Inhibitory Effects of an Antioxidant Coating on a Polylactic Acid Film on Inflammatory Cytokines from Macrophage

Ga Hyun Lee*, Sung June Lee, Sang Won Jeong, Hyun-Chul Kim, Jin Hyun Choi*,†, and Se Geun Lee†

Division of Nano and Energy Convergence Research, DGIST, 333 Techno Jangang-daero, Hyeonpung-myeon, Dalseong-gun, Daegu 711-873, Korea
*Department of Advanced Organic Materials Science & Engineering, Kyungpook National University, 80 Daehakro, Bukgu, Daegu 702-701, Korea
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Abstract: Polylactic acid (PLA) films have been widely used in medical devices for surgical treatments. However, their use has been associated with complications, such as inflammation around the PLA film implant area. Several antioxidant agents have been incorporated on the surface of PLA films to suppress initial excessive inflammatory reactions through the quenching of reactive oxygen species (ROS) released from macrophages. The suppression of inflammation was evaluated by measuring the levels of various pro-inflammatory cytokines produced by the macrophages. In addition, by measuring quercetin release behavior, we determined the optimum type and molecular weight of the film coating polymer for the effective suppression of inflammation. These results suggest that the incorporation and controlled release of quercetin could be a promising method to reduce inflammation induced by PLA films.

Keywords: antioxidant, anti-inflammatory, controlled release, PLA film, implantation.

Introduction

Polymers are an interesting class of materials that have been used in medical implants. Biocompatible and biodegradable polymers are considered suitable for use in other surgical materials such as resorbable sutures, surgical meshes, adhesion barriers, and implants for orthopedic surgeries or blood vessels. These materials eventually degrade and are replaced by body tissues. However, most implants cause acute and chronic inflammation following implantation.1-3

Inflammation at the host tissue-biomaterial interface persists for the lifetime of the medical device. Leukocytes are inflammatory cells that are particularly adept at generating and releasing reactive oxygen species (ROS), including free radicals, such as superoxide anions (·O2-), hydroxyl radicals (·OH), hydrogen peroxide (H2O2), nitric oxide (NO) and singlet oxygen (1O2).4,5 The generation of ROS is one of the most important characteristics of the inflammatory process.6 Excessive levels of ROS can injure cellular biomolecules, such as nucleic acids, proteins, carbohydrates, and lipids, causing fur-
ther damage to cells and tissues, which in turn augments inflammatory conditions. In addition to promoting general cytotoxicity, ROS upregulate pro-inflammatory gene expression by activating nuclear factor-kappa B (NF-κB), a process that is also sensitive to the cellular redox state. Furthermore, the inflammatory process, which is activated by inflammatory cells (i.e., neutrophils, eosinophils, mononuclear phagocytes, and macrophages), enhances the secretion of ROS and inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α.

Antioxidants are known to possess a number of biological activities that are considered beneficial to health, including free radical scavenging, anticancer, and antiviral activities. The involvement of the immune system in adverse reactions to implantation is well-known, and antioxidants have shown anti-inflammatory activities. Biological compounds with antioxidant properties protect cells and tissues from the deleterious effects of ROS and other free radicals generated during inflammation. The inflammatory process typically occurs within 2 weeks of implantation, and hence, the inhibition of early inflammatory responses is very important to mediating chronic inflammation. In the present study, we investigated the effects of antioxidants incorporated on the surfaces of polylactic acid (PLA) films on murine macrophages and fibroblasts subjected to acute inflammatory conditions. The suppression of the inflammatory process was evaluated by measuring the various cytokines produced by lipopolysaccharide (LPS)-stimulated macrophages. Various quercetin-coated PLA films were prepared. The reported results included quercetin release profiles and anti-inflammatory activity.

