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Engineering biodegradable polyester elastomers with antioxidant properties to attenuate oxidative stress in tissues

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ABSTRACT

Oxidative stress plays an important role in the limited biological compatibility of many biomaterials due to inflammation, as well as in various pathologies including atherosclerosis and restenosis as a result of vascular interventions. Engineering antioxidant properties into a material is therefore a potential avenue to improve the biocompatibility of materials, as well as to locally attenuate oxidative stress-related pathologies. Moreover, biodegradable polymers that have antioxidant properties built into their backbone structure have high relative antioxidant content and may provide prolonged, continuous attenuation of oxidative stress while the polymer or its degradation products are present. In this report, we describe the synthesis of poly(1,8-octanediol-co-citrate-co-ascorbate) (POCA), a citric-acid based biodegradable elastomer with native, intrinsic antioxidant properties. The *in vitro* antioxidant activity of POCA as well as its effects on vascular cells *in vitro* and *in vivo* were studied. Antioxidant properties investigated included scavenging of free radicals, iron chelation and the inhibition of lipid peroxidation. POCA reduced reactive oxygen species generation in cells after an oxidative challenge and protected cells from oxidative stress-induced cell death. Importantly, POCA antioxidant properties remained present upon degradation. Vascular cells cultured on POCA showed high viability, and POCA selectively inhibited smooth muscle cell proliferation, while supporting endothelial cell proliferation. Finally, preliminary data on POCA-coated ePTFE grafts showed reduced intimal hyperplasia when compared to standard ePTFE grafts. This biodegradable, intrinsically antioxidant polymer may be useful for tissue engineering application where oxidative stress is a concern.

1. Introduction

The biocompatibility of implantable materials has evolved in the past decades from simply meaning inertness, via the lack of a deleterious response, to the current Williams' definition of "the ability of a biomaterial to perform its desired function without eliciting any undesired effect, but generating a beneficial cellular or tissue response"[1]. Our increased understanding of the biological responses to synthetic materials has led to this change of viewpoint. An important component of a biomaterial's response that can have an impact on the performance of medical devices yet is often overlooked in the biomaterials science community is oxidative stress. When a biomaterial induces an inflammatory response, leukocytes release various cytokines and chemokines and generate reactive oxygen species (ROS) (e.g., superoxide, hydroxyl radicals and hydrogen peroxide) [2]. Pro-oxidant molecules and compounds react with and damage DNA, proteins and lipids, potentially impairing the normal function of cells. Indeed, the detection of ROS is currently being used to characterize the inflammatory host tissue response to biomaterials, both *in vitro* and *in vivo* [3]. The induction or presence of oxidative stress is particularly relevant to biodegradable polymers such as polylactides, as the local accumulation of polymer degradation products generate ROS [4,5]. In fact, excess ROS is a significant cause of toxicity for many biodegradable materials [6–9].

Oxidative stress may also be a pathophysiological response due to an imbalance between the production of oxidants and the antioxidant defense mechanism, resulting in a net increase in ROS

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[10]. For example, oxidative stress has been implicated in the progression of atherosclerosis [11,12] and restenosis, the major cause of failure of vascular interventions [13,14]. Specifically, excessive ROS lead to overproliferation of vascular smooth muscle cells [15–17]. Therefore, biomaterials that can counter the effects of oxidative stress and inhibit excessive ROS generation in a sustained manner may be a useful tool for therapies that target these medical problems.

Previous attempts to limit biomaterial-induced oxidative stress included synthesizing polymers that lead to charge-neutral degradation products [18], which does not inhibit oxidative stress induced by other factors, and conjugating antioxidant molecules to the surface of the biomaterial to provide local antioxidant therapy [19–21]. Examples of the latter include the conjugation of small molecule antioxidants such as superoxide dismutase mimetics (mSOD), vitamin E, gallic acid, catechin, ascorbic acid and glutathione to ultra-high molecular weight poly(ethylene) (UHMPE), poly(acrylic acid), gelatin, poly(methyl methacrylate) and poly(-ethylene glycol) [22–26]. Although this strategy has resulted in some suppression of oxidative stress, it results in materials that have low relative antioxidant mass. Biodegradable polymers with native intrinsic antioxidant properties may therefore provide the benefit of relatively high antioxidant content and continuous local antioxidant potential while the polymer is present. Recently such a polymer with intrinsic antioxidant capacity was synthesized [27], but despite some inhibition of ROS generation in cells, no specific effects on free radicals or excess metals, specifically iron were reported. Moreover, the authors did not report *in vivo* evidence of a functional effect of their material.

Our laboratory has previously described the synthesis and characterization of polydiolcitrates (PDC), biodegradable non-toxic polyesters that have been shown to be useful for several tissue engineering applications [28–31]. Herein we demonstrate that these polymers can be easily engineered to have enhanced, biologically relevant antioxidant activity by incorporating ascorbic acid (AA) into the polymer network (Fig. 1). Ascorbic acid is a safe and natural antioxidant that has several hydroxyl moieties that can participate in a polycondensation reaction. Citric acid, a metal chelator, is a stabilizer of AA and was expected to protect AA during the reaction conditions for the prepolymer synthesis [32–34]. The inclusion of metal-chelating citric acid is important as transition metals such as iron are well-known factors in oxidative stress, particularly involved in the Fenton reaction and implied in many ROS-related conditions [35,36]. Moreover, ascorbic acid can act as a pro-oxidant in the presence of free transition metals [37], highlighting the need for metal chelating properties. We hypothesized that these degradable poly(diolcitrate-co-ascorbate) polyesters would have both direct and indirect antioxidant properties due to the radical scavenging ability of AA and iron chelation activity of citric acid and that the copolymer would protect cells from oxidative stress conditions. Furthermore, we hypothesized that inhibition of ROS would selectively inhibit vascular smooth muscle cell proliferation and therefore result in the inhibition of neointimal hyperplasia.

