Gene Delivery

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Virion-Mimicking Nanocapsules from pH-Controlled Hierarchical Self-Assembly for Gene Delivery**

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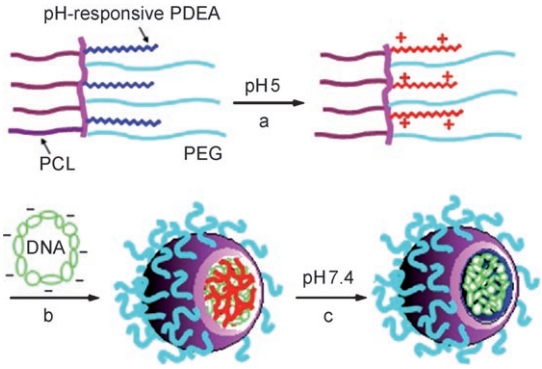
Polymer gene delivery is considered a promising alternative to viral vectors[1,2] owing to its safety and the ready availability of a variety of polymer carriers such as polyethyleneimine (PEI)[3–5] and poly(β-aminoester)s,[6] whose structures may be optimized by using high-throughput methods.[7] Nanoparticles with surface-coated ligands[8] or magnetic properties,[9] layered structures,[10] linear-dendritic polymers,[11] triblock copolymers,[12] polyion micelles,[13] surface-charge-shielded PEI polyplexes,[14] dendrimers,[15] and inorganic nanotubes[16] have also been tested as carriers.

In polymer-mediated gene delivery, cationic polymers generally complex plasmids to compact them into nanoparticles and to shield their negative charges for effective cellular internalization.[1] Tight packing is also needed for DNA trafficking to the nucleus and prevention from enzymatic degradation.[17] However, this tight complexation is one of the main barriers to efficient DNA transcription because the complexed DNA is inaccessible to the transcription machine.[18] Facilitated dissociation of the complexes using short,[19] reversibly cross-linked,[20] degradable,[4,21] or low-charge-density cationic chains[22] or charge-reversible amphiphiles[23] could significantly enhance transgenic efficiency.

We hypothesized that nanocarriers that could release free DNA into the nucleus would possess high transfection efficiency. Herein, we present the design, synthesis, and evaluation of a unique virion-mimicking polymer/DNA nanocapsule carrying free DNA fabricated from pH-controlled hierarchical self-assembly of an A/B/C-type terpolymer poly(ε-caprolactone)/poly[2-(*N*,*N*-diethyl)aminoethyl methacrylate]/poly(ethylene glycol) (PCL/PDEA/PEG) brush and DNA (Scheme 1). The pH-responsive PDEA chains were used to pack DNA into the nanocapsule at an acidic pH value and to tightly bind DNA once in lysosomes to protect it from degradation; the chains then dissociate from the DNA at a neutral pH value. The hydrophobic PCL chains were used to form the nanocapsule through their hydrophobic–hydrophobic interactions, while their degradation in the acidic lysosome was used to disintegrate the nanocapsule to release the free DNA. The PEG chains were used to impart stealth properties (see below) to the nanocapsules. The nanocapsules were very efficient in gene transfection once internalized, and were also promising for in vivo cancer gene therapy.



Scheme 1. Nanocapsule formation by a pH-controlled hierarchical self-assembly of PCL/PDEA/PEG and DNA. The PDEA chains are positively charged owing to protonation at pH 5 (a) and then complex with DNA to form a hydrophilic core. The hydrophobic PCL chains collapse and form a membrane surrounding the core; the hydrophilic PEG chains are incompatible with the hydrophobic PCL layer and thus extend into the aqueous solution as a PEG outer layer (b). After the solution pH is raised to 7.4, the PDEA chains are deprotonated, become insoluble, and are thus dissociated from the DNA, leaving free DNA in the core (c).

