A Photolithographic Method to Create Cellular Micropatterns

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Abstract

Here we describe a simple and rapid system for creation of patterned cell culture substrates. This technique is based on (1) printing a mask on a standard overhead transparency, (2) coating a thin layer of a photocrosslinkable chitosan on a slide, (3) exposing the slide and mask to ultraviolet (UV) light, and (4) rinsing the uncrosslinked polymer to expose the underlying cell-repellent patterns. Photocrosslinkable chitosan does not require photoinitiators, it is non-toxic and forms flexible, biocompatible hydrogel upon short (min) UV exposure. Patterns of various shapes (lanes, squares, triangles, circles) were created on two surfaces commonly used for cell culture: glass and tissue culture polystyrene. The pattern size could be varied with a mm resolution using a single mask and varying UV exposure time.

Cardiac fibroblasts formed stable patterns for up to 18 days in culture. Cardiomyocytes, patterned in lanes 68–99 mm wide, exhibited expression of cardiac Troponin I, well developed contractile apparatus and they contracted synchronously in response to electrical field stimulation. Osteoblasts (SAOS-2) localized in the exposed glass regions (squares, triangles, or circles; 0.063–0.5 mm2). They proliferated to confluence in 5 days, expressed alkaline phosphatase and produced a mineralized matrix.

Keywords: Photolithography; Chitin/chitosan; Micropatterning; Osteoblast; Cardiomyocyte; Fibroblast

1. Introduction

Control of the cellular microenvironment is of great importance in the development of biosensors, tissue engineering constructs, high throughput drug screening systems and for fundamental studies of cell biology. Substrate geometry is particularly important in studies involving highly complex multicellular tissues such as myocardium or bone.

Since native myocardium consists of elongated cardiomyocytes arranged into aligned myofibers, significant efforts have been focused on reproducing the elongated phenotype on two-dimensional (2D) substrates. For tissue engineering studies, spatially organized cardiomyocyte cultures have been created on biodegradable, elastomeric polyurethane films patterned by microcontact printing of laminin lanes [1]. For physiological studies, elongated phenotype was achieved by patterning photoresist lanes on glass substrate and growing the myocytes on the exposed glass [2,3]. Myocyte cultivation on patterned substrates generated important insight into the role of fibroblasts in electrical signal propagation [4] as well as the effect of electrical field signals on changes in transmembrane potential [5].
For bone tissue, very little is known about the mechanisms that initiate bone formation in vitro, although specific heterotypic cell–cell interactions are believed to play an important role [6–9]. For example, experiments comparing bone formation in vitro between monolayer and micromass cultures have shown that cells both proliferate more slowly and differentiate more rapidly in micromass cultures which led to increased levels of collagen assembly and mineralization [8]. This difference is believed to be due to an increased level of cell–cell contacts in the micromass cultures. In addition, bone nodule formation depended on the plating cell density. In limiting dilution analysis, the number of bone nodules produced was shown to follow a linear relationship only at high cell densities [6]. This was attributed to more than one cell type being limiting for stromal osteoprogenitor differentiation, and to a dependence on heterotypic cell–cell interactions. In an attempt to better understand these phenomena, experiments to date have employed “randomly distributed” cultures [6]. In this regard, patterned culture substrates, of which topographical features and chemical properties are spatial control of chemical and topographical properties of patterned cell culture substrates would provide a good opportunity to investigate the cellular structure–function relationship [10,11].

Here we describe the development of a simple, rapid system for creating patterned culture substrates. This technique is based on (1) creating a mask using a standard overhead transparency with a high resolution printer, (2) creating a thin layer of a cell-repellant photocrosslinkable chitosan on a glass slide, (3) exposing the polymer coated slide and mask to ultraviolet (UV) light, and (4) rinsing the uncrosslinked polymer to expose the underlying cell-repellent patterns. Recently, photocrosslinkable poly ethylene glycol (PEG) hydrogels, which are inherently cell repellent yet capable of modification with cell adhesive regions have been used to pattern cells on substrates [12–17]. Specifically, PEG has been used to pattern cells on a substrate [12–15] or by directly capturing cells within the hydrogel [16,17]. In this study we decided to focus on a natural photocrosslinkable material chitosan [18,19] due to the rapid crosslinking kinetics, biocompatibility and biodegradability which can be employed in creation of patterned co-cultures.