Experimental

Superoxide Radical Scavenging Activity of Antioxidants and Antioxidant-coated PLA Films. Superoxide radicals were generated by xantine/xantine oxidase (XO) and measured using the nitroblue tetrazolium (NBT) (Sigma-Aldrich, St. Louis, MO, USA) reduction method. An antioxidant solution was prepared by dispersing each antioxidant in PBS according to concentration. Also, PLA films were incubated in phosphate buffer saline (PBS, pH 7.0) at 37°C for 24 h. The extraction ratio was 0.2 g/mL. Then, the films were removed, and an extract solution was obtained. The antioxidant solution or PLA film extract solution was mixed in a PBS containing XO (1.65×10^{-2} units mL^{-1}) and NBT (133 μm). The reaction was initiated by the addition of xanthine (164 μm). After 10 min, production of the superoxide radical was measured using a ultraviolet (UV)-Visible (vis) spectrophotometer (560 nm) at 25°C. The superoxide scavenging activity was calculated according to the following formula: superoxide scavenging activity (%) = (1–[(A–B)/A])×100, where A and B represent the absorbance in the absence and presence of samples, respectively.

Preparation of Antioxidant-coated PLA Films. PLA films were cast using a recently developed hot presssing method instead of the traditional solution casting technique. PLA chips were melt-pressed at a pressure of 15 kgf/cm² and a temperature of 180°C. The chips were then sandwiched between a pair of thick metal plates in a compression molding machine. After a 5 min holding time, the film was allowed to cool under ambient conditions to room temperature.

Polyacrolactone (PCL) and PLA polymers coated with antioxidants solutions were prepared by dissolving 3 g of various molecular weight samples of PCL and PLA with 1 g of antioxidant in 100 mL of methylene chloride (MC)/tetrahydrofuran (THF; 4:1 ratio). The PLA films were dipped in the solutions for 1 min and allowed to dry inside a vented hood under ambient conditions.

Cell Culture. Murine macrophage (RAW 264.7) and murine fibroblast (L-929) cell lines were purchased from Korean Cell Line Bank. L-929 cultures were grown in 90% Dulbecco’s modified Eagle’s medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). These fibroblast cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. When the cultures reached 80% confluence, they were trypsinized with 0.25% trypsin containing 1 mM ethylenediaminetraacetic acid (EDTA, Gibco), and counted by a hemacytometer (Hausser Scientific, USA).

Cytotoxicity of Antioxidant-coated PLA Films. The cytotoxicity of the films was evaluated using a procedure adapted from the ISO 10993-5 standard test method. The viability of cells was determined by the 3-[4,-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; thiazolyl blue) assay. The yellow MTT reagent produces a dark blue formazan product when incubated with viable cells. The level of reduction of MTT to formazan reflects the level of cell metabolism. For the MTT assay, PLA films of approximately 0.2 mm thickness
were sterilized with ultraviolet (UV) irradiation for 4 h. The films were then incubated in DMEM at 37 °C for 24 h. The extraction ratio was 0.2 g/mL. At the end of this period, the films were removed, and the film extract medium was obtained. L-929 cells were cultured at 1×10⁵ cells per well in 96-well plates in 200 µL of DMEM. After incubation for 24 h, the culture medium was removed and replaced with the film extract medium, and the cells were incubated for another 24 h. Then, 100 µL of MTT solution was added to each well, followed by a 3 h incubation at 37 °C. Subsequently, 200 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The dissolved solution was swirled for approximately 10 min on a shaker until homogeneous. The optical density (OD) of the formazan solution was determined using an enzyme-linked immunosorbent assay (ELISA) reader (Multiskan EX, Thermo Scientific) at 570 nm. For negative controls, samples of cells seeded in fresh culture medium under the same seeding conditions but without film extract solution were prepared. Each assay was performed six times in triplicate.

Anti-inflammatory Activity of Antioxidant-coated PLA Films. RAW 264.7 cells were treated with PLA film extract medium and stimulated by LPS (1 µg/mL) for 24 h. The pro-inflammatory cytokines of each culture supernatant were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D System, Inc., MN, USA), according to the manufacturer’s protocol.

Release Profile of Quercetin from the PLA Film. The measurement of quercetin release from the PLA films was performed using the dipping method. The quercetin-coated PLA films (0.2 g/mL) were immersed in 100 mL of PBS at 37 °C. Samples (5 mL) was taken at different time intervals and replaced by an equal volume (5 mL) of fresh PBS. A correction factor was used to account for the dilution caused by the addition of fresh buffer at each time point. UV-vis spectrophotometry (UV, Easyconc-Varian, USA) was used to determine the concentration of quercetin released from the quercetin-coated PLA film.

