DNA Nanoparticles

DOI: 10.1002/ange.201208556

Use of Single-Site-Functionalized PEG Dendrons To Prepare Gene Vectors that Penetrate Human Mucus Barriers**

*Anthony J. Kim, Nicholas J. Boylan, Jung Soo Suk, Minyoung Hwangbo, Tao Yu, Benjamin S. Schuster, Liudimila Cebotaru, Wojciech G. Lesniak, Joon Seok Oh, Pichet Adstamongkonkul, Ashley Y. Choi, Rangaramanujam M. Kannan, and Justin Hanes**

Protective mucus layers serve as the body's first line of defense at exposed surfaces of the eyes and respiratory, gastrointestinal, and cervicovaginal tracts. These highly viscoelastic and adhesive mucus gels trap most foreign pathogens and environmental ultrafine particles, which are then removed by mucus clearance mechanisms[1] (on the order of seconds to a few hours, depending on anatomical site). However, mucus also immobilizes and rapidly clears therapeutic nanoparticles, including synthetic drug carriers[2] and clinically tested viral[3] and nonviral gene vectors,[4] and therefore, represents a critical obstacle to localized drug and gene delivery at mucosal surfaces for the treatment of a variety of diseases.[1b]

For efficient delivery to mucosal surfaces, gene vectors must be small enough to diffuse through the mucus mesh, and at the same time possess a muco-inert surface to avoid adhesion to mucus constituents.[5] We have previously demonstrated that a dense surface coating of low-molecular-weight (MW) poly(ethylene glycol) (PEG) markedly improved the transport of polymeric nanoparticles through human cervicovaginal mucus (CVM),[5a,6] chronic rhinosinusitis mucus,[7] and cystic fibrosis (CF) sputum,[8] with the latter being typically the most viscous and elastic human mucus secretion. Conventional cationic gene carriers are immobilized in CF sputum, due to their positive charge, by associating with the negatively charged sputum constituents.[9] We recently showed that the only clinically tested polymeric gene carrier, composed of poly-L-lysine conjugated to 10 kDa PEG through a single cysteine residue, $CK_{30}PEG_{10k}$, is trapped by adhesive interactions in CF sputum most likely due to insufficient PEG surface density.[4]

Achieving high PEG surface density, at levels comparable to that of muco-inert particles,[6,8a] is an important step towards developing gene vectors that can penetrate CF sputum. However, it was unclear whether a dense PEG coating could be achieved without markedly altering the stability and/or morphology of cationic polymer-based gene carriers. Conjugation of a high ratio of PEG chains to a cationic polymer reduces the number of positive charges available for, and could also sterically interfere with, DNA compaction.[10] Previous studies have shown that conjugation of PEG chains to cationic polymers resulted in less efficient DNA compaction,[10] larger particle sizes,[11] inferior protection of cargo DNA,[12] and reduced buffering capacity of the gene carriers.[13] We recently found that gene carriers formulated using polyethylenimine (PEI, 25 kDa) conjugated with a high density of PEG (5 kDa, 37:1 PEG/PEI ratio) were extensively trapped in CF sputum, which we attribute to larger particle size and/or incomplete DNA compaction.[10b] We present here a strategy that utilizes the molecular construction of cystamine core poly(amido amine) (PAMAM S-S) dendrimers to prepare densely PEG-coated gene vectors, which can readily penetrate human mucus secretions.

The synthetic strategy was inspired by the unique chemical properties of PAMAM S-S dendrimers, including the high density of primary amino groups on the dendrimer surface and a cleavable disulfide bond in the core.[14] By

[*] Prof. Dr. J. Hanes
Departments of Ophthalmology, Biomedical Engineering, Chemical & Biomolecular Engineering and Oncology
Center for Cancer Nanotechnology Excellence and Center for Nanomedicine
Johns Hopkins University School of Medicine
400 North Broadway, Baltimore, MD 21231 (USA)
E-mail: hanes@jhu.edu

