Acta Biomaterialia 6 (2010) 4208–4217

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Effects of the architecture of tissue engineering scaffolds on cell seeding and culturing

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ARTICLE INFO

Article history:
Received 20 April 2010
Received in revised form 30 May 2010
Accepted 10 June 2010
Available online 16 June 2010

Keywords:
Scaffold architecture
Computer-aided design
Cell seeding
Hypoxia
Nutrient transport

ABSTRACT

The advance of rapid prototyping techniques has significantly improved control over the pore network architecture of tissue engineering scaffolds. In this work, we have assessed the influence of scaffold pore architecture on cell seeding and static culturing, by comparing a computer designed gyroid architecture fabricated by stereolithography with a random pore architecture resulting from salt leaching. The scaffold types showed comparable porosity and pore size values, but the gyroid type showed a more than 10-fold higher permeability due to the absence of size-limiting pore interconnections. The higher permeability significantly improved the wetting properties of the hydrophobic scaffolds and increased the settling speed of cells upon static seeding of immortalised mesenchymal stem cells. After dynamic seeding followed by 5 days of static culture gyroid scaffolds showed large cell populations in the centre of the scaffold, while salt-leached scaffolds were covered with a cell sheet on the outside and no cells were found in the scaffold centre. It was shown that interconnectivity of the pores and permeability of the scaffold prolonged the time of static culture before overgrowth of cells at the scaffold periphery occurred. Furthermore, novel scaffold designs are proposed to further improve the transport of oxygen and nutrients throughout the scaffolds and to create tissue engineering grafts with a designed, pre-fabricated vasculature.

1. Introduction

In tissue engineering two major challenges are the homogeneous seeding of cells throughout a porous scaffold and providing the seeded cells with sufficient oxygen and nutrients to sustain, proliferate and generate new tissue. Several methods have been proposed for the seeding of cells into microporous scaffolds. Static seeding, although still widely used, is characterised by low cell seeding efficiencies and inhomogeneous distributions [1,2]. An improvement in both seeded cell density and uniformity of the distribution can be achieved by employing mild suction to inoculate cells into a porous scaffold [3]. A similar technique that showed good results, but is only possible when using elastomeric scaffolds, is compression-induced suction [4]. Furthermore, several dynamic seeding methods have been developed using, for example, spinner flasks [2,5] or perfusion bioreactors [6]. Besides the technique employed for cell seeding, the scaffold architecture and physical properties of the scaffolding material play an important role in the cell seeding procedure [7]. Most polymers used for tissue engineering scaffolds (such as poly(lactide), poly(ε-caprolactone) and poly(trimethylene carbonate)) are hydrophobic, which impedes the penetration of aqueous cell suspensions. These materials are often incubated in culture medium prior to cell seeding, in order to let proteins adsorb that make the scaffold surface less hydrophobic and improve cell attachment [8]. An open, permeable pore network architecture facilitates wetting by culture medium and aids in eventually achieving high densities of uniformly distributed cells upon seeding. Moreover, it can improve oxygen and nutrient transport in the culturing phase [9]. In static culture of cell seeded porous scaffolds cells that are too far from the periphery often become necrotic because of hypoxia or lack of nutrients. Due to the diffusion constraints of foams fabricated using conventional techniques,

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doi:10.1016/j.actbio.2010.06.012

such as salt leaching and phase separation/freeze drying, only the in vitro growth of tissues with cross-sections of less than 500 μm have proven successful in static culture [10,11]. Cell colonisation at the scaffold periphery can consume or act as an effective barrier to the diffusion of oxygen and nutrients into the interior of the scaffold [12]. Perfusion of medium through scaffolds in a bioreactor has been suggested to overcome cell death due to transport limitations [13]. It can increase the survival rate of cells in the centre of porous scaffolds, resulting in more homogeneous tissue engineered constructs [14]. However, perfusion alone is not always enough to ensure an adequate oxygen supply inside porous scaffolds [15]. The use of solid freeform fabrication (SFF) or rapid prototyping (RP) techniques such as stereolithography may be an attractive approach to produce scaffolds with customised external shapes and predefined and reproducible internal morphologies. The pore networks of such scaffolds may be designed such as to reduce resistance to mass transport by shortening diffusion paths (lower tortuosity) and increasing pore interconnectivity [16]. In stereolithography a computer controlled laser beam or a digital light projector is used to locally solidify a liquid resin through photo-initiated polymerisation. In combination with a computer driven building stage, a solid, three-dimensional object can be constructed in a layer-by-layer fashion [17]. Recently, we have reported the use of stereolithography and methacrylate end-functionalised poly(D,L-lactide) (PDLLA) oligomers to fabricate tissue engineering scaffolds using computer-aided design (CAD) [18]. Just like poly(D,L-lactide, which has a contact angle of 74° and takes up less than 1 wt.% water [19], these PDLLA networks were also hydrophobic and had an equilibrium water uptake of approximately 0.6 wt.%.

We aim to improve the scaffold architecture such that cell seeded scaffolds can be cultured for prolonged times under static conditions, providing the proliferating cells with sufficient oxygen and nutrients. We have used stereolithography to prepare highly accessible and permeable porous poly(D,L-lactide) scaffolds and compared their cell seeding and cell culturing characteristics with more tortuous scaffolds prepared using the salt leaching technique. The latter method is widely used to prepare porous tissue engineering scaffolds, but allows limited control of the pore network architecture [20]. Cell survival and proliferation are expected to improve for the more accessible architectures.

