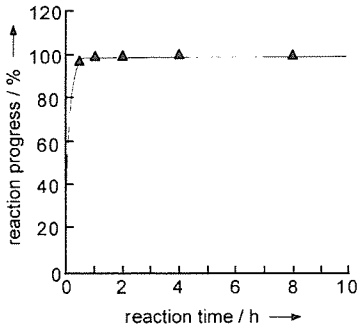

Figure 3. Time course of the aminolysis of PEG-*b*-PBLA with DET in DMF at 40 °C. The reaction progress was estimated from the change in the ratio of the proton peak integration (f over b) in the ^{1}H NMR spectrum (Figure 2).

action conditions (data not shown), highlighting a unique mechanism involved in the aminolysis of PBLA under mild conditions. Presumably, the amide groups of the main chain interact with the carbonyl group of the side chain, which may facilitate the aminolysis reaction.[14] The details of the mechanism of this unique aminolysis reaction are now under investigation in our research group and will be reported elsewhere.

The pH-dependent protonation of PEG-*b*-P[Asp(DET)] in media containing 150 mM NaCl was evaluated by potentiometric titration. The α/pH curve shown in Figure 4 clearly indicates

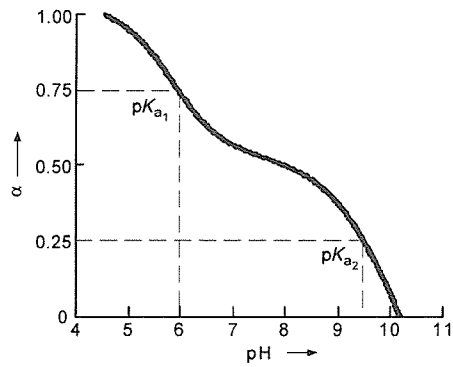

Figure 4. Degree of protonation (α) as a function of pH (α/pH curve) for the PEG-*b*-P[Asp(DET)] block catiomer (150 mM NaCl, aq, 25 °C).

the two-step protonation behavior of PEG-*b*-P[Asp(DET)], which is attributable to the two-step protonation of the ethylenediamine moiety with a distinctive *gauche–anti* conformational transition as indicated in Scheme 2. The two distinct pK_a values of the ethylenediamine moiety in the side chain of polyaspartamide were determined to be 6.0 and 9.5. Notably, this group remains nearly 100% populated by the mono-protonated state (*gauche* form) at pH 7.4, and is capable of exerting a substantial buffering effect in the pH range down to 5.0, at which point the equilibrium shifts to the di-protonated state (*anti* form) (Scheme 2).

The polyplex micelle was prepared by mixing solutions of PEG-*b*-P[Asp(DET)] and pDNA in various ratios of *N/P*, for which *N* is the total number of amine groups in the block catiomer and *P* represents the number of phosphate units in the pDNA. The formation of the polyplex, which accompanies pDNA condensation, was followed by an ethidium bromide (EtBr) dye-exclusion assay at different pH values. As shown in Figure 5, the

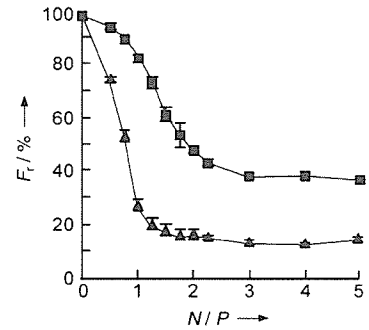

Figure 5. Effect of pH (▲: pH 5.0, ■: pH 7.4) on the relative fluorescence intensity (F_r) of EtBr in solution with pDNA and PEG-*b*-P[Asp(DET)] at various *N/P* ratios.

fluorescence intensity of EtBr decreases with an increase in the *N/P* ratio. At pH 5.0, the fluorescence of EtBr levels off at $N/P = 1$, which is consistent with approximately 95% protonation of the ethylenediamine unit, as expected from the α/pH curve in Figure 4. On the other hand, at pH 7.4, substantial quenching occurred at $N/P \approx 2.0$, which is consistent with the hypothesis that the mono-protonated form of the ethylenediamine unit in PEG-*b*-P[Asp(DET)] might be maintained even inside the polyplex. It is possible that the stabilized *gauche* conformation (Scheme 2) of the mono-protonated form may prevent the ethylenediamine unit from further protonation facilitated by the zipper effect or the local electrostatic field effect in the complexation process with anionic pDNA at pH 7.4.[15] The cumulant diameters and ζ potentials of the polyplexes prepared at different *N/P* ratios are shown in Figure 6. The cumulant diameters of the polyplex micelles were determined to be 70–90 nm throughout the range of the examined *N/P* ratios of 1–20, and the ζ potentials of the polyplexes increased with *N/P* ratios and leveled off at $N/P = 2$ (Figure 6). At $N/P > 2$, the polyplexes were observed to have small absolute ζ potentials (~8 mV), suggesting a core–shell architecture with a hydrophilic and neutral PEG shell surrounding the polyplex core.

