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FREEFORM FABRICATION OF BIOLOGICAL SCAFFOLDS BY PROJECTION PHOTOPOLYMERIZATION

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ABSTRACT

This article presents a micro-manufacturing method for direct, projection printing of 3-dimensional (3D) scaffolds for applications in the field of tissue engineering by using a digital micro-mirror-array device (DMD) in a layer-by-layer process. Multi-layered scaffolds are microfabricated using curable materials through an ultraviolet (UV) photopolymerization process. The pre-patterned UV light is projected onto the photocurable polymer solution by creating the "photomask" design with graphic software. Poly (ethylene glycol) diacrylate (PEGDA), is mixed with a small amount of dye (0.3 wt %) to enhance the fabrication resolution of the scaffold. The DMD fabrication system is equipped with a purging mechanism to prevent the accumulation of oligomer, which could interfere with the feature resolution of previously polymerized layers. The surfaces of the pre-designed, multi-layered scaffold are covalently conjugated with fibronectin for efficient cellular attachment. Our results show that murine marrow-derived progenitor cells successfully attached to fibronectin-modified scaffolds.

INTRODUCTION

Freeform fabrication technology [1-10] has become a very useful tool for the manufacturing of biological scaffolds for tissue regeneration and stem cell engineering [10-13] due to the efficient and simple process for creating three-dimensional (3D) microstructures. Pre-designed, complex, 3D geometry in the micro- and nanometer scale can be created using polymeric materials without the use of lithographical masks or mechanical molds. The resolution given by DMD projection printing (DMD-PP) can create micro-pores comparable to the size of biological cells, i.e., micrometers. Depending on the number of pixels in a micromirror array, a DMD based system is capable of simultaneously forming structures in an area of several centimeters. The high resolution and fast fabrication speed make the DMD based system one of the most promising technology for fabricating tissue engineering scaffolds.

In a typical process of DMD-based fabrication, a biological scaffold is formed on a moving-stage placed in a vat of photocurable monomer. The micro-mirror array from the DMD chip forms a pattern, created by graphic software, forming the geometry of a single layer for the 3D scaffold. An ultraviolet (UV) light source illuminates the micromirror-array, and the illuminated pattern is projected, through an optical lens, onto the surface of the photo-curable monomer to form a single-layered structure. After one layer of monomer is selectively cured, the stage moves downward, as a new layer of monomer is formed

above the previously cured layer. The cross-section pattern can be changed in between scaffold layers, and the process is repeated until the entire scaffold is formed.

The DMD-based fabrication process is, however, challenged by deep UV penetration in the solution and unwanted light-scattering. The curing depth of the UV light could be too deep such that previous layers are affected by the current photo-pattern being displayed. UV-light scattering by previously formed microstructures may polymerize the monomer within the previous layers, causing a lost in feature resolution during the fabrication process. Lowering the UV intensity could fix these problems, but it could also degrade the resolution of the exposure layer. These problems became limiting factors to fabrication in our previous study [1].

Scattering and penetration problems can be resolved by mixing UV dyes into photocurable monomers prior to irradiation. The function of the UV dye can be understood through Beer's Law [14]. Assuming that a monomer has an absorption constant α and a UV light intensity, I_0, the UV intensity becomes I at a depth z to give the following:

$$I = I_0 \exp(-\alpha \cdot z), \qquad (1)$$

Figure 1A shows the exponential decay of light intensity as z increases. I_{Cure} is a threshold intensity at which the monomer starts to cure. CD is the curing depth, $I(CD) = I_{Cure}$. When the UV dye is added, α becomes larger (α') and CD becomes smaller (CD'); the addition of the UV-dye to the monomer increases the absorption constant α and decreases the curing depth, and the structural resolution is therefore enhanced. Figures 1B and 1C show the patterned-polymerization of honeycomb-shaped structures with and without dye addition. In the absence of UV dye (Figure 1B), the monomer within the pores was partially cured by undesired scattering, and the structure had decreased resolution. With a small amount of dye in the same monomer solution, (Figure 1C), the structure is polymerized with increased resolution.

Another challenge encountered with the DMD system is the accumulation of oligomers within previously polymerized layers of the multi-layered construct. These oligomers are partially cured monomers, weakly cross-linked by unwanted scattered and penetrating UV light. To overcome this problem, it is necessary to purge the structure with new monomer solution between scaffold layers during the fabrication process.

