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A porous tissue engineering scaffold selectively degraded by cell-generated reactive oxygen species

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ABSTRACT

Biodegradable tissue engineering scaffolds are commonly fabricated from poly(lactide-co-glycolide) (PLGA) or similar polyesters that degrade by hydrolysis. PLGA hydrolysis generates acidic breakdown products that trigger an accelerated, autocatalytic degradation mechanism that can create mismatched rates of biomaterial breakdown and tissue formation. Reactive oxygen species (ROS) are key mediators of cell function in both health and disease, especially at sites of inflammation and tissue healing, and induction of inflammation and ROS are natural components of the *in vivo* response to biomaterial implantation. Thus, polymeric biomaterials that are selectively degraded by cell-generated ROS may have potential for creating tissue engineering scaffolds with better matched rates of tissue in-growth and cell-mediated scaffold biodegradation. To explore this approach, a series of poly(thioketal) (PTK) urethane (PTK-UR) biomaterial scaffolds were synthesized that degrade specifically by an ROS-dependent mechanism. PTK-UR scaffolds had significantly higher compressive moduli than analogous poly(ester urethane) (PEUR) scaffolds formed from hydrolytically-degradable ester-based diols ($p < 0.05$). Unlike PEUR scaffolds, the PTK-UR scaffolds were stable under aqueous conditions out to 25 weeks but were selectively degraded by ROS, indicating that their biodegradation would be exclusively cell-mediated. The *in vitro* oxidative degradation rates of the PTK-URs followed first-order degradation kinetics, were significantly dependent on PTK composition ($p < 0.05$), and correlated to ROS concentration. In subcutaneous rat wounds, PTK-UR scaffolds supported cellular infiltration and granulation tissue formation, followed first-order degradation kinetics over 7 weeks, and produced significantly greater stenting of subcutaneous wounds compared to PEUR scaffolds. These combined results indicate that ROS-degradable PTK-UR tissue engineering scaffolds have significant advantages over analogous polyester-based biomaterials and provide a robust, cell-degradable substrate for guiding new tissue formation.

1. Introduction

Biodegradable scaffolds made from synthetic polymers have been extensively investigated for use in tissue engineering and regenerative medicine. Examples include poly(lactic-*co*-glycolic acid) (PLGA) [1,2], poly(ε-caprolactone) (PCL) [3,4], polyanhydrides (PAA) [5,6], and polyurethanes [7,8], all of which have a history of use in products approved by the FDA [9–12]. These materials are applicable for a diverse range of regenerative applications because they offer a high degree of tunability, generate a minimal host inflammatory response, and degrade into non-cytotoxic components [13,14] that are resorbed and cleared from the body [15,16].

In situ curing, injectable scaffolds such as poly(ester urethanes) (PEURs) that support cellular infiltration and degrade into non-toxic breakdown products represent a particularly promising class of biomaterials [17]. Porous PEUR scaffolds are formed by mixing hydroxyl-functionalized polyols (*e.g.*, 900 g mol^{-1} triols comprised of caprolactone, glycolide, and D,L-lactide) [13] with isocyanate-functional precursors to form a crosslinked network. Water can be added as a blowing agent to create an inter-connected pore structure, and the mechanical, chemical, and degradation properties of the scaffold can be modified through the selection of the polyol and isocyanate precursors [18,19]. Unlike many other techniques used for fabrication of porous scaffolds, this approach

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does not require a porogen leaching step. This *in situ* foaming method, combined with the relatively short working time of the reactive liquid mixture [20], renders PEURs useful as injectable and settable scaffolds suitable for minimally invasive procedures in the clinic.

PEUR scaffolds are primarily degraded by acid-catalyzed hydrolysis of ester bonds in the amorphous soft segment, resulting in chain scission and formation of hydroxyl and carboxylic acid end groups. Residual carboxylic acids in the polymer reduce the local pH at later stages of degradation [21,22], thereby catalyzing accelerated hydrolysis of the polymer [23]. As the polymers degrade, low molecular weight and soluble α-hydroxy acids diffuse from the scaffold into the medium, resulting in mass loss. Although α-hydroxy acids are non-toxic and can be cleared from the body [13,24], autocatalytic degradation of the PEUR network driven by residual carboxylic acid groups can result in a mismatch in the rates of scaffold degradation and tissue in-growth that leads to resorption gaps and compromised tissue regeneration [25].

