DNA Binding

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"On-Off" Multivalent Recognition: Degradable Dendrons for Temporary High-Affinity DNA Binding**

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Dedicated to Professor Richard J. K. Taylor on the occasion of his 60th birthday

Design of molecules capable of binding to biological systems with high affinity is of key importance in the development of synthetic systems capable of intervening in biological pathways. Multivalent binding has emerged as one of the key principles in enhancing binding to biological targets.[1] By organizing multiple copies of a given ligand on a single molecular scaffold, the entropic cost of binding to more than one binding site on the target is decreased, and hence the extra binding affinity gained for each additional ligand can increase in a nonlinear manner.[2] Dendritic molecules, with their inherent repetitive branched structures, offer one of the most useful synthetic scaffolds for organizing multiple ligands on an accessible surface.[3] Early influential work focused on dendritic arrays of saccharides, capable of binding to lectins, for example, and demonstrated that significant multivalent effects could be achieved.[4] Recently, we chose to synthesize dendritic arrays of spermine ligands, to achieve high-affinity DNA binding.[5] Spermine is a simple tetraamine employed in nature for nonspecific minor-groove DNA binding.[6] However, it has relatively low DNA affinity, and suffers from competition with sodium cations. Indeed, it has to be present in millimolar amounts in eukaryotic cells to play its ubiquitous role. We found that dendritic arrays of spermine, such as **G2-amide** (Figure 1), could effectively bind DNA at low nanomolar concentrations and were completely unaffected by the presence of competitive sodium cations.[5] This multivalency presumably occurs because more than one of the spermine ligands on the flexible dendron support are able to simultaneously interact with the minor groove of DNA. In subsequent work, we explored the ability of these dendrons to achieve gene delivery into cells and found that, with relatively simple modifications, effective gene delivery could be achieved.[7] We also grafted these dendrons onto proteins to generate new biosynthetic constructs incorporating adhesive patches with high affinity for DNA.[8]

However, the extremely high affinity of multivalent ligands for their biological targets may cause problems if such systems are to be applied in vivo, owing to the persistence of the dendrimer and strong, almost irreversible interactions with the binding partner. Such interactions are a particular problem, for example, in gene therapy, wherein the persistence of dendrimers within cells after transfection has taken place is a known problem.[9] Indeed, the development of degradable vectors is a key frontier in current approaches to gene delivery.[10] We therefore became interested in degradable dendritic systems. There have been a number of elegant studies of degradable dendrimers, in particular from the groups of Shabat,[11] McGrath,[12] de Groot,[13] and Fréchet.[14] In general, these researchers were interested in using degradable systems to release multiple copies of a drug in a controlled way. In contrast, however, our aim is to use degradation to *switch off* multivalent interactions between a dendritic molecule and its biological binding partner, to turn a multivalent array into smaller units, such as individual monovalent ligands. In this way, after degradation, the binding affinity will drop dramatically, meaning that the ligands can no longer intervene in biological pathways. We recently reported a dendron design which made use of a UV-cleavable linker group between the scaffold and the ligands and allowed triggered conversion from multi- to monovalency.[15] UV-controlled degradation is potentially useful in,

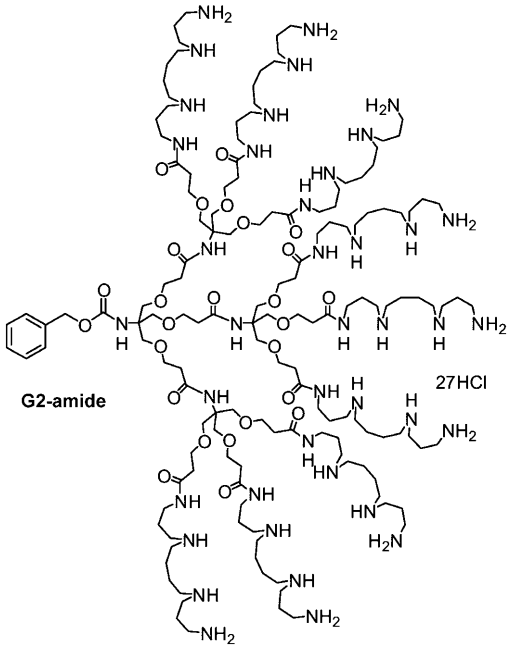

Figure 1. Structure of **G2-amide**.

