Control of cardiomyocyte orientation on a microscaffold fabricated by photopolymerization with laser beam interference

Akiko Fujita
Osaka University
Department of Frontier Biosciences
2-1 Yamadaoka, Suita
Osaka 565-0871, Japan

Katsumasa Fujita
Osaka University
Department of Applied Physics
2-1 Yamadaoka, Suita
Osaka 565-0871, Japan
E-mail: fujita@ap.eng.osaka-u-ac.jp

Osamu Nakamura
Osaka University
Department of Frontier Biosciences
2-1 Yamadaoka, Suita
Osaka 565-0871, Japan

Takehisa Matsuda
Kyushu University
Graduate School of Engineering
Division of Biomedical Engineering
3-1-1 Maidashi, Higashi-ku
Fukuoka 812-8582, Japan

Satoshi Kawata
Osaka University
Department of Applied Physics
2-1 Yamadaoka, Suita
Osaka 565-0871, Japan
and
The Institute of Physical and Chemical Research
2-1 Hirosawa, Wako
Saitama 351-0198, Japan

Abstract. We fabricate scaffolds with a fine linear grating (periodicity of 1 to 8 μm and height of 1 μm) on a glass substrate for controlling cardiomyocyte orientation. The fabrication is done by the solidification of a liquid photopolymerizable material using two laser beam interference. As the photopolymerizable material, we use acrylated trimethylene-carbonate-based oligomers initiated with trimethylolpropane (T/TMP), followed by acrylation at terminal ends. Rat cardiomyocytes cultured on the fabricated scaffolds exhibit cell elongation, orientation, and contraction along the scaffold grating. Fluorescence observation of bundles of actin filaments of the cultured cells show that the cytoskeleton of the cells is also generated and oriented parallel to the grating. With a change in grating periodicity from 8 to 1 μm, the percentage of the cells that show orientation along the grating increase from approximately 40 to 70%. The cell orientation along the grating is observed 18 h after seeding the cells on the scaffold. This result implies that the attachment between a cell and a well-defined microarchitectural substrate at an early stage of culture is a significant determinant of cell morphology. *© 2006 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2186042]

Keywords: cardiomyocyte; photofabrication; photopolymerization; micropatterning; cell orientation.

Paper 05170SSRR received Jul. 1, 2005; revised manuscript received Jan. 5, 2006; accepted for publication Jan. 6, 2006; published online Mar. 22, 2006.

1 Introduction

A cell on a culture dish is remarkably different in morphology from that in living tissue in terms of both microscopic and macroscopic scales. In culture, cells adhere to a culture dish and then elongate and proliferate in a disorderly manner. In a living heart, cardiomyocytes are oriented in the same direction as one another in contractile myofibrils and are interconnected at the end through intercalated disks. In culture, myofibrils are disorganized and cardiomyocytes become flat, resulting in the spread of intercellular junctions in the membrane. This difference in morphology between the cells on a culture dish and those in living tissue is responsible for an important functional difference in excitation and contraction of cells.[1]

There have been many reports on the reproduction of morphology of the living cell in culture.[2–17] In those reports, the main issues are surface topography,[2–4] surface chemistry,[5–10] and change of cell adhesiveness by distribution of cell-adhesive proteins[11–14] on a substrate. Lieberman et al. aligned cardiomyocytes linearly by using rat tail collagen and agar.[13] Rohr, Scholly, and Kleber elongated cardiomyocytes longitudinally by a photoresist on a glass substrate patterned with photolithography and observed electrical characteristics similar to those of cardiomyocytes in a heart.[9] McDevitt et al. used micropatterned laminin surfaces to form rod-shaped cardiomyocytes with organized myofibrils and bipolar intercalated disks.[14] In these works, cardiomyocytes are similar in

Address all correspondence to Katsumasa Fujita, Osaka University, Department of Applied Physics, 2–1 Yamadaoka, Suita, Osaka 565–0871, Japan. Tel: +816–6879–7847; Fax: +816–6879–7330; E-mail: fujita@ap.eng.osaka-u-ac.jp

