# Novel Polymeric Micelles of Amphiphilic Triblock Copolymer Poly (*p*-Dioxanone-co-L-Lactide)-*block*-Poly (ethylene glycol)

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*Received August 13, 2003; accepted September 3, 2003*

***Purpose.*** The objective of this study is to characterize the micelles of novel block copolymer of poly (*p*-Dioxanone-co-L-Lactide)-*block*-Poly (ethylene glycol) (PPDO/PLLA-b-PEG-) and evaluate its ability to induce gene transfection.

***Methods.*** The ability of the block copolymer to self-assemble was determined by viscometery, dye solublization, NMR spectra and dynamic light scattering. The Trypan blue assay for *in vitro* biocompatibility of the block copolymer was carried out with NIH 3T3, CT-26 and MCF-7 cells, and β-glactosidase assay was applied to measure the transfection efficiency of the block copolymer on MCF-7 breast cancer cell.

***Results.*** Depending on the block lengths and molecular weights, solubility of the polymeric samples in water was varied. Diluted aqueous solution properties of the copolymer were studied. 1,6-Diphenyl-1,3,5-hexatriene solubilization and $^1$H NMR spectra carried out in $CDCl_3$ and $D_2O$, were used to prove the existence of hydrophobic domains as the core of micelle. Average particle size of 60–165 nm with low polydispersity, and lower negative ξ potential of –3 to –14 mV were observed on the aqueous copolymer dispersion. Copolymer was found with almost no cytotoxic effect and was able to promote the transfection efficiency (about 3-fold) in MCF-7 cells.

***Conclusions***. The PPDO/PLLA-b-PEG copolymer has ability to assemble into nanoscopic structures in aqueous environment, which enable to enhance gene transfection.

**KEY WORDS:** Poly (p-Dioxanone-co-L-Lactide)-*block*-Poly (ethylene glycol)-; polymeric micelles, cell transfection, β-glactosidase assay.

## INTRODUCTION

Association of block copolymers in a solvent which is poor for one block (block A) and good for the other block (block B) can produce structure like micelles and physical networks (1–4), which find potential application both in industry and basic research. Therefore, model polymers of low polydispersity have been prepared. These are linear water-soluble polymers, normally poly (ethylene glycol) (PEG) with hydrophobic end groups attached via ether, ester, or urethane (5) linkages. Studies on aqueous solution of these hydrophobically end-capped PEGs using techniques such as SANs, SAXS, fluorescence, light scattering, NMR relaxation, and ESR measurements (5–7) have been carried out. It has been found that at low concentration, two hydrophobic end groups associate in water to form "flower" type micelles, similarly to the poly (propylene oxide)-poly (ethylene oxide)-poly (propylene oxide) triblock copolymers in aqueous solution (3).

The low toxicity of PEG is attractive for biomedical applications (4). A Hydrophobically modified PEG consisting of hydrophilic PEG chain covalently attached to a hydrophobic aliphatic double chain moiety has been prepared (8). These amphiphilic polymers are widely used as diblock (9), triblock (10,11), or multiblock (11) copolymers, and bioactive gel with enhanced mechanical stability (8). The uniqueness associated with these copolymer systems largely originated from the choice of the hydrophobic block for micelle formation (15). Indeed, several biodegradable (e.g., poly (D,L-lactide) or poly (L-lactide), poly (lactide-co-glycolide) and poly (ε-caprolactone)), as well as nonbiodegradable (e.g. poly (propylene) biocompatible polymers have been employing as core forming blocks of micelle (15–17). The synthesis of amphiphilic polymers consisting poly (*p*-dioxanone-co-L-lactide) (PPDO/PLLA) hydrophobic moieties attached to the two ends of a PEG chain has been described as well, and it has been also found that the random insertion of the PPDO segments, well known biocompatible and biodegradable material (12), to the PLLA chain, improves biodegradability of the resulting copolymer (13,14). However, higher conversion of the PDO monomers into copolymer is still challenged due to lower reactivity at higher temperature (12,14).

Polyelectrolyte complexes formed between DNA and polycations have been used for gene transfer in mammalian and bacteria cells (18,19). Furthermore, commercially available cationic dendrimers and lipids (here Lipofectin®) that are known to improve gene expression *in vivo*/*in vitro* test. But, due to charge neutralization, these complexes are often unstable in aqueous solutions and precipitate, thereby hindering their application in gene delivery (19–21).

