DOI 10.5012/bkcs.2011.32.2.651

Sequential Conjugation of 6-Aminohexanoic Acids and L-Arginines to Poly(amidoamine) Dendrimer to Modify Hydrophobicity and Flexibility of the Polymeric Gene Carrier

Gwang Sig Yu,[†] Ha Na Yu,[†] Yun Hui Choe,[†] Sang Jae Son,[§] Tai Hwan Ha,[‡,*] and Joon Sig Choi[†,§,*]

[†]*Department of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon 305-764, Korea*
[‡]*BioMonitoring Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Gwahangno 111 Yuseong-gu, Daejeon 305-806, Korea. [*]E-mail: taihwan@kribb.re.kr*
[§]*Graduate School of Analytical Science and Technology (GRAST), Chungnam National University, Daejeon 305-764, Korea*
[*]*E-mail: joonsig@cnu.ac.kr*
Received November 29, 2010, Accepted December 20, 2010

We synthesized a novel cationic dendrimer consisting of a poly(amidoamine) dendrimer (PAMAM, generation 4) backbone with both L-arginine (Arg) at the termini and 6-aminohexanoic acid (Ahx) between the original core polymer and the peripheral Arg units. The sequential chemical modification of PAMAM G4 with Ahx and Arg resulted in higher transfection efficiency with much less cytotoxicity. PAMAM G4-Ahx-Arg formed stable polyplexes at weight ratios of 8:1 or higher (polymer: plasmid DNA), and the mean polyplex diameter was 180 ± 20 nm. PAMAM G4-Ahx-Arg showed much higher transfection ability than PAMAM G4 or PAMAM G4-Ahx. Furthermore, PAMAM G4-Ahx-Arg was much less cytotoxic than PEI25KD and PAMAM G4-Arg. In addition to Arg grafting of the PAMAM dendrimer, which endows a higher transfection capability, the addition of Ahx spacer increased dendrimer hydrophobicity, introduced flexibility into the conjugated amino acids, and reduced cytotoxicity. Overall, it appears that the concomitant modification of PAMAM with Ahx and Arg could lead to new PAMAM conjugates with better performances.

Key Words: Dendrimer, Gene delivery, Polyplex, Transfection

Introduction

The poly(amidoamine) (PAMAM) dendrimer has been widely investigated as a non-viral vector in both *in vitro* and *in vivo* gene and drug delivery systems.[1,2] Many primary amine groups at the peripheral region of this polymer strongly interact with negatively charged molecules, such as DNA, at a physiological pH and enable the polymer to condense DNA through electrostatic interactions.[3,4] In contrast, the tertiary amines at the core of this globular and highly symmetrical polymer act as a proton sponge, which protects the biodegradation of the complexes in endosome/lysosome.[5] Despite these unique characteristics of PAMAM dendrimers, they are not widely applicable largely due to their cytotoxicity and relatively low transfection efficacy.

To achieve better gene delivery efficacy with less toxicity, PAMAM dendrimers have been subjected to various chemical modifications to prepare more stable, less toxic, and smarter polymeric gene carriers. Many strategies have been employed for these purposes, including PEGylation to prolong circulation time in the bloodstream,[6] acetylation to reduce toxicity,[7] conjugation of specific ligands to selectively target tumor cells,[8] and grafting functional peptides or amino acids to enhance transfection efficacy.[9,10] Some hydrophobic moieties have been adopted to enhance the transfection ability of cationic gene delivery carriers.[11,12] Hydrophobic functional groups are assumed to interact with cell membranes. Recently, Kono *et al*. succeeded in showing enhanced transfection ability of PAMAM dendrimers conjugated to surface amines with hydrophobic amino acids (phenylalanine and valine).[13] Similarly, other research groups have reported that introducing hydrophobic spacers into the head groups of lipids improves their gene expression levels in liposomal delivery systems,[14,15] indicating that introducing a hydrophobic moiety into the conventional vector system helps obtain a higher transfection efficiency. As the stability and dissociation behavior of the polyplex may be affected by charge density, hydrophobicity, and polymer vector flexibility,[16] the transfection efficiency could be improved by modulating the hydrophobicity, flexibility and charge density of the carriers.

