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Biological Evaluation of Polyester Dendrimer: Poly(ethylene oxide) "Bow-Tie" Hybrids with Tunable Molecular Weight and Architecture

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Abstract: High molecular weight (MW) polymers have shown promise in terms of improving the properties and the efficacy of low MW therapeutics. However, new systems that are highly biocompatible, are biodegradable, have well-defined MW, and have multiple functional groups for drug attachment are still needed. The biological evaluation of a library of eight polyester dendrimer−poly(ethylene oxide) (PEO) bow-tie hybrids is described here. The group of evaluated polymers was designed to include a range of MWs (from 20000 to 160000) and architectures with the number of PEO arms ranging from two to eight. In vitro experiments revealed that the polymers were nontoxic to cells and were degraded to lower MW species at pH 7.4 and pH 5.0. Biodistribution studies with ^{125}I-radiolabeled polymers showed that the high MW carriers (>40000) exhibited long circulation half-lives. Comparison of the renal clearances for the four-arm versus eight-arm polymers indicated that the more branched polymers were excreted more slowly into the urine, a result attributed to their decreased flexibility. Due to their essentially linear architecture that does not provide for good isolation of the iodinated phenolic moieties, the polymers with "two arms" were rapidly taken up by the liver. The biodistributions of two long-circulating high MW polymers in mice bearing subcutaneous B16F10 tumors were evaluated, and high levels of tumor accumulation were observed. These new carriers are therefore promising for applications in drug delivery and are also useful for improving our understanding of the effect of polymer architecture on pharmacokinetic properties.

Keywords: Drug delivery; polymer; dendrimer; tumor; biodistribution; biodegradable

Introduction

Many low molecular weight (MW) drug candidates are limited in their therapeutic value by properties such as low water solubility, poor bioavailability, and rapid elimination.[1] In addition, while the beneficial effects of anticancer drugs arise through their interactions with tumor cells, undesirable side effects and toxicity typically result from their exposure to other cell types.[2] The concept of conjugating low MW drugs to water soluble polymers was initially introduced by Ringsdorf [3,4] and Kopeček[5,6] and has since been developed

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and shown to result in several advantages. For example, the solubility of the drug can be improved and the circulation time of the polymer drug conjugate in the plasma can be increased upon polymeric attachment.[7,8] This enhancement in circulation time is the result of the decreased rate of renal filtration that correlates with molecular size, as molecules with larger hydrodynamic volumes are generally eliminated more slowly.[9−11] Passive targeting to solid tumors can also be achieved upon conjugation of anticancer drugs to polymers. This targeting is thought to be possible due to the increased permeability of tumor vasculature to macromolecules and limited lymphatic drainage, leading to the selective accumulation of macromolecules in tumor tissue. This phenomenon is known as the enhanced permeation and retention (EPR) effect.[12−14]

The polymer characteristics that are believed to be important for a drug delivery system include water solubility, lack of both toxicity and immunogenicity, low polydispersity, and the presence of multiple, highly accessible functional handles for drug attachment. Despite the large number of polymers available, relatively few possess all of these features. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) is a biocompatible polymer that has been extensively investigated in terms of its drug delivery capabilities.[7] While HPMA has shown promise and has many attractive characteristics,[15] relative limitations of this system include its inherent lack of biodegradability, and the early difficulties encountered in the preparation of low polydispersity HPMA.[16,17] The lack of biodegradability places an upper MW limit on the size of polymer that can be administered, thus reducing the potential enhancement in plasma circulation time and tumor targeting.[15,18] Furthermore, for systemic applications, the use of a material with a broad polydispersity index (PDI) is not favored as it may lead to irreproducible or undesired pharmacokinetic behavior due to the presence of species with vastly different MW within a given sample, or to variations in MW distribution from preparation to preparation. Poly(ethylene oxide) (PEO) is biocompatible and is widely used[19−21] though it is not biodegradable and suffers from a shortage of functional handles for drug attachment. In general, drug conjugates of linear polymers are prepared statistically, either by incorporation of a comonomer in the polymer preparation or by postsynthetic modification of a homopolymer with a functional handle, making the preparation of well-defined systems difficult. Therefore, the development of alternative, biodegradable polymers, with better-defined MWs and architectures enabling enhanced control over drug loading, will broaden the scope of applicability of polymers in therapeutic applications.

Dendrimers are very promising candidates for the development of polymeric delivery systems. In contrast to conventional polymers, dendrimers are prepared by a stepwise synthetic procedure, leading to a highly regular branching pattern, well-defined architecture, and a unique MW or at least a very low PDI.[22,23] In addition, dendrimers possess a strictly controlled number of functional groups on their periphery that can be used for the attachment of drugs,

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solubilizing groups, targeting groups, or other moieties to tune the biological properties of the system. We have recently reported the design, synthesis, and biological properties of polyester dendrimers based on the monomer 2,2-bis(hydroxymethyl)propionic acid.[24,25] Thus far, this dendrimer scaffold has proven to be very promising as it is easily prepared by either convergent or divergent synthetic methods[26,27] and is very biocompatible.[25] While the fourth-generation dendrimer, with a MW of 3800, had a plasma circulation half-life of less than 10 min, it was possible to enhance this to greater than 1 h by preparing a hybrid structure consisting of polyester dendrons conjugated to the periphery of a three-arm PEO star polymer with a MW of 22000.[25] Star-shaped PEO was chosen for its water solubility and biocompatibility and because it is available with low polydispersity (PDI = 1.02), thus providing hybrids of similar narrow polydispersity. However, an even longer circulation half-life is desirable for some applications.