Recently, to overcome this effect, the works of Kabanov's groups (19–22) have greatly enhanced the development of Pluronic® block copolymer as polycation mediated gene transfer. In their work**,** Pluronic® block copolymer has been used either as "microcontainer" (micelle) or "biologic response modifier" (uimers) or both (28,29). But, Pluronic® block copolymer itself is not biodegradable. So, for this purpose, demands of highly disperse, nonionic, nanoscopic, highly biodegradable, as well as biocompatible micelle structured polymer has not yet been achieved completely. More or less to meet these demands, we synthesized a novel block copolymer formed from PPDO/PLLA-b-PEG-. To our knowledge, although there has only been one previous report on the use of this novel block copolymer as a cytokine delivery system for inducing bone formation (13), the aqueous solution properties have not been studied.

In this study we introduce aqueous solution property of a novel block copolymer system bearing random sequence of PPDO/PLLA segments. Dihydroxyl PEG is end capped with this moiety thus making ABA type linear associative polymer where A and B represent PPDO/PLLA segment and a PEG

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0724-8741/03/1200-2021/0 

chain, respectively. The chemical structure of the associative polymer is shown in Fig. 1. Solubility and phase separation temperature in aqueous solution of the copolymer is investigated. The aqueous solution properties associated micelle formations of the new amphiphilic copolymer at low concentration are investigated by viscometry dye solubilization, NMR spectra, and dynamic light scattering. Furthermore, cytotoxicity and transfection efficiency of the block copolymer are investigated.

## MATERIALS AND METHODS

### Chemicals

Poly (ethylene glycol) (PEG) (with number average molecular weights of 1000, 1500, 2000, 3400, 4000, and 10000 g $mole^{-1}$), L-Lactide (LLA) and 1,4-dioxan-2-one (PDO) were purchased from Aldrich, Boeringer Ingelheim (Germany) and Biopolytech (Korea), respectively. Stannous octoate ($Sn(Oct)_2$; Stannous 2-ethyl hexanoate) from Aldrich was used after dissolving it in pure and dry toluene (0.0386 moles/liter). The (PPDO/PLLA)-b-PEG- bock copolymers were synthesized as previous methods (14). In brief, block copolymers were synthesized by adding monomers PDO and LLA to predried poly (ethylene glycol) in presence of stannous octoate catalyst. Triblock copolymers (A-B-A type) were formed by ring opening polymerization of PDO and LLA where A and B segments are from PPDO/PLLA and poly (ethylene glycol) PEG, respectively (see Fig. 1). A complete discussion of the molecular characterization has been reported previously (14). Phosphate-buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), RPMI1640, Fetal Bovine Serum (FBS), Antibiotics (penicillin and streptomycin), and Trypsin and Trypan blue were the products of Gibco-BRL (Grand Island, NY). CT-26, NIH3T3, and MCF-7 cells were supplied from the Korean cell Line Bank (Seoul, Korea). Commercial transfection reagent, Lipofectin® was obtained from Sigma Chemicals. Reagents used in β-galactosidase assay and X-gal staining, were obtained from Promega and prepared as recommended by respective product protocols.

### Viscometry

The viscosity measurements were carried out with Ubbelohde type viscometer with a capillary of 0.45 mm diameter. The solution was filtered prior to any measurements. The apparatus was thermostated at 25°C. Reduced viscosity ($\eta_{red}$) of the polymer solution is the ratio of specific viscosity ($\eta_{sp}$) to the concentration (C) of which is measure of the specific capacity of the polymer to increase the relative viscosity (i.e., (a) $\eta_{red} = \eta_{sp}C$ and (b) $\eta_{sp}$ measured according to following relation, $\eta_{sp} = t - t_0 / t_0$. Where, t and $t_0$ are, efflux times for the solution and pure solvent, respectively.

### Dye Solubilization

A number of aqueous solution of the copolymer concentration, ranges from $0.5 \times 10^{-3}$ to $20 \times 10^{-3}$ g/ml, were prepared. Twenty-five microliters of a 0.4-mM solution of 1,6-diphenyl-1,3,5-hexatriene (DPH) in methanol were added to each 2.5 mL of the copolymer solution. Solutions were incubated in dark for 16 h. The absorbance spectra in the range $\lambda = 300–500$ nm were recorded on a UV-VIS spectrometer (Model 550S, Perkin–Elemer, USA). Main absorption intensity peak, characteristic of DPH solubilized in a hydrophobic environment, was at 356 nm. The measurements were done at room temperature.

### Nuclear Magnetic Resonance (NMR) Spectroscopy

$^1H$ NMR spectra of the block copolymer were recorded on FT-NMR spectrometer (JNM-Ex, Japan) in $CDCl_3$ and $D_2O$ at 25°C.