In this study, the PAMAM generation 4 (G4) dendrimer, decorated with L-arginines (L-Args) through hydrophobic spacers, was synthesized, and the synergistic effect on gene transfection efficiency was estimated. 6-Aminohexanoic acid (Ahx) was introduced as a hydrophobic spacer between the PAMAM dendrimer primary amine groups and the terminal L-Args. The gene transfection efficiency and cytotoxicity of the novel PAMAM dendrimer (PAMAM G4-Ahx-Arg) were examined using human embryonic kidney 293 cells.

Materials and Methods

Materials. PAMAM G4 dendrimer, *N*,*N*-diisopropylethylamine (DIPEA), and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma-Aldrich (Seoul, Korea). *N*-hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU) were purchased from Anaspec (San Jose, CA, USA). Fmoc-Ahx-OH and Fmoc-Arg(pbf)-OH were purchased from Novabiochem (San Diego, CA, USA). Fetal

bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and 100 × antibiotic-antimycotic agent were purchased from Gibco (Gaithersburg, MD, USA). Luciferase assay kit was purchased from Promega (Madison, WI, USA).

Synthesis of the PAMAM G4-Ahx-Arg Dendrimer. PAMAM G4 was reacted with four equivalents of Fmoc-Ahx-OH, HOBt, and HBTU and eight equivalents of DIPEA in anhydrous DMF solution for 16 hr at room temperature. The coupled intermediate was washed and precipitated with excess cold ethyl ether. The precipitated product was dissolved in 30% piperidine solution and stirred for 1 hr to deprotect the Fmoc groups. After removing the Fmoc groups, the product was washed and precipitated using ethyl ether, and four equivalents of Fmoc-Arg (pbf)-OH were further reacted using the same method described above. To deprotect the Args, the product was dissolved in a deprotection reagent (95:2.5:2.5, trifluoroacetic acid:triisopropylsilane:H_2O, v/v/v) and stirred for 6 hr at room temperature. The product was precipitated with cold ethyl ether and dialyzed against distilled water using a dialysis membrane (MWCO 3500, Spectra/Por® Spectrum Laboratories, CA, USA). After dialysis, the final product was lyophilized and collected as a white solid powder (56% yield).

Agarose Gel Retardation Assay and Dynamic Light Scattering Study. Complexes between polymers and plasmid DNA were prepared in HEPES buffer (25 mM, pH 7.4). A fixed amount of plasmid DNA (pCN-Luci) was mixed with polymers at different polymer/DNA weight ratios at room temperature in HEPES buffer and incubated for 30 min. The complex samples were analyzed on a 0.7% agarose gel containing ethidium bromide (0.5 μg/μL gel). Agarose gel electrophoresis was typically performed for 30 min at 100 V. The size distribution of the PAMAM G4 derivatives/plasmid DNA polyplexes was measured using an ELS-Z2 system (Photal, Otsuka Electronics, Otsuka, Japan).

Cell Culture and Transfection Study. Human embryonic kidney 293 cells were seeded in a 24-well plate at 2×10^5 cell/well and incubated for 24 hr to reach 70 - 80% confluency. A transfection complex was prepared by mixing 1 μg of plasmid DNA with various amounts of polymers, followed by a 30-min incubation at room temperature. PEI25KD/pCN-Luci (weight ratio = 2/1) and PAMAM G4/pCN-Luci (weight ratio = 4/1) complexes were prepared as control groups to compare transfection efficiencies. Then, the cells were treated with a complex solution containing 1 μg of plasmid DNA and 10% FBS at various polymer weight ratios for 24 h. The old medium was removed and washed with DPBS (Dulbecco's phosphate buffered saline), and the cells were lysed for 30 min using Reporter lysis buffer (Promega). Luciferase activity was measured using a Lumat LB 9507 instrument (Berthold Technology, Bad Wildbad, Germany), and protein content was measured using a Micro BCA™ protein assay kit (Pierce, Rockford, IL, USA).

