Fabrication and Functionalization of Three-Dimensional Well-defined Scaffolds Using Novel Carbon Dioxide Assisted Microfabrication

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INTRODUCTION

Recent trends in tissue engineering have aimed at simulating the microenvironment *in vitro* to successfully engineer human tissue into artificial organs. Tissue scaffolds have played a major role in reconstructing this microenvironment, and the cell-scaffold interaction also affects cell proliferation, differentiation, migration and function. A variety of tissue scaffolds ranging from fibrous matrices to foams have been used. However, none of these scaffolds have a well-defined pore structure. Solid freeform fabrication allows layer-by-layer construction of complex 3D products, and has been utilized to generate scaffolds with predefined structures. However, it is a time-consuming sequential writing process with a narrow processing window and relatively low resolution.[1] Recently, micro/nanofabrication technology is attracting increased interest because it can achieve topographical, spatial, chemical, and immunological control over cells.[2, 3] However, so far this technology has been limited to 2D environments and on silicon or glass substrates because it is difficult to fabricate 3D scaffolds based on biodegradable polymer substrate. In this work, a novel carbon dioxide (CO_2) assisted microfabrication (CAMF) process was developed for the production of well-defined 3D tissue scaffolds from premolded 2D skeletons generated from microembossing. Fig. 1 shows the design concept and the process to assemble single-layer scaffold skeletons into a 3D scaffold with a prescribed pore structure.

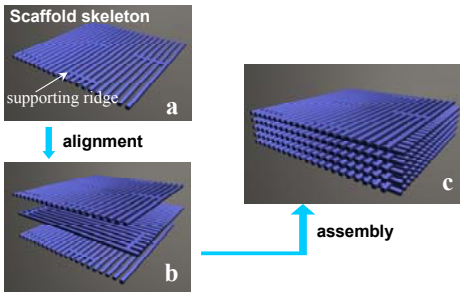

Fig. 1. Assembling of well-defined 3D tissue scaffold from microfabricated planar skeletons.

EXPERIMENTAL

Poly(DL-lactide-co-glycolide) (PLGA) nanocomposite was used as the prototypic polymer substrate to demonstrate this technique. PLGA (Medisorb® 5050 DL High IV, Alkermes, Inc.) was mixed with nano-clay (Cloisite 30B, Southern Clay Products) by 95/5 wt.% at 120°C, 150 rpm for 5 min using a DACA minicompounder. For simplicity, PLGA is used to represent this kind of PLGA nanocomposite, unless otherwise indicated.

The CAMF process involved three steps: 1) photolithography to generate the mold with the planar skeletal structure; 2) microembossing to fabricate 2D scaffold skeletons with the planar structure transferred from the mold; and 3) subcritical CO_2 bonding to assemble the laminated multiple 2D skeletons into 3D tissue scaffolds. The skeletal structure (parallel ridges with 60 μm in width, 60 μm in height, and 120 μm spacing) was first generated on a photoresist (SU-8) via photolithography, and replicated in poly(dimethylsiloxane) (PDMS) substrate via soft lithography.[4] With the inverse PDMS mold, the scaffold skeletal structure was transferred into the PLGA substrate by the developed bilayer embossing (BLE). As illustrated in Fig. 2, a PLGA film was placed on a PDMS mold, which was placed on the hot plate. When the PLGA film had melted, which usually took 1-2 min, another PDMS mold in an orthogonal orientation to the bottom mold was pressed down at a pressure of 0.5 MPa into the molten PLGA film. After holding for 1.5 min, the system was cooled, and a PLGA bilayer skeleton was then removed

