.1 ± 0.7	8.5 ± 0.6
$Lys_{16}(BDS)_{32}$	1	0.4 ± 0.0	15.8 ± 0.3	7.8 ± 0.3	7.2 ± 0.5	15.2 ± 0.6
	5	1.0 ± 0.1	190 ± 35	14.3 ± 2.2	10.9 ± 2.0	7.2 ± 1.4
	25	3.2 ± 0.5	2204 ± 142	8.8 ± 0.6	13.9 ± 1.5	3.2 ± 0.2

Table 3. Urinary Excretion of Injected ^{3}H from Rats Administered ^{3}H-Dendrimers at a Dose of 5 mg/kg (Mean ± s.d., $n = 3$)[a]

dendrimer	0–8 h	8–24 h	24–30 h	total
$Lys_8(BS)_{16}$	6.1 ± 0.8	15.3 ± 5.6	4.0 ± 3.5	25.4 ± 8.2
$Lys_{16}(BS)_{32}$	4.5 ± 4.3	21.6 ± 4.3	4.3 ± 1.0	30.4 ± 6.8
$Lys_{16}(BDS)_{32}$	0.4 ± 0.3	2.7 ± 3.6	0	3.0 ± 4.1
$Lys_{16}(Succ)_{32}$	49.4 ± 13.1	12.6 ± 5.0	1.6 ± 1.5	63.7 ± 9.0

[a] Values represent the % of injected ^{3}H excreted in urine ± s.d. per collection time.

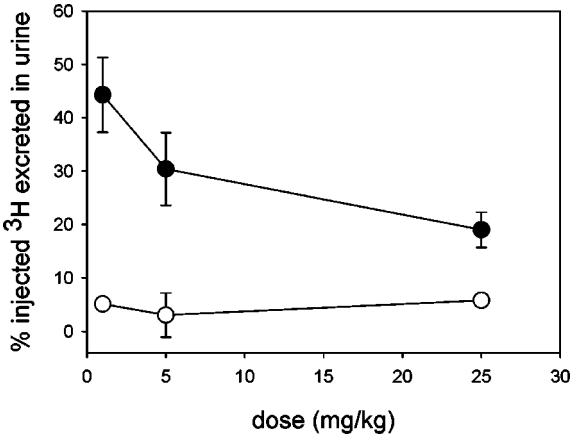


Figure 3. Correlations between the intravenous dose of $Lys_{16}(BS)_{32}$ (closed circles) or $Lys_{16}(BDS)_{32}$ (open circles) and % of injected ^{3}H excreted in urine collected over 30 h after dosing in rats. Values are mean ± s.d., $n = 3$–4.

LOQ for the arylsulfonic dendrimers in feces was high because of the low specific activity of the dendrimers (typically 0.1 μCi/mg) and because the analytical method for feces was based on the assay of relatively small sample masses (20 mg), which constituted only a small proportion of the total pooled feces mass (typically 2 g of dry weight). In contrast, the LOQ for the succinate dendrimer was lower due to the higher specific activity of the succinate dendrimer. In this case, 1.2 ± 1.0% of injected tritium was recovered in 30 h pooled feces. In all cases, however, it seems likely that elimination of radiolabel into the feces was limited.

Metabolic Profiling by Size Exclusion Chromatography of Plasma and Urine. The SEC profiles of tritiated material present in plasma and urine following administration of 5 mg/kg $Lys_{16}(Succ)_{32}$ are shown in Figure 4. The retention time for the succinate dendrimer was 23–24 min both in PBS and after in vitro preincubation for 1 h in fresh plasma (panel A). A very minor shoulder peak was also identified at 17 min in plasma incubations. Plasma taken at the completion of dendrimer infusion ($t = 0$) contained mostly intact dendrimer that eluted at 24 min (panel B). While only a small amount of tritium remained in 1 h plasma (due to the very rapid clearance of the succinate dendrimer), several peaks were present that eluted at 17, 24, 34, and 42 min. The peak at 17 min may reflect a larger species resulting from dendrimer (or radiolabel) association with plasma proteins, as this peak was also evident (albeit in very small amounts) in in vitro plasma incubations with the succinate dendrimer (panel A). The peak eluting at 42 min was also present as a low molecular weight contaminant in dendrimer stock solutions. The low-MW contaminant was present in very low proportion in the stock solution (~1% of total activity, Figure 4A) and was therefore unlikely to have had a significant effect on dendrimer pharmacokinetics, especially for rapidly eliminated systems such as the succinate dendrimer. The peak at 34 min is likely to be a product of metabolic cleavage of lysine–bis(succinate) capping groups, although this possibility was not investigated further for the succinate dendrimer since renal clearance was the predominant method of elimination. This species was also found in urine samples, along with intact dendrimer (which was the major species identified in urine, panel C).