The promising characteristics of polyester dendrimer−PEO hybrids in initial biological studies have inspired us to undertake a systematic study of the effect of MW and architecture of these carriers on their pharmacokinetic properties. Therefore, we have recently reported the design and synthesis of a new dendritic bow-tie system that allows access to a library of carriers with a range of MWs and architectures.[28] This system consists of two covalently attached and orthogonally functionalized polyester dendrons. One dendron was selectively deprotected to allow coupling of PEO moieties to one side of the system, while the other dendron provides functional handles for further derivatization or drug attachment. By varying the generations of the dendrons, both the number of solubilizing PEO arms and the drug loading can be tuned. The in vitro and in vivo evaluation of a representative sublibrary of eight bow-tie polymers with different MWs and architectures is described here. To the best of our knowledge, this work represents the first systematic study of the effect of molecular weight and architecture on pharmacokinetic properties for well-defined polymer systems.

Experimental Section

Cell Cytotoxicity Assay. MDA-MB-231 breast cancer cells were cultured by the UC Berkeley Cell Culture Facility in a medium consisting of Dulbecco's modified Eagle medium (DMEM), containing 10% FBS. The cytotoxicities of the [G-1] − 10 K, [G-1] − 20 K, [G-3] − 10 K, and [G-3] − 20 K were determined using the MTT assay.[29] Cells were seeded onto a 96-well plate at a density of 1.6×10^4 cells per well in 100 μL of medium and incubated overnight (37 °C, 5% CO_2). The medium of each well was then replaced by 100 μL of new medium (DMEM, 10% FBS, 1% penicillin−streptomycin) containing concentrations of polymer ranging from 0.04 to 10 mg/mL. The tests were conducted in replicates of four to eight for each concentration. The cells were incubated for 48 h, and then the medium was aspirated, and 100 μL of fresh medium was added, followed by 20 μL of MTT solution (5 mg/mL). The cells were incubated for 4 h, and then the medium was carefully aspirated. To the resulting purple crystals were added 200 μL of DMSO, followed by 25 μL of pH 10.5 glycine buffer (0.1 M glycine, 0.1 M NaCl). The optical densities at 570 nm were obtained using a SpectraMAX 190 microplate reader (Molecular Devices). Optical densities measured for wells containing cells that received no polymer or drug were considered to represent 100% growth.

Polymer Degradation Study. The polymer was dissolved at a concentration of 1 mg/mL in a solution containing either 30 mM acetate buffer (pH 5.0) with 70 mM $NaNO_3$ or 30 mM phosphate buffer (pH 7.4) with 70 mM $NaNO_3$. The samples were incubated at 37 °C, and the MW was determined periodically by size exclusion chromatography (SEC) over a period of a few weeks. SEC was performed at 25 °C using a Waters 2690 separation module and a Waters 410 differential refractometer with a Suprema 10μ column (10^3 Å) as the stationary phase. Purified water with 0.1 M $NaNO_3$ was used as eluent with a constant flow rate of 1 mL/min, and the instrument was calibrated using linear PEO standards.

Representative Procedure for the Introduction of Tyramine Groups for Radiolabeling. The [G-3] − 10 K (100 mg) was dissolved in 2 mL of dry CH_2Cl_2, and 10 μL (12 equiv) of pyridine was added, followed by 4-nitrophenyl chloroformate (12 mg, 6 equiv per dendrimer hydroxyl). The reaction mixture was stirred at room temperature, and then the solvent was evaporated. The resulting solid was then redissolved in 1.5 mL of 2:1 benzene/pyridine, and tyramine (25 mg, 18 equiv per dendrimer hydroxyl) was added. The reaction mixture was stirred at room temperature overnight, and then the solvent was evaporated under reduced pressure. The resulting residue was dissolved in water and filtered, and then the filtrate was purified by dialysis to remove low MW contaminants and residual PEO−NH_2 arms from the polymer synthesis procedure.[28] A Spectra/Por cellulose ester membrane with a molecular mass cutoff of 100 kDa from

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Spectrum Laboratories was used for all samples. Polymer solutions were dialyzed against distilled water, the water being changed every 12 h. Dialyses were stopped after 18 h for removal of the 5000 MW PEO−NH_2, and after 48 or 96 h for removal of 10000 MW or 20000 MW PEO−NH_2, respectively. The resulting polymer solutions were lyophilized to provide the pure tyramine-labeled polymers. The approximate numbers of phenols on the polymers were quantitated by ^{1}H NMR spectroscopy (δ = 6.6, 6.9 ppm).

Radioiodination of Tyramine-Functionalized Polymers. Tyramine-functionalized polymers were iodinated as previously described,[30] resulting in solutions of radiolabeled polymer in HBS (20 mM HEPES, 130 mM NaCl, pH 7.4). Polymers were separated from low MW radioactive contaminants by SEC on Bio-Rad 10DG desalting columns that had been equilibrated with HBS. The initial, high MW fractions were collected and pooled. A suitable quantity of radiolabeled polymer was mixed with cold polymer in sterile HBS to create a solution with 4 mg/mL polymer and specific activities ranging from 1.2 to 7.6 μCi/mL.

Biodistribution Studies. Polymer solutions (200 μL) were administered intravenously via the tail vein to 6−8-week-old CD-1 female mice. The mice were sacrificed at five or six different time points (three mice per group) ranging from 5 min to 48 h postinjection for biodistribution analyses. The blood (collected by heart puncture), heart, lungs, liver, stomach, spleen, intestines, kidney, and carcass were weighed, and the amount of radioactivity present in each organ was quantified. Periodically, blood was also collected from the retroorbital sinus at intermediate time points to determine the dose of polymer in the blood. For the 24- and 48-h time points, mice were housed in metabolic cages to allow for the collection of urine and feces. As it was not possible to remove all blood from the organs by washing, the radioactivity in the organs due to blood contamination was subtracted. The quantity of residual blood in the organs was determined using ^{51}Cr-labeled red blood cells. The following percentages of total blood were used in the calculations: lungs, 2.35; liver, 5.07; spleen, 0.84; heart, 0.70; kidneys, 2.14; stomach, 0.22; intestines, 1.19; carcass, 25.19; and in calculation of the total amount of blood it was assumed that the mice contained 7 wt % blood.