Dr. A. J. Kim,[+] Dr. N. J. Boylan,[+] Dr. J. S. Suk, T. Yu, B. S. Schuster, Dr. L. Cebotaru, Dr. W. G. Lesniak, A. Y. Choi, Prof. Dr. R. M. Kannan, Prof. Dr. J. Hanes
The Center for Nanomedicine, The Wilmer Eye Institute
Johns Hopkins University, Baltimore (USA)

Dr. A. J. Kim,[+] Dr. N. J. Boylan,[+] M. Hwangbo, Prof. Dr. J. Hanes
Department of Chemical & Biomolecular Engineering
Johns Hopkins University, Baltimore (USA)

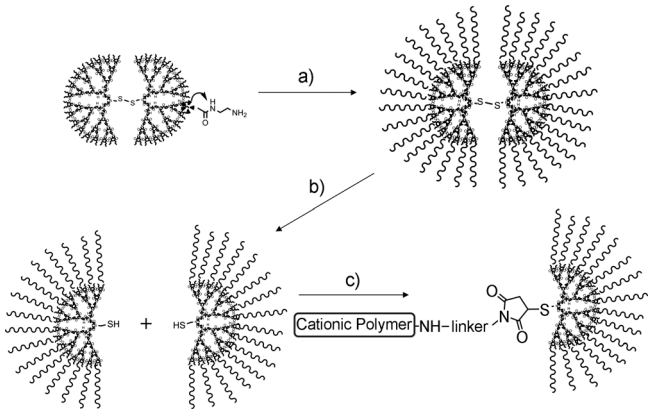
Dr. J. S. Suk, T. Yu, B. S. Schuster, J. S. Oh, P. Adstamongkonkul, Prof. Dr. J. Hanes
Department of Biomedical Engineering
Johns Hopkins University, Baltimore (USA)

[+] These authors contributed equally to this work.

[**] This project was supported by the National Heart, Lung, and Blood Institute (grant numbers P01L51811 and F32L103137 to A.J.K.) and the National Institute of Biomedical Imaging and Bioengineering (grant number R01EB003558). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute, the National Institute of Biomedical Imaging and Bioengineering, or the National Institutes of Health. We thank Dr. Michael Boyle and Meghan Ramsay at the Johns Hopkins Adult Cystic Fibrosis Center for CF sputum collection. We also thank Dr. Himat Patel and Dr. Qingguo Xu for helpful comments.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201208556.

PEGylating the terminal amino groups, cleaving the disulfide bond in the core, and coupling various cationic polymers to the free sulfhydryl group, a variety of precise nanostructures possessing cationic polymer cores conjugated with PEG-modified dendrons can be tailored for gene-delivery applications (Scheme 1; for a complete description of materials and methods, see the Supporting Information).



Scheme 1. Schematic showing preparation of PEG-dendron-conjugated PAMAM and PEI polymers. a) PEGylation step (PEG-NHS or PEG-VS), b) reduction step (TCEP, Tris-(2-carboxyethyl)phosphine), c) conjugation step (SPDP or sulfo-SMCC; PAMAM or PEI).

In the first step, we covalently conjugate 5 kDa PEG-vinyl sulfone (PEG-VS) or 5 kDa PEG-*N*-hydroxysuccinimide (PEG-NHS) onto Generation 2 or 4 PAMAM S-S dendrimers (G2 or G4 PAMAM S-S), respectively. PEG MW was chosen on the basis of our previous finding that coating polystyrene nanoparticles with 5 kDa PEG provided them with mucus-penetrating transport properties.[6b] ^{1}H NMR analysis confirmed that approximately 10 and 52 of the surface primary amino groups of G2 and G4 PAMAM dendrimers (out of 16 and 64, respectively) were conjugated with PEG (Figure S1). Following PEG conjugation and purification steps, the disulfide bond in PAMAM S-S was reduced to produce two single-site, sulfhydryl-functional PEG-dendrons (-SH), which can be subsequently conjugated with other polymers.[14a] Two cationic polymers, G4 PAMAM and branched polyethylenimine (PEI, 25 kDa), were coupled to reduced PEG-dendrons (-SH) by using hetero-bifunctional cross-linkers, succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and sulfosuccinimidyl 4-[*N*-maleimidomethycyclohexane-1-carboxylate (sulfo-SMCC), respectively. The conjugation between the reduced PEG-dendrons (-SH) and cationic polymers was confirmed by Ellman's reagent, which indicated that nearly all the free sulfhydryl groups on the PEG-dendrons (-SH) had reacted with cationic polymers (98 % and 89 % for PAMAM and PEI, respectively). This conjugation was also verified by gel permeation chromatography (GPC) (Figure S2).