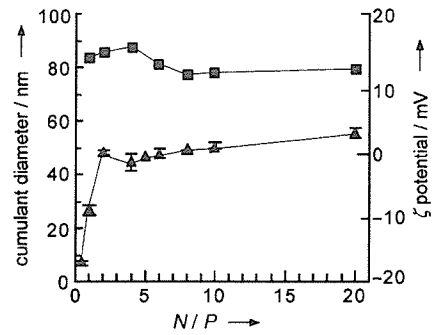

Figure 6. Cumulant diameter (■) and ζ potential (▲) of the PEG-*b*-P[Asp(DET)] polyplex micelles as a function of *N/P* ratio.

The in vitro transfection efficiency (TE) against human hepatoma HuH-7 cells was assessed by a luciferase assay (Figure 7). Notably, a similar trend in TE was also observed for human kidney 293T cells (Supporting Information). In this experiment,

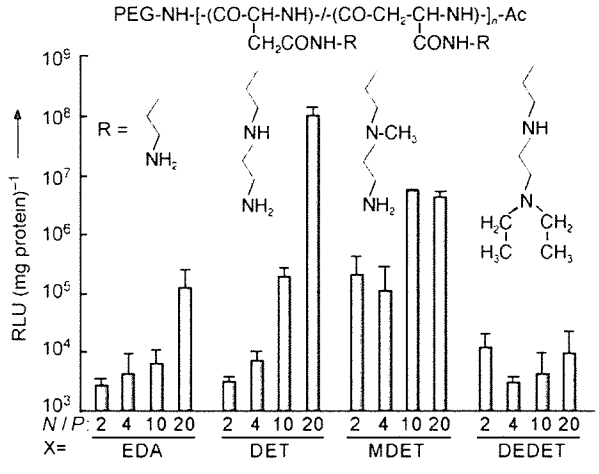

Figure 7. In vitro transfection of the luciferase gene into HuH-7 cells by polyplex micelles from PEG-*b*-polyaspartamides carrying various polyamine components in the side chain (PEG-*b*-P[Asp(X)]) with varying *N/P* ratios. Transfection is reported in relative light units (RLU) per mg protein. The cells were incubated with each polyplex in the medium containing 10% serum for 24 h, followed by incubation for a further 24 h in the absence of polyplex.

the PEG-*b*-P[Asp(DET)]–pDNA micelle was compared with the polyplex micelles from various PEG-*b*-polyaspartamide catiomers made by the similar aminolysis of PEG-PBLA with different amine compounds, with the aim to highlight the unique nature of the P[Asp(DET)] segment. Note that the polyplex micelles from each block catiomer prepared in this study showed sizes and ζ potentials similar to those of the polyplex micelle from PEG-*b*-P[Asp(DET)] (data not shown). The polyplex micelle from the block catiomer with the 2-aminoethyl group $-(CH_2)_2NH_2$ (pK_a 9.4) in the side chain (PEG-*b*-P[Asp(EDA)]), which was prepared through the aminolysis of PEG-*b*-PBLA with ethylenediamine (EDA), showed only 1/10000 of the TE compared with the PEG-*b*-P[Asp(DET)] polyplex micelle at $N/P=20$. This is presumably due to the impaired buffering capacity of the $-(CH_2)_2NH_2$ unit with the high pK_a value of 9.4 in the experimental pH range as well as to the weak ability of PEG-*b*-P[Asp(EDA)] to condense pDNA based on the EtBr exclusion assay (data not shown).

