A digital micro-mirror device-based system for the microfabrication of complex, spatially patterned tissue engineering scaffolds

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Abstract: Our ability to create precise, pre-designed, spatially patterned biochemical and physical microenvironments inside polymer scaffolds could provide a powerful tool in studying progenitor cell behavior and differentiation under biomimetic, three-dimensional (3D) culture conditions. We have developed a simple and fast, layer-by-layer microstereolithography system consisting of an ultra-violet light source, a digital micro-mirror masking device, and a conventional computer projector, that allows fabrication of complex internal features along with precise spatial distribution of biological factors inside a single scaffold. Photocrosslinkable poly(ethylene glycol) diacrylates were used as the scaffold material, and murine bone marrow-derived cells were successfully encapsulated or seeded on fibronectin-functionalized scaffolds. Fluorescently-labeled polystyrene microparticles were used to show the capability of this system to create scaffolds with complex internal architectures and spatial patterns. We demonstrate that precisely controlled pore size and shapes can be easily fabricated using a simple, computer-aided process. Our results further indicate that multi-layered scaffolds with spatially distributed factors in the same layer or across different layers can be efficiently manufactured using this technique. These microfabricated scaffolds are conducive for osteogenic differentiation of marrow-derived stem cells, as indicated by efficient matrix mineralization. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 396–405, 2006

Key words: tissue engineering scaffolds; microfabrication; digital micro-mirror device; stereolithography; layer-by-layer

INTRODUCTION

Recent advances in fabricating scaffolds for tissue engineering applications have yet to report methods in creating three-dimensional (3D) constructs that incorporate complex spatial-patterning of extracellular matrix components (ECM) and growth factors, which could provide biomimetic complex microenvironments for studying cell behavior and differentiation.[1–3] Most 3D scaffolding systems are only capable of differentiating a single progenitor cell population into one particular cell lineage due to either (a) bulk incorporation of bio-factors within the scaffolding matrix[4] or (b) exogenous delivery of hormones, chemicals, or growth factors in culture medium.[5–7] From a tissue engineering perspective, a significant advancement could be attained by creating precise, spatially distributed microenvironments within a single scaffold that would allow us to study simultaneous, patterned differentiation of stem and progenitor cells into multiple lineages and develop concepts to ultimately engineer complex, hybrid organ structures. A key step toward achieving such patterned 3D structures is the development of novel scaffold-manufacturing techniques by which distributed environments can be incorporated in a simple yet precise, reproducible fashion.

Laser micro-stereolithography (μSL) has become an accepted rapid prototyping method that allows the 3D microfabrication of solid models from images created by computer-aided design (CAD) programs.[8–10] We have recently reported a layer-by-layer laser microfabrication method for creating spatially patterned scaf-

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folds using photo-crosslinkable polymers. In this method, a motorized *x-y-z* platform immersed in a liquid photopolymer is selectively exposed to a focused ultra-violet (UV) laser light. The polymer cures and becomes solid only at the focal point whereas non-irradiated areas remain liquid. After the first layer is formed, the platform moves downward and a new layer of polymer is solidified according to the design. This layer-by-layer micro-manufacturing method enables complex internal features such as patterning of growth factors and ECM proteins. Bhatia and colleagues[11] have also provided an innovative soft lithography and a microsyringe method to fabricate biodegradable PLGA-based scaffolds with complex architectures. Multilayered structures were fabricated by thermally laminating each layer, and a classical salt leaching approach was used to create random porosities in the scaffold.

Although fine structures can be produced by the laser μSL technique, the process is usually slow because of the nature of point-by-point laser scanning. This prevents the incorporation of cells within the scaffold walls during the fabrication process and could also lead to denaturation and inactivation of biological molecules during the prolonged fabrication period. Here we report a novel, digital micro-mirror device (DMD)-based scaffold fabrication technique that allows precise, predesigned patterning of multiple molecules and allows generation of complex architectures in a high-throughput, layer-by-layer fashion.

Bertsch and colleagues reported a μSL process employing a liquid crystal display (LCD) as a dynamic mask to photopolymerize an entire layer simultaneously and demonstrated fabrication of small mechanical parts such as a turbine and spring.[12,13] Itoga and colleagues[14] have also explored LCD projectors to study two-dimensional (2D) cellular behavior through the micro-patterning of non-cytoadhesive polymers onto plasma-treated glass surfaces. However, LCD as a dynamic mask has limited optical efficiency.[15] A new technology, Digital Micro-mirror Device™ (DMD, Texas Instruments, Dallas, TX), offers better performance in terms of optical fill factor (85% with DMD vs. 64% with LCD) and light transmission (71% with DMD vs. 21% with LCD). Furthermore, computer projectors, like the ones widely used for PowerPoint presentations, are commercially available for utilizing DMD technology in image transferring.

Rather than writing the 3D microstructure point-by-point (as previously reported in our laser scanning system[16]), or using a molded structure with thermal lamination,[11] we have developed a DMD-based dynamic mask for the simultaneous photo-polymerization of partial and entire layers of a scaffold via projection. By changing bio-factors or controlled-released particles within the polymerizable resin, each layer or even partial layers were made up of a variety of controlled-release microparticles, thereby creating spatially distributed environments with micron-size resolution. In addition, precise and complex internal architectures, for example pore size and shape, were created using DMD. Cells were efficiently incorporated inside the scaffold walls during fabrication or seeded on the scaffolds following covalent modification of the surface with fibronectin. Murine marrow-derived stromal cells seeded in these DMD μSL-fabricated patterned scaffolds efficiently differentiated into osteoblasts and produced scaffold mineralization thereby demonstrating the ability of such structures to support cell proliferation and differentiation.

