Biomaterials 31 (2010) 7648–7652

Contents lists available at ScienceDirect

Biomaterials

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

Three-dimensional microstructured tissue scaffolds fabricated by two-photon laser scanning photolithography

Tseng Ming Hsieh, Chien Wei Benjamin Ng, Karthikeyan Narayanan, Andrew C.A. Wan*, Jackie Y. Ying*

Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, Singapore 138669, Singapore

ARTICLE INFO

Article history:
Received 26 April 2010
Accepted 22 June 2010
Available online 25 July 2010

Keywords:
Scaffold
Microstructure
Photolithography
Hepatocyte

ABSTRACT

Current tissue engineering scaffolds fabricated via solvent casting and porogen leaching methods suffer from the lack of control over parameters such as interconnectivity and pore geometry, properties that are a function of the fabrication process. The progress of tissue engineering would thus benefit from the ability to design scaffolds that facilitate cell–cell interactions, and provide mass transfer characteristics necessary for good cell viability and function. In this research, we have developed two-photon laser scanning photolithography (TPLSP) for the fabrication of three-dimensional (3D) microstructured scaffolds with high resolution and fidelity. Modification of our two-photon setup allowed for a scan height of 30 mm and a scan speed of 30 mm/s, making it more amenable to scaffold fabrication. Scaffold production was adapted to computer-aided design (CAD)/computer-aided manufacturing (CAM) technology, to achieve the desired length scales from the submicron level and up. A commercially available photocurable resin that exhibited favorable ultraviolet–visible (UV–vis) transparency, cell compatibility and reproducibility in fabrication was used as the scaffold material. As a proof-of-concept, a microporous, cubic scaffold was fabricated for the purpose of hepatocyte culture. Primary hepatocytes could be uniformly seeded on these scaffolds as observed by confocal fluorescence microscopy. Albumin and urea assays demonstrated that hepatocytes cultured in the 3D scaffold maintained higher levels of liver-specific function over a period of 6 days as compared to the monolayer control. These results may be attributed to the high local concentration of soluble factors within the scaffold, which is important for maintaining the hepatocyte phenotype. Our study illustrates the potential of TPLSP as a new platform for the fabrication of designed, well-controlled, 3D microstructured tissue scaffolds.

1. Introduction

To realize the promise of tissue engineering as a strategy to fabricate 3D, clinically relevant tissues, scaffolds should ideally provide the necessary degree of resolution and complexity to spatially define both cells and biomolecules. Such spatial definition is especially critical for the engineering of complex organs, such as the kidney and liver. Besides enabling cells to be cultured with well-defined spatial relationships, a scaffold with controlled pore interconnectivity would also provide good mass transfer characteristics essential for good cell viability and function.

In general, intercellular communication and interactions are necessary to achieve good cell and tissue function. For example, it has been shown that co-cultures of hepatocytes with a second non-parenchymal cell type enable the hepatocytes to maintain higher levels of liver-specific function [1]. The effect of cell interactions may be due to direct cell-to-cell contact, or mediated by paracrine factors such as growth factors and cytokines that are secreted by the cells. Scaffolds obtained by solvent casting, porogen leaching or lyophilization suffer from the lack of control over parameters such as interconnectivity and pore geometry, which are dependent on the fabrication process. The heterogeneity of the pore geometry in turn results in ill-defined cellular distribution, interaction and mass transfer characteristics. Thus, it would be difficult to further develop such scaffolds to refine the tissue architecture of the cells and produce a higher level of functionality.

Considering all the shortcomings inherent in scaffold technologies based on physical processes, much attention has been devoted to microfabrication technologies to achieve scaffolds with the required degree of resolution for control of cell–cell interactions. The microfabrication methods that have been investigated include microelectrodeposition, soft lithography and microsyringe deposition [2–6]. In general, these are approaches that involve the build-up of micropatterned layers in the vertical (z) direction.

* Corresponding authors. Tel.: +65 6824 7100; fax: +65 6478 9020.
E-mail addresses: awan@ibn.a-star.edu.sg (A.C.A. Wan), jyying@ibn.a-star.edu.sg (J.Y. Ying).

