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International Journal of Pharmaceutics 326 (2006) 169–173
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Pharmaceutical Nanotechnology

Dexamethasone nano-aggregates composed of PEG–PLA–PEG triblock copolymers for anti-proliferation of smooth muscle cells

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Received 13 April 2006; received in revised form 1 June 2006; accepted 28 June 2006
Available online 1 July 2006

Abstract

Dexamethasone nano-aggregate was prepared for the treatment of intimal hyperplasia caused by abnormal proliferation of smooth muscle cells. Triblock copolymers composed of poly(ethylene glycol) [PEG] and poly(D,L-lactic acid) [PLA] were synthesized with different chain lengths of PEG. Triblock copolymers in organic phase were mixed with dexamethasone and dexamethasone nano-aggregates was prepared by dispersing the organic phase into water. The average diameter of the nano-aggregates ranged from 200 to 300 nm. Dexamethasone was released out from the nano-aggregates and the release profile was dependent on PEG chain lengths. The dexamethasone nano-aggregates showed superior anti-proliferation effects on smooth muscle cells compared to dexamethasone. Flow cytometry showed that smooth muscle cells treated with dexamethasone nano-aggregates was arrested at a dormant phase in a dose-dependent manner. The dexamethasone nano-aggregates are expected to be a potent candidate for anti-proliferating smooth muscle tissues after a balloon-catheter treatment.

Keywords: Dexamethasone; Nano-aggregate; Smooth muscle cell; Anti-proliferation

1. Introduction

Dexamethasone, a synthetic glucocorticoid, and other corticosteroids are known to have a wide variety of biological effects, especially in modulation and suppression of immune function (Clagett et al., 1986; Lincoff et al., 1997; Radke et al., 2004; Dixon et al., 1999; Reil et al., 1999). Because dexamethasone has been shown to inhibit the function of lymphocytes, fibroblasts, macrophages and other immune cells, restenosis after vascular interventions has been recently treated with dexamethasone to eliminate neointimal hyperplasia resulted from the abnormal migration and proliferation of medial smooth muscle cells (SMC) (Dixon et al., 1999; Lincoff et al., 1997; Yoon et al., 2003). Dexamethasone, however, is hydrophobic and insoluble in an aqueous phase organic solvents are often employed to dissolve in water phase, resulting in poor drug efficacy when it is administered with a balloon-catheter. Therefore, it has been indispensably required to devise a novel carrier solubilizing dexamethasone in aqueous phase.

Copolymers consisting of both hydrophobic and hydrophilic blocks tend to form micellar aggregates in aqueous phase to reduce free energy from hydrophobic interactions between hydrophobic chains (Tang et al., 2003; Yokoyama et al., 1992; Yoo and Park, 2001). The size of micellar aggregates normally ranges from 50 nm to several hundred nanometers in a diameter. They have received much attention as pharmaceutical materials for their unique structures called ‘core and shell’. While the hydrophilic chains of the polymer are exposed to outer medium to form the shells, the hydrophobic blocks buried inside of the nano-aggregates to form the cores. Many researchers have focused on encapsulate hydrophobic drugs within this core regions to render them be solubilized in aqueous phase, enhancing drug efficiencies in clinical applications (Lee et al., 2004; Jeong et al., 2000, 1997; Tang et al., 2003; Yoo and Park, 2001). Among them, doxorubicin was widely encapsulated within micellar aggregates or nano-particulates to enhance its solubility in aqueous phase and pharmacokinetics. PEG–PLGA block copolymer was employed to prepare doxorubicin-conjugated diblock copolymer, spontaneously forming micellar aggregates in an aqueous solution (Kwon et al., 1995; Yoo and Park, 2001).

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0378-5173/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2006.06.041

In this study, A–B–A type triblock copolymers with different chain lengths were formulated to dexamethasone nano-aggregates for the purpose of anti-proliferating smooth muscle cells. Physiochemical characteristics of the nano-aggregates were evaluated in terms of their sizes and critical micelle concentrations. Feasibilities as an anti-proliferating agent were further investigated by an in vitro release study and an anti-proliferation assay on smooth muscle cells by flow cytometry and a cytotoxicity assay.

2. Materials and methods

2.1. Materials

D,L-Lactide, methoxy poly(ethylene glycol) [mPEG] were purchased from Aldrich (St. Louis, MO) and Polysciences (Warrington, PA). Dexamethasone, pyrene, hexamethylene dissocyanate (HMDI), and 3-(4,5-dimethylthiol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Carlsbad, CA). A thoracic aorta smooth muscle cell from DB1X rat was obtained from the Korea Cell Line Bank (Seoul, South Korea). All other chemicals were of analytical grades.

