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Cytotoxicity, Hemolysis, and Acute in Vivo Toxicity of Dendrimers Based on Melamine, Candidate Vehicles for Drug Delivery

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Abstract: A small library of dendrimers was prepared from a common precursor that is available in 5 g scale in five linear steps at 56% overall yield. The precursor is a generation three dendrimer that displays 48 peripheral sites by incorporating AB_4 surface groups. Manipulation of these sites provided six dendrimers that vary in the chemistry of the surface group (amine, guanidine, carboxylate, sulfonate, phosphonate, and PEGylated) that were evaluated for hemolytic potential and cytotoxicity. Cationic dendrimers were found to be more cytotoxic and hemolytic than anionic or PEGylated dendrimers. The PEGylated dendrimer was evaluated for acute toxicity in vivo. No toxicity—neither mortality nor abnormal blood chemistry based on blood urea nitrogen levels or alanine transaminase activity—was observed in doses up to 2.56 g/kg ip and 1.28 g/kg iv.

Introduction

As the products of stepwise and orchestrated synthesis, dendrimers have attracted the attention of the drug-delivery community due in part to the belief that exquisite control over composition can counter challenges to absorption, distribution, metabolism, excretion, and toxicity.[1] The field is driven by the belief that polymeric therapeutics will contribute significantly to human health by increasing the efficacy of currently used drugs as well as providing opportunities for the use of new agents currently precluded from the clinic due to challenges including low solubility and systemic toxicity.[2] This desired increase in activity could be derived from a number of effects including prolonged circulation times, reduced toxicity of drugs, increased drug solubility, and perhaps most importantly, selective delivery to tumors through either active targeting with appended ligands for tumor-specific receptors or passive targeting that results from the *enhanced permeability and retention* (EPR) effect.[3]

Our previous studies have established that dendrimers based on melamine are tractable, engineerable targets[4] that solubilize hydrophobic drugs.[5] Solubilization does not preclude drug action.[5] These dendrimers attenuate the toxicity of approved anticancer drugs (methotrexate and 6-mercaptopurine) in vivo, suggesting that higher dosing might be attainable.[6a] In vitro and in vivo studies have shown that cationic dendrimers based on melamine have acute toxicities at dosages ip of 40 mg/kg. Mortality is observed at 160 mg/kg.[5,6b] In addition, these cationic polymers are hemolytic. Here, we use synthesis to explore these toxicities as a function of surface group with the goal of identifying a candidate for iv administration. Studies of the in vitro cytotoxicity and hemolytic potential of six derivatives of **1** (Chart 1, Table 1), including two polycations, three polyanions, and a PEGylated species, are complemented with acute toxicity studies of **7** in mice.

Results and Discussion

The dendrimers examined comprise triazines linked by diamines. While we have reported extensively on these architectures, **1** and its derivatives (**2**−**7**) are unique in a number of respects. These dendrimers incorporate AB_4 terminal groups providing 48 surface sites that increase the steric bulk on the periphery. The minimum-energy structures (Figure 1) that result from gas-phase simulations of **2** and **7** show that the core of the dendrimer is confined to the center of globular architectures having diameters of 2.4 and 4.6 nm, respectively. The sizes of these architectures in aqueous solution, especially that of **7**, are likely larger.

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(2) Duncan, R. *Nat. Rev. Drug Discovery* **2003**, *2*, 347.
(3) (a) Maeda, H. *Adv. Enzyme Regul.* **2001**, *41*, 189. (b) Matsumura, Y.; Maeda, H. *Cancer Res.* **1986**, *6*, 6387. (c) Greish, K.; Fang, J.; Inutsuka, T.; Nagamitsu, A.; Maeda, H. *Clin. Pharmacokinet.* **2003**, *42*, 1089.
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(6) (a) Neerman, M. F.; Chen, H. T.; Parrish, A. R.; Simanek, E. E. Unpublished results. (b) Neerman, M. F.; Zhang, W.; Parrish, A. R.; Simanek, E. E. *Int. J. Pharm*. In press.