Cytotoxicity Study. Polymer cytotoxicity was assessed with EZ-Cytox reagent (Daeil Lab Service, Seoul, South Korea) based on the WST-1 method. Briefly, 293 cells were seeded in a 96-well plate at 2×10^4 cells/well and incubated in 100 μL of DMEM containing 10% FBS. After one day incubation, the cells were treated with various concentrations of PEI25KD, PAMAM G4, PAMAM-Ahx, and PAMAM-Ahx-Arg. After further incubation for 24 hr, 10 μL of EZ-Cytox solution was added to each well and incubated for an additional 4 hr. The absorbance was measured at 450 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Result and Discussion

Synthesis of the PAMAM G4-Ahx-Arg Dendrimer. As shown in Fig. 1, the PAMAM G4-Ahx and PAMAM G4-Ahx-Arg dendrimers were analyzed by ^{1}H NMR (400 MHz, D_2O).

PAMAM G4-Ahx: δ 1.19 (-$COCH_2C\boldsymbol{H_2}CH_2CH_2CH_2$- and -$COCH_2CH_2C\boldsymbol{H_2}CH_2CH_2$- of the Ahx unit), 1.46 (-$COCH_2CH_2CH_2CH_2C\boldsymbol{H_2}CH_2$- of the Ahx unit, 126H), 2.08 (-$COC\boldsymbol{H_2}CH_2CH_2CH_2CH_2$- of the Ahx unit), 2.24 (-$NCH_2C\boldsymbol{H_2}CO$- of the PAMAM unit, 248H of the PAMAM unit), 2.45 (-$CONHCH_2C\boldsymbol{H_2}N$- of the PAMAM unit, and -$NC\boldsymbol{H_2}C\boldsymbol{H_2}N$- of the PAMAM unit), 2.64 (-$CONHCH_2C\boldsymbol{H_2}NH_2$ of the PAMAM unit and -$NC\boldsymbol{H_2}CH_2CO$- of the PAMAM unit), 2.78 (-$CONHC\boldsymbol{H_2}CH_2N$- and -$CONHCH_2C\boldsymbol{H_2}NH_2$- of the PAMAM unit), 3.14 (-$COCH_2CH_2CH_2CH_2C\boldsymbol{H_2}N$- of the Ahx unit).

PAMAM G4-Ahx-Arg: δ 1.15 (-$COCH_2C\boldsymbol{H_2}CH_2CH_2CH_2$- and -$COCH_2CH_2C\boldsymbol{H_2}CH_2$- of the Ahx unit), 1.42 (-$COCH_2CH_2CH_2C\boldsymbol{H_2}CH_2N$- of the Ahx unit and -$HCCH_2C\boldsymbol{H_2}CH_2NH$- of the arginine unit), 1.65 (-$HCC\boldsymbol{H_2}CH_2CH_2NH$- of the arginine unit), 2.07 (-$COC\boldsymbol{H_2}CH_2CH_2CH_2CH_2$- of the Ahx unit), 2.27 (-$NCH_2C\boldsymbol{H_2}CO$- of PAMAM unit, 248H of PAMAM unit), 2.50 (-$CONHCH_2C\boldsymbol{H_2}N$- of the PAMAM unit, and -$NC\boldsymbol{H_2}C\boldsymbol{H_2}N$- of the PAMAM unit), 2.68 (-$CONHCH_2C\boldsymbol{H_2}NH_2$ of the PAMAM unit and -$NC\boldsymbol{H_2}CH_2CO$- of the PAMAM unit), 3.06 (-$CONHC\boldsymbol{H_2}CH_2N$- and -$CONHCH_2C\boldsymbol{H_2}NH_2$- of the PAMAM unit), 3.14 (-$COCH_2CH_2CH_2CH_2C\boldsymbol{H_2}N$- of the Ahx unit), 3.58 (-**H**C$CH_2CH_2CH_2NH$- of the arginine unit, 62H).

