A photolithographic method to create cellular micropatterns

Jeffrey M. Karp[a,1], Yoon Yeo[a,1], Wenlinag Geng[a], Christopher Cannizarro[b], Kenny Yan[a], Daniel S. Kohane[b,g], Gordana Vunjak-Novakovic[f], Robert S. Langer[a,b,c], Milica Radisic[d,e,*]

[a]*Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*
[b]*Harvard-MIT Division of Health Science and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*
[c]*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*
[d]*Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ont., Canada M5S 3E5*
[e]*Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Rm 407, Toronto, Ont., Canada M5S 3G9*
[f]*Department of Biomedical Engineering, Columbia University*
[g]*Pediatric Intensive Care Unit, Ellison 317, Massachusetts General Hospital, 55 Fruit St., Boston, MA 02114, USA*

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. Photocross-linkable 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 μm 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 μm 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 mm^2). They proliferated to confluence in 5 days, expressed alkaline phosphatase and produced a mineralized matrix.
© 2006 Elsevier Ltd. All rights reserved.

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].

*Corresponding author. Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Rm 407, Toronto, Ont., Canada M5S 3G9. Tel.: +1 416 946 5295; fax: +1 416 978 8605.

E-mail address: milica@chem-eng.utoronto.ca (M. Radisic).

URL: http://www.chem-eng.utoronto.ca/~milica.

[1]Constitutes equal contribution.

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 repellant 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-azidobenzoic 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 cardifibroblasts 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 mm². For patterning, 100 μl of the chitosan gel solution was evenly spread onto center of 60 mm Petri Dishes (non-treated, Costar) or 40 × 25 mm coverslips composed of glass (VWR International, West Chester, PA) or Thermonox® 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 ddH₂O 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), and 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 alpha-minimal 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.
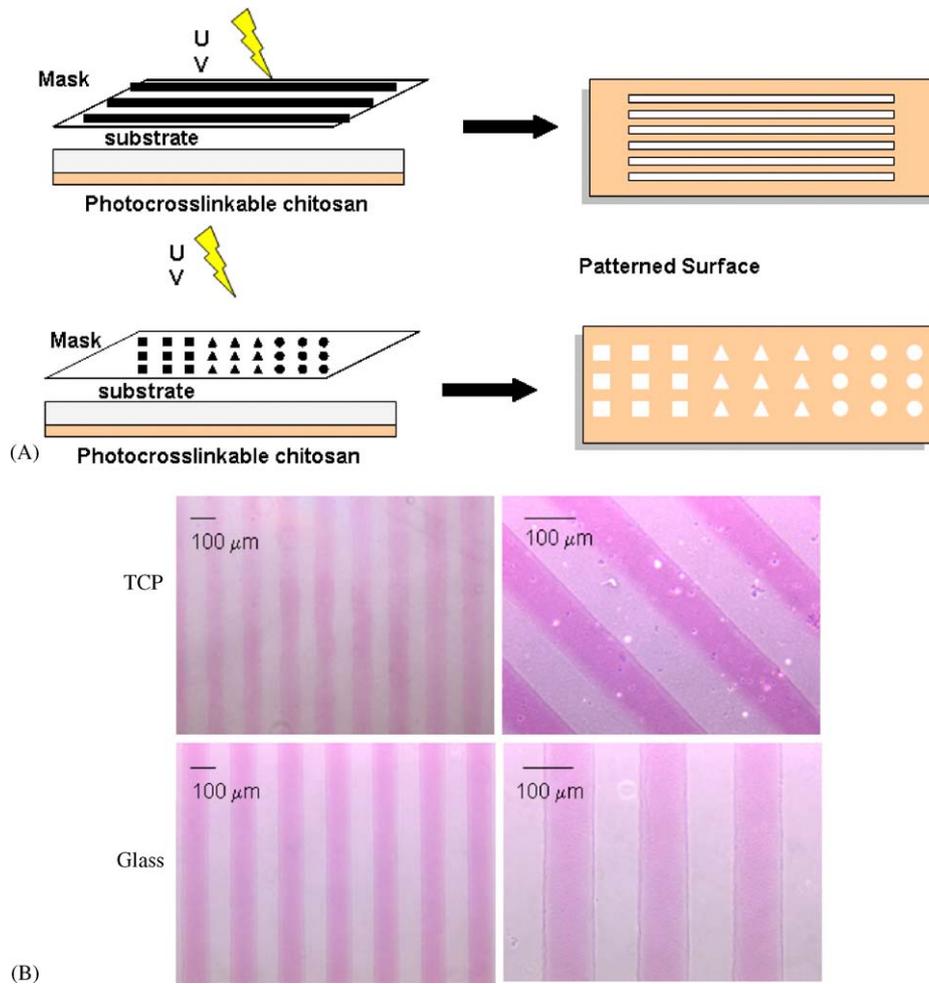
Fig. 1. Surface patterning using photocrosslinakble chitosan: (A) Fabrication method; to fabricate patterned substrates, a mask containing a variety of black patterns was placed on top of an inverted chitosan coated coverslip. UV light was focused onto the mask for 60–120 s followed by repeated washes in PBS to remove the non-polymerized gel and expose the underlying glass substrate within the patterned regions. (B) Cross-linked chitosan patterns were created on two surfaces most commonly used in tissue culture: tissue culture polystyrene (TCP) and glass. For visualization chitosan lanes were stained with eosin.