Gene vectors were assembled by compaction of plasmid DNA (pBAL, 5.1 kbp) with PEG-dendron-conjugated cationic polymers (dPEG-PAMAM and dPEG-PEI) at varying nitrogen to phosphate (N/P) ratios. We found that PEG-dendron-coated gene vectors assembled in this fashion, dPEG-PAMAM/DNA and dPEG-PEI/DNA, were highly compacted with hydrodynamic diameters comparable to that of uncoated gene vectors (Table 1). Morphological examination by transmission electron microscopy (TEM) revealed that the assembled structures of dPEG-PAMAM/DNA and dPEG-PEI/DNA gene vectors were spherical, similar to the uncoated gene vectors (Figure 1 a,b). As expected, gene vectors assembled using the "conventional" PEG-conjugation method, PEGylated PAMAM/DNA and PEGylated PEI/DNA, showed much larger particle sizes and/or incomplete particle assembly (Figure S3). All PEG-dendron-coated gene vector formulations displayed a near-neutral surface charge (as measured by ζ-potential), whereas uncoated formulations exhibited a highly positive surface charge (Table 1). In ethidium bromide exclusion (Figure S4) and heparin displacement assays (Figure 1 c,d), PEG-dendron-coated and uncoated formulations displayed comparable cargo DNA protection capability, which suggests that dense PEG coatings did not reduce the ability of cationic polymers to efficiently compact the plasmid DNA. Likewise, PEG-dendron-coated gene vector formulations protected the cargo DNA against DNase challenge as efficiently as did the uncoated gene vectors (2 h at 0.5, 1, 2 and 5 IU per μg DNA shown in Figure S5).

Table 1: Characterization and transport of gene vectors in CF sputum.

Gene vector formulation	Hydrodynamic diameter [nm][a]	ζ-potential [mV][b]	MSD_w/ ⟨MSD⟩[c]
PAMAM/DNA	52 ± 1	34 ± 2	9000
dPEG-PAMAM/DNA	73 ± 3	−0.2 ± 0.8	110
PEI/DNA	33 ± 1	32 ± 1	9700
dPEG-PEI/DNA	44 ± 4	6 ± 1	60

[a] Measured by dynamic light scattering. Error values represent the standard error of measurement (SEM) of three independent measurements. [b] Measured in 10 mM NaCl pH 7.1. Error values represent SEM of three independent measurements. [c] MSD_w is the theoretical mean squared displacement of particles in water calculated from the Stokes–Einstein equation and using the relation $MSD = 4D\tau$, at a time scale of $\tau = 1$ s. ⟨MSD⟩ is the ensemble-averaged mean squared displacement of particles in CF sputum measured at a time scale of 1 s. The ratio MSD_w/⟨MSD⟩ indicates by what multiple the average particle transport rate is slowed in CF sputum compared to that in pure water. The larger the ratio, the higher the degree of hindrance to particle motion.