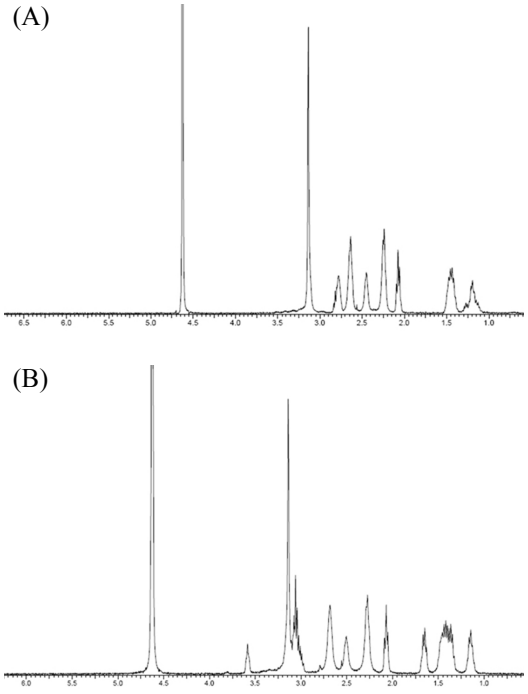

Figure 1. ^{1}H NMR spectra of poly(amidoamine) (PAMAM) generation 4 (G4) derivatives. PAMAM G4-Ahx (A), PAMAM G4-Ahx-Arg (B).

The number of conjugated Ahx and Args on the periphery of PAMAM G4 was calculated based on the ^{1}H NMR data. The NMR spectrum of PAMAM G4 revealed some unique peaks between 2.4 and 3.3 ppm, whereas PAMAM G4-Ahx showed characteristic peaks of the Ahx group at 1.19 and 1.46 ppm, indicating that approximately 63 Ahx molecules were conjugated with primary amines. PAMAM G4-Ahx-Arg had characteristic Arg group peaks at 1.65 and 3.58 ppm, indicating that approximately 62 Arg molecules were coupled with the amino-terminal of the Ahx unit. This number molecules means that approximately 98% and 97% of the surface amines were conjugated with Ahx and Ahx-Arg groups, respectively.

Agarose Gel Retardation Assay. Polyplexes with varying polymer weight ratios to plasmid DNA (pDNA) were run on a 0.7% agarose gel to obtain the optimum polymer amount for a given pDNA. Judging from the observation that the 8:1 (and higher) weight ratio complexes were clearly retarded with both PAMAM G4-Ahx and PAMAM G4-Ahx-Arg (Fig. 2), it can be concluded that free pDNA was not observed, and that all of the pDNA succeeded in forming polyplexes at 8:1 or higher ratios. The results indicate that PAMAM G4 derivatives effectively complexed with pDNA at the given weight ratios.

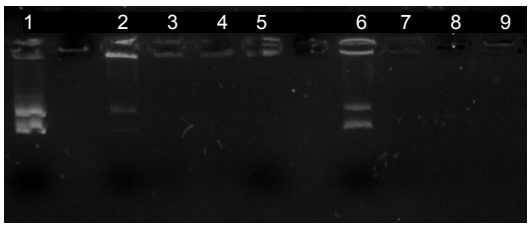

Figure 2. Agarose gel retardation assay. Lane 1 is control plasmid DNA (pDNA), lanes 2 - 5 are PAMAM G4-Ahx with weight ratios of 6:1, 8:1, 10:1, and 12:1, respectively (polymer: DNA). Lanes 6 - 9 are PAMAM G4-Ahx-Arg with weight ratios of 6:1, 8:1, 10:1, and 12:1, respectively (polymer: DNA).

