Bionanotechnology

DOI: 10.1002/anie.200603387

Transition of Cationic Dipeptide Nanotubes into Vesicles and Oligonucleotide Delivery**

*Xuehai Yan, Qiang He, Kewei Wang, Li Duan, Yue Cui, and Junbai Li**

The self-assembly of biocompatible molecules, such as peptides and proteins, into regular supramolecular structures has important implications. In a sense these molecules mimic biological systems.[1,2] Additionally, such biomimetic systems are becoming important for the delivery of drugs, genes, and proteins. Peptide-based nanostructures have attracted considerable attention owing to their biocompatibility, capability of molecular recognition, and well-defined structures.[3–10] For example, Görbitz first found that simple dipeptides can self-assemble into nanotubes, and that the resulting structures have chiral hydrophilic channels with a Van der Waals diameter of up to 10 Å.[10a] Reches and Gazit showed that very short dipeptides with the β-amyloid diphenylalanine structural motif associated with Alzheimer's disease can form long, stiff nanotubes by self-assembly, and that these nanotubes can be used as scaffolds to produce discrete silver nanowires.[6] Subsequently, Song et al. simplified the synthesis of the dipeptide nanotubes (DPNTs) and observed that DPNTs turned into vesicles when the DPNT dispersion was diluted by adding water.[8] Zhang and co-workers also reported that linear surfactant-like oligopeptides self-assembled simultaneously into nanotubes and vesicles.[7,9] Such peptide nanotubes (PNTs) can be used for intracellular-delivery applications. Herein, we report that cationic dipeptides (H-Phe-Phe-NH_2·HCl; see Figure 1) self-assemble into nanotubes at physiological pH values, and that these cationic dipeptide nanotubes (CDPNTs) can also rearrange to form vesicles upon dilution. Moreover, the CDPNTs can traverse cell membranes and be absorbed by the cells upon spontaneous conversion into vesicles. We utilized this property to deliver oligonucleotides into the interior of cells as an indication of the potential applications of the system in gene and drug delivery.

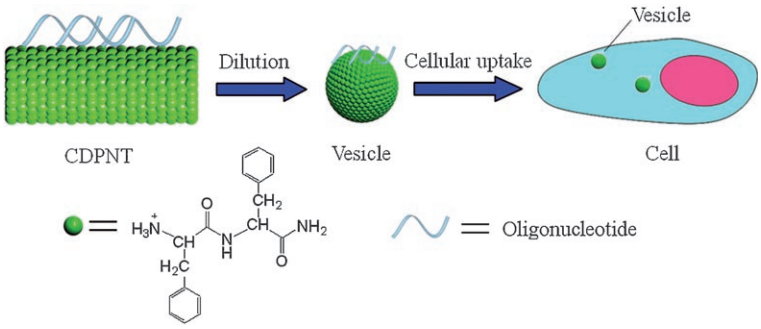

Figure 1. Proposed transition of the CDPNTs into vesicles for oligonucleotide delivery.

We selected cationic dipeptides to fabricate nanotubes so that negatively charged nucleic acids could be bound to the tubes and then delivered into cells. The CDPNTs were self-assembled at physiological pH values (see experimental section in the Supporting Information). A scanning electron microscopy (SEM) image showed that the nanotubes are quite homogeneous with lengths of tens of micrometers (Figure 2a). The inset in Figure 2a shows a typical tubular structure at a larger magnitude. A transmission electron

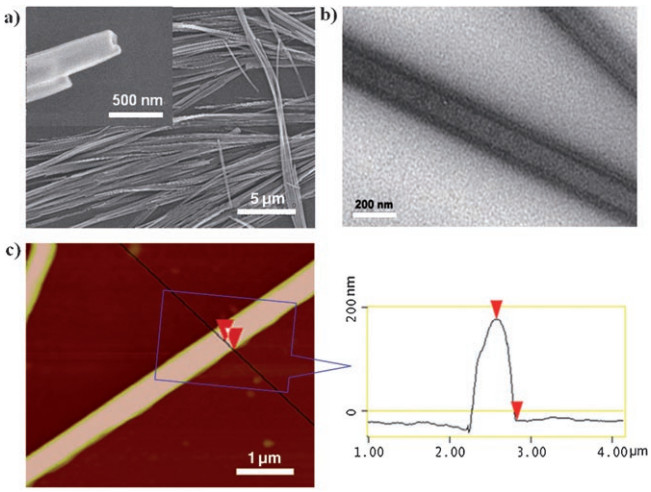

Figure 2. a) SEM image of the CDPNTs (inset: a hollow nanotube at greater magnification); b) TEM image of the CDPNTs; c) height AFM image (area: 5 μm × 5 μm) of the CDPNTs; the cross-sectional profile of a nanotube shows its height of approximately 190 nm.

