# Biodegradable Triblock Copolymer of PLGA-PEG-PLGA Enhances Gene Transfection Efficiency

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***Purpose.*** A tri-block copolymer of PLGA-PEG-PLGA was used as an excipient to enhance the gene transfection efficiency of various cationic polymeric carriers.

***Methods.*** Luciferase plasmid DNA was complexed with polyethylenimine for gene transfection. Various concentrations of PLGA-PEG-PLGA copolymer up to 0.5% were added in the transfection medium to explore whether the copolymer increased the level of gene expression. Pluronic F68 was used as a control. Various polyplexes and different cell lines were used to verify the effect of the triblock copolymer on gene transfection. The cellular uptake extent of radiolabeled plasmid was quantitatively determined as a function of PLGA-PEG-PLGA concentration.

***Results.*** PLGA-PEG-PLGA copolymer significantly enhanced gene transfection efficiency at a concentration as low as 0.25% (w/v), which was more effective than Pluronic F68 at the same concentration range. The additive effect of the triblock copolymer in the transfection medium was clearly observed for various cationic polyplexes and cell lines, although the gene expression extents largely depended on polymers and cell lines used. Five- to 10-fold increment of gene transfection levels were attained in the presence of the PLGA-PEG-PLGA tri-block copolymer. The enhanced gene transfection efficiency was attributed to the increased cellular uptake of PEI/DNA complexes in the presence of the PLGA-PEG-PLGA tri-block copolymer.

***Conclusions.*** Biodegradable PLGA-PEG-PLGA tri-block copolymer that facilitates the endocytic process can be used as a novel additive in non-viral gene transfection.

**KEY WORDS:** PLGA-PEG-PLGA triblock copolymer; gene delivery; excipient; biodegradable; nontoxic.

## INTRODUCTION

Gene delivery systems using nonviral polymeric carriers have drawn much attention recently because they have no inherent safety problems occurring for viral vectors (1,2). Polyelectrolyte nanoparticulate complexes formed between DNA and polycations have been used as major nonviral gene-delivery vehicles (3). Various polycations, including synthetic polymers (4–6), lipids (7), and polymer–lipid hybrids (8), have been introduced for the improved transfection of DNA into cells. Covalent modification of the vectors with receptor-recognizing ligands was studied to enhance the cellular uptake of the complexes, and formulations with fusogenic peptides were reported for facilitating the endosomal escape of the complexes to improve transfection efficiency to a certain level (9–11). As a more simple approach, nonionic polymers were used as an additive to enhance gene expression. Pluronics are triblock copolymers composed of polyethyleneoxide and propyleneoxide (PEO-PPO-PEO). The presence of a small amount of Pluronic copolymer in the transfection medium made naked DNA and various DNA/polymer formulations increase their *in vitro* transfection efficiencies, although the exact mechanism for the increased gene expression is still unknown (12–16). The formulation of Pluronic copolymers with naked DNA also showed significant increase in gene expression when administered intramuscularly (12).

In this study, biodegradable and nontoxic triblock copolymer, poly(D,L-lactic-co-glycolic acid)-b-poly(ethylene glycol)-b-poly(D,L-lactic-co-glycolic acid) (PLGA-PEG-PLGA), was used to explore whether it enhanced the extent of gene transfection in a similar manner to Pluronic copolymers. Pluronic triblock copolymers have a hydrophobic PPO segment in the middle block with two hydrophilic PEO segments in the side block. In contrast, PLGA-PEG-PLGA copolymers have an opposite structural configuration with a hydrophilic PEG segment in the middle block. Aqueous solutions of PLGA-PEG-PLGA triblock copolymers above a certain concentration range exhibited a unique thermosensitive sol-gel transition behavior and they have been used as novel injectable drug delivery systems for various macromolecular drugs (17). However, in a dilute condition, PLGA-PEG-PLGA self-assembles in aqueous solution and exists in a form of micelles. The micelle structure of PLGA-PEG-PLGA copolymers differs from that of Pluronic copolymers. It has a flower-type micellar shape, in which the middle PEG chain makes a loop on the surface. Thus, it is of interest to compare two structurally different triblock copolymers in terms of their effect on gene transfection efficiency. Gene transfection efficiencies of various polyplex formulations were examined in the presence of PLGA-PEG-PLGA micelles with different copolymer concentrations. In order to elucidate the possible role of PLGA-PEG-PLGA micelles in the gene transfection, physical characteristic changes of DNA/polymer complexes and differences in their cellular uptake were investigated in the presence and absence of PLGA-PEG-PLGA.