The TE of the PEG-*b*-P[Asp(DET)] polyplex micelle was further compared with those of the polyplex micelles from the PEG-*b*-polyaspartamide catiomers carrying the N-alkylated ethylenediamine units in the side chain to explore the structural features of the polyplex micelles that are important for effective gene transfection (Figure 7). These block catiomers, PEG-*b*-P[Asp(MDET)] and PEG-*b*-P[Asp(DEDET)], are prepared by the aminolysis reaction of PEG-PBLA with the corresponding amine compounds, 4-methyldiethylenetriamine (MDET) and *N,N*-diethyldiethylenetriamine (DEDET), respectively. Both the PEG-*b*-P[Asp(MDET)] and PEG-*b*-P[Asp(DEDET)] polyplex micelles showed an appreciably lower TE than the PEG-*b*-P[Asp(DET)] polyplex micelle, particularly at higher *N/P* ratios (Figure 7). This result, which highlights the critical sensitivity of TE toward subtle changes in catiomer structure, indicates that additional structural factors, besides distinct pK_a values, play a substantial role in determining the TE of the polyplex micelles constructed from the PEG-*b*-polyaspartamide catiomers carrying the derivatized ethylenediamine units as a side chain; further study is needed to clarify the detailed mechanisms.

The cytotoxicity of the polyplex-forming catiomers is also a crucial aspect for successful nonviral gene therapy. In this regard, all the polyplex micelles from each block catiomer shown in Figure 7 elicited no appreciable cytotoxicity toward HuH-7 cells under the same conditions used for gene transfection (data not shown). Notably, the polyplex micelle from PEG-*b*-P[Asp(DET)] showed remarkably low cytotoxicity despite its efficiency in gene transfection. Therefore, the intrinsic cytotoxicity of PEG-*b*-P[Asp(DET)] catiomer was further assessed against HuH-7 cells, and compared with that of branched polyethylenimine (BPEI, 25 kDa, Aldrich Chemical, USA) and linear polyethylenimine (LPEI, 22 kDa, ExGen500, MBI Fermentas, Germany). As shown in Figure 8, the PEG-*b*-P[Asp(DET)] catiomer showed >20-fold higher 50% growth-inhibitory concentration (IC_{50}) than BPEI and LPEI, highlighting the remarkably low cytotoxicity of the block catiomers synthesized in this study.

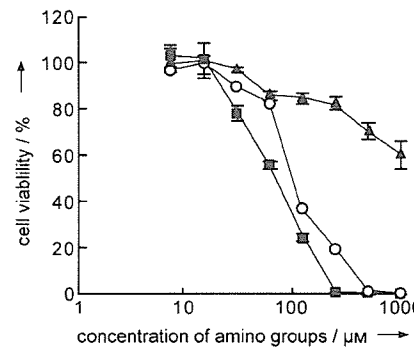

Figure 8. Cytotoxicity of branched (BPEI, ○) and linear (LPEI, ■) polyethylenimines and PEG-*b*-P[Asp(DET)] (▲) against HuH-7 cells. The cells were incubated with each catiomer with different concentrations for 48 h.

The major challenge for the practical use of synthetic vectors in gene therapy is the effective and non-cytotoxic gene transfer to primary cells with therapeutic interest. To evaluate the feasibility of the PEG-*b*-P[Asp(DET)] polyplex micelles toward such primary cells, mouse primary osteoblasts, which are the focus of clinical interest in bone regeneration,[16] were challenged with the polyplex micelles. The luciferase plasmid was transfected, and the resulting TE and cytotoxicity profiles are shown in Figure 9. Notably, the PEG-*b*-P[Asp(DET)] system with $N/P=80$ gave a TE similar to the polyplexes from ExGen500, the most effective transfection reagent based on LPEI,[17] with the optimal *N/P* ratios (Figure 9A). Nonetheless, the PEG-*b*-P[Asp(DET)] system exhibited no appreciable cytotoxicity under the conditions of gene transfection (Figure 9B).

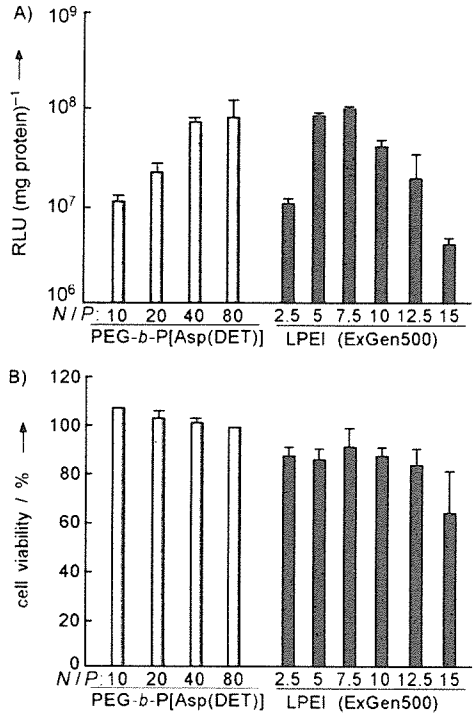

Figure 9. A) In vitro transfection efficiency and B) cytotoxicity of the PEG-*b*-P[Asp(DET)] polyplex micelles and LPEI polyplexes with varying *N/P* ratios toward mouse primary osteoblasts after a 48 h incubation.

