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molecular pharmaceutics

The Role of the Size and Number of Polyethylene Glycol Chains in the Biodistribution and Tumor Localization of Triazine Dendrimers

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Received February 26, 2008; Revised Manuscript Received June 11, 2008; Accepted June 12, 2008

Abstract: The synthesis and biodistribution of three triazine dendrimers differing in PEGylation are described. Dendrimers **1**, **2**, and **3** are derived from a common intermediate, dendrimer **4**, and vary in molecular mass from 11 to 73 kDa as a result of PEGylation with multiple (theoretically, 16) PEG groups of 0.6, 2, and 5 kDa, respectively. As expected, elimination half-lives increased with an increase in molecular mass. In light of other results, however, molecular mass proves not to be the primary determinant of elimination half-lives. Instead, these times can be more readily predicted from the number of PEG groups on the dendrimer: the size of the PEG chain contributes to a lesser extent. Tumor uptake is observed for all the three dendrimers in mice bearing prostate cancer xenografts.

Keywords: Dendrimer; biodistribution; tumor

Introduction

Over the past decade, polymeric drug delivery systems[1–5] have been extensively studied with the aim of improving the efficacy and safety of conventional drugs in clinical use by a variety of mechanisms: tailoring molecular size to prevent their premature elimination of drugs from the body,[6,7] achieving high drug payload with controllable drug release via labile linkers,[8,9] enhancing solubility and bioavailability under physiological conditions with biocompatible polymers,[10–12] and targeting specific diseased cells with ligands.[13,14] Dendrimers are attractive drug delivery vehicles because of many unique characteristics, including multivalency, a well-defined, globular structure, and monodispersity or low polydispersity.[15,16] However, pharmacokinetic behaviors of dendrimers have only recently been evaluated even though the understanding of pharmacokinetics related to dendritic structure, composition, and size is essential to achieving reproducible and desirable results in drug delivery. In 2005, Fréchet and Szoka systematically demonstrated that the degree of pegylation

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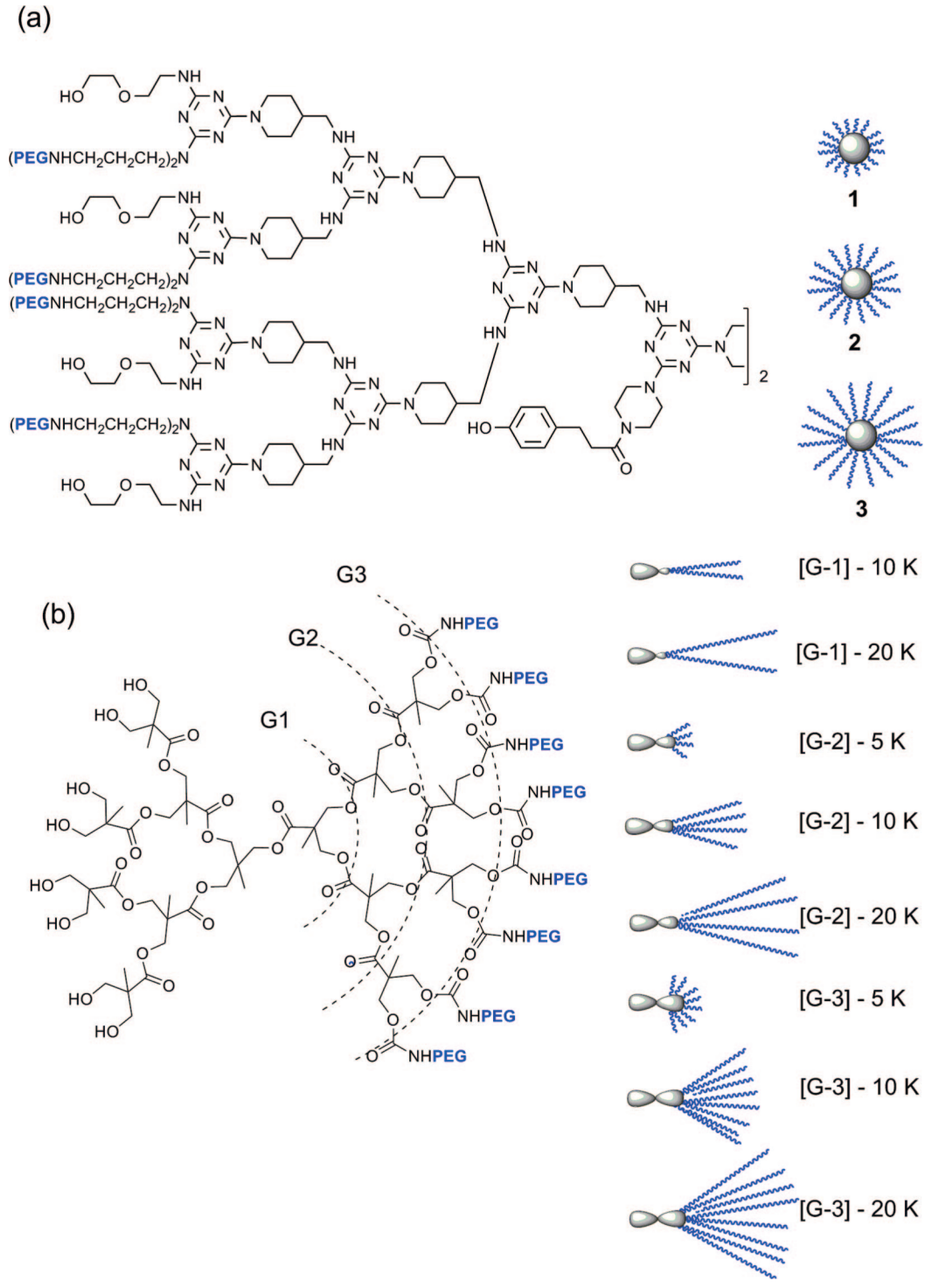
10.1021/mp8000292 CCC: $40.75
Published on Web 08/04/2008