10.1021/ja048548j CCC: $27.50

Chart 1. Dendrimer **1** (R = BOC) in Atomic Detail and **1**−**7** Shown Schematically with R Identified

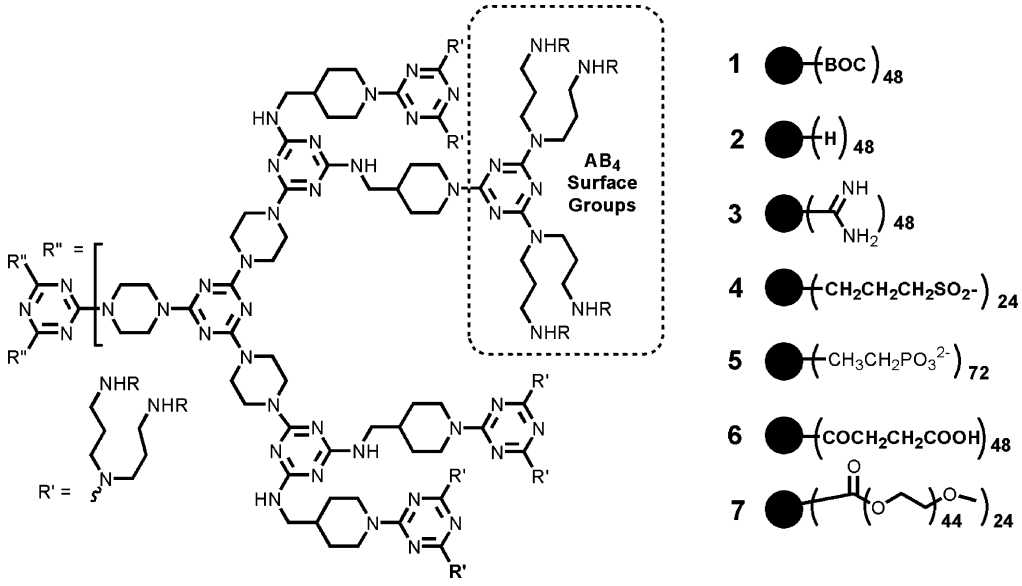
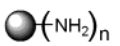
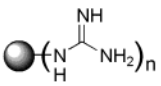
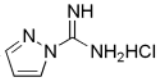
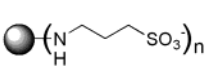
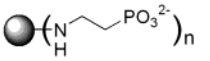
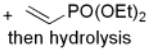
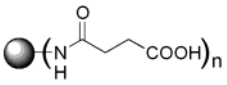
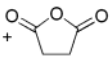
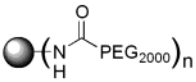


Table 1. Library of Dendrimers Used in This Study

Compounds	~n	Reagents	Conditions	M+ (m/z)
2 	48	**1**+TFA	DCM/MeOH rt, 24 hr	Calcd: 6941 Obsd: 6943
3 	48	**2** + 	MeCN/H_2O, DIPEA, rt, 5 days	Calcd: 8959 Obsd: 8989
4 	24	**2** + 	MeCN/H_2O, DIPEA rt, 5 days	Calcd: 9869 Obsd: 9787
5 	72	**2** +  then hydrolysis	MeCN/H_2O, rt, 5 days 20% HCl, reflux, 1 day	Calcd: 14718 Obsd: 14285
6 	48	**2** + 	MeCN/THF/H_2O, DIPEA, rt, 5 days	Calcd: 11742 Obsd: 11770
7 	24	**2** + PEG_{2000}-NHS	MeOH/H_2O, DIPEA rt, 5 days	Calcd: 54941 Obsd: 59500

The synthesis is convergent in the classical sense (Scheme 1).[7] Using BOC−ON, the primary amines of 3,3′-iminobispropylamine are protected to yield **8**. Reaction with cyanuric chloride produces monochlorotriazine **9**, which can undergo chemoselective reaction with the secondary amine of aminomethylpiperidine to yield **10**. Reaction with cyanuric chloride provides the monochlorotriazine that is ultimately attached to the hexavalent core. The core is available by reaction of cyanuric chloride with BOC−piperazine. Upon deprotection of **12**, triamine **13** is reacted with the protected monochlorotriazine **14**, a material available in one step in 97% yield. The protected hypercore **15** is reacted with trifluoroacetic acid before the ultimate coupling step.

