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Cell Sheet Engineering: Intelligent Polymer Patterned Surfaces for Tissue Engineered Liver

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Summary: We describe a new culture system utilizing the temperature-responsive polymer grafted surface for designing of cell position and layered tissue reconstruction. Organizing of the hepatic tissue structure by controlling the culture system, that is patterned co-culture and layered cell sheet co-culture achieved by moving the cultured cells from the culture surface, resulted in regulation of the hepatocyte function. The technique for cell sheet manipulation would promote the liver tissue engineering in quality.

Keywords: cell sheet; co-culture; hepatocyte; poly (*N*-isopropylacrylamide); temperature-responsive culture surface

Introduction

Development of tissue engineering has been accelerated with new technologies. Previously, various cell-based constructs such as cartilage, bone, blood vessel, and urinary bladder has achieved by using biodegradable polymer as culture scaffold. These constructs are randomly formed of cell-material combination. However in liver tissue engineering, it is difficult to reconstruct of functional tissue by only this relatively simple technique. Because liver has many kind of cells, more complex but systematic three-dimensional tissue structure, vascular-connection and highly metabolic cellular function. Further advanced technique is required for the basic cell culture system to achieve the controllable cell positioning, 'patterning' culture and

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reconstruction of *in vivo* like 'layered tissue' composed of cell sheet units. We here report the novel cell manipulation technique utilizing intelligent temperature-responsive culture surface.

Temperature-Responsive Cell Culture Surface

The cell culture surface we have developed responds reversibly and dynamically to temperature changes.[1] The temperature-responsive polymer, poly (*N*-isopropylacrylamide) (PIPAAm),[2] was covalently grafted to tissue culture polystyrene (TCPS) dishes by electron beam irradiation. The PIPAAm-grafted surfaces are relatively hydrophobic at 37°C compared to the TCPS dishes, because the PIPAAm chains are aggregated due to dehydration and lie compactly on the surface. At reducing temperature below the lower critical solution temperature of 32°C, the grafted PIPAAm rapidly hydrates and becomes hydrophilic. On the PIPAAm surface, cells adhere, spread and proliferate at 37°C as well as TCPS dish, and detach at the lower temperature 20°C without enzymatic digestion or divalent cation chelators. Loose interaction between the cell layer and the culture surface caused by structural change of hydrated PIPAAm resulted in cell detachment via their tractional forces[3] without cell injury. Therefore, the PIPAAm surface induced both proliferating single cell detachment together with their producing extracellular matrix (ECM), and confluent cell layer 'cell sheet' detachment with underlying ECM and remaining cell-cell junction. The detaching units including the cell and the ECM were gradually floated in culture medium as temperature decrease.

Temperature-Responsive Patterned Surface

Since electron beam is easily shielded by thin masks, patterned graft of PIPAAm is achieved. (Fig.1). In addition, the phase transition temperature can be controlled by copolymerization with other monomers. Therefore, PIPAAm-patterned surfaces have two advancements comparing with other patterning surfaces. The cells are movable from PIPAAm-patterned surfaces keeping the cell sheet shapes as well as micropatterning.[4] And various cell types co-cultured can be combined limitlessly while the cells show resemblance in adherent abilities. When electron beam was radiated through a mask having many circular holes onto tissue culture polystyrene (TCPS) dishes with the monomer solution, PIPAAm was grafted as circular domains, which is temperature-responsive, and outside was naked TCPS, which is cell adhesive temperature-independently. On

these culture dishes, parenchymal hepatocytes and nonparenchymal cells could be co-cultured as following. First, parenchymal hepatocytes were seeded and adhered on the whole surfaces at 37°C, then hepatocyte were detached only from the PIPAAm domains by reducing temperature below 32°C. Finally, nonparenchymal cells were seeded on the same surfaces at 37°C. The endothelial cells and fibroblasts adhered not on the hepatoytes but on PIPAAm domains.