The % injected dose per gram (% ID/g) of blood versus time curve was analyzed using a two-compartment model (eq 1), since ln(% ID/g) vs time curves clearly displayed two different rates of decay for early and late time points. The pharmacokinetic parameters A, α, B, and β for the two-compartment model were estimated using the residuals method.[31] The elimination half-life was calculated as (ln 2)/β, and the $AUC_{0\rightarrow\infty}$ was calculated as $A/\alpha + B/\beta$. C_p, the concentration of polymer in the blood at time t, is given by

$$C_p = A(e^{-\alpha t}) + B(e^{-\beta t}) \quad (1)$$

Biodistribution Studies in Tumored Mice. Female C57BL6 black mice were injected with B16F10 melanoma cells on the right rear flank by subcutaneous administration (3×10^5 cells per mouse in 0.1 mL of saline). The tumors were allowed to reach an average size of 330 ± 160 mg, and then the mice (three per group) were injected intravenously via the tail vein with 200 μL of radiolabeled [G-3] − 10 K or [G-3] − 20 K polymer solution prepared as described above. The mice were sacrificed at 48 h postinjection, their blood, organs, muscle, and tumor were collected and weighed, and the radioactivity in each was quantified.

Results and Discussion

Description of the Structural Features of the Evaluated Compounds. Polyester dendrimer−PEO bow-tie hybrids (Figure 1a) were prepared as previously reported.[28] A key structural feature of these molecules is that by changing the generation of one dendron, the number of PEO arms can be adjusted to provide polymers of different architectures, as illustrated in Figure 1b. By tuning the combination of the number of PEO arms and their length, a variety of MWs can be prepared from only a few common precursors. A small library of eight polymers was prepared where the PEO functionalized dendron was varied from the first to the third generation (two to eight arms). Since one goal of this study was to prepare long-circulating drug carriers, PEO arms with MWs of 5000−20000 were used to prepare polymers with MWs ranging from 20000 to 160000. For this study, the dendron having peripheral hydroxy groups was always third generation, although this could later be adjusted to tune the drug loading of the polymers. The polymers are named as [dendrimer generation] − length of PEO arms, where [G-1], [G-2], and [G-3] provide two, four, and eight arms, respectively, and 5000, 10000, and 20000 MW PEO are denoted 5 K, 10 K, and 20 K respectively. Some important characterization data previously reported for the molecules evaluated in this study are shown in Table 1. It is notable that the MWs determined using size exclusion chromatography (SEC) were more underestimated for the more branched structures due to their smaller hydrodynamic volume relative to linear PEO calibration standards.

In Vitro Cytotoxicity of the Carriers. In vitro studies were performed to evaluate the cytotoxicity of the polymeric carriers. On the basis of the known biocompatibility of PEO,[32] and the fact that the polyester scaffold is also very well tolerated, the carriers were expected to be nontoxic.[25] Four representative carriers spanning a range of MWs as well as low and high degrees of branching were selected, and

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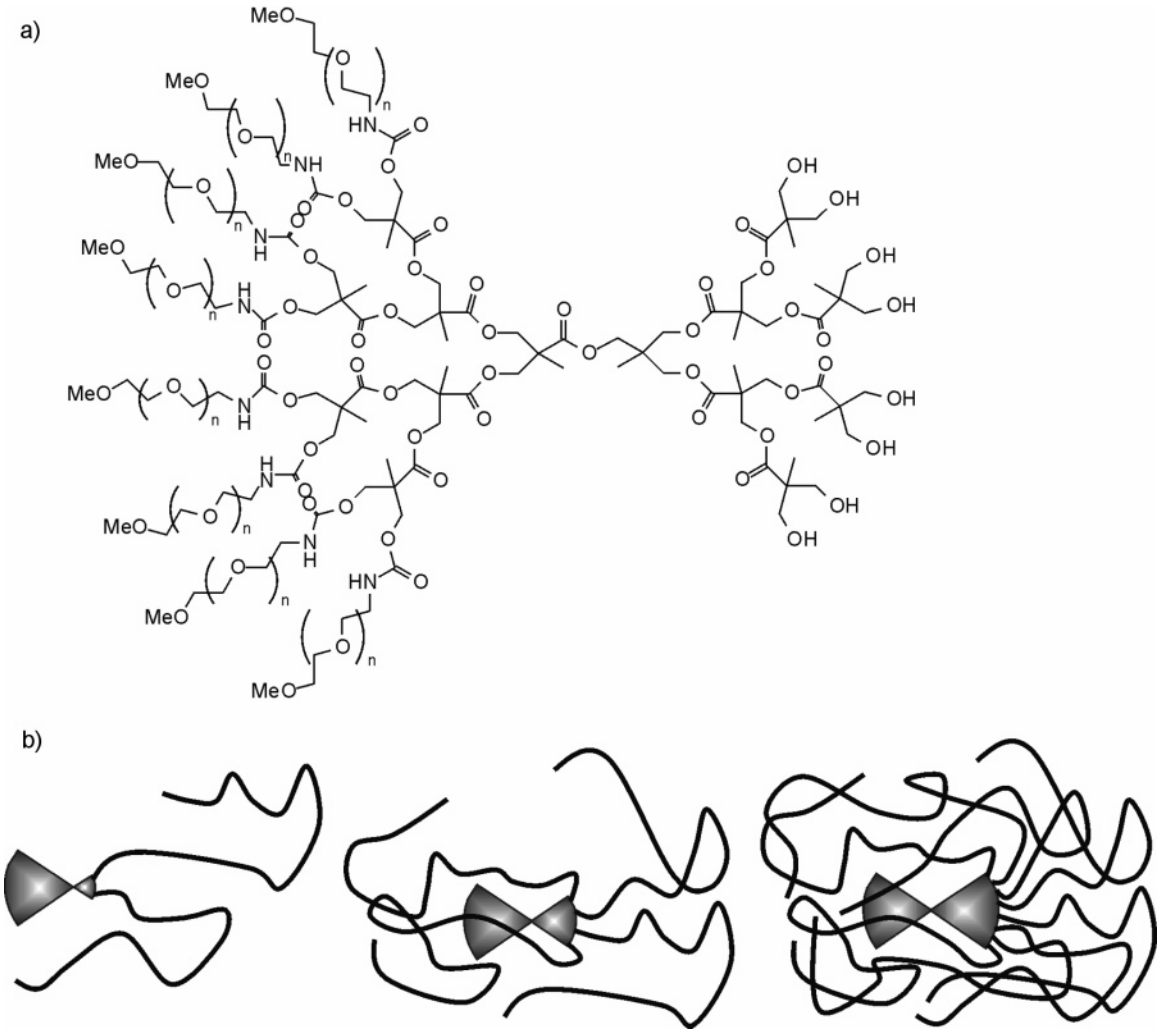