The last step in the synthesis of **1** involves the coupling of six second generation monochlorotriazine dendrons, **11**, to a core displaying six reactive piperazine groups, **16**. The core of the dendrimer is available in four synthetic steps necessitating two chromatographic purifications in 76% overall yield. The second generation dendron is available in four synthetic steps necessitating two chromatographic purifications in 75% overall yield. Thus, the synthesis can been executed in five linear steps to yield 5 g of material in 56% overall yield and requires one final chromatographic step to yield pure **1** (>95%). A single peak observed by HPLC analysis corroborates this conclusion. The efficiency of the final multicomponent coupling step is highly dependent on the base used. A survey of diisopropylethylamine, K_2CO_3, MTBD,[8] BEMP,[9] and resin-supported TBD[10] and BEMP[11] reveals that resin-supported BEMP provides the greatest yield and fewest side products.

(7) For a detailed description of dendrimer syntheses, including this hypercore approach, see: Grayson, S. M.; Fréchet, J. M. J. *Chem. Rev.* **2001**, *101*, 3819.
(8) MTBD = 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene. Schwesinger, R. *Chimia* **1985**, *39*, 269.
(9) BEMP = 2-*tert*-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine. (a) Schwesinger, R. Schlemper, H. *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 1167. (b) O'Donnell, M. J.; Delgado, F.; Hostettler, C.; Schwesinger, R. *Tetrahedron Lett.* **1998**, *39*, 8775.

