

Fabrication of an α -lipoic acid-eluting poly-(D,L-lactide-co-caprolactone) cuff for the inhibition of neointimal formation

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Abbreviations: ALA, α -lipoic acid; MethoxyPEG, methoxy polyethylene glycol; PLC, poly-(D,L-lactide-co-caprolactone) 40:60

Abstract

The purpose of this study was to develop a novel polymer cuff for the local delivery of α -lipoic acid (ALA) to inhibit neointimal formation *in vivo*. The polymer cuff was fabricated by incorporating the ALA into poly-(D,L-lactide-co-caprolactone) 40:60 (PLC), with or without methoxy polyethylene glycol (MethoxyPEG). The release kinetics of ALA and *in vitro* degradation by hydrolysis were analyzed by HPLC and field emission scanning electron microscopy (FE-SEM), respectively. *In vivo* evaluation of the effect of the ALA-containing polymer cuff was carried out using a rat femoral artery cuff injury model. At 24 h, 48% or 87% of the ALA was released from PCL cuffs with or without MethoxyPEG. FE-SEM results indicated that ALA was blended homogeneously in the PLC with MethoxyPEG, whereas ALA was distributed on the surface of the PLC cuff without MethoxyPEG. The PLC cuff with MethoxyPEG showed prolonged and controlled release of

ALA in PBS, in contrast to the PLC cuff without MethoxyPEG. Both ALA-containing polymer cuffs had a significant effect on the inhibition of neointimal formation in rat femoral artery. Novel ALA-containing polymer cuffs made of PLC were found to be biocompatible and effective in inhibiting neointimal formation *in vivo*. Polymer cuffs containing MethoxyPEG allowed the release of ALA for one additional week, and the rate of drug release from the PLC could be controlled by changing the composition of the polymer. These findings demonstrate that polymer cuffs may be an easy tool for the evaluation of anti-restenotic agents in animal models.

Keywords: coronary restenosis; delayed-action preparations; drug delivery systems; poly(ϵ -caprolactone-co-lactide); thioctic acid

Introduction

Although percutaneous transluminal coronary angioplasty (PTCA) is the most effective treatment for patients with ischemic heart disease, restenosis remains a major limitation to its long-term efficacy (Oberhoff *et al.*, 2001). During PTCA, balloon dilation of the arterial wall can lead to de-endothelialization, subsequent exposure of vascular smooth muscle cells (VSMCs) to proinflammatory cytokines in the serum, and consequent activation of these cells (Ferns *et al.*, 1992). Vascular smooth muscle cell activation and proliferation are key elements in the development and progression of neointimal hyperplasia after an intra-arterial intervention, such as balloon angioplasty or stenting (Fuster *et al.*, 1995). Recently, drug-eluting stents (DESs) containing anti-proliferative compounds, such as paclitaxel and rapamycin, have been shown to dramatically reduce the rate of restenosis from 20-30% to 1-3% at one year post-surgery (Vaina *et al.*, 2005; Garcia-Garcia *et al.*, 2006); however, researchers have been unable to consistently demonstrate whether DESs reduce restenosis, target lesion revascularization, or target vessel revascularization, when compared with bare-metal stents (Simonton *et al.*, 2005; Eisenberg *et al.*, 2006). An animal model of restenosis bearing a local drug delivery device that allows fast examination of therapeutic approaches to prevent in-stent restenosis

nosis would be extremely valuable for the elucidation of these concerns; however, these tools have yet to be developed.

One well-defined mouse model of restenosis utilizes the perivascular cuff design. In this model, a non-constrictive perivascular polyethylene cuff is placed around the mouse femoral artery, which results in a reproducible and concentric intimal thickening within 2-3 weeks, mainly caused by rapid induction of VSMC proliferation (Lardenoye *et al.*, 2000; Sasaki *et al.*, 2004). Although a perivascular cuff is used in this model for the induction of restenosis, this tool may be an ideal device to evaluate the delivery and action of anti-restenotic drugs. Perivascular cuffs indirectly contact the blood or the lumen of vessels, thereby reducing the risk of thrombosis and the loss of drug by dilution resulting from blood flow (Moroi *et al.*, 2003; Sasaki *et al.*, 2004; Nuno *et al.*, 2005; Castoldi *et al.*, 2007).