[*] X. Yan, Dr. Q. He, K. Wang, L. Duan, Dr. Y. Cui, Prof. Dr. J. Li
Beijing National Laboratory for Molecular Sciences (BNLMS)
International Joint Laboratory
Key Laboratory of Colloid and Interface Sciences
Institute of Chemistry, Chinese Academy of Sciences
Beijing 100080 (P. R. China)
Fax: (+86) 10-8261-2629
E-mail: jbli@iccas.ac.cn
Dr. Q. He
Max Planck Institute of Colloids and Interfaces
14476 Golm/Potsdam (Germany)

[**] This work was supported financially by the National Natural Science Foundation of China (Nos. 20574077, 20471063, 20403022, and 90206035) and the Chinese Academy of Sciences, as well as the German Max Planck Society collaboration project. The authors acknowledge Hans Riegler for critical reading of the manuscript.

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

microscopy (TEM) image confirmed that tubes had formed with a diameter of approximately 200 nm (Figure 2b). To obtain direct topographical information, the samples were deposited on a mica surface and investigated by atomic force microscopy (AFM; Figure 2c). The height of the nanotube shown is about 190 nm, as estimated from its cross-sectional profile. All of these results confirm that the cationic dipeptide can self-assemble into tubes at pH 7.2.

We find that the CDPNTs transform gradually into vesicles when the CDPNT dispersion is diluted under neutral conditions. As observed by optical microscopy, the CDPNTs disappear with time, and the number of vesicles increases simultaneously (Figure 3a). To verify the structures of the vesicles (the black dots in Figure 3a), the solution was diluted

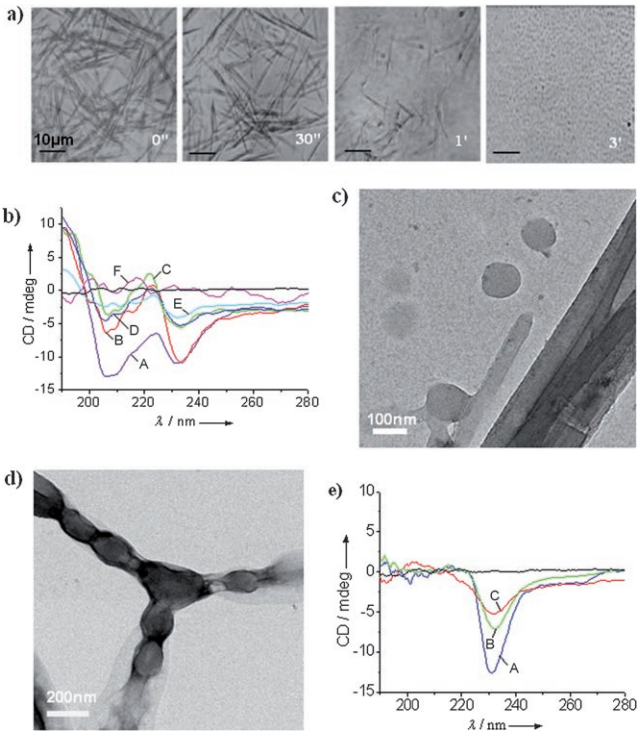

Figure 3. a) Observation through an optical microscope of the conversion of the CDPNTs (as a dispersion in PBS at pH 7.2) into peptide vesicles; b) CD spectra of the CDPNTs and vesicles at different concentrations (A: 10, B: 8, C: 7, D: 5, E: 2, F: 1 mg mL^{-1}); c),d) TEM images of negatively stained CDPNTs at a concentration of 7 and 5 mg mL^{-1}, respectively; e) CD spectra of zwitterionic DPNTs at different concentrations (A: 10, B: 5, C: 2 mg mL^{-1}).

further with pure water to a final concentration of 1 mg mL^{-1}. These diluted samples were then characterized by negative-staining TEM and AFM (see the Supporting Information). The TEM image showed that peptide vesicles are formed after dilution of the nanotube dispersion. Analysis by AFM confirmed the three-dimensional, spherical shape of the nanostructures. The cross-sectional profile of the vesicles showed that the peptide vesicles are approximately 100 nm in height, which is consistent with the TEM analysis. All of the above results confirm that the CDPNTs can convert spontaneously into vesicles when diluted at physiological pH values.

