Biomed Microdevices (2010) 12:721–725
DOI 10.1007/s10544-010-9425-2

Fabrication of three-dimensional scaffolds for heterogeneous tissue engineering

Li-Hsin Han · Shalu Suri · Christine E. Schmidt · Shaochen Chen

Published online: 15 April 2010

Abstract The development of biomedical scaffolds mimicking a heterogeneous cellular microenvironment for a specified regulation of cell-fates is very promising for tissue engineering. In this study, three-dimensional scaffolds with heterogeneous microstructure were developed using a DMD-PP apparatus. During the fabrication process, this apparatus can efficiently switch monomers to form microstructures with localized, different material properties; the resolution in the arrangement of material properties is comparable to the characteristic size of functional subunits in living organs, namely, a hundred microns. The effectiveness of this DMD-PP apparatus is demonstrated by a woodpile microstructure with heterogeneous fluorescence and also by a microporous cell-culturing scaffold with selected sites for protein adhesion. Cell-cultivation experiment was performed with the microporous scaffold, in which selective cell adhesion was observed.

Keywords Heterogeneous structure · Scaffold · Tissue engineering · Solid freeform fabrication · Poly(ethylene glycol) · Biocompatible material

L.-H. Han · S. Chen (✉)
Texas Materials Institute and Department of Mechanical Engineering, The University of Texas at Austin, C2200,
Austin, TX 78712, USA
e-mail: shaochen.chen@engr.utexas.edu

S. Suri · C. E. Schmidt
Department of Biomedical Engineering, The University of Texas at Austin, C0800,
Austin, TX 78712, USA

1 Introduction

Solid freeform fabrication (SFF) techniques, such as microsyringe stereolithography, selective laser sintering, and digital-micromirror-device-projection-printing (DMD-PP), are a collection of technologies to create three-dimensional (3-D) objects by delivering energy or materials to specified positions in 3-D space. (Madou 2001; Vozzi et al. 2003; Singamaneni et al. 2008; Han et al. 2008) For biomedical applications, SFFs are especially promising to fabricate tissue engineering scaffolds because of their flexibility in creating irregular and bio-mimetic structures, such as interconnecting-pores and branched vessels. (Palsson and Bhatia 2004a, b, c)

One major challenge faced by SFFs is the speed of fabrication. Most SFFs fabricate a continuous geometry by creating connected volumetric pixels (or voxels); the time for scanning a 3-D object with voxels is proportional to the object's volume and also the complexity of its internal structure. This method can be very slow at making a large tissue engineering scaffold with microstructures. Fortunately, this issue is eliminated by DMD-PP.

To create a 3-D object, instead of making numerous voxels, DMD-PP develops one cross-sectional slice at a time by projecting a cross-sectional image onto a photo-crosslinkable monomer and curing a thin slice from the monomer according to the projected image. The cross-sectional image is generated on a DMD chip which has on its surface several hundred thousands of micro-mirrors arranged in a rectangular array. The micro-mirrors are rotated electrically to an "on" or "off" state according to the colors of pixels (dark or bright) of the cross-sectional image to be generated. The micromirrors in the "on" state reflect an ultraviolet (UV) light toward a lens; the lens projects the reflected light from the

micromirrors as a bright image onto the monomer, creating the cross-sectional slice. Consequently, a 3-D structure is created by a sequential development of cross-sectional slices according to a series of cross-sectional images generated on the DMD chip.

Limited only by the quality of the projection lens, the smallest feature a DMD-PP apparatus can create is comparable to the size of biological cells, i.e., in micrometers. In our previous study, a poly-ethylene-glycol (PEG) based biological scaffold with regular pores (200 μm) and thin networks (20 μm) was fabricated for cell study. Results from the cell-cultivation experiment demonstrated the promise of DMD-PP (Han et al. 2008).