The terpolymer brush PCL/PDEA/PEG was synthesized by copolymerization of the macromonomers PCL (M_n 3 KDa), PEG (M_n 2.1 KDa), and PDEA (M_n 2.5 KDa). Its molecular weight was 10.8 KDa with a polydispersity of 1.8 (see the Supporting Information). PDEA is a pH-sensitive polymer[24] that is insoluble in water at pH values above 6.5 but becomes soluble by protonation and carries cationic charges at pH values less than 6.[25] The copolymer brush

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formed micelles with a critical micelle concentration of $4\,mg\,L^{-1}$ at pH 7.2 and $15.8\,mg\,L^{-1}$ at pH 5. The micelles had a diameter of about 82 nm at pH 7.4 and 88 nm at pH 5. At pH 5, they formed stable complexes with pEGFP DNA when the N/P ratio (defined as the ratio of nitrogen atoms of the polymer to phosphate units of the plasmid DNA) was greater than 2 (Figure S1 in the Supporting Information). The TEM images confirm that at this pH the DNA was tightly packed into small particles (Figure 1a). The particles became

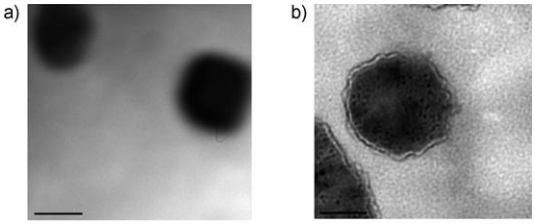

Figure 1. High-resolution TEM images of PCL/PDEA/PEG/DNA complexes at pH 5.0 (a) and pH 7.2 (b); N/P = 10; scale bars: 50 nm.

less tightly packed at pH 7.2 and had a membrane surrounding the core (Figure 1b). At pH 7.2, the particles in solution were about 200 nm in diameter depending on the N/P ratio. The particles became neutrally charged at an N/P ratio of 10 (Figure S3 in the Supporting Information). The neutral particles slightly aggregate, and thus the size measured by laser light scattering was larger, and the PDI of the particles was about 0.5 at an N/P ratio of 10. The neutral complexes, however, are especially suitable for in vivo gene transfection.[26] The polymer exhibited very low toxicity (Figure S4 in the Supporting Information).

The state of the DNA in the complexes, free or complexed, was probed by an ethidium bromide (EB) exclusion assay using fluorescence microscopy.[27] EB is positively charged and can bind free DNA plasmids to form complexes having strong fluorescence, but it cannot bind the plasmids that are already complexed with cationic polymers. Free EB has weak fluorescence,[27] and its fluorescence is not pH-dependent in the range of pH 5–7.2.[28] The particles with EB were invisible from the fluorescence channel at pH 5 but very bright at pH 7.2 (Figure 2). This observation indicates that the DNA was complexed with positively charged PDEA at pH 5 but was free or only very weakly associated with the uncharged PDEA at pH 7.2.

The presence of a hydrophobic membrane surrounding the DNA core was observed using confocal laser scanning microscopy. The complexes were prepared from the terpolymer brush and DNA in the presence of the hydrophobic dye PKH26. Under the fluorescence channel, a bright layer surrounding a core without any fluorescence was clearly visible (Figure 3). As a control, the terpolymer brush alone was observed to assemble into spherical micelles with a hydrophobic core (Figure S5 in the Supporting Information). This result is consistent with the TEM result that at pH 7.2 the particle had a polymeric membrane surrounding the DNA core. This hydrophobic membrane consisted of the PCL chains and the PDEA chains dissociated from the plasmids

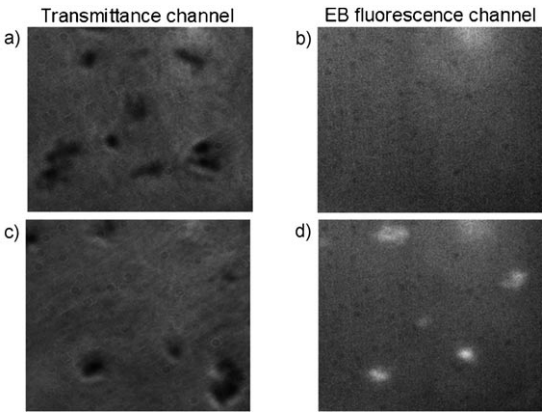

Figure 2. Ethidium bromide exclusion assay of the DNA in the PCL/PDEA/PEG/DNA complexes at pH 5.0 (a,b) and pH 7.2 (c,d) observed from the transmittance (a,c) and EB fluorescence (b,d) channels using fluorescence microscopy; N/P = 10. Original magnification is 400×.