1083-3668/2006/11(2)/021015/5/$22.00 © 2006 SPIE

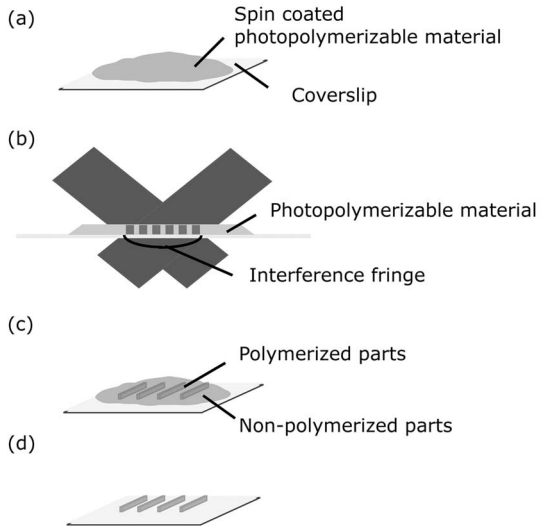

Fig. 1 The procedure of fabrication of a scaffold for cell growth by using laser interference. (a) Make a thin layer of a photopolymerizable material by spin coating, (b) polymerize the material by laser interference, (c) complete the structure, and (d) remove nonsolidified parts.

morphology (i.e., shape, alignment, orientation, and distribution of proteins in intercalated disks) to those in an actual heart.[9,10,13,14,17] Additionally, their contractile activity is found to be nearly the same as that of cells in a heart.[9,10,13]

In this work, we discuss a scaffold with fine linear grating for controlling cell shape, orientation, and cell growth. We fabricated the fine linear gratings, with different periodicity (1 to 8 μm), made of acrylated trimethylene-carbonate-based oligomers initiated with trimethylolpropane[18] (T/TMP) on a glass substrate using interferometric fabrication.[19] Rat cardiomyocytes were cultured on the fabricated scaffold and observed under a phase contrast microscope and fluorescence microscope. From the observation, we found that more cells oriented parallel to the grating with smaller periodicity, and the cell orientation along the grating was observed within 1 day postseeding.

2 Methods and Materials

2.1 *Fabrication of Scaffolds Using Photopolymerization by Laser Beam Interference*

Photofabrication is used to make a microstructure by solidification of a photopolymerizable material with light exposure. In photopolymerization by laser beam interference, a photopolymerizable material is exposed to light distribution that results from the interference fringes.[19,20] This method produces a microstructure in the region of light irradiation that has a periodicity of the order of the laser wavelength. With this technique, it is possible to fabricate a structure in a large dimension with a single laser irradiation exposure.

Figure 1 shows the procedure of fabricating scaffolds by photopolymerization using laser beam interference. An $Nd:YVO_4$ laser (BL6S-355Q, wavelength of 355 nm, Spectra-Physics, Osaka, Japan) was used as the light source. The output laser beam was divided into two collimated beams, and the two beams were made to interfere on a photopolymerizable material that was spin coated on a coverslip.

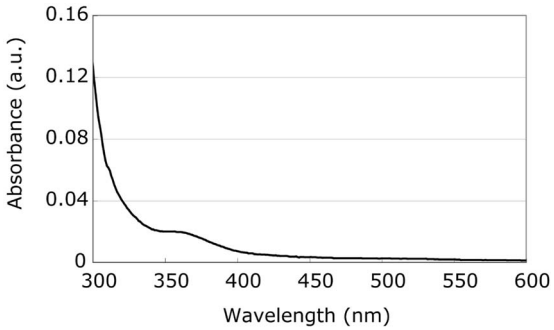

Fig. 2 Absorption spectrum of the photopolymerizable material (T/TMP) before photopolymerization.

The material was exposed to the interference pattern and hence solidified into structures with stripes or a grating pattern corresponding to the interference fringes. After completion of solidification, the nonsolidified material was removed by soaking in acetone for 3 days. The periodicity of a solidified stripe (the distance between the centers of the solidified part of the material) can be selected by tuning the angle between the two beams and the wavelength of the laser, and can be minimized to approximately half of the laser wavelength.