2. Materials and methods

2.1. Scaffolds

A liquid photo-polymerisable resin based on PDLLA was synthesised in a similar way to that previously described [18]. In short, 1,6-hexanediol (Sigma–Aldrich) and D,L-lactide (Purac) were reacted at 130 °C for 40 h under an argon atmosphere using stannous octoate (Sigma–Aldrich) as a catalyst. The hydroxyl termini of the oligomers were reacted with methacrylic anhydride (Sigma–Aldrich) in the presence of triethyl amine (Sigma–Aldrich) (both in a 20 mol.% excess) in dried dichloromethane for 5 days at room temperature to yield methacrylate end-functionalised lactide macromers. These were purified by precipitation from isopropanol followed by washing with water and freeze drying. ^{1}H NMR ($CDCl_3$, Varian 300 MHz) indicated high purity, a molecular weight of 5 kg mol^{-1} and a degree of functionalisation of 92–99%. The macromers were used to prepare a photo-polymerisable liquid resin with 40 wt.% dry *N*-methylpyrrolidone (NMP) (Fluka) as a non-reactive diluent, 2 wt.% ethyl-2,4,6-trimethylbenzoylphenylphosphinate (Lucirin TPO-L photo-initiator, a gift from BASF), 0.15 wt.% Orasol Orange G dye (Orasol Orange G is also known as CI Solvent Orange 11, CAS No. 61725-76-6, and was a gift from Ciba SC) to control the cure depth and 0.2 wt.% tocopherol (Fluka) to prevent preliminary cross-linking reactions.

A scaffold design with an open and accessible gyroid architecture was generated using K3dSurf v. 0.6.2 software (http://k3dsurf.sourceforge.net). The surface of the gyroid pore architecture is described by a triply periodic function, closely approximating the minimal surfaces of Schwarz and Schoen [21,22]. Addition of an offset value to the implicit function allows the design of porous structures having specific porosities. A value of −0.60 was chosen for the offset, corresponding to 70% porosity. The following equation was used to describe the scaffold design:

$$G : \cos(x)\sin(y) + \cos(y)\sin(z) + \cos(z)\sin(x) - 0.60 = 0$$

with boundary conditions $x^2 + y^2 < (14\pi)^2$ and $|z| < 10\pi$. A commercial stereolithography apparatus (Envisiontec Perfactory Mini Multilens SLA) was employed to build the designed gyroid scaffolds using the PDLLA-based resin. The building process involved subsequent projections of 1280 × 1024 pixels, each 32 × 32 μm^2 in size. Layers with a thickness of 25 μm were cured by irradiating for 30 s with blue light (intensity 16 mW cm^{-2}). Uncured excess resin was washed out and the diluent, non-reacted macromer, dye and photo-initiator were extracted from the structures with acetone. The complete extraction of these compounds was confirmed by NMR analyses. The extracted structures with a height of 5 mm and a diameter of 8 mm were then dried at 90 °C for 2 days under a nitrogen flow. Under these conditions post-curing also took place, as no unreacted double bonds could be detected in the network structure after the drying procedure.

A similar resin, without dye and containing camphorquinone instead of Lucirin TPO-L, was used to prepare porous PDLLA network structures by a salt leaching process. The camphorquinone concentration was also 2 wt.%. The resin was mixed with NaCl salt particles sieved to sizes of 425–710 μm, in a 1:3.5 weight ratio. The size range and weight ratio was chosen such that a porosity and average pore size similar to that of the stereolithography built scaffolds was obtained. Then the mixture was placed in tubular polypropylene moulds (inner diameter 14 mm) and cured by irradiation through the tube wall with a Kerr dental light (mono-chromatic blue LED light, wavelength 470 nm, intensity 1000 mW cm^{-2}) for 40 s. The specimens were frozen in liquid nitrogen, cut to the desired dimensions and post-cured by heating to 90 °C for 12 h. The salt-containing composites were extracted in acetone for 2 days and the salt fraction was leached out with water over a period of 7 days. Then, the porous structures with the same final approximate dimensions as the built gyroid scaffolds were dried at 90 °C for 2 days under a nitrogen flow. No detrimental effects of the leaching and drying procedure on the network properties were observed, and the conversion of double bonds was complete.

Structural analyses were performed on the fabricated scaffolds by micro-computed tomography (μ-CT) using a General Electric eXplore Locus SP scanner at 14.3 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. The scanned data was reconstructed with a Feldkamp-based algorithm to obtain three-dimensional images and for the calculation of porosities, pore size distributions and pore accessibility.

The water flow resistance was determined for both scaffold types by measuring the time required for a fixed volume of water to flow through a fully pre-wetted scaffold inserted in a silicone rubber tube. Permeabilities were calculated using Darcy's law:

$$Q = -\kappa \frac{\Delta P}{v} \frac{A}{L}$$

where κ is the scaffold permeability (mm^2), Q is the flow rate (mm^3 s^{-1}), μ is the viscosity of water (0.001 Ns mm^{-2} at room temperature), ΔP the pressure difference (N mm^{-2}), A the cross-section surface area (mm^2) and L the scaffold height (mm).