## MATERIALS AND METHODS

### Materials

PLGA-PEG-PLGA tri-block copolymer (MW = 4200, Mn/MW = 1.3, PEG MW 1450) was obtained from Macromed (Sandy, UT, USA). Polyethylenimine (branched PEI, MW 25000) was purchased from Aldrich (Milwaukee, WI, USA). Poly(L-lysine) (MW 25000), protamine, and (3-(4,5-dimethylthyazolyl-2)-2,5-diphenyl tetrazolium bromide) were from Sigma (St. Louis, MO, USA). Pluronic F-68 was obtained from BSAF (Parsippany, NJ, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA).

### Methods

#### *Cell Culture*

293 cells were cultured in DMEM supplemented with 10% FBS, streptomycin at 100 μg/ml, penicillin at 100 IU/ml,

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and 2 mM L-glutamine. The cells were maintained at 37°C in a humidified 5% $CO_2$ atmosphere. One hundred thousand cells per well were plated in a six-well plate in 1.5 ml DMEM with 10% FBS 24 h before transfection.

*Formulation of Complexes and Transfection*

Luciferase plasmid with SV40 promoter (pLuc, Promega, Madison, WI, USA) was purified from *Escherichia coli* DH5α using a Qiagen column (Qiagen, Hilden, Germany). The plasmid DNA and the desired amount of cationic polymers were diluted separately in 10× volume of DMEM. The diluted polymer solution was mixed with DNA and left for 15 min at room temperature. Final DNA concentration was 4 μg/ml. PLGA-PEG-PLGA copolymer dissolved in PBS was then added to the DNA/polymer complexes at a desired concentration and stored for 15 min at room temperature. The size and zeta potential value of the PEI/DNA complex in the presence and absence of PLGA-PEG-PLGA copolymer were measured by dynamic light scattering equipment (ZetaPlus, Brookhaven Instruments Co., Holtsville, NY, USA). A MASOPTION software with multimodal size distribution option was used for data fitting. Cells (n = 293) were transfected in 0.8 ml of serum-free DMEM or DMEM with 10% FBS by adding the transfection formulation. After 3-h incubation, the medium was removed and supplemented with fresh DMEM containing 10% FBS. Luciferase gene expression was determined after 48 h post-transfection by using a commercial luciferase assay kit (Promega). The luciferase activity was monitored for 15 s in a Lumat LB 9501 luminometer (Berthold, Wilbach, Germany). Each transfection experiment was performed in triplicate and was expressed as relative light unit per mg of cell protein. Protein concentration per well was measured by using Micro-BCA assay (Pierce, Rockford, IL, USA). Each transfection experiment was repeated at least three times to ensure its reproducibility.

*DNA Uptake Study*

Plasmid DNA (pLuc) was labeled with [α-$^{32}$P]dATP (400 Ci/mmol, 10 mCi/ml, Amersham, Piscataway, NJ, USA) by using a nick-translation method under the manufacturer's recommended condition. One hundred thousand cells per well were plated in a six-well plate in 1.5 ml of DMEM with 10% FBS and incubated for 24 h. The medium was removed and supplemented with serum-free medium. The labeled plasmid DNA formulated with PEI and PLGA-PEG-PLGA as described above was added to the cells and incubated for 3 h at either 4°C or 37°C. The cells were washed extensively with PBS and lysed by adding cell-culture lysis buffer (Promega). Cellular uptake of DNA was measured by using liquid-scintillation counter (Tri-Carb 1900TR, Packard, Groningen, The Netherlands). The amount of internalized DNA was expressed as a percent radioactivity by relating the cells treated with the PLGA-PEG-PLGA formulation and ones with PEI/DNA complexes alone.