2.2 *Photopolymerizable Material*

We used trimethylene-carbonate-based oligomers with trimethylolpropane as an initiator, followed by acrylation at terminal ends (T/TMP) as a photopolymerizable material.[18] Acrylate-end-capped liquid prepolymers were subjected to photopolymerization by ultraviolet light irradiation. Figure 2 shows the absorption spectrum of T/TMP before photopolymerization. When light irradiated T/TMP, radicals were generated, which attacked the acrylate. Radical polymerization of acrylate groups resulted in production of nonwater-swellable crosslinked solid polymers. T/TMP was spin coated on a glass substrate to a thickness of about 1 μm. The T/TMP used in the experiments is hydrophobic and shows a water contact angle of 60 deg.[18] This hydrophobicity induces better cell-adhesive property compared to a glass substrate. Cells seeded on a fabricated scaffold attached to the T/TMP more efficiently than to a glass substrate.

2.3 *Cell Culture*

Neonatal rat cardiomyocytes were isolated and purified as described in a previous paper.[21] Briefly, the hearts of 1-day-old Wistar rats (Nippon Doubutsu Corporation, Osaka, Japan) were removed under ether anesthesia. Neonatal rat cardiomyocytes were isolated by collagenase and purified from other cells (i.e., fibroblasts and endothelial cells) by the Percoll density gradient centrifugation method. The isolated cells were seeded on a glass substrate with fabricated scaffolds at a cell density of 1.2×10^5 cells/cm^2. As a control, cells were also seeded on a glass substrate without fabricated scaffolds. All cells were cultured in an incubator at 37°C in the presence of a humidified atmosphere of 95% air and 5% CO_2.

2.4 *Cell Observation*

Cell shape, alignment, orientation and contractile activity of cells cultured on a scaffold were observed with a phase con-

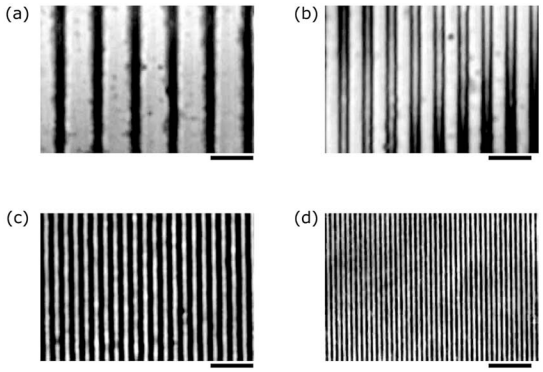

Fig. 3 Top view of T/TMP fabricated scaffolds by phase contrast microscopy. The periodicity of each grating was (a) 8, (b) 4, (c) 2, and (d) 1 μm (bar=10 μm).

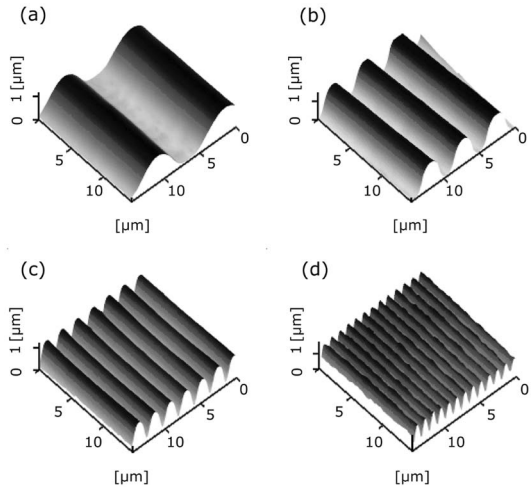

Fig. 4 Topographic images of T/TMP fabricated scaffolds by atomic force microscopy. The periodicity of each grating was (a) 8, (b) 4, (c) 2, and (d) 1 μm.