Figure 1. (a) Chemical structure of a polyester dendrimer−PEO bow-tie hybrid. (b) Illustration of different architectures available by tuning the dendrimer generation.

Table 1. Characterization Data for the Evaluated Polymers

polymer	MW[a] (MALDI-TOF MS)[b]	M_n[c] (SEC)[d]	PDI[e] (g/mol)
[G-1] − 10 K	21800	17900	1.11
[G-1] − 20 K	44200	30900	1.16
[G-2] − 5 K	23000	18400	1.06
[G-2] − 10 K	43000	34900	1.06
[G-2] − 20 K	86800	63700	1.09
[G-3] − 5 K	44700	28300	1.08
[G-3] − 10 K	84900	52100	1.08
[G-3] − 20 K	na (theor 160000)	109000	1.12

[a] MW = molecular weight. [b] MALDI-TOF MS = matrix-assisted laser desorption ionization spectroscopy. [c] M_n = number average molecular weight. [d] SEC = size exclusion chromatography. [e] PDI = polydispersity index.

their toxicities were evaluated. The polymers were incubated with MDA-MB-231 cancer cells for a period of 48 h, and then the cell viability was evaluated using the MTT assay.[29] As shown in Figure 2, no significant toxicity was observed up to 10 mg/mL, the highest concentration evaluated, with cell viabilities exceeding 85% relative to controls at all

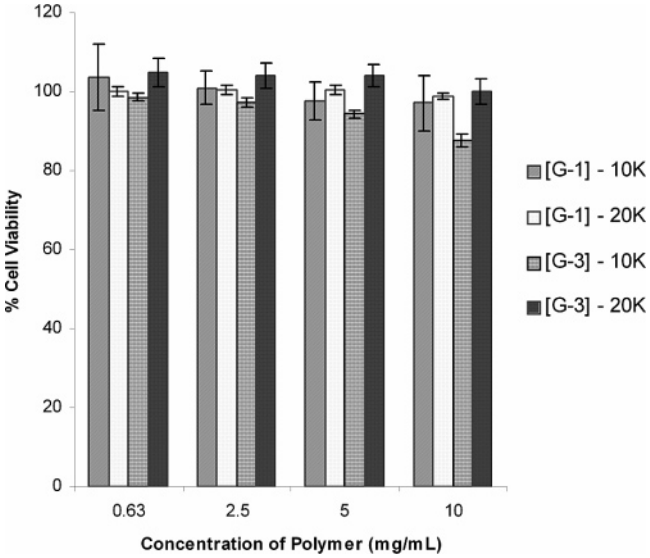

Figure 2. Cell viability results upon treatment of MDA-MB-231 cells with representative bow-tie carriers after 48 h of incubation (MTT assay).

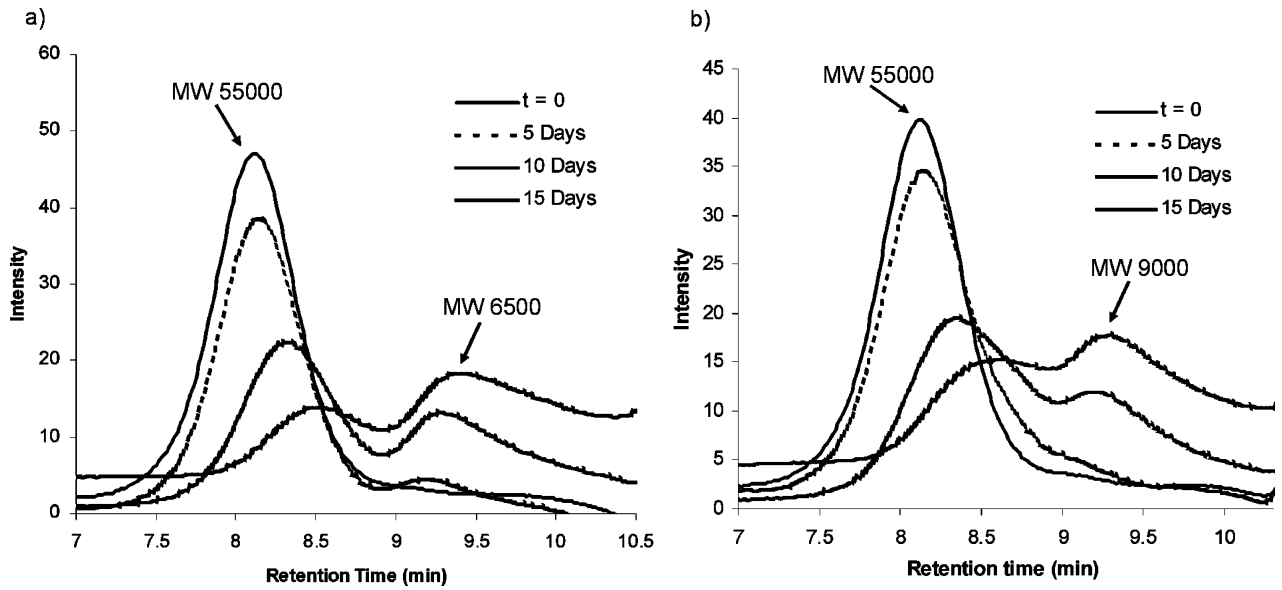

Figure 3. Time-dependent size exclusion chromatography profiles of the [G-3] − 5 K polymer upon incubation in (a) pH 5.0 and (b) pH 7.4 buffer at 37 °C.