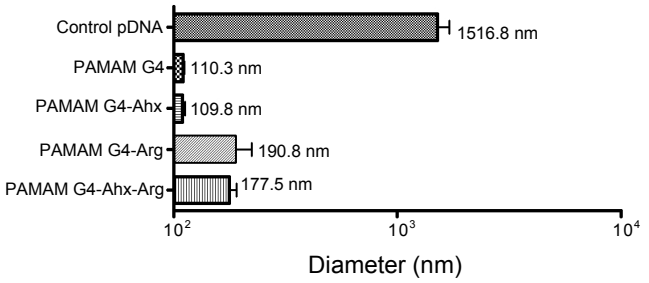

Figure 3. Size distribution of PAMAM G4 derivatives/plasmid DNA polyplexes.

Size Distribution of the PAMAM-G4-Ahx-Arg/pDNA Complexes. The synthesized PAMAM dendrimers with distinct functional groups (such as Ahx, Arg, and Ahx-Arg) were complexed with target pDNA that expresses luciferase, and the resulting sizes were measured by dynamic light scattering (Fig. 3). Compared to the intact pDNA, the reduced sizes of the polyplexes with cationic polymers were attributed to the electrostatic interactions between the phosphate groups in the DNA and the amine/Arg groups. As demonstrated in Fig. 3, the hydrodynamic sizes of the polyplexes decreased remarkably compared to the original pDNA (110 nm *vs*. 1520 nm). By forming polyplexes with cationic polymers, the pDNA not only neutralized the highly negative charges but also reduced their hydrodynamic sizes, which facilitated entry into cell membranes by endocytosis. Furthermore, the polyplexes were assumed to be resistant to enzymatic degradation by DNases, which dissociate the functional pDNA into DNA fragments, before expressing any useful protein it may code.

The newly synthesized PAMAM derivatives and the intact PAMAM effectively condensed the pDNA forming nanoparticles with sizes from 110 to 190 nm. Interestingly, Arg- and Ahx-Arg-terminated PAMAM derivatives showed a relatively larger size distribution compared to the amine-terminated PAMAM dendrimers (-NH_2 and/or Ahx-NH_2). The effect of hydrophobic chains (Ahx) on the size distributions in the amine-terminated PAMAM appeared negligible but demonstrated a slight downsizing of the polyplexes with Arg terminals (190 nm *vs.* 177 nm). It is presumed that the hydrophobic interactions between spacers may have facilitated the formation of more compact polyplexes compared to those with PAMAM G4-Arg.

In 2000, Okano *et al.* reported that transfection efficiency increased by incorporating hydrophobic monomer units into

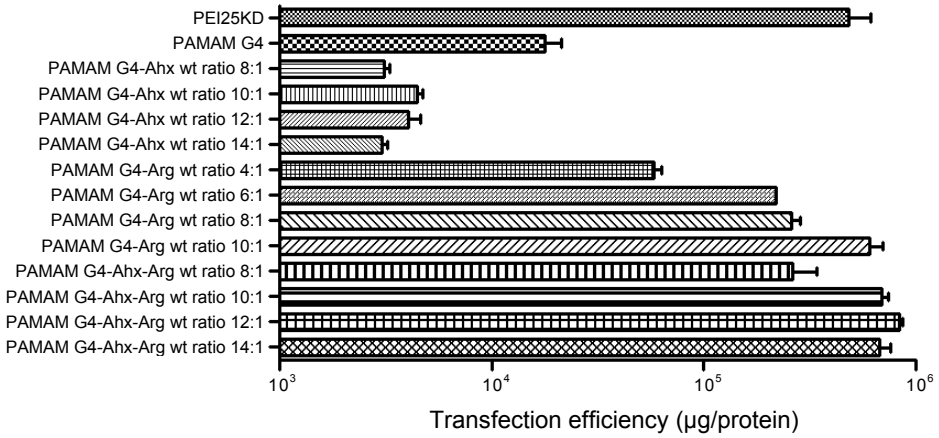

Figure 4. *In vitro* transfection efficiency of PAMAM derivatives in human embryonic kidney 293 cells.