### Determinations of Particle Size

Aqueous dispersions of the copolymer were prepared by precipitation/solvent evaporation technique. A solution of the copolymer (3–100 mg $ml^{-1}$) in acetonitrile was added to the aqueous phase, and the acetonitrile allowed to evaporate overnight under slight vacuum. All dispersions were filtered using disposable 1.2 μm filter (Sartorus JAG Co, Germany). Thus, prepared aqueous dispersion of the copolymer was used for all experiments. Size of the particles was determined by dynamic light scattering, using a Malvern System 4700 instrument with vertically polarized light supplied by an argon-ion laser (Cyonics), operated at 20 mW. All experiments were performed at 25°C with measuring angle of 90° to incident beam. The correlation decay functions were analyzed by the cumulants method to determine average particle diameter and polydispersity (defined as the variance of the log normal distribution of particle sizes). Constrained regularized CONTIN method was used to obtain particle size distribution.

### Determination of ζ Potential

The ζ potential of the particle aqueous dispersion was determined by Electrophoretic light scattering spectrophotometer (ELS 8000/6000 Otsuka electronics Co., Japan). Measurements were performed at 20°C on samples appropriately diluted with deionized water (four times of the initial volume).

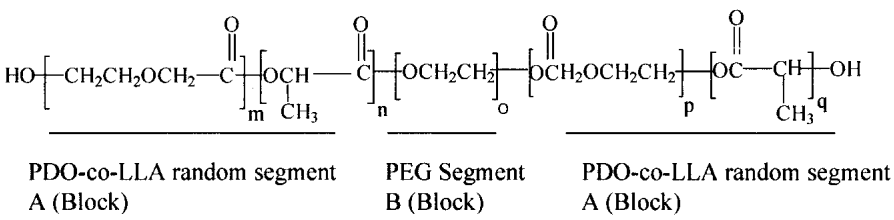


**Fig. 1.** Molecular structure of the block copolymer chain. The subscripts m, n, o, p and q represent the variable number units.

### Amplification and Purification of Plasmid DNA (pcDNA3.1/His/Myc/lacZ)

Plasmid DNA encoding lacZ gene was used as the reporter gene in this study. The plasmid (pcDNA3.1 - LacZ) was transformed into *E. coli* JM109 bacterial strain. The transformed cells were grown in large quantities of LB broth supplemented with Ampicillin (100 μg/ml). The plasmid DNA was purified by phenol-chloroform and was diluted with sterilized water. Purity was conformed by 1% Agarose gel electrophoresis followed by Ethedium bromide staining, and DNA concentration was measured by UV absorption at 260 nm.

### Cytotoxicity Study

Toxicity evaluation of the aqueous copolymer dispersion was determined by cell viabilities of NIH 3T3; MCF-7 and CT-26 cell, *in vitro*. Various cell suspensions containing $1 \times 10^4$ cells in RPMI-1640 for NIH 3T3 cell and DMEM for MCF-7 and CT-26, containing 10% FBS were distributed in a 24-well plates, and incubated in a humidified atmosphere containing 5% $CO_2$ at 37°C for 24 h. After removing the medium, different concentrations of the block copolymer dispersion were added to the 24 well plate, and were incubated in a humidified atmosphere containing 5% $CO_2$ at 37°C for 3 h. The cell culture was washed with PBS solution, and fresh respective media solution was added to the plates. Amount of live cells was counted by Trypan blue method, after 24 h. The extent of cytotoxicity on the aqueous block copolymer suspension was defined on the relative viability, which correlates with the amount of viable cells in relation to the cell control (=100%).

### *In Vitro* Transfection and β-Galctosidase Activity

MCF-7 cells (breast cancer cell) were used to measure transfection efficiency with DNA/Lipofectin® and DNA/Lipofectin®/block copolymer. MCF-7 cells were cultured in complete DMEM medium at 37°C and 5% $CO_2$. These cells were seeded in 10 cm dishes ($2.5 \times 10^5$ cells per dish), and incubated for 24 h before the transfection test. The monolayer of cells (70–80% confluence) was washed with PBS (pH 7.4) shortly before transfection. For analysis, stock solution of plasmid DNA (0.6 mg/ml), Lipofectin® (1mg/ml) and block copolymer dispersion (33.3 mg/ml) were used. Various amounts of Lipofectin® were added to 5 μg aliquot of plasmid DNA to optimize ratio (w/w) between Lipofectin® and DNA. A mixture of Lipofectin® and DNA was equilibrated for 30 min at room temperature and the mixture was subjected to electrophoresis in 1% agarose gel. The optimal ratio of 1/3 (w/w) between DNA and Lipofectin® was determined where DNA did not migrate into the gel because of the complex formation with Lipofectin®.

