Gene Therapy

DOI: 10.1002/ange.200502152

A Family of Hierarchically Self-Assembling Linear-Dendritic Hybrid Polymers for Highly Efficient Targeted Gene Delivery**

Kris C. Wood, Steven R. Little, Robert Langer, and Paula T. Hammond**

To fully realize the potential for new medical advances in the postgenomic era, safe and efficient delivery systems for nucleotide-based drugs must be developed.[1] Ideally, such systems will be nontoxic, nonimmunogenic, and made from versatile building blocks that allow optimal delivery to a range of cells or tissues of interest. Herein, for the first time, we present the design, synthesis, and evaluation of a unique family of hierarchically structured linear-dendritic hybrid polymers that self-assemble with DNA to form stable nanoparticles with a series of concentric, functional "shells" that display independently tuneable properties necessary for effective targeted delivery. The resultant ligand-functionalized systems demonstrate receptor-mediated delivery to targeted cells with robust serum stability, transfection efficiencies that exceed the most efficient commercially available polymer poly(ethylenimine) (PEI), and low toxicity at concentrations one to two orders of magnitude higher than those at which PEI is toxic. These systems may find potential utility as targeted in vivo gene delivery systems for DNA- or RNA-based therapies.

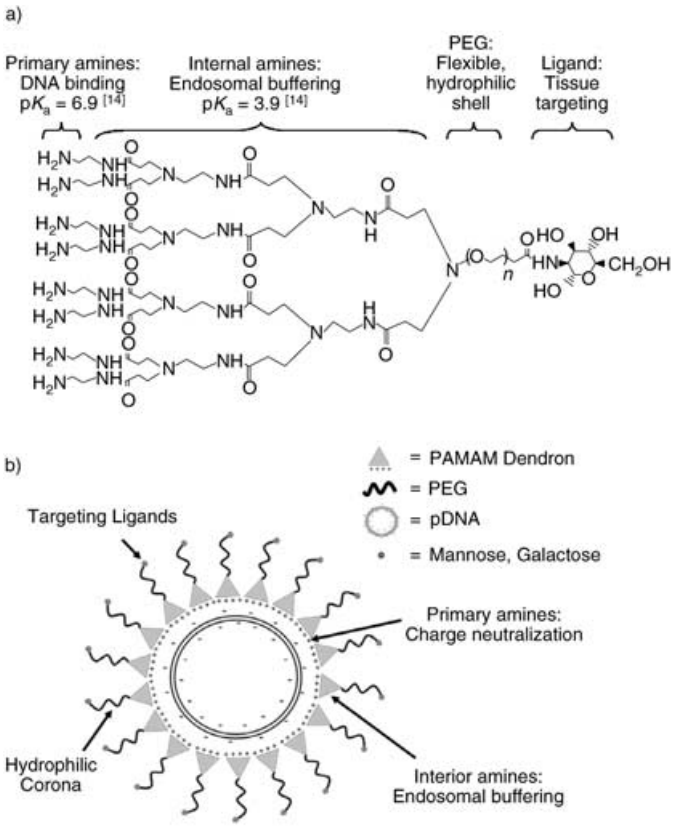
The success of gene-based therapies is dependent upon the ability to deliver genes that express key proteins when and where they are needed. As of yet, no such therapies have been approved for clinical use, primarily because of the lack of versatile, safe, and efficient gene delivery systems.[2,3] A suite of electrical, mechanical, and modified viral delivery systems have been investigated with some success, but these systems suffer from significant drawbacks.[4–7] Notably, modified viruses often elicit severe immunogenicity, are prone to insertional mutagenesis, and are refractory to repeated administrations. Chemical delivery systems, such as cationic linear polymers, dendrimers, or lipid-based reagents, while generally safer than their viral counterparts, typically lack the high efficiency or multiple functionalities required for in vivo administration. Moreover, even subtle synthetic modifications to these systems can dramatically influence existing biological properties.[8–10] Herein, we present a new family of multifunctional gene delivery polymers based on dendritic poly(amidoamine) (PAMAM) and linear poly(ethylene glycol) (PEG) with a wide array of properties (namely, blood stability, cellular targeting, DNA binding, and endosomal buffering capacity) that can be independently tuned in a modular fashion to address each of the barriers to effective gene delivery. As a proof of concept, we demonstrate the ability to independently modulate targeting and expression levels by choice of the ligand and dendrimer species, respectively. Furthermore, these systems represent a platform on which additional functionalities may be added to impart properties such as vector unpackaging and nuclear targeting.[11,12]