2 Heterogeneous microfabrication

Microstructures with localized, distinct material properties are common in natural biological systems. (Palsson and Bhatia 2004a, b, c) The formation of these heterogeneous microstructures is guided by the interactions between cells and their surrounding environment, including the properties of extracellular matrix (ECM), gradient of biochemical cues, and the signals from other cells. These interactions regulate the migration, division, differentiation, and apoptosis of cells, leading to the formation of functional subunits in living organs. Cell-culturing scaffolds mimicking these heterogeneous microenvironments will be extremely useful for tissue engineering. For example, a tubular microstructure with encapsulated bio-molecules of a spatially changing concentration may stimulate the extension of neurons, which respond to biochemical gradients (Dodla and Bellamkonda 2006; Gallo et al. 1997; Cao and Shoichet 2001, 2003). Moreover, localization of different cues (biochemical or topographical) in micro-scale might cause the formation of distinct cell-types from the differentiation of a common stem-cell and thus make possible the manufacture of artificial tissues at an organ level. (Palsson and Bhatia 2004a, b, c) However, there is no well-developed fabrication scheme to construct heterogeneous microstructures in a resolution comparable to the characteristic size of cellular subunits, which is approximately 100 μm. (Sharma and Elisseeff 2004)

This article reports our development of three-dimensional, heterogeneous tissue engineering scaffolds using the DMD-PP apparatus. The DMD-PP apparatus has a mechanism to switch monomers for making different parts of a scaffold. In the first part of our experiment, a heterogeneous woodpile microstructure was developed from two fluorescent monomers. Also developed was a honeycomb scaffold with selected protein-adhesion sites. Cell-cultivation was performed using the second scaffold.

3 Setup of the DMD fabrication system

Our DMD-PP fabrication system (Fig. 1) has a linearly-moving stage (with actuator CMA-25-CCCL & ESP300, Newport), multiple syringe pumps, a UV-transparent window, a UV-grade project lens (NT 57-542, Edmund Optics), and a DMD chip (Discovery 1100, Texas Instruments) illuminated by an ultraviolet (UV) light source (200 W, S2000, EXFO). The DMD chip has a micromirror array (1024 by 768 pixels) on its surface to create cross-sectional images. Illuminated by UV light, the DMD chip image is projected by the lens onto the stage, where monomers are loaded from the syringe pumps. The window is located at the focus of the projection lens, right above the stage, to keep the injected monomer flat. Upon UV exposure, the monomer below the window is crosslinked and becomes a thin slice resembling the cross-sectional image on the DMD chip. The window is coated by a fluorinated agent (1H,1H,2H,2H-perfluorooctyltrichlorosilane) to release the cured slice. (Sun et al. 2008)

4 Material preparation

Fluorescent particles (green and red fluorescent dyes in polystyrene, 1 μm in average diameter) were purchased from Bands' Laboratory. Poly (ethylene glycol) diacrylate (PEGDA, Mw 700), acrylic acid (AA), and 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO, free-radical quencher) were purchased from Sigma-Aldrich and used as received. Photo-initiator Irgacure 2959 and UV dye TINUVIN 234 were given by Ciba Chemistry; both chemicals were used without

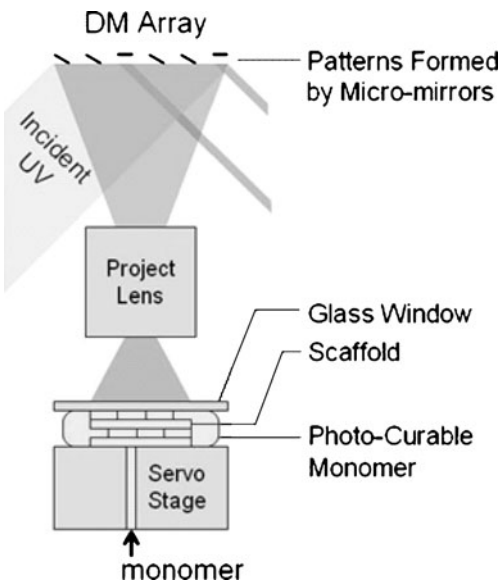

Fig. 1 Illustration of the DMD-PP apparatus for developing 3-D scaffolds with heterogeneous material properties

further purification. TINUVIN 234 is a UV-absorbing agent to reduce the curing depth of monomers and to adjust the thickness of microstructures. (Palsson and Bhatia 2004a, b, c) TEMPO, on the other hand, enhances the contrast of the UV-curing and optimizes the structural resolution at the projection plane.