We next used high-resolution multiple-particle tracking[1c,15] (MPT) to quantify the transport rates of individual gene vectors in sputum freshly expectorated by CF patients (for a complete description of materials and methods, see the Supporting Information). To visualize the gene vectors in sputum, coated and uncoated formulations were prepared using fluorescent Cy3 and Cy5-labeled DNA, respectively, and their morphologies were confirmed by TEM (Figure S6). As expected, uncoated gene vectors, PAMAM/DNA and PEI/DNA, were immobilized in CF sputum (Figure 2 a,b). In contrast, PEG-dendron-coated gene vector formulations displayed markedly enhanced transport in the same sputum samples (Figure 2 a,b). The difference in the transport behavior of gene vectors is summarized in the plots of the mean-squared displacement (MSD) versus time scale (Figure 2 c). The ensemble-averaged MSD (⟨MSD⟩) of dPEG-PAMAM/DNA and dPEG-PEI/DNA gene vectors were 75 and 160-

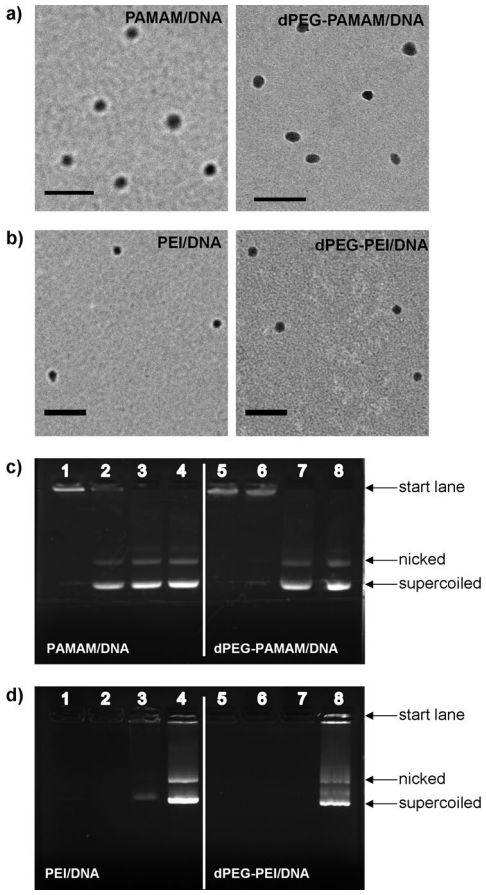


Figure 1. Physicochemical properties of gene vectors. Transmission electron micrographs (TEM) of uncoated and PEG-dendron-coated gene vectors formulated using a) PAMAM and b) PEI. Scale bars: 200 nm. Results of heparin displacement assays demonstrating the DNA compaction stability of c) PAMAM/DNA (lanes 1–4) and dPEG-PAMAM/DNA (lanes 5–8), and d) PEI/DNA (lanes 1–4) and dPEG-PEI/DNA (lanes 5–8). Gene vectors were incubated with increasing amounts of heparin (0, 0.02, 0.2, and 2 IU per μg DNA).

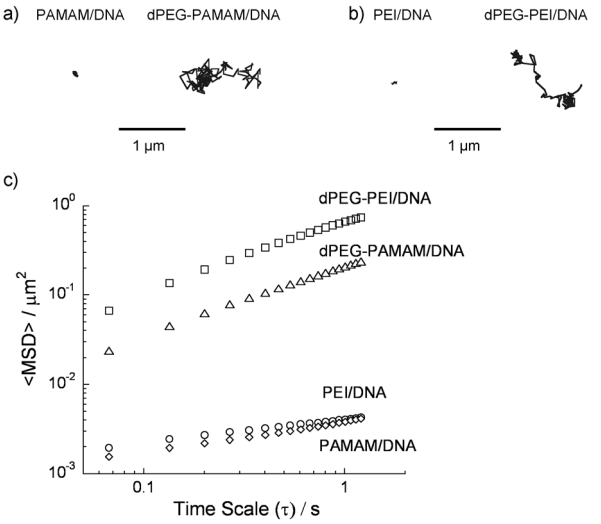


Figure 2. Transport rates of gene vectors in undiluted human airway sputum spontaneously expectorated by CF patients. Representative trajectories of uncoated and PEG-dendron-coated gene vectors formulated using a) PAMAM and b) PEI during 20 s movies. The effective diffusivities (D_{eff}) of individual traces shown are within one standard deviation of the $\langle D_{eff} \rangle$. c) Ensemble-averaged geometric mean squared displacement ($\langle MSD \rangle$) of gene vectors as a function of time scale (τ). Data represent three independent experiments with $n \geq 100$ particles per experiment.

fold greater than that for uncoated gene vectors, respectively, at a time scale of 1 s (Figure 2c). PAMAM/DNA and PEI/DNA gene vectors were slowed 9000- and 9700-fold, respectively, compared to their theoretical MSD in water, also at a time scale of 1 s (Table 1 and Movies S1 and S2). In contrast, dPEG-PAMAM/DNA and dPEG-PEI/DNA gene vectors were slowed only 110- and 60-fold, respectively, compared to their theoretical MSD in water (Table 1 and Movies S3 and S4).