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 $500 \times 500 \, \mu m$ mask for 90, 150, 180 and 240 s; or through $100 \times 100 \, \mu m$ 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 $40 \times$ and $100 \times$ 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 the 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 $500 \times 500 \, \mu m$ mask for 180 s, followed by rinsing in PBS for 24 h in 60 mm Petri dishes. 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 $100 \, \mu m$ wide ($100 \times 100 \, \mu m$ mask), followed by rinsing in PBS for 24 h. Subsequently, 3 million of neonatal rat cardio-myoctytes 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 $20,000 \, cells/cm^2$. Once the cells were confluent (after 5 days in culture), media was supplemented with $50 \, \mu g/ml$ 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 cardiomyoyctes and vimentin for cardiofibroblasts. 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 coverslipped (Vectorshield mounting medium with DAPI) and imaged using 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 ddH_2O and then left in ddH_2O for 15 min. Plates were then stained for alkaline phosphatase (ALP) by incubating for 40 min in a solution containing 5-chloro-4-benzamido-2-methylbenzene-diazonium chloride hemi(zinc chloride) salt (fast red violet LB salt) as previously reported [23]. Plates were then rinsed 3–4× in ddH_2O, and stained with 2.5% silver nitrate for 30 min. After rinsing 3–4× in ddH_2O, 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$\pm$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 photoreactive 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 photoreactive 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 N_2); (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 (SiO^- 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 $500 \times 500 \, \mu m$ mask we were able to increase the width of chitosan lanes from 440 ± 11 to $752 \pm 7 \, \mu m$ 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 $260 \pm 7 \, \mu m$ ($n = 4$–6, $p < 0.001$, Tukey test). Similarly, using a $100 \times 100 \, \mu m$ mask we increased the width of chitosan lanes from 99 ± 4 to $134 \pm 2 \, \mu m$ 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 $68 \pm 3 \, \mu m$ with the increase in the exposure time from 50 to 135 s ($n = 4$–6, $p < 0.001$, Dunn's test). Thus the
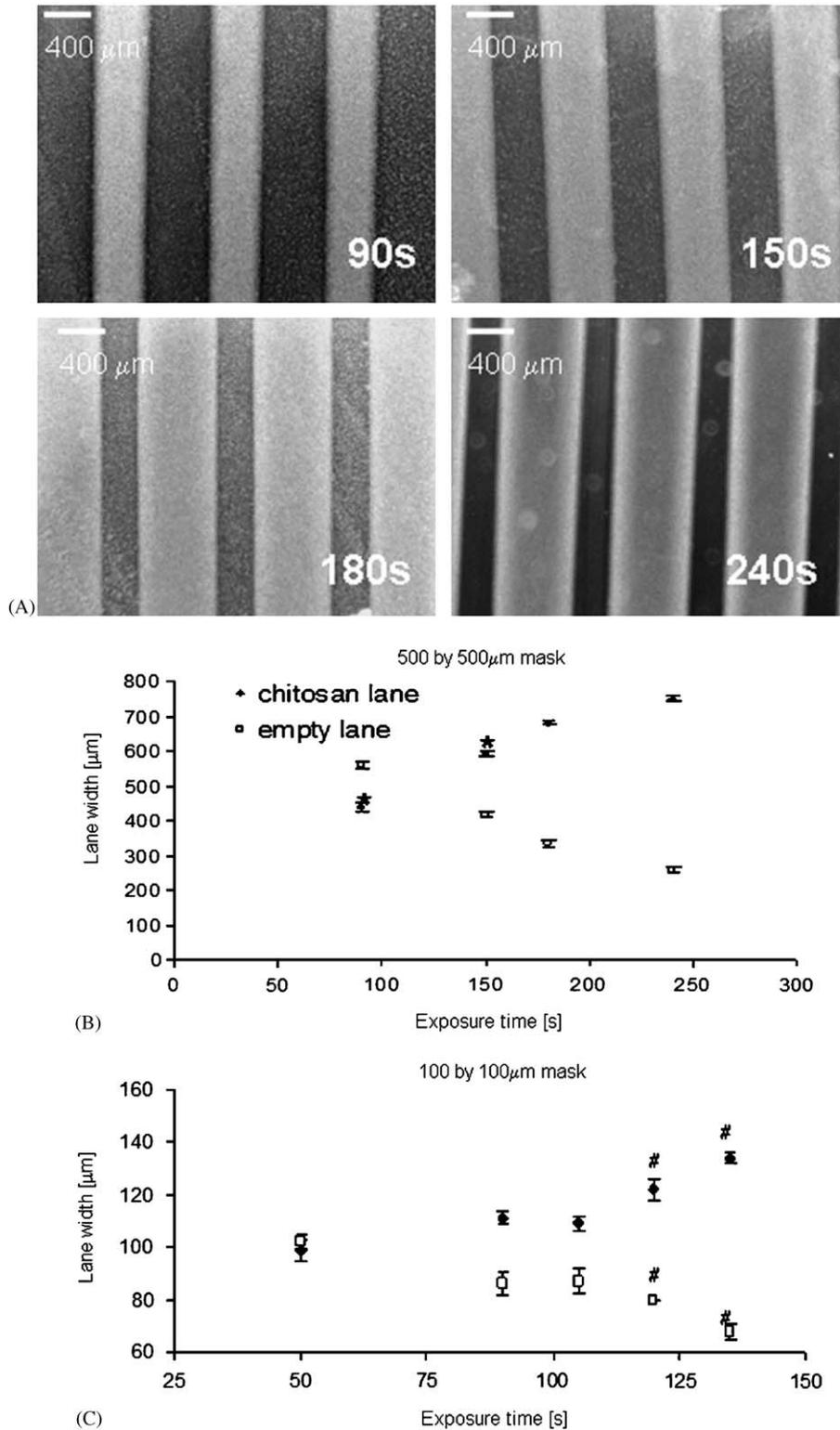