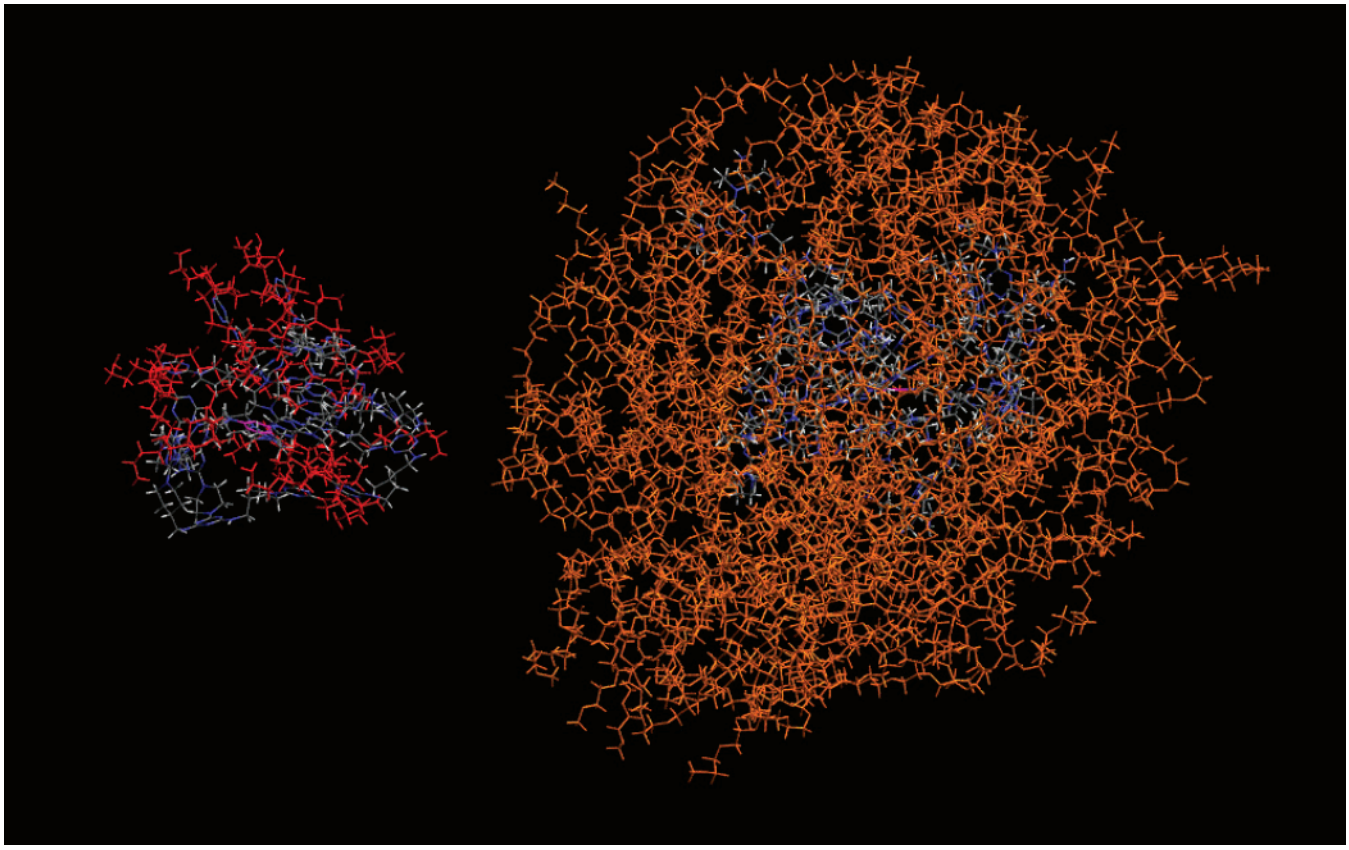
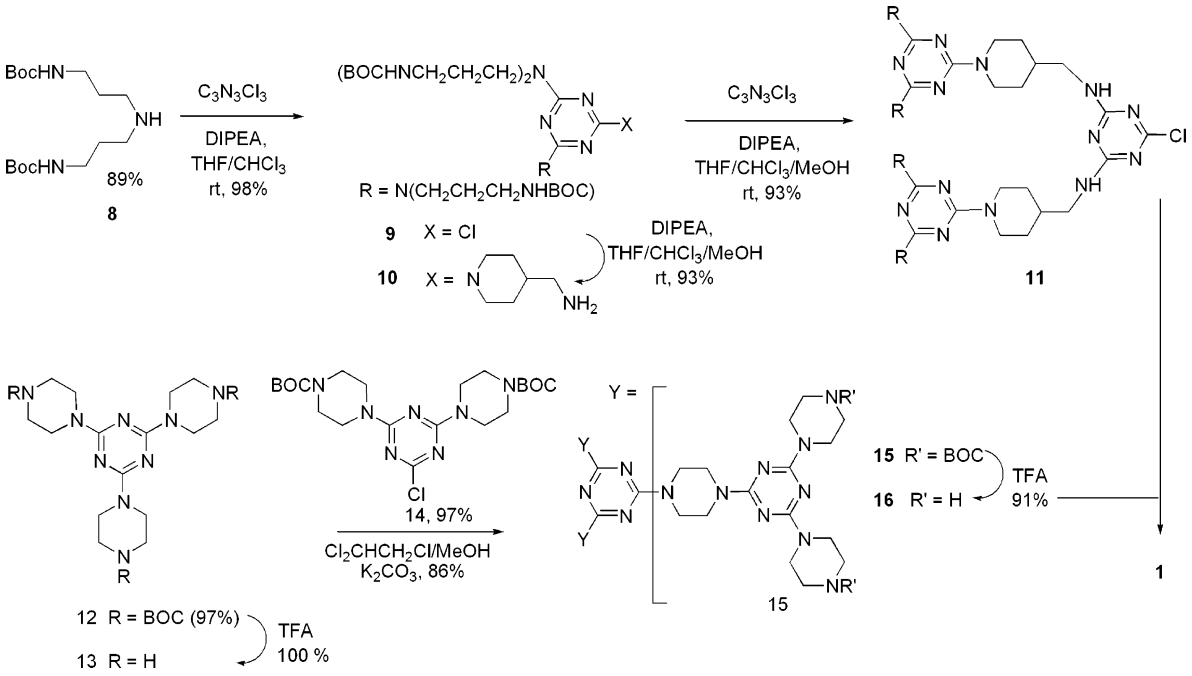


Figure 1. Gas-phase simulations of **2** and **7** show globular structures with diameters of 2.4 and 4.6 nm, respectively. The dendrimers are shown in blue (nitrogen) and gray (carbon) with the central triazine in maroon. Surface groups are shown as red (AB_4 amines) or orange (PEG_{2000}).