In order to prepare cell non-adhesive domains, highly hydrophilic polymers such as poly(*N,N'*-dimethylacrylamide) and polyacrylamide are grafted by electron beam irradiation. As shown in Figure 1b, square patterned PIPAAm surfaces were achieved by using square glass coverslips for the graft of highly hydrophilic polymers onto PIPAAm-grafted surfaces. The glass slips shielded electron beam so that highly hydrophilic polymers were grafted only outside the slip. With the patterned surfaces, square cell sheets are prepared. The cultured cell positioning including the area shape and size could be regulated by combination of the temperature-responsive polymer and mask patterns.

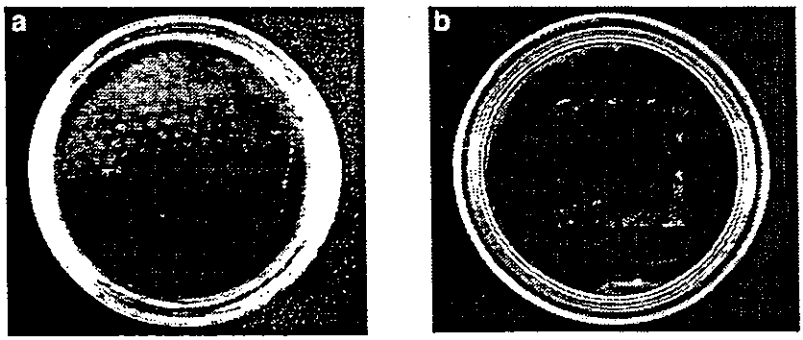

Figure 1. Macroscopoic views of patterned cell culture. Cells were cultured on PIPAAm grafted culture dish surface with porous (a) and square (b) patterns.

Co-culturing of parenchymal hepatocyte with nonparenchymal cells had positive effect on continuous hepatic albumin expression. Interestingly the different culture conditions of hepatocytes co-cultured with endothelial cells, and with fibroblasts, and without co-cultured cells,

resulted in different hepatic albumin expression (Figure 2). Higher albumin expression was observed when hepatocytes were co-cultured with endothelial cells. This culture condition would reflect the environment of hepatocytes along sinusoids in liver lobules.

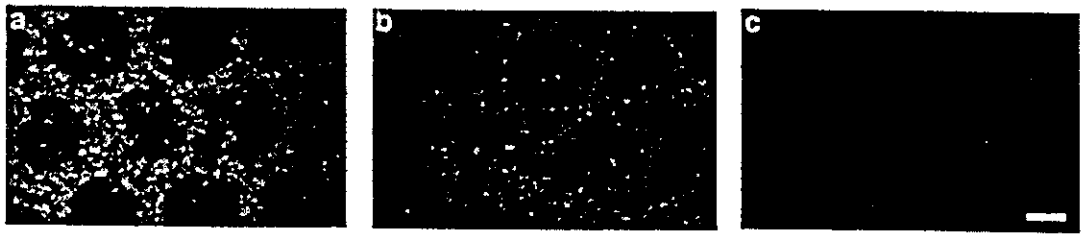

Figure 2. Immunostaining of albumin in hepatocytes. Hepatocytes were co-cultured with endothelial cells (a) or fibroblasts (b) on day 7. These co-cultured cells are present in the circular domains having 1-mm diameter. Homotypic culture shows the basal albumin expression (c). Bar, 100 μm.