To further clarify this transition process, circular dichroism (CD) spectroscopy was used to determine changes in the secondary structure during the conversion of the self-assembled CDPNTs into vesicles. CD spectra of the CDPNTs and vesicles at different concentrations show that the CDPNT-to-vesicle transition causes a change in the secondary structure of the peptide aggregations (Figure 3b). The observed CD curve of the assembled CDPNT (10 mg mL^{-1}) has some similarities with the CD signature of α-helical polypeptides, and the observed extrema may be indicative of transitions found in α-helical peptides. The maximum at 192 nm and the minimum at 206 nm correspond to the α-helical $\pi\rightarrow\pi^*$ transitions. Another absorption peak at 232 nm may correspond to $n\rightarrow\pi^*$ transitions of helical arrangements of the PNTs.[11,12] The CD signal reflects the interactions between the three-dimensional aromatic stacking arrangement and the hydrogen-bonded cylinders of peptide main chains.[13] When the CDPNT dispersion was diluted to a concentration of 7 mg mL^{-1}, the minimum peaks for the β sheets of the peptide nanostructures were observed at 206 nm and 212 nm.[14,15] When the concentration was decreased further, these peaks disappeared gradually. At 1 mg mL^{-1}, the secondary structure of CDPNT vanished.

Although the CD spectra of the self-assembled structures have not yet been assigned completely, they demonstrate that dilution indeed results in a change in the peptide nanostructures. Furthermore, TEM images show that at a concentration of 8 mg mL^{-1}, the system maintains a tubular structure (see the Supporting Information). However, when the solution is diluted to 7 mg mL^{-1}, vesicles start to form and coexist with the tubular structures (Figure 3c). The ratio of peptide vesicles to nanotubes becomes larger upon a further decrease in the concentration to 5 mg mL−1. The intermediate state of the conversion of CDPNTs into vesicles can be observed directly (Figure 3d). When the concentration of the system was decreased to 1 mg mL^{-1}, the transition is complete, and the system is composed entirely of vesicles. (see the Supporting Information). The transition from tube to vesicle occurs at a concentration of approximately 7 mg mL^{-1}, which is consistent with the CD results.

For comparison, we chose a zwitterionic dipeptide, diphenylalanine (L-Phe-L-Phe), for similar experiments under the same conditions. We found that this dipeptide could self-assemble into nanotubes at a concentration of 10 mg mL^{-1} (see the Supporting Information). This self-assembly is similar to results reported previously.[6] A clear Cotton effect is observed at 232 nm in the CD spectra of these zwitterionic DPNTs at a concentration of 10 mg mL^{-1} (Figure 3e). Upon a decrease in the concentration of these zwitterionic DPNTs, the intensity of the CD signal at 232 nm decreases dramatically. When the concentration of the system is decreased to 2 mg mL^{-1}, no new peak appears. This result indicates that within this concentration range the zwitterionic dipeptide forms mainly tubular structures rather than vesicles, unlike the cationic dipeptide, for which the transition from nanotubes to vesicles can be observed at a concentration of 7 mg mL^{-1}.

To test the CDPNTs for applications in intracellular delivery, negatively charged single-stranded DNA (ssDNA)

labeled with 5-((5-aminopentyl)thioureidyl)fluorescein was bound to the CDPNTs through electrostatic interactions. Figure 4a shows the green fluorescent CDPNTs detected by confocal laser scanning microscopy (CLSM). The CDPNTs are stable after binding with ssDNA, and the nanotube