Fig. 2. Pattern dimensions can be controlled by varying the exposure time to UV light using a single mask. (A) Photomicrographs of chitosan patterns of TCP created by varying the exposure time through 500 μm by 500 μm mask, 40 × . (B) Graph representing the width of chitosan lanes and empty lanes on 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.
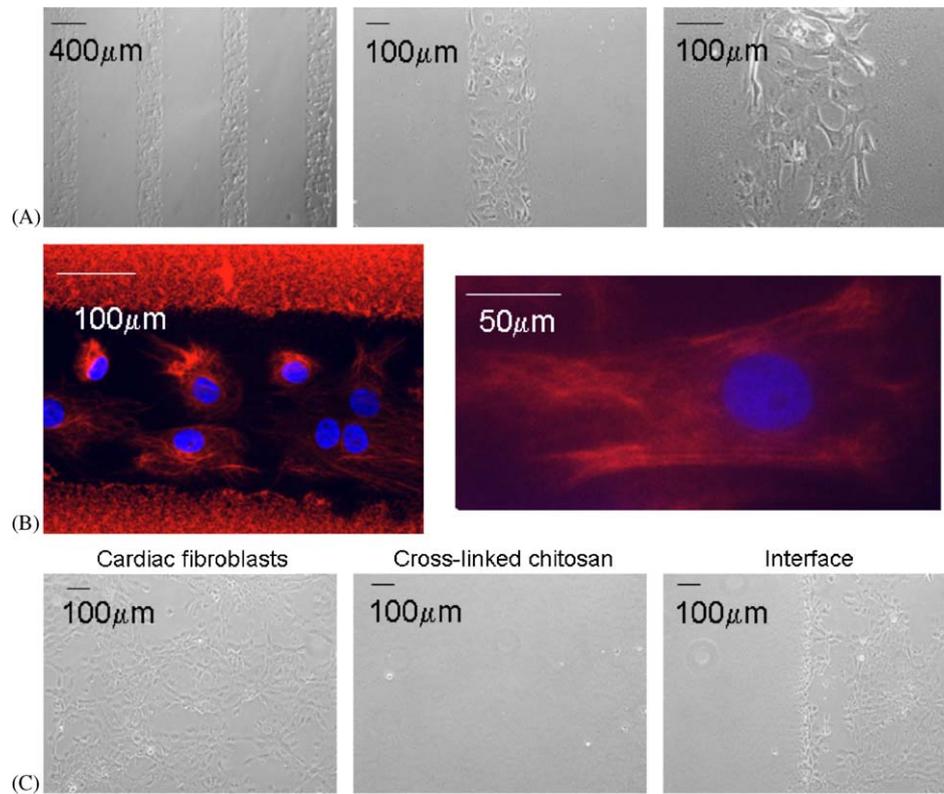
Fig. 3. Patterning of cardiac fibroblasts: (A) Cardiac fibroblasts were seeded on chitosan patterned TCP and maintained for 8 days of culture. The cells attached to the surface of empty lanes, elongated and proliferated to confluence. (B) Immunofluorescent staining for intermediate filament vimentin (red). Nuceli are couterstained with DAPI (blue). Chtiosan 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.
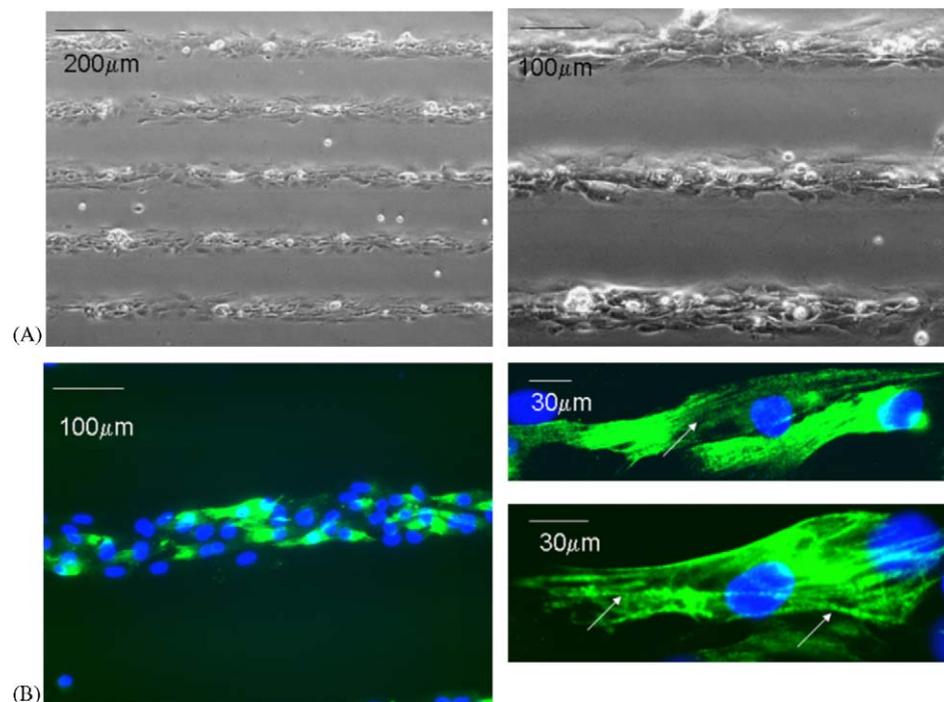
Fig. 4. Patterning of cardiomyocytes: (A) Neonatal rat cardiomyocytes were seeded on chitosan patterned glass surfaces at 8 days of culture. The cells adhered to glass and formed confluent cell lanes that exhibited spontaneous contractions. (B) Patterned cardiomyocytes express cardiac troponin I (green) 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.