MATERIALS AND METHODS

PEGDA solutions and photoinitiator

Macromer solutions used to fabricate hydrogel scaffolds were formulated using 100% (w/v) poly(ethylene diacrylate) (PEGDA, M_w 3400, Nektar Therapeutics, AL) dissolved in phosphate buffered saline (PBS). To induce chain polymerization through the generation of free radicals, an UV photoinitiator, 2-hydroxy-1-[4(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, Ciba Geigy, USA), was used at a concentration of 0.1 wt %. The photoinitiator was first dissolved in PBS at a concentration of 0.7 wt % to ensure complete solubility before adding to the PEGDA solution. Prepared macromer solutions were kept in a dark environment to prevent precrosslinking of the polymer by incidental exposure to ambient light.

DMD μSL and scaffold fabrication

The micro-stereolithographic system was developed based on a commercial projector (PB2120, BenQ, Taiwan) coupled with a digital micro-mirror device (DMD™, Texas Instruments). Similar to a conventional stereolithography process, the DMD μSL created 3D microstructures in a layer-by-layer fashion. The shapes of the constructed layers were determined by slicing the desired 3D scaffold design into a series of evenly spaced planes. Patterns of each layer were drawn in a series of PowerPoint slides, which were then executed on the DMD chip to generate a dynamic mask. The illuminated light is modulated according to the defined mask on the DMD chip and then goes through a reduction-projection lens assembly to form an image on the surface of the resin or macromer solution. The illuminated area was solidified simultaneously under one exposure, while the dark regions remained in the liquid phase. After one layer was patterned, the substrate was lowered and the as-patterned layer was then covered by fresh macromer solution. Microstructures with complex geometries were created by sequentially polymerizing the layers. To fabricate scaffolds with multiple material compositions, solidified areas were rinsed thoroughly before immersing with a different macromer composition. To demonstrate the ability of this sys-

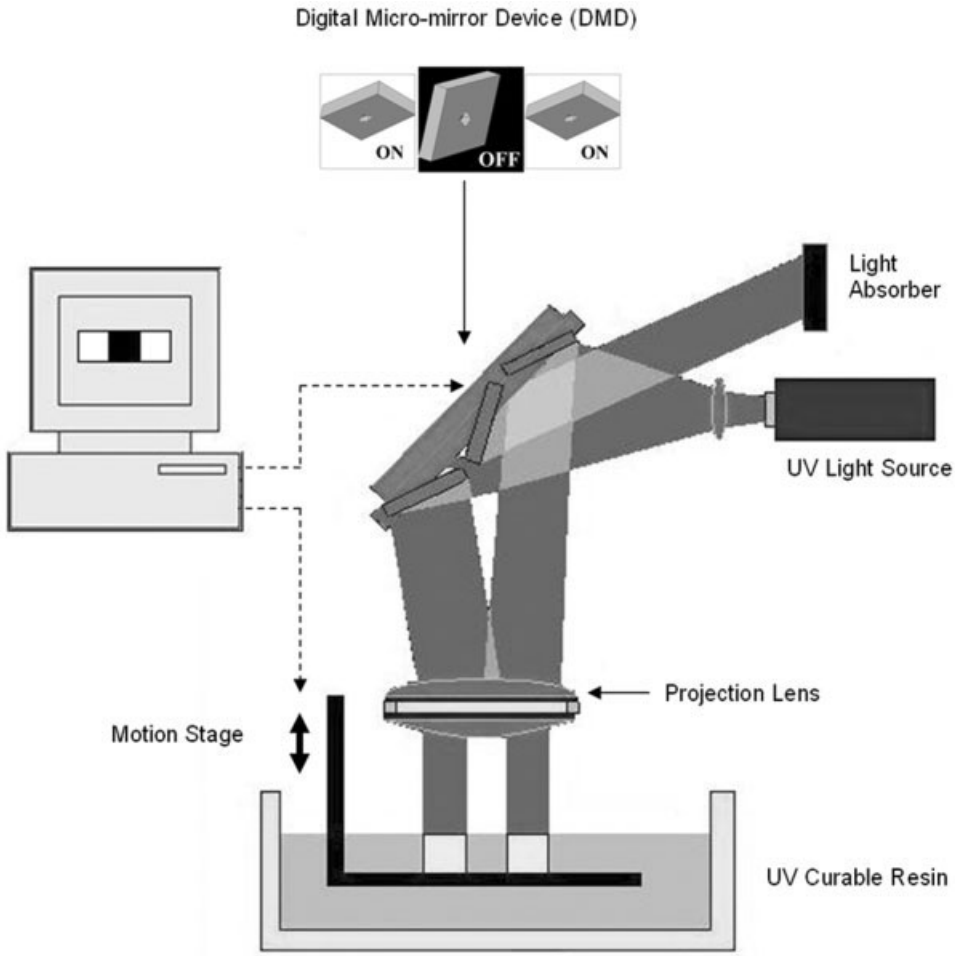

Figure 1. Schematic of the Digital Micro-mirror Device Micro-stereolithography (DMD-μSL) set-up.

tem to create spatially-patterned, multi-layered scaffolds, fluorescently-labeled polystyrene microparticles (1.0 μm Cy5-labeled and 1.0 μm FITC-labeled, Molecular Probes, Eugene, OR) were added separately to PEGDA solutions at a final concentration of 0.03 wt % prior to irradiation. These constructs were analyzed using either a confocal microscope for fluorescence patterning (Leica SP2 AOBS) or by scanning electron microscopy (Phillips 515 SEM) for multi-layered scaffolds.