α -lipoic acid (ALA) has been demonstrated to induce cell cycle arrest in non-transformed cell lines, to mediate apoptosis in tumor cell lines, and to inhibit expression of TNF α -induced cell adhesion molecules through inhibition of NF- κ B activity (Zhang and Frei, 2001; Lee *et al.*, 2006). Because of its strong antioxidant and redox-regulating properties, ALA has been proposed for the treatment of diseases mediated by free radicals, including heavy metal poisoning, liver disease, radiation poisoning, and diabetes (Biewenga *et al.*, 1997; Jürgen *et al.*, 1997). Furthermore, ALA is currently being used clinically for the treatment of diabetic neuropathy (Ziegler *et al.*, 1995).

Here, we used biodegradable and biocompatible polymers to fabricate a polymeric cuff for the sustained and local release of the anti-proliferative drug ALA. We examined the efficacy and safety of the ALA-polymer cuff *in vitro* and *in vivo*, using a cuff injury animal model. In the present study, the polymer cuff used to induce restenosis was fabricated from a polymeric formulation of poly-(D,L-lactide-co-caprolactone) (PLC), with or without methoxy polyethylene glycol (MethoxyPEG). PLC is a biodegradable synthetic polymer that has elasticity and toughness adequate for surrounding blood vessels and delivering of ALA. MethoxyPEG is a surfactant predicted to improve the cuff's ALA holding capacity by enhancing the incorporation and fixation of the compound into the PLC.

Our results show that the novel polymer cuffs induced reproducible intimal hyperplasia and simultaneously allowed local delivery of ALA to the vessel wall. The ALA-eluting PLC cuff containing MethoxyPEG yielded a sustained release of the drug for at least one week *in vitro*. Furthermore,

this sustained release resulted in a reduced neointimal formation *in vivo*, with no adverse effects. Although ALA was released from the PLC cuff without MethoxyPEG almost completely within 48 h *in vitro*, this cuff still effectively inhibited neointimal formation *in vivo*. Therefore, these polymer cuffs could become a tool to evaluate the effects of anti-restenosis compounds on neointimal formation, as well as to assess potential side effects.

Results

In vitro release profile of ALA from polymer cuffs

Figure 1 shows the *in vitro* release profiles of ALA from each polymer. The ALA release kinetics differed from polymer to polymer. Eighty-six percent (144 μ g/ml) of the total ALA embedded in the PLC only polymer cuffs was released during the first 24 h, followed by a slow sustained release phase that lasted for one week. Conversely, ALA release from the PLC polymer with MethoxyPEG was slower, with an ALA release within the first 24 h of 44% (130 μ g/ml), and a total release of 54% of the compound after one week. These results demonstrate that the addition of MethoxyPEG to PLC enhanced the controlled release of ALA.

In vitro changes of the surface of the polymer cuffs

Observation of the PLC only cuffs by FE-SEM indicated that the cuff surface was rough, and that ALA particulates were distributed on the surface of

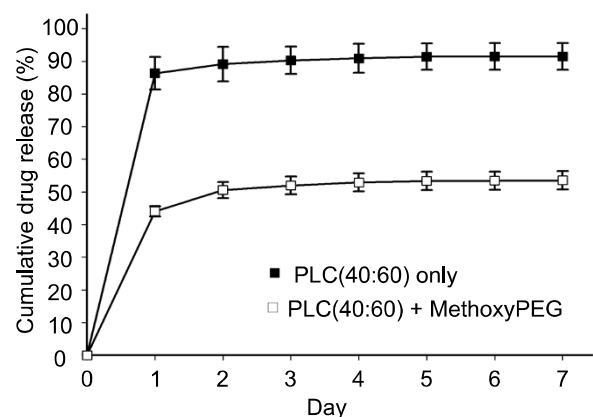


Figure 1. *In vitro* drug release profile from polymer. The ALA release kinetics varied from polymer to polymer. Eighty-six percent (144 μ g/ml) of the total ALA embedded in the PLC only cuffs was released in the first 24 h, followed by a slow sustained release phase that lasted for one week. ALA release from the PLC cuffs containing MethoxyPEG was slower, with an ALA release within the first 24 h of 44% (130 μ g/ml), and a total release of only 54% in seven days.