2.2. Preparation of PEG–PLA–PEG triblock copolymers

Triblock copolymer was synthesized as described previously in literatures with a minor modification (Jeong et al., 1997). Briefly, ring opening polymerization of D,L-lactide onto mPEG followed by coupling of resulting diblock copolymer using HMDI resulted in PEG–PLA–PEG triblock copolymers. For PLA–PEG diblock copolymer, 4 g of D,L-lactide and 1 g of mPEG were completely dissolved at 140 °C under nitrogen atmosphere for complete melting in the presence of 0.03% of stannous octoate. After 6 h, the reaction mixture was diluted with acetone and precipitated twice in an ice-cold diethylether. The synthesized diblock copolymer (1 g) dissolved in methylene chloride (1 ml) was coupled to triblock copolymers in the presence of HMDI as previously described (Jeong et al., 1997). The synthesized triblock copolymer was precipitated in an ice-cold diethylether and dried under vacuum. ^{1}H NMR was performed to confirm number average molecular weights of triblock copolymers and a gel permeation chromatography was performed to measure weight average molecular weight of triblock copolymers. Chloroform was used as a mobile phase and a refractive index detector was used to detect block copolymers in a mobile phase. The numbers of PEG repeating units used in this study were 16, 48 and 121.

2.3. Critical aggregation concentration of triblock copolymers

Critical aggregation concentration was monitored by employing pyrene as an extrinsic probe as previously described (Yoo and Park, 2001). Varying amounts of three triblock copolymers in methanol (1 ml) were mixed with 9 ml of distilled deionized water (DDW) in the presence of 10 μg of pyrene. After completely evaporating the organic phase, fluorescence intensity ratio (391 nm/374 nm) was monitored at an excitation wavelength of 280 nm.

2.4. Preparation of nano-aggregates containing dexamethasone

Nano-aggregates were prepared by a spontaneous solvent diffusion and evaporation method as previously described with a minor modification (Yoo and Park, 2001). Ten milligrams of dexamethasone was completely dissolved in 1 ml of methanol and mixed with 90 mg of triblock copolymers dissolved in 9 ml of acetone. The organic phase was directly dispersed in distilled water with vigorous stirring at 3000 rpm. After evaporating the organic phase for 3 h with gentle stirring, insoluble dexamethasone aggregates were removed by centrifugation at 1000 rpm for 5 min. The supernatant was recovered and stored less than 4 °C for further use.

2.5. Characterization of dexamethasone nano-aggregates

In order to measure loading amounts of dexamethasone, freeze-dried dexamethasone nano-aggregates was dissolved in acetone and gently centrifuged at 1000 rpm for 5 min to collect dexamethasone. After discarding the supernatant and drying out the organic solvent, dexamethasone was redissolved in methanol and the absorbance was subsequently measured at 239 nm. Dynamic light scattering confirmed average diameters of dexamethasone nano-aggregates in aqueous phase (Zetaplus, Brookhaven Instruments Inc., UK). All experiments were performed in a triplicate manner.

2.6. In vitro release of dexamethasone from dexamethasone nano-aggregates

The release profile of dexamethasone form dexamethasone nano-aggregates was examined by a dialysis method. One milliliter of dexamethasone nano-aggregates (1 mg/ml) was poured into a dialysis membrane (SpectraPor 6, M_w cut-off = 10,000) and subsequently incubated in 100 ml of phosphate buffered saline (PBS) at 37 °C with gentle stirring. Released dexamethasone in outer medium was completely freeze-dried for 24 h and redissolved in 0.1 ml of methanol. The released amount of dexamethasone was measured at 293 nm.