concentrations. Although the toxicity of these carriers in vivo has not been explicitly tested in this study, we expect these polymers to also be well tolerated in vivo, on the basis of previous in vivo studies of the polyester dendrimer−PEO star hybrids.[25]

***In Vitro* Biodegradability of the Polymers**. The dendritic backbone of the bow-tie polymers is a polyester, and this is advantageous as polyester bonds are typically characterized by their susceptibility to hydrolysis.[33,34] Therefore, this provides the opportunity for a high MW, biodegradable PEO-based system, which had previously been challenging to prepare.[35,36] However, any degradation that might take place is expected to be slow since the sterically hindered ester linkages formed by this monomer make it relatively stable to acid- and base-catalyzed hydrolysis. Another potential source of degradability for these polymers is the carbamate bonds that link the PEO to the dendritic scaffold. While complete ester hydrolysis alone should release 2,2-bis-(hydroxymethyl)propionic acid monomers having two PEO chains attached, carbamate hydrolysis should lead to release of the individual PEO arms.

To determine whether these polymeric carriers are susceptible to degradation under conditions similar to those expected to be encountered in a physiological environment, a representative polymer, the [G-3] − 10 K bow tie, was incubated in pH 5.0 and pH 7.4 buffers at 37 °C and the MW was monitored over time by SEC. As shown in Figure 3a, at pH 5.0, over a period of 15 days there is an increase in the retention time of the peak corresponding to the PEO−dendrimer hybrid indicating a decrease in MW. In addition, a new peak appears with a longer retention time corresponding to a MW of approximately 6500. As the PEO arms of this [G-3] − 10 K polymer have a MW of 10000, it is clear that hydrolysis of the carbamate linkages must occur at mildly acidic pH, thus releasing free PEO arms, and decreasing the MW of the resulting bow tie. It is possible that some further degradation of the PEO arms also occurs over a prolonged period under these conditions, leading to a decrease in apparent MW of the PEO arm to 6500.

At pH 7.4, a similar decrease in the polymer MW is observed, and a new peak appears with a retention time corresponding to a MW of 9000. Therefore, at pH 7.4 carbamate hydrolysis must also occur. However, as ester hydrolysis was determined to be the dominant mode of degradation under mildly basic conditions (pH 9.0, data not shown) with release of polymers of MW 20000, corresponding to two PEO arms, it is likely that both carbamate and ester hydrolysis occur at pH 7.4.

Biodistribution Studies. After determining the promising nature of the bow-tie carriers in terms of their biocompatibility and degradability, we were interested in investigating their time-dependent biodistribution profiles to examine the effect of MW and architecture. In order to track the polymers in vivo, a number of dendrimer hydroxy groups of each bow-tie polymer were statistically converted to tyramine carbamates as shown in Scheme 1, by activation of the polymers with a limiting amount of 4-nitrophenyl chloroformate, followed by excess tyramine. The numbers of phenols on the polymers were quantified by ^{1}H NMR spectroscopy

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Scheme 1

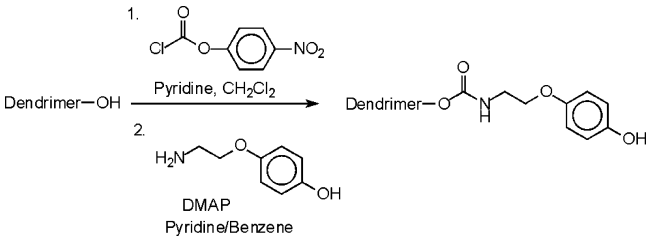

Table 2. Pharmacokinetic Data for the Evaluated Polymers

polymer	av no. of tyramine moieties per molecule	half-life (h)	$AUC_{0-\infty}$ (% dose·h/g)[a]
[G-1] − 10 K	1	8 ± 1	36 ± 10
[G-1] − 20 K	2.5	1.4 ± 0.4	22 ± 10
[G-2] − 5 K	1	11 ± 3	180 ± 70
[G-2] − 10 K	0.5	26 ± 6	850 ± 300
[G-2] − 20 K	trace	25 ± 8	1190 ± 600
[G-3] − 5 K	3.5	31 ± 2	1370 ± 140
[G-3] − 10 K	2	40 ± 4	1880 ± 270
[G-3] − 20 K	7	50 ± 10	2250 ± 580

[a] $AUC_{0-\infty}$ = area under the % injected dose/g of blood curves from zero to infinity.

(Table 2), and then the polymers were radiolabeled with ^{125}I using the chloramine T method as previously described.[30]

Polymer solutions (200 μL) were administered intravenously to CD-1 female mice at a dose of approximately 40 mg/kg. The mice were sacrificed at different time points following injection for biodistribution analyses (three mice per group). The blood (collected by heart puncture), heart, lungs, liver, stomach, spleen, intestines, kidney, and carcass were weighed, and the amount of radioactivity present in each organ was quantified. Radioactivity due to residual blood in the organs was subtracted after the blood content of each organ was determined using ^{51}Cr-labeled red blood cells. For the 24- and 48-h time points, mice were housed in metabolic cages to allow for the collection of urine and feces.