Environmentally-responsive polymers have been heavily investigated for the development of smart materials that respond to specific biological stimuli [26]. In particular, biomaterials that degrade by cell-mediated mechanisms, such as materials with protease-cleavable peptides, have been successfully utilized to synthesize environmentally-sensitive nanoparticles [27,28], hydrogels [29,30], and polymeric scaffolds [31,32]. However, it is difficult to establish this approach as a generalizable tissue engineering platform because these peptide sequences are cleaved by specific enzymes that are upregulated in specific pathological environments [33] and feature highly variable levels across patient populations [34]. Also, manufacturing peptides on the scale necessary to fabricate large tissue scaffolds is both expensive and time-consuming with current technology [35]. Development of degradable polymers that can be affordably synthesized in large scales, similar to polyesters, but that target a ubiquitous cell-mediated signal for scaffold degradation may provide a more generalizable and better-performing biomaterial. Scaffolds degraded by cell-generated reactive oxygen species (ROS) are a promising candidate because ROS serve as important biological mediators in many normal biological processes [36], and elevated ROS, or "oxidative stress", is a hallmark of inflammation and the pathogenesis of myriad diseases [37]. Polymeric biomaterial implants have also been shown to elicit a stable three-fold increase in ROS production at surgery sites over a four week timeframe [38], further highlighting the potential utility of this cell-generated signal as a trigger for material degradation. This has motivated the recent emergence of new classes of ROS-responsive polymer-based nanoparticles [39–44] and development of salt-leached, porous scaffolds composed of a combination of the polyester PCL and ROS-sensitive, proline-based peptides [45].

Here we sought to develop a generalizable, cell-degradable polyurethane scaffold formulated from polyols exhibiting ROS-dependent degradation. To do so, we synthesized a class of polyols based on ROS-degradable poly(thioketals) (PTKs). PTKs were recently applied for development of orally-delivered nanoparticles that remain stable in transit through the stomach and specifically release their cargo "on demand" at sites of ulcerative colitis [40]. To date, however, this unique polymer chemistry has solely been utilized in targeted nanoparticle drug delivery applications [40,44]. Herein, we report the development and testing of PTK macrodiols amenable to synthesis of injectable, porous poly(thioketal)-urethane (PTK-UR) tissue engineering scaffolds that are selectively degraded by cell-generated ROS. These fully synthetic, non-peptide based scaffolds have been developed to further explore utilization of an ROS-dependent degradation mechanism in order to yield scaffolds with better matched rates of cellular infiltration and degradation.

2. Methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (Milwaukee, WI) except the following. 2-mercaptoethyl ether (MEE), glutaraldehyde, and cobalt chloride were purchased from Fisher Scientific (Pittsburgh, PA), and the tertiary amine catalyst (TEGOAMIN33) was obtained from Goldschmidt (Hopewell, VA). Glycolide and D,L-lactide were obtained from Polysciences (Warrington, PA). Coscat83, an organo-bismuth urethane catalyst, was supplied by ChasChem, Inc. (Rutherford, NJ). Hexamethylene diisocyanate trimer (HDIt, Desmodur N3300A) was received as a gift from Bayer Material Science (Pittsburgh, PA). Cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were supplied by Gibco Cell Culture (Carlsbad, CA). All materials were used as received unless otherwise indicated.