To study the gene transfection efficiency of the block copolymer, different volumes of the aqueous copolymer solution (20 to 1000 μl) from the stock (33.3 mg/ml) were added to fixed amount of Plasmid DNA/Lipofectin® complex (1/3 w/w). Before the cell transfection, plasmid DNA/Lipofectin®/block copolymer at various weight ratios were diluted in serum and antibiotic free DMEM media with final volume 3 ml so that the concentration of the copolymer ranged from 0.22–11 mg/ml. After adding this solution to the culture plate, cells were incubated for 3 h. Then, the incubation medium was replaced by a complete DMEM medium and the cells were cultured for an additional 48 h (37°C and 5% $CO_2$). After 48 h of transfection, monolayers of cell in 10 cm plates were washed with PBS and fixed by fixing solution for 5 min. Expression of LacZ gene was established by incubation of fixed cells with X-gal staining solution for 2 h at 37°C. Using a light microscope, transfected cells were made visible as blue spots. To quantify the DNA uptake or transfection yield, the β-galactosidase assay method was used. The total protein concentration of the cell lysates was determined using the Bradford method (23).

## RESULTS AND DISCUSSIONS

Various compositions of amphiphilic copolymers, comprised hydrophobic poly(p-dioxanone-co-L-lactide) PPDO/PLLA and hydrophilic poly(ethylene glycol) PEG, were previously synthesized via ring opening polymerization. Table I lists the number average molecular weights (Mn), polydispersity (Mw/Mn), thermal characteristics, and water solubility of the copolymer corresponding to their compositions. Copolymers showed the different trend of solubility in water at 15°C up to the concentrations 25 wt% depending upon the block

**Table I.** Triblock Copolymers of PDO and L-lactide with PEG

| Copolymer (PDO/LLA/PEG) | Mn of parent PEG | $M_n$ (a) | PDI (b) | $T_m$ (°C) (c) | Solubility (d) | 2nd run DSC data of the copolymer (°C) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | $T_m$ | $T_g$ | $T_c$ |
| B8 (20/20/60) | 1000 | 2100 | 1.43 | 15 | Soluble | 11 | –35 | –12 |
| B10 (17/18/65) | 1500 | 3300 | 1.42 | 20 | Soluble | 22 | –35 | –10 |
| B12 (15/18/67) | 2000 | 5000 | 1.21 | 25 | Soluble | 28 | –36 | — |
| B14 (14/19/67) | 3400 | 8000 | 1.13 | 45 | Soluble | 48 | –35 | — |
| B15 (15/20/65) | 4000 | 10000 | 1.11 | 46 | White suspension | 37 | –37 | — |
| B16 (10/20/70) | 10000 | 19000 | 1.32 | 48 | White suspension | 47 | –35 | — |
| 1F (30/30/40) | 1500 | 8000 | 1.2 | — | — | — | — | — |
| LPL1[(e)] (0/50/50) | 10000 | 17000 | 1.3 | — | — | — | — | — |

Copolymer composition ratio measured form 1H NMR. (a) and (b) are the number average molecular weights of the copolymers and polydispersity index (Mw/Mn) of the copolymers evaluated by GPC using polystyrene narrow standards (16), respectively (c) is the melting points of the copolymer at the first heating scan of DSC. (d) is the solubility of triblock copolymer (25%) in water at 15°C (e) Copolymer of PLLA-b-PEG- with mole ratio 50:50. Tm, Tg, Tc represent the melting temperature, glass transition temperature and crystallization temperature of the copolymers, respectively.

lengths and copolymer molecular weights. Aqueous solution of the copolymers (i.e. B8 to B14) having both short PPDO/PLLA and PEG segments were transparent, whereas the copolymers (B15 and B16) having relatively longer segment of PPDO/PLLA and PEG gave white suspension at the same conditions. Transparent aqueous solutions of B10, B12, and B14 below room temperature were also turned to white suspension at elevated temperature (14). In case of white dispersions, precipitation was formed after 1–2 h. According to the $^1$H-NMR spectra, the precipitation did not result from the composition changes because precipitate and corresponding original compounds showed the similar spectra. These unique behaviors of the triblock copolymer in an aqueous medium are based on the fact of an increase in temperature promotes hydrophobic interactions between the hydrophobic moieties (PPDO/PLLA) in the polymer chain resulting in the phase separation. The appearance of turbidity reflects the micelle aggregation, as expected from amphiphilic triblock copolymer with convenient block lengths (1,11,24).