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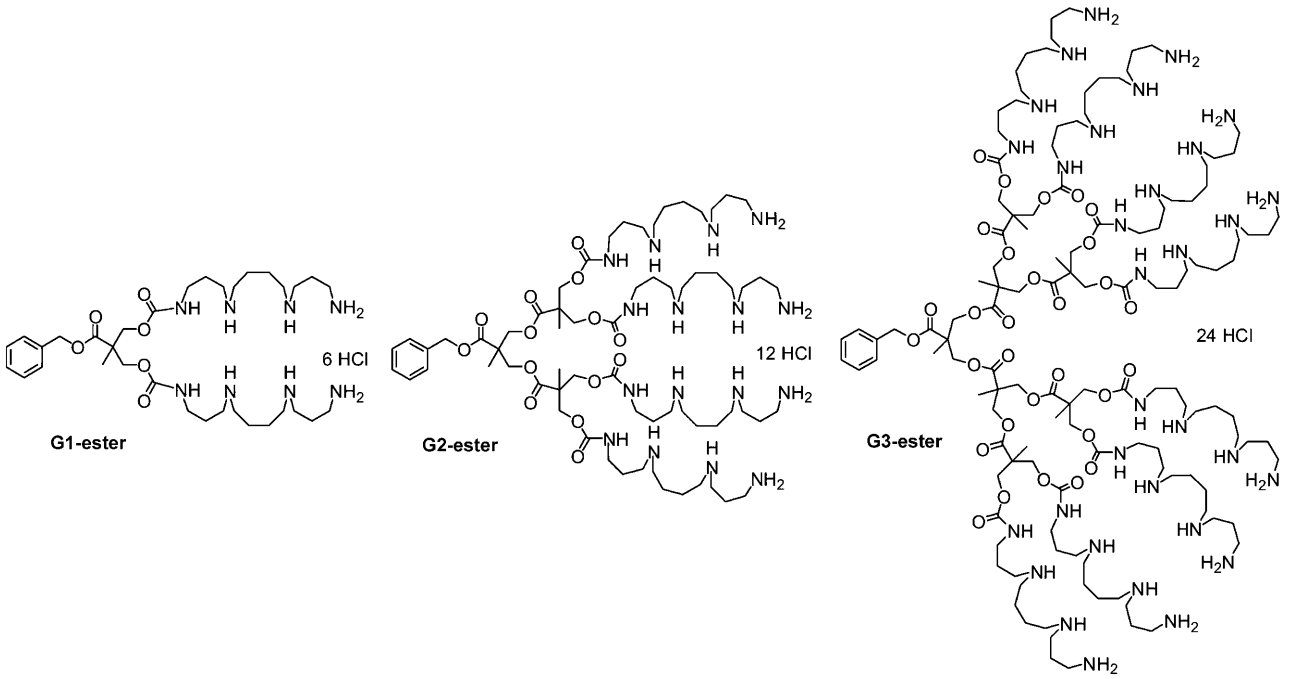

Figure 2. Structures of the chemically (bio)degradable ester-derived dendrons synthesized for this study.

for example, the development of controllable chip surfaces in which high-affinity binding can be switched off. However, it is less applicable in biological systems. We sought to demonstrate that (bio)chemically degradable dendrons could be used in this way—the results are reported herein.

