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Micropatterning of proteins and mammalian cells on biomaterials

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ABSTRACT

Controlling the spatial organization of cells is vital in engineering tissues that require precisely defined cellular architectures. For example, functional nerves or blood vessels form only when groups of cells are organized and aligned in very specific geometries. Yet, scaffold designs incorporating spatially defined physical cues such as microscale surface topographies or spatial patterns of extracellular matrix to guide the spatial organization and behavior of cells cultured in vitro remain largely unexplored. Here we demonstrate a new approach for controlling the spatial organization, spreading, and orientation of cells on two micropatterned biomaterials: chitosan and gelatin. Biomaterials with grooves of defined width and depth were fabricated using a two-step soft lithography process. Selective attachment and spreading of cells within the grooves was ensured by covalently modifying the plateau regions with commercially available protein resistant triblock copolymers. Precise spatial control over cell spreading and orientation has been observed when human microvascular endothelial cells are cultured on these patterned biomaterials, suggesting the potential of this technique in creating tissue culture scaffolds with defined chemical and topographical features.

Key words: microcontact printing • chitosan • scaffold • microfabrication

Engineering capabilities for regenerating diseased or damaged tissues and restoring organ function either through implantation of tissues grown outside the body or stimulating cells to grow into implanted matrices (1–5) have advanced rapidly in the last few years. Engineered tissue analogs, composed of cultured cells, biomaterials, or composites combining cells and biomaterials, have achieved some clinical success as substitutes for skin (6). There has also been steady progress in strategies for fabricating cardiovascular components (7), cartilage (8), bone (9), and liver (10) tissues in vitro. However, only a few functional products (11) have become available for clinical use at affordable cost.

A major challenge in engineering tissues in vitro is establishing the proper microenvironment around cells to mimic the natural chemical and physical cues that guide the spatial organization, growth, proliferation, and differentiation of cells. Most efforts in tissue engineering have focused on the design and preparation of novel scaffold materials that are modified with bioactive factors, such as cell adhesion peptides (12–15), or growth factors (16–18). Other studies have explored methods for forming robust structured biomaterials with sufficient strength to provide temporary support against the in vivo forces exerted by surrounding tissues (19). Scaffold designs

incorporating spatially defined physical cues such as microscale surface topographies or spatial patterns of extracellular matrix to guide the spatial organization and behavior of cells cultured in vitro remain largely unexplored. For the most part, these studies have been hampered by the lack of suitable techniques for patterning cells directly onto biocompatible and biodegradable materials.

Controlling the spatial organization of cells is vital in engineering tissues that require precisely defined cellular architectures. For example, functional nerves or blood vessels form only when groups of cells are organized and aligned in very specific geometries. A variety of techniques have been used to fabricate micron-size patterns of protein to spatially control the adhesion of cells on substrates. Some examples include classical photoresist patterning of nerve cells with silanes (20), photochemical approaches for patterning endothelial cells (21), and self-assembled monolayers for patterning hepotocytes (22). Among these proven cell patterning techniques, self-assembled monolayers have taken the lead as the preferred method because of its simplicity and high spatial resolutions.

Singhvi et al. (23) pioneered the use of microcontact printing for patterning cells over defined areas. In this technique, self-assembled monolayers (SAMs) of alkanethiols and oligo-ethylene glycol terminated alkanethiols are patterned on gold surfaces. The hydrophobic alkanethiolate SAMs promote the adhesion of extracellular matrix (ECM) and attachment of cells while the background area, covered by the oligo-ethylene glycol terminated SAMs, resists protein adsorption. Complementary patterns of these two SAMs define the size and shape of cell adhesive islands.

These surface microtopographies have been shown to alter cellular attachment (23), alignment (24), cytoskeletal arrangement (25), metabolism, and gene activity (26). However, most efforts in investigating and controlling cell behaviors using microfabrication techniques rely on micropatterned silicon or glass substrates. More recent work has used flexible polymeric materials such as polydimethylsiloxane (PDMS) that allow surface patterning of micron-sized features (27). Although these synthetic polymer materials are inert and easy to manufacture, they are nonbiodegradable and have only limited applications in tissue engineering. Thus, despite the high degree of control micropatterning techniques offer, there have only been a few reports of their use in tissue engineering.

To address this deficiency, we have extended the soft lithography/microcontact printing methodology to form chemical and topographical micropatterns directly onto biocompatible and biodegrade biomaterials. Similar efforts have been taken for patterning mammalian cells on biomaterials. Lu et al. (28), for example, have developed an elegant technique for patterning cells on poly(lactic-co-glycolic acid) substrates by printing patterns of cell-resistant diblock copolymers of poly(ethylene glycol) and poly(lactic acid).

Advantages of our method include *1*) ease in forming micrometer-scale topographical features on biomaterials; *2*) effective control on the spatial organization, spreading, and orientation of cells; *3*) permitting direct attachment of cells without the need for precoating substrates with extracellular matrix; and *4*) the substrates are transparent and attached cells can be readily visualized using conventional light microscopy.

Here, we report the first use of chemical and topographical patterns to control the spatially organized attachment and proliferation of cells on two model biomaterials: chitosan and gelatin. We have chosen these two materials due to their high biocompatibility, low toxicity, and wide use in medical applications. Chitosan is a high molecular weight cationic polysaccharide derived from crustacean shells by deacetylation of naturally occurring chitin. It is a linear polymer composed of glucosamine and residual *N*-acetyl glucosamine units linked in a β(1-4) manner. Gelatin is obtained from the thermal denaturation or physical and chemical degradation of collagen. Chitosan and gelatin have both been used widely for a variety of biomedical applications including sealants for vascular prostheses, wound dressing and adsorbent pads for surgical use, as well as three-dimensional scaffolds for tissue regeneration (29–32).