The amphiphilic nature of these copolymers, and their capability to aggregate into nano-sized structure were further evaluated. The variation of the reduced viscosities, $\eta_{red}$ of the block copolymers, and its unmodified PEG 10,000 [PEG10K] in dilute aqueous solution is shown in Fig. 2. The concentration dependence of $\eta_{red}$ of PEG10K was linear and gave an intercept, corresponding to the limiting viscosity of 16 ml/g. In contrast to the PEG10K, the concentration dependence of the $\eta_{red}$ of the copolymer was not linear; at low concentration its viscosity was slightly lower than that of PEG10K, whereas at higher concentrations it increases. Such behavior could be explained by assuming that at low concentrations, ring-shaped macromolecules were formed due to intra-chain interactions. These types of macromolecules have smaller dimension than the linear ones at equal molecular weight (25). In higher concentration, formation of polymolecular aggregates took place. The aggregates were assumed to consist of hydrophobic interior surrounded by a hydrophilic corona built up of PEG chains in a looping conformation. On a further increase of the concentration, the viscosity increased due to bridging interaction between aggregates (1).

A dye solubilization method was used to prove the existence of hydrophobic domains. DPH was a probe molecule. DPH solubilization has been used previously for determination of the critical micelle concentration (CMC) of nonionic amphiphilies (26). DPH has a significantly lower absorptivity at 356 nm in an aqueous environment compared with that in the hydrophobic environment. Therefore, with the formation of micelles, the hydrophobic dyes are preferentially partitioned into the hydrophobic core of the micelles, which results in the increase in absorbency of the dye. A typical plot of DPH absorbance at 356 nm vs. log of copolymer concentration is presented in Fig. 3. A CMC of $7.8 \times 10^{-3}$ g/ml was determined. The value fits nearly close to the onset of the viscosity increase ($6.5 \times 10^{-3}$ g/ml).

To further characterize micelle formation, the $^1$H-NMR spectra of the triblock copolymer taken in $D_2O$ and $CDCl_3$ were compared (Fig. 4). All the characteristic peaks resulted at Fig. 5A are based on the basic chemical structure of the triblock copolymer PPDO/PLLA-b-PEG- (16). The spectrum of Fig. 5A can be compared to that of suspension of the copolymer in $D_2O$, shown in Fig. 5B. In the later spectrum, the peaks correspond to PDO and LLA repeating units are not visible. The absence of peaks for the hydrophobic segment indicates limited mobility owing to association of these segments. This is in agreement with the structural model that these residues were part of the solid core of the micelle particles. Of particular interest for the purpose of this study is the ethylene peak of the PEG residues. This peak is highly visible in both spectra presented. It is sharp and well resolved both in $CDCl_3$ and $D_2O$. Chloroform is a good nonselective solvent for both PEG and PPDO/PLLA segments whereas water is a good selective solvent for PEG but poor for PPDO/PLLA. The characteristic features in the Fig. 4 are consistent with the view that the structure of the miceller particles, when suspended in an aqueous solution, is indeed of the core-corona type with the PEG chains extending out into aqueous environment (16).

Average particle size and polydispersity of the PPDO/PLLA-b-PEG- particles are presented in Table II. All of the copolymer produced with low polydispersity particulate dispersion with a clear trend of increasing particle size with increased concentration of the polymer in organic phase. This is

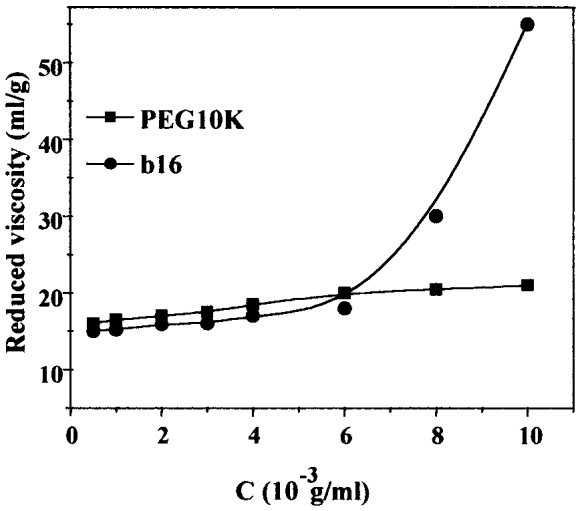


**Fig. 2.** Variation of the reduced viscosity of PEG10K and the copolymer B16 as a function of concentration in aqueous solution at 25°C.