Cardiomycoytes 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
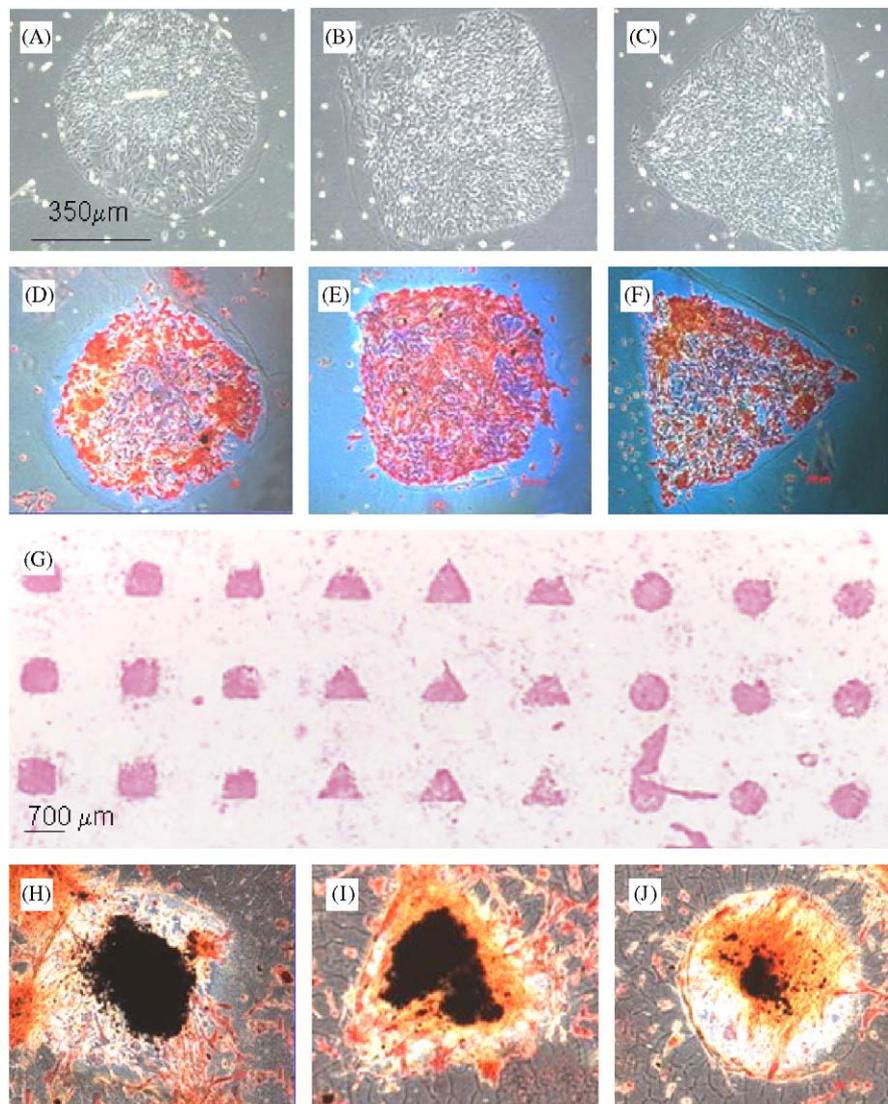
Fig. 5. Patterning of osteoblasts: (A–C) SAOS-2 cells localized within the patterned regions and became confluent after 5 days. (D–F) Cells within the patterned regions stained for alkaline phosphatase (red/pink color). (G) Macroscopic view of alkaline phosphatase stained patterned osteogenic cells immobilized 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 rom 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).
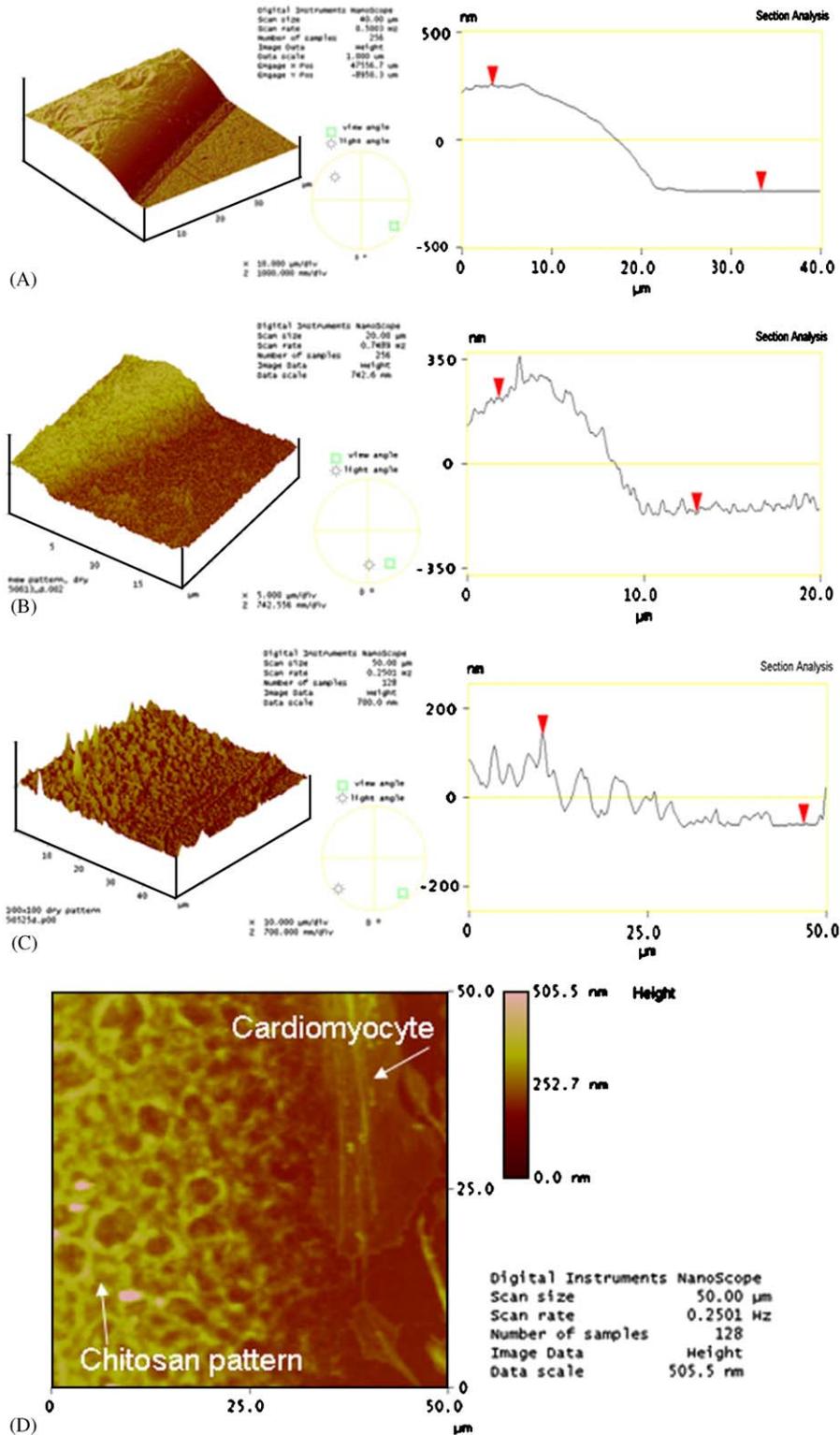
Fig. 6. AFM characterization of chitosan patterns. (A) 500 × 500 μm lanes prepared on TCP had well defined shape and average height of 490 nm (dry). (B) 100 × 100 μm lanes prepared on glass have a well-defined shape and average height of 380 nm. (C) 100 × 100 μm lanes on glass after cell culture. The 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[TM] 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 $500 \times 500\,\mu m$ mask had well defined shape and the average height of 490 nm, while the lanes prepared using a finer mask ($100 \times 100\,\mu m$) 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 $\sim$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 co-culture 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 $\sim$μm resolution using a single mask and varying UV exposure time. Cardiac fibroblasts, cardiomycoytes 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.