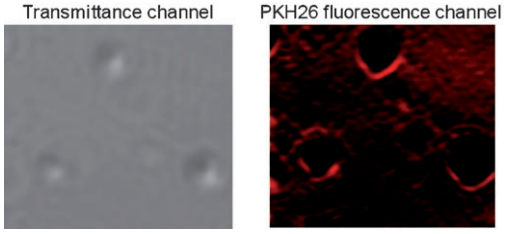

Figure 3. Confocal microscopy images of PCL/PDEA/PEG/DNA nanocapsules (N/P = 10) at pH 7.2 labeled with PKH26. Original magnification is 2016×.

after the solution pH was adjusted to 7.2 (Scheme 1). The tightness of the hydrophobic membrane was probed by using EB. When EB was added to the nanocapsule solution at pH 7.2, it took about 20 min for the fluorescence of EB/DNA to reach its maximum, while the fluorescence of the free DNA with EB in solution reached the maximum immediately (Figure S6 in the Supporting Information). This observation suggests that cationic hydrophilic EB could only slowly diffuse and cross the hydrophobic membrane, and therefore, the membrane must be continuous and tight. Thus, the as-prepared PCL/PDEA/PEG/DNA complexes are indeed nanocapsules with free DNA in the core, a tight hydrophobic middle layer, and a PEG outer layer. The hydrophobic middle layer membrane protects the free plasmids from degradation and keeps the nanocapsule integrated to hold the negatively charged DNA after it dissociates from the PDEA at pH 7.2.

The cellular uptake of the nanocapsules was measured by flow cytometry and compared with that of the polyplexes of PEI (branched, $M = 25$ KDa), a standard for polymer gene transfection. Figure 4 shows that the nanocapsules enter the cells much more slowly than the PEI polyplexes. This result was further confirmed by confocal microscopy (Figure S7 in the Supporting Information). PEI/DNA polyplexes are positively charged and thus are easily taken up by cells through electrostatically adsorptive endocytosis.[5,29] The nanocapsules are neutral and have a PEG outer layer, as was designed for

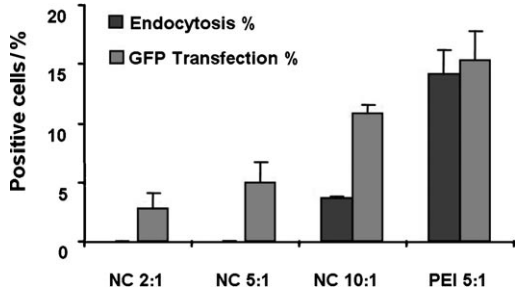

Figure 4. Cellular internalization and transfection of the nanocapsules (NC) at varied N/P ratios and the PEI/DNA polyplexes at an N/P ratio of 5:1 measured by flow cytometry. For the cellular-internalization measurements, the plasmids were labeled with Cy3, and the nanocapsules or PEI polyplexes were cultured with SKOV-3 ovarian cancer cells at a DNA concentration of 2 μg mL^{-1} at 37 °C for 1 h. For gene transfection, the cells were transfected for 48 h, and 10000 cells were counted per sample. The reporter gene was pEGFP. The corresponding blank polymer controls were used as the background in the measurements.

providing in vivo "stealth properties" by minimizing the nanocapsule–cell interaction, and thus exhibit a slowed endocytosis.[30]

The subsequent lysosomal and nuclear colocalization was observed by confocal microscopy using a multifluorescence-labeling method (Figure 5).[31] After 4 h culture, most nanocapsules were localized in lysosomes (yellow spots in Figure 5d), but some nanocapsules were already associated with the nuclei (pink dots; see Figure S8 in the Supporting Information for an enlargement of Figure 5d).

The in vitro gene transfection of the nanocapsules to SKOV-3 cancer cells was tested using the pEGFP gene as a reporter gene. The gene transfection efficiency was measured in terms of the percentage of cells expressing GFP. The data were plotted together with the cellular uptake results in Figure 4 for a direct comparison. Nanocapsules at an N/P ratio of 10 were most efficient in transfection. About 11% of the cells transfected with the nanocapsules expressed GFP. In contrast, the cells transfected with the PEI polyplexes under optimal transfection conditions had only 15% GFP-positive cells despite a cellular uptake rate of about four times that of the nanocapsules. Similar results were found in gene transfection to neonatal mouse cardiomyocytes using pALDH2 as a reporter gene and western immunoblotting assay as a quantitation method (Figures S9 and S10 in the Supporting Information). Thus, it can be concluded that once internalized, the nanocapsules are more efficient in gene transcription than the PEI polyplexes.