Results and Discussion

Although biocompatible polymers are known to be inert materials, it has been experimentally shown that implantable polymer medical devices generate inflammatory responses in tissues at implant sites. In normal tissues, implantation triggers a number of simultaneous events, including the activation of the immune system, the inflammation process, regeneration of the epithelium and endothelium, tissue fibrosis, and remodeling.14

Polyphenolic compounds have been demonstrated to exhibit strong anti-inflammatory, antitumor, and antioxidant properties.15 Therefore, we studied the feasibility of using various antioxidants and conducted bioactive characterizations of the following substances:

1. Morin : a yellow pigment isolated from plants of the Moraceae family
2. Quercetin : a natural plant pigments, that constitutes the most abundant flavonoid in the human diet
3. Caffeic acid : a yellow solid, that consists of both phenolic and acrylic functional groups
4. Curcumin : a yellow pigment from Curcuma longa

Superoxide radicals are ROS and are formed during normal aerobic metabolism and by activated phagocytes. Free radical scavenging and metal chelation are two commonly proposed mechanisms to explain the action of antioxidant components. Therefore, we evaluated the superoxide radical scavenging activity of each antioxidant. In the NBT test, the antioxidants reduced the radicals to the violet-colored, blue chromogen formazan, which is indicative of their scavenging ability. The free radical scavenging activities of morin, quercetin, caffeic acid, and curcumin are shown in Figure 1. The four antioxidants scavenged the superoxide anions in a concentration-dependent manner. Quercetin was shown to be the strongest scavenger, followed by caffeic acid; morin and curcumin were the weakest scavengers. Compared with the ROS levels in the control samples, 300 µM of quercetin exhibited a scavenging effect of

![Figure 1](image-url). Radial scavenging activities of various antioxidants.
96.3%, while the antioxidants in morin, caffeic acid, and curcumin showed scavenging rates of 45.4, 77.6, and 42.5%, respectively. It has been suggested that the 3',4'-diphenolic group is required for flavonoids to be effective free radical scavengers. Quercetin possesses a 3',4'-diphenolic group, whereas morin and curcumin do not. However, caffeic acid, which does not possess this group, also presented a rather strong activity. Although the 3',4'-diphenolic group is important for effective superoxide anion scavenging activity, the antioxidant activities of flavonoids may be attributable to other factors.

Figure 2 shows the radical scavenging activities of the antioxidant-coated PLA films. Uncoated PLA films did not exhibit antioxidant activity, whereas the PLA films coated with morin, quercetin, caffeic acid, and curcumin showed scavenging activities of 7.1, 57.5, 76.4, and 20.4%, respectively. The antioxidant-coated PLA films had slightly lower radical scavenging activities than their respective antioxidants alone. The activity of the antioxidant-coated PLA films appeared to decrease with the coating agent. In the radical scavenging test, the amount of released antioxidant was considerably lower. The radical scavenging activity may be correlated with the diffusion of antioxidants from the PLA films. Equimolar antioxidant-coated PLA film and free antioxidants showed similar activities.

Cell viability correlates with the degree of MTT reduction. Figure 3 shows the cell viability effects of the antioxidant-coated PLA films compared with controls. Sample OD measurements were normalized to the control (100% viability). A high cell viability index was shown for the PLA film samples.

Compared with the control, cell viability did not fall below 100%. The antioxidant-coated PLA films were evidently non-toxic. Furthermore, the results revealed that cell viability slightly increased in response to treatment with the antioxidant alone. Therefore, all PLA films exhibited biocompatibility.