To ensure that the observed rapid transport for PEG-dendron-coated gene vectors was not biased by a small fraction of fast-moving outliers, we examined the distribution of individual particles' MSDs at a time scale of 1 s (Figure S7).[5b,16] A substantial fraction of PEG-dendron-coated gene vectors diffused rapidly in CF sputum. The fastest 70% of dPEG-PAMAM/DNA and dPEG-PEI/DNA gene vectors exhibited uniformly rapid transport, with MSD only approximately 80- and 45-fold slower than that of the same particles in water, respectively. In contrast, the fastest 70% of uncoated gene vectors were slowed 8000-fold or more compared to their theoretical speeds in water.

Based on the particle diameter and the N/P ratio necessary to fully compact plasmid DNA,[17] we estimated PEG surface densities of roughly 0.33 and 0.28 PEG per nm^2 for dPEG-PAMAM/DNA and dPEG-PEI/DNA gene vectors, respectively (Table S1). The estimated PEG densities are roughly 6- to 8-fold higher than that of $CK_{30}PEG_{10k}$ DNA nanoparticles (0.04 PEG per nm^2),[4] which were unable to diffuse through CF sputum, and comparable to those of model muco-inert nanoparticles that rapidly penetrated human CVM and CF sputum.[6,8a] In comparison to muco-adhesive $CK_{30}PEG_{10k}$ DNA nanoparticles, the improved PEG coverage on dPEG-PAMAM/DNA and dPEG-PEI/DNA gene vectors likely provides better protection of the cationic polymeric core from adhesive interactions with anionic and/or hydrophobic sputum constituents. Our results indicate that a critical threshold of PEG surface density exists for polymeric gene carriers, where PEG density in excess of 0.28 PEG per nm^2 may be required to achieve penetration in CF sputum. However, it is likely that the exact threshold of PEG surface coverage required to achieve mucus penetration may depend on the specific system of interest.

We next investigated whether PEG-dendron-coated gene vectors can mediate efficient gene expression of functional proteins in vitro. In human bronchial epithelial (BEAS-2B) cells, dPEG-PAMAM/DNA and dPEG-PEI/DNA gene vectors displayed 2000- and 15000-fold higher luciferase activity compared to the plasmid DNA control, respectively (Figure 3a). However, dPEG-PAMAM/DNA and dPEG-PEI/DNA gene vectors showed lower gene transfection efficiencies than their uncoated counterparts, PAMAM/DNA and PEI/DNA gene vectors, most likely due to the reduced cellular uptake (Figure S8). In cystic fibrosis bronchial