Linear-dendritic hybrid polymers were designed based on the hypothesis that these unique polymer architectures, which contain functionalities that are both chemically orthogonal and physically separate, could self-assemble with DNA to yield nanoparticles with an outer shell of targeting ligands accessible to cell-surface receptors, a flexible hydrophilic corona designed to prevent protein opsonization, plasma clearance, and nonspecific uptake, and an interior of amine groups to promote DNA binding and escape from endosomal vesicles into the cytoplasm[13,14] (see Scheme 1 and the Supporting Information). Ligand-functionalized linear-dendritic polymers were synthesized as follows: Fmoc-PEG-NHS

[*] S. R. Little, Prof. R. Langer
Department of Chemical Engineering, Room E25-342
Massachusetts Institute of Technology
Cambridge, MA 02139 (USA)
Fax: (+1) 617-258-8827
E-mail: rlanger@mit.edu
K. C. Wood, Prof. P. T. Hammond
Department of Chemical Engineering, Room 66-546
Massachusetts Institute of Technology
Cambridge, MA 02139 (USA)
Fax: (+1) 617-258-5766
E-mail: hammond@mit.edu

[**] This work was supported by the National Institutes of Health (EB 00244), the Division of Materials Research of the National Science Foundation (DMR 9903380), the Office of Naval Research, and the Mark Hyman, Jr. Professorship in Chemical Engineering at MIT. S.R.L. thanks the National Science Foundation for a graduate research fellowship. We thank Gregory Zugates and Kristoffer Stokes for helpful discussions and Ryan Bennett for assistance with the transmission electron microscopy.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 1. Rational design and hierarchical self-assembly of linear-dendritic polymers with plasmid DNA (pDNA). a) Molecular structure–function relationship in a mannose-PEG-PAMAM G3.0 system. b) Structure of linear-dendritic polyplexes show the relative positions of the functional elements (not to scale).

($M_n = 3500$, $n = 72$, polydispersity index (PDI) = 1.01; Fmoc = 9-fluorenylmethoxycarbonyl, NHS = *N*-hydroxysuccinimide) was dissolved in 0.1 M $NaHCO_3$ buffer (37.5 mg mL^{-1}) and adjusted to pH 8.5 with 1 M NaOH. Amine-modified mannose and galactose ligands were dissolved separately in 0.1 M $NaHCO_3$ buffer (30 mg mL^{-1}), adjusted to pH 8.5, and added to an aliquot of dissolved polymer in solution at a molar excess of 10:1 (24 h, 25 °C, under N_2). The polymers were recovered by filtration and lyophilization, dissolved in dimethylformamide (DMF; 100 mg mL^{-1}), and added dropwise to a solution of 20% piperidine in DMF to remove the Fmoc protecting group (30 min, 25 °C, under N_2). Following this step, the polymers were recovered by precipitation with diethyl ether and dried overnight under vacuum. The dendrimer synthesis proceeded by serial Michael addition and amidation steps by the addition of methyl acrylate and ethylene diamine, respectively, as described previously (see Supporting Information).[15] In general, the ligand-functionalization and deprotection steps proceeded at 80–85%, and all the dendrimer synthetic steps proceeded with conversions of 90–100% (see Supporting Information). The physical properties of these polymers are listed in Table 1. The growth of the amide (3200–3400 cm^{-1}) and carbonyl (1600–1800 cm^{-1}) bands during dendrimer synthesis can be seen qualitatively in Figure 1.