In vivo gene transfection was also conducted to evaluate the nanocapsules as an in vivo gene carrier. Athymic mice were xenografted with subcutaneous tumors using human SKOV-3 ovarian cancer cells (Figure 6). The nanocapsule solution in PBS buffer (15 μg DNA) was injected into the tail vein of the mice. After 48 h, the GFP expressions in the tumor and several key organs were observed using fluorescence microscopy. Under the GFP–fluorescence channel, the tumor transfected with the nanocapsules had many very bright zones as shown in Figure 6c (marked with a dotted line; see also Figure S11 in the Supporting Information), thus suggesting that the nanocapsules extravasate from the tumor's porous blood vessels as a result of the enhanced permeation and retention effect,[32] enter the cells, and efficiently express GFP. There was no sign of GFP in the muscle (Figure S13 in the Supporting Information) and little expression in the liver (Figure 6d), which is the major organ that sequestrates PEGylated neutral particles.[33] Thus, the nanocapsules are promising as in vivo cancer gene transfection carriers.

The intracellular release of free DNA is proposed as the reason for the high transfection efficiency of the nanocapsules

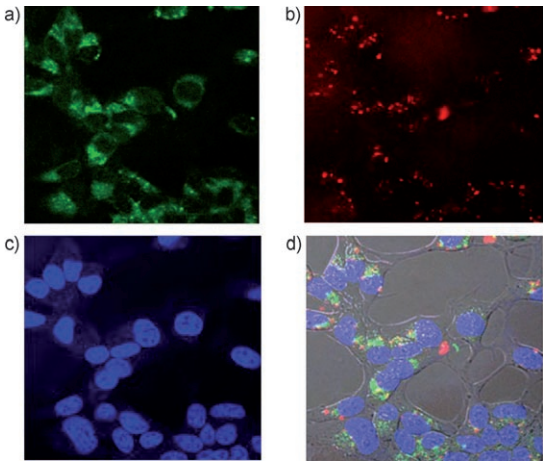

Figure 5. Subcellular colocalization of PCL/PDEA/PEG/DNA nanocapsules labeled with PKH26 after 4 h incubation with SKOV-3 cells observed by confocal microscopy from a) LysoTracker channel, b) PKH26 channel, c) nuclear dye Draq5 channel. d) Overlap of the transmittance channel image with (a–c); see Figure S8 in the Supporting Information for a larger image. Original magnification is 63×.

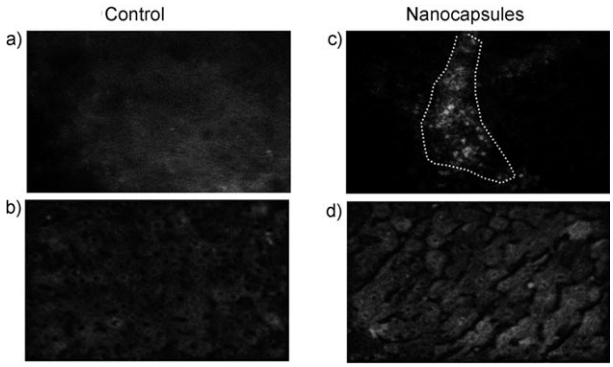

Figure 6. In vivo GFP gene expression in tissues of athymic mice inoculated with SKOV-3 ovarian tumors and transfected with the nanocapsules. Control mouse (a,b) and transfected mouse (c,d) injected with the nanocapsule solution containing 15 μg pEGFP plasmid through the tail vein. After 48 h, tissues were isolated. Representative images of tumor (a,c) and liver (b,d) were taken using fluorescence microscopy. Original magnification is 100×. See Figure S11 in the Supporting Information for a larger image of (c), Figure S12 in the Supporting Information for the hematoxylin and eosin staining image of the tumor, and Figure S13 in the Supporting Information for the corresponding images of muscle.