2. Experimental

2.1. Synthesis and characterization of the photocrosslinkable chitosan

Photocrosslinkable chitosan (Az-chitosan) was synthesized as described elsewhere [19]. Briefly, 200 mg (1.24 mmol) of chitosan glutamate (Protasan UP G113; Mw: <200 kDa; degree of deacetylation: 75–90%; Novamatrix, Norway) was dissolved in 15 ml distilled water. N,N,N’,N’-tetramethylethylenediamine (TEMED) 116.2 mg, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) 70 mg (0.451 mmol), and 4-azidobenzonic acid (ABA, TCI America, Portland, OR, USA) 40 mg (0.245 mmol) were added to the chitosan solution. The reaction was conducted at pH 5 overnight, and the modified chitosan was purified by ultrafiltration. The modification degree was confirmed using $^1$H NMR (Varian Mercury 300 MHz).

2.2. Preparation of the chitosan gel

The photocrosslinkable chitosan macromonomer was dissolved in 0.9% NaCl solution (Baxter Healthcare, Deerfield, IL) to a concentration of 20 mg/ml. The gel was protected from light and placed on a shaker plate for 12 h. The final solution was stored at 4°C for no longer than 1 week prior to use.

2.3. Micropatterning of the chitosan gel

 Masks with desired patterns, created using Adobe Illustrator, were printed onto an acetate transparency using a 5080 dpi herkules imagesetter printer (Pageworks, Cambridge, MA). The following patterns were used: alternating black and transparent stripes for patterning of cardiomyocytes and cardiacfibroblasts as well as patterns consisting of triangles, squares and circles for patterning of osteoblasts. The stripes consisted of 500 µm wide black lanes separated by 500 µm transparent lanes (referred to as 500 × 500 µm mask) or 100 µm wide black lanes separated by 100 µm transparent lanes (referred to as 100 × 100 µm mask). Patterns of circles, squares and triangles (normalized for area) were created having areas of 0.5, 0.25, 0.125 and 0.063 mm$^2$. For patterning, 100 µl of the chitosan gel solution was evenly spread onto a 60 mm Petri Dishes (non-treated, Costar) or 40 × 25 mm coverslips composed of glass (WVR International, West Chester, PA) or Thermoxon$^®$ brand vinyl (Nalge Nunc International, Rochester, NY). To ensure that the gel was spread evenly, each materials was placed on a spincoater for approximately 10 s at 2000 rpm.

To fabricate patterned surfaces (Fig. 1), a mask was suspended 1 mm above an inverted chitosan coated coverslip and an UV lamp (Black-Ray, UVP Inc., radiation range 315–400 nm, peak at 365 nm) was positioned above the mask and exposed for a desired time period. A stain consisting of 1% Eosin Y (Aldrich Chemical Co., Milwaukee, WI) in dd$_2$H$_2$O was used to visualize the chitosan gel. The dark regions on the mask served as a barrier between the incoming UV light and the photoreactive gel. Thus, the area surrounding the patterned regions crosslinked, while the chitosan macromonomer under the black patterns remained in solution. Repeated washes with PBS cleared away the fluid residue between the lanes or within the microwells, resulting in a patterned gel.

2.4. Cell culture

Cardiomyocytes were obtained from 1–2-day-old neonatal Sprague Dawley (Charles River) rats according to procedures approved by the Institute’s Committee on Animal Care, as previously described [20]. In brief, ventricles were quartered, incubated overnight at 4°C (in a 0.06% (w/v) solution of trypsin in Hank’s Balanced Salt Solution (HBSS, Gibco), subjected to a series of digestions (3 min, 37°C, 150 rpm) in 0.1% (w/v) solution of collagenase type II in HBSS. To separate myocytes and fibroblasts the cell suspension was centrifuged (750 rpm, 5 min), and the pellet was resuspended in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 4.5 g/l glucose supplemented with 10% FBS, 10 mM HEPES, 2 mM l-glutamine and 100 unit/ml penicillin. The cells from the pellet were pre-plated in T75 flasks for one 75 min period to enrich for cardiomyocytes. Cells that remained unattached were labeled “cardiomyocytes.” The attached cells were propagated for 3–7 days in culture, trypsinized as described previously [21] and labeled “fibroblasts.”