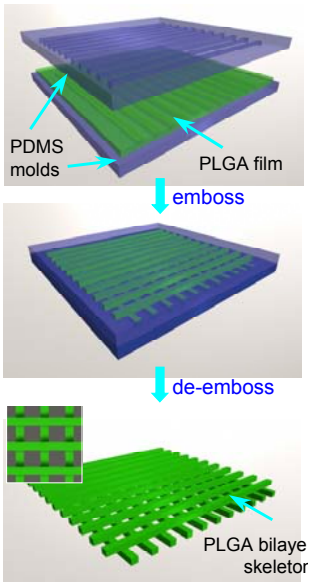

Fig. 2. Schematics of bilayer process. The inset shows the top-view of the skeleton.

from the molds. Typically, 4 PLGA bilayer skeletons were orthogonally aligned and stacked between two glass slides. A contact pressure of 0.06 MPa was applied by placing weights on the top glass slide. The assembly was placed in a pressure vessel that was immersed in a water bath at 35°C, and an ISCO 500C syringe pump was used to deliver and control the CO_2 pressure at 0.69 MPa. After saturation with CO_2 for 1 h, the pressure was quickly released and the bonded scaffold removed from the vessel.

Human brain astrocytoma CCF-STTGI (ATCC: CRL 1718) was used to evaluate cytocompatibility of the PLGA scaffold. The astrocytes were grown in RPMI 1640 medium (Irvine Scientific) containing 1.5 g/l sodium carbonate, 2 mM L-glutamine, 4.5 g/l glucose, 10 mM HEPES and 1.0 mM sodium pyruvate along with 10% FBS. The sterilized PLGA scaffolds were kept in 12-well plates and soaked with 2 ml of growth medium overnight. The cells were initially grown in T-flasks (Corning), then trypsinized and resuspended in medium at 1-2x10^6 cells/ml. One milliliter of this suspension was used to seed each scaffold in the 12-well plates. Cells were allowed for 8 h to attach to the scaffolds, which were then transferred to new wells containing fresh media and incubated in a 5% CO_2 environment at 37°C. After 4 days, the total cell number in each scaffold was counted and attachment and growth of cells in the scaffolds were also examined using a Philips XL 30 scanning electron microscope (SEM).

RESULTS

Photolithography precisely transferred the designed micropattern to the photoresist (SU-8). The profilometric analysis of planar SU-8 skeletal structure using Veeco NT 3300 showed that the dimensions of ridges were 60.0±1.5 μm in width and 60.5±2.8 μm in height, with a spacing of 120.3±1.6 μm, which agreed well with the design (60x60x120 μm^3). Fig. 3 shows a bilayer skeleton with open structure generated in a BLE process. The orthogonal orientation with numerous contacts between two layers of PLGA skeletons reinforced the open structure and made the bilayer skeletons strong. Dimensional analysis revealed a slight 8.7% deformation of the microstructure (ridge width 65.2±0.4 μm). This was due to a high embossing pressure resulting in deformation of the PDMS mold. To improve the replication accuracy, metal (e.g., nickel) molds, instead of PDMS molds, may be applied. The skeletons produced in BLE were bonded at 35°C and 0.69 MPa CO_2 pressure to construct a large 3D scaffold. Fig. 4 shows the PLGA skeletons before and after CO_2 bonding, which essentially preserved the original dimensions of the skeletons and yielded an invisible interface between the bonded layers. Lap-shear measurements showed that the bond strength increased with the bonding time, and approached 1 MPa after 0.5 h.[5] Bonding was strong enough to maintain the 3D structure even throughout the cell culture experiments (see Fig. 6). By increasing CO_2 pressure to 2.07 MPa at 35°C, it was observed that there were interconnected

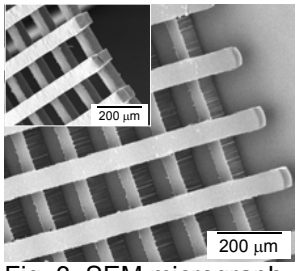

Fig. 3. SEM micrograph of scaffold skeleton made via BLE. The inset shows detailed structure.