in the cells. The hydrophobic interaction among the PCL chains in the tight hydrophobic membrane layer acts as physical cross-linkers to keep the nanocapsules integrated and hold negatively charged DNA in the core at neutral pH. After the nanocapsules are internalized and transferred to lysosomes (Figure 5), the nanocapsules are exposed to a pH of about 4–5.[34] Discher et al. showed that PCL in PCL–PEG worm micelles or polymersomes degrade much faster than their bulk materials, particularly at low pH values (e.g., at pH 5).[35] Thus, the thin PCL layer of the nanocapsules quickly hydrolyzes in lysosomes, thereby weakening its hydrophobic–hydrophobic interactions. In lysosomes, the PDEA is positively charged and thus binds the DNA tightly (Scheme 1). Therefore, the DNA is not released into the lysosome even with hydrolysis of the PCL membrane, and is thereby still protected. Protonation of the PDEA chains also induces the influx of chloride ions and subsequent osmotic swelling/lysis of the lysosomes (proton-sponge effect[36]), thus releasing the nanocapsules into the cytosol. Once in the cytosol or in the nucleus, the PDEA is deprotonated and dissociated from the DNA. The degraded PCL membrane can not hold the free DNA any more owing to its strong electrostatic repulsion, thereby releasing the free DNA.

In conclusion, we demonstrate a novel concept for fabricating nanocarriers carrying free DNA for gene delivery. The pH-controlled hierarchical self-assembly of a pH-responsive terpolymer brush and DNA can form virion-like nanocapsules. The plasmids in free form are encapsulated in the core of the nanocapsule, which is surrounded by a tight hydrophobic membrane that acts like the capsid of a virion protecting the DNA from degradation and keeping the nanocapsule integrated after the plasmids and PDEA dissociate at neutral pH. The fluffy PEG outer layer and the neutral surface provide the nanocapsules with "stealth properties". The in vitro and in vivo transfection experiments indicate that the nanocapsules are very efficient in gene transfection once internalized and are very promising for in vivo cancer gene therapy. A detailed intracellular-trafficking study of the nanocapsules, optimization of the polymer properties such as the degradation rate of the hydrolyzable chains, and functionalization of the carrier to enhance its cellular uptake and further increase the transfection efficiency are currently underway.

Experimental Section

Polymer synthesis: Preparation of the PDEA macromonomer and its copolymerization with the macromonomers of PCL and PEG to synthesize the PCL/PDEA/PEG terpolymer brush by radical copolymerization are given in the Supporting Information.

Nanocapsule preparation: Plasmids (2 μg) in 25 mM sodium acetic acid buffer at pH 5.0 (50 μL) were added to a gently vortexing solution of the terpolymer (50 μL in a pH 5.0 buffer; the polymer concentration was adjusted to yield a designated N/P ratio, defined as the ratio of nitrogen atoms of the polymer to phosphate units of the plasmid DNA). The mixture was incubated at room temperature for 30 min for complex formation. The solution pH was adjusted to 7.2 by using 1.5 M Tris buffer. PEI/DNA polyplexes were prepared at pH 7.4 at an N/P ratio of 5 without adjusting the pH.

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[1] a) H. C. Kang, M. Lee, Y. H. Bae in *Nanotechnology in Therapeutics* (Eds.: N. A. Peppas, J. Z. Hilt, J. B. Thomas), Horizon Bioscience, Wymondham, UK, **2007**, p. 131; b) E. Wagner, J. Kloeckner, *Adv. Polym. Sci.* **2006**, *192*, 135; c) D. Putnam, *Nat. Mater.* **2006**, *5*, 439; d) T. G. Park, J. H. Jeong, S. W. Kim, *Adv. Drug Delivery Rev.* **2006**, *58*, 467; e) D. W. Pack, A. S. Hoffman, S. Pun, P. S. Stayton, *Nat. Rev. Drug Discovery* **2005**, *4*, 581.

[2] R. Haag, F. Kratz, *Angew. Chem.* **2006**, *118*, 1218; *Angew. Chem. Int. Ed.* **2006**, *45*, 1198.