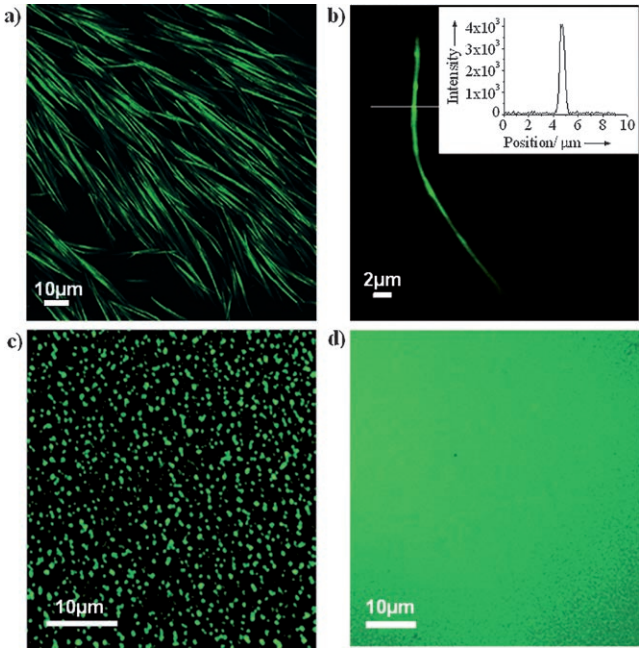

Figure 4. CLSM images of a) the CDPNTs with bound fluorescently labeled ssDNA; b) a selected single nanotube, which was used for the intensity measurements (inset: intensity profile of a fluorescent CDPNT); c) peptide vesicles from the reassembly of the peptides derived from the CDPNTs with bound fluorescently labeled ssDNA; d) a solution of fluorescently labeled ssDNA.

structure can be still observed after four weeks (see the Supporting Information). Analysis of the fluorescence-intensity distribution on an individual nanotube (Figure 4b) shows that the fluorescence remains mainly on the sidewalls of the nanotubes (see inset in Figure 4b). Furthermore, peptide vesicles formed from CDPNTs retain the green fluorescence (the green dots in Figure 4c, in contrast with ssDNA alone in Figure 4d), which indicates that the ssDNA is localized on the peptide vesicles after the conversion of the CDPNTs. We also investigated the ability of CDPNTs to inhibit the migration in gel electrophoresis of ssDNA. When the volume ratio of CDPNTs to ssDNA was changed to 3:1 (see the Supporting Information), the migration of ssDNA in gel electrophoresis was inhibited. This observation indicates that ssDNA can be immobilized securely on the cationic dipeptide nanostructures through electrostatic interactions. In contrast, zwitterionic DPNTs and cationic dipeptide molecules in solution without aggregation have no effect on the migration of ssDNA in gel electrophoresis at different volume ratios (see the Supporting Information), which indicates that ssDNA does not bind to zwitterionic DPNTs and free cationic dipeptides. These results confirm that ssDNA can be bound to the cationic dipeptide nanostructures through charge interactions, which are very important for the immobilization on the nanostructures and intracellular release of DNA.[16,17]

The dispersion of the CDPNT/ssDNA complexes was dialyzed for 2 h with constant stirring in phosphate buffer saline (PBS) to eliminate cytotoxic hexafluoro-2-propanol. We then studied the interaction of the resulting complexes with HeLa cells. CDPNTs labeled with Congo red (CR-CDPNTs) were used for the control experiment of cellular uptake. It was found that Congo red does not influence the tubular structure of the cationic dipeptide (see the Supporting Information). Both TEM and confocal-microscopy images confirmed the stability of CDPNTs in the presence of Congo red. The HeLa cells were incubated with CR-CDPNTs and CDPNTs/ssDNA (c(CDPNTs) $\approx 0.5\ mg\,mL^{-1}$) in a cell-growth medium with 5% CO_2 at 37°C for 24 h. The medium containing CR-CDPNTs or the complexes was then removed, the cells were rinsed gently twice, and a fresh culture medium was added. Subsequently, the cells were imaged directly in glass-bottomed Petri dishes by CLSM. CLSM scans normal to the dish plane revealed that fluorescence originated mainly from the interior of the cells (Figure 5; see also the Supporting Information). This result indicates that the CR-CDPNTs and CDPNT/ssDNA enter the cells by converting into vesicles and accumulate in the cytoplasma of the cells. No significant cell death was observed when HeLa cells were incubated under the conditions described above. As negative-control experiments, cells were incubated with a solution that contained zwitterionic