In this research, we built multilayered 3D structures for cellular behavior studies and for tissue engineering applications using the DMD-based fabrication system. The monomer that forms the scaffold was loaded with a small amount of UV absorbing dye to enhance the structural resolution. Our fabrication equipment also includes a simple purging system to prevent the accumulation of oligomers. Upon fabrication, scaffolds were biologically

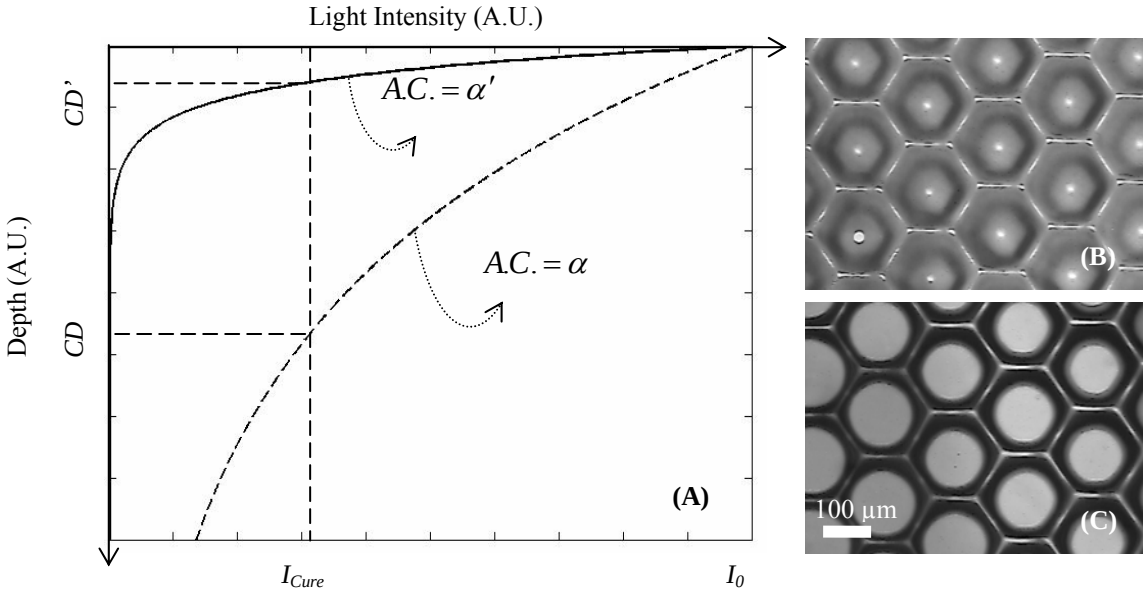

Figure 1. The curing of monomer with and without the addition of the UV dye, under the same intensity of exposure, (A) Theoretical model: The curing depth CD decreases when the absorption constant is increased after the addition of dye. (B) Photo-patterning of poly(ethylene glycol) diacrylate on a glass slide. The pores of a honeycomb structure are sealed by unwanted curing caused by scattered light. (C) Patterning the same monomer with the addition of UV dye TINUVIN 234 at a concentration of 0.2wt%. The geometry of the honeycomb structure had increased feature resolution.

modified for cell attachment. We show the intricate architecture of the 3D scaffold and also demonstrate efficient cell attachment within the porous tissue engineering construct.

THE DMD PROJECTION PRINTING SYSTEM

This scaffold fabrication system (Figure 2A) consists of a vat containing the polymer, a servo-stage (CMA-25-CCCL & ESP300, Newport) that supports the construct, a syringe pump, a DMD system (Discovery 1100, Texas Instruments), a UV lamp (200W, S2000, EXFO), and a fixed-focal lens (Edmond Optics). The syringe pump is created by connecting a 3mL disposable syringe barrel to the servo-stage and is used to inject fresh photocurable monomer solution to an outlet located on the stage. The pump is automatically operated and is used in between layer fabrication. The DMD chip is composed of an array of micro-mirrors that project the patterned photomask onto the photocurable monomer. These micro-mirrors are illuminated by the UV light from the lamp using an 8mm light guide, and the fixed focal lens focuses and projects the images onto the translational stage. The moving-stage, syringe-pump, DMD system, and the UV lamp are connected to a personal computer, and the DMD-base system is controlled through a driver interface supplied by Texas Instruments.