trast microscope and fluorescence microscope. To confirm the cellular generation of cytoskeleton, bundles of actin filaments were observed after staining with fluorescein-phalloidin. For staining, cells were rinsed with phosphate buffered saline (PBS) to remove the culturing medium and then fixed with 1% paraformaldehyde in PBS for 10 min, followed with permeabilization by using 5% Triton X-100 for 10 min. Thereafter, the cells were blocked with 3% BSA in PBS for 20 min and then incubated in fluorescein-phalloidin (F-432, Molecular Probes, Tokyo, Japan) for 30 min at room temperature. The cells were rinsed with PBS and mounted with glycerol and coverslips. Fluorescent images were obtained with a laser scanning confocal microscope (LSM510, Zeiss, Osaka, Japan).

Cell orientation on the fabricated grating was evaluated by calculating the ratio of cells oriented along the grating stripes. The procedure is as follows. For each cell on a grating stripe, a rectangle was drawn around the entire cell with the smallest dimensions that could completely encompass the cell. The direction of cell orientation was defined to be in the same direction as the long axis of the rectangle. The cells with an angle between the long axis and grating stripes of up to 10 deg were then defined as cells oriented along the grating stripes. The cells with a surrounding rectangle that had an aspect ratio less than 2 were not defined as oriented, because orientation is not clear for those cells. The number of the oriented cells was counted and calculated as the percentage of cell orientation for all the cells on the grating stripes.

3 Results

Figure 3 shows phase contrast images of fabricated scaffolds using photopolymerization of T/TMP. The periodicities of the stripes were 8, 4, 2, and 1 μm, and the height of each grating was estimated to be approximately 1 μm by atomic force microscopy (Fig. 4). These different gratings were fabricated by changing the angles between the two beams. The laser power needed for solidification was 12 mW and the exposure time was 1/4 s. The irradiation area was about 3 mm^2.

Figure 5 shows phase contrast images of cardiomyocytes on the fabricated scaffolds at 3 days postseeding. Cells on the scaffold attached to the solidified T/TMP. On each scaffold, most of the cells showed an elongation and orientation parallel to the grating stripe, and a small fraction of the cells did not elongate or orient. Contraction of individual cells was first observed within 24-h postseeding. By the second day, the contraction of the cells was seen to synchronize along the direction of the grating stripe. Figure 6 shows a series of fluorescent images of the distribution of bundles of actin filaments in the cells at each sized grating at 4-days postseeding. The fluorescent images were taken at the region where cells exist at higher density and more cells are oriented along the grating stripes. It is observed that bundles of actin filaments were generated parallel to the grating stripe in one direction. On the contrary, the control group cells without a particular scaffold did not show elongation of cytoskeleton in any definite direction, and the shape of the cells was not uniform. The bundles of actin filaments in the control group cells were distributed radially, centering on the nucleus.

To understand the relationship between the periodicity of the grating and cell orientation, the number of oriented cells was counted. The percentage of cells that showed orientation along the grating stripe was calculated from the results ob-

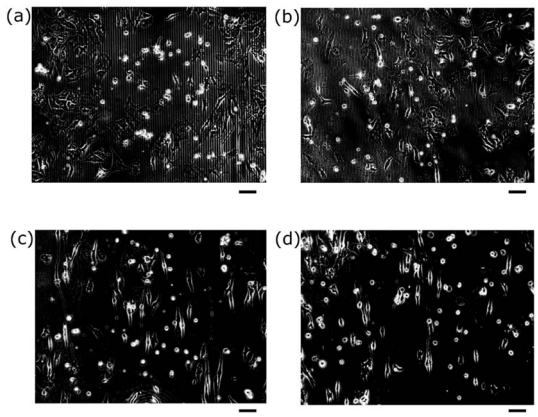

Fig. 5 Phase contrast images of cultured cardiomyocytes on the T/TMP fabricated scaffolds. Dimension of the periodicity is (a) 8, (b) 4, (c) 2, and (d) 1 μm (bar=30 μm).