Human osteogenic sarcoma cells (SAOS-2 cells), an osteoblast cell line (Advanced Type Culture Collection, ATCC), were grown in alphaminimal essential media (Gibco Invitrogen Co, Grand Island, NY) supplemented with Gentamicin (Sigma–Aldrich, St. Louis, MO), Fungizone and Penicillin (Sigma–Aldrich). Cells were subcultured at 50–70% confluency using trypsin and media was changed every 2–3 days.
2.5. Effect of gelation time on patterning

To determine the effect of gelation time on pattern dimensions, chitosan solution was applied to the tissue culture polystyrene (TCP) or glass cover slips as described above. The UV light was applied through the 500 μm mask for 90, 150, 180 and 240 s; or through 100 μm mask for 50, 90, 105, 120 and 135 s. Uncross-linked chitosan was removed by rinsing in phosphate buffered saline (PBS) followed by rinsing in 75% ethanol. Subsequently, the patterns were air dried and visualized at 40× and 100× by bright field and phase contrast microscopy (Nikon). The lane width was measured at five different locations for each sample using the built in eye piece.

2.6. Plating cells on the micropatterns

2.6.1. Cardiac fibroblasts
Lanes of chitosan were patterned onto the surface of 60 mm Petri dish by UV light illumination through 500 μm mask for 180 s, followed by rinsing in PBS for 24 h. Subsequently, 1 million of primary cardiac fibroblasts were inoculated into the dish using 5 ml of culture medium. The medium was replaced by 100% after 1 day in culture and the cells were maintained for total of 8 days.

2.6.2. Cardiomyocytes
Lanes of chitosan were patterned onto the surface of glass slides by UV light illumination for 50 s, through a mask consisting of alternating black and transparent lanes 100 μm wide (100 μm mask), followed by rinsing in PBS for 24 h. Subsequently, 3 million of neonatal rat cardiomyoctytes were inoculated onto the patterns using 3 ml of culture medium. The medium was replaced by 100% after 1 day in culture and the cells were maintained for total of 8 days.

2.6.3. Osteoblasts
Glass and Thermanox coverslips patterned with a series of circles, squares and triangles were placed in a separate 60 mm Petri dish and disinfected with repeated washes of 70% ethanol and sterile PBS. SAOS-2 cells were inoculated into each dish at a density of 20,000 cells/cm². Once the cells were confluent (after 5 days in culture), media was supplemented with 50 μg/ml ascorbic acid and 5 mM beta-glycerophosphate to initiate the formation of mineralized matrix.

2.7. Pattern stability

To test stability of chitosan surfaces a pattern consisting of 2 mm chitosan lanes separated by 5.6 mm was prepared on the surface of 100 mm
Petri dishes. Cardiac fibroblasts (2.5 million) were added to the dishes and cultured for 18 days.

2.8. Atomic force microscope (AFM) characterization of the patterns

Topographs of glass surfaces patterned with chitosan lanes were obtained using a Nanoscope® IIIa AFM (Digital Instruments, Santa Barbara, CA). Silicon and silicon nitride cantilevers (Veeco Probes, Santa Barbara, CA) were used for dry samples (in a tapping mode) and wet samples (in a contact mode), respectively. The data was analyzed with Nanoscope® III software.

2.9. Histochemical analysis of cells

2.9.1. Cardiac fibroblasts and cardiomyocytes

The cells were fixed overnight using 10% neutral buffered formaline and stained for phenotypic markers: cardiac troponin-I for cardiomyocytes and vimentin for fibroblasts. The slides were blocked with 10% horse serum (Vector Laboratories) and incubated with the solution containing polyclonal rabbit troponin I (Chemicon 1:200) or mouse anti-vimentin Cy3 conjugated (clone V9, Sigma, 1:100). Subsequently, the slides were rinsed in PBS and incubated for 30 min at 37°C with fluorescein conjugated goat anti-rabbit IgG (1:200, Vector Laboratories) for TnI visualization as described[22]. All antibodies were diluted in PBS containing 0.5% Tween 20 and 1.5% horse serum. The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with an inverted microscope (Axioplan, Zeiss).

