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A functional polyester carrying free hydroxyl groups promotes the mineralization of osteoblast and human mesenchymal stem cell extracellular matrix

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ABSTRACT

Functional groups can control biointerfaces and provide a simple way to make therapeutic materials. We recently reported the design and synthesis of poly(sebacoyl diglyceride) (PSeD) carrying a free hydroxyl group in its repeating unit. This paper examines the use of this polymer to promote biomineralization for application in bone tissue engineering. PSeD promoted more mineralization of extracellular matrix secreted by human mesenchymal stem cells and rat osteoblasts than poly(lactic-*co*-glycolic acid) (PLGA), which is currently widely used in bone tissue engineering. PSeD showed in vitro osteocompatibility and in vivo biocompatibility that matched or surpassed that of PLGA, as well as supported the attachment, proliferation and differentiation of rat osteoblasts and human mesenchymal stem cells. This demonstrates the potential of PSeD for use in bone regeneration.

1. Introduction

Functional groups such as hydroxyls (–OH), amines ($–NH_2$), carboxyls (–COOH) and phosphates ($–PO_3H_2$) are ubiquitous in biomolecules such as proteins, nucleic acids and polysaccharides. Functional groups have a well-documented impact on cellular behavior, including adhesion, spreading, proliferation and differentiation [1–7]. For example, surfaces with amine groups ($–NH_2$) were shown to support cell adhesion, spreading and growth of Chinese hamster ovary cells compared with surfaces with other functional groups including carboxylic acid (–COOH), hydroxyl ($–CH_2OH$) and amide ($–CONH_2$, neutral) groups [3]. Recently, it was demonstrated that functional groups can facilitate multilineage differentiation of human mesenchymal stem cells (hMSCs) in three-dimensional culture, even in the absence of biological stimuli [5]. Thus, functional groups can modulate biointerfaces and may provide a simple and cost-effective way to synthesize biomaterials for clinical application [5,8].

Synthesis of biodegradable polymers containing free functional groups, however, remains a challenge [9–12]. Usually, synthetic biodegradable polymers contain labile chemical linkages such as ester, urethane, anhydride and carbonate bonds [9,13]. Active functional groups likely interfere with reactions such as polycondensation and ring-opening polymeriztion that form these labile bonds and need to be protected during polymerization [14–16]. The tedious protection and deprotection steps necessary for successful polymerization of these materials greatly compromise the overall synthetic efficiency and yield, thus limiting their availibility and utilization.

Recently, we developed an efficient method to prepare poly(sebacoyl diglyceride) (PSeD) (Fig. 1) [17–20], a polyester that contains numerous free hydroxyl groups. Hydroxyl groups may facilitate bone cell adhesion, proliferation and differentiation, thus promoting bone tissue formation and growth [1,21,22]. Hydroxyl groups are also believed to be essential for in vivo biomineralization [23–26]. The calcium phosphate layer deposited on hydroxyl-containing materials changes the material biointerface with surrounding proteins, cells and tissues, facilitating the integration of orthopedic implants such as bioactive glass and titanium with host tissues. Hydroxyl groups may account for the excellent osteocompatibility of these bone implants [27,28]. Bone extracellular

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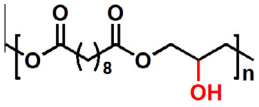

Fig. 1. The structure of poly(sebacoyl diglyceride) (PSeD) with the free hydroxyl group highlighted in red.

matrix (ECM) contains many hydroxyl groups (protein and the mineral phase) [29]. For these reasons, PSeD has good potential for applications in bone regeneration.

Many synthetic polymers, including poly(lactic-*co*-glycolic acid) (PLGA), poly(propylene fumarate) and polyurethanes have shown promise in bone regeneration [11,12,30–33]. However, they are usually biologically inert and lack free functional groups. The goal of this study is to elucidate the potential of PSeD, which contains many free hydroxyl groups, in bone regeneration. We adjusted synthetic conditions to produce physiologically osteomimetic PSeD, and investigated its physical, mechanical and biological properties, both in vitro and in vivo. We demonstrated the in vitro osteocompatibility of PSeD using primary hMSCs and rat osteoblasts, and in vivo biocompatibility via subcutaneous implantations in rats. The osteogenic capability and biocompatibility of PSeD was evaluated using PLGA as a control. The results indicated that PSeD was a promising biodegradeable polymer for bone regeneration.

2. Materials and methods

2.1. Polymer synthesis, characterization, in vitro degradation and polymer surface preparation for cell culture

PSeD was synthesized via a method modified from our previous reports [20]. Briefly, an equimolar amount of diglycidyl sebacate and sebacic acid were reacted in the presence of 0.6 mol.% bis(tetrabutylammonium) sebacate in N,N-dimethyl formamide (DMF) at 95 °C for 26 h. The reaction mixture was purified via precipitation in ethyl ether and vacuum-dried to give PSeD.