Scheme 1. Synthesis of **1**



Dendrimers **2**−**7** were prepared by quantitatively deprotecting **1** with trifluoroacetic acid before subjecting the resulting material, **2**, to a range of reaction conditions (Table 1) to provide the small library of dendrimers. These manipulations sacrifice monodispersity of the resulting dendrimer populations in order to efficiently address the biological challenges. Monodisperse populations will be prepared when in vivo tumor-inhibition activity warrants this action. The table provides the calculated molecular ions for the indicated number of surface reactions

(10) TBD = 1,5,7-triazabicyclo[4.4.0]dec-5-ene. (a) Xu, W.; Mohan, R.; Morrissey, M. M. *Tetrahedron Lett.* **1997**, *38*, 7337. (b) Weidner, J. J.; Parlow, J. J.; Flynn, D. L. *Tetrahedron Lett.* **1999**, *40*, 239.

(11) Shuttleworth, S. J.; Nasturica, D.; Gervais, C.; Siddiqui, M. A.; Rando, R. F.; Lee, N. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2501.

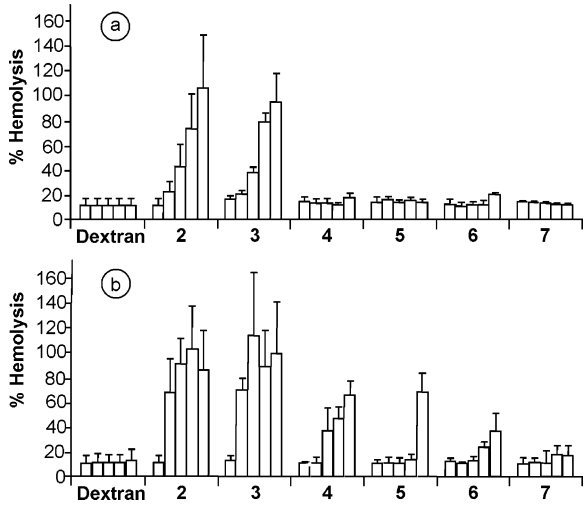


Figure 2. Impact of surface modification on the hemotoxicity of **2**−**7** at 1 (panel a) and 24 h (panel b). Doses increase for each species from left to right: 0.001, 0.01, 0.1, 1, and 10 mg/mL.

and the center of the observed distribution of products. On the basis of analysis of the mass spectra, we conclude that reactions to produce **3** (with 1H-pyrazole-1-carboxamidine·HCl)[12] and **6** (with succinic anhydride[13]) proceed almost quantitatively. Reaction with sultone[14] yields **4** with ∼24 of the 48 peripheral amines functionalized. Reaction of **2** with the vinylphosophonate diester[15] followed by hydrolysis[16] leads to formation of tertiary and quarternary amines. The population is centered at 72 reactions, corresponding to 1.5 reactions for every primary amine of **2**. PEGylation with the NHS ester of a 2000 Da *N*-succinimidyl propionate PEG that is terminated with a methyl group shows the greatest dispersity.[17] Molecular weights range from species with >12 PEG chains (>25%) to dendrimers to almost fully PEGylated architectures. For these studies, we chose a mixture whose center of mass corresponds to approximately half of the 48 surface groups (26) reacting. This mean mass exceeds the perceived 40 kDa threshold for the EPR effect that tumors show for polymeric molecules.[3] PEGylation can be driven to completion. Dendrimers **2**−**7** are soluble in phosphate-buffered saline at physiological pH.

Red blood cell lysis precludes direct intravenous delivery of desired agents and often enhances the toxicity of these agents when administered by other routes. Due to the number of groups, the periphery of a dendrimer can greatly influence the physical and biological properties, and accordingly, by changing these groups, hemolysis can be attenuated. The results of these studies appear in Figure 2. We examined the hemolytic potential of a dextran control and **2**−**7** over a 10^3 concentration range.[18,19] Hemolysis was recorded spectrophotometrically at 1 and 24 h.