The DMD μSL system is schematically shown in Figure 1. The system consists of five major components: a DMD chip embedded in the projector as a dynamic mask, a light source, a projection lens assembly, a translation stage with a micrometer, and a vat containing macromer solution. All the components cooperate to ensure correct exposure, resolution, and layer thickness. To ensure cell viability through the use of a biocompatible UV photoinitiator, the original high intensity white light source was replaced with a UV light source (Green Spot, UV Source, CA). The light was guided through a ¼ inch (6.35 mm) liquid-filled fiber optics. Two bi-convex lenses (18 mm diameter, 40 mm focal length) with 5 mm spacing were used to converge the light emanating from the fiber optics. The projection lens assembly with adjustable aperture and focus consisted of two equal plano-convex lenses (25 mm diameter, 25 mm focal length). Each lens was oriented with the convex surface toward the longer conjugate distance. The aperture was placed in-between two lenses. All lenses were made of UV grade fused silica (Edmund Optics, Barrington, NJ). The average exposure intensity was determined to be 2 mW/cm^2. Throughout the experiments, the magnification (size of the scaffold/size of the pattern) was fixed at 1/90.

The working principle of the DMD chip is also detailed in Figure 1. The DMD chip serves as an array of reflective aluminum micro-mirrors, which can be tilted with two bias electrodes to form angles of either $+10°$ or $-10°$ with respect to the surface. Illumination from the light source reflects into the projection lens only when the micro-mirror is in its $+10°$ state. In the $-10°$ state, the pixel appears dark because the illuminated light is not reflected into the projection lens. The reflected light from the $-10°$ micro-mirror is collected by a light absorber. When the micro-mirror is in $+10°$ state, it is classified as "tilt on" or ON. Conversely, when the micro-mirror is in $-10°$ state, it is classified as "tilt off" or OFF.

Though the schematic in Figure 1 depicts only three micro-mirrors for illustrative purposes, the actual DMD chip contains more than 442,000 switchable mirrors on a 5/8-in (15.875 mm) wide surface. For instance, in Figure 1, there are three pixels of white, black, white color, respectively, on the screen. The DMD chip is signaled by the computer to tilt the first micro-mirror to $+10°$ state, the second micro-mirror to $-10°$ state, and the third micro-mirror to $+10°$ state. The first and third micro-mirror, which is in ON condition, reflects

the illuminated light to the projection lens. Subsequently, the light reflected by the DMD into the projection lens is directed into the resin to cure the macromer solution. Conversely, the second mirror, which is in OFF condition, reflects the illuminated light to the light absorber.

DMD μSL cell encapsulation and viability

Murine OP-9 marrow stromal cells (a gift from T. Reid, University of Toronto) were cultured in Minimum Essential Medium-Alpha (MEM, Gibco Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum, 30 m*M* sodium bicarbonate (Sigma-Aldrich), and 1% (v/v) penicillin–streptomycin (Gibco Invitrogen). Cell passaging and medium exchange were carried out every 2–3 days.

Before the 10th passage, OP-9 cells were trypsinized and centrifuged to a pellet. PEGDA solution (formulated as described earlier) was filter sterilized and added to the cell pellet at concentration of 5×10^6 OP-9 cells/mL macromer. A 15 μL suspension of cell-macromer solution was then pattern-polymerized using DMD μSL for ~3 min. Unpolymerized solution was rinsed away extensively with sterile PBS, and scaffolds were transferred into tissue culture plates with medium and placed into an incubator. Cell encapsulation efficiency was determined in triplicate by counting cells in the unpolymerized solution using a hemocytometer. After a 24-h incubation (5% CO_2, 37°C) period, 2 μ*M* calcein AM (Molecular Probes, Eugene, OR) in PBS was added to the hydrogel-cell constructs, following the manufacturer's protocol, which stains viable cells green through intracellular esterase activity. Scaffolds were observed using fluorescence microscopy, and images were captured.

Surface modification of scaffolds for cellular attachment

Surfaces of hydrogels were modified to be cyto-adhesive by covalently conjugating fibronectin (from Bovine serum, Sigma Aldrich, St. Louis, MO) to patterned scaffolds. Before photo-polymerization, methacrylic acid (Sigma Aldrich, St. Louis, MO) was added to the macromer (PEGDA) solutions, as described earlier, at a molar ratio of 1:4 (methacrylic acid:PEGDA). Upon vortexing to ensure thorough mixing, solutions were irradiated with DMD μSL for the patterned polymerization of a 3D scaffold. The carboxylate groups tethering from the surfaces of the scaffolds (because of the presence of methacrylic acid) was activated through 1-ethyl-3-(3-dimethylaminopropyl)/*N*-hydroxysulfosuccinimide chemistry (EDC/Sulfo-NHS, Pierce Biotechnology Inc., Rockfold, IL) to form stable amide bonds with fibronectin.[17] Briefly, a 500 μL solution consisting of 0.1*M* MES buffer [2-(*N*-morpholino) ethane sulfonic acid), pH 6.5], 30 μ*M* of Sulfo-NHS, and 30 μ*M* of EDC was added to each scaffold. The conversion of the carboxyl groups to amine-reactive Sulfo-NHS esters was performed for 2 h at room temperature on a plate rotator before the addition of 1.5 mL fibronectin (10 μg/mL), using low-adhesion protein binding microtubes (Fisher Scientific). Fibronectin conjugation to the surfaces of the scaffolds was performed for a 24-h period at room temperature. The negative control scaffolds did not contain methacrylic acid, but were treated in the same method as the experimental scaffolds. X-ray photoelectron spectroscopy (XPS, PHI 5700) was used to determine the presence of nitrogen (N) elements due to amide linkages of the conjugated fibronectin. Scaffolds were rinsed extensively with sterile PBS before cell seeding experiments.