2.7. Flow cytometry of smooth muscle cells

Thoracic aorta smooth muscle cells (SMC) from DB1X rat (1×10^4 cell/ml) were incubated in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C. The SMC at a logarithm phase was synchronized by serum deprivation for 24 h. The synchronized SMC was subsequently recovered to a normal cell cycle by supplementing 10% FBS in DMEM. After 1 h, 0.1 mg of dexamethasone nano-aggregates were added to SMC in six-well culture dishes. After 3 h of further incubation, SMC

Table 1
Triblock copolymers composed of PEG and PLA

Samples	Polymer composition	M_n	M_w	M_w/M_n	Composition ratio of PLA/mPEG
PEG16	mPEG16–PLA153–mPEG16	26200	33800	1.29	33.50/8.34
PEG48	mPEG48–PLA153–mPEG48	29600	34900	1.18	22.61/15.81
PEG121	mPEG121–PLA153–mPEG121	31000	38000	1.2	24.71/22.28

The number of repeating units is shown in polymer compositions.

was removed from the dish by trypsinization and washed with PBS three times. Flow cytometry was performed as described previously (Reil et al., 1999). Briefly, 100 μl of propidium iodide (1 mg/ml) was added to each sample and SMC was examined under a FACSCalibur (BD Biosciences, NJ, USA) to assess cell cycle stage. SMC with FSC and SSC values between 100 and 300 was selectively gated to further analysis.

2.8. Thymidine uptake

The proliferation of SMC was quantitated by determining the amount of incorporated [^{3}H] thymidine (GE Life Sciences, NJ, USA) during a DNA synthesis. SMC was synchronized by feeding them with DMEM without FBS. After 24 h of synchronization, the synchronized SMC was fed again with DMEM supplemented with 10% FBS to proliferate SMC. After 1 h, 0.1 mg of dexamethasone nano-aggregates and 0.1 μCi of [^{3}H] thymidine was added to each well of SMC in a six-well culture dish. After 12 h, SMC was washed twice with cold PBS and 1% cold trichloroacetic acid (TCA). SMC was transferred to a scintillation vial and a relative radioactivity was measured by a scintillation counter (Beckman, USA).

3. Results and discussion

Synthesized triblock copolymers used in this study was summarized as shown in Table 1. A number average molecular weight and a weight average molecular weight were determined by ^{1}H NMR and gel permeation chromatography, respectively. Three triblock copolymers had the same length of PLA having different length of PEG segments at both ends.

Upon contacting with an aqueous phase, block copolymers spontaneously form nano-sized aggregates, exposing hydrophilic segments outside while hiding hydrophobic ones inside of the nano-aggregates (Kwon et al., 1995; Yoo and Park, 2001). Therefore, critical aggregation concentrations (CAC) of triblock copolymers were investigated to assess the effects of PEG chain lengths on CAC, as shown in Fig. 1. Although three triblock copolymers seemed to have the similar CAC, the significant difference was still shown among those three polymers. PEG16 showed the lowest CAC, followed by PEG48 and PEG121 (6.1, 6.6 and 7.2 μg/ml, respectively). This could be attributed to the increase of the PEG chain length. In order to form nano-aggregates in aqueous solution, hydrophobic segments should be associated to form a thermodynamically stable complex. However, PEG at each end of PLA, a hydrophobic segment inhibited their associations because of its hydrodynamic volume in aqueous solution. Considering PEG is widely used as a repellent for protein adsorption to biocompatible materials, PEG chains in the triblock copolymers delayed formations of nano-aggregates in an aqueous solution.

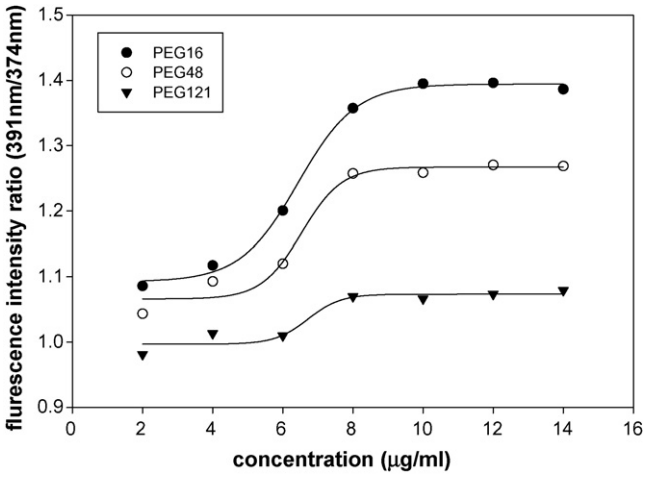

Fig. 1. Critical aggregation concentration of triblock copolymers in aqueous solution.

Average diameters of dexamethasone nano-aggregates measured by dynamic light scattering were shown in Table 2. Their size ranged from 186 to 295 nm, which was regarded within the same particle size range considering their standard deviations.