the PLC only polymer (Figure 2A). In contrast, the surface of the PLC cuffs containing MethoxyPEG

was smooth, with no indication of phase separation (Figure 2F, K). Furthermore, the surface of the

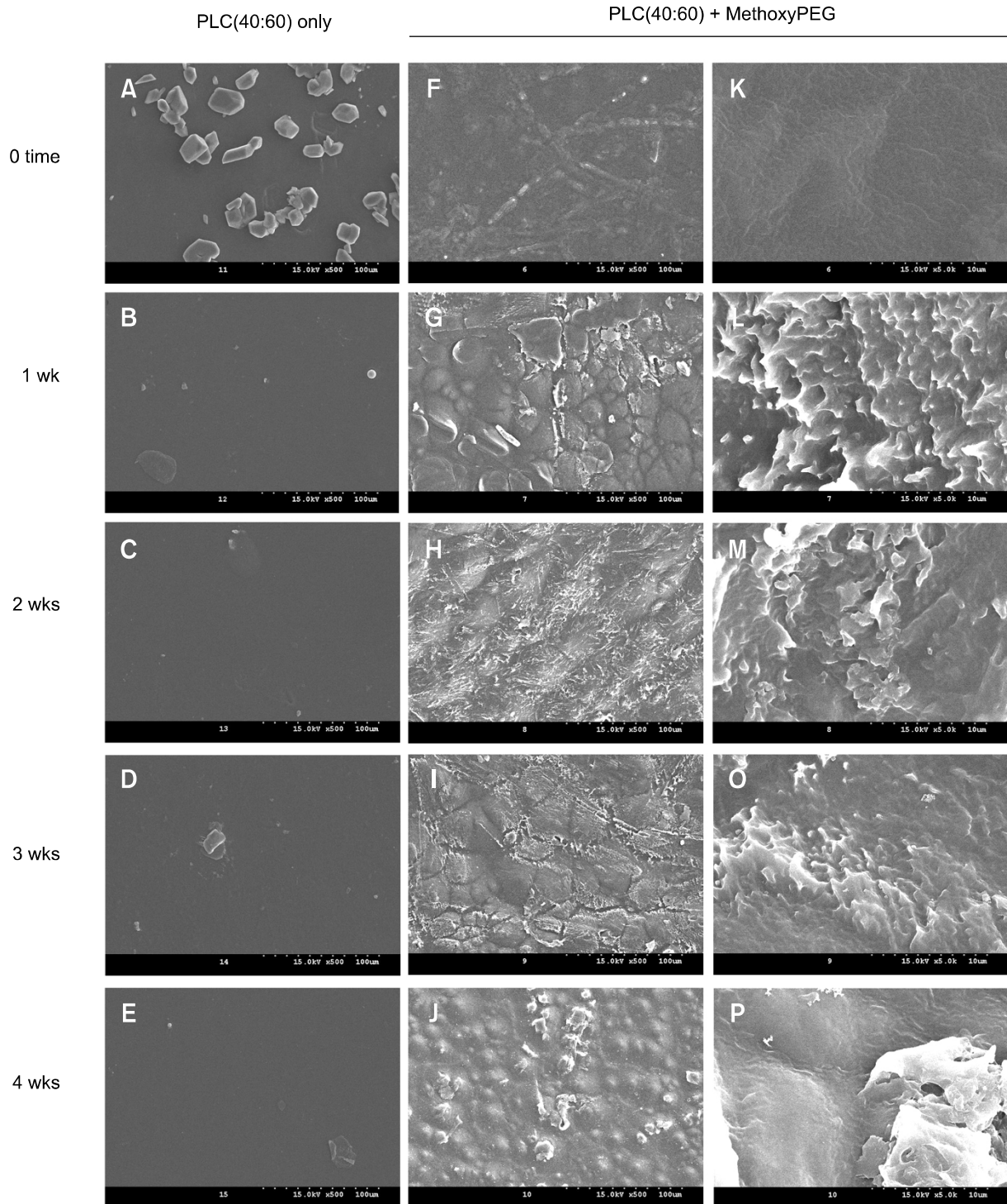


Figure 2. FE-SEM micrographs of the surface of each polymer. Three pieces of ALA-loaded PLC polymer cuffs with or without MethoxyPEG were prepared for *in vitro* degradation assay. The degradation study was carried out over four weeks. The surface characteristics of each polymer cuff were examined for degradability using a Field Emission Scanning Electron Microscope. A, F, K: 0 weeks; B, G, L: 1 week; C, H, M: 2 weeks; D, I, O: 3 weeks; and E, J, P: 4 weeks. A-J: original magnification was 500 \times ; K-P: original magnification was 5,000 \times .

MethoxyPEG-containing cuffs peeled off with time (Figure 2F-P), when compared with the negligible surface changes observed in the PLC only cuffs (Figure 2A-E).

In vivo inhibition of neointimal formation by polymer cuffs containing ALA

We examined the effects of ALA release from the polymer cuff on neointimal formation by calculating the ratio of the intimal area to the medial area (IA/MA ratio) of the vessels. ALA-free polymer cuffs induced and ALA-loaded polymer cuffs inhibited neointimal formation (Figure 3A-E). The IA/MA ratio was significantly reduced in arteries cuffed with the ALA-containing polymer: the IA/MA ratio in arteries treated with ALA-free PLC only cuffs was 1.15 ± 0.13 , vs. 0.20 ± 0.005 in arteries with ALA-containing cuffs ($P < 0.05$, $n = 3$). The IA/MA ratio in arteries treated with ALA-free PLC cuffs containing MethoxyPEG was 1.36 ± 0.11 , vs. 0.20 ± 0.005 in arteries with ALA-containing cuffs ($P < 0.05$, $n = 3$) (Figure 3F). These results show that ALA-containing polymer cuffs completely inhibited intimal formation in a rat femoral artery cuff injury model.

In vivo biocompatibility test of polymer cuffs

To test the biocompatibility of the polymers, each cuff was inserted under the dorsal skin of a mouse.

Grafted tissues were examined after 1 and 4 weeks of cuff insertion, using the histological approach outlined in the Methods. The region containing PLC only cuffs were slightly thicker than the normal skin at 1 week, but by 4 weeks no trace of inflammation or skin tissue transformation could be seen (Figure 4A-C). Skin areas where PLC cuffs containing MethoxyPEG were inserted did not exhibit any abnormal changes at either time point (Figure 4D, E). Absorption or disappearance of the polymer was not observed over time. The results of this biocompatibility test demonstrated that an acute inflammatory reaction was not prominent in skin areas containing either polymer, and histological examination revealed no specific abnormal findings, indicating that neither polymer yielded any prominent effects of acute inflammation or histological changes.

Discussion

The placement of ALA-eluting PLC polymer cuffs with or without MethoxyPEG effectively inhibited neointimal formation in a rat femoral artery cuff injury model. This indicates that the controlled release of ALA from the polymer cuff was effective, and that the released ALA exerted its pharmacological effects on the cuffed area of the vessel.

The *in vitro* drug release patterns differed between the PLC only and the PLC with MethoxyPEG