The SEC profiles of tritiated material in plasma and urine following administration of 5 mg/kg $Lys_8(BS)_{16}$ are shown in Figure 5. The retention time for the Lys_8 benzene sulfonate dendrimer was 28–29 min both in PBS and after preincubation for 1 h in fresh plasma (panel A). Following incubation of the dendrimer in fresh plasma an additional, albeit minor, peak again appeared in SEC profiles at 18 min. This peak was also present in the $t = 0$ and 2 h plasma samples (panel B) collected from rats intravenously administered 5 mg/kg $Lys_8(BS)_{16}$ (but not in incubations with PBS) and therefore represents a plasma-derived product. A single low-MW product that eluted at 36–38 min was the only product identified in 0–8 and 8–24 h urine (panel C) and likely represents the ^{3}H-Lys–bis(benzene sulfonate) surface

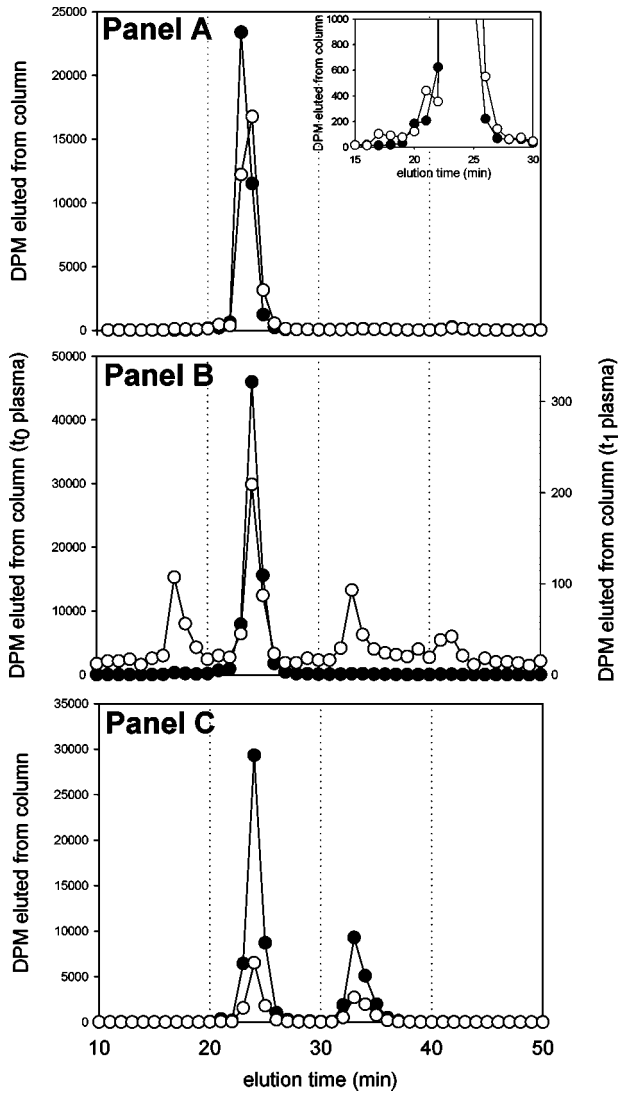


Figure 4. Size exclusion profiles of ^{3}H in the plasma and urine of rats dosed IV with $Lys_{16}(Succ)_{32}$. Panel A: SEC profile of $Lys_{16}(Succ)_{32}$ incubated for 1 h in PBS (closed circles) or fresh plasma (open circles). Panel A, inset: expanded profile showing formation of a smaller peak at 17 min in dendrimer incubations in fresh plasma. Panel B: SEC profile of $Lys_{16}(Succ)_{32}$ in plasma at t_0 (closed circles) and 1 h (open circles). Panel C: SEC profile of ^{3}H in 0–8 h urine (closed circles) and 8–24 h urine (open circles).