2.9.2. Osteoblasts

Cell culture plates were fixed in 10% formalin buffered saline for 20–30 min, washed once with ddH₂O and then left in ddH₂O for 15 min. Plates were then stained for alkaline phosphatase (ALP) by incubating for 40 min in a solution containing 5-chloro-4-benazamido-2-methylbenzenediazonium chloride hemi(zinc chloride) salt (fast red violet LB salt) as previously reported[23]. Plates were then rinsed 3–4 times in ddH₂O, and stained with 2.5% silver nitrate for 30 min. After rinsing 3–4 times in ddH₂O, plates were incubated in sodium carbonate formaldehyde for 1–2 min, rinsed, air dried and examined by light microscopy.

2.10. Cardiomyocyte contractile response

Upon cultivation the patterned slides were placed in between two parallel electrodes (carbon rod) spaced 1 cm apart and connected to the cardiac stimulator (Nikon-Kohden). Cardiomyocytes were paced using square pulses 2 ms in duration. The stimulating voltage was varied to determine excitation threshold (minimum voltage necessary to induce synchronous contractions) and maximum capture rate (maximum beating frequency) as described[22].

2.11. Statistical analysis

Statistical significance in pairwise comparisons was determined by one way ANOVA in conjunction with Tukey or Dunn’s test, using Sigma Stat 3.1. Normality and equality of variance was tested. p<0.05 was considered significant. Data are represented as avg±SD.

3. Results and discussion

The main objective of this study was to develop a simple and rapid method of surface patterning using a naturally occurring polymer chitosan. Chitosan is produced through deacetylation of chitin, a glycoaminoglycan (GAG) that is one of the most abundant polysaccharides found in nature and is present in the exoskeleton of shellfish like shrimp, lobster and crabs[24]. The amino groups in chitosan allow for modification of the polymer with useful functional groups, such as photoactive ABA[18,19]. The modified chitosan forms a flexible yet durable cross-linked hydrogel which can be used for surgical adhesives[18,19].

In particular, we chose to investigate photocrosslinkable chitosan as a model material for obtaining patterned cell cultures for the following reasons: (i) the kinetics of cross-linking is rapid enough so that hydrogel forms within minutes of UV exposure; (ii) the cross-linked chitosan is biodegradable[25], essentially non-toxic[18,19] and does not support cell attachment[18,19], which is ideal for the patterning application; (iii) the photoactive functional groups eliminate the use of additional photoinitiators, of which toxicity often becomes a concern, and the crosslinking reaction does not leave toxic by-products (the only by-product is N₂); (iv) upon crosslinking a flexible hydrogel forms thus allowing easy handling of patterned surfaces.

The shape and spacing of the micropatterns could be easily controlled by exposing the gel precursor to the light source through patterned masks (Fig. 1A). Using the described methods we were able to create micro-patterns on two surfaces most commonly used for tissue culture: glass and TCP (Fig. 1B). In this method, a solution of the gel precursor was spread on a solid platform, and the UV light was applied over a patterned mask to cross-link the precursor on the exposed side. In forming a thin layer of the gel precursor solution, it was important to use relatively hydrophilic surface (i.e., a substrate with low contact angle) such as glass or TCP. Once cross linked, the chitosan pattern remained stable (without peeling off) on the platform for up to 14 days. The stability most likely comes from electrostatic interactions between positively charged amine groups in chitosan and negatively charged surfaces (SO₄⁻ on the glass or COO⁻ on the TCP). The shape and spacing of the micropattern could be easily modified using various laser-printed patterns and UV exposure time.