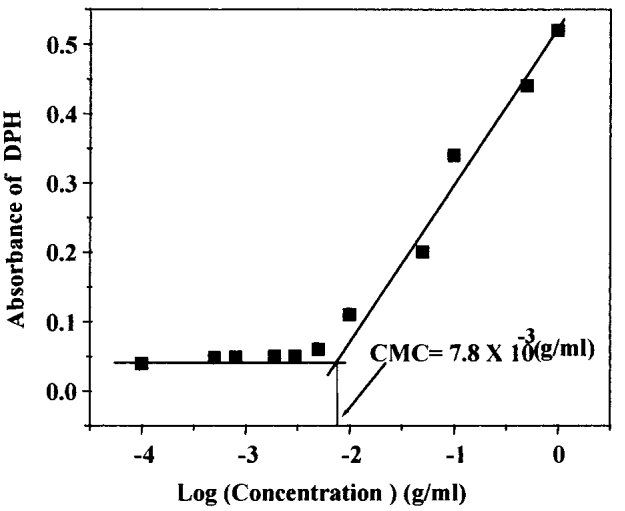


**Fig. 3.** Variation of the absorbance of DPH at 356 nm as a function of the triblock copolymer (B16) concentration in aqueous solution.

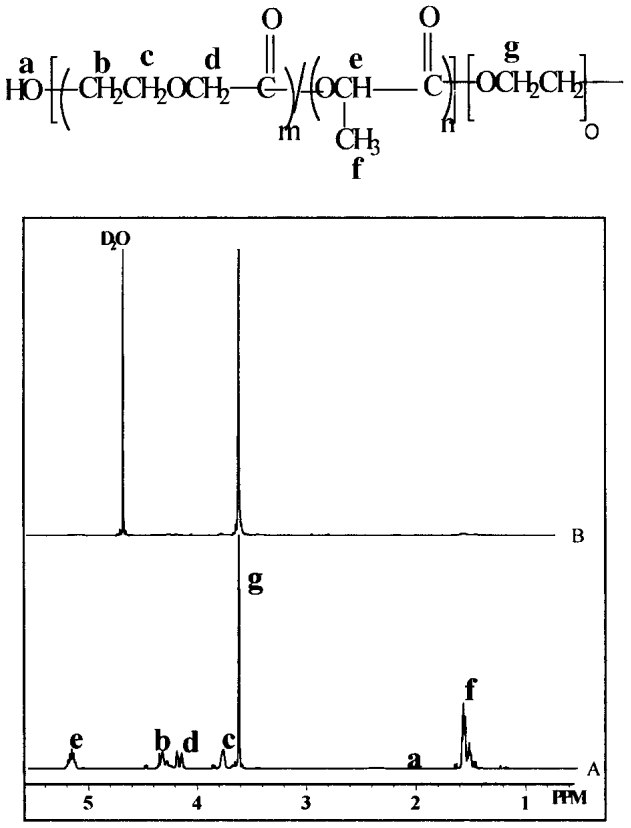


**Fig. 4.** $^1$H-NMR spectra of PPDO/PLLA-b-PEG- of B16 (A) in $CDCl_3$ and (B) in $D_2O$.

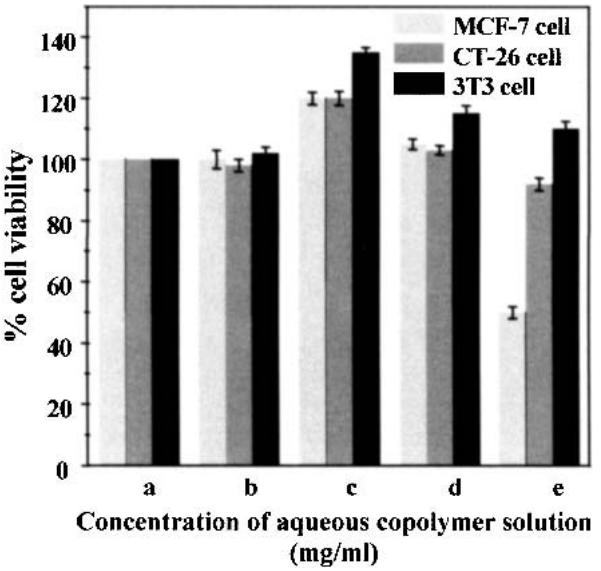


**Fig. 5.** Relative cell viability: $1 \times 10^4$ of three different cells in respective medium containing 10% FBS were incubated with block copolymer suspension (i.e. B16) in 24 well plate in a humidified atmosphere containing 5% $CO_2$ at 37°C for 48 h, (a) control, (b) 0.3 mg/ml, (c) 0.6 mg/ml, (d) 1.2 mg/ml, and (e) 3 mg/ml copolymer, respectively. Final volume of the copolymer suspension used was 500 μl in respective media solution. Mean ± sd (n = 3).

expected based on theoretical approaches to the structure of polymeric micelles (1,2), and can be related with the change of association mode of the copolymer chains as described earlier. It can also be concluded from the results of Table II that the particle sizes depended roughly on the ratio of PPDO/PLLA segment length to PEG segment (i.e. B17, B15, and 1F).