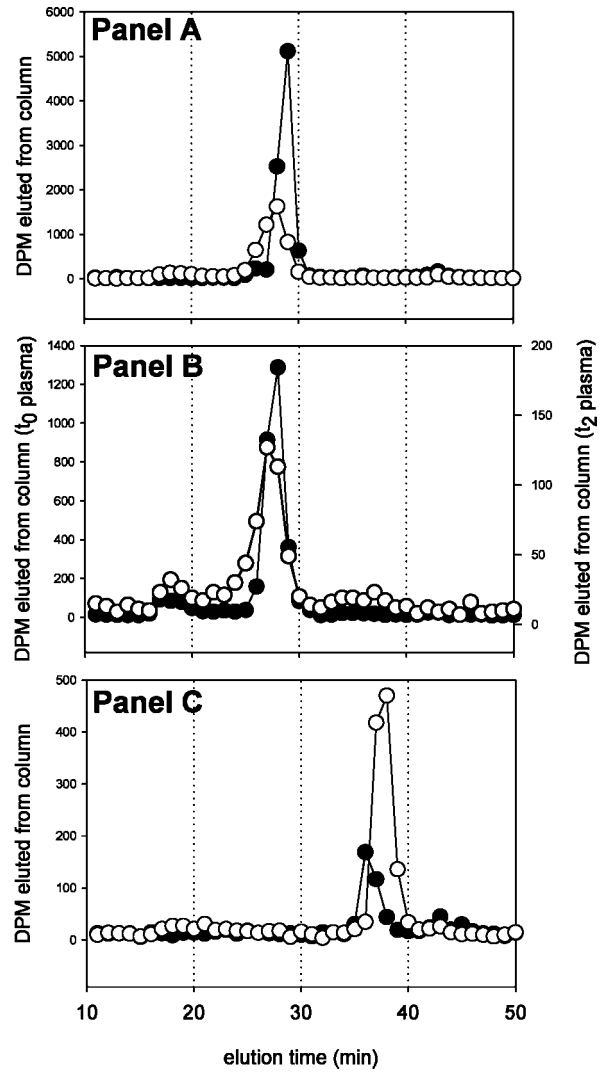


Figure 5. Size exclusion profiles of ^{3}H in the plasma and urine of rats dosed IV with $Lys_8(BS)_{16}$. Panel A: SEC profile of $Lys_8(BS)_{16}$ incubated for 1 h in PBS (closed circles) or fresh plasma (open circles). Panel B: SEC profile of $Lys_8(BS)_{16}$ in plasma at t_0 (closed circles) and 2 h (open circles). Panel C: SEC profile of ^{3}H in 0–8 h urine (closed circles) and 8–24 h urine (open circles).

cleavage product. Characterization of the urinary elimination product is described in more detail in the following section. Importantly, this product was only evident in plasma samples at very low levels and appeared in urine at the highest concentrations at times considerably after the disappearance of intact dendrimer from plasma (i.e., more than 8 h after dosing and greater than 7 h after plasma radioactivity levels had declined to less than 10% of C_p^0). Also, the product was not formed ex vivo as a breakdown product of the intact dendrimer in urine, as incubation of dendrimer in urine for several hours did not result in liberation of this species (data not shown).

The SEC profiles of $Lys_{16}(BDS)_{32}$ and $Lys_{16}(BS)_{32}$ in plasma and urine are shown in Figure 6. The retention times for intact $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$ were 24 and 22 min, respectively (Figure 6, panels A and B, respectively). Incubation of either dendrimer in fresh plasma for 1 h at room temperature resulted in a shift in these retention times to 19 and 18 min, respectively. Incubation of $Lys_{16}(BDS)_{32}$ in a 20 mg/mL solution of albumin in PBS (to reflect the approximate concentration of albumin in rat plasma) also resulted in a similar shift in retention time (to approximately 17–22 min, data not shown), providing some evidence to support the suggestion that these high molecular weight species represent products of dendrimer binding to plasma proteins rather than a metabolic product. This suggestion was confirmed further using a phenol/chloroform extraction of

