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Zirconium amine tris(phenolate): A more effective initiator for biomedical lactide

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ABSTRACT

Here a zirconium amine tris(phenolate) is used as the initiator for the production of polylactide for biomedical applications, as a replacement for a tin initiator (usually tin octanoate). The ring opening polymerization (ROP) was carried out in the melt at 130 °C. The zirconium-catalyzed PLA (PLA-Zr) required 30 min, resulting in a polydispersity index (PDI) of 1.17, compared to 1 h and PDI = 1.77 for tin-catalyzed PLA (PLA-Sn). PLA-Zr and PLA-Sn supported osteosarcoma cell (MG63) culture to the same extent (cell number, morphology, extracellular matrix production and osteogenic function) until day 14 when the PLA-Zr showed increased cell number, overall extracellular matrix production and osteogenic function. To conclude, the reduction in reaction time, controllable microstructure and biologically benign nature of the zirconium amine tris(phenolate) initiator shows that it is a more effective initiator for ROP of polylactide for biomedical applications.

1. Introduction

Linear aliphatic polyesters are routinely used in biomedical research [1,2]. Ring-opening polymerization (ROP) of cyclic monomers (i.e. lactide or glycolide) is a commonly used method to synthesize linear aliphatic polyesters as it provides good control over polymer molecular characteristics such as predictable molecular weight, end group and polymer stereochemistry control [3]. ROP requires an initiator, and tin compounds are the most widely used in industry as they have the advantages of being cheap, soluble in common organic solvents and relatively easy to handle [4]. Concerns have been raised regarding the toxicity of tin initiators for the synthesis of polymers for biomedical applications [5,6]. Tin compounds tend to accumulate in lung tissue and brain, which may cause neurotoxicity and slow penetration into blood circulation systems [5,7]. To avoid these potential side-effects, the amount of tin residue in a standard biomedical polymer is limited to below 20 ppm according to the US Food and Drug Administration [8]; it is impossible to completely remove the residual tin from the resulting polymers, so sophisticated purification processes have to be used in order to minimize the metal residue [8]. Continuous efforts have been made to develop initiators based on more biologically benign metals as alternatives to tin initiators, for example aluminum [9–12], magnesium [13], zinc [13–18] and calcium [19,20] for production of biomedical polymers. In addition, Group 4 metal complexes, have also shown great promise to give polymers with desired properties [21–24]. The polymerization has been shown to proceed via a traditional coordination insertion mechanism, this has been proven via end group analysis (MALDI-ToF mass spectrometry and NMR spectroscopy) [25]. It has been shown that an unusual combination of high stereoregularity and reactivity can be achieved using a zirconium initiator supported by amine tris(phenolate) ligands for ROP of lactide under solvent-free conditions [25]. The application of solvent-free conditions eliminates the hazard of using a solvent to carry out polymerization; furthermore it has been previously noted that a solvent-free method is necessary for industry and allows for a highly pure product [25]. In addition to this, zirconium compounds typically exhibit 10–20 times fewer toxic effects compared with tin [26]. Reduced processing conditions, e.g. temperature and time, will help minimize manufacture cost which is favorable for mass production, as is a reduction in batch-to-batch variation which it can be hypothesized to be achieved by a more controlled polymerization mechanism. Despite the great success in developing relatively biocompatible initiators for well-controlled ROP, to date, it is unclear how cells will respond to polymers prepared from these initiators compared to the commercial tin initiator. In this study, PLA prepared from the zirconium-based amine tris(phenolate) initiator [25] (Zr) and the commercial tin-based initiator, namely tin octanoate (Sn), were characterized. A comparative investigation of the osteoblast-like osteosarcoma MG63 cell line attachment, proliferation and differentiation on smooth PLA films was performed to establish the effects of initiator residue on cell behavior.

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2. Experimental section

2.1. Polymerization

D,L-Lactide was purchased from Sigma-Aldrich and was purified by recrystallisation from toluene and dried under vacuum overnight. Tin octanoate (Sigma) was used as received. The zirconium amine tris(phenolate) initiator was prepared according to the method reported previously [25]. Polymerizations were performed at a temperature of 130 °C under solvent-free conditions with a monomer to initiator (Zr or Sn) ratio [M]/[I] of 200:1. In all cases 3 g of D,L-lactide was used. After the reaction time (0.5 or 1 h) an excess of methanol was used to quench the reaction and the resulting solid was dissolved in dichloromethane. The solvents were then removed under vacuum. The resulting solid was further purified by washing with methanol and dried in vacuo overnight. The conversions were determined by ^{1}H NMR spectroscopy (Bruker 300 MHz spectrometer, solvent: CDCl3), and molecular weights (M_n) and polydispersity index (PDI) of the polymers were determined by gel permeation chromatography (GPC, Polymer Laboratories GPC 50, solvent: THF, 1 mL/min flow rate at 35 °C and referenced to polystyrene standards). Three polymers were made: two using the tin initiator and a reaction time of 0.5 h or 1.0 h, denoted as PLA-Sn-0.5 and PLA-Sn-1.0, and one using the zirconium initiator and a reaction time of 0.5 h, denoted as PLA-Zr-0.5.