Layered Cell Sheets Co-Culture

Volume increase of tissue engineered constructs is needed for clinical use. However, little is developed about systematic technique for three-dimensional hepatic tissue reconstruction. For example, it is well known that hepatic spheroids show lower cell viability in the inner mass when the size is increased. Therefore, the novel technique for reconstructing the three-dimensional tissue structures have been required. Beyond the plane limitation of the co-culture by extending the PIPAAm-grafted surfaces, we established a contiguous layered co-culture system formed by cell sheets of hepatocytes and endothelial cells to achieve the functional units like liver lobules. The vascular endothelial cells were cultured on the PIPAAm-grafted dishes to obtained the movable endothelial cell sheets. The endothelial cell sheets recovered from the PIPAAm-grafted surfaces are moved onto the cultured hepatocytes directly (Figure 3). In this co-culture system, the double layered structures and the hepatic albumin expression were maintained during prolonged culture more than one months.[5] The endothelial cells overlaid on the hepatocytes also showed

higher uptake of acetylated-LDL than the homotypically cultured control. The cell sheet manipulation and layered tissue reconstruction could not be limited to the combination of these cell types. These techniques to modulate spatial cell positioning and cell-cell interactions, and cell functions would open the novel way in tissue engineering toward the ideal tissue reconstruction.

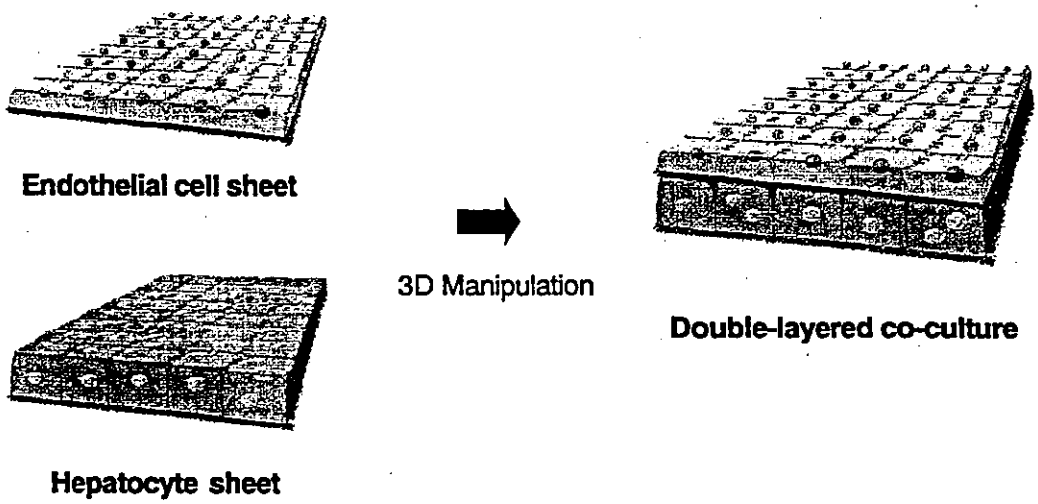

Figure 3. Schematic drawing of the double layered cell sheets co-culture by three-dimensional manipulation of the endothelial cell sheet onto the hepatocyte monolayer.

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TRANSPLANTATION

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FUNCTIONAL BIOENGINEERED CORNEAL EPITHELIAL SHEET GRAFTS FROM CORNEAL STEM CELLS EXPANDED EX VIVO ON A TEMPERATURE-RESPONSIVE CELL CULTURE SURFACE

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Background. **Limbal stem-cell deficiency by ocular trauma or diseases causes corneal opacification and visual loss. Recent attempts have been made to fabricate corneal epithelial graft constructs, but the technology is still evolving. We have developed a novel cell-sheet manipulation technology using temperature-responsive culture surfaces to generate functional, cultivated corneal epithelial cell sheet grafts.**

Methods. **Human or rabbit limbal stem cells were cocultured with mitomycin C -treated 3T3 feeder layers on temperature-responsive culture dishes at 37°C. Cell sheets were harvested from the dishes after 2 weeks by reducing temperature to 20°C. Histologic analyses, immunoblotting, and colony-forming assay were performed to characterize the cell sheets. Autologous transplantation was undertaken to reconstruct the corneal surfaces of rabbits with experimentally induced limbal stem cell deficiencies.**