SCAFFOLD FABRICATION

Preparation of the Photocurable Monomer

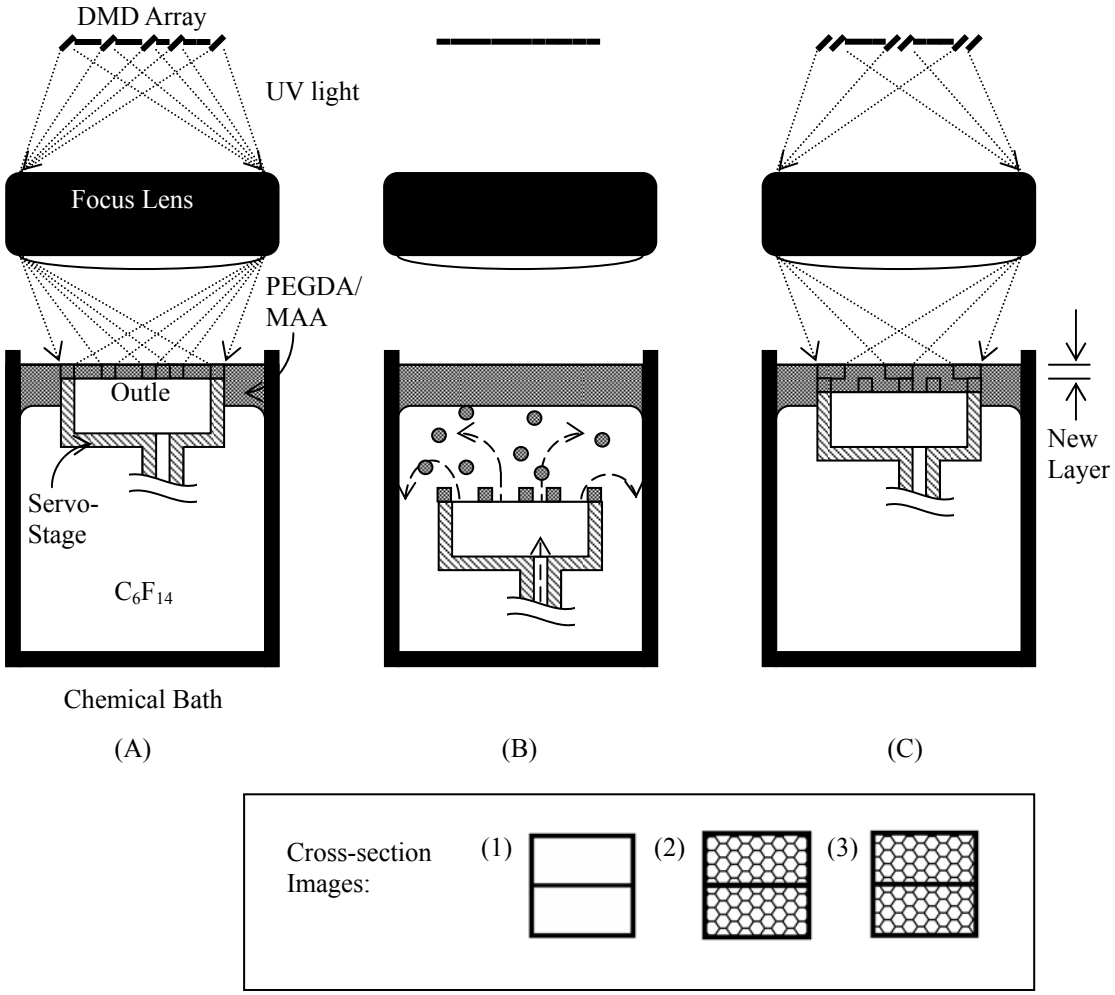

Figure 2. The schematics of the DMD fabrication system showing the fabrication scheme (A-C), and the patterns of the scaffold cross-section (inset). (A) The forming of one structural layer by a patterned UV-cross-section image. (B) A purging process to remove partially cured monomer. (C) Forming the next structural layer; the stage is positioned 50 um below the liquid surface to define the thickness of a new layer.

Perflorohexane (99.5%) and poly (ethylene glycol) diacrylate (PEGDA, Mn 258) were purchased from Sigma-Aldrich and used as received. Methacrylic acid (MAA) from Sigma-Aldrich was distilled before use to remove inhibitors. The photo-initiator, Irgacure 2959, and UV dye, TINUVIN 234, were provided by Ciba Chemistry; both chemicals were used without further purification.

To prepare the photocurable monomer solution, 10 wt% of Irgacure 2959 and 0.2 wt% of TINUVIN 234 were added into a 4:1 (volume ratio) mixture of PEGDA and MAA. The monomer solution was sonicated for 30 minutes and degassed for 15 minutes.