As shown in Figure 4, all of the eight-arm polymers, [G-3] − 5 K, [G-3] − 10 K, and [G-3] − 20 K, ranging in MW from 40000 to 160000 had long plasma circulation times. Elimination half-lives ($t_{1/2\beta}$) and areas under the % dose/g versus time curve from $t = 0$ to infinity ($AUC_{0-\infty}$) were calculated using the residuals method with the two-compartment model and are shown in Table 2.[31] As expected, with increasing MW of these eight-arm polymers from [G-3] − 5 K to [G-3] − 10 K to [G-3] − 20 K, both $t_{1/2\beta}$ (31 ± 2, 40 ± 4, 50 ±10 h, respectively) and $AUC_{0-\infty}$ values (1370 ± 140, 1880 ± 270, 2250 ± 580% dose·h/g, respectively) increase as well. No specific organ accumulation was observed for these molecules, with a significant portion of the dose (35−46%) found in the carcass after 48 h (Table 3). Less than 4% of the dose was found in the urine for each of these polymers, indicating that their effective sizes are above the threshold for renal filtration. After 48 h, 6−16% of the dose was excreted in the feces. Therefore, the primary route for elimination of these molecules was via intestinal excretion, believed to be the primary route by which large molecules that cannot be excreted through the kidney can escape the body.[37]

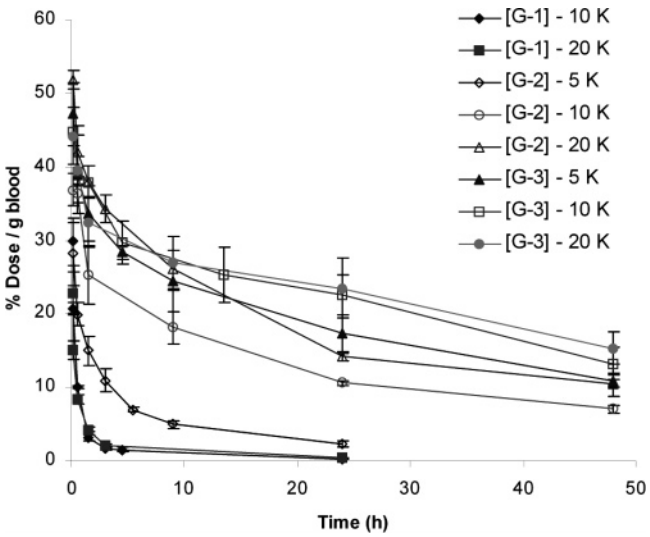

Figure 4. Plot of polymer concentration in the blood with respect to time.

Table 3. Polymer Found in the Urine, Feces, and Carcass over Periods of 24 and 48 h

	% dose in urine		% dose in feces		% dose in carcass	
polymer	24 h	48 h	24 h	48 h	24 h	48 h
[G-1] − 10 K	33		37		6 ± 3	
[G-1] − 20 K	20		47		4 ± 1	
[G-2] − 5 K	22		33		15 ± 2	
[G-2] − 10 K	16	18	13	22	27 ± 1	29 ± 1
[G-2] − 20 K	10		11	25	35 ± 4	34 ± 3
[G-3] − 5 K	1	2	2	7	40 ± 5	46 ± 5
[G-3] − 10 K		2		6	20 ± 4	34 ± 5
[G-3] − 20 K	3	4	12	16	31 ± 1	35 ± 5

The four-arm polymers [G-2] − 5 K, [G-2] − 10 K, and [G-2] − 20 K have MWs of 20000, 40000, and 80000, respectively. As shown in Figure 4, both the [G-2] − 10 K and [G-2] − 20 K are long circulating as predicted with $t_{1/2\beta}$ of 26 ± 6 and 25 ± 8 h, respectively, and $AUC_{0-\infty}$ of 850 ± 300 and 1200 ± 600% dose·h/g, respectively. After 48 h, 29−34% of the doses were found in the carcass, close to that found for the eight-arm polymers of similar MW. In contrast, the [G-2] − 5 K with a MW of only 20000 has a $t_{1/2\beta}$ of only 11 ± 3 h and an $AUC_{0-\infty}$ of 180 ± 70% dose·h/g. The rapid elimination of this polymer is not surprising considering that the MW is well below the nominal MW cutoff for renal filtration of 30000−40000 previously reported for linear PEO.[38] After 24 h, 22% of the dose of the [G-2] − 5 K was excreted into the urine. In comparison, 16% and 10% of the injected doses were found in the urine for the [G-2] − 10 K and [G-2] − 20 K, respectively.

(37) Drobnik, J.; Rypácek, F. Soluble synthetic polymers in biological systems. *Adv. Polymer Sci.* **1984**, *57*, 23−49.

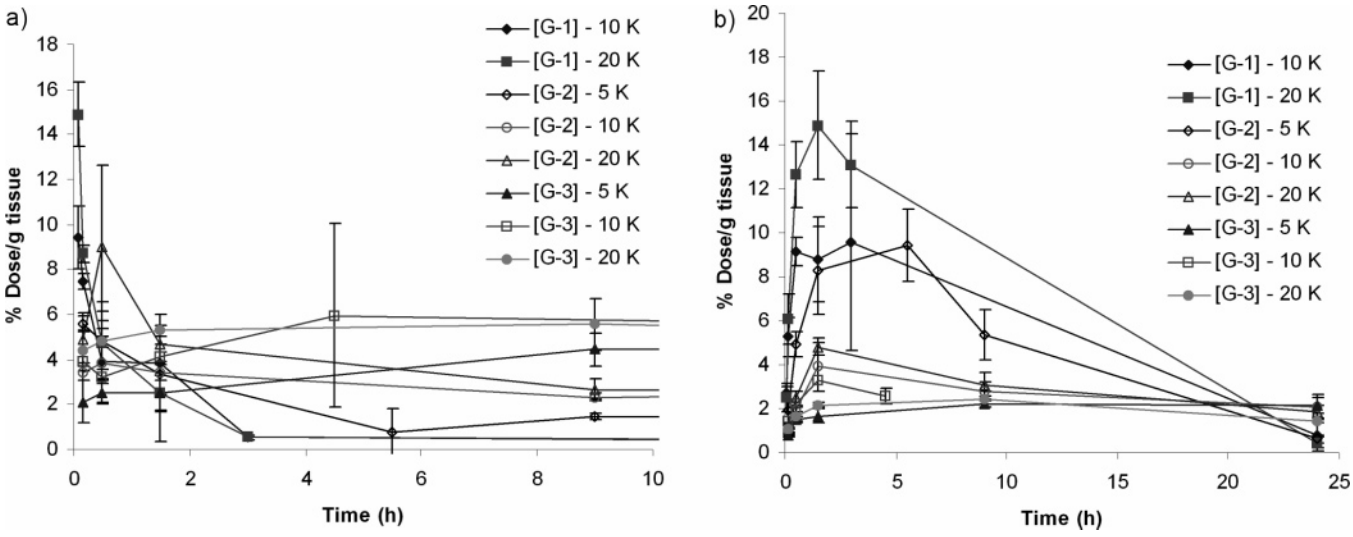

Figure 5. Concentration of polymer over time in (a) the liver and (b) the intestines.