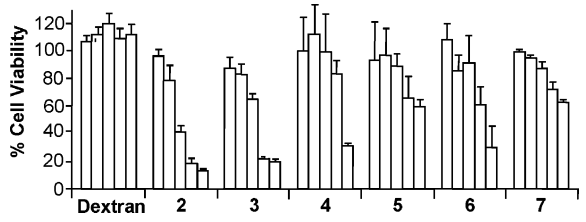


Figure 3. Cell viability measured using the MTT assays at concentrations that increase from left to right: 0.001, 0.01, 0.1, 1, and 10 mg/mL.

All the molecules show a concentration-dependent and time-dependent hemolysis. Hemolysis is more pronounced in cationic dendrimers than in anionic dendrimers. The PEGylated dendrimer is the least hemolytic in these experiments.

This trend in hemolytic potential is mirrored in cell viability assays with Clone 9 cells (Figure 3) using the MTT spectrophotometric assay.[20,21] The cationic dendrimers (**2** and **3**) displayed significant cytotoxicity, even at low concentrations. Anionic surface modification (**4**−**6**) afforded protection against cytotoxicity; however, decreases in viability were observed at higher concentrations. The PEG-modified dendrimer **7** showed little cytotoxicity over the range of concentrations surveyed. We conclude from these experiments that the modified dendrimers are less toxic to normal cells than is **2**.

Microscopic evaluation at 32× of Clone 9 cells after exposure to unmodified and modified dendrimers bears out the spectrophotometric assays of viability. Control cells (Figure 4, panel a) were compared with cells treated with 1 or 10 mg/mL of cationic dendrimer **2** (panels b and c, respectively) and 1 or 10 mg/mL of the PEGylated dendrimer **7** (panels d and e, respectively). After 3 h of exposure, both concentrations of **2** induced morphological changes in the cells. Cells treated with **7** appear more similar to untreated cells.

To establish the validity of these in vitro analyses and the dosage for future tumor inhibition studies, the in vivo toxicity of **7** was determined. Male C3H mice were administered doses up to 2.56 g/kg in saline solution by ip injection. No mortality was observed. After 48 h, serum samples obtained from each animal showed no significant increases in either urea nitrogen levels or liver enzyme (alanine transaminase) activity. Accordingly, we conclude that the in vivo toxicity to liver and kidneys is low enough to warrant further study and that this architecture is suitable for tumor inhibition studies. Dendrimer **7** was administered iv in doses up to 1.28 g/kg. Again, serum samples collected after 24 h showed no significant increases in either urea nitrogen levels or liver enzyme (alanine transaminase) activity.

Conclusions

A library of six dendrimers that vary in surface chemistry was prepared from a common precursor available in five steps in 56% overall yield. The in vitro evaluation of cytotoxicity and hemolytic potential of this library identified a candidate, **7**, for further in vivo toxicology studies. No toxicity of this polyPEGylated architecture was observed in mice at doses up to 2.56 g/kg ip or 1.28 g/kg iv based on blood chemistry analysis

(12) As per Larsen, S. D.; Connell, M. A.; Cudahy, M. M.; Evans, B. R.; May, P. D.; Meglasson, M. D.; O'Sullivan, T. J.; Schostarez, H. J.; Sih, J. C.; Stevens, F. C.; Tanis, S. P.; Tegley, C. M.; Tucker, J. A.; Vaillancourt, V. A.; Vidmar, T. J.; Watt, W.; Yu, J. H. *J. Med. Chem.* **2001**, *44*, 1217.
(13) As per Trester-Zedlitz, M.; Kamada, K.; Burley, S. K.; Fenyö, D.; Chait, B. T.; Muir, T. W. *J. Am. Chem. Soc.* **2003**, *125*, 2416.
(14) As per Adamczyk, M.; Chen, Y.-Y.; Mattingly, P. G.; Pan, Y.; Rege, S. *J. Org. Chem.* **1998**, *63*, 5636.
(15) Rosowsky, A.; Forsch, R. A.; Moran, R. G.; Kohler, W.; Freisheim, J. H. *J. Med. Chem.* **1988**, *31*, 1326.
(16) Palacios, F.; Aparicio, D.; Vicario, J. *Eur. J. Org. Chem.* **2002**, *24*, 4131.
(17) As per Kojima, C.; Kono, K.; Maruyama, K.; Takagishi, T. *Bioconjugate Chem.* **2000**, *11*, 910.
(18) Carreño-Gómez, B.; Duncan, R. *Int. J. Pharm.* **1997**, *148*, 231.
(19) Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. *Biomaterials* **2003**, *24*, 1121.
(20) Mosmann, T. *J. Immunol. Methods* **1983,** *65*, 55.
(21) (a) Sgouras, D.; Duncan, R. *J. Mater. Sci.: Mater. Med.* **1990**, *1*, 61. (b) Malik, N.; Wiwattanapatapee, R.; Klopsch, R.; Lorenz, K.; Frey, H.; Weener, J. W.; Meijer, E. W.; Paulus, W.; Duncan, R. *J. Controlled Release* **2000**, *65*, 133.