Table 1: Theoretical molecular weights and number of amine end groups for ligand-functionalized PEG–PAMAM hybrid polymers.

Polymer	M_n (theoretical)	Number of amine end groups
ligand-PEG-PAMAM-G0.0	3344	1
ligand-PEG-PAMAM-G1.0	3572	2
ligand-PEG-PAMAM-G2.0	4028	4
ligand-PEG-PAMAM-G3.0	4940	8
ligand-PEG-PAMAM-G4.0	6764	16
ligand-PEG-PAMAM-G5.0	10412	32
ligand-PEG-PAMAM-G6.0	17708	64

An array of techniques was used to probe the biophysical character of polymer/DNA complexes, or "polyplexes" (see Figure 2). Gel electrophoresis demonstrates binding and

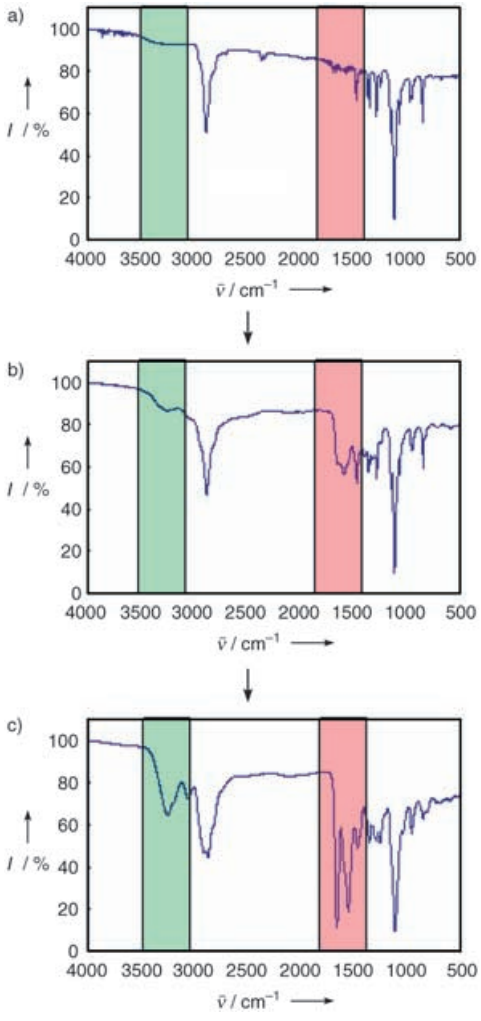


Figure 1. FTIR spectroscopic analysis demonstrates exponential dendron growth in sugar-PEG-PAMAM a) G0.0, b) G2.0, and c) G4.0 systems. Growth of the amide and carbonyl signals are highlighted in green and pink, respectively. (Conversion at the deprotection step was approximately 80%, and conversion at each branching step was 90–100%.) Complete NMR and FTIR spectroscopic analysis is given in the Supporting Information.