## RESULTS AND DISCUSSION

Biodegradable and nontoxic PLGA-PEG-PLGA triblock copolymers have an amphipathic structure, and above a certain concentration, they self-assembled to form micelles in an aqueous solution. PEI was used as a primary cationic polymeric carrier to form DNA/polymer complexes with luciferase plasmid DNA. PEI and DNA were complexed at an N/P (nitrogen/phosphate) ratio of 8.0. A series of transfection experiments was carried out in the presence and absence of the PLGA-PEG-PLGA tri-block copolymer. When 293 cells were transfected with PEI/DNA complexes in the presence of PLGA-PEG-PLGA as an excipient, enhanced luciferase gene expression was observed as shown in Fig. 1. The concentration of the triblock copolymer was varied from 0.01 to 0.5% (w/v) to identify the optimal condition for enhanced transfection. The level of transfection increased up to 0.25% of the triblock copolymer concentration, but it tended to decrease thereafter. The DNA/PEI complexes formulated with PLGA-PEG-PLGA shows that the transfection efficiency increased 3-fold at the concentration of 0.25% than those without PLGA-PEG-PLGA. Although the complexes formulated with the Pluronic® F68 also showed some enhanced transfection in 293 cells, the increment of transfection level was not as significant as that of PLGA-PEG-PLGA. Pluronic® F68 was used as an emulsifier of cationic lipid for gene delivery and showed positive effect on transfection (18). Pluronic® F68 also showed increased luciferase expression by about three times when co-injected with naked DNA into muscle tissue (19). The enhancement of transfection level by the addition of PLGA-PEG-PLGA as an excipient was also observed in the presence of 10% fetal bovine serum (data not shown). It is noted that higher transfection level was observed at above the critical micelle concentration of PLGA-PEG-PLGA. The critical micelle concentration of PLGA-PEG-PLGA was 0.005 mg/ml as determined by a fluorescence probe technique using pyrene. The result suggests that the formation of polymeric micelles seems to be one of the important factors for the polyplex gene delivery formulations. In previous study on eye-drop gene delivery system, the formulation of PEO-PPO-PEO tri-block copolymer with naked DNA showed higher level of gene expression above the critical micelle concentration (13). It was previously reported that the transfection efficiency of the plasmid DNA complexed with poly(*N*-ethyl-4-vinylpyridinium) increased in the presence of 0.1% Pluronic® P85 (15). Pluronic® P85 exists as a micellar form at that concentration (20). It was suggested that PEO-PPO-PEO tri-copolymer could interact with naked DNA to condense it