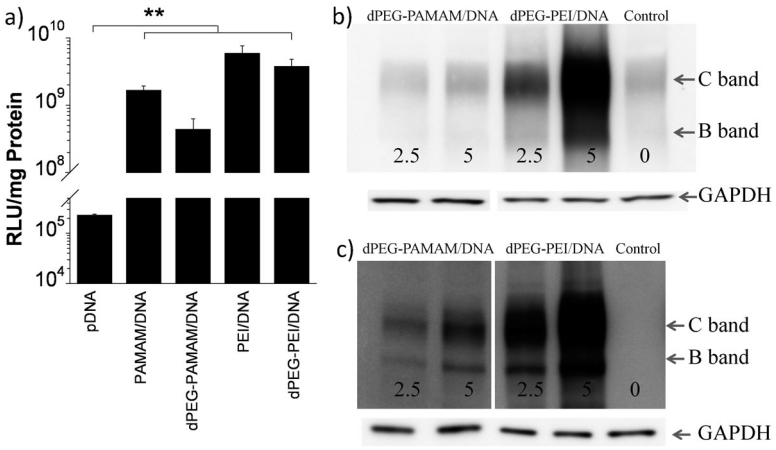


Figure 3. Gene transfer in vitro. a) Luciferase activity in human bronchial epithelial (BEAS-2B) cells. ** denotes statistical significance ($p < 0.01$). RLU, relative light unit. Western blot images showing CFTR protein expressions in b) cystic fibrosis bronchial epithelial (CFBE41o-) cells stably expressing wild-type CFTR and in c) COS7 cells. Numbers on each panel represent dose of gene vectors in μg of plasmid DNA. C and B bands show mature (fully glycosylated) and immature CFTR proteins, respectively.

epithelial (CFBE41o-) cells that stably express wild-type cystic fibrosis transmembrane conductance regulator (CFTR), the level of detectable C bands (fully glycosylated CFTR) was increased following the treatment with gene vectors (Figure 3b). To confirm that the C bands originated from the gene transfer mediated by gene vectors carrying pcDNA 3.1 WT-CFTR plasmid DNA, we also transfected COS7 cells that do not express endogenous CFTR. While no bands were detected in untreated cells, prominent expression of fully glycosylated CFTR was observed following the treatment with gene vectors (Figure 3c).

We have presented a novel synthetic strategy, using single-site-functionalized dendrons, to achieve a dense PEG coating on the surface of cationic polymer-based gene vectors. The resulting carriers could condense DNA into compact nanoparticles that were able to readily penetrate human CF sputum and provide gene transfer in various cell lines. This general scheme enables preparation of precise core–shell nanostructures, each with distinct chemical and physical properties, without compromising DNA compaction and protection capability. In addition to potentially treating CF lung airway disease, this simple design principle may facilitate the development of treatments for various mucosal diseases in the respiratory, gastrointestinal, and female reproductive tracts.

Received: October 24, 2012
Published online: March 4, 2013

Keywords: cystic fibrosis · DNA nanoparticles · gene therapy · mucus-penetrating particles · nanotechnology

[1] a) R. A. Cone, *Adv. Drug Delivery Rev.* **2009**, *61*, 75; b) M. R. Knowles, R. C. Boucher, *J. Clin. Invest.* **2002**, *109*, 571; c) S. K. Lai, Y. Y. Wang, J. Hanes, *Adv. Drug Delivery Rev.* **2009**, *61*, 158.

[2] a) A. S. Hoffman, *J. Controlled Release* **2008**, *132*, 153; b) A. Kumari, S. K. Yadav, S. C. Yadav, *Colloids Surf. B* **2010**, *75*, 1; c) F. Mohamed, C. F. van der Walle, *J. Pharm. Sci.* **2008**, *97*, 71; d) D. Putnam, *Nat. Mater.* **2006**, *5*, 439.

[3] K. Hida, S. K. Lai, J. S. Suk, S. Y. Won, M. P. Boyle, J. Hanes, *PLoS One* **2011**, *6*, e19919.

[4] a) J. S. Suk, N. J. Boylan, K. Trehan, B. C. Tang, C. S. Schneider, J. M. Lin, M. P. Boyle, P. L. Zeitlin, S. K. Lai, M. J. Cooper, J. Hanes, *Mol. Ther.* **2011**, *19*, 1981; b) N. J. Boylan, J. S. Suk, S. K. Lai, R. Jelinek, M. P. Boyle, M. J. Cooper, J. Hanes, *J. Controlled Release* **2011**, *0*, 0.

[5] a) S. K. Lai, Y. Y. Wang, K. Hida, R. Cone, J. Hanes, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 598; b) B. C. Tang, M. Dawson, S. K. Lai, Y. Y. Wang, J. S. Suk, M. Yang, P. Zeitlin, M. P. Boyle, J. Fu, J. Hanes, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 19268.