While within the [G-2] series the amount of polymer excreted into the urine decreases with increasing MW as expected, a comparison with the [G-3] series shows that much more polymer is cleared by renal filtration for the [G-2] series, even for those polymers with sizes above the renal threshold. Initially this result was somewhat surprising considering the lower hydrodynamic volumes measured by SEC for the eight-arm polymers due to their more compact globular structure (e.g., M_n = 28300 for the [G-3] − 5 K, compared to 34900 for the [G-2] − 10 K). It has been reported that, for PEO-functionalized proteins, it is primarily the hydrodyamic radius of the resulting molecule rather than the length and number of the attached PEO chains that is important in determining the circulation half-life and renal filtration rate.[39,40] However, hydrodynamic volume is not the only factor governing the rate of renal clearance for polymers. Researchers have suggested that polymers such as poly(vinylpyrrolidone), HPMA, and PEO with MWs greater than the renal threshold can pass through glomerular pores mediated by an "end-on" motion as long as they possess a flexible structure.[10,41] Comparisons of the glomerular filtration rates of relatively elongated polymers such as dextran with highly cross-linked and almost spherical molecules such as ficoll have shown that, for molecules with the same hydrodynamic radius, the more elongated molecules are cleared more rapidly.[42,43] As the differences in hydrodynamic volume are quite small for the eight-arm versus four-arm bow ties, it is likely that the flexibility factor dominates and the decreased flexibility of the eight-arm polymers slows their rate of renal filtration relative to the four-arm series.

Examination of the biodistribution results for the [G-1] − 10 K and [G-1] − 20 K reveals anomalous behavior. Despite their MWs of 20000 and 40000, both molecules are very rapidly cleared from circulation, as shown in Figure 4. Although significant amounts of polymer were found in the urine after 24 h (33% for [G-1] − 10 K and 20% for [G-2] − 20 K), consistent with the trend related to flexibility discussed above, even larger amounts ranging from 37% to 47% of the dose were found in the feces. Closer inspection of the time-dependent concentrations of these polymers in the liver (Figure 5a) and intestines (Figure 5b) reveals that, in contrast to the other polymers evaluated in this study, significantly higher liver values are observed at early time points such as 5 and 10 min postinjection, followed by high values in the intestines. Accumulation of these polymers in other organs such as the heart, lungs, spleen, or stomach was not observed. These results are consistent with the rapid accumulation of the polymers in the liver, likely due to the interaction of exposed iodophenols with complementary receptors, followed by excretion via the bile duct into the intestines, and finally the feces. Previous biodistribution experiments in which radiolabeled phenols were placed at the periphery of a PEO star polymer or on the end of a linear

(38) Yamaoka, T.; Tabata, Y.; Ikada, Y. Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. *J. Pharm. Sci.* **1994**, *83*, 601−606.

(39) Knauf, M. J.; Bell, D. P.; Hirtzer, P.; Luo, Z.-P.; Young, J. D.; Katre, N. V. Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2 chemically modified with water-soluble polymers. *J. Biol. Chem.* **1988**, *263*, 15064−15070.

(40) Nakaoka, R.; Tabata, Y.; Yamaoka, T.; Ikada, Y. Prolongation of the serum half-life of superoxide dismutase by poly(ethylene glycol) modification. *J. Controlled Release* **1997**, *46*, 253−261.

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(42) Ohlson, M.; Sörensson, J.; Lindström, K.; Blom, A. N.; Fries, E.; Haraldsson, B. Effects of filtration rate on the glomerular barrier and clearance of four differently shaped molecules. *Am. J. Physiol. Renal Physiol.* **2001**, *281*, F103−F113.

(43) Bohrer, M. P.; Deen, W. M.; Robertson, C. R.; Troy, J. L.; Brenner, B. M. Influence of molecular configuration on the passage of macromolecules across the glomerular capillary wall. *J. Gen. Physiol.* **1979**, *74*, 583−593.

PEO showed similar results.[25,30] The fact that the [G-2] and [G-3] polymers do not exhibit similar liver accumulation, despite having more phenols per molecule in many cases (Table 1), can likely be attributed to a significant architectural effect. The presence of numerous PEO chains surrounding the dendrimer core where the iodinated phenol is located probably provides steric shielding preventing the iodophenols from being recognized by receptors. In contrast, the [G-1] polymers with only two arms behave much like linear PEO with an exposed dendron in the middle. Although the [G-2] − 5 K polymer does not have exceptionally high amounts of radioactivity in the liver for any of the time points measured, the high intestinal values may indicate that the liver-mediated excretion process also occurs for this molecule to some extent. The steric protection provided by the more highly branched and higher MW structures may have important implications for their applications in drug delivery, as they may be capable of shielding highly sensitive drug payloads at their core.