To detect surface-conjugated fibronectin, immunostaining was performed using biotinylated anti-fibronectin antibody (Abcam, Cambridge, MA) and streptavidin-FITC (Southern Biotechnology Associates, Birmingham, AL). The incubation time for the antibody was 1 h and for the streptavidin-FITC was 30 min at room temperature using a plate rotator. For a 24-h period, scaffolds were incubated and rinsed several times with sterile PBS at room temperature. Fluorescence microscopy was used to detect surface-conjugated fibronectin. Furthermore, enzyme-linked immunosorbent assay (ELISA) was used to determine the amount of fibronectin conjugated to surfaces of the scaffolds. Biotinylated anti-fibronectin, streptavidin horseradish peroxidase, and tetramethylbenzidine (R&D Systems, Minneapolis, MN) were used to detect for unconjugated protein in the reaction supernatant of four different scaffolds. Absorbance was determined at 450 nm using a microplate reader (Opsys MR, Thermo Labsystems, Chantilly, VA) and plotted against a known standard curve.

Mesenchymal stem cell isolation and osteogenic induction

Bone marrow was obtained from BALB/c mice (7–30-weeks-old, Charles River Laboratories, Wilmington, MA). NIH guidelines for the care and use of laboratory animals (NIH Publication #85–23 Rev. 1985) have been observed and all animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at Austin. Upon sacrificing mice through carbon dioxide asphyxiation, bone marrow was flushed out of the tibias and femurs using a 27½-gauge needle and barrel with basal medium. Basal medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin–streptomycin (Gibco Invitrogen, Carlsbad, CA). After washing cells via centrifugation (1000 rpm, 5 min), viable cells were counted using trypan blue and a hemocytometer. To initiate murine mesenchymal stem cell culture (mMSC), cells were plated at a density of 2×10^6 cells/cm^2. The culture was placed in a humidified incubator (5% CO_2, 37°C) for 72 h when non-adherent cells were removed via medium exchange. Cell passaging upon plate confluence and medium exchange were performed every 2–3 days.[18]

Primary mMSCs, 2 weeks in culture, were trypsinized for 10 min at 37°C, centrifuged, counted, and seeded onto fibronectin-modified scaffolds at a density of 50,000 cells per scaffold. Sterile parafilm was placed in a non-treated cell culture plate to form a hydrophobic surface when seeding the cells, and the scaffolds were placed on top of the film. Cell attachment onto scaffolds was performed by suspending the cells in 100 μL of medium onto the constructs,

thereby forming a "ball" because of an increased contact angle with the parafilm surface. Medium was added to the culture plate after a 4-h incubation period. Cell attachment was quantified in triplicate by washing the cell-scaffold structures with sterile PBS and counting unattached cells with a hemocytometer. Osteogenic differentiation was initiated by culturing seeded mMSCs onto scaffolds for up to 4 weeks in basal medium supplemented with 10 mmol/L β-glycerophosphate, 10^{-8} mol/L dexamethasone, and 5 μg/mL ascorbic acid 2-phosphate.

Histology

At 2-week and 4-week time points, scaffolds were removed from *in vitro* culture and fixed overnight in 4% paraformaldehyde at 4°C. Scaffolds were dehydrated by adding 80% ethanol, 90% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, 50% ethanol/50% Citrisolve (Fisher Scientific), 100% Citrisolve, 100% Citrisolve, and molten paraffin at 60°C in 1-h sequential steps.[7] The scaffolds were then left overnight in molten paraffin at 60°C. Paraffin embedded samples were allowed to harden for a 24-h period, sectioned at 10 μm using a microtome (American Optical Spencer Rotary Microtome Model 820), and then placed on glass slides. The slides were warmed for 5 min at 60°C and dried overnight at room temperature. Slides were dried an additional 30 min at 60°C, rehydrated, and stained. Nuclear fast red and von Kossa staining were used to visualize cell nuclei and scaffold calcification, respectively. Scaffolds that did not contain cells were also von Kossa stained to determine if the PEG scaffolds alone stained black. Brightfield microscopy was used to observe the samples (Olympus IX70, Olympus America, Melville, NY; Optronics MagnaFire digital camera, Goleta, CA).

RESULTS

Single and multi-layered scaffolds with defined architectures created by DMD μSL

The DMD μSL method was used to create polymer scaffolds with pores and channels having wide variety of shapes and dimensions. The configuration of the scaffold pores was dictated simply by altering the "mask" drawn on a PowerPoint slide, thus illustrating the powerful capability of this system to design features of any shape or form. As shown in the scanning electron microscopic (SEM) micrographs of Figure 2(a–d), different pore geometries (hexagons, triangles, honeycombs with triangles, and squares) can be included within a single scaffold (pore size dimensions range from ~165 to 650 μm, scale bars shown). Precise internal features of the scaffolds were fabricated with one single 90-s exposure to the UV light of the DMD μSL. Additionally controlled internal architectures can be generated in parallel. Figure 2(d) shows scaffolds fabricated in a multi-layered fashion. The pore dimensions used in this scaffold are 250 μm by 250 μm with a measured wall thickness of 100 μm. Construct edges appeared to be slightly rounded, due to swelling of the hydrogel structure. Keeping the light intensity (2 mW/cm^2) and exposure time constant, the smallest feature size attainable with DMD μSL was measured to be approximately 20 μm (data not shown). This system is fundamentally limited by optical diffraction and diffusion of free radicals in the polymer solutions.