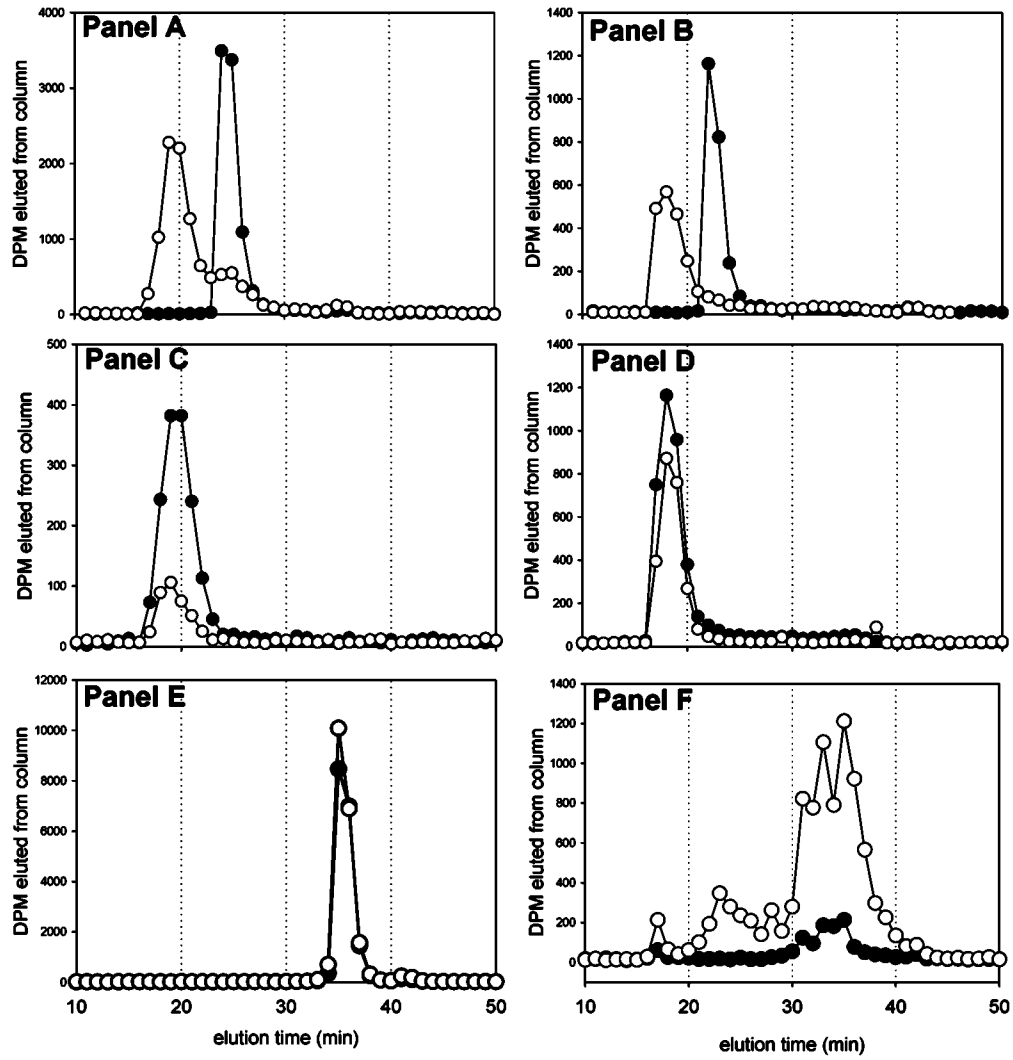


Figure 6. Size exclusion profiles of ^{3}H in the plasma and urine of rats dosed IV with 5 mg/kg $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$. Panel A: SEC profile of $Lys_{16}(BS)_{32}$ incubated for 1 h in PBS (closed circles) or fresh plasma (open circles). Panel B: SEC profile of $Lys_{16}(BDS)_{32}$ incubated for 1 h in PBS (closed circles) or fresh plasma (open circles). Panel C: SEC profile of $Lys_{16}(BS)_{32}$ in plasma at t_0 (closed circles) and 2 h (open circles). Panel D: SEC profile of $Lys_{16}(BDS)_{32}$ in plasma at t_0 (closed circles) or 2 h (open circles). Panel E: SEC profile of ^{3}H in 0–8 h urine (closed circles) and 8–24 h urine (open circles) after administration of 5 mg/kg $Lys_{16}(BS)_{32}$. Panel F: SEC profile of ^{3}H in 0–8 h urine (closed circles) and 8–24 h urine (open circles) after administration of 25 mg/kg $Lys_{16}(BDS)_{32}$ to allow detection of radiolabeled products in urine samples by SEC.

the dendrimer from the plasma protein complex. In these studies, extraction with phenol/chloroform resulted in liberation of intact dendrimer from the complex, resulting in the loss of the high molecular weight peak at 18 min in the SEC profile and the reappearance of a prominent peak at the elution time of the intact dendrimer (see Supporting Information).

SEC profiles of plasma collected from rats dosed with 5 mg/kg $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$ are shown in Figure 6, panels C and D, respectively. Both profiles show that at the completion of intravenous dendrimer infusion (i.e., $t = 0$) and 2 h after infusion both dendrimers elute at times corresponding to those of the large molecular weight species present after incubation of dendrimer in fresh plasma (shown in panels A and B). Smaller metabolic products are not clearly evident in the plasma profiles.

The major radiolabeled product in urine collected from animals dosed with $Lys_{16}(BS)_{16}$ eluted at 35 min (panel E) in a pattern reflecting the urinary elimination of radiolabel after administration of $Lys_8(BS)_{16}$ (Figure 5, panel C), that is, at a time corresponding to that of the cleavage product ^{3}H-Lys–bis(benzene sulfonate).

There was insufficient radiolabel present in the urine collected after administration of 5 mg/kg $Lys_{16}(BDS)_{32}$ to produce an accurate SEC profile. Therefore, urine collected after administration of 25 mg/kg $Lys_{16}(BDS)_{32}$ was used to better identify the possible radiolabeled species excreted into

urine (Figure 6F). Several small peaks eluted at 17 and 23 min which were likely due to excretion of small amounts of protein-bound dendrimer and intact dendrimer, respectively. A cluster of peaks also eluted at 31, 33, and 35 min, consistent with small quantities of metabolic cleavage products. However, since only 3–5% of the radioactivity associated with $Lys_{16}(BDS)_{32}$ was excreted in 30 h urine and breakdown product related peaks were not identified in plasma, it is apparent that the larger disulfonate capping groups prevented extensive metabolism of the $Lys_{16}(BDS)_{32}$ dendrimer.

Characterization of the Urinary Metabolite of Benzene Sulfonate Dendrimers. Because of the relatively high quantities of nondendrimer material recovered in the urine of animals administered the benzene sulfonate derived dendrimers (Figure 5C, Figure 6E), an attempt was made to identify the nature of the species recovered. This is reported in full in the Supporting Information. In brief, LCMS was used to identify a molecular ion of 513 Da in the urine of animals administered $Lys_{16}(BS)_{32}$, consistent with the presence of $Lys(BS)_2$. ^{3}H-$Lys(BS)_2$ was also synthesized and shown by LCMS (mass selective detection at 513 Da) and SEC to coelute with the radiolabeled urinary metabolite. The synthesized ^{3}H-$Lys(BS)_2$ was also shown to be rapidly cleared from plasma via the urine following IV dosing to a rat, consistent with the urinary elimination profile of the $Lys_{16}(BS)_{32}$ metabolite.