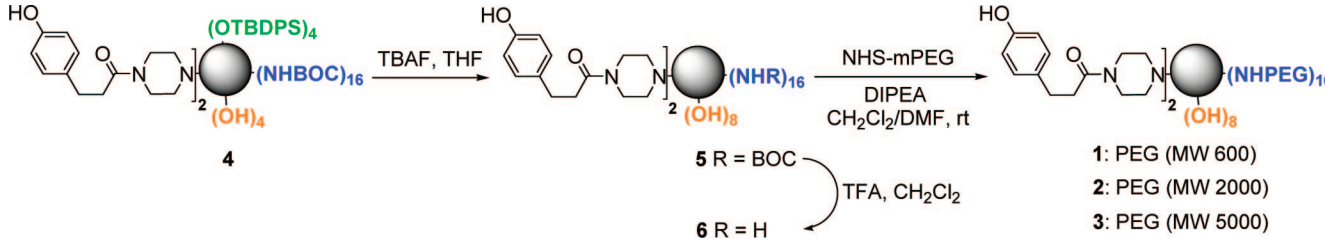
Chart 1. Structures of (a) Triazine Dendrimers and (b) Fréchet and Szoka's Bow Tie Polymers with the Original Nomenclature



of a dendrimer is reflected in blood circulation time and that these dendrimers accumulate in tumors.[17] PEGylation is a convenient way to optimize the pharmacokinetics and evade the sequestration by the reticuloendothelial system (RES), thereby increasing the longevity of the carriers in the bloodstream.[18–21] Prolonged circulation times may even lead to passive targeting via the enhanced permeability and retention (EPR) effect resulting from the leaky vasculature and poor lymphatic drainage in solid tumors.[22,23]

The structures of our PEGylated dendrimers, **1**, **2**, and **3**, and Fréchet and Szoka's PEGylated bow tie polymers are shown in Chart 1. These architectures vary significantly in composition and in the number and size of PEG chains attached. Triazine dendrimers **1**−**3** are derived from a common core and differ only in the size of the PEG chain attached. Ideally, 16 PEGs with molecular masses of 600, 2000, and 5000 Da are attached to **1**, **2**, and **3**, respectively. Indeed, throughout this work, we will refer to these constructs

Scheme 1. PEGylation of Dendrimers with Bolton−Hunter Type Groups



in this way. It is clear from mass spectrometry that pegylation is not quantitative in any case, but rather 10, 13, and 14 PEG chains are better estimates for **1**−**3**, respectively. The bow tie constructs display two, four, and eight PEG chains (theoretically), respectively, as a result of generation. The depiction of these molecules is not intended to communicate three-dimensional structure or reflect favored disposition of groups. Instead, it serves to unambiguously attribute architectures to specific classes.