Single- and multi-layered scaffolds can be spatially patterned using DMD μSL

The DMD μSL system can fabricate both single layer and multi-layered scaffolds with pre-designed, spatially-patterned molecules and particles. The feasibility of such precise spatial patterning was demonstrated using PEGDA solutions containing either Cy-5 or FITC-labeled polystyrene particles that were encapsulated in a predesigned pattern during the polymerization process. As shown in Figure 3(a), solutions containing different particles can be patterned in a quadrant-specific geometry, in which the solution with Cy-5 particles were polymerized in the upper left and lower right regions, and the solution with FITC particles were polymerized in the upper right and lower left regions. This figure demonstrates the ability of the DMD μSL system to pattern multiple agents within a single layer through sequential steps of polymerization and rinsing of unpolymerized solutions. We also demonstrated spatial patterning in multi-layered scaffolds, as shown in Figures 3(b,c), by creating constructs that specifically consisted of two layers, each containing either Cy-5 or FITC-labeled particles. The bottom layer was pattern-polymerized with a single 90-s exposure using Cy-5 particle-polymer solution, and then rinsed extensively to remove unpolymerized polymer and particles. The second layer, containing FITC particle-polymer solution, was then polymerized in the same method on top of the first layer using the same patterning mask.

Covalent modification of scaffold surfaces with fibronectin

Since PEG polymers have hydrophilic and non-ionic properties, the scaffold surface must be modified to mediate efficient cell seeding. Here we demonstrate the covalent conjugation of fibronectin, an extracellular matrix component that signals for cell anchorage and spreading, to scaffolds via EDC/Sulfo-NHS chemistries. Figure 4 shows fluorescence images of immunostained scaffolds. Scaffolds containing methacrylic acid in the

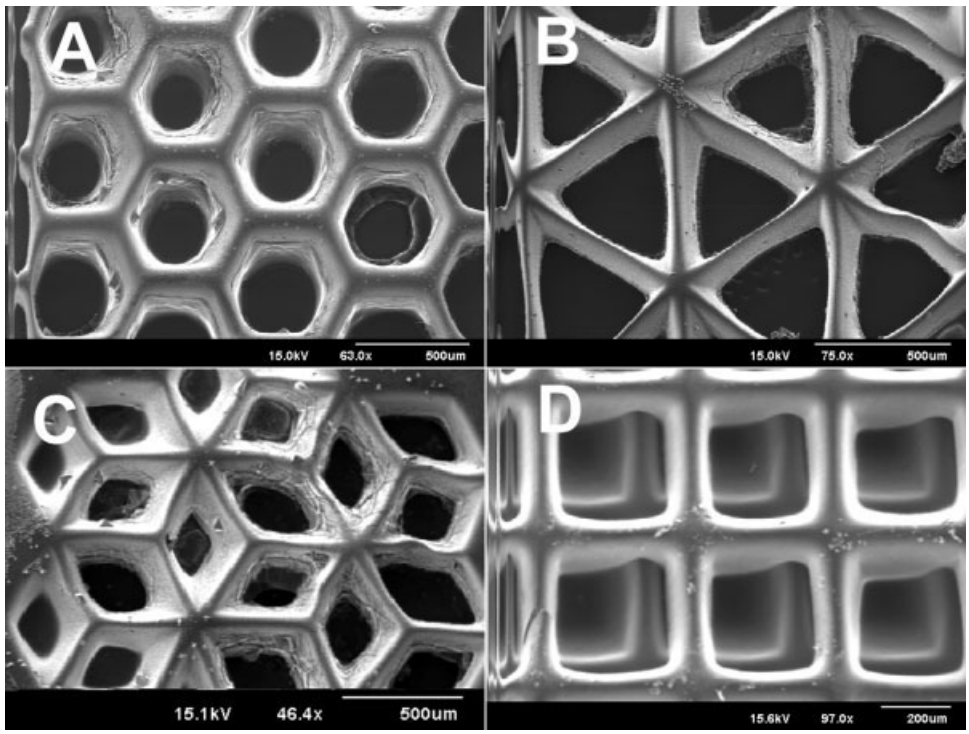

Figure 2. Scanning electron microscopy (SEM) illustrate that DMD-μSL can create scaffolds with intricate pore geometries. Hexagons (honeycomb), triangles, triangles inside hexagons, and squares shaped pores were created by directly drawing in PowerPoint files and using the DMD as a dynamic "mask." Geometrical side dimensions of the pores range from approximately 165–650 μm (scale bars shown). Scaffolds depicted in (D) specifically show a two-layered scaffold. All scaffolds were irradiated for 90 s per layer and formulated using 100% (w/v) PEGDA in PBS and 0.1 wt % Irgacure 2959.

macromer solution (left) fluoresce green, indicating the successful covalent conjugation of the fibronectin, whereas the scaffold without methacrylic acid (right) did not show any adsorption or conjugation of fibronectin. ELISA studies indicated that fibronectin attached to scaffold surfaces at a concentration of approximately 9.2 $\mu g/cm^2$ ($\pm$ 0.3 $\mu g/cm^2$, $n = 4$).