Biodistribution of Radiolabel in Major Organs 30 h after Dosing. The distribution of residual tritium in major organs 30 h after administration of the anionic dendrimers is shown in Figure 7. Between 20 and 50% of the injected radiolabel from the arylsulfonic dendrimers was recovered in the liver, kidneys, and spleen collectively (panel A). Levels of radioactivity in the pancreas, heart, lungs, and brain were below the LOQ of the assay for the arylsulfonate dendrimers (which for all organs was <0.1% of the dose). Only 2% of injected radiolabel from the succinate dendrimer was recovered in the major organs, presumably as a result of extensive renal elimination. As a proportion of the dose, residual tritium was found predominantly in the liver for all the arylsulfonate dendrimers. Normalizing the distribution data for tissue mass suggests that for $Lys_8(BS)_{16}$ radioactivity also accumulated in the kidneys; however, in general for the Lys_{16} arylsulfonic dendrimers uptake into the reticuloendothelial organs (spleen and liver) was most prevalent (panel B). Panels A and B of Figure 7 also suggest a trend toward increasing uptake into the liver and spleen with increasing size and charge of the arylsulfonate dendrimers, with a corresponding decrease in the proportion of the radiolabel detected in kidneys.

To examine the relative distribution of the arylsulfonate dendrimers at early time points $Lys_8(BS)_{16}$, $Lys_{16}(BS)_{32}$, and $Lys_{16}(BDS)_{32}$ (5 mg/kg) were intravenously administered, and animals were sacrificed at different times (1 or 4 h later) to provide distribution patterns after similar extents of dendrimer elimination from plasma for systems with varying elimination kinetics. Four hours after dosing, approximately 52% and 63% of $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$, respectively,

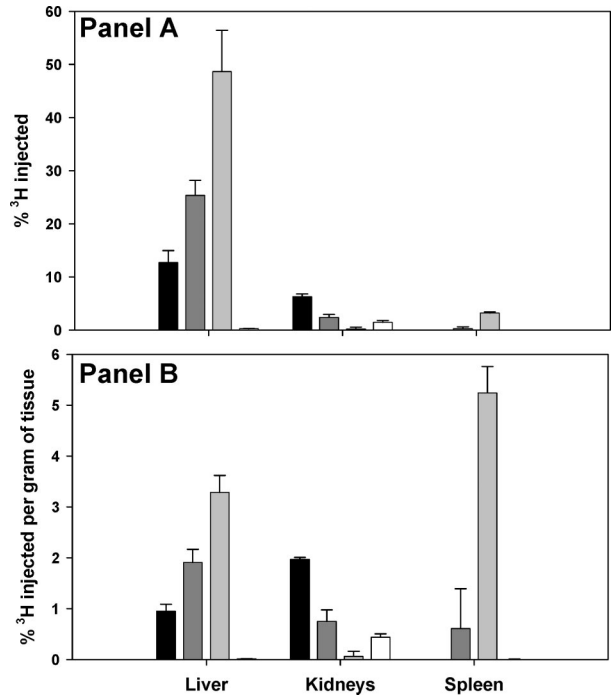


Figure 7. Distribution of residual ^{3}H in major organs 30 h after intravenous administration of anionic ^{3}H-dendrimers (5 mg/kg) to rats. Panel A represents the % of injected radiolabel retained in each organ, whereas panel B provides data normalized for tissue mass (mean ± s.d., $n = 3$). Black bars represent data for $Lys_8(BS)_{16}$ dosed rats, dark gray bars depict data for $Lys_{16}(BS)_{32}$ dosed rats, light gray bars represent data for $Lys_{16}(BDS)_{32}$ dosed rats, and white bars represent data for $Lys_{16}(Succ)_{32}$ dosed rats.

were found in liver (results not shown), indicating similar and rapid initial accumulation of these dendrimers in the liver. A further 5% was detected in kidneys and spleen (where 4% of $Lys_{16}(BS)_{32}$ was located in the kidneys and 1% in the spleen and roughly equal proportions of $Lys_{16}(BDS)_{32}$ were present in both organs). In contrast, 1 h after administration of $Lys_8(BS)_{16}$ only 18, 7, and 2.2% of injected radiolabel was recovered in the liver, kidneys, and spleen, respectively.