We prepared two different monomers to fabricate a scaffold with selected sites for cell-adhesion: a binding-monomer to be modified by proteins, and a blank-monomer that is not modifiable. To prepare the binding-monomer, 1 wt% of Irgacure 2959, 0.01 wt% of TEMPO, and 0.15 wt% of TINUVIN 234 were added into a 4:1 (volume ratio) mixture of PEGDA and AA. The carboxylic groups from AA molecules are for absorbing proteins. PEGDA serves as a cross-linker which provides no reactive group. The blank monomer was prepared by mixing 0.15 wt% of TINUVIN 234, 0.01 wt% of TEMPO, and 1 wt% of Irgacure 2959 into PEGDA. Experiments showed that our DMD-PP apparatus can form parallel lines with 10 μm line-width and 50 μm line-spacing by using both of the monomers. The curing depth of this monomer was about 50 μm under a UV illumination of 50 mW/cm^2 for 10 s.

Monomer G (monomer of green fluorescence) and monomer R (monomer of red fluorescence) were prepared by adding 0.01 wt% of green fluorescence particles and 0.01 wt% of red fluorescence particles into the blank monomer, respectively.

5 Scaffold fabrication

As shown in Fig. 2, the servo-stage was positioned 100 μm below the glass window to start the fabrication process. Monomers were injected into the gap between the window and the servo-stage. Because only one drop (about 10 μL) of monomer is used to fabricate each cross-sectional slice, this system aids to minimize material consumption and to facilitate monomer switch. The intensity of the UV image was approximately 50 mW/cm^2. In order to achieve a desired thickness of 100 μm, the exposure time of the UV pattern for each layer was set to 20 s. After a layer was formed (Fig. 2b), the stage was repositioned downward 300 μm, pulling the structure away from the window. The release of the scaffold was aided by the low surface energy of the fluoride coating. A mild solvent (isopropanol alcohol) was pumped into the gap to purge uncured monomers. The used solvent was aspirated by a specified syringe-pump.

To create a new layer above the just-formed structure, as shown in Fig. 2(C1 to D1), the stage was repositioned upward until the top of the structure was situated 100 μm below the glass-slide. Fresh monomer was pumped into the 100 μm gap, and the UV exposure was repeated to create the next slice. To fabricate a new structure with another material at the same slice (Fig. 2C2 to D2), monomers were switched after the purge by solvent, and the stage was moved back to the position it held before the previous exposure step. The