Results. **Multilayered corneal epithelial sheets were harvested intact simply by reducing the temperature, without the use of proteases. Cell-cell junctions and extracellular matrix on the basal side of the sheet, critical to sheet integrity and function, remained intact. A viable population of corneal progenitor cells, close in number to that originally seeded, was found in the sheets. Harvested sheets were easily manipulated, transplantable without any carriers, and readily adhesive to corneal stroma so that suturing was not required. Corneal surface reconstruction in rabbits was highly successful.**

Conclusions. **Cell sheet engineering technology allows us to create intact, transplantable corneal epithelial cell sheets that retain stem cells from limbal stem cells expanded ex vivo. Our research indicates highly promising clinical capabilities for our bioengineered corneal epithelial sheet.**

Corneal epithelial stem cells reside in the basal layer of the limbus (*1, 2*), the transitional zone between cornea and bulbar conjunctiva. They govern the renewal of corneal epithelium (*3*) by generating transient amplifying cells that migrate from the limbus into the corneal basal layer (*4*). Complete loss of limbal epithelial stem cells because of severe trauma (e.g., thermal and chemical burns) or eye diseases (e.g., Stevens-Johnson syndrome, ocular pemphigoid) prompts adjacent conjunctival tissues to completely cover the cornea, leading to corneal vascularization and opacification

Regarding financial conflict of interest, Kohji Nishida and Masayuki Yamato are consultants for Cell Seed, Ltd, Tokyo, Japan and the inventor/developer designated on the patent for the presently described graft. Teruo Okano is an investor in Cell Seed, Ltd, Tokyo, Japan and an inventor/developer designated on the patent for the temperature-responsive culture surface.

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with severe visual loss. Patients with limbal stem cell deficiencies can be treated with limbal transplantation (*5, 6*), but because of limited donor materials and the high risk of rejection with allogeneic transplantation, recent attempts have been made to fabricate corneal epithelium graft constructs ex vivo from expanded donor or autologous limbal stem cells on different carriers such as collagen (*7*), amniotic membrane (*8–11*), fibrin gel (*12*), and cross-linked gel of fibronectin and fibrin (*13*). Choice of carrier materials can affect the prognosis after grafting. In addition, risk of infection from these biologic materials cannot be excluded. Therefore, we now show a novel method of carrier-free cell sheet transplantation that overcomes many of these obstacles and improves the availabilities of grafting materials for this therapeutic potential.

The key to our current developments is the use of temperature-responsive cell culture surfaces. These surfaces are covalently grafted with a temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), which facilitates cell adhesion, spreading, and growth in culture conditions at 37°C. This is above the polymer's lower critical solution temperature (LCST) of 32°C, meaning that the culture surface is dehydrated and collapsed to support cell culture. Reducing the temperature below the LCST causes the surface to rapidly hydrate and swell, prompting complete detachment of all adherent cells without typical proteolytic enzymes or EDTA treatments (*14*). Confluent cell cultures on these surfaces can then be harvested as a single contiguous cell sheet, retaining cell-cell junctions as well as deposited extracellular matrix (ECM) on the basal sheet surface (*15*). This permits the harvested cell sheets to be readily manipulated, transferred, layered, or fabricated because they adhere rapidly to other surfaces such as culture dishes (*16*) and other cell sheets (*17*). For example, stratified cardiac myocyte sheets harvested by this method rapidly integrate into multilayer tissue-like viable laminates connected to each other both physically and biochemically and exhibiting uniform synchronous pulsation (*17*).

Our approach produces robust, viable, multilayered corneal epithelial cell sheets without any substrates or carriers and promotes strong, rapid adhesion of the transplanted sheets onto corneal stroma in vivo without the need for suturing. Therapeutic advantages for this approach over current transplantation methods are considerable.