Fréchet, Hult, and co-workers reported the synthesis of degradable dendritic molecules constructed from 2,2-bis(hydroxymethyl)propionic acid,[16] and we therefore decided to make use of this dendritic motif in synthesizing our new multivalent DNA binders. We adapted the synthetic procedure (see the Supporting Information) to generate first-, second-, and third-generation dendron structures with spermine groups on the surface and a benzyl ester protecting group at the focal point (**G*n*-ester**, Figure 2). The synthesis of these dendrons was efficient and high yielding (see the Supporting Information). Importantly, these dendrons were constructed from repeated ester groups, with the surface groups connected by carbamates. Both of these linkages have potential chemical (bio)degradability which should enable degradation under biologically relevant conditions.[14]

Initially, we investigated the ability of these dendrons to bind calf thymus DNA. To gain a comparative quantification of the DNA binding abilities of these dendrons, we employed an ethidium bromide (EthBr) displacement fluorescence assay, which is commonly used to investigate the binding of polyammonium cations to DNA.[17] When EthBr is displaced from its complex with DNA, the fluorescence intensity decreases, which allows quantification of the amount of dendron required to effectively bind DNA and displace EthBr, most usually expressed as the charge excess of dendron relative to DNA required for 50% EthBr displacement (CE_{50}, Table 1). This experiment also allows calculation of the concentration of dendron required for effective DNA binding (C_{50}). In each case in this study, we employed a biologically relevant amount of NaCl (150 mM). In analogy with our previous results, which used Newkome-type[18] dendritic scaffolding (e.g. **G2-amide**), we expected these dendrons to exhibit multivalent binding effects.[5,8,15] It should be noted that the Fréchet–Hult ester-type branching consists of a 1→2 branching motif, whereas the Newkome system has 1→3 branching. Therefore **G2-amide** should be roughly comparable with **G3-ester** in terms of the number of surface ligands (nine versus eight).

Encouragingly, as the number of surface spermine units increased from two (**G1-ester**) to four (**G2-ester**) to eight (**G3-ester**), a smaller charge excess of dendron was required to displace 50% of the EthBr, that is, the CE_{50} value decreased from 41.3 to 2.8 to 0.93 (Table 1, Figure 3).

Table 1: DNA binding data extracted from ethidium bromide (EthBr) displacement assays. Assays were performed using EthBr (2.54 μM) and calf thymus DNA (1.00 μM base pair concentration; the concentration of each negatively charged phosphate is therefore 2.00 μM). CE_{50} represents the charge excess (positive/negative ratio) required to decrease EthBr fluorescence by 50%. C_{50} represents the concentration of dendron required to displace 50% of EthBr. $[Spermine]_{50}$ represents the effective (normalized) concentration of spermine ligating group required to displace 50% of EthBr.

Dendron	CE_{50}	C_{50}[a] [μM]	$[Spermine]_{50}$[b] [μM]
G1-ester	41.3[c]	13.8	27.6
G2-ester	2.8	0.467	1.87
G3-ester	0.93	0.078	0.624
G2-amide	0.76	0.056[d]	0.504

[a] $C_{50} = (CE_{50} \times 2.00\ \mu M)/(\text{number of protonatable amines in the dendron})$. [b] $[Spermine]_{50} = C_{50} \times \text{number of spermine ligands on dendron}$. [c] This value was estimated by linear extrapolation of the data points. [d] This value is higher than that previously reported[5] as the DNA and EthBr concentrations have both been doubled.

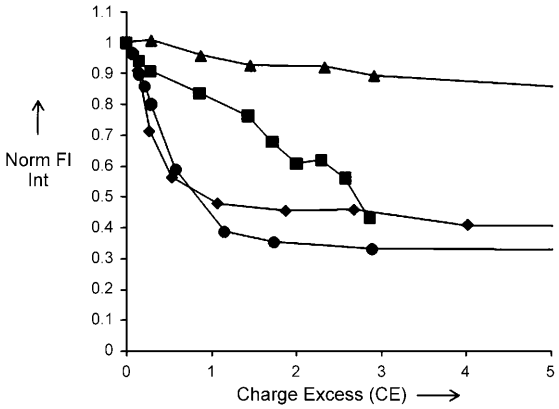

Figure 3. Fluorescence titration of DNA (1 μM per base pair) and EthBr (2.54 μM) with dendrons. The normalized fluorescence intensity decreases as EthBr is displaced from its complex with DNA. ▲ **G1-ester**; ■ **G2-ester**; ◆ **G3-ester**; ● **G2-amide**. Measurements in buffered water at pH 7.4, with 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES; 2 mM), ethylenediaminetetraacetic acid (EDTA; 0.05 mM), and NaCl (150 mM).