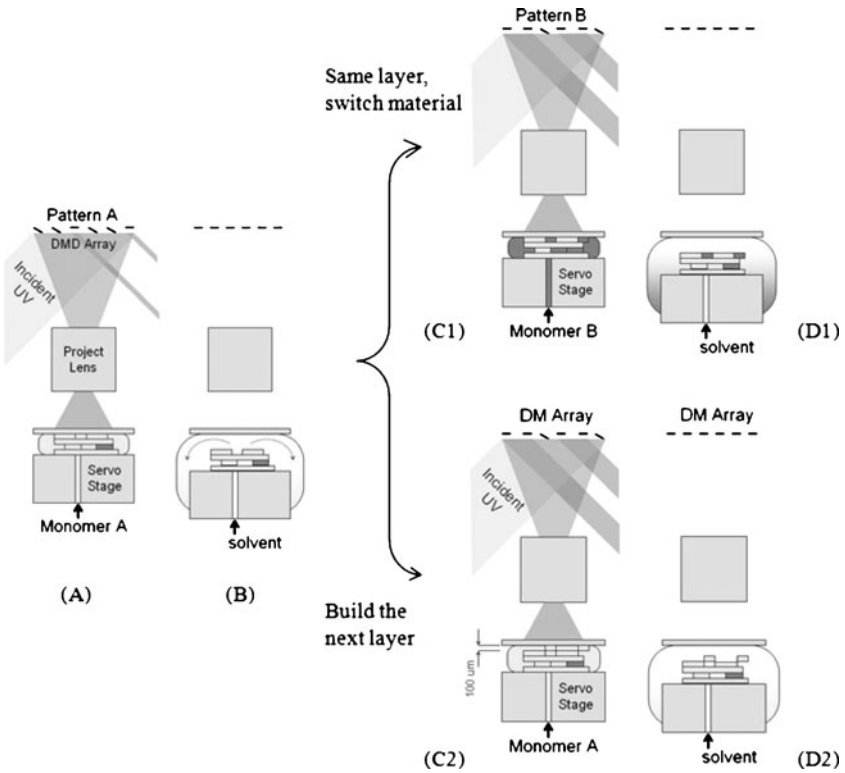

Fig. 2 The DMD-PP fabrication processes include (I) developing a new slice (A-B-C1-D1) and (II) switching materials for the same slice (A-B-C2-D2)

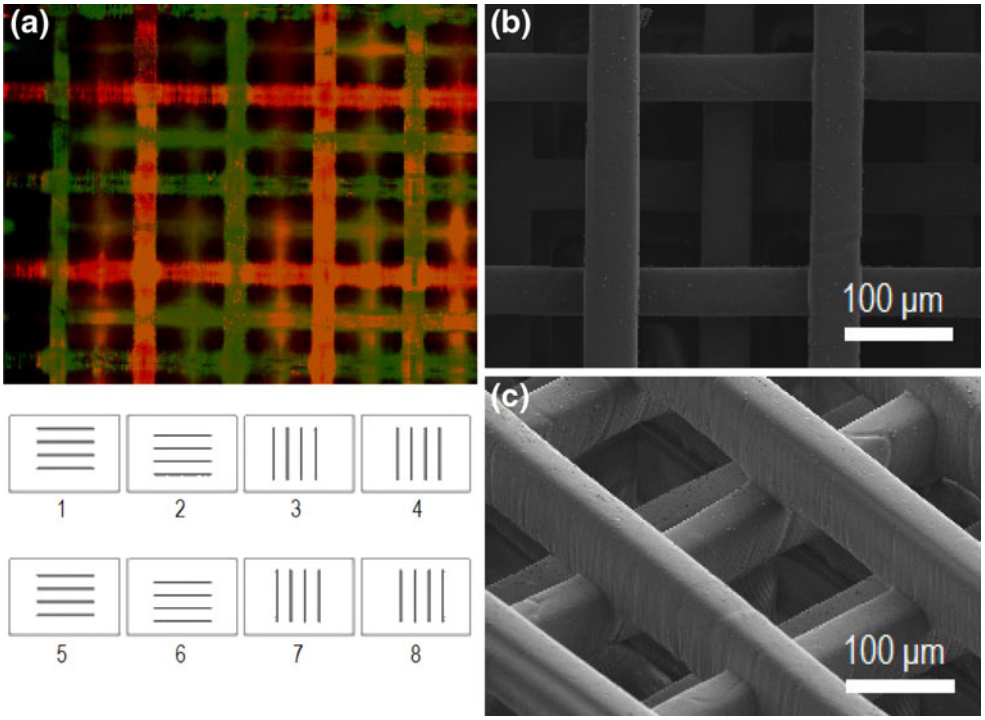

Fig. 3 The woodpile microstructure includes green-fluorescent beams (pattern 1, 3, 5, 7) and red-fluorescent beams (pattern 2, 4, 6, 8)

UV-exposure, solvent purging, and monomer switching processes were repeated until the entire scaffold was fabricated.