Table 2
Average diameters of dexamethasone nano-aggregates composed of triblock copolymers according to initial loading (0%, 1%, 5% and 10%) amounts of dexamethasone (average diameter ± S.D.)

Samples	Effective diameter (nm)
PEG16	
0%	211 ± 25
1%	295 ± 17
5%	214 ± 21
10%	211 ± 19
PEG48	
0%	228 ± 31
1%	214 ± 12
5%	211 ± 14
10%	198 ± 11
PEG121	
0%	209 ± 16
1%	231 ± 17
5%	197 ± 11
10%	186 ± 13

The concentration of dexamethasone nano-aggregates was 1 mg/ml.

Table 3
Loading efficiency of dexamethasone in nano-aggregates composed of triblock copolymers

Samples	Loading efficiency (%)
PEG16	
1%	125.8 ± 17.1
5%	111.9 ± 13.8
10%	94.2 ± 25.5
PEG48	
1%	104.1 ± 16.3
5%	75.4 ± 14.2
10%	66.7 ± 11.0
PEG121	
1%	66.4 ± 9.7
5%	54.3 ± 12.2
10%	42.3 ± 13.4

Loading efficiency is defined as: (actual loading amount)/(initial loading amount (1%, 5% and 10%)) and presented in average value ± S.D.

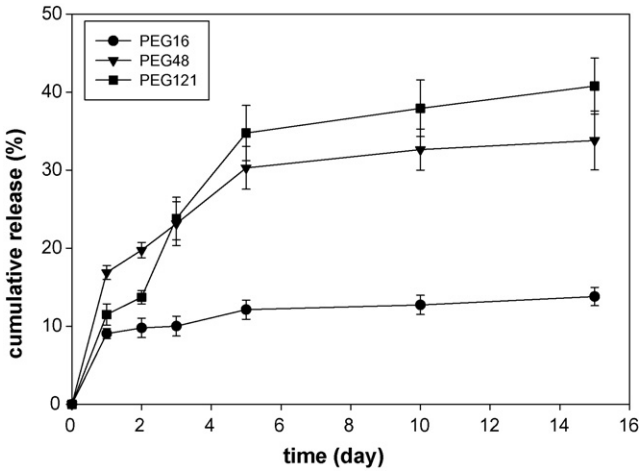

Fig. 2. Release profile of dexamethasone from dexamethasone nano-aggregates made of triblock copolymers.

Therefore, the size of nano-aggregates remains same irrespective of PEG chain lengths in the triblock copolymers. This is of interest because each triblock copolymer showed different CACs according to their different PEG chain lengths. Therefore, upon forming nano-aggregates, PEG did not render any effects on structures of nano-aggregates in solution because PEG chains in nano-aggregates did not play any other roles except making nano-aggregates soluble in an aqueous phase. However, it should be mentioned that the balance of hydrophobic and hydrophilic block does affect the aggregate structure therefore the release profile of dexamethasone as indicated in many studies (Sheihet et al., 2005).

Table 3 compares loading amounts of dexamethasone in dexamethasone nano-aggregates made of triblock copolymers. On increasing loading amounts up to 10%, loading efficiency significantly decreased in all nano-aggregates. This result can be attributed that hydrophobic regions of nano-aggregates are too small to encapsulate all initially loaded dexamethasone within it. In addition, the PEG length also affected the encapsulation efficiency, showing that a long chain of PEG prevented dexamethasone from being encapsulated within the nano-aggregates. Considering PEG16 showed the lowest CAC among three triblock copolymers tested in this study (Fig. 1), dexamethasone is easily encapsulated in the nano-aggregates when the same amount of triblock copolymer was used. Therefore, loading efficiency of dexamethasone was significantly increased as a short PEG chained triblock copolymer was used.

In vitro release profile of dexamethasone nano-aggregates was investigated as shown in Fig. 2. A hydrophobic drug including dexamethasone tends to release out to aqueous phase relatively for a long period because of the low solubility in water. According to the length of PEG chains in triblock copolymer, different release profiles were obtained, showing that PEG played an important role in dexamethasone release. Upon increasing PEG length in triblock polymer, more dexamethasone was release out from the dexamethasone nano-aggregates. Specifically, 40% of dexamethasone in the nano-aggregates made of PEG121 triblock copolymer was release out for 15 days. Because PEG chain has a role as a repellent against each nano-aggregate, nano-aggregates with a long chain of PEG had less chance of forming a large aggregates from many dexamethasone nano-aggregates than those with a short PEG chain. Considering dexamethasone easily forms insoluble aggregates for its low solubility in water, released dexamethasone around nano-aggregates can act as a core to aggregate nano-sized particles, which subsequently leaded to large aggregates. Therefore, dexamethasone entrapped in a large aggregate cannot be easily released out to the medium for dexamethasone nano-aggregates with a short PEG chain. In addition, it should be noticed that a sink condition is not satisfied in this release study because dexamethasone has low solubility in the release medium (Suh et al., 1998).