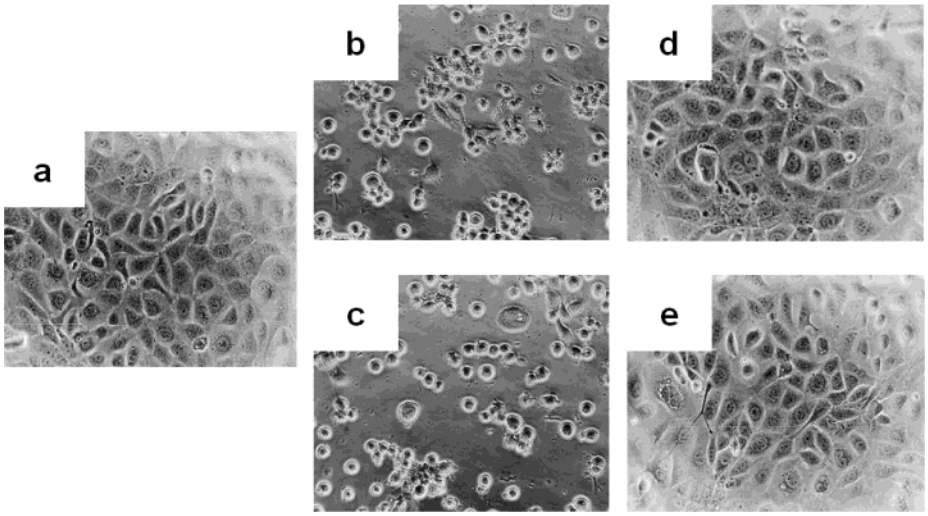


Figure 4. Clone 9 cells grown to confluence (panel a) undergo morphological changes in the presence of 1 mg/mL of **2** (panel b) or 10 mg/mL of **2** (panel c) but are relatively unaffected by similar concentrations of **7** (panels d and e).

of blood urea nitrogen levels (kidney toxicity) or alanine transaminase activity (liver toxicity). It is important to note that units of mg/mL are used throughout these experiments as is tradition in the toxicology literature. The molecular weights of dendrimers **2**−**6** are approximately the same, ranging from 7 to 12 kDa. Dendrimer **7** is significantly larger, with a molecular weight of ∼60 kDa. Comparisons of hemolysis and cell viability on a mole:mole basis suggest that **7** is much more similar to **4**−**6** than casual inspection would suggest. Our choice of limiting the acute in vivo studies to only the PEGylated dendrimer **7** (instead of including **4**−**6**) was based primarily on reports that anionic polymers have anti-coagulant activity and can stimulate cytokine release.[2] The acute toxicity levels observed for **7** are similar to those observed by Fréchet and co-workers in a three-arm poly(ethylene oxide) star polymer, bearing generation two polyester dendrons with a molecular weight of 23.5 kDa.[22] It should be noted that our toxicity studies are the result of a single (acute) injection. The chronic toxicity of the species has not been established and may be different than the acute toxicity as a result of dendrimer metabolism and toxicity of the degraded products. Accordingly, these issues, as well as the biodistribution and excretion of candidate **7** and similar architectures, will be pursued in due course to satisfy our aim of rationally approaching in vivo tumor assays.

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Supporting Information Available: Complete description of biological, computational, and synthetic methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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