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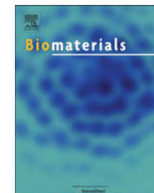

A poly(D,L-lactide) resin for the preparation of tissue engineering scaffolds by stereolithography

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ABSTRACT

Porous polylactide constructs were prepared by stereolithography, for the first time without the use of reactive diluents. Star-shaped poly(D,L-lactide) oligomers with 2, 3 and 6 arms were synthesised, end-functionalised with methacryloyl chloride and photo-crosslinked in the presence of ethyl lactate as a non-reactive diluent. The molecular weights of the arms of the macromers were 0.2, 0.6, 1.1 and 5 kg/mol, allowing variation of the crosslink density of the resulting networks. Networks prepared from macromers of which the molecular weight per arm was 0.6 kg/mol or higher had good mechanical properties, similar to linear high-molecular weight poly(D,L-lactide). A resin based on a 2-armed poly(D,L-lactide) macromer with a molecular weight of 0.6 kg/mol per arm (75 wt%), ethyl lactate (19 wt%), photo-initiator (6 wt%), inhibitor and dye was prepared. Using this resin, films and computer-designed porous constructs were accurately fabricated by stereolithography. Pre-osteoblasts showed good adherence to these photo-crosslinked networks. The proliferation rate on these materials was comparable to that on high-molecular weight poly(D,L-lactide) and tissue culture polystyrene.

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1. Introduction

Tissue engineering scaffolds prepared by rapid prototyping techniques have several advantages when compared to those fabricated by conventional techniques such as porogen leaching, phase-separation/freeze-drying and gas-foaming. These conventional techniques often result in inhomogeneous scaffolds with broad pore size distributions, poor pore interconnectivity and inferior mechanical properties. Rapid prototyping allows computer-designed architectures to be built in a reproducible manner. This enables the preparation of scaffolds with optimal properties regarding pore structure and connectivity, geometry, mechanical properties, cell-seeding efficiency, and transport of nutrients and metabolites [1]. Of the rapid prototyping techniques, stereolithography is the most versatile method with the highest accuracy and precision [2]. Its working principle is based on spatially controlled solidification of a liquid photo-polymerisable resin. Using a computer-controlled laser beam or a digital light projector with a computer-driven building stage, a solid, three-dimensional object can be constructed in a layer-by-layer fashion.

The number of stereolithography resins available for use in biomedical applications is limited. Poly(ethylene glycol)-dimethacrylate has been used to fabricate non-degradable cell-containing hydrogels in pre-designed shapes [3–5]. In tissue engineering however, resorbable constructs are desired. The only biodegradable macromers that have been applied are based on trimethylene carbonate and ε-caprolactone oligomers [6,7], or on poly(propylene fumarate). The latter requires a reactive diluent such as diethyl fumarate to obtain an appropriate reaction rate and viscosity of the resin [8]. Upon photo-polymerisation of these resins, networks are formed with low glass transition temperatures and low E-modulus values under physiological conditions. For tissue engineering of hard tissues such as bone, strong and rigid biodegradable materials are desired. Polylactide is such a material, and it has a long track record of successful application in the clinic and in the preparation of tissue engineering scaffolds. The amorphous form, poly(D,L-lactide) (PDLLA), has successfully been applied in resorbable bone fixation devices clinically [9,10] and for scaffolds that proved well suited for bone tissue engineering [11–13]. PDLLA has a glass transition temperature of approximately 55 °C, and an elasticity modulus close to 3 GPa; it is one of the few biodegradable polymers with mechanical properties that approach those of bone (the E-modulus of bone is 3–30 GPa) [14]. The ability to process PDLLA-based materials by stereolithography would allow to significantly advance the field of bone tissue engineering as optimised

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structures with regard to mechanical properties, cell seeding and culturing can then be prepared.

PDLLA networks can be formed by (photo-initiated) radical polymerisation of poly(lactide) oligomers end-functionalised with an unsaturated moiety such as a methacrylate- [15], acrylate- [16] or fumarate- [17] group. To be able to apply PDLLA macromers in stereolithography, the macromer must be in the liquid state. This can be achieved by heating or diluting. Reactive diluents such as methyl methacrylate, butane-dimethacrylate and N-vinyl-2-pyrrolidone have been used in regular photo-polymerisation reactions [15,18] and in stereolithography [19,20]. This, however, introduces significant amounts of a non-degradable component.