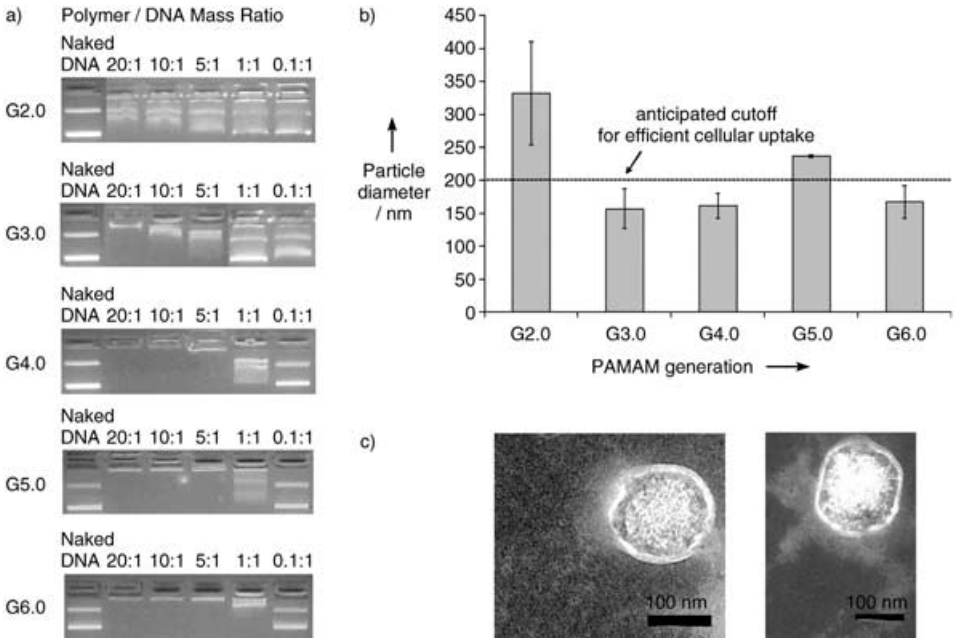


Figure 2. Biophysical characterization of linear-dendritic polyplexes. a) Electrophoresis on 1% agarose gel demonstrates DNA binding at the indicated mass ratios. b) Particle diameter measured with dynamic light scattering (DLS). c) Transmission electron microscopy (TEM) images depict narrowly dispersed, roughly spherical particles.

charge neutralization of DNA by linear-dendritic polymers incubated at mass ratios of >20:1, 10:1, 5:1, 1:1, and 1:1 for generations 2.0 (G2.0), 3.0 (G3.0), 4.0 (G4.0), 5.0 (G5.0), and 6.0 (G6.0), respectively (an acetate buffer of pH 5.1 was used in all cases to ensure complete protonation of the primary amines on the dendrimer periphery). The nature of this trend is consistent with intuition, as the exponentially increasing number of the amine functionalities with increasing dendrimer generation results in higher charge density with increasing dendrimer size. Dynamic light scattering (DLS) suggests that polyplexes of G3.0, G4.0, and G6.0 are around 150 nm in diameter on average, which is under the reported cut-off point of around 200 nm that is required for efficient cellular uptake (Figure 2b).[16] G5.0 polyplexes form larger particles with DNA, a seemingly anomalous result that was nevertheless highly repeatable. The large size of G2.0 polyplexes reflects the fact that little DNA binding and charge neutralization occurred in these systems. In all cases, mass ratios in the range 0.1:1–200:1 were tested, and polyplex size was shown to be relatively insensitive to mass ratio above the point at which complexation occurs in each system, thus suggesting that in all cases the polyplexes consist of a single DNA plasmid and that excess polymers remain dispersed in solution. Thus, particle diameters given in Figure 2b represent average diameters for an evenly weighted range of mass ratios up to 200:1. Finally, transmission electron micrographs of G6.0 polyplexes show narrowly dispersed, roughly spherical complexes with an outer corona of approximately 6–8 nm, which is consistent with the expected size of PEG–PAMAM G6.0 (Figure 2c).[17]

In all of the above cases, complexes were formed prior to assay by incubation of the dilute solutions of the plasmid-DNA-encoding firefly luciferase (6.2 kb, 2.05×10^6 g mol^{-1}, 0.1 mg mL^{-1}, 25 mM acetate buffer, pH 5.1) with equal volumes of solutions that contain polymers (in 25 mM acetate buffer (pH 5.1) at appropriate concentrations to achieve the indicated mass ratios) for 20 min at room temperature.[18]