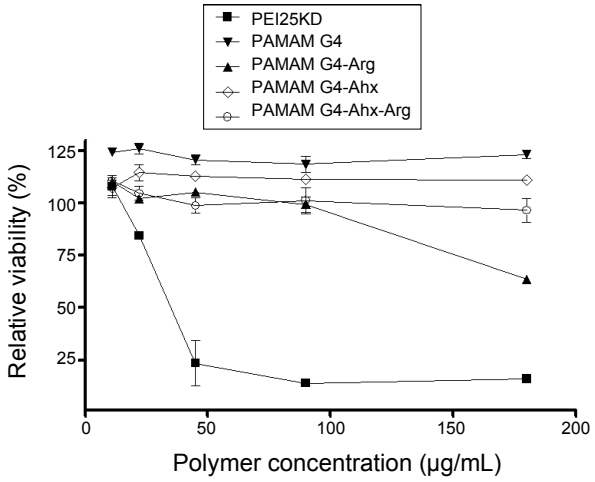

Figure 5. Cytotoxicity of PAMAM G4 derivatives in human embryonic kidney 293 cells.

polymeric gene carriers.[17] Recently, Epand *et al.* reported that hydrophobic amino acid-modified PAMAM dendrimers located near or in lipid-head groups interact with lipid phosphate groups.[18] More recently, Gupta *et al.* reported that the presence of a hydrophobic domain at the N-terminus of the peptide influences peptide complexes with pDNA.[1] These reports indicate that modification with hydrophobic molecules affects the hydrophobicity, size and stability of polyplexes and it may play an important role in the enhanced interaction with cellular membranes. All of these studies indicate that modifying the hydrophobicity of polymeric gene carriers could improve gene transfection efficacy.

Transfection and Cytotoxicity Assay. Human embryonic kidney 293 cells were incubated with PAMAM derivatives complexed with pDNA at various weight ratios in the presence of 10% serum, and the expressed luciferase activity was measured to evaluate *in vitro* transfection efficiency of the synthesized PAMAM derivatives. Transfection efficiency was evaluated as the relative light unit divided by the amount of cellular protein and shown in Fig. 4. As revealed in the previous study,[10] the remarkable increase in transfection efficiency was observed for PAMAM G4-Arg.

Interestingly, PAMAM derivatives coupled with only Ahx (PAMAM G4-Ahx) displayed less transfection ability than the original PAMAM G4, suggesting that the resulting decrease of charge density and lack of functional residues could be the major disadvantages minimizing the advantage of introducing hydrophobicity to the carrier. However, PAMAM G4-Ahx-Arg showed much higher transfection efficiency than native PAMAM G4 dendrimer and even than PEI25KD, which is an efficient but highly cytotoxic polymeric vector. As the weight ratio increased from 8:1 to 10:1, the transfection efficiency of PAMAM G4-Ahx-Arg increased by two fold, and reached a plateau at higher weight ratios (12:1 and 14:1). This may be explained by the synergistic effects of Ahx with Arg. At the molecular level, the Ahx spacer unit may play a role as a flexible linker to Arg and its hydrophobic nature may have affected the physicochemical properties of the complexes with pDNA. Due to the increased flexibility and hydrophobicity of complexes, surplus Arg residues may be located on the surface of polyplexes and could interact more efficiently with the negatively charged plasma membrane by electrostatic interaction. Thus, the increased mobility and hydrophobicity of the dendrimer by Ahx-Arg residues could explain the enhanced transfection efficiency of PAMAM G4-Ahx-Arg compared to the native PAMAM G4 and PAMAM-Arg.

The cytotoxicity of the synthesized PAMAM G4 derivatives was evaluated using an EzCytox cell viability assay kit based on the WST-1 assay. As is well recognized, cationic charge density and biodegradability are known to be important factors closely related with the cytotoxicity of polymeric gene carriers.[19] As shown in Fig. 5, PEI25KD becomes highly toxic as polymer concentration increases, whereas PAMAM derivatives showed much less toxicity over broad concentration ranges. Interestingly, PAMAM derivatives bearing Ahx spacers were less toxic than intact PAMAM G4 or PAMAM G4-Arg over a wide range of polymer concentrations. PAMAM G4-Ahx-Arg displayed much less toxicity than PAMAM G4-Arg even at a dosage of 180 μg/mL, indicating that introducing a spacer between PAMAM G4 and an Arg unit may lower the charge density compared to PAMAM G4-Arg.