OP-9 cell encapsulation and viability

Patterned encapsulation of cells within the scaffold walls was achieved by the addition of OP-9 cells to the macromer solution prior to DMD μSL UV irradiation. Figure 5(a) shows a fluorescence micrograph of cells overlaid onto a transmitted micrograph of the scaf-

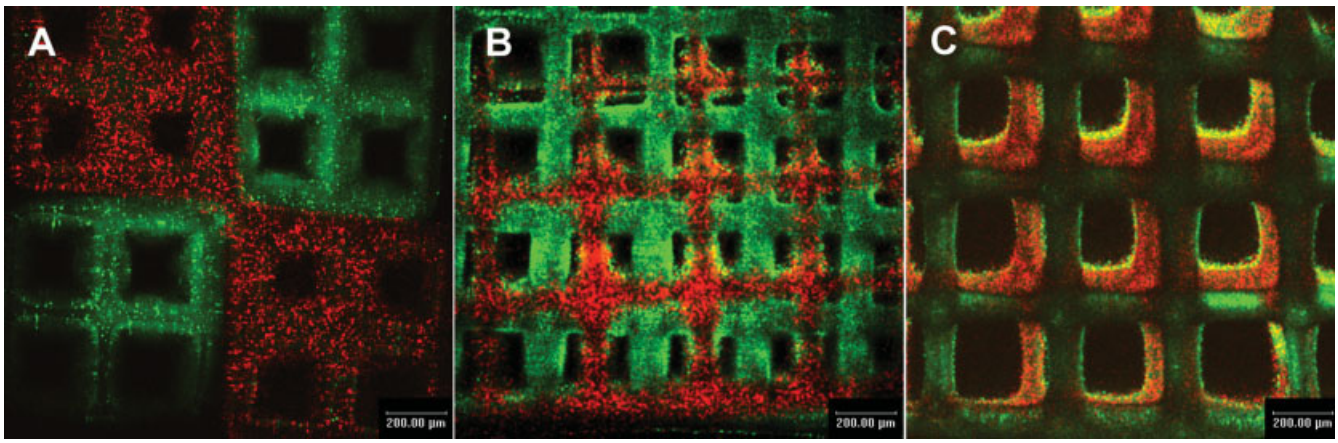

Figure 3. DMD-μSL can create pre-designed, spatially-patterning inside scaffold structures. Fluorescence confocal microscopy of scaffolds formulated with 100% (w/v) PEGDA in PBS, 0.1 wt % Irgacure 2959, and 0.03 wt % carrying either FITC- or Cy5- labeled polystyrene particles. A: Spatial patterning of a single-layer in a "quadrant" specific pattern. B–C: Spatial patterning in multi-layered scaffolds. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

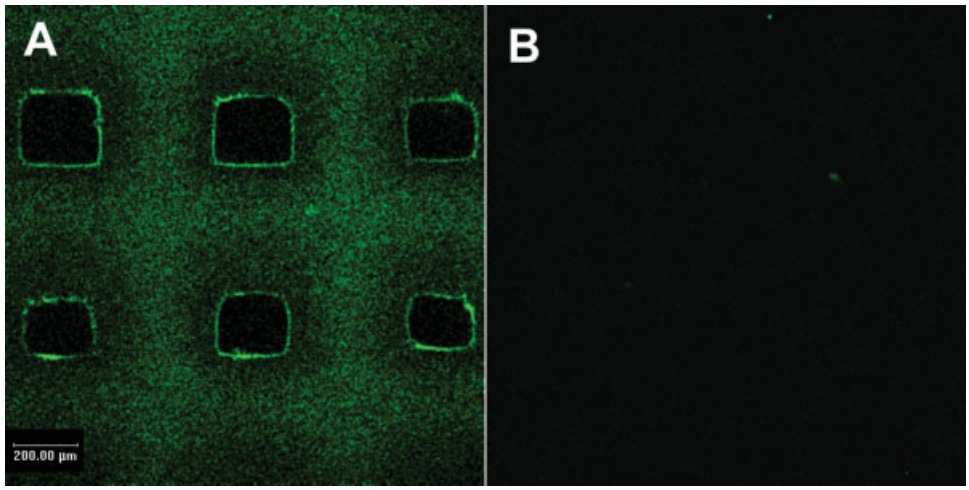

Figure 4. Fibronectin was covalently conjugated to scaffolds surfaces. Fluorescence micrographs showing scaffolds immuno-stained for fibronectin. Biotinylated anti-fibronectin antibody and streptavidin-FITC were used to stain the microfabricated scaffolds following fibronectin conjugation. A: The successful covalent conjugation of fibronectin through the methacrylic acid macromer added during the scaffold fabrication process. The scaffold illustrated in (B) does not contain methacrylic acid and shows no conjugation (negative control). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

fold, and demonstrates the viability of encapsulated cells within a single-layered scaffold (~150 μm thick layer). Most of the encapsulated cells within the hydrogel structures remained viable after a 24-h incubation period as indicated by calcein staining, indicating a cytocompatible fabrication process. Cell encapsulation efficiency for this particular patterned-scaffold was determined to be approximately 73% (±9.6%, $n = 3$). Encapsulation efficiency is, however, dependent on scaffold channel or pore dimensions, the volume of cell-macromer solution used, and the presence of a container to hold the cell-macromer solution during the photo-polymerization process to prevent solution spreading.