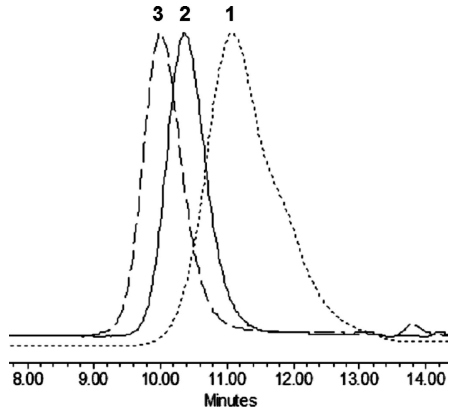


Figure 1. GPC traces of pegylated dendrimers.

Experimental Section

Materials and Instruments. All reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise noted. ^{125}I ($Na^{125}I$ in 0.1 N NaOH) was purchased from Perkin-Elmer (Shelton, CT). Milli-Q water (18 MΩ cm) was obtained from a Millipore (Billerica, MA) Gradient Milli-Q water system. Centricon filters (YM-10, molecular mass cutoff of 10 kDa; YM-30, molecular mass cutoff of 30 kDa) were purchased from Millipore. Dialysis tubing was purchased from Spectrum Laboratories (Rancho Dominguez, CA). Instant thin-layer chromatography (ITLC) SG plates were purchased from PALL Life Sciences (East Hills, NY). IODO-GEN precoated iodination tubes were purchased from Pierce (Rockford, IL). The ammonium acetate buffer (0.1 M, pH 6.5) was pretreated with Chelex 100 resin (Bio-Rad, Hercules, CA) before use. The T-medium, fetal bovine serum (FBS), and Matrigel were purchased from Invitrogen Corp. (Grand Island, NY), Gemini Bio-Products (Woodland, CA), and BD Biosciences (Bedford, MA), respectively. Male balb/c and nu/nu mice (5−7 weeks of age) were purchased from Harlan (Indianapolis, IN). Radio-TLC analysis was performed on a Rita Star (Straubenhardt, Germany) radioisotope TLC analyzer. High-

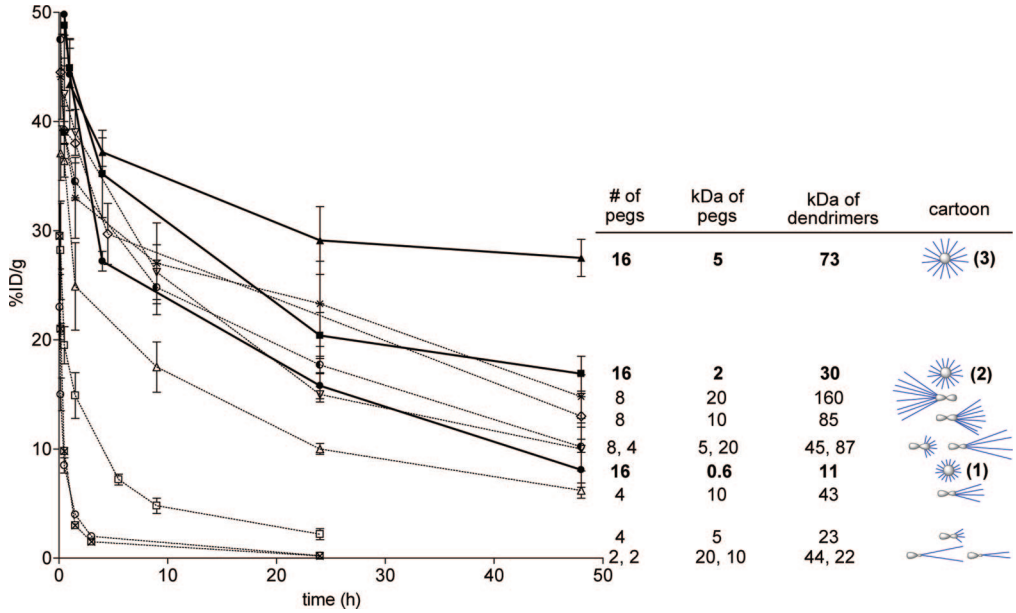
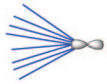
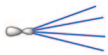


Figure 2. Time−concentration plot of triazine dendrimers (bold, solid lines) and Fréchet and Szoka's bow tie polymers (dotted lines) in the blood. The indicated number of PEGs reflects the theoretical number and not necessarily the exact number of PEG chains attached.