To evaluate the ability of polyplexes to transfect target cells by receptor-mediated uptake, we transfected two cell types, P388D1 murine macrophages bearing a mannose receptor and HepG2 human hepatocytes bearing an asialoglycoprotein receptor (for galactosylated ligands).[19–22] The transfections were performed in quadruplicate in a 96-well plate format. The polymers and DNA were combined for 20 min at mass ratios in the range 1:1–200:1 (polymer/DNA) in 25 mM acetate buffer, added to a serum-free or 10% serum-containing medium, and incubated with cells for 4 h (587 ng DNA well^{-1}), after which the polyplex-containing medium was removed and replaced with growth medium. The cells were assayed for expression of the luciferase reporter gene after 72 h.[18] Figure 3a shows the levels of luciferase-reporter-gene expression in macrophages (in the absence of serum) with optimized formulations of ligand-functionalized polyplexes, control polyplexes bearing no ligand, ligand-functionalized polyplexes in the presence of excess soluble ligand, and PEI. The G6.0 mannose-bearing polyplexes demonstrate transfection 1.6- to 1.8-fold higher than PEI, the most efficient commercially available polymer for in vitro transfections. The G5.0 polyplexes mediate

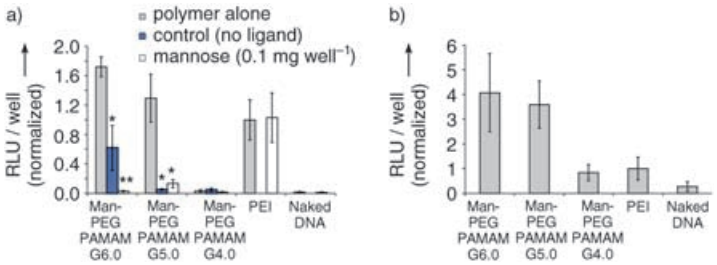


Figure 3. Transfection of P388D1 macrophages bearing the mannose receptor. a) Transfection by linear-dendritic polyplexes with and without the mannose ligand and in the presence of soluble mannose (0.1 mg well^{-1}); * indicates $p<0.04$, ** indicates $p<0.002$ (two-tailed, unpaired Student's T-Test). Results normalized to an optimized formulation of PEI (2:1 PEI/DNA, serum free, no free mannose added). b) Serum stability is demonstrated through transfection in the presence of serum proteins. Results normalized to an optimized formulation of PEI (2:1 PEI/DNA, 10% serum, no free mannose added). All results are given as an average ± standard error.

reporter expression levels approximately 1.3-fold higher than PEI, whereas the G4.0 polyplexes (as well as G3.0 and G2.0, data not shown) transfect at low levels comparable to naked DNA. The highest transfection levels were observed in polymer/DNA ratios under 50:1 in all systems (under 20:1 in G6.0), presumably because of the effects of toxicity at high concentrations. Polyplexes with no mannose ligand exhibited significantly lower transfection efficiencies, and competitive inhibition of mannose receptors by an excess of soluble ligand virtually silenced reporter-gene expression without affecting expression levels in the positive and negative controls (Figure 3a). These data further support the hypothesis of cellular internalization by means of specific receptor-mediated endocytosis. Finally, macrophages were transfected in the presence of a 10% serum-containing medium to probe the serum stability of pegylated polyplexes (Figure 3b). Fourfold transfection enhancements were observed relative to PEI, most likely because of the "stealth" effect imparted by PEG, which is known to lower particle agglomeration by attenuation of opsonization of serum proteins.[23]

Transfection of HepG2 hepatocytes by linear-dendritic polyplexes bearing the galactose ligand is shown in Figure 4. In the absence of serum, optimized formulations of G6.0 and G4.0 ligand-functionalized polyplexes transfect significantly more efficiently ($p < 0.06$) than control polymers with no ligand (Figure 4a). Moreover, G6.0-, G5.0-, and G4.0-targeted systems mediate transfection levels within one order of magnitude of PEI in the absence of serum and as much as eightfold more than PEI in the presence of serum (Figure 4a,b). Optimal polymer/DNA mass ratios were in the range 100:1–200:1 for all the systems studied. Taken together, these data suggest that the hepatocyte-targeted polyplexes are serum-stable and demonstrate enhanced transfection because of a cell-specific receptor-mediated process. Interestingly, expression levels were unaffected by the presence of an excess of soluble galactose, a finding that may arise from the multivalent nature of ligand binding by the asialoglycoprotein receptor; thus, it is suggested that multivalent ligand presentation through synthetic multimeric galactose ligands may yield enhanced targeting relative to the monomeric species.[24,25]