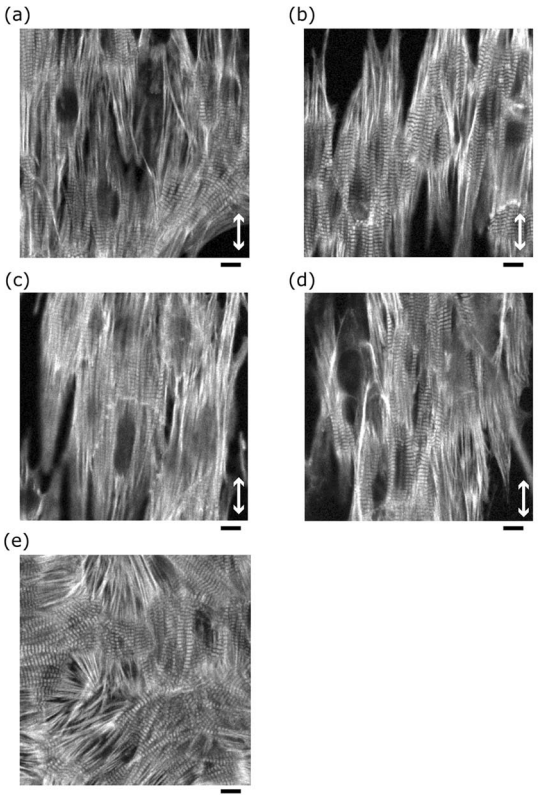

Fig. 6 Fluorescent images of the distribution of bundles of actin filaments stained with fluorescein-phalloidin taken by a LSM. The periodicity of each grating was (a) 8, (b) 4, (c) 2, (d) 1 μm, and (e) without any structure. (The arrows are parallel to the grating stripes, bar=10 μm.)

served at 1-day postseeding (18 h), 3-days postseeding, and 5-days postseeding (Fig. 7). The periodic scaffold-orientation relationship showed that when the periodicity between grating stripes was smaller, more cells oriented parallel to the grating stripes. The cell orientation along the grating stripe was observed at 18-h postseeding on the scaffold. The percentage of the cells showing orientation did not change during the first 3-days postseeding and dropped by approximately 10% at 5-days postseeding.

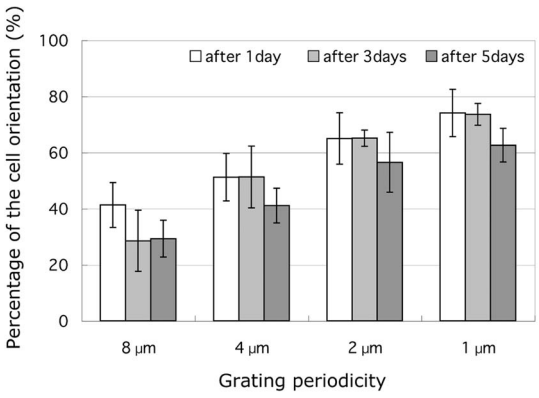

Fig. 7 The percentage of cells oriented on the scaffolds of T/TMP.