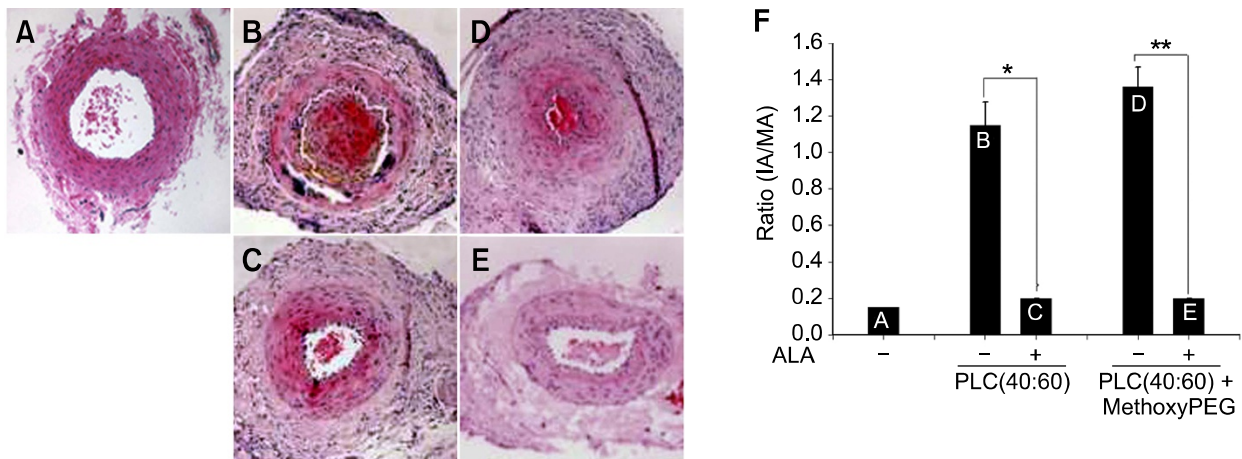


Figure 3. Inhibitory effect of ALA-containing polymer on neointimal formation *in vivo*. Three weeks after cuff placement, rat femoral arteries were harvested and embedded in paraffin. Vessels were stained with H&E and images of the stained vessels were acquired using a digital camera (Zeiss, AxioCam, Germany) attached to a microscope (Zeiss, Axiover 200 M, Germany). Image analysis was performed using the Scion image analysis software (Scion Co., Frederick, MA). Intimal and medial areas were measured with a digitizing system (model INTUOS 6.8, Wacom, Vancouver, WA). The average ratio of the intimal/medial areas of the femoral artery in animal groups corresponding to the different types of cuffs is presented in (F). Bars represent the intimal/medial ratio of femoral arteries after cuff injury in each group of animals studied ($n = 3$ /group). Values represent the mean \pm S.D. with significance set as $*P < 0.01$, when compared with PLC only, and $**P < 0.05$ when compared with PLC containing MethoxyPEG without ALA. Figures are presented at the original magnification of $25 \times$ (A-E). A: Normal femoral artery; B: PLC without ALA; C: PLC with ALA; D: PLC:MethoxyPEG without ALA; E: PLC:MethoxyPEG with ALA; F: Quantification of microphotographs.