Pre-Designed Scaffold Fabrication

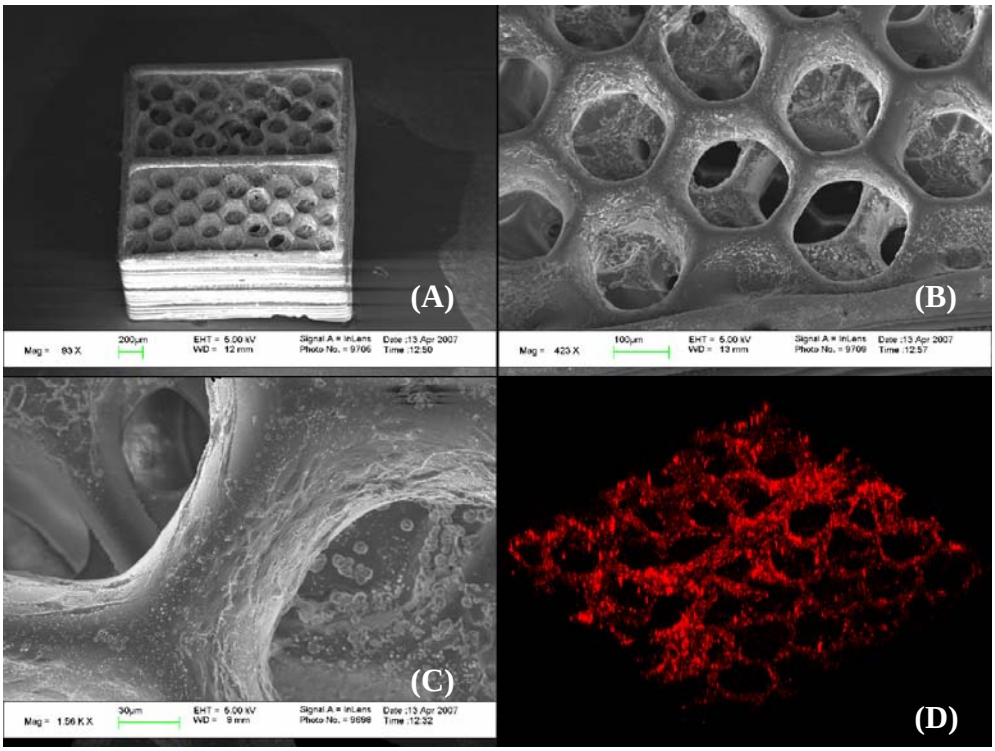

Figure 3. SEM pictures (Figure 3A-C) and fluorescence (Figure 3D) micrographs show that the D1 cells attach and secrete extracellular matrix onto the surfaces of the fibronectin-modified scaffold.

The polymer vat was filled with perfluorohexane (C_6F_{14}), an inert chemical with high density (1.685 g/cm^3) and a molecular polarity significantly lower than the photo-curable monomer. Because of these properties, the photocurable monomer forms a layer on top of perfluorohexane within the polymer vat. The majority of the stage is immersed in perfluorohexane. A key advantage of using an inert, heavy liquid like perfluorohexane is that only a small amount of photocurable monomer solution is necessary during fabrication as the perfluorohexane acts as a "filler" material for the bottom region of the stage. The syringe pump was also filled with perfluorohexane to be used as the purging agent. The focal plane of the DMD-PP was determined, and the stage was set accordingly. The height of the polymer vat was adjusted to allow a thin layer of the photocurable monomer to form on the outlet of the syringe pump. A UV photomask from the DMD system selectively solidifies the monomer into a thin layer, creating the first layer of the structure. The shape of the outlet was properly designed, such that the scaffold layers completely cover the outlet.

The power of the UV image was determined to be approximately 5mW/cm^2, and UV exposure time was set for 60 seconds. After the first layer was built (Figure 2B), the stage moves the structure below the perfluorohexane/monomer interface, and the syringe pumps perfluorohexane through the microstructure on the outlet. The perfluorohexane solution pushes the uncured material (which includes oligomers) back to the monomer layer. After

the purging step, the stage moves up and locates the top of the structure slightly higher than the perfluorohexane/monomer interface. The syringe aspirates monomer at a volume equal to the amount purged by perfluorohexane, and the structure is filled with fresh monomer. Following this step, the stage moves to 50 μm below the position where the previous layer was created (Figure 2C), and the next layer is then created. The patterned-polymerization and purging cycles are repeated until the entire, pre-designed scaffold is built.