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.06.029

TPLSP presents an alternative to these layer-by-layer methods. For two-photon excitation, a molecule must absorb two or more photons simultaneously to reach the excited state. The high intensity required for excitation can be achieved by using a pulsed laser emitting photons of half the energy required for excitation. As the laser focal point is the only location along the optical path where the two-photon excitation occurs, photoreactive processes such as polymer crosslinking can be confined to the microscaled focal volume. In the field of tissue engineering, TPLSP has been used to fabricate micropatterns of biomolecules for the purpose of guiding cell migration within 3D PEG-based hydrogels [7], and to fabricate crosslinked extracellular matrix (ECM) microstructures [8]. In this study, we aim to exploit the advantages of TPLSP with respect to microfabrication, i.e. its high fidelity and resolution, but adopt instead an alternative approach of creating solid, porous scaffolds that are amenable to cell seeding. Specifically, this work aims to develop TPLSP as a method to produce porous microstructured scaffolds as a 3D cell culture system for tissue engineering and medical device applications. A commercially available photocurable polymer was investigated as the material for free-form fabrication of scaffolds by TPLSP. The resulting scaffold material was seeded with primary hepatocytes and assayed for liver-specific functions, and compared to monolayer controls.

2. Materials and methods

2.1. Fabrication of 3D scaffolds by TPLSP

The photocurable polymer (Accura™ SI10) was obtained from 3D Systems (Rock Hill, SC, USA). The desired scaffold was designed using CAD software (Fig. 1), and generated in a stereolithography system with a galvanometric mirror scanner (Scanlabs, Munich, Germany). An isolator was placed in front of the laser aperture to prevent reflected laser light from returning to the laser cavity. An acousto-optic modulator (AOM) served as a high-speed shutter for the system. The beam expander (Scanlabs, Munich, Germany) acted as the on-the-fly focusing module to automatically correct for any plane distortion. Axial control of the scanned structures was provided by a high-resolution elevation stage (Newport, Irvine, CA, USA) that stepped with each slice of exposure. Localized polymerization would occur on the laser spot. The structures were built layer-by-layer through a laser scanning process. The device was developed for 1 h in acetone and rinsed with isopropanol. UV–vis spectra of polymerized and non-polymerized samples were acquired on an Agilent 8453 UV–Visible Spectrophotometer (Santa Clara, CA, USA).

2.2. Primary rat hepatocyte isolation and cell culture

Primary hepatocytes were harvested from 7 to 8 week old male Wistar rats weighing 250–300 g by a two-step *in situ* collagenase perfusion method. The animals were handled according to the IACUC protocol. Viability of the hepatocytes was determined to be >90% by Trypan Blue exclusion assay (Invitrogen, Carlsbad, CA, USA). Freshly isolated hepatocytes were seeded onto collagen-coated substrates at a density of 2×10^5 cells/cm^2 in a 24-well plate (3.5×10^5 cells/well), and cultured in Hepatozyme (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1 μM of dexamethasone (Sigma, St. Louis, MO, USA), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were incubated with 5% of CO_2 at 37 °C and 95% humidity for 24 h.

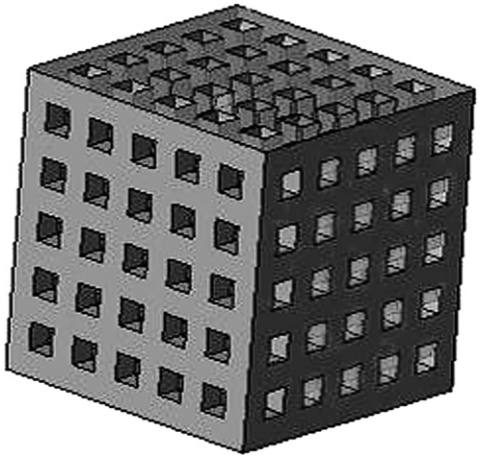

Fig. 1. CAD of a 3D microstructured scaffold (2.5 mm × 2.5 mm × 2.5 mm) used in this study.