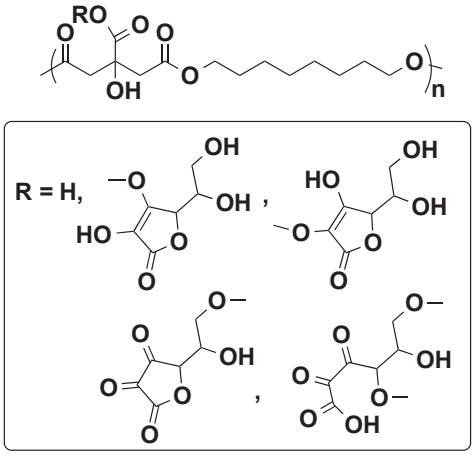

Fig. 1. Proposed structure and scheme of poly(1,8-octanediol-co-citrate-co-ascorbate) (POCA). In case of R = H, the copolymer is poly(1,8-octanediol-co-citrate)(POC).

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma and used without further purification except where indicated otherwise.

2.2. Polymer synthesis

Poly(1,8-octanediol-co-citrate-co-ascorbate) (POCA) was synthesized by mixing 1,8-octanediol, citric acid and ascorbic acid at a ratio of 5:5:1 and heated at 160 °C for 10 min, followed by 140 °C for an additional 60 min. Increasing the relative amount of ascorbate further led to the formation of a viscous liquid rather than a solid elastomer, but a viscous liquid that lacked structural integrity (data not shown). POC was synthesized as a reference polymer by copolymerizing 1,8-octanediol and citric acid in equimolar amounts. After prepolymerization, prepolymers were dissolved in ethanol and purified by precipitation in 5× excess distilled water (MQ, Millipore, Billerica, MA). The resulting purified prepolymer precipitate was lyophilized for 3 days. Prepolymers were subsequently post-polymerized for 4 days at 80 °C for all experiments. After post-polymerization, polymers were gas sterilized with ethylene oxide gas according to manufacturer's instructions (Anprolene AN74i, Andersen Products, Haw River, NC). DMEM cell culture media was added to sterilized polymers to leach out acidic products at 37 °C, with replacement of media upon yellow discoloration. When no more discoloration for at least one day was observed, the polymer was rinsed 3× with phosphate buffered saline (PBS) and used for cell culture experiments. For other *in vitro* experiments, polymers were washed thoroughly with MQ water and lyophilized before use.

2.3. Polymer characterization

Tensile testing was performed according to American Society for Testing and Materials (ASTM) 412A on an Instron 5544 equipped with 500-N load cell (Instron, Norwood, MA). Briefly, dumbbell-shaped samples were pulled to failure at a rate of 500 mm/min. Young's modulus and maximum loads were obtained from stress–-strain data. The polymers' density ρ was measured using the liquid displacement test using ethanol as test liquid. The crosslink density n was calculated using Equation (1) [38]:

$$n = \frac{E_0}{3RT} = \frac{\rho}{M_c} \quad (1)$$

where n represents the number of active network segments; M_c the molecular weight between crosslinks; R the universal gas constant (8.3144 J mol^{-1} K^{-1}); T the absolute temperature (K) and E_0 the Young's modulus.

The proton nuclear magnetic resonance (NMR) spectrum of prepolymers was recorded with an Ag500 NMR spectrometer (Bruker, Billerica, MA) at ambient temperature, using dimethylsulfoxide-*d6* as solvent, and trimethylsilane as the internal reference. The spectra were obtained with a free induction delay resolution of 0.157 Hz/point, corresponding to a sweep width of 10.33 kHz, acquisition time was 3.17 s.

Fourier transform infrared (FT-IR) transmission spectra were recorded in attenuated total reflectance mode on a Nicolet Nexus 870 spectrometer (Thermo Scientific, Waltham, MA) by accumulation of 32 scans, with a resolution of 8 cm^{-1}. Matrix-assisted laser desorption/ionization (MALDI) was performed on POC and POCA prepolymer solutions, as well as supernatants after several hours of incubation of polymer films in MQ at 37 °C. For supernatants, solutions were directly plated without use of a matrix using a matrix-free laser desorption/ionization (LDI-MS) method. For prepolymer solutions, diluted solutions were used in a matrix of cinnamic acid in tetrahydrofuran (THF) (10 mg/mL) at a 1:10 ratio, final polymer concentration of 3 mg/mL. Spectra were collected with a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). A 355 nm Nd:YAG laser was used as a desorption/ionization source, and all spectra were acquired with 20 kV accelerating voltage using positive reflector mode. The weight-average and number-average molecular mass of pre-POC and pre-POCA was measured by MALDI-MS. The peaks from the main distribution and all the sub-distributions were taken in consideration in the calculation of molecular weight of the pre-polymers. The intensity threshold for inclusion of peaks was set to 1%, while spectra were corrected for matrix-specific peaks.