Table 1. Pharmacokinetic Data of Fréchet and Szoka's Bow Ties and Triazine Dendrimers (**1**−**3**)

Compound	MW (kDa) of compounds	av. no. of PEG arms	MW (kDa) of PEGs	half-lives (h)		$AUC_{0\rightarrow\infty}$ (%ID·h/g)[a]
				$t_{1/2\alpha}$	$t_{1/2\beta}$	
 **(3)**	**73**	**14**	**5**	**0.9 ± 0.1**	**100 ± 7**	**5410 ± 670**
	NA (theor. 160)	8	20	NA	50 ± 10	2250 ± 580
 **(2)**	**30**	**13**	**2**	**0.8 ± 0.2**	**43 ± 1**	**2160 ± 190**
	85	8	10	NA	40 ± 4	1880 ± 270
	45	8	5	NA	31 ± 2	1370 ± 140
 **(1)**	**11**	**10**	**0.6**	**0.8 ± 0.1**	**27 ± 4**	**1200 ± 170**
	87	4	20	NA	25 ± 8	1190 ± 600
	43	4	10	NA	26 ± 6	850 ± 300
	23	4	5	NA	11 ± 3	180 ± 70
	22	2	10	NA	8 ± 1	36 ± 10
	44	2	20	NA	1.4 ± 0.4	22 ± 10

[a] $AUC_{0\rightarrow\infty}$ equals the area under the %ID/g of blood curve from zero to infinite time.

Table 2. Cumulative Excretion Data of **1**, **2**, and **3** Found in the Urine and Feces

	%ID in urine				%ID in feces			
dendrimer	1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h
1	8.84	14.18	15.85	17.10	0.01	0.20	0.80	1.28
2	0.61	5.04	10.13	11.89	0.01	0.04	0.93	2.76
3	1.57	4.36	9.14	12.19	0.00	0.02	0.30	1.84

performance liquid chromatography (HPLC) and radio-HPLC were performed on a Waters 600 multisolvent delivery system equipped with a Waters 2996 photodiode array (PDA) detector and an in-line Shell Jr. 2000 radiodetector (Fredericksburg, VA).

Radiolabeling of 1, 2, and 3 with ^{125}I and Purification. IODO-GEN precoated iodination tubes were gently rinsed with 10 mM PBS before the labeling. To an IODO-GEN tube containing 200 μL of a dendrimer (15.0−17.0 nmol) solution in 10 mM PBS (pH 7.4) was added 300−500 μCi of ^{125}I in 0.1 N NaOH. The resulting solution was kept at room temperature for 20 min with periodic gentle swirling. The reaction mixture was loaded onto a Millipore Centricon filter (**1**, YM-10; **2** and **3**, YM-30), which was then centrifuged at 5000 rpm to remove the free ^{125}I activity. The product was washed with three portions of 2 mL of 10 mM PBS buffer, then collected, and constituted with the PBS buffer for radio-HPLC analysis and biodistribution studies.

Radio-TLC and Radio-HPLC Analysis. Radio-TLC was used to monitor the radiolabeling reactions in IODO-GEN tubes. Briefly, it was done by spotting the reaction mixture on an ITLC SG strip, which was developed in 10 mM PBS buffer (pH 7.4) and analyzed with a TLC scanner. Under the TLC condition, ^{125}I-labeled dendrimers remained at the origin, while the free radioactivity migrated to the solvent front. The radiochemical purity of the products was determined by radio-HPLC. Briefly, 20 μL of each radiolabeled dendrimer (~5 μCi) was injected into a Kromatek PSS SUPREMA GPC/SEC column, which was eluted with 20 mM HEPES and 150 mM NaCl buffer at an isocratic flow rate of 1.0 mL/min. The UV wavelength was 240 nm for the dendrimer detection, and the radioactivity was determined with an in-line radiodetector.

Tissue Culture and Animal Model. The PC-3 cell line was obtained from the American type Culture Collection (ATCC, Manassas, VA). PC-3 cells were cultured in T-medium at 37 °C in an atmosphere of 5% CO_2 and were passaged at 75% confluence in P150 plates. T-Medium was supplemented with 5% fetal bovine serum (FBS) and 1× penicillin/streptomycin. Cultured PC-3 cells were harvested from the monolayer using PBS and trypsin/EDTA and suspended in T-medium with 5% FBS. The cell suspension was then mixed 1:1 with Matrigel and injected subcutaneously (2.5×10^6 cells per injection, injection volume of 100 μL) into both rear flanks of 24 nu/nu mice. After the cell injection, the animals were monitored three times a week

by general observation. The tumor grew in the first week and was allowed to grow for 3 weeks to reach a palpable size for biodistribution studies. The weight of most tumors (38 of 48) was 25−90 mg.