The DMD-PP apparatus fabricated the woodpile scaffold by four slices according to eight cross-sectional images (Fig. 3), which were delivered to the DMD chip in numeric order. Images (1,3,5,7) were used with the G-monomer while images (2,4,6,8) were used with the R-monomer. Each slice of the woodpile scaffold was heterogeneous: images (1,2) plus monomer-R & G are used for the 1st slice, images (3,4) plus monomer-R & G for the 2nd slice, and so on. The honeycomb scaffold for selected cell-adhesion was developed in a similar way, except that it was formed by two heterogeneous slices and two homogeneous slices based on six cross-sectional images (Fig. 4). Images (1), (2), (4), and (5) were used with the blank-monomer, and images (3) and (6) were with the binding-monomer. Image pairs (2,3) and (5,6) were used to create two heterogeneous slices with both the binding- and blank-monomers; images (1) and (4) were used to create two homogeneous slices with only the blank-monomer.

6 Heterogeneous fluorescence in the woodpile scaffold

The micrograph of Fig. 3(a) shows the heterogeneous fluorescence (pseudo-colored) from the woodpile scaffold. The fluorescence colors were switched between different beams. The width of the beams was 50 μm and the spacing between neighboring beams was also 50 μm (Fig. 3b). The thickness of the beams was about 100 μm (Fig. 3c). This result demonstrates the capability of our fabrication technique for heterogeneous scaffolds, by which different materials are

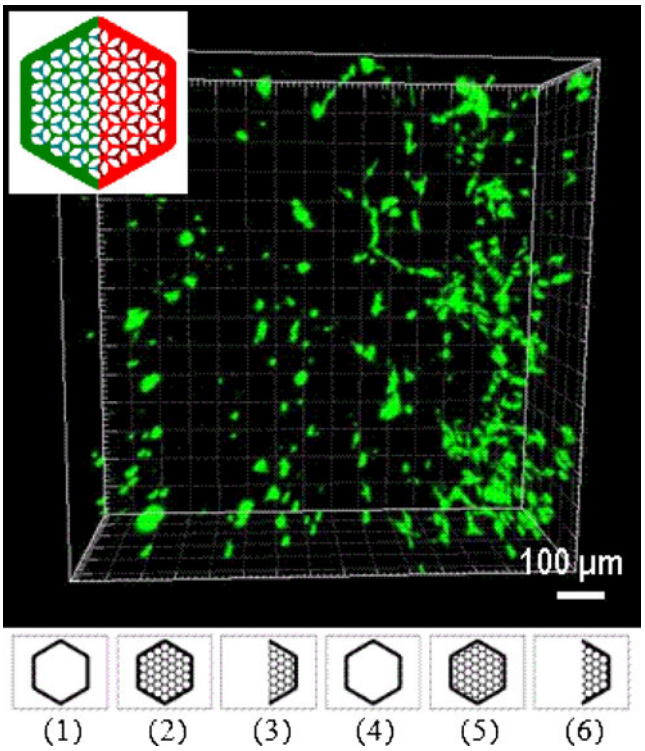

Fig. 4 The fabricated honeycomb microstructure is composed of cell-adhesion sites (pattern 3, 6) and blank structures (pattern 1, 2, 4, 5). Cell aggregation and adhesion are observed in the half of the scaffold where protein was conjugated

arranged in a resolution comparable to the characteristic dimension of functional subunits for living tissues.

7 Modification of the honeycomb scaffold with cell-adhesive proteins

The cell-adhesive biomolecules were attached to the scaffold using 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride, and N-hydroxysuccinamide (EDC-NHS) chemistry. Scaffolds were briefly incubated in a working solution of 0.15 M EDC and 0.12 M NHS in DI water (pH 5) for 2 hrs at room temperature. Following incubation, the EDC solution was aspirated and a 100 μg/mL laminin solution was added. The scaffolds were then incubated in a protein solution for 24 hrs at 4°C. After conjugation, excess and unreacted protein was removed by washing the scaffolds three times with phosphate buffer saline (PBS, pH 7.4). The scaffolds were sterilized by exposure to a germicidal UV lamp for 2 hrs.

8 Cell adhesion studies

Schwann cells were employed to investigate the cell adhesive properties of the double material scaffold. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin-amphotericin (Sigma). The cells were maintained in standard tissue culture dishes (TCPS) coated with poly-L-lysine (PLL) and passaged 1:8 when the cells reached 70% confluency.