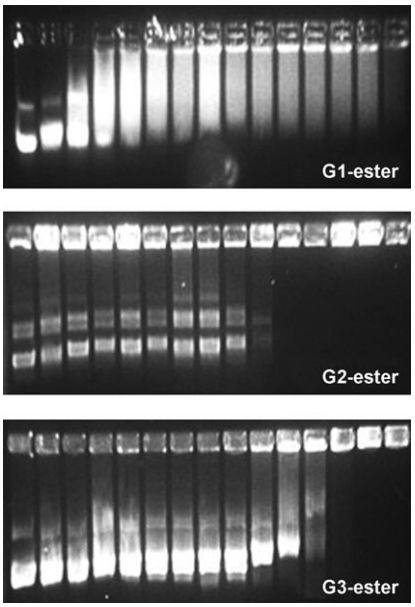

Figure 4. Gel electrophoresis at increasing dendron/DNA ratio (w/w). 1 μg pGL3 DNA used per well. Ratios (polyamine/DNA, w/w): lane 1, 0:1; lane 2, 0.02:1; lane 3, 0.04:1; lane 4, 0.06:1; lane 5, 0.08:1; lane 6, 0.1:1; lane 7, 0.2:1; lane 8, 0.3:1; lane 9, 0.4:1; lane 10, 0.5:1; lane 11, 0.6:1; lane 12, 0.7:1; lane 13, 0.8:1; lane 14, 0.9:1; lane 15, 1:1.

Converting the CE_{50} values into concentrations (C_{50} values) indicated that **G1-ester** binds at micromolar levels (13.8 μM), whereas for **G3-ester**, relatively low nanomolar amounts of the dendron were required (78 nM). Normalizing the data to yield the effective concentration per spermine unit demonstrated a clear multivalency effect on DNA binding, with the largest enhancement on going from **G1-ester** to **G2-ester** (15-fold) with a smaller improvement on going from **G2-ester** to **G3-ester** (3-fold). Importantly, **G3-ester** achieves effective DNA binding with a total spermine loading of just over 0.6 μM—significantly less than the typical millimolar amounts of free spermine present in eukaryotic cells.

Gel electrophoresis was also used to provide complementary insight into DNA binding (Figure 4). However, on this occasion, to obtain good electropherograms, the DNA used was a pGL3 plasmid instead of standard calf thymus DNA. Using this approach, **G1-ester** exhibited much smearing at higher dendron loadings, indicating that, although some DNA binding was taking place, the DNA was not sufficiently well condensed to fully prevent its migration. On the other hand, compounds **G2-ester** and **G3-ester** were both able to prevent migration of the DNA at relatively low loadings. **G2-ester** fully retards DNA mobility in with a polyamine/DNA ratio of 0.6:1 w/w (approximately 0.33 nmol **G2-ester**; Figure 4), whereas **G3-ester** condenses and stops the movement of DNA completely with a ratio of 0.8:1 w/w (approximately 0.22 nmol **G3-ester**; Figure 4). Clearly, these results are in agreement with **G3-ester** being a better DNA binder than **G2-ester** in molar terms. The binding is slightly less effective than that previously detected using **G2-amide** (retardation at 0.5:1 w/w, corresponding to 0.12 nmol).[7b] Again this result is in agreement with the EthBr displacement assay. These studies therefore indicate that, irrespective of the DNA binding assay employed, or the nature of the DNA being bound, these new dendrons bind DNA very well, with **G3-ester** being measurably more effective than **G2-ester**.