4 Discussion

Cardiomyocytes cultured on a fine linear grating scaffold exhibit elongation and orientation along the grating stripe, as similarly shown in previous reports.[9,10,13,14,17] The difference in cell elongation and orientation is due to the difference of cell adhesiveness between T/TMP and glass. T/TMP is more hydrophobic than a glass substrate. The hydrophobicity of T/TMP helps cell adhesion, and cell elongation and orientation. The scaffold with finer periodicity showed higher orientation along the grating stripe. This orientation parallel to the direction of grating is called "contact guidance," by which a majority of anchorage-dependent cells behave. When the periodicity is 1 μm, more than 70% of cells oriented parallel to the grating stripe. This periodicity is more than ten times smaller than the values mentioned in previous reports.[9,10,13,14,17] This result indicates that the grating stripes influence the distribution of focal adhesions and cell growth. Focal adhesions distribute in the cell membrane on a scale of several hundreds of nanometers and are related to generation of the cell skeleton[22] (e.g., action filaments), which determines cell shape.[23] The existence of grating stripes limits possible locations for cell adhesion. As the periodicity of grating stripes is smaller, positions for focal adhesions become more limited, and the distribution of focal adhesions along the grating stripes is produced. To clarify the distribution of focal adhesions related to cell orientation, it is necessary to investigate the distribution of focal adhesions depending on the grating periodicity by immunostaining of cell adhesion proteins, and examine the relationship between the distribution of focal adhesions and cell skeleton generation.[24] In our experiment, the cell orientation along a fabricated scaffold was observed at 18-h postseeding. The percentage of orientation did not change for at least 3 days. This result indicates that the attachment between a cell and a substrate at an early stage of culture has a significant influence on the morphology of the cell after its growth. It also indicates that a structure smaller than cells affects the distribution of focal adhesions at the early stage of cell culture, and induces a more efficient elongation and orientation of the cells. To clarify this point, it is necessary to examine the relationship between cell attachment at an early stage and later morphology of the cell by time-lapse observation. It is also important to investigate cell behavior depending on the change in periodicity of the scaffold, especially for scaffolds smaller than those used in our experiments.

Photopolymerization by laser interference was used to produce scaffolds with micron-scale periodic gratings on large dimensional substrates in this research. The periodicity up to approximately half of the laser wavelength can be made, and the total size of the fabricated structure is determined by the diameter of the laser beam, which is limited by the power of the laser light source versus the laser power needed for the solidification of the material. The height of the grating is controlled by the thickness of the material, but the maximum possible height is limited by the absorption of light in the material. If the wavelength of light is chosen to allow sufficient penetration of light into the material, multibeam interference can produce 3-D structures. For urethane acrylate resin (KC1077B, UV absorbing), it has been reported that a structure with approximately 150-μm height was fabricated

by holographic patterning.[19] Since the interferometric fabrication method is suitable for producing periodic structures, the method presented here can be applied to reproduce tissues with a periodic alignment of cells, such as heart, skeletal muscle, and blood vessels.

In this work, we use interferometric photopolymerization to fabricate scaffolds for growing cells in culture. The elongation and orientation of cardiomyocytes parallel to the grating stripe in the scaffolds is observed. Photopolymerization makes it possible to fabricate a scaffold with fewer step processes, and is flexible to change the design and the dimension of a scaffold. One disadvantage of this method is the availability of materials, which are limited to photopolymerizable types. However, by coating with cell-adhesive proteins[25] (e.g., collagen, laminin, fibronectin), the outer surface of the scaffold can be modified to suit the cell type of interest.