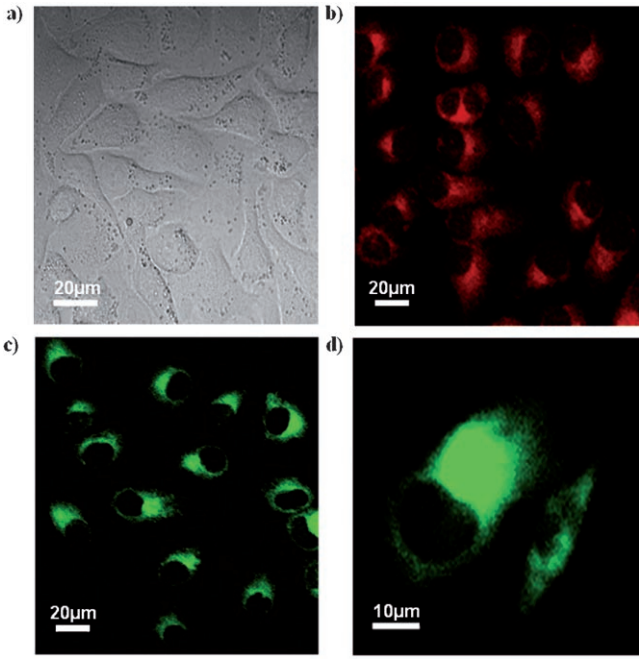

Figure 5. Uptake of the CR-CDPNTs and CDPNTs/ssDNA by HeLa cells: a) bright-field image of cells; b) fluorescence image of positive-control cells after incubation for 24 h with the CDPNTs labeled with Congo red; c),d) fluorescence images of cells after incubation for 24 h with the complexes of CDPNTs and fluorescently labeled ssDNA; the cell nuclei (dark circular regions) appear free of fluorescence; the complexes of peptide vesicles/ssDNA are accumulated within the cytoplasm of the cell.

dipeptide or only fluorescently labeled ssDNA. No fluorescence of the cells was detected by CLSM (see the Supporting Information), which means that the CDPNTs can traverse cell membranes by converting spontaneously into vesicles, and that they can transport ssDNA into the cells.

The self-assembly of cationic dipeptides is driven by hydrogen bonding and by π–π stacking interactions, as described by Reches and Gazit for the self-assembly of aromatic dipeptides into nanotubes.[6] Zhang and co-workers pointed out previously that the self-assembly and disassembly processes of linear surfactant-like peptide nanostructures are dynamic over time.[7a] On the basis of molecular simulations of the D-Phe-D-Phe dipeptide, Song et al. suggested that the concentration of the peptide is key to the self-assembly process of PNTs.[8] The conversion of CDPNTs into vesicles also appears to be a dynamic process. When the concentration of the CDPNTs is decreased, their disassembly occurs probably as a result of electrostatic repulsion rather than hydrogen bonding or π–π stacking interactions between peptide molecules. As observed under the optical microscope, vesicles of relatively incompact stacking are formed rapidly by peptide reassembly. Modeling and simulation studies of this process are still required for further understanding. As for the mechanism of cellular uptake of the CDPNTs, we propose that their intracellular internalization occurs by endocytosis of the reassembled vesicles (Figure 1), which is a well-known mechanism for many species.[18–22]

In summary, self-assembled positively charged DPNTs underwent spontaneous conversion into vesicles upon dispersion dilution. CDPNTs with fluorescently labeled ssDNA bound electrostatically could enter cells readily, most likely after conversion into vesicles with attached ssDNA. The fluorescently labeled ssDNA accumulates in the cytoplasm of the cells after internalization. Thus, because of their biocompatibility, bioabsorption, and recyclability, it may be possible to exploit the peptide nanostructures as a new class of molecular transporter for the delivery of a wide range of foreign substances, such as drugs, genes, and proteins.

Received: August 18, 2006
Published online: February 28, 2007

Keywords: cells · oligonucleotides · peptide nanotubes · self-assembly · vesicles

[1] a) S. Fernandez-Lopez, H. S. Kim, E. C. Choi, M. Delgado, J. R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D. A. Weinberger, K. M. Wilcoxen, M. R. Ghadiri, *Nature* **2001**, *412*, 452–455; b) J. S. Quesada, M. P. Isler, M. R. Ghadiri, *J. Am. Chem. Soc.* **2002**, *124*, 10004–10005; c) S. Zhang, *Nat. Biotechnol.* **2003**, *21*, 1171–1178; d) M. G. Ryadnov, D. N. Woolfson, *Angew. Chem.* **2003**, *115*, 3129–3131; *Angew. Chem. Int. Ed.* **2003**, *42*, 3021–3023; e) G. A. Silva, C. Czeisler, K. L. Niece, E. Beniash, D. A. Harrington, J. A. Kessler, S. I. Stupp, *Science* **2004**, *303*, 1352–1355.