We also investigated the ability of the dendrons to condense DNA as visualized by transmission electron microscopy (TEM). Condensed spherical aggregates were formed on mixing higher generation dendrons with calf thymus DNA (see the Supporting Information). These aggregates ranged in size from approximately 100–1000 nm.

We then investigated the stability of these complexes under physiologically relevant conditions. Fréchet and co-workers reported that dendrons with this type of structure can degrade on standing in aqueous solution.[14] In particular, they reported that at pH 7.4, both ester and carbamate hydrolysis occur over a time period of days, whereas at pH 5.0, ester hydrolysis was no longer detected but carbamate hydrolysis could still take place. We therefore wished to determine whether this kind of degradation process could be used to 'switch off' multivalent binding, and convert these multivalent dendritic ligands into smaller, more weakly binding fragments.

We made a 10 μM solution of **G3-ester** in 1,4-piperazine bis(ethanesulfonic acid) buffer (PIPES; pH 7.4) and left it to incubate at 37 °C for the period of the experiment. At time zero, we took a solution of DNA and EthBr and then added sufficient dendron to displace approximately 50% of the EthBr. At each time point (intervals of one day), we made up an equivalent fresh solution of DNA/EthBr, to avoid any potential problems which might arise from any instability of this complex in solution, and then added the same amount of **G3-ester**. In each case, we then monitored the ability of **G3-ester** to bind DNA, as reflected in its ability to displace EthBr and decrease fluorescence (Figure 5). As a control, we employed **G2-amide**, which has similar relative DNA binding affinity to **G3-ester**. We reasoned that, because **G2-amide** is

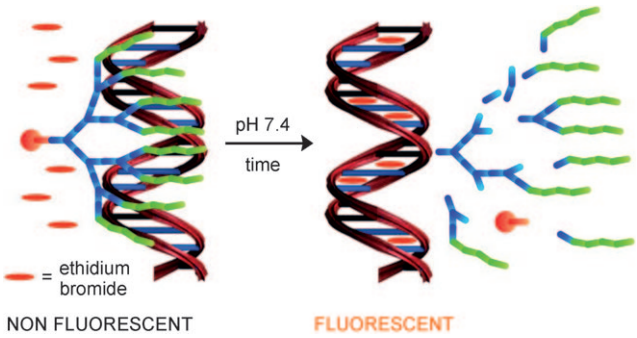

Figure 5. Experiment designed to assay the ability of the dendron to release DNA on degradation. Ethidium bromide is used as a fluorescent probe of the availability of "free" DNA in solution.

composed of ether and amide linkages, it should be stable at pH 7.4. The detected fluorescence intensity was plotted against dendron incubation time (Figure 6). As can clearly be seen, the fluorescence intensity on addition of **G3-ester**

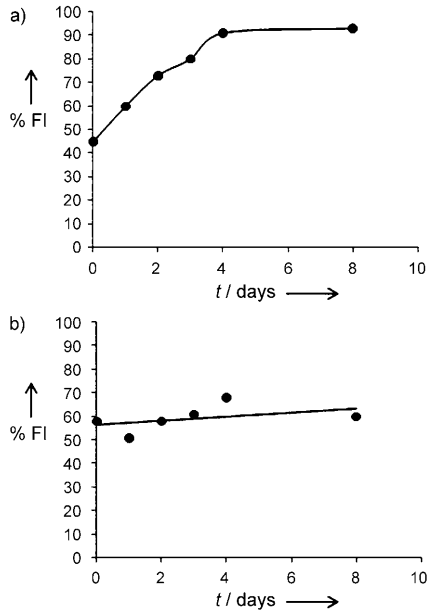

Figure 6. Effect of standing a solution of dendrons in pH 7.4 (PIPES) buffer on the DNA binding ability as assayed by the EthBr displacement assay. Graph (a) shows how **G3-ester** degrades over time, and can no longer bind DNA (i.e., EthBr re-intercalates), whereas graph (b) shows how **G2-amide** retains its strong DNA binding ability over the course of the experiment.