Biodistribution Studies in Normal Balb/c Mice. All animal studies were performed in compliance with guidelines set by the University of Texas Southwestern Institutional Animal Care and Use Committee. The injection doses were prepared by diluting the purified ^{125}I-labeled dendrimers with 10 mM PBS buffer. Normal 4−5-week-old male healthy balb/c mice (Harlan) were anesthetized with isoflurane, and then 100 μL of each ^{125}I-labeled dendrimer (ca. 6 μCi/mouse) was injected via the tail vein. The animals were anesthetized again prior to being sacrificed 30 min, 1 h, 4 h, 24 h, and 48 h postinjection (p.i.) ($n = 4$ at each time point). Organs of interest (blood, heart, lung, liver, spleen, kidney, fat, muscle, intestine, stomach, and thyroid) were removed, weighed, and counted. Standards were prepared and counted along with the samples to calculate the percent injected dose per gram of tissue (%ID/g) and percent injected dose per organ (%ID/organ). The animals of the last time point groups were housed in metabolic cages (four mice per cage) to collect urine and feces 1 h, 4 h, 24 h, and 48 h p.i. For the evaluation of pharmacokinetic parameters, 5−10 μL of blood was collected from the retroorbital sinuses of the animals 5 min, 10 min, and 20 min p.i. The unpaired *t* test on the biodistribution data was performed using Prism, version 4.00 (Graphpad, San Diego, CA).

Biodistribution Studies in Tumor-Bearing Mice. The 24 PC-3 tumor-bearing mice were randomly separated into six groups (two groups per dendrimer), and ~6 μCi of ^{125}I-labeled dendrimer (100 μL) was injected intravenously via the tail vein. The animals were sacrificed 4 and 24 h p.i. ($n = 4$). The blood, heart, lung, liver, spleen, stomach, intestines, kidneys, fat, muscle, thyroid, and tumors were harvested and weighed, and the radioactivity was quantified. Standards were prepared and counted along with the samples.

Results and Discussion

Synthesis of PEGylated Triazine Dendrimers. The chemoselective reactivity of chlorotriazines with amine nucleophiles allows precise control over composition and structure.[24,25] In previous studies, pegylated triazine dendrimers displayed little cytotoxicity even at high concentrations (0.1−10 mg/mL).[26,27] Here, we utilize dendrimer **4** to prepare dendrimers **1**, **2**, and **3** for biodistribution studies (Scheme 1). Dendrimer **4** displays four free hydroxyl groups, four *tert*-butyldiphenylsilyl (TBDPS) ether groups, 16 *tert*-butoxycarbonyl (BOC) protected amine groups, and two Bolton−Hunter type groups for radioiodination.[28,29] After removal of both the TBDPS and BOC groups, pegylation is used to tailor the molecular size and to improve the solubility

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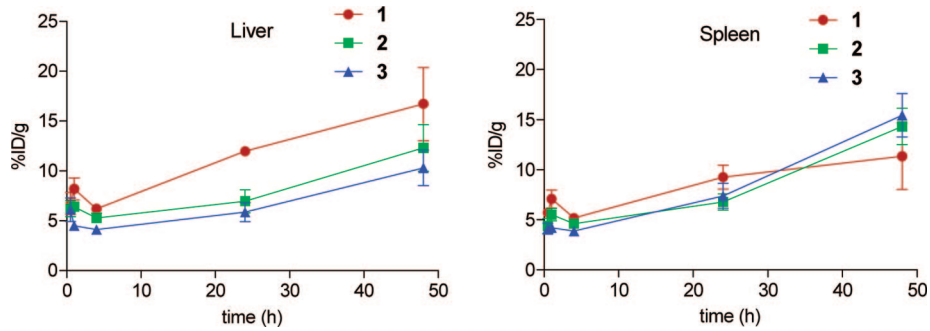

Figure 3. Biodistribution of **1**, **2**, and **3** in the liver and spleen.