Even though Arg- and Ahx-Arg-terminated PAMAM derivatives demonstrated almost comparable transfection efficiency, PAMAM G4-Ahx-Arg seems to be more advantageous in terms of cytotoxicity than PAMAM G4-Arg. So, these observations could suggest that through the synergistic effect of the sequential hydrophobic and Arg-modifications, the enhanced transfection efficacy could be retained and improved for the Ahx-Arg modification considering that higher weight ratios are also tolerable and applicable compared with PAMAM G4-Arg.

Conclusion

We synthesized PAMAM G4 dendrimer derivative containing a hydrophobic flexible spacer and a cationic amino acid as a non-viral gene delivery vector. Conjugating only Ahx to the PAMAM G4 dendrimer reduced transfection efficiency substantially, but the additional conjugation of Arg residues to the dendritic N-terminal of Ahx chains resulted in more enhanced transfection capability. Moreover, the synthesized PAMAM G4 derivatives with Ahx-Arg were much less cytotoxic than PEI25KD or PAMAM G4-Arg. It is expected that the present strategy will be widely applicable to other drug delivery as well as gene delivery systems.

Acknowledgments. This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (KRF-2008-313-D00293), and by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-611-C00003).

References and Notes

1. Cloninger, M. J. *Curr. Opin. Chem. Biol.* **2002**, *6*, 742.

2. Lee, C. C.; MacKay, J. A.; Frechet, J. M.; Szoka, F. C. *Nat Biotechnol.* **2005**, *23*,1517.
3. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297.
4. Wu, G. Y.; Wu, C. H. *J. Biol. Chem.* **1987**, *262*, 4429.
5. Behr, J. P. *Chimia.* **1997**, *51*, 34.
6. Cheng, Y. Y.; Xu, Z. H.; Ma, M. L.; Xu, T. W. *J. Pharm. Sci-Us* **2008**, *97*, 123.
7. Kolhatkar, R. B.; Kitchens, K. M.; Swaan, P. W.; Ghandehari, H. *Bioconj. Chem.* **2007**, *18*, 2054.
8. Shukla, R.; Thomas, T. P.; Peters, J.; Kotlyar, A.; Myc, A.; Baker, J. R. *Chem. Commun.* **2005**, 5739.
9. Kang, H. M.; DeLong, R.; Fisher, M. H.; Juliano, R. L. *Pharm. Res.* **2005**, *22*, 2099.
10. Choi, J. S.; Nam, K.; Park, J.; Kim, J. B.; Lee, J. K.; Park, J. *J. Control Release* **2004**, *99*, 445.
11. Goparaju, G. N.; Satishchandran, C.; Gupta, P. K. *Int. J. Pharm.* **2009**, *369*, 162.
12. Obata, Y.; Saito, S.; Takeda, N.; Takeoka, S. *Bba-Biomembranes* **2009**, *1788*, 1148.
13. Kono, K.; Akiyama, H.; Takahashi, T.; Takagishi, T.; Harada, A. *Bioconj. Chem.* **2005**, *16*, 208.
14. Palmer, L. R.; Chen, T.; Lam, A. M. I.; Fenske, D. B.; Wong, K. F.; MacLachlan, I.; Cullis, P. R. *BBA-Biomembranes* **2003**, *1611*, 204.
15. Bajaj, A.; Kondaiah, P.; Bhattacharya, S. *Biomacromolecules* **2008**, *9*, 991.
16. Kakizawa, Y.; Kataoka, K. *Adv. Drug Deliv. Rev.* **2002**, *54*, 203.
17. Kurisawa, M.; Yokoyama, M.; Okano, T. *J. Control Release* **2000**, *68*, 1.
18. Klajnert, B.; Epand, R. M. *Int. J. Pharm.* **2005**, *305*, 154.
19. Mintzer, M. A.; Simanek, E. E. *Chem. Rev.* **2009**, *109*, 259.