To keep the cells from flowing off the scaffold, a hydrophobic surface was created by placing a piece of parafilm on a TCPS plate and the scaffold was placed on the film. Cells were seeded on the scaffolds at a concentration of 2×10^5 cells/mL using 200 μl cell solution. After 4 hrs, the dish was filled with culture medium. After 3 days of cell culture, the scaffolds were rinsed with prewarmed PBS and stained using 2 μm calcein AM (Molecular Probes) in PBS. Calcein stains the live cells green because of the presence of intracellular esterase activity. The cells were imaged using confocal microscopy, and z stacks were reconstructed to obtain 3D images using Imaris software (Bitplane, Zurich, Switzerland).

9 Results of cell-adhesion experiment

As mentioned above, the heterogeneous, honeycomb scaffold was composed of two regions: one synthesized from only PEG, and the other consisting of PEG with acrylic acid. When this heterogeneous scaffold was modified with EDC-NHS chemistry, the protein was conjugated only to the part composed of PEG-acrylic acid. Figure 4 shows the 3D distribution of cells in the scaffold. As evidenced by the figure, high cell density is clearly shown in the half of the scaffold where protein was conjugated. There were very few rounded cells in the other half of the scaffold because of negligible conjugation of cell adhesive proteins on the scaffold surface. The figure also demonstrates that the cells were spreading and making cell-cell contacts in the protein-conjugated region. Cell spreading and cell-cell interactions are important for vital cell function i.e., cell proliferation, migration, ECM production, and bioactive factors secretion.

10 Conclusion

In summary, we have successfully developed two 3-D scaffolds with heterogeneous material properties by using the DMD-PP apparatus, which is able to switch monomers during scaffold fabrication. Our technique in creating heterogeneous material properties in 3-D is demonstrated by fabricating the multi-fluorescence woodpile structure and a scaffold with selected cell-adhesive sites. This study aims to explore the future development of tissue engineering scaffolds, which may include a heterogeneous ECM to induce a heterogeneous differentiation of stem cells.

Acknowledgement This work is supported by grants to SC from the Office of Naval Research and the National Science Foundation. We appreciate the donation of the DMD tool kit from Texas Instruments. We also thank the computer support from Intel's High Education Program.

References

X. Cao, M.S. Shoichet, Neuroscience **103**, 831 (2001)
X. Cao, M.S. Shoichet, Neuroscience **122**, 381 (2003)
M.C. Dodla, R.V. Bellamkonda, J. Biomed. Mater. Res. A **78**, 213 (2006)
G. Gallo, F.B. Lefcort, P.C. Letourneau, J. Neurosci **17**, 5445 (1997)
L. Han, G. Mapili, S.C. Chen, K. Roy, J. Manuf. Sci. Eng. **130**(1), 021005 (2008)
M.J. Madou, *Fundamentals of Microfabrication: The Science of Miniaturization* (CRC, New York, 2001), p. 379
B.O. Palsson, S.N. Bhatia, *Tissue Engineering* (Prentice Hall, Upper Saddle River, 2004a). Ch16
B.O. Palsson, S.N. Bhatia, *Tissue Engineering* (Prentice Hall, Upper Saddle River, 2004b). Ch2
B.O. Palsson, S.N. Bhatia, *Tissue Engineering* (Prentice Hall, Upper Saddle River, 2004c). 31
B. Sharma, J.H. Elisseeff, Ann. Biomed. Eng **32**, 148 (2004)
S. Singamaneni, M.C. LeMieux, H.P. Lang, C. Gerber, Y. Lam, S. Zauscher et al., Adv. Mater. **20**, 653 (2008)
H. Sun, J. Liu, P. Gu, D. Chen, Appl. Surf. Sci. **254**, 2955 (2008)
G. Vozzi, C. Flaim, A. Ahluwalia, S. Bhatia, Biomaterials **24**, 2533 (2003)