Pro-inflammatory cytokines have been suggested to play important roles in inflammation. Activated macrophages can produce several pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6, and TNF-α. IL-1β is an important mediator of the inflammatory response and is involved in a variety of cellular activities including cell proliferation, differentiation, and apoptosis. A low level of IL-1β is necessary for host defense and wound healing, whereas its overproduction can hinder the early phase of wound healing. IL-6 is one of the most important mediators of fever and the acute phase response. TNF-α is another central mediator of the inflammatory response and plays important roles in antimicrobial defense, wound healing, and defense against malignant disorders. Although low levels of TNF-α are necessary for the host defense to ward off infections, its overproduction can be detrimental. The pro-inflammatory properties of the antioxidant-coated PLA films were determined using immunoassays. The amounts of IL-1β, IL-6, and TNF-α released by macrophages treated with the antioxidant-coated PLA films were reduced, as shown in Figure 4. A dramatic decrease in pro-inflammatory cytokine production was observed in RAW 264.7 cells, which varied based on the specific antioxidant, especially curcumin. RAW 264.7 cells exposed to a curcumin-coated PLA film showed 80.0, 94.5, and 69.1% decreases in IL-1β, IL-6, and TNF-α levels, respectively. When treated with other antioxidants, IL-1β levels were maintained at approximately 20%. IL-6 levels were reduced by...
94% or more in the presence of morin or curcumin. TNF-α was less affected than the other cytokines; quercetin and curcumin reduced TNF-α levels by only 44.6% and 69.1%, respectively. These results were correlated with other observations that showed that the antioxidants efficiently inhibited cytokine release by the LPS-stimulated macrophages and reduced inflammation. Compared with the superoxide radical scavenging activities, there was no direct correlation. For example, caffeic acid had a very strong superoxide radical scavenging activity, but its efficiency in reducing pro-inflammatory cytokines was very weak. Conversely, curcumin showed a strong efficiency in reducing pro-inflammatory cytokines but had a weak superoxide radical scavenging activity. Therefore, superoxide radical scavenging is not the only mechanism responsible for inhibiting pro-inflammatory cytokines.

To determine the effects of polymer type and molecular weight on the release property, different molecular weight samples of PCL and PLA were studied under the same conditions. PCL and PLA of various molecular weights that contained quercetin were coated onto the surfaces of the PLA film. The release characteristics of each quercetin-coated PLA films were measured at 37 °C in PBS over a 2-week period. The results of these drug release studies are shown in Figure 5. The results show that for days 2-3, the dominant quercetin release mechanism was burst release from the PCL-coated surface. Over the two weeks following the burst release, sustained release profiles were observed for the various PCL samples. The amount of quercetin released increased with decreasing molecular weights of the PCL coating. Compared with PLA, the PCL matrices contained higher amounts of quercetin, which may provide for an accelerated drug release rate. The drug release rates increased in the following order: PCL 10 k > PCL 80 k > PLA 2 k > PLA 100 k > PLA 300 k. With respect to treating inflammation, short-term pain management or long-term alleviation may be required. The drug release profiles reported herein may provide significant insights into managing a range of inflammatory conditions.

We examined the effects of pro-inflammatory cytokine production in RAW 264.7 cells for various molecular weights of quercetin-loaded PCL and PLA coatings on PLA films. Figure 6 showed a dramatic decrease in the pro-inflammatory cytokine production in RAW 264.7 cells in quercetin-loaded PCL-
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Based coatings compared with PLA coatings. Furthermore, the reduction in TNF-α was negligible between the 100 k and 300 k PLA samples. The reductions in IL-1β, IL-6, and TNF-α levels were more effective with PCL. A quercetin-coated PLA film using a 10 k PCL polymer decreased IL-1β, IL-6, and TNF-α levels by 67.5, 86.4, and 49.4%, respectively, compared with the control.

Conclusions

A study of the effects of antioxidants on a PLA film showed a potentially novel therapeutic option for the prevention of early complications. Cytokines, including IL-1β, IL-6, and TNF-α, play significant roles in inflammation. In our study, significantly lower IL-1α, IL-6, and TNF-α levels were observed with the antioxidant-coated implantable polymer medical devices compared with controls. The molecular weights of PCL and PLA can be adjusted to control quercetin release patterns. This intervention could be helpful in ameliorating the adverse response associated with the integration of implantable polymer medical devices.

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