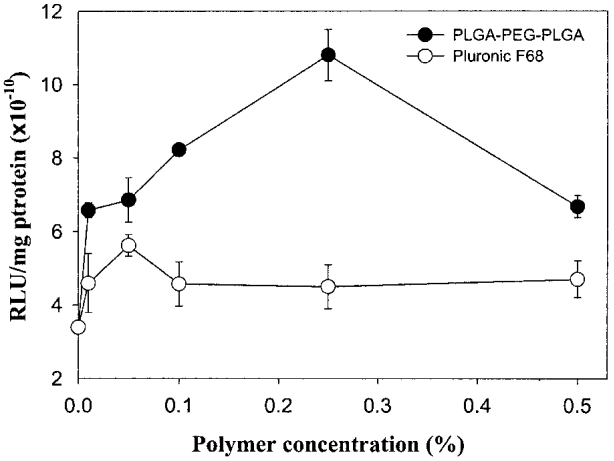


**Fig. 1.** Transfection efficiency of DNA/PEI complexes at different concentrations of PLGA-PEG-PLGA and Pluronic® F68.

to small size (approx. 150 nm) and mask the negative charges of the DNA (13). In this study, however, no detectable changes were found in the size (115.7–120 nm) and the surface zeta-potential (38.6 ± 0.76 mV) of PEI/DNA complexes in the presence of 0.25% PLGA-PEG-PLGA. Although the amount of PLGA-PEG-PLGA micelles (0.25%) would be greater than that of PEI/DNA complexes in the solution, two distinct intensities of light scattering at ca. 40 nm and at ca. 120 nm were still observed; they were caused by the individual presence of PLGA-PEG-PLGA micelles and PEI/DNA complexes, respectively. This is probably due to the nature of PLGA-PEG-PLGA micelles that have neutral and sterically repulsive PEG chains present on their surface, which minimizes their physical interaction with the complexes.

The formulations with PLGA-PEG-PLGA micelles also exhibited enhanced transfection efficiency when formulating with different cationic polymers such as poly(L-lysine) and protamine (Fig. 2). In the case of protamine, a natural cationic polypeptide, the transfection level of complexes formulated with PLGA-PEG-PLGA showed about 10-fold higher than that of complexes alone (Fig. 2C). The substantial increment of transfection in various cells by PLGA-PEG-PLGA was also observed with hepatoma cell line (HepG2), fibroblast (NIH3T3), and smooth muscle (A7R5) cell lines as shown in Fig. 3. Although there were differences in transfection level among the cell lines, the formulation with PLGA-PEG-PLGA micelles showed much higher level of transfection compared to the formulation with Pluronic® F68 and the complexes alone. These results reveal that PLGA-PEG-PLGA micelles can be used as a versatile excipient for enhancing transfection efficiency of the cationic polymer-mediated non-viral delivery systems. It should be noted that the PEI/DNA complexes formulated with PLGA-PEG-PLGA tri-block copolymer showed superior transfection efficiency to commercially available LipofectAMINE® formulations. The luciferase transfection efficiency of the PEI/DNA formulations in the absence of the copolymer was about approx. $10^{10}$ RLU/mg protein, which was comparable to that of LipofectAMINE® formulation. However, by simply mixing PEI/DNA complexes with the copolymer micelles, the transfection efficiency could reach to approx. $10^{11}$ RLU/mg protein under the same conditions.

Because there were no apparent differences in physical characteristics of the complexes with or without PLGA-PEG-PLGA as determined by dynamic light scattering, it was assumed that PLGA-PEG-PLGA micelles might interact with cells to some extent. Because amphiphilic block copolymers have a surface active and detergent-like activity, it was reasonable to expect that they might affect cell viability. But, there was no detectable change in cell viability up to the concentration of 0.5% (data not shown). In order to see whether PLGA-PEG-PLGA micelles aided the intra-cellular uptake of DNA/polymer complexes and played a key role in intra-cellular trafficking of DNA, the internalization of the DNA/polymer complexes was investigated by using $^{32}$P-labeled plasmid DNA. The level of plasmid uptake increased by 2.5-fold in the presence of 0.25% PLGA-PEG-PLGA at 37°C (Fig. 4). However, no significant changes in plasmid uptake were observed when the experiment was carried out at 4°C, at which temperature the cellular endocytosis process is suppressed (15). The relative amount of cellular DNA uptake was calculated by relating the radioactivity of the groups with PLGA-PEG-PLGA formulation to that of the group without the formulation (PEI/DNA complexes only). This result suggests that PLGA-PEG-PLGA micelles could facilitate the internalization of the complexes by promoting an endocytosis process. It is well agreed with the previous study with Pluronic® P85 (15). Figure 5 shows that the level of reporter gene expression is not enhanced when the cells were pretreated with PLGA-PEG-PLGA micelles before transfection.