Osteogenic differentiation of mMSCs in DMD μSL-fabricated scaffolds

Seeded mMSCs successfully attached onto patterned fibronectin-conjugated scaffolds created by

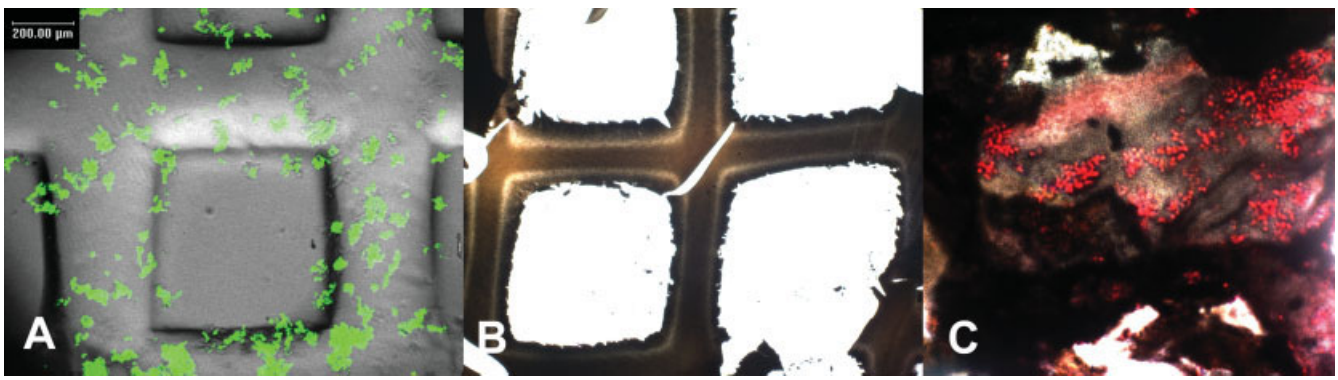

Figure 5. A: Marrow-derived stromal cells remain viable following encapsulation in DMD-μSL fabricated scaffolds. Scaffolds depicted in this figure were formulated with 100% (w/v) PEGDA in PBS and 0.1 wt % Irgacure 2959. Before photo-polymerization, OP-9 marrow stromal cells were added to the macromer solution at a concentration of 5×10^6 cells/mL. After a 24-h incubation period, 2 μ*M* calcein was added to cell-scaffold constructs, which stains viable cells green. Transmitted and fluorescent images captured from confocal microscopy were overlaid and shown in (A). B–C: Patterned scaffolds are capable of osteogenic differentiation of bone-marrow derived progenitor cells. B–C: Fibronectin-conjugated scaffolds seeded with primary mMSCs at a density of 50,000 cells per scaffold. At 2-week and 4-week time points, scaffolds were removed from culture, paraffin embedded, and sectioned. These sections were stained with nuclear fast red and von Kossa stains. Brightfield microscopic micrographs (B; with a ×10 objective at 2-week time point and (C) with a ×40 objective at 4-week time point) show extensive scaffold mineralization (Von Kossa stain: black) indicating osteogenic differentiation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DMD μSL, with a 70% (±3%, $n = 3$) attachment efficiency, and were then exposed to osteogenic medium. Figures 5(b,c) show nuclear fast red and von Kossa staining in constructs cultured for 2 and 4 weeks. Figure 5(b) shows von Kossa staining on scaffolds removed at 2-week time period (10× objective), and Figure 5(c) shows nuclear fast red and von Kossa staining of scaffolds removed at 4-week time period (40× objective). Cell cytoplasm stains pink and the nuclei stain bright red. Mineralized areas of the constructs stained black. These histological data show high levels of mineralization throughout the microfabricated scaffold matrix, indicating that seeded mMSCs transform into osteoblasts with the presence of osteogenic medium. Additionally, as a negative control, PEG scaffolds alone did not stain black when von Kossa was applied (data not shown). PEG-based scaffolds incorporating MSCs cultured only in basal medium do not show any mineralization when stained via von Kossa, as presented by Nuttelman et. al.[7]

DISCUSSION

A key limitation in tissue engineering is the fabrication of predesigned, spatially-patterned microenvironments that physiologically mimic what is observed by stem and progenitor cells during organ development. Creating such complex microenvironments inside scaffold constructs would allow us to better study cell behavior and differentiation under controlled, biomimetic settings. The ability to develop such constructs that combine both spatial and temporal-patterning of physical and biochemical factors could ultimately lead to creating multiple tissue types from a single stem cell population inside a 3D structure. We have developed a versatile layer-by-layer microfabrication system consisting of a DMD-based dynamic masking technology to create polymer scaffolds that integrate complex micro-architectures and spatially patterned bio-factors for studies in progenitor cell differentiation.

Similar to the laser-based stereolithography method we have previously reported,[16] DMD μSL can also build 3D scaffold structures through a layer-by-layer photo-polymerization technique. In addition, the key features achieved using the laser-based system, for example, creation of precise gradients of bio-factors or fabrication of specific, pre-designed pore sizes can be easily obtained using the DMD system (data not shown). The main advantage of using this novel scaffold fabrication system over the laser-based system or other stereolithography methods is that the photo-polymerization of an entire single layer is achieved simultaneously, and ultimately a 3D construct is achieved by "building" subsequent layers in the same fashion by exchanging polymer solution in between. In addition, more complex internal architectures as well as efficient cell encapsulation can be achieved using DMD. Although PEGDA was used in this present study, because the DMD μSL system is based on a photo-initiated, free-radical chain polymerization process, any polymer-photoinitiator solution undergoing such a reaction can be used. To achieve degradable scaffold structures, enzymatically or hydrolytically degradable moieties, as reported by Anseth and colleagues or Hubbell and colleagues[19–22] could also be used in the DMD μSL system.