MATERIALS AND METHODS

Preparation of Cell Culture Surfaces Grafted with a Temperature-Responsive Polymer

Specific procedures for the preparation of PIPAAm-grafted cell culture surfaces (provided by Cell Seed, Ltd, Tokyo, Japan) were described previously (*16*). *N*-isopropylacrylamide monomer (kindly provided by Kohjin, Tokyo, Japan) in 2-propanol solution was spread onto polystyrene cell culture dishes (Falcon 3001, Becton Dickinson, Franklin Lakes, NJ). These dishes were then subjected to irradiation with a 0.3 MGy electron beam using an Area Beam Electron Processing System (Nissin-High Voltage, Kyoto, Japan). The PIPAAm-grafted dishes were rinsed with cold distilled water to remove non-grafted monomer and sterilized with ethylene oxide gas.

Primary Culture of Limbal Corneal Epithelial Stem Cells on Temperature-Responsive Culture Surfaces

For preparation of lethally treated National Institutes of Health (NIH)/3T3 feeder layers, subconfluent NIH/3T3 fibroblasts were incubated with 16 μg/mL of mitomycin C (MMC) for 2 hours at 37°C, then trypsinized and seeded onto 35 mm temperature-responsive culture dishes at a density of 2×10^4 cells/cm^2. We used more than 50 USA eye-bank eyes. First, we used the 7.5 mm-diameter central regions for penetrating keratoplasty. From the remaining corneoscleral rims, including limbal and peripheral regions, epithelial cells were collected by peeling off all epithelial layers after treatment with dispase II (3 mg/mL, Roche Diagnostics GmbH, Mannheim, Germany) at 37 °C for 1 hour. Collected materials were placed in trypsin/EDTA for 15 minutes to make single cell suspensions. Aliquots (1×10^4 cells/cm^2) were cultured independently of each other (i.e., cells from different eyes were not pooled) in the same dishes as lethally treated NIH/3T3 feeder layers for 2 weeks (*18*). For colony-forming assay, trypsin-EDTA treatment was used to isolate single cells from the limbal and peripheral epithelium and from corneal epithelial sheets harvested from temperature-responsive culture surfaces by reducing the temperature after 2 weeks. Cells were counted, seeded onto temperature-responsive culture surfaces (35 mm in diameter), and cultured with MMC-treated NIH/3T3 feeder layers. After 10- to 12-day cultivation, dishes were fixed and stained with rhodamine B. Colony formation was screened on the entire dish under a dissecting microscope.

Immunohistochemistry

Indirect immunofluorescence microscopy was performed using monoclonal anti-keratin 3 (AE5, Progen, GmbH, Heidelberg Germany), monoclonal anti-p63 (4A4, Santa Cruz Biotechnology, Santa Cruz, CA), and polyclonal anti-type IV collagen (Southern Biotechnology Associates, Birmingham, AL) antibodies. BrdU uptake was examined with a detection kit (no. 1296736, Roche Diagnostics, GmbH, Mannheim, Germany). Fluorescein isothiocyanate- or rhodamine-labeled secondary antibodies were from Jackson Immuno-Research Laboratories (West Grove, PA). Five specimens were examined by all the antibodies. For cross-sectional observation, 10 μm cryosections were subjected to immunostaining. In some specimens, nuclei were costained with a DNA-binding dye (Hoechst 33342, Molecular Probes, Leiden, The Netherlands) or propidium iodide (Sigma, St. Louis, MO). They were observed using a confocal laser scanning microscope (LSM-510, Zeiss, Jena, Germany).