References

[1] McDevitt TC, Angello JC, Whitney ML, Reinecke H, Hauschka SD, Murry CE, et al. In vitro generation of differentiated cardiac myofibers on micropatterned laminin surfaces. J Biomed Mater Res 2002;60:472–9.

[2] Rohr S, Scholly DM, Kleber AG. Patterned growth of neonatal rat heart cells in culture. Morphological and electrophysiological characterization. Circ Res 1991;68:114–30.

[3] Thomas SP, Bircher-Lehmann L, Thomas SA, Zhuang J, Saffitz JE, Kleber AG. Synthetic strands of neonatal mouse cardiac myocytes: structural and electrophysiological properties. Circ Res 2000;87:467–73.

[4] Gaudesius G, Miragoli M, Thomas SP, Rohr S. Coupling of cardiac electrical activity over extended distances by fibroblasts of cardiac origin. Circ Res 2003;93:421–8.

[5] Fast VG, Rohr S, Ideker RE. Nonlinear changes of transmembrane potential caused by defibrillation shocks in strands of cultured myocytes. Am J Physiol Heart Circ Physiol 2000;278:H688–97.

[6] Aubin JE. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell–cell interactions in osteoblast differentiation. J Cell Biochem 1999;72:396–410.

[7] Gerber I, ap Gwynn I. Influence of cell isolation, cell culture density, and cell nutrition on differentiation of rat calvarial osteoblast-like cells in vitro. Eur Cell Mater 2001;2:10–20.

[8] Gerber I, ap Gwynn I. Differentiation of rat osteoblast-like cells in monolayer and micromass cultures. Eur Cell Mater 2002;3:19–30.

[9] Purpura KA, Aubin JE, Zandstra PW. Sustained in vitro expansion of bone progenitors is cell density dependent. Stem Cell 2004;22:39–50.

[10] Bhatia SN, Balis UJ, Yarmush ML, Toner M. Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions. Biotechnol Progr 1998;14:378–87.

[11] Bhatia SN, Balis UJ, Yarmush ML, Toner M. Probing heterotypic cell interactions: hepatocyte function in microfabricated co-cultures. J Biomater Sci Polym Ed 1998;9:1137–60.

[12] Revzin A, Russell RJ, Yadavalli VK, Koh WG, Deister C, Hile DD, et al. Fabrication of poly(ethylene glycol) hydrogel microstructures using photolithography. Langmuir 2001;17:5440–7.