As shown in the inset of figure 2, we used three different cross-sectional images (1-3) to pattern the scaffold with hexagonal-shaped porosity. The images were used in a sequence: 1-1-1-2-1-1-1-3-1-1-1-2-1-1-1-3-1. The layered structure of the scaffold includes four honeycomb layers, neighboring honeycomb layers are spaced by three rectangular rims, and the space between two honeycomb layers was measured to be 150 μm.

By using UV dye TINUVIN 234, we successfully adjusted the curing depth of monomer to about 50 μm. There is little scattering interference between the new layer and previously formed layer. Under a brightfield microscope, we found that the geometry of each of the honeycomb layers was clearly defined, and the pores of the honeycomb structures were free from unwanted polymerization or oligomer accumulation.

Surface Modification of the Preformed Scaffold with Fibronectin

After the scaffold was fabricated, tethered carboxyl groups from the methacrylic acid (pre-mixed in the monomer) was activated using 1-ethyl- 3- [3-dimethylaminopropyl] carbodiimide hydrochloride/ *N*- hydroxysulfosuccinimide chemistry (EDC/ sulfo-NHS, Pierce Technologies, Rockford, IL). EDC is a commonly used crosslinker in bioconjugation chemistries to create amide bonds. Carboxyl groups are converted to amine-reactive sulfo-NHS esters by EDC with the presence of sulfo-NHS. 0.4*M* EDC and 0.4*M* sulfo-NHS in 0.1*M* MES buffer [(2-(N-morpholino) ethane sulfonic acid), pH 6.5], were added to the scaffolds at a total volume of 1.5 mL and incubated on a rotator for 2 h at room temperature. Using a low-affinity microcentrifuge tube, a 1.5 mL solution of fibronectin (10 g/mL) was then added to the scaffolds upon carboxyl activation, and incubated at room temperature for a 24 h period. Scaffolds were then sterilized using 70% ethanol for 30 minutes prior to cell seeding, and rinsed several times with PBS to remove unconjugated fibronectin and ethanol.

Murine Marrow-derived Progenitor Cells

A bone marrow progenitor cell line derived from mice, D1 ORL UVA (ATCC, Manassas, VA), was seeded onto the fibronectin-modified scaffolds created by the DMD-based system. Prior to seeding the cells onto the scaffolds, an amine-reactive fluorescent dye, CellTrace™ Far Red DDAO-SE (Molecular Probes, Eugene, OR) was used to stain the cells red, following the manufacturer's protocol. Scaffolds were placed on sterilized parafilm within a tissue culture plate and then suspended in primary medium that contained 250,000 D1 cells. The suspended cell solution forms a "ball" on top of the scaffold, thereby

localizing the cells onto the fibronectin-modified scaffold, due to the hydrophobic surface of the parafilm. After a 4 h seeding period, the rest of the primary medium was added to the tissue culture plate. Primary medium used to culture D1 was composed of 10% w/v FBS (ATCC) and 1% w/v penicillin streptomyocin in Dulbecco's Modified Eagle's Medium (ATCC). The cell-scaffold construct was placed in a humidified incubator (5% CO_2, 37°C) and kept in culture for a 48 h period before fixing in a 10% formalin solution. Scaffolds were then prepared for scanning electron and fluorescence confocal microscopic analyses.

Scanning electron (Figure 3A-C) and fluorescence (Figure 3D) micrographs show that D1 cells attach and secrete extracellular matrix onto the surfaces of the fibronectin-modified scaffold. Fibronectin is a highly characterized protein known to bind to cell integrins, causing cell anchorage onto surfaces bound to this particular protein. Figure 3D is a three-dimensional compiled fluorescence micrograph of attached cells created from individual images obtained through confocal microscopy. The scaffold depicted here is composed of four layers with a wall thickness of 50 μm and a hexagonal pore geometry of approximately 150 μm.

CONCLUSION

A porous, multilayered, 3D scaffold for studies in cellular behavior was successfully created by patterning a photocurable monomer using a DMD-based fabrication tool. The monomer includes a small amount of absorbing dye (TINUVIN 234, 0.2wt%) which enhances the geometric resolution of the scaffold and reduces the curing depth of the monomer. The fabrication tool also includes a perfluorohexane-based purging mechanism to remove oligomers within the pores of the curing structure. The surfaces of the 3D scaffolds were biochemically modified with fibronectin for efficient cellular attachment. Data from SEM and confocal fluorescence micrographs confirm the successful seeding, attachment, and proliferation of D1 cells onto these pre-designed, micro-fabricated porous structures.

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