The methods we use in this report for fabricating micron-scale patterns on chitosan and gelatin are simple and generally applicable to the micropatterning of cells and proteins on a variety of biomaterials. We have confirmed the efficacy of these methods by monitoring the selective adsorption of fluorescently labeled proteins and spatially controlled attachment, spreading, and orientation of human microvascular endothelial cells. The ease with which spatially well-defined cultures can be formed on biocompatible and biodegradable materials should encourage further development of this technique for direct applications in tissue engineering.

MATERIALS AND METHODS

Materials

PDMS (Sylgard 184) was obtained from Dow Corning (Midland, MI). Microvascular endothelial cell growth medium and fetal bovine serum (FBS) were purchased from Cambrex Biosciences (Walkersville, MD). Pluronic F127 was a gift from BASF (Whitehouse, OH). Chitosan of medium molecular weight was purchased from Fluka (St. Louis, MO). Gelatin and paraformaldehyde were purchased form Sigma (St. Louis, MO). Alexra488-phalloidin and 4′,6–diamidino-2-phenylindole (DAPI) were purchased form Molecular Probes (Eugene, OR).

Microfabrication of the silicon master pattern and transfer of the topological patterns onto PDMS

Silicon patterns with a series of 10 μm high parallel grooves of varying width (10, 20, 30, and 50 μm) were fabricated on silicon wafers using standard photolithographic techniques. From this silicon master pattern, complementary PDMS replicas were formed by pouring PDMS prepolymer (mixed in a 10:1 ratio with a cross linking catalyst) over the Si master and curing at 55°C for 2 h. Compared with the silicon master, the PDMS replicas are durable, optically transparent, inexpensive, and allow biomaterials formed in the subsequent steps to be peeled away easily. Direct patterning of biomaterials using the silicon master with the photoresist features while possible is not recommended due to progressive reduction of feature heights after each molding operation due to gradual dissolution of the photoresist.

Transfer of the topological pattern onto chitosan or gelatin

Micropatterned chitosan films were formed by introducing a small volume of 2% chitosan solution with acetic acid as solvent onto the PDMS mold. After drying, the chitosan films with complementary topological features to the PDMS mold can be peeled off easily from the PDMS

mold. Before use in cell culture, residual acetic acid in the chitosan film was neutralized by Hanks' buffered saline solution (HBSS, Life Technologies) and air dried. Patterned gelatin films were prepared by adding gelatin solutions (0.2 M acetic acid) drop wise to the PDMS substrates. After drying, the gelatin film was removed from PDMS using tweezers and crosslinked with 0.5% glutaraldehyde solution. The chitosan and gelatin films were rinsed in deionized water, air dried, and then sterilized in 70% ethanol solution (Aaper Alcohol and Chemical Co., Shelbyville, KY) before use in cell cultures. The mechanical properties of the chitosan and gelatin substrates were measured using the earlier method used by Wang et al. (33) and shown in Table 1. The surface topography on the biomaterials was imaged with Hitachi S4000 field emission scanning electron microscope and measured using Dektak II profilometer.

Chemical patterning nonadhesive (Pluronic F127) regions

Pluronic F127 is a triblock copolymer consisting of $[(PEO)_{100}\text{-}(PPO)_{65}\text{-}(PEO)_{100}]$ with a total molecular weight of 12,600 g/mol. Trichlorovinylsilane (TCVS) was used as a coupling agent to link Pluronic F127 to chitosan. Two percent TCVS was reacted with 20% Pluronic F127 solution under UV light for 3 h. Topographical plateaus on the surface of the chitosan films were selectively modified with Pluronic F127 by contacting the chitosan surface with glass slides coated with the Pluronic/TCVS mixture. This procedure leaves the grooves of the chitosan substrate unmodified. Spatial control of protein adsorption onto Pluronic F127 modified chitosan films was tested by incubating the substrates with fluorescently labeled bovine serum albumin (BSA; Molecular Probe, Eugene, OR). In all cases, fluorescence microscopy (Nikon TE-2000) revealed that fluorescently labeled BSA adsorbs exclusively within the uncoated grooves of the micropatterned chitosan substrates.

Culture of endothelial cells

Human microvascular endothelial cells (HMVEC-d, purchased from Cambrex Biosciences) were cultured in endothelial basal medium (Cambrex Biosciences) containing 5% FBS, 1 μg/ml hydrocortisone, 10 μg/ml epidermal growth factor (EGF), 10 μg/ml bovine brain extract, 50 μg/ml gentamycin, and 50 μg/ml amphotericin-B under 5% CO_2. Before incubation with the micropatterned biomaterials, cells were dissociated from the culture dish with trypsin, resuspended in endothelial basal medium containing 10% serum, and allowed to attached onto 1 cm^2 square pieces of micropatterned biomaterials.

Immunostaining

After 72 h of incubation, the attached cells were fixed with 4% paraformaldehyde for 10 min, washed in phosphate buffered saline, and then permeabilized for 5 min with 0.2% solutions of Triton X-100. Samples were then rinsed with PBS and incubated with Alexra488-phalloidin and DAPI to stain F-actin and the nuclei. Immunofluorescence microscopy was carried out with a Nikon TE-2000 inverted microscopy.