References

1. E. A. Johnson and M. Lieberman, "Heart: excitation and contraction," *Annu. Rev. Physiol.* **33**, 479–529 (1971).
2. P. Clark, P. Connolly, A. S. G. Curtis, J. A. T. Dow, and C. D. W. Wilkinson, "Topographical control of cell behavior. 2. Multiple grooved substrata," *Development* **108**, 635–644 (1990).
3. A. Curtis and C. Wilkinson, "Topographical control of cells," *Biomaterials* **18**, 1573–1583 (1997).
4. R. Singhvi, G. Stephanopoulos, and D. I. C. Wang, "Effects of substratum morphology on cell physiology - Review," *Biotechnol. Bioeng.* **43**, 764–771 (1994).
5. S. Britland, P. Clark, P. Connolly, and G. Moores, "Micropatterned substratum adhesiveness: a model for morphogenetic cues controlling cell behavior," *Exp. Cell Res.* **198**, 124–129 (1992).
6. T. Matsuda and T. Sugawara, "Development of surface photochemical modification method for micropatterning of cultured cells," *J. Biomed. Mater. Res.* **29**, 749–756 (1995).
7. D. Kleinfeld, K. H. Kahler, and P. E. Hockberger, "Controlled outgrowth of dissociated neurons on patterned substrates," *J. Neurosci.* **8**, 4098–4120 (1998).
8. A. Ohl and K. Schroder, "Plasma-induced chemical micropatterning for cell culturing applications: a brief review," *Surf. Coat. Technol.* **119**, 820–830 (1999).
9. S. Rohr, D. M. Scholly, and A. G. Kleber, "Patterned growth of neonatal rat heart cells in culture morphological and electrophysiological characterization," *Circ. Res.* **68**, 114–130 (1991).
10. S. P. Thomas, L. Bircher-Lehmann, S. A. Thomas, J. Zhuang, J. E. Saffitz, and A. G. Kleber, "Synthetic strands of neonatal mouse cardiac myocytes: structural and electrophysiological properties," *Circ. Res.* **87**, 467–473 (2000).
11. P. Clark, S. Britland, and P. Connoly, "Growth cone guidance and neuron morphology on micropatterned laminin surfaces," *J. Cell. Sci.* **105**, 203–212 (1993).
12. R. Singhvi, A. Kumar, G. P. Lopez, G. N. Stephanopoulos, D. I. Wang, G. M. Whitesides, and D. E. Ingber, "Engineering cell shape and function," *Science* **264**, 696–698 (1994).
13. M. Lieberman, A. E. Roggeveen, J. E. Purdy, and E. A. Johnson, "Synthetic strands of cardiac muscle: growth and physiological implication," *Science* **175**, 909–911 (1972).
14. T. C. McDevitt, J. C. Angello, M. L. Whitney, H. Reinecke, S. D. Hauschka, C. E. Murry, and P. S. Stayton, "*In vitro* generation of differentiated cardiac myofibers on micropatterned laminin surfaces," *J. Biomed. Mater. Res.* **60**, 472–479 (2002).
15. A. Curtis and M. Riehle, "Tissue engineering: the biophysical background," *Phys. Med. Biol.* **46**, R47–R65 (2001).
16. T. A. Desai, "Micro- and nanoscale structures for tissue engineering constructs," *Med. Eng. Phys.* **22**, 595–606 (2000).
17. J. Deutsch, D. Motlagh, B. Russell, and T. A. Desai, "Fabrication of microtextured membranes for cardiac myocyte attachment and orientation," *J. Biomed. Mater. Res.* **53**, 267–275 (2000).
18. T. Matsuda, I. K. Kwon, and S. Kidoaki, "Photocurable biodegradable liquid copolymers: synthesis of acrylate-end-capped trimethylene carbonate-based prepolymers, photocuring, and hydrolysis," *Biomacromolecules* **5**, 295–305 (2004).
19. S. Shoji and S. Kawata, "Photofabrication of three-dimensional photonic crystals by multibeam laser interference into a photopolymerizable resin," *Appl. Phys. Lett.* **76**, 2668–2670 (2000).
20. S. Shoji, H. B. Sun, and S. Kawata, "Photofabrication of wood-pile three-dimensional photonic crystals using four-beam laser interference," *Appl. Phys. Lett.* **83**, 608–610 (2003).
21. T. Matsushita, M. Oyamada, H. Kurata, S. Masuda, A. Takahashi, T. Emmoto, I. Shiraishi, Y. Wada, T. Oka, and T. Takamatsu, "Formation of cell junctions between grafted and host cardiomyocytes at the border zone of rat myocardial infarction," *Circulation* **100**, II262–II268 (1999).
22. B. Geiger, E. Schmid, and W. W. Franke, "Spatial distribution of proteins specific for desmosomes and adhaerens junctions in epithelial cells demonstrated by double immunofluorescence microscopy," *Differentiation* **24**, 189–205 (1983).
23. C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, and D. E. Ingber, "Geometric control of cell life and death," *Science* **276**, 1425–1428 (1997).
24. D. E. Ingber, "Cellular tensegrity- defining new rules of biological design that govern the cytoskeleton," *J. Cell. Sci.* **104**, 613–627 (1993).
25. R. S. Bhati, D. P. Mukherjee, K. J. McCarthy, S. H. Rogers, D. F. Smith, and S. W. Shalaby, "The growth of chondrocytes into a fibronectin-coated biodegradable scaffold," *J. Biomed. Mater. Res.* **56**, 74–82 (2001).