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Polyhydroxyalkanoates: Waste glycerol upgrade into electrospun fibrous scaffolds for stem cells culture

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ABSTRACT

This integrated study shows that waste glycerol can be bio-valorized by the fabrication of electrospun scaffolds for stem cells. Human mesenchymal stem cells (hMSC) provide an interesting model of regenerating cells because of their ability to differentiate into osteo-, chrondro-, adipo- and myogenic lineages. Moreover, hMSC have modulatory properties with potential on treatment of immunologic diseases. Electrospun fiber meshes offer tunable mechanical and physical properties that can mimic the structure of the native extracellular matrix, the natural environment where cells inhabit. Following a biorefinery approach, crude glycerol directly recovered from a biodiesel post-reaction stream was fed as major C source to *Cupriavidus necator* DSM 545 to produce polyhydroxyalkanoates at polymer titers of 9–25 g/L. Two of the P(3HB-4HB-3HV) terpolymers produced, one containing 11.4% 4HB and 3.5% 3HV and the other containing 35.6% 4HB and 3.4% 3HV, were electrospun into fibers of average diameters of 600 and 1400 nm, respectively. hMSC were cultured for 7 days in both fiber meshes, showing their ability to support stem cell growth at acceptable proliferation levels. Comparative results clearly demonstrate that scaffold topology is critical, with electrospun PHA fibers succeeding on the support of significant cell adhesion and proliferation, where planar PHA films failed.

1. Introduction

Industrial efforts for microbial synthesis of polyhydroxyalkanoates (PHA) started with the pilot plant trial of Imperial Chemical Industries in 1980 with the aim of bringing environmental sustainability to the polymer sector, producing a biodegradable polymer to replace the petroleum based ones. PHAs are biological macromolecules that can be produced from different C sources besides refined sugars, namely sugar cane syrup molasses and vegetable oils [1]. Since 2003, as biodiesel production has become more prominent in the energy sector, the volumes of available glycerol in the European and US market increased, while the respective spot price decreased [2]. From 1995 to 2007, the price of refined glycerol dropped from US$1 to US$0.34 per pound and, in 2006, crude glycerol by-product of biodiesel was US$0.02 per pound [3], calling for new uses of this commodity. As far as bioprocess robustness and cost efficiency are concerned, it is crucial to assess crude grade instead of highly purified glycerol as C source, since many microbial cultures fail due to traces of toxic compounds carried out from biodiesel production. Following a biorefinery approach, Cavalheiro et al. [4,5] have demonstrated the efficient use of crude glycerol as major C source for the biological synthesis of PHAs.

Synthetic polyesters such as polycaprolactone, polyglycolate and polylactate are polymers approved by regulatory agencies for medical applications [6,7]. Due to their ester moiety, these polymers are biodegradable into products tolerable by human

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metabolism at low concentrations and they have, therefore, been extensively tested as materials for the development of scaffolds for cell and tissue engineering, as reviewed elsewhere [8], two specific examples with clinical applications include cartilage regeneration [9] and engineered bladders [10]. Polyester hydrophobicity, elasticity, and biodegradability depend on the alkyl moiety, and block co-polymers, e.g. poly(lactic-co-glycolic acid) with different glycolide and lactide composition have been chemically synthesized to tailor such properties to biomedical requirements. Bio-produced PHAs are also polyesters composed by different monomers including the R-3-hydroxybutyrate (3HB), R-4-hydroxybutyrate (4HB), R-3-hydroxyvalerate (3HV), R-3-hydroxyhexanoate (3HHx) and R-3-hydroxyoctanoate (3HO). According to their monomeric composition, polymers with different hydrophobicity and elasticity are obtained. In general, the Young's modulus and tensile strength increase with the increase of the 3HB molar fraction in co-polymers such as P(3HB-3HV) and P(3HB-4HB), whereas elasticity decreases [11–13]. Contrarily to other biopolymers with biomedical uses, such as collagen and hyaluronate, PHAs are thermoplastic and thus suitable to be processed by a wide range of techniques usually applied to synthetic polymers [14].

In 2007, the FDA approved the clinical application of P(4HB) ("TephaFLEX® Absorbable Suture" and "BioTREK™ Bioabsorbable Septal Repair"). Since then, there has been an increasing interest in testing PHAs for the manufacture of scaffolds for cell and tissue engineering applications. Homopolymers (P(3HB) and P(4HB)), as well as co-polymers (P(3HB-4HB), P(3HB-3HV) and P(3HB-3HHx)) and blends of different PHA types have been used for the fabrication of scaffolds with different properties [14–19]. An alternative strategy has been the use of PHAs combined with other materials such as polyvinyl alcohol, polylactatic acid [20] or chitosan [21]. Terpolymers had been successfully used as planar surfaces for cell cultivation [22,23], but not processed in 3D structures. Recent studies to provide 3D structures using PHAs include the fabrication of microspheres by solvent-water and solvent evaporation based protocols [15]. Plus, techniques such as particulate leaching [24], salt leaching [16], freeze drying [17] and thermal induced solid–liquid phase separation [18] have been used for the fabrication of porous scaffolds with different pore sizes according to the protocol and polymer used. Flat sheets have also been prepared and rolled [25]; in addition, fibrous structures have been processed by extrusion [12] and more often, by electrospinning [19–21]. Different cell types have been cultured on 2D or 3D PHA-based scaffolds, including L929 mouse fibroblast cells [21], used for cytotoxicity tests, other animal and human cell lines [15,20,24], and stem cells [17–19]. In addition, different in vivo studies show good biocompatibility of these materials, their ability to support cell growth and their low level of inflammatory response. Examples with animal trials that show no adverse effects of PHAs are (i) a study where PHB and P(3HB-3HV) were implanted as sutures to treat muscle facial cuts in Wistar rats [11] and (ii) PHB being used as an artificial nerve conduit with adipose-derived stem cells transplanted into rat sciatic nerve model, with the aim of releasing growth factors for stimulation of endogenous Schwann cells activity [14].