Determination of cell areas

Digital fluorescent microscope images were captured using a Spot II CCD camera and analyzed using Metamorph image processing software (Universal Imaging Co.) to quantify cell and nuclear areas through interactive tracing of the cell edges.

RESULTS

Unlike previous studies, which have used micropatterned silicon, glass, or PDMS substrates to alter cell behaviors, we have focused our efforts on creating topographical and chemical micropatterns directly on biomaterials. Figure 1*A* illustrates the procedure used to create these topographically patterned biomaterials. The features of interest are initially patterned onto a silicon master using the traditional photolithography technique. PDMS precursor solution is poured over the mold and cured to form a flexible and reusable PDMS mold from which micropatterned chitosan films can be formed. Figure 2*A-D* shows a phase contrast micrograph of a chitosan film patterned with a series of 10 μm deep parallel grooves 10, 20, 30, and 50 μm wide. The spacing between grooves is 50 μm for the chitosan film shown in Fig. 2*A-C* and 10 μm for the film shown in Fig. 2*D*. The sharp edges (Fig. 2*E* and *F*) of the grooves demonstrate the effectiveness of this soft lithography technique for creating topographical patterns on chitosan films.

To spatially control the attachment and proliferation of endothelial cells exclusively within the grooves, we have developed a simple technique for chemically modifying the plateaus of the chitosan film and rendering these regions cell resistant. The classical cell micropatterning techniques pioneered by Whitesides make use of protein resistant oligo-ethyleneglycol terminated thiols on gold, silver, or palladium substrates. While effective, widespread use of this technique for forming micropatterned materials for tissue engineering has been limited by the nonbiodegradibility of the metal substrates. To form micropatterned biomaterials that may be use for future implantation, we use a new approach to selectively deposit commercially available protein resistant triblock polyethylene glycol/polypropylene glycols (Pluronic F127) directly onto biomaterials. Figure 1*B* shows the schematic diagram for microcontact printing the TCVS modified PEO/PPO/PEO block copolymers exclusively onto the plateaus of the patterned chitosan and gelatin substrates leaving the surface of the grooves unmodified.

This chemical modification procedure used to immobilize Pluronic F127 on chitosan or gelatin surfaces has previously been used to graft Pluronic F127 onto glass and pyrolytic carbon surfaces (34). Our studies show that trichlorovinylsilane coupled Pluronic F127 remains robustly attached to the chitosan substrate and resists protein adsorption or cell attachment after more than 2 wk in culture medium. On the other hand, Pluronic F127 physically adsorbed onto the chitosan substrates without TCVS gradually dissolves in aqueous solution, losing its capability to resist protein or cell adsorption after <24 h.

The efficacy of this approach for patterning proteins on biomaterials was monitored by fluorescence microscopy. Pluronic coated chitosan films with 10, 20, 30, and 50 μm grooves were incubated with fluorescently labeled BSA protein for 30 min then rinsed with water to wash off nonadsorbed BSA. Resulting protein patterns on the chitosan surface (Fig. 3) show that BSA adsorbs exclusively within the grooves not coated with Pluronic F127. The sharp edges demarcating the borders of regions where BSA protein adsorbs reveal the exceptional spatial control of protein adsorption possible using this technique.

To demonstrate that our method can be used to control the spatial distribution of cells, human microvascular endothelial cells were plated onto Pluronic coated biomaterials. Figure 4*A* shows that endothelial cells selectively attach and spread along the 20 μm grooves. In contrast, cell culture studies performed on identical topographically patterned chitosan films without selective

Pluronic coating on the plateaus show completely random attachment of cells on both the groove and plateau regions (Fig. 4*B*).

Cell culture studies on these micropatterned chitosan substrates also revealed that confinement within the grooves significantly alters the spreading of endothelial cells. Human microvascular endothelial cells were found to spread most on the unpatterned chitosan substrates (Fig. 4*C*) with a mean cell area of 2558 ± 295 μm^2. As Fig. 5 shows, cell spreading within the grooves decreases as the grooves become narrower, with average areas of 2550 ± 220, 2240 ± 190, 1830 ± 200, and 1280 ± 220 μm^2 on 50, 30, 20, and 10 μm wide grooves, respectively. The studies also reveal that the width of the 10 and 20 μm grooves is never spanned by more than a single cell.

The cytoskeleton of the patterned cells visualized using fluorescence microscopy is shown in Fig. 6. F-actin components of the cytoskeleton was labeled using Alexra488 linked phalloidin, while the cell nuclei were visualized by DAPI staining. Compared with cells on unpatterned chitosan, cells patterned within the 10, 20, 30, and 50 μm grooves become oriented along the grooves after 72 h. This technique is also equally effective for patterning cells on gelatin (Fig. 6*F* and *G*). The patterned cell morphologies on gelatin surfaces are similar to that observed on chitosan surfaces.

Previous studies (35) using flat glass substrates coated with adhesive islands of extracellular matrix revealed that capillary endothelial cells can be geometrically switched between growth, differentiation, and apoptosis depending on cell spreading area. Endothelial cells cultured on single islands >1500 μm^2 spread and progressed through the cell cycle, whereas cells restricted to areas <500 μm^2 failed to extend and underwent apoptosis. Cells spreading to intermediate degree (~1000 μm^2) differentiate. The capability of our method in controlling the spatial organization, spreading, and orientation of cells supports our confidence in using this technique for developing a new generation of micropatterned and biodegradable scaffolds for tissue engineering applications. Future work will explore the use of these topographically and chemically patterned biomaterials to promote the differentiation of microvascular endothelial cells to form capillary tube-like structures.