In order to compare anti-proliferation effects of dexamethasone nano-aggregates, smooth muscle cells incubated with the nano-aggregates were examined for their cell cycle phases, G_0–G_1 phase. Because SMCs were synchronized to G_0 phase, adding serum to the medium could stimulate cell cycle transition from G_0–G_1 phase to S phase. Fig. 3 shows flow cytometry of SMC treated with dexamethasone nano-aggregates. In comparison with dexamethasone, dexamethasone nano-aggregates highly arrested SMC cycle in G_0–G_1 phase with a statistical significance ($p < 0.05$). This result clearly shows that nano-aggregates could efficiently transport dexamethasone than native dexamethasone did. This can be attributed to endocytic uptakes of the smooth muscle cells. Animal cells are generally known to uptake small particles through an endocytosis and this behavior was frequently employed to transport anti-cancer drug to tumor cells (Kwon et al., 1995; Yoo and Park, 2001). While most free drugs can enter cells by diffusion, nano-particulates can be uptaken by cells by endocytosis. However, it should be noted that PEG length of dexamethasone nano-aggregates did not alter flow cytometry results. While release profile was examined during the 15-day period, the cells were treated with dexamethasone nano-aggregates for 3 h. Therefore, release rate did not affect dexamethasone uptake within the cells. Considering endocytic uptake rates are affected by particles size, where smaller particle

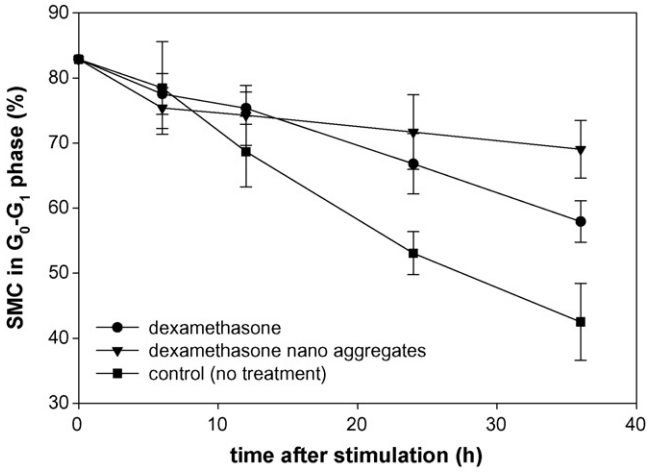

Fig. 3. Anti-proliferation effects of dexamethasone nano-aggregates on SMC by measuring G_0–G_1 cell cycle arrest in flow cytometry. PEG121 nano-aggregate with 5% of dexamethasone was used.

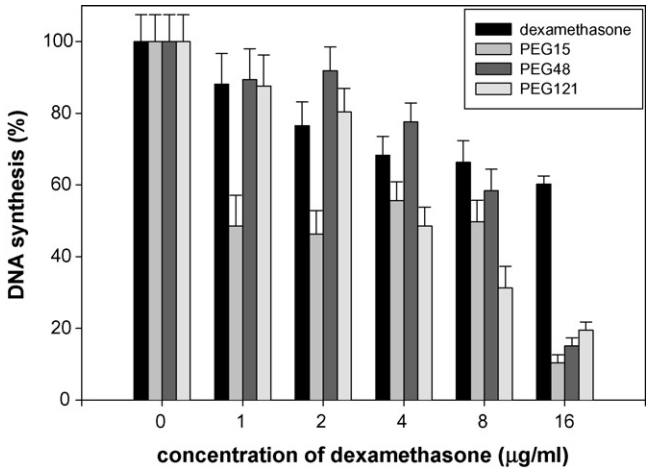

Fig. 4. The amount of synthesized DNA after stimulation of SMC growth on administrating dexamethasone nano-aggregates according to the concentration of dexamethasone.

shows better uptake rates, it is plausible that all nano-aggregates showing similar sizes show no difference of endocytic uptake rate.