Thus, we have successfully obtained highly transfection-efficient and less toxic polyplex micelles in this study. Particularly, the less toxic nature of the block catiomers compared with conventional catiomers of high transfection efficiency, as observed in Figure 8, should be of great significance for in vivo nonviral gene therapy. Indeed, bone regeneration in critical-size cranial defects based on in vivo transduction of osteogenic factors was recently carried out by our research group by using the PEG-*b*-P[Asp(DET)] polyplex micelle with plasmids expressing the optimized combination of osteogenic factors to facilitate cellular differentiation in situ.[18] Furthermore, polyplex micelles with the PEG palisade seem to be suitable for systemic gene delivery,[7,11] and the engineering the constituent block catiomers to construct polyplex micelles with integrated smart functions such as environment sensitivity[8,10] and tissue targetability[9] will maximize the efficacy of nonviral gene therapy. Thus, the PEG-*b*-P[Asp(DET)] polyplex micelle is expected to be a biocompatible vector system applicable toward various aspects of gene medicine.

Conclusion

We have established a simple and novel synthetic route for the generation of biocompatible block catiomers through the quantitative aminolysis of PEG-*b*-PBLA. The construction of a library of block catiomers, the PEG-*b*-polyaspartamides carrying a series of amine compounds in the side chain, revealed the importance of the ethylenediamine unit for enhanced and less toxic gene transfection by the polyplex micelles made from pDNA and the block catiomers. The availability of the polyplex micelles developed in this study for the transfection of primary osteoblasts will facilitate the use of this type of block catiomer for the construction of synthetic vectors suitable for nonviral gene therapy.

Experimental Section

Materials: β-Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) and α-methoxy-ω-amino poly(ethylene glycol) (MeO-PEG-NH_2) (M_r = 12000) were obtained from Nippon Oil and Fats (Tokyo, Japan). Ethylenediamine (EDA), diethylenetriamine (DET) and 4-methyldiethylenetriamine (MDET) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and distilled over CaH_2 under decreased pressure. *N,N*-Diethyldiethylenetriamine (DEDET) was purchased from Lancaster Synthesis, (Lancashire, England) and distilled over CaH_2 under decreased pressure. *N,N*-Dimethylformamide (DMF), dichloromethane, and acetic anhydride were purchased from Wako Pure Chemical Industries, (Osaka, Japan) and purified by general methods before use.

Synthesis of PEG-*b*-polyaspartamide catiomers: The PEG-*block*-poly(β-benzyl L-aspartate) (PEG-*b*-PBLA) copolymer was prepared as previously reported.[19] Briefly, BLA-NCA was polymerized in DMF at 40 °C by the initiation from the terminal primary amino group of MeO-PEG-NH_2, followed by acetylation of the *N*-terminus of PBLA to obtain PEG-*b*-PBLA. PEG-*b*-PBLA was confirmed to have a unimodal molecular weight distribution (M_w/M_n: 1.17) by gel-permeation chromatography (GPC) measurement (columns: TSK-gel G4000HHR + G3000HHR, eluent: DMF + 10 mM LiCl, T = 40 °C, detector: RI) (data not shown). The degree of polymerization (DP) of PBLA was calculated to be 68 based on ^{1}H NMR spectroscopy (data not shown).

Lyophilized PEG-*b*-PBLA (300 mg, 11.6 μmol) was dissolved in DMF (10 mL), followed by reaction with DET (50 equiv to benzyl group of PBLA segment, 4.0 g, 39.4 mmol) under mild anhydrous conditions at 40 °C to obtain PEG-*b*-P[Asp(DET)]. After 24 h, the reaction mixture was slowly added dropwise into a solution of acetic acid (10% *v/v*, 40 mL) and dialyzed against a solution of 0.01 N HCl and distilled water (M_r cutoff: 3500 Da). The final solution was lyophilized to obtain the polymer as the chloride salt form, and the yield was approximately 90%. Similarly, other block catiomers, PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(MDET)], and PEG-*b*-P[Asp(DEDET)] were synthesized by the aminolysis reaction of PEG-*b*-PBLA with EDA, MDET, and DEDET, respectively. The structures of these block catiomers were confirmed by ^{1}H and ^{13}C NMR measurements and size-exclusion chromatography (SEC).