clearly increased over time (Figure 6a), reflecting that after incubation, this ligand was no longer able to effectively displace EthBr from its complex with DNA. In other words, the interaction between **G3-ester** and DNA was diminished. We propose that this weakened interaction is a consequence of the dendron being degraded at pH 7.4 on the time scale of several days, in agreement with Fréchet's previous studies.[14] On degradation of the dendron, the multivalent array of ligands would be converted into smaller (mono-, di-, or tetravalent) fragments. When this fragmentation occurs, we reason that the associated decrease in binding affinity combined with the reversibility of DNA binding increases the "off" rate and decreases the "on" rate for binding, which would lead to the dendron being unable to bind the DNA, which would undergo re-intercalation from the EthBr. In this way, multivalency is "switched off". In contrast, the fluorescence intensity detected on the addition of **G2-amide** varied relatively little over time (within experimental error; Figure 6b). We propose that this demonstrates that this dendron is stable in pH 7.4 buffer, and that the multivalent array of spermine ligands retains its high-affinity DNA binding properties for at least eight days.

We then performed the same experiment at pH 5.0 (2-morpholinoethanesulfonic acid (MES) buffer). Under these conditions with **G3-ester**, only very limited degradation occurred (ca. 5% increase in fluorescence intensity), which was somewhat surprising, as we had expected that, in analogy with the previous data,[14] although the esters are stable under these conditions, the carbamates may hydrolyze, leading to the release of monovalent spermine units. Unlike those in Fréchet's previous studies, however, our surface groups (spermine) have significant amounts of positive charge, as a result of protonation, which, we suggest, may change the pK_b of the C=O group, thus hindering acid-promoted carbamate cleavage. Clearly therefore, the degradation of these dendrons is a pH-dependent process.

In summary, we have demonstrated for the first time that using a chemically degradable dendron framework allows multivalent binding to be switched off, with subsequent loss of affinity for a biological target, in this case DNA. Such degradable systems which exhibit temporary high-affinity binding are of great potential interest, for example, in gene therapy applications. Dendritic polyamines can persist in cells and bind RNA within the cytoplasm after gene delivery.[9] These results offer an approach by which this problem can be avoided. In future work, we intend to optimize the structure of these dendrons to enable effective gene delivery,[19] an approach we have previously successfully employed with our Newkome-type amide–ether dendritic materials.[7] We will also characterize the dendron degradation products more fully, explore the effect of pH, enzymes, and serum on dendron stability, and elucidate the kinetics of the degradation process. In general, we propose that using temporary multivalency effects, in the way outlined in this paper, offers a unique and novel approach through which it is possible to gain the benefits of high-affinity binding to biological targets, with slow degradation of the dendron under biological conditions helping to avoid longer term negative effects of such high-affinity recognition, once the desired bio-intervention is complete.

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[1] a) M. Mammen, S.-K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908 – 2953; *Angew. Chem. Int. Ed.* **1998**, *37*, 2754 – 2794; b) A. Mulder, J. Huskens, D. N. Reinhoudt, *Org. Biomol. Chem.* **2004**, *2*, 3409 – 3424; c) J. D. Badjic, A. Nelson, S. J. Cantrill, W. B. Turnbull, J. F. Stoddart, *Acc. Chem. Res.* **2005**, *38*, 723 – 732.

[2] J. Huskens, A. Mulder, T. Auletta, C. A. Nijhuis, M. J. W. Ludden, D. N. Reinhoudt, *J. Am. Chem. Soc.* **2004**, *126*, 6784 – 6797.