Discussion

Dendrimers are receiving increasing interest for their potential as both drug carriers and therapeutic entities in their own right. Despite this level of interest, few systematic studies of dendrimer pharmacokinetics are evident in the literature, and little is known of the impact of variations in structure and chemical functionality on systemic pharmacokinetics and biodistribution patterns. As a first step toward building a level of knowledge for poly-L-lysine-based dendrimers, we have previously reported the pharmacokinetic and biodistribution behavior of "uncapped" cationic lysine dendrimers.[12] Linear polylysine has been employed for many

years as a vector for the cellular delivery of DNA,[14,15] and in blood, the polypeptide has a very short terminal half-life of only 1.5 min and is hydrolyzed by cellular peptidases to monomeric lysine.[16,17] Consistent with these data, our previous studies with dendritic poly-L-lysine suggested that the exposed lysine surface and strong cationic surface charge of the dendrimers resulted in rapid binding to the vasculature and subsequent breakdown and reincorporation of liberated lysine into endogenous protein synthetic pathways. The next stage in these investigations has been to introduce capping groups on the surface of these dendrimers. The first of these investigations is described in this report and has examined the use of anionic benzene sulfonate, disulfonate, and succinate capping groups on poly-L-lysine cores. The primary hypothesis underpinning the current study was that, by capping the surface of cationic poly-L-lysine dendrimers with anionic functionalities, binding to the vasculature would be reduced or eliminated and that metabolism of the dendrimer to free lysine would be similarly reduced. This hypothesis stemmed from previous observations that poly-L-lysine dendrimers capped with nonbiologically active D-lysine resulted in reduced metabolism of the dendrimer.[12]

$Lys_{16}(Succ)_{32}$. After intravenous administration the half-life of the succinate-capped dendrimer was not significantly extended when compared with the uncapped poly-L-lysine core.[12] However, unlike the uncapped dendrimers, the plasma profiles did not show any evidence of reincorporation of tritium label into plasma protein resynthetic pathways and the majority of the administered radiolabel was rapidly recovered in urine as intact dendrimer. It seems likely therefore that capping with anionic succinate groups avoided the previously described interaction of the uncapped cationic dendrimer with the vascular wall but that this subsequently led to efficient renal elimination of this relatively small (7.4 kDa) dendrimer. Although the plasma profiles suggested an increase in plasma stability for the succinate-capped poly-lysine dendrimer, the presence of a low molecular weight metabolite in urine (34 min, Figure 6, panel C) provided some evidence of metabolism (although this represented a relatively small proportion of the renally excreted radiolabel).

$Lys_8(BS)_{16}$. The data obtained after intravenous injection of the $Lys_8(BS)_{16}$ dendrimer revealed a reduction in plasma clearance and an increase in half-life when compared with the succinate-terminated species. Interestingly, the proportion of the $Lys_8(BS)_{16}$ radiolabel that was recovered in urine was considerably lower than that observed for the succinate dendrimer (25% vs 64%), and most of the radiolabel in urine was excreted at time points following the initial disappearance of radiolabel from plasma. The radiolabel recovered in urine was also almost entirely attributable to a small molecular weight metabolic product that eluted at ~36 min on the SEC column. This urinary metabolite showed similar pharmacokinetic and chromatographic characteristics to $Lys(BS)_2$ which is expected to be liberated via cleavage of lysine–lysine bonds between the two outermost lysine layers of the dendrimer. The data therefore suggest that at least 25% of the surface $Lys(BS)_2$ units were cleaved from the $Lys_8(BS)_{16}$ dendrimer over the 30 h postdose period. Following liberation of the labeled $Lys(BS)_2$ surface layer, the poly-L-lysine core is expected to be further metabolized to free lysine; however, in the current experiments the remainder of the core was unlabeled, and therefore this was not evident in the pharmacokinetic profiles.

$Lys_{16}(BS)_{32}$. The most remarkable distinction between the smaller $Lys_8(BS)_{16}$ dendrimer and the larger $Lys_{16}(BS)_{32}$ dendrimer was the rapid opsonisation and RES uptake of the larger, more highly charged species. The data suggest that the increase in size and surface charge for the $Lys_{16}(BS)_{32}$ dendrimer facilitated increased binding to plasma opsonic factors and subsequent recognition and uptake of the dendrimer by phagocytic cells in the liver. This is consistent with the rapid accumulation of ~50% of the injected dose of radiolabel in the liver by 1 h postdose. Increasing the core size of the dendrimer from Lys_8 to Lys_{16} increased both molecular weight and surface charge; however, it is most likely that differences in surface charge were responsible for the differences in opsonization (and therefore clearance profile) since significant opsonization was not seen with the $Lys_{16}(Succ)_{32}$ dendrimer (which was almost equivalent in size to the $Lys_{16}(BS)_{32}$ dendrimer but had a weaker base as a surface capping group (calculated pK_a of the terminal carboxyl (modeled on $Lys(Succ)_2$) ~ 4.67 vs pK_a of terminal sulfonate (modeled on $Lys(BS)_2$) ~ −2.0 to −2.61; MarvinSpace, www.chemaxon.com).

Consistent with the data obtained for the smaller $Lys_8(BS)_{16}$ dendrimer, $Lys_{16}(BS)_{32}$ was extensively metabolized as evidenced by the urinary excretion of 30% of the injected radiolabel as nonparent dendrimer (and presumably $Lys(BS)_2$). However, in the case of the $Lys_{16}(BS)_{32}$ dendrimer, the later time scale of urinary elimination and the decrease in radiolabel in the liver over the 4–30 h postdose period suggested that the dendrimer was first taken up by the liver and subsequently metabolized leading to release and renal clearance of the $Lys(BS)_2$ capping group, rather than the systemic liberation of $Lys(BS)_2$ as seems likely in the case of $Lys_8(BS)_{16}$.