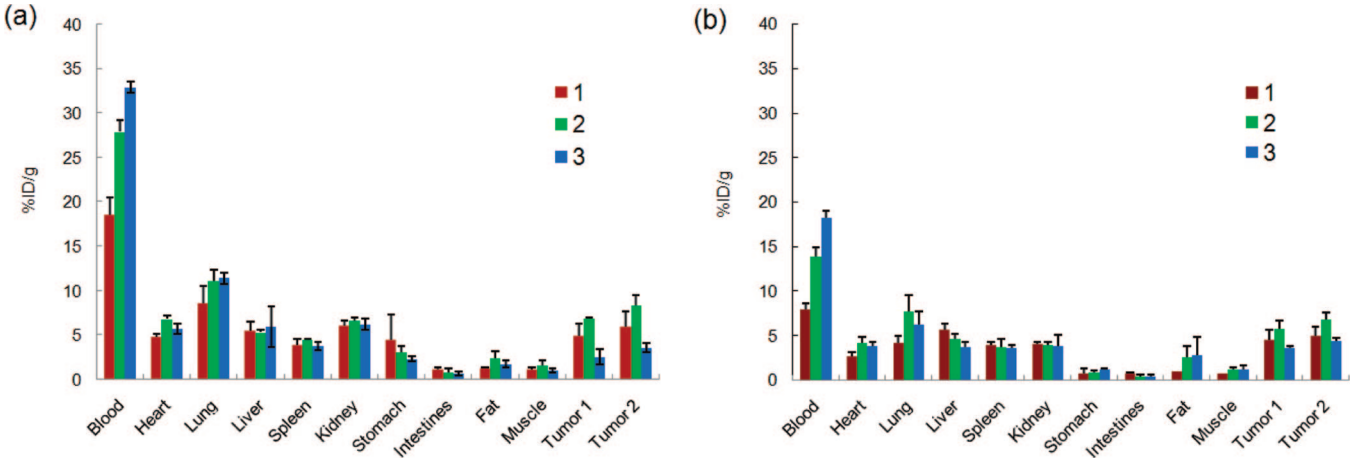

Figure 4. Biodistribution and tumor localization of **1**, **2**, and **3** in nude mice bearing PC-3 xenografts 4 h (a) and 24 h (b) p.i.

and biocompatibility of the dendrimer. Silyl ethers are cleaved with tetrabutylammonium fluoride to yield **5**. Treatment of **5** with trifluoroacetic acid (TFA) produces **6**. MALDI-TOF MS is useful for following this deprotection. A ladder of lines corresponding to partially deprotected intermediates can be observed between the lines corresponding to **5** and **6**. The free amines of **6** are PEGylated with activated mPEGs (*N*-hydroxysuccinimide esters) with different molecular masses (600, 2000, or 5000 Da) to afford architectures with molecular masses of 11 kDa (**1**), 30 kDa (**2**), and 73 kDa (**3**). Complete PEGylation is not achieved. Instead, between 10 and 14 of the 16 amines react, presumably as a result of incomplete reaction. The PEGylated dendrimers are purified by dialysis in deionized water over a week and analyzed using GPC in 0.1 M $NaNO_3$ (aqueous) with a refractive index (RI) detector (Figure 1). We cannot definitively ascribe the significant shoulder that appears on **1** to any specific chemical entity, including free PEG; indeed, conclusions drawn from this architecture must be treated as potentially suspect.

Biodistribution Studies in Normal Balb/c Mice. Dendrimers **1**, **2**, and **3** were radiolabeled using Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril), an effective oxidation reagent, and $Na^{125}I$ in 0.1 N NaOH.[30,31] The radiochemical yields as determined by radio-TLC were 67, 80, and 59% for **1**, **2**, and **3**, respectively, at 20 min (a time judged to be optimal). The purification via Millipore Centricon filters afforded nearly 100% radiochemical purity for the three ^{125}I-labeled dendrimers as determined by radio-HPLC. The specific activity of the ^{125}I-labeled dendrimers was in the range of 12.4−20.3 μCi/nmol. The radiolabeled dendrimer solutions (100 μL, 6 μCi per mouse) were intravenously administered to healthy male balb/c mice (four mice per

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group). The mice were sacrificed at the scheduled time points (30 min, 1 h, 4 h, 24 h, and 48 h p.i.). Blood, heart, lung, liver, spleen, kidney, fat, muscle, intestine, stomach, and thyroid were weighed, and the radioactivity of each organ was quantified.