[3] a) C. C. Lee, J. A. MacKay, J. M. J. Fréchet, F. C. Szoka, *Nat. Biotechnol.* **2005**, *23*, 1517 – 1526; b) U. Boas, J. B. Christensen, P. M. H. Heegard, *Dendrimers in Medicine and Biotechnology*, RSC, Cambridge, **2006**; c) S. Svenson, D. A. Tomalia, *Adv. Drug Delivery Rev.* **2005**, *57*, 2106 – 2129; d) D. K. Smith, *Curr. Top. Med. Chem.* **2008**, *8*, 1187 – 1203.

[4] a) Y. M. Chabre, R. Roy, *Curr. Top. Med. Chem.* **2008**, *8*, 1237 – 1285; b) R. J. Pieters, *Med. Res. Rev.* **2007**, *27*, 796 – 816.

[5] M. A. Kostiainen, J. G. Hardy, D. K. Smith, *Angew. Chem.* **2005**, *117*, 2612 – 2615; *Angew. Chem. Int. Ed.* **2005**, *44*, 2556 – 2559.

[6] a) C. W. Tabor, H. Tabor, *Annu. Rev. Biochem.* **1984**, 740 – 790; b) V. Vijayanathan, T. Thomas, A. Shirahata, T. J. Thomas, *Biochemistry* **2001**, *40*, 13644 – 13651; c) K. Igarashi, K. Kashiwagi, *Biochem. Biophys. Res. Commun.* **2000**, *271*, 559 – 564.

[7] a) S. P. Jones, N. P. Gabrielson, D. W. Pack, D. K. Smith, *Chem. Commun.* **2008**, 4700 – 4702; b) J. G. Hardy, M. A. Kostiainen, D. K. Smith, N. P. Gabrielson, D. W. Pack, *Bioconjugate Chem.* **2006**, *17*, 172 – 178; c) J. G. Hardy, C. S. Love, N. P. Gabrielson, D. W. Pack, D. K. Smith, *Org. Biomol. Chem.* **2009**, *7*, 789 – 793.

[8] a) M. A. Kostiainen, G. R. Szilvay, D. K. Smith, M. B. Linder, O. Ikkala, *Angew. Chem.* **2006**, *118*, 3618 – 3622; *Angew. Chem. Int. Ed.* **2006**, *45*, 3538 – 3542; b) M. A. Kostiainen, G. R. Szilvay, J. Lehtinen, D. K. Smith, M. B. Linder, A. Urtti, O. Ikkala, *ACS Nano* **2007**, *1*, 103 – 113.

[9] J.-H. S. Kuo, Y.-L. Lin, *J. Biotechnol.* **2007**, *129*, 383 – 390.

[10] For examples see: a) J. Luten, C. F. van Nostruin, S. C. De Smedt, W. E. Hennink, *J. Controlled Release* **2008**, *126*, 97 – 110; b) J. A. Wolff, D. B. Rozema, *Mol. Ther.* **2008**, *16*, 8 – 15; c) B. Martin, M. Sainlos, A. Aissaoui, N. Oudrhiri, M. Hauchecorne, J.-P. Vigneron, J.-M. Lehn, P. Lehn, *Curr. Pharm. Des.* **2005**, *11*, 375 – 394; d) A. Roosjen, J. Smisterova, C. Driessen, J. T. Anders, A. Wagenaar, D. Hoekstra, R. Hulst, J. B. F. N. Engberts, *Eur. J. Org. Chem.* **2002**, 1271 – 1277; e) J. B. Wong, S. Grosse, A. B. Tabor, S. L. Hart, H. C. Hailes, *Mol. Biosyst.* **2008**, *4*, 532 – 541; f) H. G. Chen, H. Z. Zhang, C. M. McCallum, F. C. Szoka, X. Guo, *J. Med. Chem.* **2007**, *50*, 4269 – 4278.