Comparison of the plasma concentration–time profiles of the $Lys_{16}(BS)_{32}$ and $Lys_8(BS)_{16}$ dendrimers at the 5 mg/kg dose

(14) Fisher, K. J.; Wilson, J. M. Biochemical and Functional Analysis of an Adenovirus-Based Ligand Complex for Gene Transfer. *Biochem. J.* **1994**, *299*, 49–58.

(15) Kataoka, T.; Yamamoto, S.; Yamamoto, T.; Tokunaga, T. Immunotherapeutic Potential in Guinea-Pig Tumor Model of Deoxyribonucleic Acid from Mycobacterium Bovis Bcg Complexed with Poly-L-Lysine and Carboxymethylcellulose. *Jpn. J. Med. Sci. Biol.* **1990**, *43*, 171–182.

(16) Quong, D.; Yeo, J. N.; Neufeld, R. J. Stability of Chitosan and Poly-L-Lysine Membranes Coating DNA-Alginate Beads When Exposed to Hydrolytic Enzymes. *J. Microencapsulation* **1999**, *16*, 73–82.

(17) Johnston, T. P.; Kuchimanchi, K. R.; Alur, H.; Chittchang, M.; Mitra, A. K. Inducing a Change in the Pharmacokinetics and Biodistribution of Poly-L-Lysine in Rats by Complexation with Heparin. *J. Pharm. Pharmacol.* **2003**, *55*, 1083–1090.

revealed evidence of nonlinearity for the larger $Lys_{16}(BS)_{32}$ dendrimer when compared with the smaller $Lys_8(BS)_{16}$ construct (Figure 2A). The nonlinearity of the $Lys_{16}(BS)_{32}$ dendrimer was subsequently confirmed by administration at lower and higher doses where clearance at the lower dose was increased and the urinary excretion of radiolabeled metabolite was higher (Table 2, Figure 3). It is interesting to speculate, therefore, that the nonlinearity of $Lys_{16}(BS)_{32}$ dendrimer pharmacokinetics may have reflected the differing mode of clearance when compared with the $Lys_8(BS)_{16}$ dendrimer. Thus, the larger dendrimer was cleared via opsonization and hepatic uptake followed by breakdown in the liver (processes that might be expected to be relatively low capacity and therefore saturable), whereas the smaller $Lys_8(BS)_{16}$ dendrimer was metabolized systemically, presumably via nonspecific esterase or protease enzymes in what might be expected to be a relatively high capacity and less readily saturable process.

Our findings with $Lys_8(BS)_{16}$ and $Lys_{16}(BS)_{32}$ are therefore in contrast to those of Kobayashi et al.,[18,19] where no differences were observed in whole blood retention on increasing the size of anionic PAMAM dendrimers (half generation and carboxylate terminus) from G2.5 to G5.5. However, in the latter study comparisons were based only on a single blood sample obtained 1 h after intravenous administration. It is possible that more significant differences would have become evident after longer time periods.

$Lys_{16}(BDS)_{32}$. Comparison of the differences in pharmacokinetic profiles of $Lys_8(BS)_{16}$ and $Lys_{16}(BS)_{32}$ dendrimers was confounded by the inherent increases in both size and surface charge resulting from the increase from 16 to 32 benzene sulfonate surface capping groups. In an attempt to more clearly delineate the influence of surface charge, we subsequently prepared dendrimers comprising a Lys_{16} dendrimer core capped with 32 benzene disulfonate (as opposed to benzene sulfonate) groups in order to proved additional anionic surface charge.

In a similar fashion to the $Lys_{16}(BS)_{32}$ dendrimer, $Lys_{16}(BDS)_{32}$ was opsonized in plasma, rapidly cleared by the reticuloendothelial organs, and showed similar evidence of nonlinear plasma pharmacokinetics. However, significant differences in the in vivo behavior of the two Lys_{16} arylsulfonate dendrimers were evident in the differences in urinary metabolite elimination (Table 3) and the time scale of dissipation of radiolabel from the liver. Thus, both $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$ were taken up into the liver

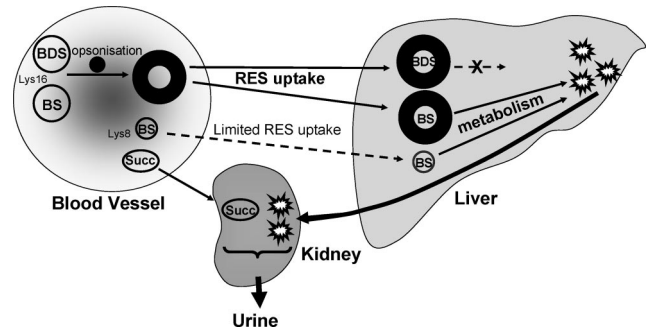


Figure 8. Schematic diagram summarizing the biopharmaceutical behavior of anionic poly-L-lysine dendrimers following intravenous administration to rats. The diagram shows the conversion of large dendrimers $Lys_{16}(BS)_{32}$ (BS) and $Lys_{16}(BDS)_{32}$ (BDS) to opsonized products that are taken up by the liver and metabolized (in the case of $Lys_{16}(BS)_{32}$) to products that are excreted via the kidneys. The smaller dendrimers ($Lys_8(BS)_{16}$ (BS) and $Lys_{16}(Succ)_{32}$ (Succ)) are not opsonized or appreciably targeted toward the liver but are also metabolized to products that are eliminated via the kidneys. Only Succ is excreted unchanged in urine.