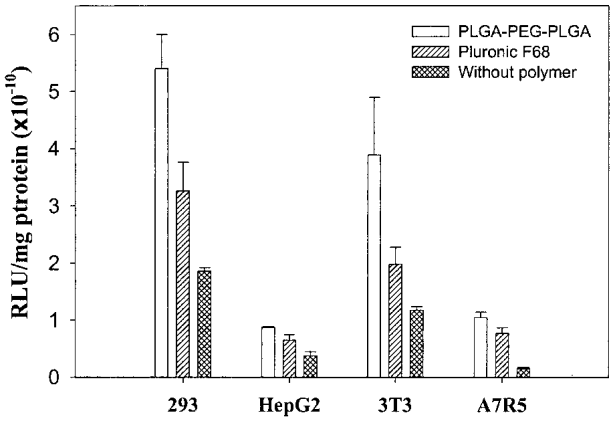


**Fig. 3.** Effect of PLGA-PEG-PLGA on the transfection efficiency of DNA/PEI complexes for different cell lines.

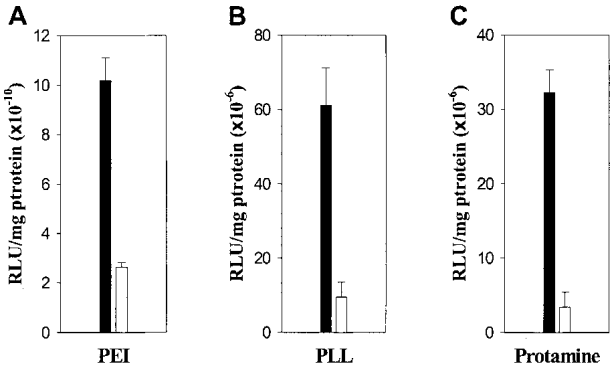


**Fig. 2.** Effect of PLGA-PEG-PLGA on the transfection efficiency of various DNA/polymer complexes. Black bar and white bars represent the presence and the absence of PLGA-PEG-PLGA, respectively.

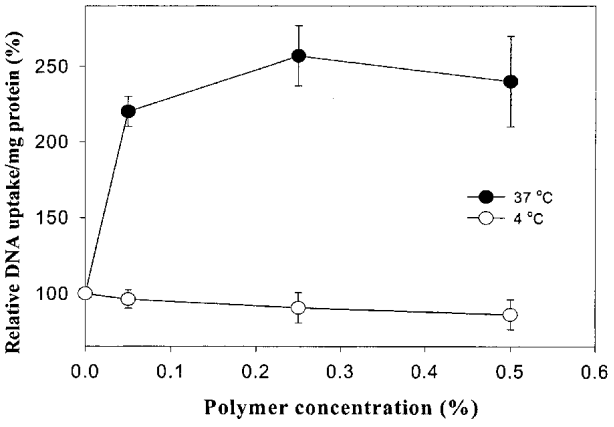


**Fig. 4.** Relative cellular uptake of DNA as a function of PLGA-PEG-PLGA concentration at 37°C and 4°C. Plasmid DNA was labeled with $^{32}$P and complexed with PEI.

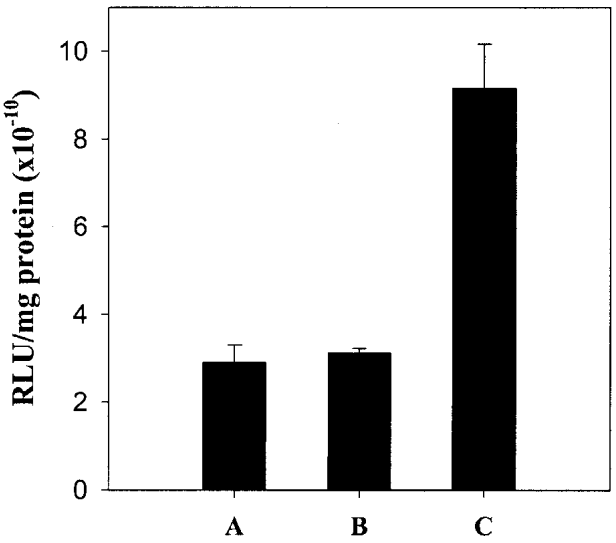


**Fig. 5.** Expression of luciferase in 293 cells by DNA/PEI complexes: (A) no PLGA-PEG-PLGA, (B) pretreatment of PLGA-PEG-PLGA, and (C) cotreatment of PLGA-PEG-PLGA.