[13] Koh WG, Revzin A, Simonian A, Reeves T, Pishko M. Control of mammalian cell and bacteria adhesion on substrates micropatterned with poly(ethylene glycol) hydrogels. Biomed Microdev 2003;5:11–9.

[14] Suh KY, Seong J, Khademhosseini A, Laibinis PE, Langer R. A simple soft lithographic route to fabrication of poly(ethylene glycol) microstructures for protein and cell patterning. Biomaterials 2004;15:557–63.

[15] Khademhosseini A, Jon S, Suh KY, Tran TNT, Eng G, Yeh J, et al. Direct patterning of protein- and cell-resistant polymeric monolayers and microstructures. Adv Mater 2003;15:1995–2000.

[16] Koh WG, Revzin A, Pishko MV. Poly(ethylene glycol) hydrogel microstructures encapsulating living cells. Langmuir 2002;18:2459–62.

[17] Liu VA, Bhatia SN. Three-dimensional photopatterning of hydrogels containing living cells. Biomed Microdev 2002;4:257–66.

[18] Ono K, Saito Y, Yura H, Ishikawa K, Kurita A, Akaike T, et al. Photocrosslinkable chitosan as a biological adhesive. J Biomed Mater Res 2000;49:289–95.

[19] Levy AP, Levy NS, Loscalzo J, Calderone A, Takahashi N, Yeo KT, et al. Regulation of vascular endothelial growth factor in cardiac myocytes. Circ Res 1995;76:758–66.

[20] Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, et al. Cardiac tissue engineering: cell seeding, cultivation parameters and tissue construct characterization. Biotechnol Bioeng 1999;64:580–9.

[21] Radisic M, Euloth M, Yang L, Langer R, Freed LE, Vunjak-Novakovic G. High density seeding of myocyte cells for tissue engineering. Biotechnol Bioeng 2003;82:403–14.

[22] Radisic M, Yang L, Boublik J, Cohen RJ, Langer R, Freed LE, et al. Medium perfusion enables engineering of compact and contractile cardiac tissue. Am J Physiol Heart Circ Physiol 2004;286:H507–16.

[23] Purpura KA, Aubin JE, Zandstra PW. Two-color image analysis discriminates between mineralized and unmineralized bone nodules in vitro. Biotechniques 2003;34:1188–92.

[24] Freier T, Koh HS, Kazazian K, Shoichet MS. Controlling cell adhesion and degradation of chitosan films by *N*-acetylation. Biomaterials 2005;26:5872–8.

[25] Singla AK, Chawla M. Chitosan: some pharmaceutical and biological aspects—an update. J Pharm Pharmacol 2001;53:1047–67.

[26] Aubin JE, Liu F, Malaval L, Gupta AK. Osteoblast and chondroblast differentiation. Bone 1995;17:77S–83S.

[27] Pavesio A, Abatangelo G, Borrione A, Brocchetta D, Hollander AP, Kon E, et al. Hyaluronan-based scaffolds (Hyalograft® C) in the treatment of knee cartilage defects: preliminary clinical findings. In: Bock G, Goode J, editors. Tissue engineering of cartilage and bone. Novartis foundation symposium no. 249. Chichester, UK: Wiley; 2003. p. 203–17.

[28] Ishihara M, Obara K, Ishizuka T, Fujita M, Sato M, Masuoka K, et al. Controlled release of fibroblast growth factors and heparin from photocrosslinked chitosan hydrogels and subsequent effect on in vivo vascularization. J Biomed Mater Res A 2003;64:551–9.

[29] Fukuda S, Yasu T, Predescu DN, Schmid-Schonbein GW. Mechanisms for regulation of fluid shear stress response in circulating leukocytes. Circ Res 2000;86:E13–8.

[30] Khademhosseini A, Suh KY, Yang JM, Eng G, Yeh J, Levenberg S, et al. Layer-by-layer deposition of hyaluronic acid and poly-L-lysine for patterned cell co-cultures. Biomaterials 2004;25:3583–92.