(and spleen in the case of $Lys_{16}(BDS)_{32}$) at early time points (1 h postdose); however only the benzene sulfonate dendrimer was subsequently cleared from the liver, resulting in recovery of dendrimer breakdown products in the urine at later time points. In contrast, the benzene disulfonate dendrimer appeared to be stable to metabolic breakdown in the liver and plasma as evidenced by the large amount of radiolabel remaining in the liver and spleen at 30 h postdose (Figure 7) and the relatively small (only 3–5%) quantity of radiolabel excreted into the urine.

The reason for the difference in metabolic lability of $Lys_{16}(BS)_{32}$ and $Lys_{16}(BDS)_{32}$ is not entirely clear but may reflect either the greater overall surface charge (64 negative charges vs 32 negative charges) or increased steric hindrance to enzymatic approach provided by the additional sulfonic acid groups associated with the surface of the $Lys_{16}(BDS)_{32}$ dendrimer when compared to the $Lys_{16}(BS)_{32}$ dendrimer.

The plasma pharmacokinetics of the $Lys_{16}(BDS)_{32}$ dendrimer were markedly nonlinear (Table 2), and in contrast to $Lys_{16}(BS)_{32}$, clearance continued to reduce with dose even between doses of 5 and 25 mg/kg doses. Since the $Lys_{16}(BDS)_{32}$ dendrimer was relatively resistant to metabolism and was not renally cleared, these data suggest that the nonlinear pharmacokinetics may reflect saturation of liver uptake (likely phagocytic) processes.

Conclusions

The data in the current study support the hypothesis that capping the surface of poly-L-lysine dendrimers with anionic arylsulfonate or succinate groups reduces vascular binding and increases metabolic stability when compared with the corresponding cationic poly-L-lysine cores. The substantial differences in the pharmacokinetics of anionic dendrimers with changes to size and charge after intravenous administra-

(18) Kobayashi, H.; Kawamoto, S.; Saga, T.; Sato, N.; Hiraga, A.; Konishi, J.; Togashi, K.; Brechbiel, M. W. Micro-MR Angiography of Normal and Intratumoral Vessels in Mice Using Dedicated Intravascular MR Contrast Agents with High Generation of Polyamidoamine Dendrimer Core: Reference to Pharmacokinetic Properties of Dendrimer-Based MR Contrast Agents. *J. Magn. Reson. Imaging* **2001**, *14*, 705–713.

(19) Kobayashi, H.; Sato, N.; Hiraga, A.; Saga, T.; Nakamoto, Y.; Ueda, H.; Konishi, J.; Togashi, K.; Brechbiel, M. W. 3D-Micro-MR Angiography of Mice Using Macromolecular MR Contrast Agents with Polyamidoamine Dendrimer Core with Reference to Their Pharmacokinetic Properties. *Magn. Reson. Med.* **2001**, *45*, 454–460.

tion further indicate that there is latitude to tailor the biopharmaceutical behavior of anionic poly-L-lysine dendrimers, even with similar surface functionalities. In the current study large arylsulfonate-capped dendrimers ($Lys_{16}(BDS)_{32}$ and $Lys_{16}(BS)_{32}$) were opsonized, leading to the production of larger species that were extensively taken up by the liver and spleen, while smaller benzene sulfonate ($Lys_8(BS)_{16}$) and succinate ($Lys_8(Succ)_{16}$) capped dendrimers were not appreciably opsonized and avoided RES uptake (Figure 8). For the smaller Lys_8 dendrimers, elimination of tritium label was primarily renal, although in the case of $Lys_8(Succ)_{16}$ the dendrimer was rapidly excreted unchanged, whereas the $Lys_8(BS)_{16}$ was metabolized prior to renal elimination. In all cases, dendrimer metabolism was reduced by capping the surface of poly-L-lysine dendrimers with anionic surface groups, and metabolism was reduced even further when the surface of these dendrimers was capped with strongly anionic benzene disulfonate groups. The increase in metabolic stability of the benzene disulfonate dendrimer may reflect either increased surface charge or enhanced steric hindrance associated with the additional sulfonic acid groups.

Acknowledgment. The authors thank Mike Campbell from the Centre for Drug Candidate Optimisation for providing the method for separating intact dendrimer from protein complexes. We also acknowledge the Australian Research Council-Linkage grant scheme for providing financial support to conduct this work.

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.

MP070047S