Potentiometric titration of block catiomers: PEG-*b*-P[Asp(DET)] (30 mg) was dissolved in 50 mL 0.01 N HCl and titrated with 0.01 N NaOH. An automatic titrator (TS-2000, Hiranuma, Kyoto, Japan) was used for titration. In this experiment, the titrant was added in quantities of 0.063 mL after the pH values were stabilized (minimal interval: 30 s). The α/pH curves were determined from the obtained titration curves.

Dye exclusion assay: Polyplex solutions with a pDNA concentration of 33 μg mL^{-1}, prepared by mixing pDNA and block catiomers at different *N/P* ratios (*N* = total amines in block catiomer; *P* = total phosphate anions in pDNA), were diluted to 10 μg pDNA mL^{-1} with ethidium bromide (EtBr, 2.5 mg mL^{-1}) in 10 mM Tris-HCl (pH 7.4) or 10 mM sodium acetate (pH 5.0) buffer. The sample solutions were incubated at ambient temperature overnight. The fluo-

rescence intensity of the samples at $\lambda = 590$ nm (excitation at $\lambda =$ 510 nm) was measured at 25 °C with a spectrofluorometer (FP-6500, Jasco, Tokyo, Japan). The relative fluorescence intensity was calculated as: $F_r = (F_{sample} - F_0)/(F_{100} - F_0)$, for which F_{sample}, F_{100}, and F_0 represent the fluorescence intensity of the samples, free pDNA, and background, respectively.

Dynamic light scattering (DLS) and ζ potential measurements: In the DLS measurements, polyplex solutions with various *N/P* ratios in 10 mM Tris–HCl buffer (pH 7.4) were adjusted to have pDNA concentrations of 33.3 $\mu g\,mL^{-1}$. DLS measurements were then performed at 25 ± 0.2 °C with a DLS-7000 instrument (Otsuka Electronics, Osaka, Japan) with a vertically polarized incident beam of $\lambda = 488$ nm from an Ar ion laser. The ζ potential of the polyplex micelles was measured at 25 ± 0.2 °C with an ELS-6000 instrument (Otsuka Electronics, Osaka, Japan) equipped with a He–Ne ion laser ($\lambda = 633$ nm). The scattering angle was fixed at 20°. From the obtained electrophoretic mobility, the ζ potential was calculated by using the Smoluchowski equation: $\zeta = 4\pi\eta v/\varepsilon$ in which η is the electrophoretic mobility, v is the viscosity of the solvent, and ε is the dielectric constant of the solvent. The results are expressed as the average of five experiments.

In vitro transfection of HuH-7 cells: Human hepatoma HuH-7 cells were seeded on 6-well culture plates and incubated overnight in 1.5 mL Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) before transfection. The cells were then incubated with the polyplex micelles from PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(MDET)], and PEG-*b*-P[Asp(DEDET)] (3 μg pDNA/well) with various *N/P* ratios in DMEM containing 10% FBS for 24 h, followed by an additional incubation for 24 h in the absence of polyplexes. Luciferase gene expression was evaluated using the Luciferase Assay System (Promega, Madison, USA) and a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The results were expressed as light units per milligram of cell protein determined by a BCA assay kit (Pierce, Rockford, USA).

Mouse primary osteoblast culture and transfection: Osteoblasts were isolated from calvariae of neonatal littermates. The experimental procedures were handled in accordance with the guidelines of the Animal Committee of the University of Tokyo. Calvariae were digested for 10 min at 37 °C in an enzyme solution containing 0.1% collagenase and 0.2% dispase for five cycles. Cells isolated by the final four digestions were combined as an osteoblast population and cultured in DMEM containing 10% FBS. For luciferase transfection assays, primary osteoblasts were inoculated at a density of 2×10^4 cells/well in a 24-multiwell plate, cultured for 24 h, and, after changing to fresh culture medium containing 10% FBS, pDNA polyplex solution (33.3 $\mu g\,mL^{-1}$, 22.5 μL) was applied to each well. Luciferase gene expression was measured 48 h later by using the Luciferase Assay System (Promega) and a Lumat LB9507 luminometer (Berthold). For the cytotoxicity assay, primary osteoblasts were plated into a 96-multiwell plate (6×10^3 cells/well). After 24 h incubation, 6 μL of each pDNA polyplex solution was added, followed by further incubation for 24 h. The viability of the cells was evaluated by an MTT assay (Cell Counting Kit-8, Dojindo, MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Each well was measured by reading the absorbance at $\lambda =$ 450 nm according to the protocol provided by the manufacturer.