2.2. Preparation and characterization of PLA films

5% (*w*/*v*) PLA was first dissolved in chloroform. 200 μL of the solution was placed onto a cover slip using 1 mL syringe at room temperature (20 °C) and allowed to spread. A thin layer of transparent polymer film was obtained after complete evaporation of solvent. The thin film was then washed with excess of distilled water and further dried in vacuo overnight. The Zr and Sn initiator metal residues in the PLA films prior to cell culture were measured by inductively coupled plasma optical emission spectrometry (ICP-OES, MEDAC Ltd., UK). The PLA films were sterilized in 1% antibiotic/antimycotic solution (Sigma) over night at 4 °C prior to cell culture [27]. After sterilization, the antibiotic/antimycotic solution was removed and the PLA films washed with excess of phosphate buffer saline (PBS). Metal content of the films was measured by inductively coupled plasma optical emission spectroscopy. To measure the water contact angle on the surface, 5 μL of distilled water was placed onto the film surface and the measurement taken after 5 min using a goniometer (NRL 100-00).

2.3. Cell culture

The human osteosarcoma cell line MG63 was obtained from the European Collection of Cell Culture (ECACC). The MG63 cells were maintained at 37 °C and 5% CO2 in a standard cell culture medium which contained Dulbecco's Modified Eagle's medium (DMEM, Sigma), supplemented with 10% (*v*/*v*) fetal calf serum (FCS, Sigma), 1 mM sodium pyruvate (Sigma), 1% (*v*/*v*) antibiotic-antimycotic and 1% (*v*/*v*) non-essential amino acids (NEAA, Sigma) for proliferation. To differentiate MG63 cells, the standard cell culture medium was switched to an osteogenic medium after 3 days which contained 10 mM β-glycerophosphate (Sigma), 100 nM dexamethasone (Sigma) and 50 μg/mL ascorbic acid-2-phosphate (Sigma) [28]. The medium was refreshed every 2–3 days. The PLA films were placed in a 24-well tissue culture treated plates (Nuclon™ Δ Surface) and secured with customized silicon rings. MG63 cells were seeded onto the samples with a density of 20,000 cells/cm^2 and maintained at 37 °C and 5% CO2. Tissue culture polystyrene (TCPS) was used as control. MG63 cells were cultured for 21 days on the smooth PLA-Zr and PLA-Sn, and TCPS. They were assessed in a standard culture medium up to 3 days then an osteo-differentiation medium up to 21 days.

2.4. Cell number

Cell number was determined using the PicoGreen assay kit (Invitrogen Ltd., Paisley UK) as per the manufacturer's instructions. Briefly, samples were prepared by rinsing three times with PBS then lysing with 0.05% Triton X-100 and freeze-thawed at −80 °C for 2–3 cycles. The fluorescence was read at excitation and emission 485/528 nm (BioTek Synergy HT) and readings compared to a standard curve to calculate cell number.

2.5. Cell morphology

Cell morphology on the PLA films was observed by f-actin staining and scanning electron microscopy (SEM). For cytoskeletal organization, FITC-phalliodin (Sigma) was used to label f-actin filaments. Cells were washed with PBS, fixed with 3.7% formaldehyde in PBS for 5 min and permeabilized with 0.1% Triton X-100 for 10 min before staining with 50 μM FITC-phalloidin for 40 min at room temperature. Cells were observed with a Leica DMI4000B fluorescent microscope. For SEM, at each time point, the culture medium was removed and the samples were fixed in 2.5% glutaraldehyde for 2 h. The samples were then washed with serum free culture medium and post-fixed in 1% osmium tetroxide in PBS buffer for 1 h. After thoroughly washing with serum free culture medium, the samples were stained with 2% aqueous uranyl acetate in water for 1 h in dark. Then the samples were freeze-dried over-night. A thin layer of gold was sputter-coated onto the samples before examining by SEM (JEOL JSM 6480LV SEM).

2.6. Osteogenic function

The alkaline phosphatase (ALP) activity of cells was measured using the *p*-nitrophenyl phosphate reduction method. Cells were lysed using 0.05% Triton X-100 solution then the samples were frozen and thawed at −80 °C for 2–3 cycles, and sonicated for 15 min. 50 μL of samples were added into 50 μL of *p*-nitrophenyl phosphate substrate in a 96-well plate and incubated for 1 h at 37 °C. The reaction was stopped by the addition of 50 μL of 1 N NaOH and absorbance measured at 405 nm. The ALP activity of cells was normalized to the cell number measured by the Picogreen assay. The osteocalcin (OC) solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA, Invitrogen, UK) was used to quantify OC production as per the manufacturer's instructions. Standards and samples were pipetted into 96-well multi-plate and reacted with pre-coated monoclonal antibody labeled with horseradish peroxidase (HRP). Wash solution was used to remove unbound enzyme labeled antibody after 2 h incubation. The bound enzyme labeled with antibody can be detected by conducting a chromogenic reaction for a further 30 min. The absorbance measurements were performed at 450 nm and OC production calculated using a standard curve. Mineralization was analyzed using Alizarin Red S to detect the deposited calcium ions on the PLA films and TCPS as described previously [29]. Briefly, MG63 cells were fixed in 10% (*v*/*v*) formaldehyde and washed with PBS for three times. Samples were immersed in 40 mM Alizarin Red S (pH = 4.2) solution for 20 min, with gentle shaking before being washed with PBS for 5 min. Insoluble red colour calcium ion-Alizarin Red S complex was observed microscopically (Leica DMI4000B) followed by using Image J analysis software (http://rsb.info.nih.gov/ij) to analyze the total area of mineralization stained by Alizarin Red S [30].

2.7. Statistical analysis

Statistical analysis was conducted using one way or two-way ANOVA with Bonferroni post-tests using Prism 4.0 (GraphPad Software Inc., San Diego CA, USA).