In order to monitor DNA synthesis after treatment of dexamethansone, synchronized cells treated with dexamethasone nano-aggregates were subjected to a thymidine uptake experiment (Fig. 4). DNA synthesis against untreated cells slowly decreased in a dose-dependent manner for all samples. At a higher concentration of dexamethasone (16 μg/ml), all dexamethasone nano-aggregates showed superior anti-proliferation effects against synchronized cells in comparison with native dexamethasone. This result coincide with the flow cytometry result shown in Fig. 3, strongly indicating dexamethasone nano-aggregates are superior in suppressing the proliferation of SMC.

4. Conclusion

Dexamethasone nano-aggregates showed different release profiles of dexamethasone according to their PEG chain lengths. The dexamethasone nano-aggregates showed superior anti-proliferation effects to native dexamethasone against SMC.

Acknowledgement

This research was supported by Kangwon National University.

References

Clagett, G.P., Robinowitz, M., Youkey, J.R., Fisher Jr., D.F., Fry, R.E., Myers, S.I., Lee, E.L., Collins Jr., G.J., Virmani, R., 1986. Morphogenesis and clinicopathologic characteristics of recurrent carotid disease. J. Vasc. Surg. 3, 10–23.

Dixon, E.R., Weinberg, J.A., Lew, D.B., 1999. Effect of dexamethasone on bovine airway smooth muscle cell proliferation. J. Asthma 36, 519–525.

Jeong, B., Bae, Y.H., Kim, S.W., 2000. In situ gelation of PEG–PLGA–PEG triblock copolymer aqueous solutions and degradation thereof. J. Biomed. Mater. Res. 50, 171–177.

Jeong, B., Bae, Y.H., Lee, D.S., Kim, S.W., 1997. Biodegradable block copolymers as injectable drug-delivery systems. Nature 388, 860–862.

Kwon, G.S., Naito, M., Yokoyama, M., Okano, T., Sakurai, Y., Kataoka, K., 1995. Physical entrapment of adriamycin in AB block copolymer micelles. Pharm. Res. 12, 192–195.

Lee, J., Cho, E.C., Cho, K., 2004. Incorporation and release behavior of hydrophobic drug in functionalized poly(D,L-lactide)-*block*-poly(ethylene oxide) micelles. J. Control. Release 94, 323–335.

Lincoff, A.M., Furst, J.G., Ellis, S.G., Tuch, R.J., Topol, E.J., 1997. Sustained local delivery of dexamethasone by a novel intravascular eluting stent to prevent restenosis in the porcine coronary injury model. J. Am. Coll. Cardiol. 29, 808–816.

Radke, P.W., Weber, C., Kaiser, A., Schober, A., Hoffmann, R., 2004. Dexamethasone and restenosis after coronary stent implantation: new indication for an old drug? Curr. Pharm. Des. 10, 349–355.

Reil, T.D., Sarkar, R., Kashyap, V.S., Sarkar, M., Gelabert, H.A., 1999. Dexamethasone suppresses vascular smooth muscle cell proliferation. J. Surg. Res. 85, 109–114.

Sheihet, L., Dubin, R.A., Devore, D., Kohn, J., 2005. Hydrophobic drug delivery by self-assembling triblock copolymer-derived nanospheres. Biomacromolecules 6, 2726–2731.

Suh, H., Jeong, B., Rathi, R., Kim, S.W., 1998. Regulation of smooth muscle cell proliferation using paclitaxel-loaded poly(ethylene oxide)–poly(lactide/glycolide) nanospheres. J. Biomed. Mater. Res. 42, 331–338.

Tang, Y., Liu, S.Y., Armes, S.P., Billingham, N.C., 2003. Solubilization and controlled release of a hydrophobic drug using novel micelle-forming ABC triblock copolymers. Biomacromolecules 4, 1636–1645.

Yokoyama, M., Kwon, G.S., Okano, T., Sakurai, Y., Seto, T., Kataoka, K., 1992. Preparation of micelle-forming polymer–drug conjugates. Bioconjug. Chem. 3, 295–301.

Yoo, H.S., Park, T.G., 2001. Biodegradable polymeric micelles composed of doxorubicin conjugated PLGA–PEG block copolymer. J. Control. Release 70, 63–70.

Yoon, J.J., Kim, J.H., Park, T.G., 2003. Dexamethasone-releasing biodegradable polymer scaffolds fabricated by a gas-foaming/salt-leaching method. Biomaterials 24, 2323–2329.