As expected, the larger the dendrimer, the longer the blood retention time. At 48 h, we observe **1** (8.12 ± 1.64%ID/g), **2** (16.95 ± 1.64%ID/g), and **3** (27.50 ± 1.73%ID/g). When the results are examined in the context of Szoka and Fréchet's data (Figure 2), the importance of both PEG number and size emerges. Said differently, the parameter judged to be least useful for predicting circulation times is molecular mass. Increased retention times are favored by a large number of PEG groups of sizes that range from large to moderate. For example, dendrimer **2** with the sixteen 2 kDa PEG chains behaves like a bow tie with eight 20 kDa PEG molecules. This disparity in molecular mass (32 kDa of PEG vs 160 kDa of PEG) is significant: solely on the basis of molecular mass, one might predict renal elimination for the former and hepatic elimination for the latter.

The role that the number of PEG groups plays in biodistribution is also reflected by the in vivo pharmacokinetic parameters (Table 1),[17] which were calculated on the basis of a two-compartment open model.[32] The distribution half-lives ($t_{1/2\alpha}$) of all the three triazine dendrimers with respect to other organs were similar (<1 h), while the elimination half-lives ($t_{1/2\beta}$) and $AUC_{0\rightarrow\infty}$ values were significantly prolonged as a factor of their peripheral PEG chain length and number: **1** (27 h, 1200%ID h g^{-1}), **2** (43 h, 2160%ID h g^{-1}), and **3** (100 h, 5410%ID h g^{-1}).

All the three dendrimers were excreted primarily through urine (Table 2). Less than 3% ID was found in the feces for these dendrimers. As outlined in Figure 3, the level of uptake of dendrimers in liver and spleen dropped to the lowest values 4 h p.i. and then increased likely as the result of the sequestration of the dendrimers from the bloodstream by the RES. The thyroid uptake observed for these dendrimers ranged between 0.25 and 3.5%ID/organ. In other organs (e.g., lung, kidney, muscle, heart, and fat), the three dendrimers demonstrated more or less similar accumulation and clearance patterns (Supporting Information).

Biodistribution Studies in Tumor-Bearing Mice. Dendrimers **1**, **2**, and **3** were also evaluated in a PC-3 tumor-bearing animal model, where each mouse carried two solid tumors in both rear flanks (Figure 4). Compared to the results in healthy balb/c mice, the three dendrimers showed significantly more efficient clearance from the major organs (e.g., blood, lungs, liver, kidneys, and spleen) in nude mice bearing PC-3 xenografts. Their tissue distribution patterns in the two animal models, however, were similar. As expected, at both 4 and 24 h p.i., the blood retention of the three compounds followed the same trend as in normal mice. Dendrimer **2** showed the highest level of tumor uptake (8.35 ± 1.13%ID/g at 4 h p.i.; 6.74 ± 0.86%ID/g at 24 h p.i.), whereas **1** exhibited the highest tumor:muscle uptake ratio (8.10 ± 2.34 at 24 h p.i.). This difference may be due to the more efficient clearance of **1**. The level of uptake of **3** in tumors increased from 4 to 24 h p.i., while the tumor deposition of **1** and **2** decreased during the same time period. This observation is consistent with the slow pharmacokinetics of **3**.

Conclusions

The postsynthetic manipulations of a generation three dendrimers containing two Bolton−Hunter type groups, four free hydroxyl groups, four TBDPS groups, and 16 BOC groups provide a common intermediate for the generation of a series of targets for examining biodistribution and tumor localization. PEGylation of this dendrimer produced targets with a range of molecular masses, from ~11 to 73 kDa. Prolonged circulation in the blood was seen in architectures with longer PEG chains. Other studies anchor these beliefs. Hashida and co-workers showed significant plasma concentration levels that appear largely unchanged over 60 h when a sixth-generation lysine dendrimer labeled with ^{111}In has ~76 of the existing 128 surface groups derivatized with PEG chains with a molecular mass of 5 kDa.[33] This architecture, with a molecular weight approaching 400 kDa, showed 75% of the original dose in the circulation and 25% in the liver over this time period. No renal accumulation was observed. At the other extreme, Porter and co-workers found that smaller poly(lysine) dendrimers bearing arylsulfonate groups were rapidly cleared from the circulation primarily through the kidneys.[34] This rapid clearance of small macromolecules was also seen in an earlier report by Fréchet and Szoka.[35]