[3] a) M. Neu, D. Fischer, T. Kissel, *J. Gene Med.* **2005**, *7*, 992; b) Y. Liu, D.-C. Wu, W.-D. Zhang, X. Jiang, C.-B. He, T. S. Chung, S. H. Goh, K. W. Leong, *Angew. Chem.* **2005**, *117*, 4860; *Angew. Chem. Int. Ed.* **2005**, *44*, 4782.

[4] M. L. Forrest, J. T. Koerber, D. W. Pack, *Bioconjugate Chem.* **2003**, *14*, 934.

[5] W. T. Godbey, K. K. Wu, A. G. Mikos, *J. Controlled Release* **1999**, *60*, 149.

[6] a) G. T. Zugates, D. G. Anderson, S. R. Little, I. E. B. Lawhorn, R. Langer, *J. Am. Chem. Soc.* **2006**, *128*, 12726; b) D. M. Lynn, D. G. Anderson, A. Akinc, R. Langer in *Polymeric Gene Delivery* (Ed.: M. M. Amiji), CRC, Boca Raton, **2005**, p. 227; c) J. J. Green, J. Shi, E. Chiu, E. S. Leshchiner, R. Langer, D. G. Anderson, *Bioconjugate Chem.* **2006**, *17*, 1162.

[7] a) A. Akinc, D. M. Lynn, D. G. Anderson, R. Langer, *J. Am. Chem. Soc.* **2003**, *125*, 5316; b) D. G. Anderson, D. M. Lynn, R. Langer, *Angew. Chem.* **2003**, *115*, 3261; *Angew. Chem. Int. Ed.* **2003**, *42*, 3153; c) D. G. Anderson, W. Peng, A. Akinc, N. Hossain, A. Kohn, R. Padera, R. Langer, J. A. Sawicki, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16028; d) D. M. Lynn, D. G. Anderson, D. Putnam, R. Langer, *J. Am. Chem. Soc.* **2001**, *123*, 8155.

[8] a) J. J. Green, E. Chiu, E. S. Leshchiner, J. Shi, R. Langer, D. G. Anderson, *Nano Lett.* **2007**, *7*, 874; b) G. Zuber, L. Zammut-Italiano, E. Dauty, J.-P. Behr, *Angew. Chem.* **2003**, *115*, 2770; *Angew. Chem. Int. Ed.* **2003**, *42*, 2666.

[9] T.-J. Yoon, J. S. Kim, B. G. Kim, K. N. Yu, M.-H. Cho, J.-K. Lee, *Angew. Chem.* **2005**, *117*, 1092; *Angew. Chem. Int. Ed.* **2005**, *44*, 1068.

[10] M. A. W. Eaton, T. S. Baker, C. F. Catterall, K. Crook, G. S. Macaulay, B. Mason, T. J. Norman, D. Parker, J. J. B. Perry, R. J. Taylor, A. Turner, A. N. Weir, *Angew. Chem.* **2000**, *112*, 4229; *Angew. Chem. Int. Ed.* **2000**, *39*, 4063.

[11] K. C. Wood, S. R. Little, R. Langer, P. T. Hammond, *Angew. Chem.* **2005**, *117*, 6862; *Angew. Chem. Int. Ed.* **2005**, *44*, 6704.

[12] a) S. Fukushima, K. Miyata, N. Nishiyama, N. Kanayama, Y. Yamasaki, K. Kataoka, *J. Am. Chem. Soc.* **2005**, *127*, 2810; b) M. Oishi, K. Kataoka, Y. Nagasaki, *Bioconjugate Chem.* **2006**, *17*, 677.

[13] M. Oishi, F. Nagatsugi, S. Sasaki, Y. Nagasaki, K. Kataoka, *ChemBioChem* **2005**, *6*, 718.

[14] L. Y. Qiu, Y. H. Bae, *Biomaterials* **2007**, *28*, 4132.

[15] D. Joester, M. Losson, R. Pugin, H. Heinzelmann, E. Walter, H. P. Merkle, F. Diederich, *Angew. Chem.* **2003**, *115*, 1524; *Angew. Chem. Int. Ed.* **2003**, *42*, 1486.