To assess the cellular toxicity of linear-dendritic hybrid-polymer-based systems, a methylthiazolyldiphenyltetrazolium bromide (MTT) assay was performed to measure the relative viability of cells treated with various polymer/DNA mass ratios. Cells were seeded in clear 96-well plates and transfected exactly as previously described. A range of polymer/DNA mass ratios were studied that corresponded to concentrations equal to and above those at which the optimal transfection levels were observed. In P388D1 macrophages (Figure 5a), cells that we have found to be highly sensitive to environmental conditions in culture, no measurable toxicity was observed in the G4.0-based systems over the entire concentration range studied. More significant toxicity was observed at high mass ratios in G5.0 (60–80% viability relative to untreated controls) and the G6.0 systems (50–70%), though these toxicities were primarily observed at concentrations higher than those optimal for transfection. In HepG2 hepatocytes (Figure 5b), no measurable toxicity was observed in the G4.0 and G5.0 systems at polymer/DNA mass ratios up to 200:1; in G6.0, moderate toxicity became apparent at ratios of 150:1 and above. In all cases, linear-dendritic systems failed to display toxicity until concentrations reached one to two orders of magnitude greater than those at which PEI was toxic.

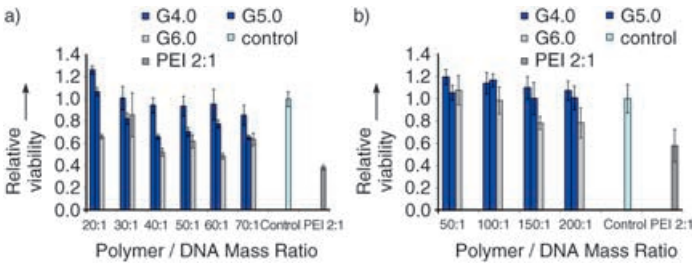


Figure 5. Relative viability of a) P388D1 macrophages and b) HepG2 hepatocytes 72 h after transfection at indicated polymer/DNA mass ratios (control cells untreated). All results are given as an average ± standard error.

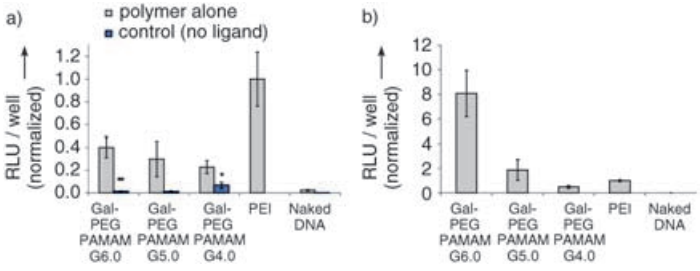


Figure 4. Transfection of HepG2 hepatocytes bearing the asialoglycoprotein receptor. a) Transfection by linear-dendritic polyplexes with and without the galactose ligand; * indicates $p < 0.06$; ** indicates $p < 0.03$ (two-tailed, unpaired Student's T-Test). Results normalized to PEI = 1.0 (serum free, no free galactose added). b) Serum stability is demonstrated through transfection in the presence of serum proteins. Results normalized to PEI = 1.0 (10% serum, no free galactose added). All results are given as an average ± standard error.

Herein, we have described the design, synthesis, and evaluation of a new family of linear-dendritic hybrid polymers for their ability to deliver DNA to two distinct mammalian cell lines. These hierarchically self-assembling polymers have functionalities that are physically and chemically distinct and can be independently modified. The modular nature of these systems makes them a platform from which further, serial modifications may be added to impart additional desired characteristics without any substantial alteration of the existing properties. As a proof of concept, we have demonstrated the condensation of DNA by linear-dendritic polymers into nanoparticle structures with small size and robust serum stability appropriate for systemic delivery. Moreover, through the presentation of an outer shell of targeting ligands, these particles can transfect cells bearing targeted surface receptors with low toxicities and efficiencies that exceed the best commercially available polymer PEI.