The DMD-μSL system has the powerful capability of (a) generating complex internal architectures and (b) entrapping multiple biochemical factors (soluble or in controlled-release particles) in precise, pre-designed spatial patterns by their addition into the macromer solution prior to photo-polymerization. Since the masks used for polymerization were designed to take on any shape or size using PowerPoint, the geometries of the porosities, as well as the scaffold, can virtually be of any pattern. Entrapped degradable particles, carrying biological or chemical factors and having various release kinetics, could also allow for temporal patterning necessary for the prolonged and sequential signaling of progenitor cell differentiation. This stereolithography process could eventually lead to developing elaborate scaffolds conducive of creating more complex tissue-like structures, a major obstacle in current tissue engineering efforts.

The effects of exposure time, light intensity, polymer, and photoinitiator concentrations were investigated to optimize the resolution and patterning of the scaffold structures. A crucial task in the optical set-up of DMD μSL is ensuring that the masking pattern irradiates uniformly by utilizing the appropriate light source. Though optical fibers could potentially provide uniform light, its intensity profile could result in a quasi-Gaussian distribution. Another option that would provide a uniform light source is the use of an optical homogenizer, which simply homogenizes the intensity profile. Instead of using either of these methods, we took advantage of the versatility of DMD μSL by simply adjusting the gray scale of the masking patterns that were irradiated onto the macromer solutions. This process was achieved by darkening the gray scale of the pixels that received the highest intensity from the light source to balance the non-uniformity. Throughout our experiments, we observed that the required exposure time to photo-polymerize the macromer solution decreased as the light intensity increased.

Generally, in fabricating photo-polymerized tissue engineering scaffolds, a short exposure time is desired for two critical reasons: (a) viability of encapsulated cells decreases as exposure time increases and (b) increased diffusion of free radicals from the photo-initi-

ator causes distorted patterns with longer exposure times. Exposure time could be reduced by increasing light intensity to avoid such issues. Doing this, however, simultaneously enhances diffraction, thereby reducing pattern resolution. Furthermore, both diffraction from the masking pattern and diffusion of the free radicals could limit scaffold resolution. Increasing both polymer and photoinitiator concentrations resulted in more resolved patterning of the scaffolds, only under an optimum light intensity and a decreased exposure time. Otherwise, as reported by Bhatia and colleagues, keeping the parameters of exposure time and light intensity constant, polymer and photoinitiator concentration does not affect patterning resolution.[23] We limited the photoinitiator concentration in the macromer solutions to no more than 0.1 wt % with our experiments based on previous photoinitiator cyto-toxicity studies done by Bryant et al.[24]

Cell viability during encapsulation within 3D constructs is dependent upon the biocompatibility of the materials used and their by-products, scaffold fabrication process, and nutrient/waste diffusion throughout the porous matrix. DMD μSL was shown to be a bio-compatible scaffold fabrication technique when encapsulating cells during the photo-polymerization of the PEGDA macromer solution. UV light intensity, exposure time for scaffold polymerization, and free radicals generated from the photo-initiator did not affect the viability of the encapsulated cells even after 24 h in culture. Because of its cross-linking nature from a liquid to a solid phase and the ability to imbibe water up to more than 90%,[25] PEGDA is an ideal polymer to encapsulate cells and sustain their viability. The cross-linking network of PEG-based hydrogels also provides efficient transport of nutrient and waste delivery, thus contributing to the sustainability of encapsulated cells. We hypothesize that the channels serving as macro-structural pores of the scaffolds created by DMD-μSL further act to increase nutrient/waste diffusion of cells as opposed to the "bulk" polymerization of cell and PEG-based polymer solutions as reported by others.[6,7] Cell viability would vary along the cross-sections (*x*-*y*-*z* directions) of thicker hydrogels that were bulk-polymerized due to inefficient nutrient/waste diffusion within the interior of the matrix (i.e. only surfaces of the gels would get optimum diffusion). Because DMD-μSL has the capability of patterning channels as pores all throughout the hydrogel scaffolds, the viability of encapsulated cells only depends on wall thickness (*x*-*y* directions) and not the entire thickness (height or *z*-direction) of the 3D construct. Scaffold wall thickness is easily controlled through the design of the masking patterns irradiated by DMD-μSL and would only have to be a certain thickness to encapsulate a sufficient amount of cells. Furthermore, because unpolymerized solutions can be washed away in between UV irradiations, multiple cell types could also be patterned within a single construct, leading to a more intricate tissue engineering construct.

In addition to cell encapsulation, scaffold surfaces can be functionalized to conjugate fibronectin, thus allowing efficient cell seeding, attachment, and proliferation. Because fibronectin is covalently-conjugated post-fabrication, the possibility of denaturation due to UV exposure is eliminated. Seeded murine MSCs attached efficiently to fibronectin functionalized scaffolds. The differentiation of MSCs on patterned scaffolds was achieved through the addition of osteogenic culture medium and confirmed through standard histological staining for matrix calcification. Mineralization of the scaffold over a 4-week period in culture illustrates that these DMD micro-fabricated, patterned scaffolds are capable of allowing mMSCs to adhere, proliferate, and transform into osteoblasts.

In conclusion, we have demonstrated DMD-μSL to be a powerful technology in creating pre-designed, spatially patterned scaffolds for applications in cell and tissue engineering. This novel stereolithography system has the capability of creating precise distributions of chemical and biological factors within a 3D scaffolding structure. The scaffolds are also suitable for the encapsulation of single or multiple cell types in a spatially distributed fashion. Functionalizing the patterned scaffolds with fibronectin creates a microenvironment suitable for the attachment, proliferation, and differentiation of mMSCs. These micro-fabricated, spatially patterned scaffolds could ultimately consist of intricate architectures that combine both spatial and controlled-release kinetics of biochemical factors, creating a suitable environment for studying hybrid tissue formation from a single stem cell population.

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