[16] N. W. S. Kam, Z. Liu, H. Dai, *Angew. Chem.* **2006**, *118*, 591; *Angew. Chem. Int. Ed.* **2006**, *45*, 577.
[17] a) H. Pollard, J.-S. Remy, G. Loussouarn, S. Demolombe, J.-P. Behr, D. Escande, *J. Biol. Chem.* **1998**, *273*, 7507; b) C. Arigita, N. J. Zuidam, D. J. A. Crommelin, W. E. Hennink, *Pharm. Res.* **1999**, *16*, 1534.
[18] a) D. V. Schaffer, N. A. Fidelman, N. Dan, D. A. Lauffenburger, *Biotechnol. Bioeng.* **2000**, *67*, 598; b) A. U. Bielinska, J. F. Kukowska-Latallo, J. R. Baker, Jr., *Biochim. Biophys. Acta* **1997**, *1353*, 180.
[19] S. Choosakoonkriang, B. A. Lobo, G. S. Koe, J. G. Koe, C. R. Middaugh, *J. Pharm. Sci.* **2003**, *92*, 1710.
[20] C. Pichon, E. LeCam, B. Guerin, D. Coulaud, E. Delain, P. Midoux, *Bioconjugate Chem.* **2002**, *13*, 76.
[21] M. Bikram, C.-H. Ahn, S. Y. Chae, M. Lee, J. W. Yockman, S. W. Kim, *Macromolecules* **2004**, *37*, 1903.
[22] a) M. L. Forrest, G. E. Meister, J. T. Koerber, D. W. Pack, *Pharm. Res.* **2004**, *21*, 365; b) N. P. Gabrielson, D. W. Pack, *Biomacromolecules* **2006**, *7*, 2427.
[23] C. A. H. Prata, Y. Zhao, P. Barthelemy, Y. Li, D. Luo, T. J. McIntosh, S. J. Lee, M. W. Grinstaff, *J. Am. Chem. Soc.* **2004**, *126*, 12196.
[24] S. Liu, S. P. Armes, *Angew. Chem.* **2002**, *114*, 1471; *Angew. Chem. Int. Ed.* **2002**, *41*, 1413.
[25] P. Xu, E. A. VanKirk, W. J. Murdoch, Y. Zhan, D. D. Isaak, M. Radosz, Y. Shen, *Biomacromolecules* **2006**, *7*, 829.
[26] A. Prokop, E. Kozlov, W. Moore, J. M. Davidson, *J. Pharm. Sci.* **2002**, *91*, 67.
[27] a) M. X. Tang, F. C. Szoka, *Gene Ther.* **1997**, *4*, 823; b) M. L. Read, T. Bettinger, D. Oupicky in *Nonviral Vectors for Gene Therapy: Methods and Protocols* (Ed.: M. A. Findeis), Humana, New Jersey, **2001**, p. 131.
[28] F. Pohl, T. M. Jovin, W. Baehr, J. J. Holbrook, *Proc. Natl. Acad. Sci. USA* **1972**, *69*, 3805.
[29] O. Harush-Frenkel, N. Debotton, S. Benita, Y. Altschuler, *Biochem. Biophys. Res. Commun.* **2007**, *353*, 26.
[30] F. De. Jaeghere, E. Allemann, J. K. Feijen, T. , E. Doelker, R. Gurny, *J. Drug Targeting* **2000**, *8*, 143.
[31] R. Savic, L. Luo, A. Eisenberg, D. Maysinger, *Science* **2003**, *300*, 615.
[32] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, *J. Controlled Release* **2000**, *65*, 271.
[33] Q. le Masne de Chermont, C. Chaneac, J. Seguin, F. Pelle, S. Maitrejean, J.-P. Jolivet, D. Gourier, M. Bessodes, D. Scherman, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 9266.
[34] A. Barret, M. Heath, *Lysosomes: A laboratory handbook*, 2nd ed., North-Holland, New York, **1977**.
[35] a) Y. Geng, D. E. Discher, *Polymer* **2006**, *47*, 2519; b) F. Ahmed, R. I. Pakunlu, G. Srinivas, A. Brannan, F. Bates, M. L. Klein, T. Minko, D. E. Discher, *Mol. Pharm.* **2006**, *3*, 340.
[36] N. D. Sonawane, F. C. Szoka, Jr., A. S. Verkman, *J. Biol. Chem.* **2003**, *278*, 44826.