[6] a) S. K. Lai, D. E. O'Hanlon, S. Harrold, S. T. Man, Y. Y. Wang, R. Cone, J. Hanes, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 1482; b) Y. Y. Wang, S. K. Lai, J. S. Suk, A. Pace, R. Cone, J. Hanes, *Angew. Chem.* **2008**, *120*, 9872; *Angew. Chem. Int. Ed.* **2008**, *47*, 9726.

[7] S. K. Lai, J. S. Suk, A. Pace, Y. Y. Wang, M. Yang, O. Mert, J. Chen, J. Kim, J. Hanes, *Biomaterials* **2011**, *32*, 6285.

[8] a) J. S. Suk, S. K. Lai, Y. Y. Wang, L. M. Ensign, P. L. Zeitlin, M. P. Boyle, J. Hanes, *Biomaterials* **2009**, *30*, 2591; b) J. S. Suk, S. K. Lai, N. J. Boylan, M. R. Dawson, M. P. Boyle, J. Hanes, *Nanomedicine* **2011**, *6*, 365.

[9] S. Ferrari, C. Kitson, R. Farley, R. Steel, C. Marriott, D. A. Parkins, M. Scarpa, B. Wainwright, M. J. Evans, W. H. Colledge, D. M. Geddes, E. W. Alton, *Gene Ther.* **2001**, *8*, 1380.

[10] a) M. Neu, D. Fischer, T. Kissel, *J. Gene Med.* **2005**, *7*, 992; b) J. S. Suk, A. J. Kim, K. Trehan, C. S. Schneider, L. Cebotaru, O. M. Woodward, N. J. Boylan, M. P. Boyle, S. K. Lai, W. B. Guggino, J. Hanes, unpublished results.

[11] H. Petersen, P. M. Fechner, A. L. Martin, K. Kunath, S. Stolnik, C. J. Roberts, D. Fischer, M. C. Davies, T. Kissel, *Bioconjugate Chem.* **2002**, *13*, 845.

[12] A. Kichler, M. Chillon, C. Leborgne, O. Danos, B. Frisch, *J. Controlled Release* **2002**, *81*, 379.

[13] A. Beyerle, O. Merkel, T. Stoeger, T. Kissel, *Toxicol. Appl. Pharmacol.* **2010**, *242*, 146.

[14] a) C. R. DeMattei, B. H. Huang, D. A. Tomalia, *Nano Lett.* **2004**, *4*, 771; b) M. Berna, D. Dalzoppo, G. Pasut, M. Manunta, L. Izzo, A. T. Jones, R. Duncan, F. M. Veronese, *Biomacromolecules* **2006**, *7*, 146; c) R. Qi, Y. Gao, Y. Tang, R. R. He, T. L. Liu, Y. He, S. Sun, B. Y. Li, Y. B. Li, G. Liu, *Aaps J.* **2009**, *11*, 395; d) G. R. Newkome, K. K. Kotta, A. Mishra, C. N. Moorefield, *Macromolecules* **2004**, *37*, 8262; e) A. M. Caminade, R. Laurent, J. P. Majoral, *Adv. Drug Delivery Rev.* **2005**, *57*, 2130; f) M. Hayder, M. Poupot, M. Baron, D. Nigon, C. O. Turrin, A. M. Caminade, J. P. Majoral, R. A. Eisenberg, J. J. Fournie, A. Cantagrel, R. Poupot, J. L. Davignon, *Sci. Transl. Med.* **2011**, *3*, 81ra35.

[15] J. Suh, M. Dawson, J. Hanes, *Adv. Drug Delivery Rev.* **2005**, *57*, 63.

[16] M. Yang, S. K. Lai, Y. Y. Wang, W. Zhong, C. Happe, M. Zhang, J. Fu, J. Hanes, *Angew. Chem.* **2011**, *123*, 2645; *Angew. Chem. Int. Ed.* **2011**, *50*, 2597.

[17] a) E. Dauty, J. S. Remy, T. Blessing, J. P. Behr, *J. Am. Chem. Soc.* **2001**, *123*, 9227; b) T. Blessing, J. S. Remy, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1427.