Although the liver uptake effect precludes the comparison of our results for the [G-1] polymers with those of the [G-2] and [G-3] polymers, our results can be compared with those previously reported for linear PEO of MWs ranging from 6000 to 170,000.[38] The $t_{1/2\beta}$ values reported for PEO of MWs 20000 and 50000 are 169 ± 20 min and 16.5 ± 1.3 h respectively, while the corresponding $AUC_{0-\infty}$ values are 110 ± 7 and 600 ± 12% dose·h/g. These values indicate that the linear PEOs are cleared more rapidly than our four-arm and eight-arm polymers of equal or lower MW. Thus the trend of increased branching leading to longer plasma circulation times still holds. Furthermore, the circulation half-life of 170000 MW linear PEO was reported to be only 23 ± 1 h, while that of [G-3] − 20 K bow tie was 50 ± 10 h. However, due to the high polydispersity of this particular linear PEO sample, it is difficult to say for certain whether the difference can be assigned to an architectural effect or to the rapid elimination of lower MW contaminants in the sample.

Biodistribution Studies in Tumored Mice. Due to the promising characteristics of the long-circulating carriers in the biodistribution studies, preliminary biodistribution studies in tumored mice were performed. Two of the highest MW bow-tie polymers, the [G-3] − 10 K and [G-3] − 20 K, were selected for the study because enhanced levels of tumor accumulation have previously been reported for higher MW, long-circulating polymers.[18,44] C57BL6 mice were injected subcutaneously with B16F10 melanoma cells, and the tumors were allowed to grow to an average size of 330 ± 160 mg. Radiolabeled polymer was injected intravenously at a dose of 40 mg/kg, and then the mice were sacrificed 48 h postinjection. The blood, organs, tumor, and muscle were collected and weighed, and the amount of radioactivity present was quantified.

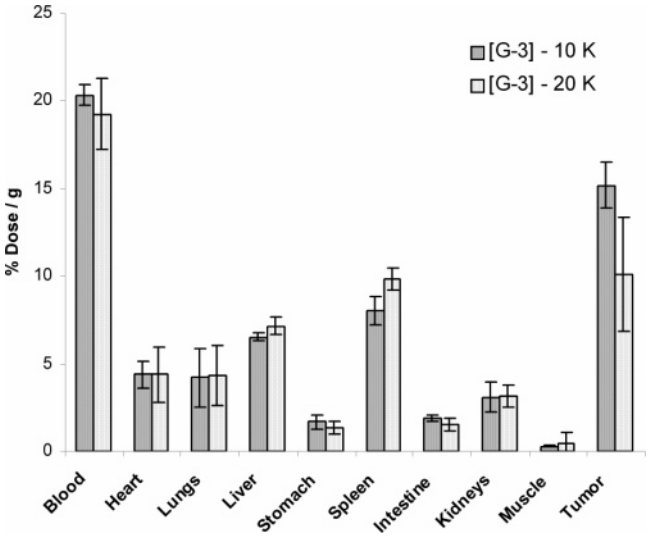

Figure 6. Biodistribution of [G-3] − 10 K and [G-3] − 20 K in mice bearing subcutaneous B16F10 tumors.

As shown in Figure 6, the highest concentrations of polymer are found in the blood and tumor with 15 ± 1 and 10 ± 3% dose/g in the tumor for the [G-3] − 10 K and [G-3] − 20 K, respectively. These values compare favorably with the tumor accumulations reported for other polymers such as HPMA and PEO.[18,44] While the % dose per gram of tumor is lower for the higher MW [G-3] − 20 K polymer than for the [G-3] − 10 K, the absolute amount of polymer is higher for the [G-3] − 20 K with 4.1 ± 1.9% of the dose in the tumor compared with 3.5 ± 0.6% for the [G-3] − 10 K. This can be explained by the larger average tumor size of 400 ± 200 mg for the mice that received the [G-3] − 20 K compared with 220 ± 40 mg for those mice that received the [G-3] − 10 K as it has been previously reported that, above 300 mg, the volume of vasculature per gram of tumor decreases with increasing tumor size, thus limiting the accumulation of circulating polymer in larger tumors.[44]

Other organs with relatively high levels of polymer after 48 h include the liver and spleen. As the accumulation of polymer in these organs was not observed during the biodistribution studies in nontumored mice, this result may be explained by the tendency of the B16F10 melanoma to metastasize to these organs.[45,46] In addition, no correction was made for the presence of blood in the organs in this experiment. As a significant amount of polymer is still found in the blood after 48 h, and these organs contain relatively high levels of blood, this may also explain the significant amounts of polymer in these organs. The tumor:muscle ratios

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were found to be 50:1 for the [G-3] − 10 K, and 21:1 for the [G-3] − 20 K on a % dose/g basis. This suggests differential permeability between the tumor endothelium and that serving leg muscle (a representative normal tissue), and confirms that the EPR effect is operative for this system. The high levels of tumor accumulation observed for these polymers should be highly beneficial in the development of these polymers as delivery vehicles for chemotherapeutics.

Conclusions

New polyester dendrimer−PEO bow-tie hybrids were evaluated for their potential as drug delivery vehicles, and to explore the effect of MW and architecture on their pharmacokinetic properties. In vitro experiments showed that these polymers were nontoxic to MDA-MB-231 cells, and that they were degraded to lower MWs at the normal physiological pH of 7.4, and at the mildly acidic pH of 5.0 which may be encountered upon uptake of these molecules by endocytosis and subsequent trafficking to endosomes and lysosomes. Biodistribution studies showed that all carriers with MWs of 40000 and greater had long plasma circulation times, while those with lower MWs were cleared more rapidly with significant quantities excreted in the urine. In general, it was found that the more branched [G-3] polymers exhibited increased circulation times and decreased renal clearance relative to the less branched polymers, a property likely attributable to their decreased flexibility and resulting difficulty in passing through glomerular pores. It was also demonstrated that the more branched structures provide increased levels of steric protection for the payload on the drug-carrying dendron at the core of the molecule. High levels of tumor accumulation were found for two [G-3] polymers in mice bearing subcutaneous B16F10 melanoma. Overall, the features of these new branched carriers including degradability, lack of toxicity, long circulation half-lives, and high levels of tumor accumulation make them very promising polymers for therapeutic applications.

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Supporting Information Available: Complete biodistribution results for each evaluated polymer. This material is available free of charge via the Internet at http://pubs.acs.org.

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