In addition, by varying the exposure time to UV light we were able to get patterns of various sizes using a single mask (Fig. 2) and taking advantage of light scattering through the substrate. Using a 500×500 μm mask we were able to increase the width of chitosan lanes from 440±11 to 752±7 μm by increasing the UV exposure time from 90 to 240 s (Figs. 2A and B, n=4–6, p<0.001, Dunn’s test). In the same system, the width of empty lanes varied inversely with the increase of the UV exposure (90–240 s) time from 560±11 to 260±7 μm (n=4–6, p<0.001, Tukey test). Similarly, using a 100×100 μm mask we increased the width of chitosan lanes from 99±4 to 134±2 μm by increasing UV exposure time from 50 to 135 s (Fig. 2C, n=4–5, p<0.001, Dunn’s test). The empty lanes exhibited a significant decrease in width from 102±3 to 68±3 μm with the increase in the exposure time from 50 to 135 s (n=4–6, p<0.001, Dunn’s test). Thus the
Fig. 2. Pattern dimensions can be controlled by varying the exposure time to UV light using a single mask. (A) Photomicrographs of chitosan patterns of TCP created by varying the exposure time through 500 μm by 500 μm mask, 40 × . (B) Graph representing the width of chitosan lanes and empty lanes on TCP as a function of exposure time through 500 μm by 500 μm mask. Avg ± SD. p < 0.05 considered significant. For empty lanes all pair-wise comparisons were significantly different. For chitosan lanes: *—significantly smaller than the lane width at 240 s. (C) Graph representing the width of chitosan lanes and empty lanes on glass as a function of exposure time through 100 μm by 100 μm mask. Avg ± SD. p < 0.05 considered significant. #—Significantly different than the width of the lane at 50 s.
Fig. 3. Patterning of cardiac fibroblasts: (A) Cardiac fibroblasts were seeded on chitosan patterned TCP and maintained for 8 days of culture. The cells attached to the surface of empty lanes, elongated and proliferated to confluence. (B) Immunofluorescent staining for intermediate filament vimentin (red). Nuclei are counterstained with DAPI (blue). Chitosan lanes appear red. (C) Pattern stability; chitosan lanes of 2 mm width were created on TCP, seeded with fibroblasts and maintained for 18 days in culture. The chitosan lanes retained the ability to resist cell adhesion during this period and the interface remained stable.

Fig. 4. Patterning of cardiomyocytes: (A) Neonatal rat cardiomyocytes were seeded on chitosan patterned glass surfaces at 8 days of culture. The cells adhered to glass and formed confluent cell lanes that exhibited spontaneous contractions. (B) Patterned cardiomyocytes express cardiac troponin I (green) and exhibit a developed contractile apparatus (arrows).
resolution of this technique is on the order of μm. It appears that the prolonged UV exposure allowed for further propagation of the photocrosslinking reaction by increasing the number of reactive nitrene intermediates, which otherwise would have had extremely short life span. In the standard soft lithography, the same number of patterns would require patterning of multiple silicon wafers in a clean room.

Cardiac fibroblasts seeded onto chitosan patterned surfaces (Fig. 3) attached to the spaces in between the chitosan lanes (i.e. empty lanes), and assumed an elongated morphology (Fig. 3). They proliferated and filled the empty lanes to 100% confluence, without attachment to the chitosan lanes (Fig. 3A). Immunohistochemical staining indicated typical fibroblastic morphology and the presence of the intermediate filament vimentin (Fig. 3B). Since fibroblasts are known for their high proliferative capacity, we tested the ability of cross-linked chitosan to repel fibroblast adhesion during extended periods in culture (Fig. 3C). Fibroblasts were maintained in chitosan patterned Petri dishes for 18 days. In the first 4 days of culture, fibroblast proliferated and filled the empty lanes in between the chitosan lanes. For the remainder of the culture (days 4–18) the cells did not spread to the chitosan lanes, and the interface between cell-filled and cell-free region remained well defined, thus illustrating a high degree of stability of the created patterns.

Cardiomyocytes were patterned onto chitosan lanes to create strands between 99 and 68 μm in width (Fig. 4, data for 99 μm only shown). Over 8 days of culture, no signs of pattern destabilization were observed. The cells assumed an elongated morphology typical of differentiated cardiomyocytes, and were capable of spontaneous contractions. Using electrical field stimulation we were able to pace the

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Fig. 5. Patterning of osteoblasts: (A–C) SAOS-2 cells localized within the patterned regions and became confluent after 5 days. (D–F) Cells within the patterned regions stained for alkaline phosphatase (red/pink color). (G) Macroscopic view of alkaline phosphatase stained patterned osteogenic cells immobilized within various substrate shapes. (H–J) After 10–14 days in culture, the osteogenic cells within the patterned regions produced a mineralized matrix as identified by von Kossa staining (black).
cells using 2 ms long pulses at 4.3 V/cm and frequency from 60 to 220 bpm. Immunohistochemical staining indicated the presence of cardiac differentiation marker: cardiac troponin I (Fig. 4B). Higher magnification images indicated the presence of developed contractile apparatus (Fig. 4B).