2.2. PTK dithiol synthesis

The condensation polymerization protocol for PTK prepolymer synthesis was adapted from Wilson et al. [40]. Briefly, p-toluenesulphonic acid monohydrate (PTSA) was added to a tri-necked boiling flask equipped with an attached addition funnel. The vessels were placed under vacuum for 15 min before being purged with nitrogen. The boiling flask was charged with anhydrous acetonitrile and batch-specific amounts of MEE (x molar eq) and 1,4 butanedithiol (BDT) ($1-x$ molar eq) where $x = 1, 0.75, 0.5, 0.25$, and 0 for the synthesized PTKs, respectively. The addition funnel was also charged with anhydrous acetonitrile and 2,2-dimethoxypropane (DMP) (0.83 M eq relative to the total amount of dithiol monomer). A molar excess of dithiol monomers was utilized relative to DMP to ensure the formation of polymers with free terminal thiols. Both the addition funnel and boiling flask's solutions were purged with flowing nitrogen for 30 min before submerging the boiling flask into an oil bath at 80 °C. After 15 min of temperature equilibration, the addition funnel stopcock was set so that the acetonitrile-DMP solution was added drop-wise into the continuously stirring boiling flask over a period of 16 h. Post synthesis, the acetonitrile was removed by rotary evaporation and the resultant PTKs were isolated by precipitation into cold ethanol and dried under vacuum. To evaluate polymer compositions, samples of the respective PTKs were dissolved in deuterated chloroform ($CDCl_3$) and analyzed with ^1H nuclear magnetic resonance spectroscopy (NMR, Bruker 400 MHz Spectrometer). ^1H NMR chemical shifts were reported as δ values in ppm relative to the deuterated $CDCl_3$ ($\delta = 7.26$). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), and m (multiplet). The number of protons (n) for a given resonance is indicated as nH and is based on integration values. ^1H NMR (400 MHz, $CDCl_3$): $\delta = 3.67–3.61$ (m, 4H), $\delta = 2.83$ (t, 4H), $\delta = 2.63$ (t, 4H), $\delta = 1.72$ (t, 4H), $\delta = 1.60$ (s, 6H).

2.3. Polyester polyol synthesis

Trifunctional or bifunctional polyester polyols were synthesized as previously documented [13]. To synthesize the trifunctional polyol, glycerol was vacuum dried for 48 h at 80 °C and then added to a 100 mL three neck flask. By molar amount, 60% ε-caprolactone, 30% glycolide, and 10% D,L-lactide were added to the glycerol starter along with a stannous octoate catalyst to yield a 900 g mol^{-1} triol (900t), a 1000 g mol^{-1} diol (1000d), and a 1500 g mol^{-1} triol (1500t).

2.4. PTK hydroxyl functionalization

The hydroxyl-functionalization of the PTK dithiols was completed [46] in order to generate polyols compatible with standard polyurethane synthesis. Briefly, PTK dithiol polymers were transferred to a boiling flask, placed under vacuum, and then exposed to a nitrogen atmosphere. The flask was charged with anhydrous dichloromethane (DCM) before adding a 10× molar excess of β-mercaptoethanol to the solution. This solution was stirred continuously at room temperature to reduce any disulfide bonds and recover the reactive thiol end groups. After 3 h of stirring, the DCM was evaporated and the residue was washed three times in cold ethanol to remove residual β-mercaptoethanol. The reduced PTK polymers were dissolved in anhydrous tetrahydrofuran (THF) before adding a 10× molar excess of cesium carbonate ($CsCO_3$) under nitrogen and stirring for 30 min at room temperature. A 5× molar excess of 2-bromoethanol was next added to the solution and stirred for 18 h under nitrogen at room temperature. After stirring, the solution was added to a separation funnel with an excess of deionized water to effectively separate the PTK-solubilizing THF layer from the water-soluble $CsCO_3$ catalyst. The hydroxyl-functionalized PTKs were extracted in THF before removing the solvent by rotary evaporation, followed by precipitation three times in cold ethanol before vacuum drying for 24 h. Molecular weights and polydispersities of the five synthesized PTK diols were analyzed by gel permeation chromatography (GPC, Agilent Technologies, Santa Clara, CA) using a mobile phase of *N,N*-dimethylformamide (DMF) with 0.1 M LiBr. Polymer molecular weights were quantified using a calibration curve generated from poly(ethylene glycol) (PEG) standards (400–4000 g mol^{-1}). Hydroxyl-functionalization was confirmed by ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.74$ (t, 4H) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR; Bruker Tensor 27 FTIR, Billerica, MA). For ATR-FTIR, thiol-terminated and hydroxyl-terminated PTK polymers were placed in contact with a ZnSe ATR crystal to quantify