CONCLUSIONS

We have demonstrated here a new approach for controlling the spatial organization, spreading, and orientation of cells on two micropatterned biomaterials: chitosan and gelatin. Unlike traditional cell patterning techniques that make use of gold, silver, palladium, or silicone substrates, cells patterned on these biomaterials have much broader tissue engineering applications. Biomaterials with grooves of defined width and depth were fabricated using a two-step soft lithography process. Selective attachment and spreading of cells within the grooves were ensured by covalently modifying the plateau regions with protein resistant triblock copolymers. The approach can be used to create many other micron-size patterns on biomaterials to suit specific tissue engineering applications.

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Table 1

Mechanical parameters of the chitosan and gelatin films

	Catalog number	Thickness (μm)	Size (cm×cm)	Young's modulus (MPa)	Tensile strength (MPa)
Chitosan	Fluka 22742	68±15	1.5×1.5	53±11	1.15±0.39
Gelatin	Sigma G2500	98±10	1.5×1.5	50±17	1.30±0.94

Values are means ± SE; $n = 5$.

Fig. 1

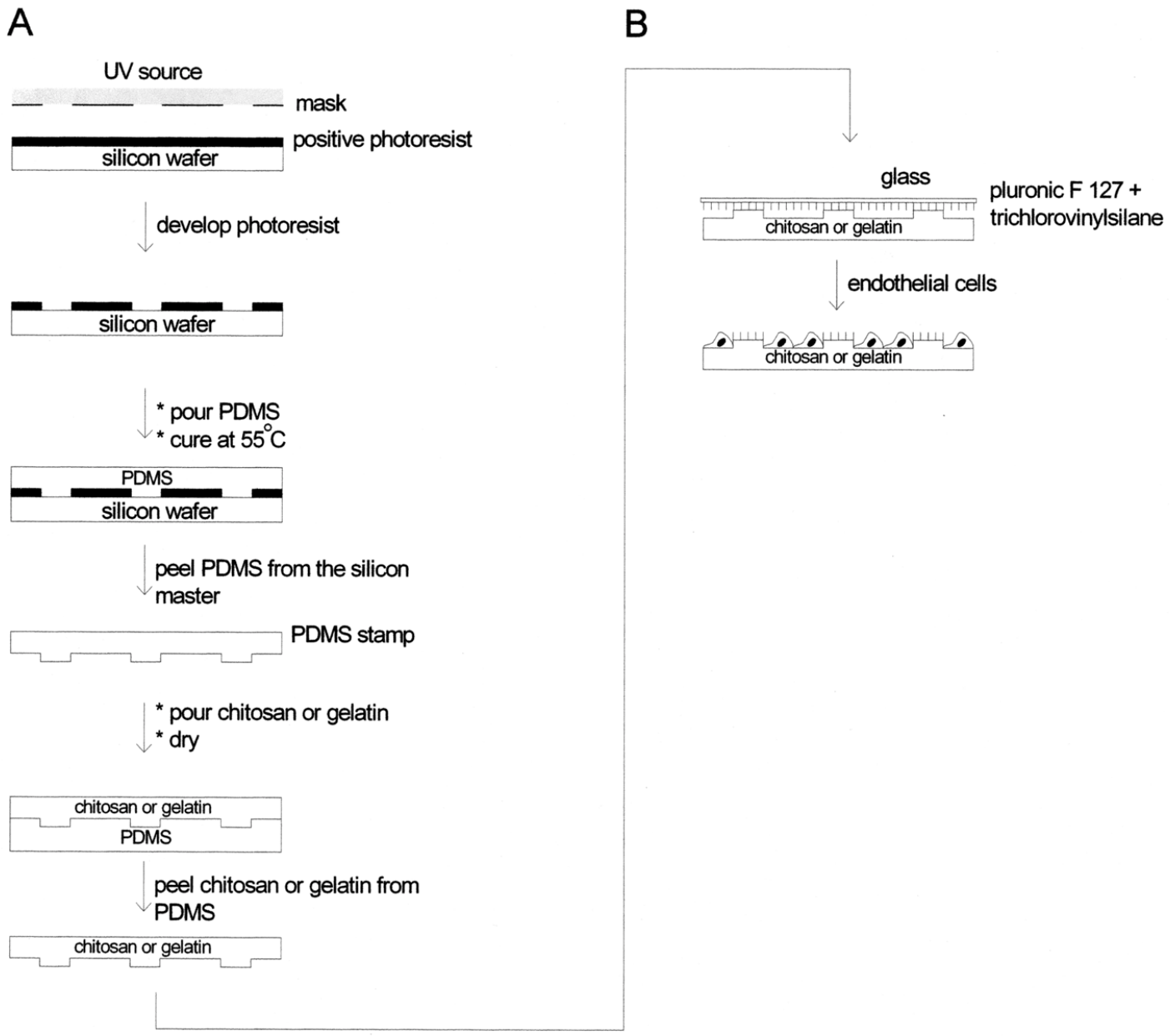

Figure 1. Schematic of the 2-step soft lithography procedure for creating topographical patterns on biomaterials (*A*) and the microcontact printing procedure to coat the plateau regions with trichlorovinylsilane modified Pluronic F127 (*B*). Contacting the Pluronic F127 coated glass slides onto the topographical patterned chitosan film coats only the plateau regions with Pluronic F127.