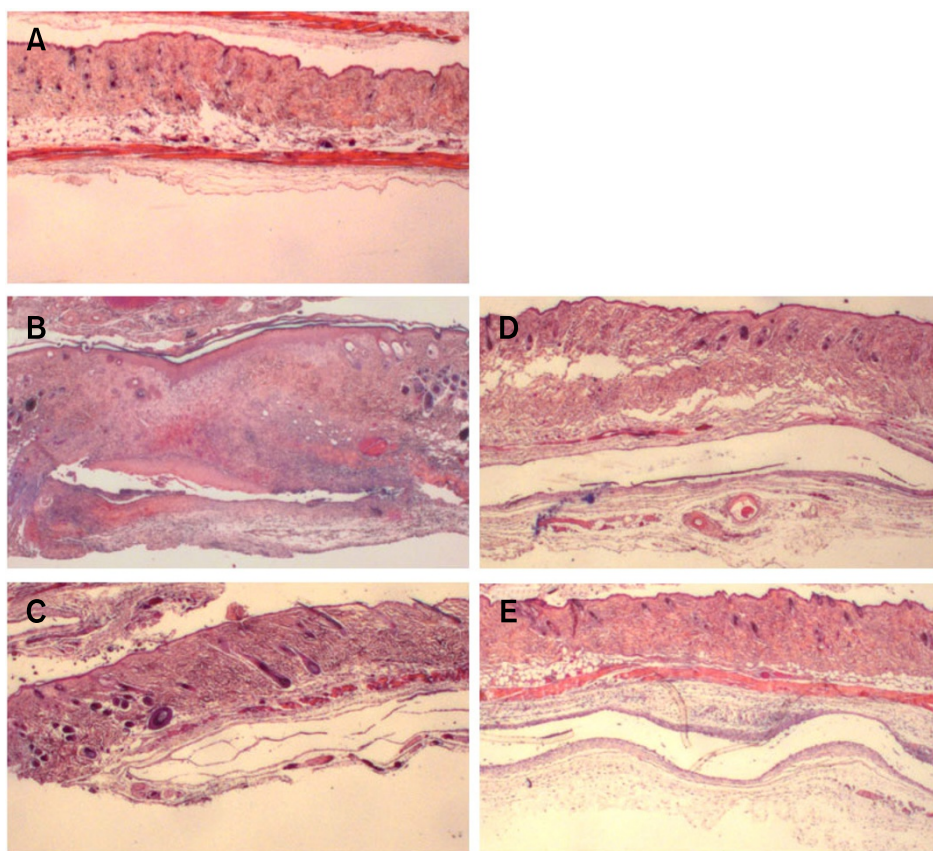


Figure 4. Histological assessment of the biocompatibility of each polymer. Each polymer cuff was grafted under the dorsal skin of a mouse. Tissues were examined with H&E stain 1 and 4 weeks after insertion of the polymers. The areas containing PLC only cuffs were slightly thicker than normal skin at 1 week, but by 4 weeks no trace of inflammation or skin tissue transformation could be detected (A-C). The areas where PLC with MethoxyPEG cuffs were inserted did not exhibit any abnormal changes at either time point (D, E). Figures are presented at the original magnification of $40\times$ (A-E) A: Normal skin tissue; B: PLC-inserted tissue (1 week); C: PLC-inserted tissue (4 weeks); D: PLC: MethoxyPEG-inserted tissue (1 week); E: PLC: MethoxyPEG-inserted tissue (4 weeks).

cuffs. As shown in Figure 1A, 90% and 50% of the ALA was released from the PLC only or PLC with MethoxyPEG cuffs during the first 48 h, respectively, which means that in the PLC only cuffs, ALA release was nearly completed by 48 h. We speculate that this fast release of ALA from PLC only cuffs may be due to diffusion. As shown in the FE-SEM results (Figure 2A), ALA was distributed on the surface of the polymer; therefore, the drug could easily diffuse into the PBS. As might be expected, ALA release from PLC cuffs containing MethoxyPEG was steady for one week, probably as a consequence of homogeneous incorporation of the drug into the polymer matrix.

As previously mentioned, ALA was incorporated homogeneously in PLC polymers containing MethoxyPEG (Figure 2F), while ALA residue was detected on the surface of the PLC only cuffs (Figure 2A); therefore, ALA was quickly released from the PLC only matrix. These results demonstrate that the MethoxyPEG contributed to the homogenous blending of the ALA into the PLC polymer phase. Microphotographs also show differences in the surface characteristics of each polymer: surface changes were not detected over time in PLC only cuffs, whereas PLC polymer con-

taining MethoxyPEG showed marked peeling with time.

These differences in surface alteration between PLC only and PLC polymer containing MethoxyPEG may be attributed to the presence of MethoxyPEG, which acts as a surfactant, thus helping with the incorporation and maintenance of the two molecules in one phase. In general, PEG decreases the toxicity and immunogenicity of a polymer, and increases the solubility and stability of drugs, which lead to improvements in drug pharmacokinetics and pharmacodynamics (Zhang *et al.*, 2005). The structure of PEG in solution is such that each ethylene glycol subunit is tightly linked with two or three water molecules. PEGylated drugs are more stable over a larger range of pH and temperature changes, when compared with their unPEGylated counterparts. Consistently, we observed an improved ALA holding capacity and an enhancement in the controlled release of ALA in the presence of PEG.