For the hepatocyte culture, 3D scaffolds were fabricated as a cube of 2.5 mm × 2.5 mm × 2.5 mm with a pitch size of 250 μm, and coated with Type I collagen. A 40-μm Nylon Cell Strainer membrane (BD Falcon, San Jose, CA, USA) was glued (Dow Corning, Midland, MI, USA) to 5 sides of the cube to create a capillary force to encapsulate the hepatocytes homogeneously in the scaffold, as well as to allow medium and waste exchange. 4×10^6 hepatocytes were seeded onto the 3D scaffold via the uncovered side of the cube. The cell-seeded scaffold was then placed on a rotator (Biosan Laboratories, Warren, MI, USA) in an incubator overnight to enhance homogeneous cell seeding.

To prepare a monolayer control for the hepatocyte culture experiment, 2D polymeric substrates were prepared by coating a photopolymer (Accura™ SI10) on Nunc treated 24-well cell culture plates (Thermo Fisher Scientific, Waltham, MA, USA). The monomers were polymerized with a 600-W UV irradiator (Newport, Irvine, CA, USA) for 30 min. 70% ethanol and isopropanol were used overnight to sterilize the coated polymer and to remove photochemical waste, respectively. Each substrate was washed at least three times with 1000 μl of 1× phosphate buffered saline (PBS). 200 μl of 1.5 mg/ml of Type I collagen were coated on the polymer for 4 h before aspiration. 4×10^6 hepatocytes were seeded onto each 2D polymeric substrate, and the plates were placed in the incubator for further culture.

To assess the viability and distribution of cells seeded on the scaffold, HepG2, a liver cancer cell line with green fluorescence protein (GFP), was seeded on the scaffold. The scaffold was transplanted to a cell culture plate after 4 h of cell seeding, and cultured for 7 days in Dulbecco's modified eagle medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 1% of penicillin–streptomycin (PS). HepG2 morphology was observed under an LSM 5 DUO inverted confocal microscope (Zeiss, Jena, Germany). Cell viability was determined qualitatively using a fluorescence microscope (Olympus, IX71) by emission of green fluorescence at an excitation wavelength of 395 nm. Stereo projection was observed slice-by-slice at steps of 20 μm for 64 slices in total, using the LSM 5 DUO inverted confocal microscope.

2.3. Assays of liver-specific function

1 mL and 4 mL of Hepatozyme were collected for the quantification of albumin levels in 2D culture and 3D scaffold, respectively. 500 μl and 4 mL of 5 mM of NH_4Cl were added to each well of the 2D culture and 3D scaffold, respectively, and incubated for 90 min for the urea assay.

Culture medium was assayed for albumin and urea secretion. The albumin production of hepatocytes was measured every 24 h using the rat albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX, US). The urea level of hepatocytes incubated with 5 mM of NH_4Cl was measured using the urea nitrogen kit (Stanbio Laboratory, Boerne, TX, US). Albumin absorbance and urea absorbance were measured at 450 nm and 520 nm, respectively, with a microplate reader (Tecan Safire, Männedorf, Switzerland). Concentration values were normalized against the nutrient medium volume and the number of seeded cells.

Immunofluorescence was used to qualitatively demonstrate hepatocyte viability and function. DAPI (Invitrogen, Carlsbad, CA, USA), Texas Red (Invitrogen, Carlsbad, CA, USA) and FITC (Abcam, Cambridge, MA, USA) were used to stain the nuclei, fibronectin and albumin of the hepatocytes. Image J (National Institute of Health, USA) was used to superimpose the images.

2.4. Statistics and data analysis

All data were presented as mean ± standard error of the mean (SEM). Statistical significance was evaluated using the *t*-test, with the significance level set at $p < 0.05$.

3. Results

The SI10 photopolymer was characterized by UV–vis spectroscopy (Fig. 2). Absorbance of the liquid monomer in the visible wavelength (400–700 nm) was negligible with reference to the control (an empty cuvette). After polymerization, the absorbance of the solid monomer was still negligible, rendering the entire device almost transparent and easily observed with a fluorescence microscope.

Our TPLSP system demonstrated excellent fabrication of microstructures with feature resolution in the micron or submicron range (see example in Fig. 3). The fabrication time for the 2.5 mm × 2.5 mm × 2.5 mm cubic scaffold depicted in Fig. 1 took only ~2 h. HepG2 cells attached and proliferated well on the