Fig. 2

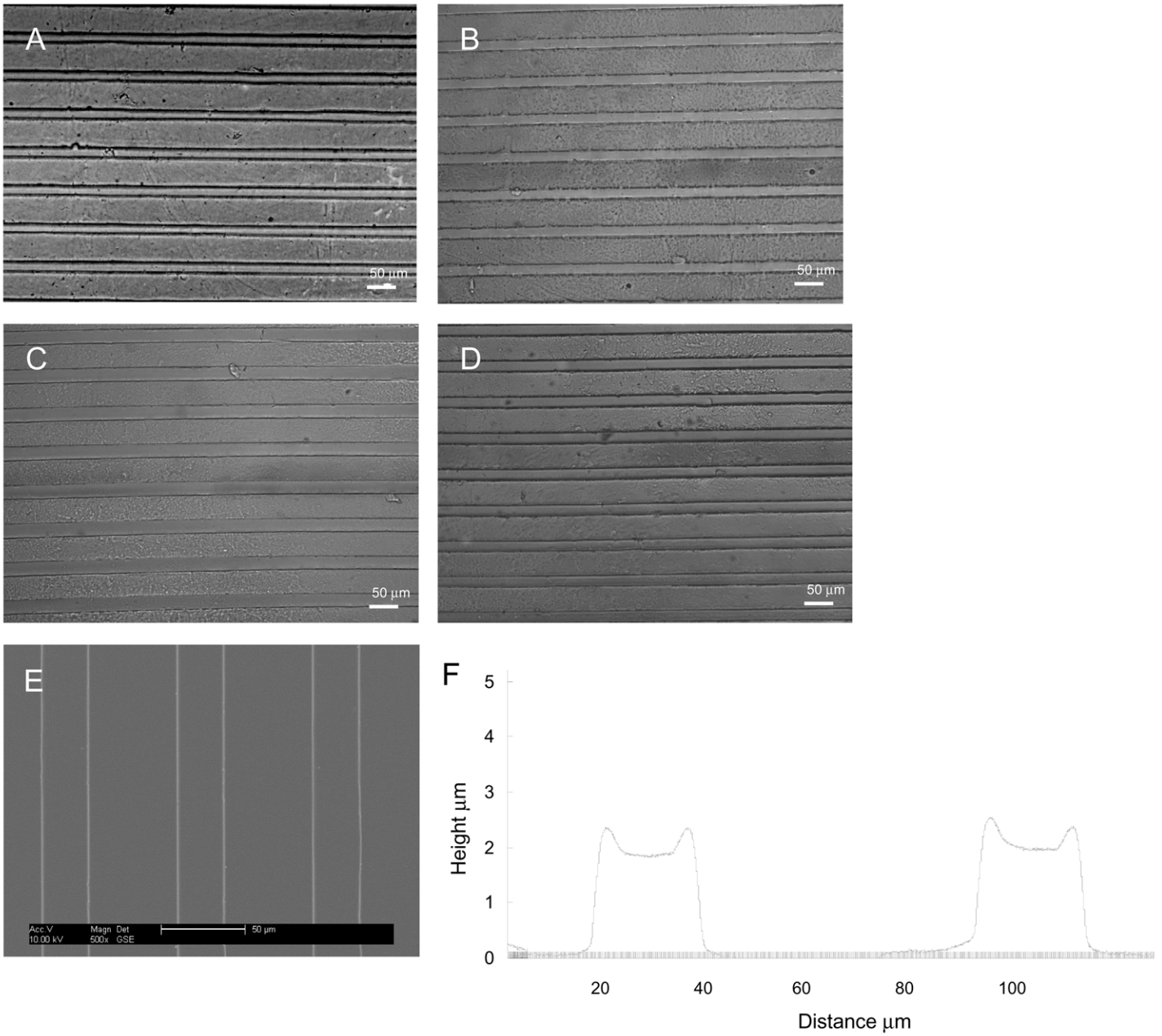

Figure 2. Topographically patterned chitosan films. Patterns consist of multiple 10 (***A***), 20 (***B***), 30 (***C***), and 50 (***D***) µm grooves. Spacing between grooves is 50 µm for ***A-C*** and 10 µm for ***D***. Edges of the grooves appear darker in this phase contrast microscope image. Surface topography of a patterned chitosan film examined by scanning electron microscope (***E***) and profilometer (***F***).