This work aims at developing a photo-curable PDLLA-based resin that is free of reactive diluents, and applying it in stereolithography. Ethyl lactate was employed as a non-reactive diluent. This poses a challenge to the optimisation of the resins, as shrinkage upon extraction of the photo-polymerised networks can be quite significant. Methacrylate end-functionalised poly(D,L-lactide) oligomers of varying molecular architectures were synthesised and photo-crosslinked in the presence of ethyl lactate. Suitable resin compositions were used in stereolithography to prepare porous structures with pre-designed architectures at high resolution. Cell attachment and proliferation on PDLLA networks prepared by stereolithography were assessed.

2. Materials and methods

2.1. Materials

D,L-Lactide was obtained from Purac Biochem, The Netherlands. Hexanediol, glycerol, stannous octoate, methacryloyl chloride (MACl), hydroquinone, vitamin E, sodium pyruvate, N-methyl dibenzopyrazine methyl sulfate (PMS) and HistoChoice tissue fixative were purchased from Sigma–Aldrich, USA and used without further purification.

Sorbitol, triethyl amine (TEA), eosin–hematoxylin solution (Fluka, Switzerland), ethyl lactate (Merck, Germany), and technical grade isopropanol and acetone (Biosolve, The Netherlands) were used as received. Irgacure 2959 (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone) and Orasol Orange G were gifts from Ciba Specialty Chemicals, Switzerland. Lucirin TPO-L (ethyl-2,4,6-trimethylbenzoylphenylphosphinate) was a gift from BASF, Germany. Analytical grade dichloromethane (Biosolve, The Netherlands) was distilled from calcium hydride (Acros Organics, Belgium).

Foetal bovine serum (FBS), L-glutamine, penicillin–streptomycin and trypsin were obtained from Lonza, Belgium. Alpha-Modified Eagle Medium (αMEM) and RPMI 1640 medium without phenol red were bought from Gibco, USA. Sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) was obtained from PolySciences, USA.

2.2. Polymer syntheses

Star-shaped oligomers were synthesised on a 30 g scale by ring opening polymerisation of D,L-lactide for 40 h at 130 °C under an argon atmosphere, using stannous octoate as a catalyst. Hexanediol, glycerol and sorbitol were used as initiators, to prepare 2-armed, 3-armed and 6-armed oligomers, respectively. The molecular weights and arm lengths were varied by adjusting the monomer to initiator ratio. Proton-nuclear magnetic resonance spectroscopy (^{1}H NMR, $CDCl_3$, Varian 300 MHz) was used to determine lactide conversion and oligomer molecular weights.

Oligomers were functionalised by reacting the terminal hydroxyl groups with methacryloyl chloride (MACl) in dry dichloromethane under an argon atmosphere. The formed HCl was scavenged with triethyl amine (TEA). An excess of 20–50 mol% MACl and TEA per hydroxyl end group was used.

The macromer solutions were filtered and precipitated into cold isopropanol. The isolated macromers were then washed with water and freeze-dried. Macromers with molecular weights lower than 1 kg/mol are soluble in isopropanol. These macromers were purified by washing the dichloromethane solutions with a saturated aqueous sodium bicarbonate solution, drying with magnesium sulfate, filtrating and evaporating the solvent under reduced pressure. The yield was 70–90%, depending on macromer molecular weight.

^{1}H NMR was used to determine the degrees of functionalisation of the macromers. Throughout this paper, all macromers are labelled as MA × B, in which A stands for the number of arms and B for the arm length. For example, M3 × 0.6 k stands for a 3-armed methacrylated PDLLA macromer with each arm having a molecular weight of 0.6 kg/mol. The corresponding non-functionalised oligomer is designated as O3 × 0.6 k, and the network obtained after photo-polymerisation of the macromer is designated as N3 × 0.6 k.

As a reference material, high-molecular weight poly(D,L-lactide) (HMW PDLLA) was synthesised by ring opening polymerisation under similar conditions. The polymer was purified by precipitation from acetone into water, and vacuum dried. Molecular weights were determined using a Viscotek GPCmax gel permeation chromatography setup with Viscotek 302 Triple Detection Array and $CHCl_3$ as an eluent with a flow of 1 mL/min. The following values were obtained: $\overline{M}_w = 4.3 \times 10^5$ g/mol, $\overline{M}_n = 3.6 \times 10^5$ g/mol and $[\eta] = 5.5$ dL/g.

2.3. Resin formulation and network preparation

To formulate liquid polymerisable poly(D,L-lactide) resins, the different macromers were diluted with ethyl lactate. The resin viscosity was determined over a range of diluent concentrations at 25 °C using a Brookfield DV-E rotating spindle viscometer, equipped with a small sample adapter. The shear rate was varied between 0.56 and 56 s^{-1} (Brookfield s21 spindle, rotating at 0.6–60 rpm). To prevent premature crosslinking 0.2 wt% hydroquinone was added to the liquid.