Fig. 6. AFM characterization of chitosan patterns. (A) 500 μm lanes prepared on TCP had well-defined shape and average height of 490 nm (dry). (B) 100 × 100 μm lanes prepared on glass have a well-defined shape and average height of 380 nm. (C) 100 × 100 μm lanes on glass after cell culture. The cells filled-in the space between chitosan lanes, creating a surface of approximately uniform height. AFM scan rates were 0.5 Hz (A), 0.75 Hz (B), and 0.25 Hz (C). (D) AFM characterization of cells/chitosan interaction on the patterned glass surface. 100 × 100 μm lanes on glass after cardiomyocyte culture for 8 days. The patterns are stabilized topographically, with approximately equal height of the chitosan lanes and the thickest cell regions.
We investigated the potential for osteogenic cells to express hallmarks of bone formation in this system. SAOS-2 osteoblasts were seeded onto patterned glass coverslips with a series of squares, circles and triangles. After 5 days in culture, cells became confluent within all patterns (squares, triangles and circles, Figs. 5A–C) and expressed the osteoblast functional phenotype [26] as demonstrated by ALP staining (Figs. 5D–F). After 10–14 days in culture, a mineralized matrix formed that stained positive for von Kossa which stains the phosphate component of the inorganic component of bone (Figs. 5H–J). In comparison to many standard 2D micropatterning techniques, where patterns are generally only stable for a few days [27], we show that patterns of osteogenic cells within the chitosan microstructures remain stable for up to 14 days (Figs. 5H–J). Similar results were obtained for ThermaNox™ substrates (data not shown).

To examine pattern morphology we utilized AFM (Fig. 6). There were no significant differences in pattern size between the dry and wet (immersed in PBS) patterns, indicating that hydrogel swelling was not significant. Lanes prepared using 500 × 500 μm mask had well defined shape and the average height of 490 nm, while the lanes prepared using a finer mask (100 × 100 μm) had a slightly lower height of 380 nm. In addition to UV exposure time and mask features, the pattern height was a function of the volume of chitosan precursor solution and the rotational velocity during spin coating. The two parameters determined the thickness of the initial liquid film and were kept constant in all experiments. The patterns imaged after cell culture, indicated that the cells tightly filled in the space between chitosan lanes, creating a surface of approximately uniform height. Thus the stability of these patterns can be attributed to topographic stabilization, where the cells are confined within ~400 nm deep wells (Fig. 6).

The described method can be used to rapidly (50–240 s) create stable patterns of various shapes: lanes, squares, circles, triangles for cell patterning on various surfaces. Utilization of biodegradable chitosan in comparison to non-degradable photocrosslinkable polymers such as PEG has potential advantages in creating patterned co-cultures. Chitosan patterns can be removed by addition of chitosan degrading enzyme lysozyme. It has been demonstrated previously that a lysozyme concentration of 10 mg/ml applied for 24 h does not affect cell viability while it results in degradation of photocrosslinked chitosan hydrogel [28]. In the envisioned co-culture scenario, the first cell type can be applied to the surface patterned using a mask printed on transparency and photocrosslinkable chitosan. When the confluence is reached, chitosan can be removed using lysozyme and another cell type can be added, creating distinct cell domains. Lysozyme can also be used to release the cells confined to the regions of defined size. In another envisioned scenario, this approach can be used for coculture in a layer-by-layer approach by switching the surface from cell non-adhesive to adhesive [29,30]. For example, after plating one cell type into regions that are devoid of the chitosan (the initial cell adhesive regions), the properties of the cell-repellant chitosan can be rendered cell adhesive to allow patterning of a second cell type. Given that chitosan is positively charged, a negatively charged polymer such as hyaluronic acid can be ionically bound to the chitosan followed by positively charged cell adhesive poly l-lysine [30].

4. Conclusions

We developed a simple and rapid method to create patterned surfaces using photocrosslinkable chitosan. Patterns of various shapes (lanes, squares, circles, triangles) were created on two surfaces most commonly used for cell culture: glass and TCP. The pattern size was varied with ~μm resolution using a single mask and varying UV exposure time. Cardiac fibroblasts, cardiomyocytes and osteoblasts formed arrays on chitosan patterned surfaces and remained stable for up to 18 days. This method can be utilized to create patterned cell co-cultures by utilization of lysozyme or layer-by-layer surface switching.

Acknowledgments

This work was supported by funding from Ontario Research Development Challenge Fund (MR), Connaught Start-up Award (MR) and NSERC Postdoctoral Fellowship (JK) and by P41 EB002520-01A1 (GV-N).

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