Fig. 3

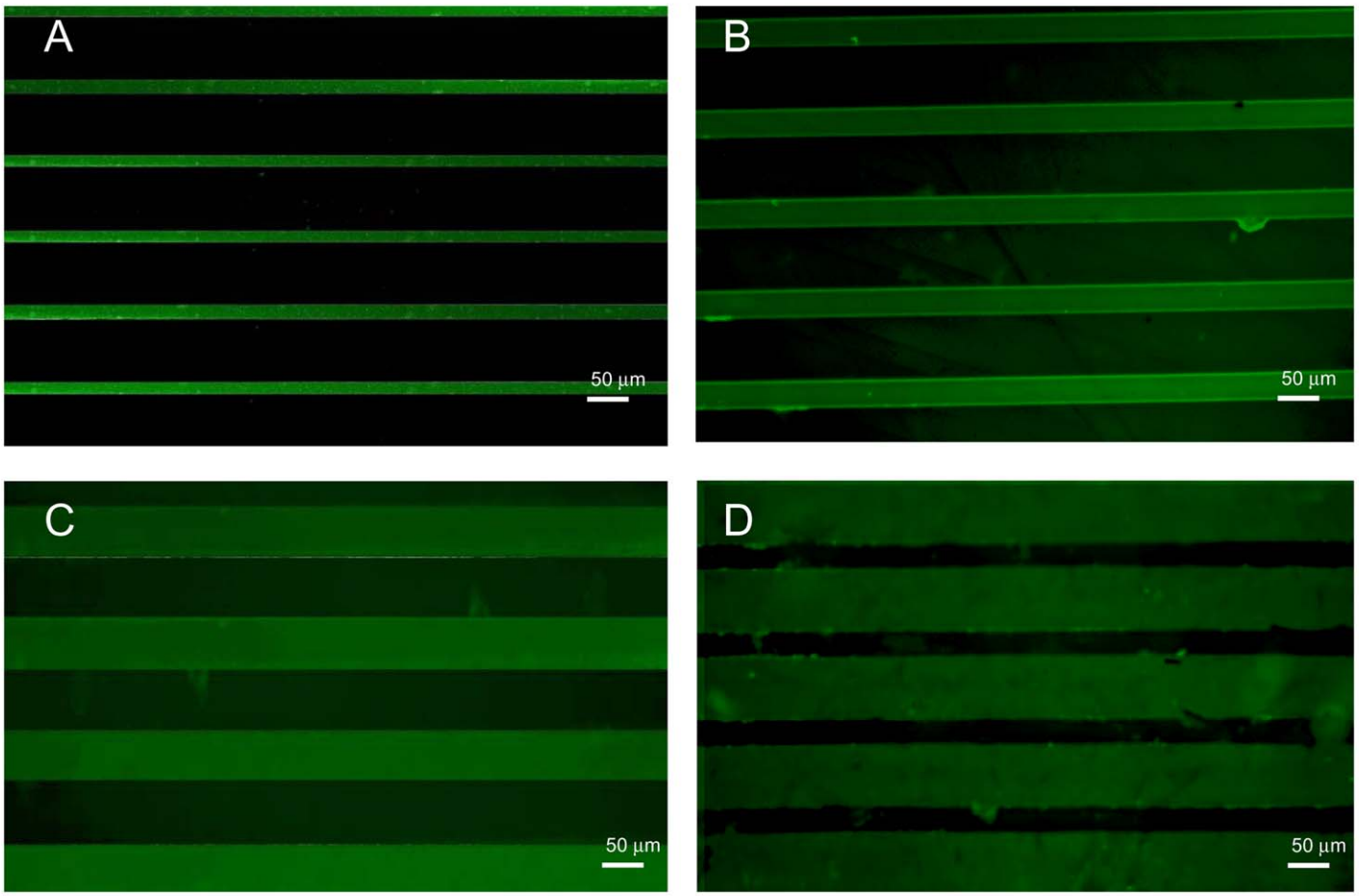

Figure 3. Fluorescence microscopy image of 10 (*A*), 20 (*B*), 30 (*C*), and 50 (*D*) µm grooves adsorbed with fluorescently labeled BSA. BSA selectively adsorbs onto the non-Pluronic coated grooves.