The ξ potentials of the triblock copolymers are presented in Table II. It was not possible to measure the ξ potential of the aqueous copolymer suspension with particle size less than 60 nm in diameter, due to an insufficient scatter light intensity. It is obvious that, the large negative ξ potential of PLLA (i.e. –50 mV), PLGA (–52 mV) and PLLA-b-PEG block copolymer with higher fraction of PLLA segment has been attributed to the presence of ionized carboxyl groups on the nanoscopic sphere surface (3,4,30). Nanoparticles produced from the triblock copolymers were expected to have negligible surface charge, because the carboxyl acid end groups of the PDO/LLA segments are capped by the PEG segment. Presence of hydrophilic PEG steric barrier shifts the hydrophobic PPDO/PLLA core away, and this would also result in a reduced ξ potential. The copolymer B16 shows lower negative ξ potential than the copolymer 1F and LPL1. This could be attributed to the presence of few carboxyl groups on the surface of the particles of B16.

The ability of the aqueous dispersion of the copolymer with ξ potential –3 to –5 mV was further studied as enhancer for Lipofectin® mediated cell transfection. Plasmid DNA has negative surface charge ranges from –17 to –20 mV and the recently available nonionic surfactant copolymer (triblock copolymer of propylene oxide and ethylene oxide) with the surface charges –4.8 ± 1.7 mV (29) can effectively enhance the gene transfections *in vivo*. Although positive surface charge of the transfecting carrier is believed to be an important factor in the complexes association with the plasma membrane, our block copolymer dispersion in aqueous medium with

**Table II.** Hydrodynamic Diameter, Polydispersity and ξ Potential of the PPDO/PLLA-b-PEG particles in Aqueous Solution

| Copolymers[a] | Particle size (nm) mean ± SD | Polydispersity mean ± SD | ξ Potential (mV) mean ± SD |
|---|---|---|---|
| B16 (3 mg/ml) | 60 ± 2.5 | 0.15 ± 0.02 | — |
| B16 (10 mg/ml) | 70 ± 2.7 | 0.12 ± 0.03 | –3 ± 0.8 |
| B16 (100 mg/ml) | 152 ± 4 | 0.09 ± 0.01 | –5 ± 1.1 |
| B15 (3 mg/ml) | 80 ± 1.5 | 0.13 ± 0.03 | — |
| 1f (3 mg/ml) | 150 ± 3.5 | 0.16 ± 0.04 | –10 ± 1.5 |
| LPL1 (3 mg/ml) | 165 ± 5 | 0.11 ± 0.01 | –14 ± 1.8 |

[a] Different weights of the block copolymer dissolved in 1 ml of organic phase.

lower ξ potential (–3 to –5 mV), could still enhance the Lipofectin® mediated transfection.

The application of the present block copolymer as enhancer can be limited if cytotoxic side effects caused by either the materials itself or their degradation products. The present copolymer has been known to be biodegradable. Thus, they were appropriate as model transport systems, especially if the side effects from the carrier itself were intended to be negligible. Figure 5. shows the representative data of cytotoxicities from three different experiments with increasing concentration of the copolymer suspension per cell line. The copolymer showed no significant toxicity on the cells. The cell viabilities in the presence of copolymer suspension ranged between 75–110% of the control in all experiments. At a maximum copolymer concentration 3 mg/ml, the mean cell viabilities of the three different cell lines showed about 75–110% viability compared with that of the control. It was shown that the copolymer suspension was not toxic to the cell. It has been investigated that the cytotoxicity correlates with membrane damage effect. Most of the polycations can bind to the negatively charged plasma membrane and destabilize them. To overcome this effect, nonionic amphiphilic copolymer like the present copolymer PPDO/PLLA-b-PEG may reduce membrane toxicity through suppression of the interaction of the cell membrane. But, in high concentration (>3 mg/ml) of the block copolymer may aggregate, and accumulate around the cell membrane, and interfere the normal biologic process, which may lead the cytotoxic effect. Moreover, present biodegradable block copolymer that can be hydrolyzed in aqueous phase, and may not prolong the cytotoxicity even in high concentration.