Acknowledgements

The authors are grateful to the Health and Labor Sciences Research Grants in Research on Advanced Medical Technology in Nanomedicine Area from the Ministry of Health, Labor and Welfare (MHLW), Japan. They also express their thanks for the Grant-in-Aid for Scientific Research, the Special Coordination Funds for Promoting Science and Technology, and the Project on the Materials Development for Innovative Nano-Drug Delivery Systems from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The authors thank Professor Yukio Nagasaki and Dr. Motoi Oishi (University of Tsukuba) for use of the SEC instruments and their helpful suggestions, and Mr. Masataka Nakanishi for valuable discussions.

Keywords: aminolysis · block copolymers · gene delivery · polycations · polymeric micelles

[1] I. M. Verma, N. Somia, *Nature* **1997**, *389*, 239.
[2] M. Ogris, E. Wagner, *Drug Discovery Today* **2002**, *7*, 479.
[3] A. K. Salem, P. C. Searson, K. W. Leong, *Nat. Mater.* **2003**, *2*, 668.
[4] N. Nishiyama, A. Iriyama, W.-D. Jang, K. Miyata, K. Itaka, Y. Inoue, H. Takahashi, Y. Yanagi, Y. Tamaki, H. Koyama, K. Kataoka, *Nat. Mater.* **2005**, *4*, 934.
[5] S. O. Han, R. I. Mahato, S. W. Kim, *Bioconjugate Chem.* **2001**, *12*, 337.
[6] Y. Liu, L. Wenning, M. Lynch, T. M. Reineke, *J. Am. Chem. Soc.* **2004**, *126*, 7422.
[7] K. Itaka, K. Yamauchi, A. Harada, K. Nakamura, H. Kawaguchi, K. Kataoka, *Biomaterials* **2003**, *24*, 4495.
[8] K. Miyata, Y. Kakizawa, N. Nishiyama, A. Harada, Y. Yamasaki, H. Koyama, K. Kataoka, *J. Am. Chem. Soc.* **2004**, *126*, 2355.
[9] D. Wakebayashi, N. Nishiyama, Y. Yamasaki, K. Itaka, N. Kanayama, A. Harada, Y. Nagasaki, K. Kataoka, *J. Controlled Release* **2004**, *95*, 653.
[10] S. Fukushima, K. Miyata, N. Nishiyama, N. Kanayama, Y. Yamasaki, K. Kataoka, *J. Am. Chem. Soc.* **2005**, *127*, 2810.
[11] M. Harada-Shiba, K. Yamauchi, A. Harada, I. Takamisawa, K. Shimokado, K. Kataoka, *Gene Ther.* **2002**, *9*, 407.
[12] Y.-Y. Kim, H.-C. Chang, Y. T. Lee, U.-I. Cho, D. W. Boo, *J. Phys. Chem. A* **2003**, *107*, 5007.
[13] J.-P. Behr, *Chemia* **1997**, *51*, 34.
[14] V. Saudek, H. Pivcova, J. Drobnik, *Biopolymers* **1981**, *20*, 1615.
[15] A. V. Kabanov, T. K. Bronich, V. A. Kabanov, K. Yu, A. Eisenberg, *Macromolecules* **1996**, *29*, 6797.
[16] R. T. Franceschi, S. Yang, R. B. Rutherford, P. H. Krebsbach, M. Zhao, D. Wang, *Cells Tissues Organs* **2004**, *176*, 95–108.
[17] C. L. Gebhart, A. V. Kabanov, *J. Controlled Release* **2001**, *73*, 401.
[18] K. Itaka, N. Kanayama, N. Nishiyama, S. Fukushima, Y. Yamasaki, S. Oba, U.-I. Chung, H. Kawaguchi, K. Nakamura, K. Kataoka, *Proceedings of the 12th International Symposium on Recent Advances in Drug-Delivery Systems and CRS Winter Symposium, Salt Lake City, Utah, USA, February 21–24*, **2005**, 9.
[19] A. Harada, K. Kataoka, *Macromolecules* **1995**, *28*, 5294.

Received: December 21, 2005
Published online on February 24, 2006