Fig. 4

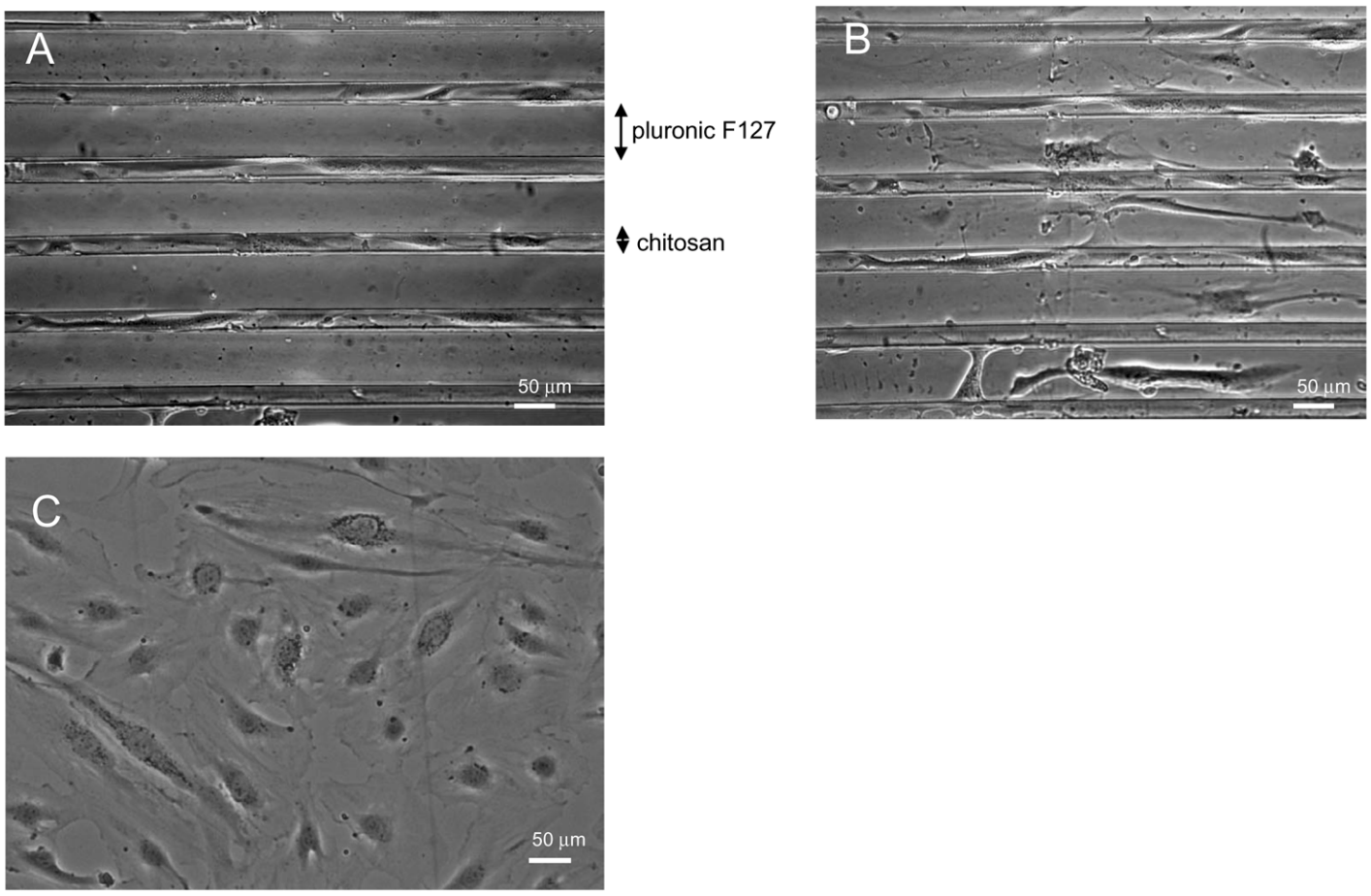

Figure 4. Spatially defined attachment of endothelial cells. Optical micrograph of human microvascular endothelial cells on 20 µm grooves (***A***). The plateaus regions in ***A*** are coated with Pluronic F127 that resists cell attachment. ***B***) Topographically patterned chitosan with 20 µm grooves without Pluronic F127 coating. ***C*)** Cells on unpatterned chitosan film.