[11] a) R. J. Amir, N. Pessah, M. Shamis, D. Shabat, *Angew. Chem.* **2003**, *115*, 4632 – 4637; *Angew. Chem. Int. Ed.* **2003**, *42*, 4494 – 4499; b) M. Shamis, H. N. Lode, D. Shabat, *J. Am. Chem. Soc.* **2004**, *126*, 1726 – 1731; c) R. Perry, R. J. Amir, D. Shabat, *New J. Chem.* **2007**, *31*, 1307 – 1312; d) A. Sagi, E. Segal, R. Satchi-Fainaro, D. Shabat, *Bioorg. Med. Chem.* **2007**, *15*, 3720 – 3727; e) E. Sella, D. Shabat, *Chem. Commun.* **2008**, 5701 – 5703.

[12] a) M. L. Szalai, R. M. Kevwitch, D. V. McGrath, *J. Am. Chem. Soc.* **2003**, *125*, 15688 – 15689; b) R. M. Kevwitch, D. V. McGrath, *New J. Chem.* **2007**, *31*, 1332 – 1336.

[13] F. M. H. de Groot, C. Albrecht, R. Koekkoek, P. H. Beusker, H. W. Scheeren, *Angew. Chem.* **2003**, *115*, 4628 – 4632; *Angew. Chem. Int. Ed.* **2003**, *42*, 4490 – 4494.

[14] a) E. R. Gillies, E. Dy, J. M. J. Fréchet, F. C. Szoka, *Mol. Pharm.* **2005**, *2*, 129 – 138; b) A. Almutairi, S. J. Guillaudeu, M. Y. Berezin, S. Achilefu, J. M. J. Fréchet, *J. Am. Chem. Soc.* **2008**, *130*, 444 – 445; c) A. Almutairi, W. J. Akers, M. Y. Berezin, S. Achilefu, J. M. J. Fréchet, *Mol. Pharm.* **2008**, *5*, 1103 – 1110.

[15] M. A. Kostiainen, D. K. Smith, O. Ikkala, *Angew. Chem.* **2007**, *119*, 7744 – 7748; *Angew. Chem. Int. Ed.* **2007**, *46*, 7600 – 7604.

[16] a) H. Ihre, A. Hult, E. Soederlind, *J. Am. Chem. Soc.* **1996**, *118*, 6388 – 6395; b) H. Ihre, A. Hult, J. M. J. Fréchet, I. Gitsov, *Macromolecules* **1998**, *31*, 4061 – 4068; c) H. Ihre, O. L. Padilla de Jesus, J. M. J. Fréchet, *J. Am. Chem. Soc.* **2001**, *123*, 5908 – 5917; d) E. R. Gillies, J. M. J. Fréchet, *J. Am. Chem. Soc.* **2002**, *124*, 14137 – 14146; e) C. C. Lee, E. R. Gillies, M. E. Fox, S. J. Guillaudeu, J. M. J. Fréchet, E. E. Dy, F. C. Szoka, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 16649 – 16654.

[17] a) B. F. Cain, B. C. Baguley, W. A. Denny, *J. Med. Chem.* **1978**, *21*, 658 – 668; b) H. Gershon, R. Ghirlando, S. B. Guttman, A. Minsky, *Biochemistry* **1993**, *32*, 7143 – 7151; c) R. Zadmard, T. Schrader, *Angew. Chem.* **2006**, *118*, 2769 – 2772; *Angew. Chem. Int. Ed.* **2006**, *45*, 2703 – 2706.

[18] a) G. R. Newkome, X. Lin, *Macromolecules* **1991**, *24*, 1443 – 1444; b) J. K. Young, G. R. Baker, G. R. Newkome, K. F. Morris, C. S. Johnson, Jr., *Macromolecules* **1994**, *27*, 3464 – 3471.

[19] In their own right, these dendrons (e.g. **G2-amide**) are unable to effectively transfect cells, primarily owing to inefficient escape from endosomal vesicles. However, we recently reported (Ref. [7a]) that modification of the dendron focal point of **G2-amide** with hydrophobic groups can significantly enhance the gene delivery profile. We are therefore currently in the process of synthetically modifying these new degradable dendrons in an analogous manner, so that their gene delivery characteristics can be fully investigated.