The results imply that the influence of PLGA-PEG-PLGA micelles on cells is temporary and negligible, and the coexistence of cationic polymer/DNA complexes and PLGA-PEG-PLGA micelles is essentially required for enhancing gene transfection activity by PLGA-PEG-PLGA micelles. A previous report showed that the existence of free Pluronic copolymers was critical for the improved transfection with Pluronic® P123-grafted PEI/DNA complexes (14,21). Pluronic copolymers were reported to increase membrane permeability and drug release from endosomes in multi-drug resistant cells (22). Hydrophobic poly(propylene oxide) (PPO) segment of Pluronic copolymer is supposed to interact with cell membrane although the mechanism is still obscure (23). Similarly, the hydrophobic PLGA segment in PLGA-PEG-PLGA copolymers, like Pluronic copolymers, may play a crucial role in the interaction with cell membrane for enhancing the cellular uptake of PEI/DNA nanoparticulates. It is conceivable that PLGA-PEG-PLGA tri-block copolymers and their micelles somehow lower the activation energy necessary for the energy-dependent endocytosis process that includes the formation of a clathrin coated pit vesicle on the cell membrane. In a separate experiment, we also confirmed that the cellular uptake of fluorescent dye labeled polystyrene nanoparticles (mean diameter, 200 nm) was enhanced in the presence of PLGA-PEG-PLGA copolymers. Thus, it can be expected that the cellular uptake of various natural and synthetic nanoparticulate materials including liposomes might be increased in the presence of polymeric micelles that play a crucial role in helping the cellular uptake by unclarified cell-polymer interactions. The detailed mechanism and the role of PLGA-PEG-PLGA micelles on cellular uptake of polyplexes remain to be explored.

In this study, we demonstrated that non-ionic PLGA-PEG-PLGA micelles could substantially increase the transport of cationic polymer/DNA complexes and the level of gene expression. The characteristics of PLGA-PEG-PLGA copolymers such as biodegradability and no cellular toxicity (24) would be advantageous in *in vivo* gene delivery experiments.