Fig. 5

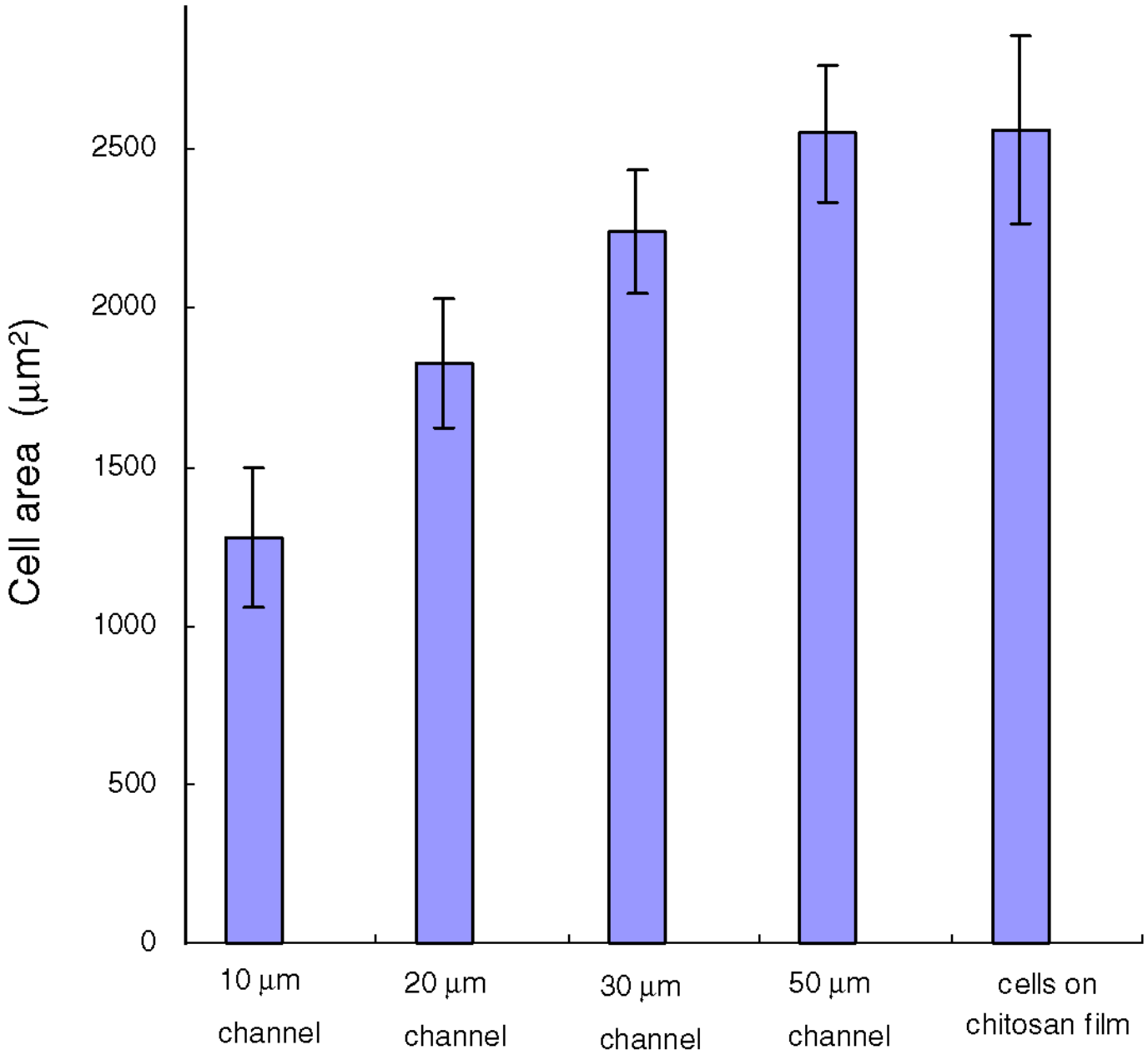

Figure 5. Geometric control of endothelial cell spreading. Cell areas, measured 72 h after seeding, increase with the width of the grooves. Cells on unpatterned chitosan films spread to the largest area.

Fig. 6

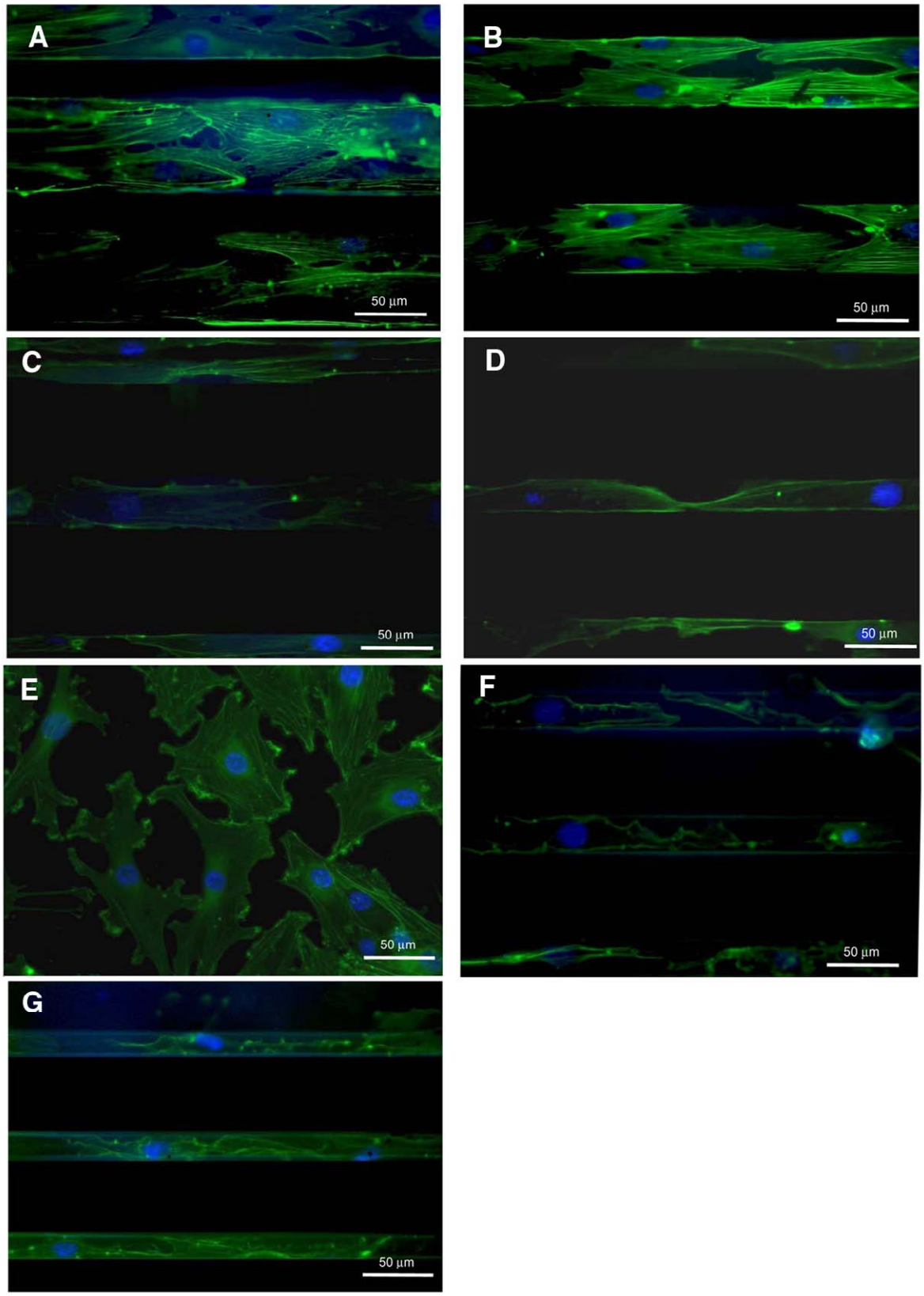

Figure 6. Cytoskeletal alignment in microvascular endothelial cells cultured in 50 (*A*), 30 (*B*), 20 (*C*), and 10 (*D*) µm grooves on the chitosan film, unpatterned chitosan (*E*), 20 (*F*), and 10 (*G*) µm grooves on the gelatin film. Microfilaments aligned parallel to the grooves within 72 h. Actin microfilament (green) were visualized by Alexra-488-labeled phalloidin. Cell nuclei were visualized by DAPI (blue).