However, the number of PEG chains may also be a critical determinant in the prediction of elimination half-lives. Combining the data from this triazine study with Frechet and Szoka's polyesters as well as PEGylated lysine dendrimers recently described by Porter (collected in rats)[36] is interesting when short 200 Da PEG chains are omitted. Figure 5 summarizes this data. The data are organized by the number of PEG chains attached to the dendrimer: 4 (blue), 8 (red), 16 (green), or 32 (violet). Dotted trend lines have been added to guide the eye. The class of dendrimer is designated with a letter: polyester (E), polytri-

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azine (T), or polylysine (L). While the combined data set is limited by the number of overlapping data points (not to mention different animal models), the picture that emerges is compelling. A strict correlation of molecular mass and elimination half-life is not observed, based on data derived from two significant outliers, both a lysine and triazine construct.

The data suggest that elimination half-life is not dependent on the composition of the dendrimer (to a first approximation). Our conceptual model presents a sea of PEG surrounding the dendrimer, making the composition largely irrelevant (unless that sea is shallow as a result of low-molecular mass PEG groups which are not included in this chart). This model is comforting. If discrimination of the cores occurs, the implications for drug delivery would be substantial: different drugs would be expected to behave differently, and universality of the vehicle's behavior in vivo would become less predictable. The model may not be able to be generalized to proteins or other constructs. Margerum's Gd-bearing generation three PAMAM dendrimers with multiple copies (7−12) of PEG chains of 2 or 5 kDa are cleared more rapidly from rats than the model would predict.[37] Knauf's work with rIL-2 with five PEG chains also is inconsistent with what would be predicted: these constructs are cleared more rapidly from rats as well.[38] We conclude that further experiments and data mining from the literature describing PEGylation of protein targets are required and warranted.

The extent of tumor localization observed for all three dendrimers (**1**, **2**, and **3**) in the PC-3 tumor model leads us to pursue architectures with therapeutic groups. However, differences in elimination half-lives for the tumored and nontumored animals are clearly evident at 4 and 24 h. As a function of %ID/g, all three dendrimers exhibit lower plasma concentrations in the tumored animals than in healthy animals at 4 h (healthy → tumor): **1** (27% → 19%), **2** (35% → 28%), and **3** (37% → 33%). These difference were more pronounced at 24 h: **1** (16% → 8%), **2** (20% → 14%), and **3** (29% → 18%). Regardless, we (and others) remain optimistic about the future of dendrimers as polymer therapeutics.[39]

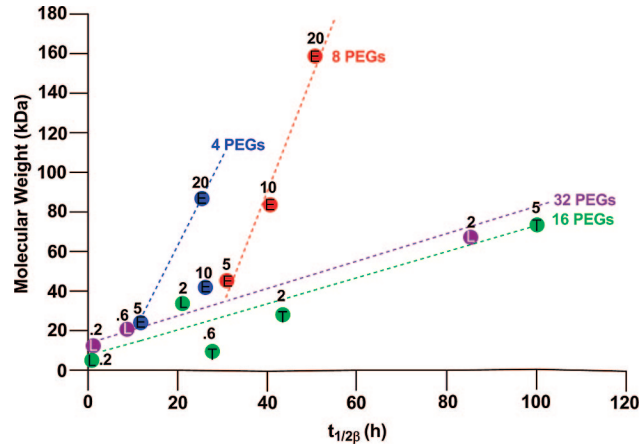

Figure 5. Plotting molecular mass against elimination half-life, $t_{1/2\beta}$, suggests that the number of PEG chains plays an important role. Dendrimers with 4 (blue), 8 (red), 16 (green), and 32 (violet) PEG chains are indicated, and colored trend lines have been added to guide the eye. The molecular mass of the PEG chains is indicated above each data point. The dendrimer class is indicated with a letter: polyester (E), polylysine (L), and polytriazine (T).

Acknowledgment. We thank the National Institutes of Health (Grant GM 64560) and the Robert A. Welch Foundation (Grant A 1439) for support. X.S. thanks the Harold C. Simmons Comprehensive Cancer Center and the Department of Radiology of the University of Texas Southwestern Medical Center for support. We also thank Dr. Christopher Porter of the Victorian School of Pharmacy in Melbourne, Australia, for providing data on polylysine dendrimers fit to a two-compartment model and many insightful suggestions.

Supporting Information Available: Biodistribution tables, synthetic experimental procedures, mass spectrometry data, and additional comments on the model. This material is available free of charge via the Internet at http://pubs.acs.org.

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