[2] a) V. Percec, A. E. Dulcey, V. S. K. Balagurusamy, Y. Miura, J. Smidrkal, M. Peterca, S. Nummelin, U. Edlund, S. D. Hudson, P. A. Heiney, H. Duan, S. N. Maganov, S. A. Vinogradov, *Nature* **2004**, *430*, 764–768; b) V. Percec, A. E. Dulcey, M. Peterca, M. Ilies, M. J. Sienkowska, P. A. Heiney, *J. Am. Chem. Soc.* **2005**, *127*, 17902–17909; c) V. Percec, A. E. Dulcey, M. Peterca, M. Ilies, S. Nummelin, M. J. Sienkowska, P. A. Heiney, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 2518–2523; d) M. Peterca, V. Percec, A. E. Dulcey, S. Nummelin, S. Korey, M. Ilies, P. A. Heiney, *J. Am. Chem. Soc.* **2006**, *128*, 6713–6720; e) V. Percec, A. E. Dulcey, M. Peterca, M. Ilies, J. Ladislaw, B. M. Rosen, U. Edlund, P. A. Heiney, *Angew. Chem.* **2005**, *117*, 6674–6679; *Angew. Chem. Int. Ed.* **2005**, *44*, 6516–6521.

[3] a) M. R. Ghadiri, J. R. Granja, L. K. Buehler, *Nature* **1994**, *369*, 301–304; b) D. T. Bong, T. D. Clark, J. R. Granja, M. R. Ghadiri, *Angew. Chem.* **2001**, *113*, 1016–1041; *Angew. Chem. Int. Ed.* **2001**, *40*, 988–1011.

[4] a) J. D. Hartgerink, E. Beniash, S. I. Stupp, *Science* **2001**, *294*, 1684–1688; b) J. D. Hartgerink, E. Beniash, S. I. Stupp, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5133–5138.

[5] X. Y. Gao, H. Matsui, *Adv. Mater.* **2005**, *17*, 2037–2050.

[6] M. Reches, E. Gazit, *Science* **2003**, *300*, 625–627.

[7] a) S. Vauthey, S. Santoso, H. Gong, N. Watson, S. Zhang, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5355–5360; b) S. G. Zhang, D. M. Marini, W. Hwang, S. Santoso, *Curr. Opin. Chem. Biol.* **2002**, *6*, 865–871.

[8] Y. J. Song, S. R. Challa, C. J. Medforth, Y. Qiu, R. K. Watt, D. Pena, J. E. Miller, F. van Swol, J. A. Shelnutt, *Chem. Commun.* **2004**, *6*, 1044–1045.

[9] S. Santoso, W. Hwang, H. Hartman, S. G. Zhang, *Nano Lett.* **2002**, *2*, 687–691.

[10] a) C. H. Görbitz, *Chem. Eur. J.* **2001**, *7*, 5153–5159; b) C. H. Görbitz, *New J. Chem.* **2003**, *27*, 1789–1793.

[11] Y. Zhang, H. Gu, Z. Yang, B. Xu, *J. Am. Chem. Soc.* **2003**, *125*, 13680–13681.

[12] V. Jayawarna, M. Ali, T. A. Jowitt, A. F. Miller, A. Saiani, J. E. Gough, R. V. Ulijn, *Adv. Mater.* **2006**, *18*, 611–614.

[13] C. H. Görbitz, *Chem. Commun.* **2006**, 2332–2334.

[14] E. Luccio, A. Matavel, S. Opi, I. Regaya, G. Sandoz, S. M'Barek, E. Carlier, E. Esteve, L. Carrega, Z. Fajloun, H. Rochat, E. Loret, M. Waard, J. M. Sabatier, *Biochem. J.* **2002**, *361*, 409–416.

[15] L. L. Burns-Hamuro, P. M. Dalessio, I. J. Ropson, *Protein Sci.* **2004**, *13*, 1670–1676.

[16] D. Pantarotto, R. Singh, D. McCarthy, M. Erhardt, J.-P. Briand, M. Prato, K. Kostarelos, A. Bianco, *Angew. Chem.* **2004**, *116*, 5354–5358; *Angew. Chem. Int. Ed.* **2004**, *43*, 5242–5246.

[17] 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*, 4860–4863; *Angew. Chem. Int. Ed.* **2005**, *44*, 4782–4785.

[18] N. W. S. Kam, T. C. Jessop, P. A. Wender, H. Dai, *J. Am. Chem. Soc.* **2004**, *126*, 6850–6851.

[19] I. Kopatz, J.-S. Remy, J.-P. Behr, *J. Gene Med.* **2004**, *6*, 769–776.

[20] P. L. Felgner, G. Rhodes, *Nature* **1991**, *349*, 351–352.

[21] D. M. Lynn, D. G. Anderson, D. Putman, R. Langer, *J. Am. Chem. Soc.* **2001**, *123*, 8155–8156.

[22] D. Luo, K. Haverstick, N. Belcheva, E. Han, W. M. Saltzman, *Macromolecules* **2002**, *35*, 3456–3462.