Although we designed three different cell lines (NIH 3T3, CT-26, and MCF-7) to evaluate the transfection efficiency of the block copolymer either with plasmid DNA or with DNA/Lipofectin®, results were taken only from MCF-7 cell line. We found higher β-galactosidase activity on MCF-7 cells than that observed from two other cell lines (results not shown). The internalization of the plasmid DNA correspondence to the expression of blue color after fixing (5 min) and staining (2 h) or X-gal activity was observed on MCF-7 cell. We observed the blue color through light microscope when cells were incubated with DNA/Lipofectin® or DNA/Lipofectin® with different concentration of the block copolymer suspension, but that color was not found either with free DNA or DNA with copolymer incubated cell (results are not shown) (n = 5).

High internalization (plasmid uptake) corresponds to the higher value of X-gal activity which was significantly increased when the Plasmid DNA/Lipofectin® complex was mixed with 1.2 to 1.4 mg/ml aqueous copolymer solution as compared with the DNA/Lipofectin® (as control) in MCF-7 cells (Fig. 6). At optimum concentrations of the block copolymer suspension, the internalization or plasmid uptake was about 3-fold than that of the control. This is our promising result; so far we are unable to predict the mechanism of action of the present block copolymer, which remains to be further explored. However, the copolymer concentration that showed significant enhancement for the transfection was below CMC value (7.8 mg/ml). Like Pluronic® block copolymer unimers, the present novel block copolymer unimers may probably involve an important role, either interaction with the cell membrane resulting in the nonspecific changes on membrane

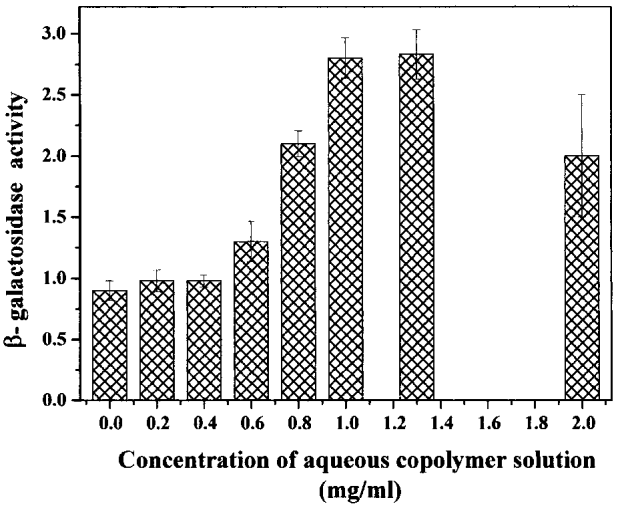


**Fig. 6.** Transfection efficacy using β-galactosidase assay on MCF-7 cells with different concentration of aqueous copolymer suspension with fixed ratio of DNA (5 μg)/Lipofectin® (15 μg). Activity of β-galactosidse was assessed by the ONPG method. Control means (absence of copolymer) DNA/Lipofectine® treatment on the cells. Transfection efficacy with the copolymer concentration above 2 mg/ml was not significant and is not shown in the figure. Mean ± s d (n = 3).

properties (such as ion transport potential and possibly fluidity) or destabilizing the endosomal environment (27,28).

In this study however, only the B16 copolymer is presented in detail, one can adjust the copolymer system for best performance as transfection enhancer and delivery situation from the given set of copolymers (see Table I) differing in hydrophobic/hydrophilic segment length and CMC.

## CONCLUSION

This article describes the self-association behavior of a novel PEG-based amphiphilic triblock copolymer, which consists of PPDO/PLLA as hydrophobic moieties joined at the two ends of the chain. Depending on the block length and the copolymer molecular weights, water solubility of the copolymer was varied from transparent clear solution to white dispersion solution. Various experimental methods were studied to prove the micelle-forming tendency of the copolymer. Clear trend of increasing the particle size with copolymer concentration in organic phase was found from DLS results. Copolymer dispersions in water showed nanoscopic structures with low polydispersity, lower negative ξ potential and almost no cytotoxic effect. The bock copolymer (PPDO/PLLA-b-PEG-) efficiently enhanced the polycation (Lipofectin®) mediated cell transfection at the concentration below the CMC, about 3-fold on MCF-7 breast cancer cell. Based on earlier mentioned results, the bock copolymer (PPDO/PLLA-b-PEG-) with the appropriate characteristic feature may challenge as enhancer material next to the Pluronic® block copolymer, and could be applied to improve current protocol (precipitation/aggregation) for polycation mediated cell transfection.

## ACKNOWLEDGMENTS

This study was supported by a grant from the Korea Health 21 R and D Project, Ministry of Health and Welfare, Republic of Korea (HMP 01PJ1PG3 31400 0013)

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