Elution of ALA from either type of polymer led to the inhibition of cuff-induced neointimal formation in rat femoral arteries. α -lipoic acid, also known as thioctic acid, is produced naturally in trace quantities in mammalian cells. This compound effectively

inhibits TNF- α -induced expression of vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, and fractalkine, via the inhibition of NF- κ B transcriptional activity in cultured VSMCs. In a rat model of neointimal hyperplasia induced by balloon injury, I.P. administration of ALA, starting 3 days before injury and up to 14 days after injury, reduced (25 or 50 mg/day/kg) or completely prevented (100 mg/day/kg) neointimal formation (Lee *et al.*, 2006). Although ALA was completely released from the PLC only cuff within 24 h, the drug still successfully inhibited neointimal formation. This implies that ALA strongly suppresses various kinds of regulatory factors related to inflammation, cell proliferation, and migration, at early vessel injury stages. In addition, the *in vitro* condition did not mimic the *in vivo* environment, and there were individual variations among experimental animals; various physiological conditions, such as biological fluids, enzyme levels, movement of muscle, and mechanical stress, may affect degradation of the polymers. Furthermore, the number of animals used in these experiments was not sufficient to significantly assess the differences between the group using PLC alone and the group using PLC containing MethoxyPEG, and their impact on the inhibition of neointimal formation; therefore, additional experiments using a larger number of animals are required to obtain conclusive evidence regarding the mechanisms of action of PLC cuffs with or without methoxyPEG.

Based on these results, we suggest that the ALA-eluting polymer cuffs described here may work efficiently to prevent the process of neointimal formation, regardless of the presence of MethoxyPEG.

The ALA dose used in for human applications is 600 mg/day, delivered by intravenous injection. In our previous study (Lee *et al.*, 2006), neointimal formation was inhibited by 100 mg/kg of ALA, administered intraperitoneally. The quantity of ALA applied to vessels in the present study was less than 1 mg/kg, based on the calculated amount of ALA loaded into the polymers (300 μ g) and on the area of the cuffs (0.2 \times 0.2 cm square, 0.4 mg in weight). These data demonstrate the major advantage of using drug eluting polymer cuff devices, which are very efficient in delivering drugs to a targeted region. The polymer cuffs fabricated in the course of this study may serve as a useful and convenient device for the study of vascular complications by minimizing the time, energy, and unreliability inherent to I.P. or oral drug administration.

The adequate selection of matrices and fabrication procedure are essential to the successful

fabrication of drug delivery polymers, as these parameters determine the delivery efficiency of the drug to the target site over an ideal time period (Holland *et al.*, 1986; Le Ray *et al.*, 2005; Simonton *et al.*, 2005). The development of polymer cuffs containing ALA has a clear clinical potential for the treatment of restenosis, if the problems inherent to the prolonged presence of the polymers inside the body are resolved. Finally, the cuff-induced rat model of neointimal formation has major potential for the *in vivo* evaluation of novel anti-restenotic drugs.

Methods

Materials

Poly-(D,L-lactide-co-caprolactone) 40:60 (PLC 40:60, MW 88,000 g/mol) was purchased from Boehringer Ingelheim (Germany), and methoxy polyethylene glycol 350 (MethoxyPEG, MW 350 g/mol) was purchased from Sigma-Aldrich. α -lipoic-acid (ALA) was kindly provided by Dalim Medical Co. (Korea). Supplemental Data Figure S1 shows the chemical structures of these compounds.

Fabrication of polymer cuffs containing ALA

To fabricate the cuffs, 40 mg of PLC were dissolved in 500 μ l of methylene chloride (Sigma-Aldrich) with vigorous vortexing, followed by addition of 20 mg of ALA. The mixture was slowly aspirated with a glass pipette, spread onto a glass slide to form a thin film, and then allowed to evaporate in a vacuum oven (Aldrich) for 4 h at 24°C. The polymer film was cut into 0.2 \times 0.2 cm squares and stored in a Petri dish at room temperature until further use. The same fabrication procedure was followed, whether MethoxyPEG was used or not. For PLC cuffs with MethoxyPEG, PLC and MethoxyPEG were mixed at a ratio of 4:1 (w/w) and dissolved in methylene chloride, and then processed as described above. Polymer cuffs without ALA were used as control.