To obtain networks, the resins containing 2.0 wt% Irgacure 2959 as a biocompatible UV photo-initiator [21,22], were irradiated with 365 nm UV-light for 15 min (Ultralum crosslinking cabinet, intensity 3–4 mW/cm^2). Silicone rubber moulds, covered with fluorinated ethylene–propylene (FEP) films to avoid oxygen inhibition, were employed to prepare specimens measuring 45 × 30 × 1.5 mm^3.

2.4. Network characterisation

The obtained PDLLA networks were extracted with 3:1 mixtures of isopropanol and acetone, and dried at 90 °C under a nitrogen flow for 2 d. Gel contents were determined in duplicate from the mass of the dry network after extraction (m_{dry}) and the macromer mass initially present in the resin (m_0). The specimens were then swollen in ethyl lactate for 2 d. The volume degrees of swelling of the networks were calculated using the swollen mass (m_{wet}) and the densities of PDLLA (1.25 g/mL) and ethyl lactate (1.03 g/mL):

$$Q = 1 + \frac{m_{wet} - m_0}{m_0} \times \frac{\rho_{PDLLA}}{\rho_{EL}}$$

Mechanical properties were determined in 5-fold in 3-point bending tests and in tensile tests using a Zwick Z020 universal tensile tester. The dimensions of the extracted and dried PDLLA networks for the bending tests were approximately 30 × 20 × 1 mm^3. According to the ISO 178 norm used, the span-width and strain rate were adjusted to the specimen thickness. For the tensile tests, dumbbell-shaped samples were used according to the ISO 37-2 norm.

Water uptake of the different networks was assessed by conditioning extracted and dried samples in demineralised water at 37 °C for 15 h. Water uptake was defined as the relative increase in weight.

2.5. Stereolithography

To fabricate (porous) PDLLA structures by stereolithography, a resin comprising 75 wt% M2 × 0.6 k PDLLA macromer, 19 wt% ethyl lactate, 6 wt% Lucirin TPO-L visible light photo-initiator, 0.025 wt% hydroquinone inhibitor and 0.2 wt% Orasol Orange G dye was formulated.

Tensile test specimens (ISO 37-2), films measuring 70 × 24 × 0.5 mm^3 and a scaffold with a gyroid architecture were designed using Rhinoceros 3D (McNeel Europe) and K3DSurf (freeware obtainable from http://k3dsurf.sourceforge.net) computer softwares. The designs were built using an EnvisionTec Perfactory Mini Multilens stereolithography apparatus. This stereolithography apparatus (SLA) is equipped with a digital micro-mirror device [23] which enables projections of 1280 × 1024 pixels, each measuring 32 × 32 μm^2. Using a build platform step height of 25 μm, layers of resin were sequentially photo-crosslinked by exposure to a blue light pattern for 40 s. The intensity of the light was 20 mW/cm^2 and the wavelength ranged from 400 to 550 nm, with a peak at 440 nm.

After building, the scaffolds were extracted with 3:1 isopropanol and acetone mixtures and dried at 90 °C for 2 d. Imaging of gold-sputtered scaffolds was performed by scanning electron microscopy (SEM) employing a Philips XL30 FEG device with a 5.0 kV electron beam. Structural analysis was performed using micro-computed tomography (μCT) scanning on a GE eXplore Locus SP scanner at 6.7 μm resolution. The scan was carried out at a voltage of 80 kV, a current of 80 μA and an exposure time of 3000 ms. No filter was applied.

2.6. Cell culturing

Disk-shaped specimens (diameter 15 mm) of HMW PDLLA were punched out from compression-moulded (140 °C, 250 kN) films. PDLLA network (N2 × 0.6 k) disks were punched out from the films prepared by stereolithography. Prior to seeding, the samples were disinfected in 70% isopropanol for 5 min, rinsed 3 times in phosphate-buffered saline (PBS) and incubated overnight in medium. Mouse pre-osteoblasts (MC3T3 cell line) were cultured in αMEM supplemented with 10% FBS, 2 mM, L-glutamine, 1 mM sodium pyruvate and 2% PennStrep (penicillin–streptomycin). The cells were detached from the culture flask using 0.10% (w/v) trypsin/0.050% (w/v) EDTA, after which 12 × 10^3 cells in 2 mL medium were pipetted onto each disk, in

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