The molecular weight of PSeD was determined via gel permeation chromatography (GPC) on a Viscotek GPCmax VE2001 system equipped with a Viscotek 270 dual detector (differential refractive index and right-angle light scattering). The measurement was performed on a PSS GRAM 100 Å and 1000 Å two-column system using a dimethylacetamide (PHARMCO-AAPER, HPLC) solution of 3 g l^{-1} lithium bromide (Alfa Aesar, 99.9%) and 6 ml l^{-1} acetic acid (EDM, HPLC) at 80 °C. Polystyrene (American Polymer Standards PS34 K) was used for calibration. Differential scanning calorimetry of the polymer was performed on a TA DSC Q200 at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere. Static air–water contact angle of the PSeD slab (thickness = 1.3 mm) was recorded on KSV Instruments Theta Lite Optical Tensiometer TL100 at room temperature. Four measurements were performed and averaged. The zeta potential of a methanol solution of PSeD was recorded on a Malvern Zetasizer Nano-ZS90 instrument. Six measurements were performed and averaged.

For mechanical testing, dog-bone-shaped specimens of PSeD were made by melt molding the polymer in an aluminum mold (D142-06a die A design scaled by 1/4, length × width × thickness = 14.75 × 3 × 1.3 mm). PSeD specimens were preconditioned at 37 °C in a humidified incubator overnight and subjected to tensile strength testing immediately upon removal from the incubator. Tensile strength testing was conducted on an MTS insight mechanical analyzer equipped with a 50 N load cell according to ASTM standard D142-06a. Three specimens were tested and averaged. Deflection speed was kept at 125 mm min^{-1}.

To investigate in vitro degradation, PSeD slabs (5 × 3 × 1.3 mm) were weighed and submerged in 10 ml Dulbecco's phosphate buffered saline (DPBS) containing 0.5% fetal bovine serum (FBS; Lonza, Walkersville, MD) and incubated at 37 °C for a predetermined period of time. The samples were then retrieved, washed with deionized water and dried. The degree of degradation was determined by dry-weight change. Three specimens were used for each time point.

To prepare polymer surface for cell culture, tissue culture treated polystyrene (TCPS) surfaces were coated with PSeD as follows. A methanol solution of PSeD (1 g l^{-1}) was filtered through a 0.2 μm filter and added to 24-well (80 μl per well) or 96-well (20 μl per well) TCPS plates. The plates were vacuum dried overnight after evaporation of the solvent, sterilized by ultraviolet (UV) light for 30 min, washed with phosphate buffered saline three times and washed with culture medium once (1 ml per well). PLGA (5050 DLG 5E, Lakeshore Biomaterials, Birmingham, AL, USA) coating on TCPS was used as a control and was prepared in the same manner as PSeD coating except that 2,2,2-trifluoroethanol was used as a solvent; the concentration was identical (1 g l^{-1}).

2.2. Polymer–hMSC interaction

2.2.1. hMSC culture

hMSCs (passage 1 from the vertebrae of cadaveric bodies, kindly provided by Prof. Albert D. Donnenberg at the University of Pittsburgh School of Medicine [34]) were cultured on PSeD and PLGA surfaces with α-modified minimal essential medium (α-MEM, Cellgro) supplemented with 15% FBS, 10,000 U ml^{-1} penicillin and 10,000 U ml^{-1} streptomycin (Sigma, USA) in a fully humidified incubator (37 °C, 5% CO_2). The medium was changed every 2 days, and within 5–7 days cells were passaged at ~80% confluence. hMSCs in the second passage were used for the following experiments.

2.2.2. hMSCs differentiation test

Osteogenic differentiation of hMSCs was assessed by immunofluorescent detection of the osteogenic biomarkers Runt-related transcription (Runx2) and Collagen type I (Col I). hMSCs were seeded onto PSeD and PLGA polymer surfaces at a density of 1×10^4 cells cm^{-2} and cultured with α-MEM containing 10% FBS, 10^{-8} M dexamethasone and 5 mM β-glycerophosphate for 21 days. The hMSCs were then replated on 3.5 cm glass bottom petri dishes at a density of 2×10^4 cells ml^{-1} for 24 h prior to staining.

hMSCs were fixed, permeabilized with 0.1% triton-X 100 for 5 min and blocked with 2% BSA for 1 h at room temperature. The cells were then incubated with primary antibodies against human Runx2 (Santa Cruz, USA) and Col I (Millipore, USA) (1:100 dilution in DPBB (0.5% BSA in DPBS)) overnight in a wet chamber at 4 °C. hMSCs were then incubated with CY3-fab2-conjugated donkey anti-goat IgG secondary antibody (Runx2) and FITC-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) (Col I) for 60 min at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 20 min. Cells were rinsed with DPBS 3 times and DPBB twice between each antibody incubation. Fluorescent micrographs were taken using a Nikon Eclipse Ti microscope. Five high power fields were used for each condition and the representative images were presented in this paper.

2.2.3. Mineralization of ECM

To detect ECM mineralization of hMSCs cultured on PSeD and PLGA, cells were stained with Alizarin Red S. Briefly, after 21 days in culture, hMSCs were washed twice with DPBS and fixed with 2% paraformaldehyde for 20 min. The cells were then incubated with 1% Alizarin Red S dissolved in distilled water (PH 4.1–4.3) for