Taken together, our data suggest that this new family of materials may find use as safe and highly efficient in vivo delivery agents for DNA, siRNA, or other nucleotide-based therapeutics.

Received: June 21, 2005
Published online: September 20, 2005

Keywords: dendrimers · DNA · gene therapy · self-assembly

[1] I. M. Verma, N. Somia, *Nature* **1997**, *389*, 239.
[2] I. M. Verma, M. D. Weitzman, *Annu. Rev. Biochem.* **2005**, *74*, 711.
[3] E. Wagner, *Pharm. Res.* **2004**, *21*, 8.
[4] E. Neumann, M. Schaefer-Ridder, Y. Wang, P. Hofschneider, *EMBO J.* **1982**, *1*, 841.
[5] S. Mehier-Humbert, R. H. Guy, *Adv. Drug Delivery Rev.* **2005**, *57*, 733.
[6] D. Luo, W. Saltzman, *Nat. Biotechnol.* **2000**, *18*, 33.
[7] D. J. Glover, H. J. Lipps, D. A. Jans, *Nat. Rev. Genet.* **2005**, *6*, 299.
[8] D. Luo, K. Haverstick, N. Belcheva, E. Han, W. M. Saltzman, *Macromolecules* **2002**, *35*, 3456.
[9] D. G. Anderson, A. Akinc, N. Hossain, R. Langer, *Mol. Ther.* **2005**, *11*, 426.
[10] D. G. Anderson, D. M. Lynn, R. Langer, *Angew. Chem.* **2003**, *115*, 3261; *Angew. Chem. Int. Ed.* **2003**, *42*, 3153.
[11] F. M. Munkonge, D. A. Dean, E. Hillery, U. Griesenbach, E. W. Alton, *Adv. Drug Delivery Rev.* **2003**, *55*, 749.
[12] D. V. Schaffer, N. A. Fidelman, N. Dan, D. A. Lauffenburger, *Biotechnol. Bioeng.* **2000**, *67*, 598.
[13] N. D. Sonawane, F. C. Szoka, A. S. Verkman, *J. Biol. Chem.* **2003**, *278*, 44826.
[14] D. A. Tomalia, A. M. Naylor, W. A. Goddard, *Angew. Chem.* **1990**, *102*, 119; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 138.
[15] J. Iyer, K. Fleming, P. T. Hammond, *Macromolecules* **1998**, *31*, 8757.
[16] J. Rejman, V. Oberle, I. S. Zuhorn, D. Hoekstra, *Biochem. J.* **2004**, *377*, 159.
[17] D. A. Tomalia, *Prog. Polym. Sci.* **2005**, *30*, 294.
[18] The experimental methods are described in the Supporting Information.
[19] S. S. Diebold, C. Plank, M. Cotten, E. Wagner, M. Zenke, *In Synthetic DNA Delivery Systems* (Eds.: D. Luo, W. M. Saltzman), Kluwer, New York, **2003**.
[20] B. Hoflack, S. Kornfeld, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 4428.
[21] J. S. Remy, A. Kichler, V. Mordvinov, F. Schuber, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 1744.
[22] R. J. Stockert, *Physiol. Rev.* **1995**, *75*, 591.
[23] J. M. Harris, R. B. Chess, *Nat. Rev. Drug Discovery* **2003**, *2*, 214.
[24] D. T. Connolly, R. R. Townsend, K. Kawaguchi, W. R. Bell, Y. C. Lee, *J. Biol. Chem.* **1982**, *257*, 939.
[25] Y. C. Lee, R. T. Lee, *Acc. Chem. Res.* **1995**, *28*, 321.