In vitro ALA release from polymer cuffs

A PLC polymer cuff (PLC only, 0.2 \times 0.2 cm square, 0.4 mg in weight) containing ALA (0.13 mg) and a PLC:MethoxyPEG polymer cuff (PLC with MethoxyPEG, 0.2 \times 0.2 cm square, 1.4 mg in weight) containing ALA (0.3 mg) were used for the *in vitro* ALA release assays. Each piece of polymer was placed in a 1.5 ml centrifuge vial containing 1 ml of PBS (pH 7.4). The tubes were capped and incubated at 37°C. PBS aliquots were collected everyday for 7 days at a designated time, and replaced with fresh PBS. Collected samples were stored at 4°C until further analysis. Each polymer cuff sample was prepared in triplicate. The quantity of ALA released at a designated time was analyzed by HPLC (C18 Novapak Waters column, Waters; mobile phase 10 mM phosphoric acid:acetonitrile = 6:4, flow rate: 0.8 ml/min, detection at 200 nm, 20°C).

***In vitro* degradation rate of polymer cuffs**

Four pieces of ALA-containing PLC polymer cuffs with or without MethoxyPEG were prepared for *in vitro* degradation assay. Each polymer cuff was placed in a 1.5 ml centrifuge tube with 1 ml of PBS. The tubes were capped and incubated at 37°C. The degradation study was carried out over 4 weeks. Each week, a polymer cuff was removed from its tube, washed twice with distilled water, and dried at room temperature. The surface characteristics of each polymer cuff were examined for degradability using a Field Emission Scanning Electron Microscope (FE-SEM, Hitachi, S-4300, Japan).

Biocompatibility test of polymer cuffs in mouse

Polymer cuffs of 0.2 × 1 cm in size were inserted subcutaneously under the dorsal skin of a mouse. The condition of the dorsal skin was observed for 4 weeks. At two time-points (once during the first week and once during the fourth week) regions of the polymer-grafted dorsal skin were harvested and a histological study of the tissue was carried out. For this, tissues were fixed in 10% formalin, processed, and embedded in paraffin. Paraffin-embedded tissues were sectioned to a thickness of 4 µm and sections were stained with Hematoxylin and Eosin (H&E). Stained tissues were digitized with a digital camera (Zeiss, AxioCam, Germany) attached to a microscope (Zeiss, Axiovert 200 M, Germany).

***In vivo* efficacy study of polymer cuffs by placement on rat femoral artery**

Adult male Sprague-Dawley rats (280-300 g in weight) were anesthetized by intraperitoneal (I.P.) injection of 50 mg/kg of sodium pentobarbital (Entorbar, Hanlim Pharmacy). At the time of surgery, animals were grouped as follows: PLC only, ALA-loaded PLC, PLC with MethoxyPEG and ALA-loaded PLC with MethoxyPEG ($n = 3$ in each group). The femoral artery was isolated from surrounding tissues, and polymers were wrapped around the femoral artery and sutured in place with Prolene 5-0 silk sutures (Johnson & Johnson). In this way, polymers were placed edge-to-edge around the femoral artery to completely cover the designated segment. The cuffs were larger than the vessels, to avoid obstructing blood flow. The wounds were closed and the animals were monitored for 3 weeks. The schematic diagram of polymer cuff placement is shown in Supplemental Data Figure S2.

Histological analysis of cuffed vessels

Three weeks after cuff placement, animals were anesthetized with ether (C₂H₅OC₂H₅, Junsei, Japan) and sacrificed. Femoral arteries were harvested, kept overnight in 10% formalin, and then vessels were embedded in paraffin wax. The paraffin-embedded vessels were sectioned to a thickness of 4 µm, and sections were stained with H&E. Images of the stained vessels were obtained with a digital camera (Zeiss, AxioCam, Germany) attached to a microscope (Zeiss, Axiovert 200 M, Germany), and then analyzed using the image analysis software from Scion (Scion Co., Frederick, MA). Intimal and medial areas

were measured with a digitizing system (model INTUOS 6.8, Wacom, Vancouver, WA). Supplemental Data Figure S3 is a schematic depiction of the method of measurement of the intimal and medial areas.

Statistical analyses

Data were represented as mean ± S.D. All statistical tests were carried out using the SPSS 11.0 for Windows software. The significance of differences between two groups was assessed using Student's *t*-test. The threshold of significance was set at $P < 0.01$ and 0.05.

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-41-1-04.pdf.

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