The mechanical properties and the biocompatibility of PHAs significantly vary with their chemical composition and the processing mode. Therefore, in the current study, the homopolymer P(3HB) and three terpolymers directly produced from crude waste glycerol are firstly assessed for material bioprocessing cytotoxicity and biocompatibility using L929 mouse fibroblast. Then, PHAs ability to support tissue engineering strategies was assessed through seeding and cultivation of human bone marrow (BM) derived mesenchymal stem cells (hMSC) on the PHA scaffolds. MSC are anchorage dependent stem cells with a high potential for regenerative due to their potential to differentiate in osteo-, chrondro-, adipo- [26] and myogenic [27] lineages, as well as their immune modulatory properties, which can be explored, for example on treatment of graft-vs-host disease and auto-immune disorders [28]. Taking into account the small amount of available hMSC in vivo and the large numbers usually required for their therapeutic application, a stage of ex vivo cultivation of hMSC involving seeding, expansion and, in some cases, differentiation is often required. Then, cells can be harvested for administration or an ex vivo seeded scaffold can be implanted in vivo. In the latter case, focusing tissue engineering settings, the scaffold biodegradability rate is an important issue.

In this work, scaffolds were prepared by electrospinning of a polymer solution. This method allows the production of continuous fiber meshes with high specific surface areas and diameters ranging from 40 to 4000 nm [29]. Electrospinning has been widely used in the manufacture of non-woven fiber meshes for tissue engineering applications. Electrospun fibers meshes offer tunable mechanical and physical properties that can mimic the structure of the native extracellular matrix (ECM), the natural environment where cells reside. It is noteworthy that one of the main structural components of the ECM is collagen, with structure fiber bundle diameters ranging in the nanoscale [30]. Electrospun nanofibers of biocompatible polymers can provide a good synthetic scaffold for hMSC cultivation by mimicking the ECM in size and structure. Indeed, the BM niche where MSC are found is characterized by an interplay of soluble factors, cell-to-cell, and cell to ECM components interactions [31]. Previous studies using electrospun scaffolds for hMSC show the ability to improve cell adhesion and proliferation, as well as the effect of scaffold architecture in cell response [32,33] and differentiation toward endothelial lineages [19].

PHA properties (e.g. stiffness, hydrophobicity and biodegradability) can be tuned according to the structure of the biomacromolecules produced. Importantly, the current work uses crude glycerol as carbon source for the production of PHA terpolymers, which are then, for the first time, employed on the manufacturing of electrospun scaffolds for stem cells proliferation. The approach taken illustrates the potential to combine PHA bioproduction conditions to tailor polymer properties and electrospinning technique to provide a scaffold 3D structure that mimics the stem cell niche. The results are expected to contribute toward sustainability of both biodiesel and biodegradable plastics production (Fig. 1).

2. Materials and methods

2.1. PHA production and recovery

PHAs were bioproduced in a 2 L reactor (B. Braun M2 culture vessel coupled to a Biostat MD 884402/0 and a digital control unit 884201/9) by two stage fed-batch cultivation of *Cupriavidus necator* DSM 545. Cell growth was performed under balanced conditions (growth stage) and PHA synthesis was subsequently promoted by nitrogen starvation. The glycerol-rich-phase (GRP) from a biodiesel plant (Fábrica Torrejana de Biocombustíveis, S.A., Portugal) was utilized as major C source. Gamma-butyrolactone (GBL) and propionic acid (PA), both from ACROS, purity 99%, were used as precursors for the 4HB and 3HV monomers, respectively. GBL and PA were fed to the system in amounts 10 and 100 fold lower, respectively, than those of biodiesel glycerol. Four batches were carried out and labeled from A to D. The specific conditions used for each batch concerning C sources, dissolved oxygen concentrations (DOC) and cultivation time per stage are shown in Table 1. Cultivation protocols for the production of P(3HB) and P(3HB-4HB-3HV) have been previously described [4,5]. In the current study minor changes were introduced: (i) during the growth phase, the glycerol concentration was controlled within the range 20–60 g/L; (ii) in batch B the GBL pulse at the beginning of the nitrogen starvation stage was 6 g/L. Information on monomers quantification, strain storage, inoculum preparation, composition