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## REFERENCES

1. F. D. Ledley. Nonviral gene therapy: the promise of genes as pharmaceutical products. *Hum. Gene Ther.* **6**:1129–1144 (1995).
2. A. Rolland and P. Felgner. (eds). Non-viral gene delivery systems. *Adv. Drug Del. Rev.* **30**:1–227 (1998).
3. S. C. De Smedt, J. Demeester, and W. E. Hennink. Cation polymer based gene delivery systems. *Pharm. Res.* **17**:113–126 (2000).
4. O. Boussif, F. Lezoualc'h, M. A, Zanta, M. D. Mergny, D. Scheman, B. Demeneix, and J. P. Behr. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. USA* **92**:7297–7303 (1995).
5. J. H. Jeong, S. H. Song, D. W. Lim, H. Lee, and T. G. Park. DNA Transfection Using Linear Poly(ethylenimine) Prepared by Controlled Acid Hydrolysis of Poly(2-ethyl-2-oxazoline). *J. Control. Release* **73**:391–399 (2001).
6. R. J. Mumper, J. G. Duguid, K. Anwer, M. K. Barron, H. Nitta, and A. P. Rolland. Polyvinyl derivatives as novel interactive polymers for controlled gene delivery to muscle. *Pharm. Res.* **13**:701–709 (1996).
7. P. L. Felgner, T. R. Gader, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Nerthrop, G. M. Ringold, and M. Danielsen. Lipofection: a highly efficient, lipid-mediated DNA transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:7412–7417 (1987).
8. S. Han, R. I. Mahato, and S. W. Kim. Water-soluble lipopolymer for gene delivery. *Bioconjug. Chem.* **12**:337–345 (2001).
9. Y. H. Choi, F. Liu, J. S. Park, and S. W. Kim. Lactose-poly(ethylene glycol)-grafted poly-L-lysine as hepatoma cell targeted gene carrier. *Bioconjug. Chem.* **9**:708–718 (1998).
10. H. Lee, J. H. Jeong, and T. G. Park. PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity. *J. Control. Release* **79**:283–291 (2002).
11. H. Lee, J. H. Jeong, and T. G. Park. A New Gene Delivery Formulation of polyethyleneimine/DNA complexes coated with PEG conjugated fusogenic peptide. *J. Control. Release* **76**:138–192 (2001).
12. P. Lemieux, N. Guerin, G. Paradis, R. Proulx, L. Chistyakova, A. Kabanov, and V. Alakhov. A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. *Gene Ther.* **7**:986–991 (2000).
13. J. Liaw, S.-F. Chang, and F.-C. Hsiao. *In vivo* gene delivery into ocular tissues by eye drops of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) polymeric micelles. *Gene Ther.* **8**:999–1004 (2001).
14. H.-K. Nguyen, P. Lemieux, S. V. Vinogradov, C. L. Gebhart, N. Guerin, G. Paradis, T. K. Bronich, V. Y. Alakhov, and A. V. Kabanov. Evaluation of polyether-polyethylenimine graft copolymers as gene transfer agents. *Gene Ther.* **7**:986–991 (2000).
15. I. Astafieva, I. Maksimova, E. Lukanidin, V. Alakhov, and A. Kabanov. Enhancement of the polycation-mediated uptake and cell transfection with Pluronic P85 block co-polymer. *FEBS Lett.* **389**:278–280 (1996).
16. C. W. Cho, Y. S. Cho, H. K. Lee, Y. I. Yeom, S. N. Park, and D. Y. Yoon. Improvement of receptor-mediated gene delivery to HepG2 cells using an amphiphilic gelling agent. *Biotechnol. Appl. Biochem.* **32**:21–26 (2000).
17. B. Jeong, Y. H. Bae, D. S. Lee, and S. W. Kim. Biodegradable block copolymers as injectable drug delivery systems. *Nature* **388**: 860–862 (1997).
18. F. Liu, J. Yang, L. Huang, and D. Liu. Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer. *Pharm. Res.* **13**:1642–1646 (1996).
19. J. Hartikka, L. Sukhu, C. Buchner, D. Hazard, V. Bozoukova, M. Margalith, W. K. Nishioka, C. J. Wheeler, M. Manthorp, and M. Sawdey. Electroporation-facilitated delivery of plasmid DNA in skeletal muscle: plasmid dependence of muscle damage and effect of poloxamer 188. *Mol. Ther.* **4**:407–415 (2001).

20. M. Y. Kozlov, N. S. Melik-Nubarov, E. V. Batrakova, and A. V. Kabanov. Relationship between Pluronic block copolymer structure, critical micellization concentration and partitioning coefficients of low molecular mass solutes. *Macromolecules* **33**:3305–3313 (2000).
21. A. V. Kabanov, P. Lemieux, S. Vinogradov, and V. Alakhov. Pluronic® block copolymers: novel functional molecules for gene therapy. *Adv. Drug Del. Rev.* **54**:223–233 (2002).
22. V. Alakhov, E. Moskaleva, E. V. Batrakova, and A. V. Kabanov. Hypersensitization of multidrug resistant human ovarian carcinoma cells by Pluronic P85 block copolymer. *Bioconjug. Chem.* **7**:209–216 (1995).
23. V. Alakhov and A. V. Kabanov. Block copolymeric biotransport carriers as versatile vehicles for drug delivery. *Exp. Op. Invest. Drugs* **7**:1453–1473 (1998).
24. G. M. Zentner, R. Rathi, C. Shih, J. C. McRea, M.-H. Seo, H. Oh, B. G. Rhee, J. Mestecky, Z. Moldoveanu, M. Morgan, and S. Weitman. Biodegradable block copolymers for delivery of proteins and water-insoluble drugs. *J. Control Release.* **72**:203–215 (2001).