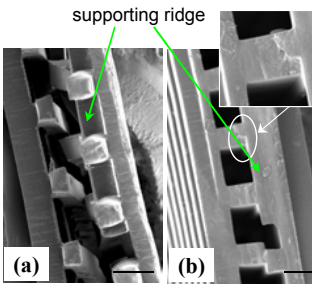

Fig. 4. SEM micrographs of PLGA scaffolds (a) before and (b) after CO_2 bonding.

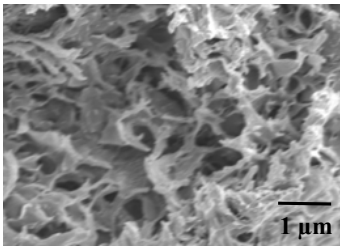

Fig. 5. SEM micrograph of interconnected nanosized porous structure.

nanosized pores formed within the scaffold skeletons (Fig. 5), while the well-defined scaffold structure was maintained.

Astrocytes were cultured in three dimensions in the PLGA scaffolds and in two dimensions on PLGA films for comparison. In two dimensions, the astrocytes usually exhibited an elongated and polygonal morphology but very fine strands were not observed. The cells spread out in only one plane when growing on a flat surface and there was little or no overlapping of cells (Fig. 6a) due to contact inhibition. However, when grown in the scaffolds they exhibited very fine substructures, which aided in attachment and stretching of the cells (Fig. 6b&c). The cells also spread in 3D instead of only on one plane (Fig. 6c). More interestingly, astrocytes (30-35 μm in diameter) can see the 60 μm ridge as a 2D surface and can grow along its width and length. But they also show extensive bridging of the 120 μm gaps (Fig. 6b). They can stretch between ridges in all three directions (Fig. 6c). As a result, the scaffolds can support cell densities as high as 2.8×10^7 – 5.0×10^7 cells/ml of the scaffold volume, which also indicated that the scaffolds with open structure and a relatively high porosity pose no nutrient limitation to cell growth.

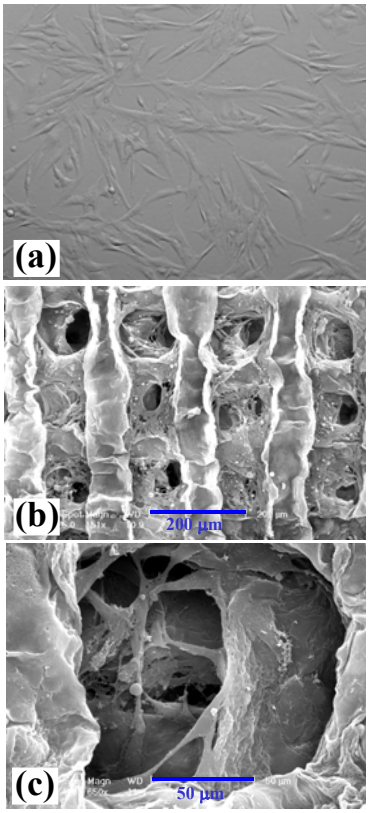

Fig. 6. Astrocytes grown on 2D PLGA substrate (a) and 3D PLGA scaffolds (b & c).

DISCUSSION

A novel biologically benign CAMF process was used to construct 3D scaffolds with well-defined microstructure. Compared with conventional techniques, CAMF could achieve precise dimensional control via photolithography and microembossing. In fact, the embossing technique has demonstrated replication at a scale as small as 10 nm.[6] Common polymer bonding methods involve either organic solvents or a temperature above the glass transition temperature (T_g) of polymer substrate. Both are undesirable for biomedical applications and they tend to deform microstructures. In this work, low pressure CO_2 was introduced to increase the free volume among the polymer chains, leading to the depression of the T_g and higher chain mobility in the proximity of the polymer surface, thus enhancing interfacial bonding of polymers and achieving 3D assembly of polymer microstructures at low temperatures.[5] The smallest feature size achieved to date using this technique is 200 nm (unpublished data). The influence of nanoporous structure within scaffold skeletons on nutrient transport and cell growth is under investigation.

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