Immunoblotting

Corneal epithelial cells were cultured on temperature-responsive culture dishes for 2 weeks. They were then recovered by one of three different methods: low temperature treatment, dispase treatment, or physical scraping with a rubber blade. All the culture in each experiment was prepared from a single donor eye. Three separate cultures from three different eyes were examined. For low temperature treatment, polymer-grafted surfaces were incubated at 20°C for 30 minutes, after which corneal epithelial cell sheets were detached. For dispase treatment, corneal epithelial cell sheets were harvested after incubation with dispase II (2.5 mg/mL, Roche Diagnostics, GmbH, Mannheim, Germany) at 37°C for 30 minutes. These were washed three times with Dulbecco's phosphate-buffered saline containing a protease inhibitor cocktail (Wako, Osaka, Japan) and 1 mM phenylmethylsulfonyl fluoride and lysed in lysis buffer (20 mM Tris-buffered saline [pH 7.4] containing 0.1% sodium dodecyl sulfate, 8 M urea, and the protease inhibitors). For physical scraping, the cell layers were removed from dish surfaces with a rubber blade in the lysis buffer. The whole cell lysates were electrophoresed on a 7.5% polyacrylamide gel with a 4.5% stacking gel on top in a discontinuous buffer system. Resolved proteins on a polyacrylamide gel were electrophoretically transferred to poly(vinylidene difluoride) (PVDF) transfer membrane (60 V for 3 hours, Immobilon-P, Millipore Corporation, Bedford, MA). The membrane was blocked with 5% bovine serum albumin fraction V and probed with either

rabbit anti-occludin polyclonal antibody (Zymed Laboratories, Inc., San Francisco, CA), mouse anti-desmocolin 3 monoclonal antibody (Dsc3-U114, Progen, GmbH, Heidelberg Germany), or mouse anti-E-cadherin monoclonal antibody (HECD-1, R&D Systems, Inc., Minneapolis, MN). Bands were detected by chemiluminescence of the product of peroxidase reaction using the ECL system (Amersham Biosciences, Buckinghamshire, UK).

Electron Microscopy

Three separate epithelial cell sheets from three different donor eyes were harvested from polymer-grafted surfaces by low temperature treatment. These were dissected, fixed in 2.5% glutaraldehyde for 2 hours, washed with cacodylate buffer, postfixed in 1.0% osmium tetroxide, and dehydrated through graded alcohols. The samples were embedded in Quetal 812 (Nisshin, Tokyo, Japan), and semithin sections (500 nm thick) were cut, stained a few seconds with toluidine blue, and observed under a conventional microscope. Ultrathin sections (90 nm), stained with uranyl acetate and lead citrate, were examined at 80 kV on a H-7000 transmission electron microscope (TEM) (Hitachi, Tokyo, Japan).

Optical Transparency of Transplantable Tissue Constructs

Three types of transplantable tissue constructs were subjected to optical transparency analysis. These were the carrier-free sheets, epithelial cells cultured on fibrin gels (*12*), or amniotic membranes (*8*). All three constructs were prepared from a single-donor cornea in each experiment, and four different sets of these constructs from four different donor eyes were examined. The average thickness of fibrin gels was 44.1±11.7 μm (SD). Amniotic membrane thickness was between 10 and 20 μm. Transmittance at 656 nm was obtained with a multichannel spectrophotometer (PMA-11, Hamamatsu Photonics, Hamamatsu, Japan). Three arbitrary points on each sample were measured. Mean±SD of four different sets of experiments were expressed. Statistical analysis was performed with one-factor analysis of variance (ANOVA) and the Bonferroni-Dunnett method.

Corneal Epithelial Cell Sheet Transplantation in a Rabbit Model

Twenty rabbits were anesthetized and underwent keratectomies of the entire corneal surface including the limbus and surgical excision of all conjunctival tissue within 5 mm of the limbus. A 7.5 mm-diameter trephine punched out the central keratectomized corneal tissue. From the remaining peripheral cornea including limbal tissue, epithelial cells were collected as described above, seeded with 1×10^3 cells/cm^2, and cultured for 3 weeks on temperature-responsive cell culture dishes. Three weeks postsurgery, the entire corneal surface of all rabbits was covered by conjunctival tissue with neovascularization, leading to severe corneal opacity. At this time, the conjunctivalized ocular surface was surgically cleared and reconstructed as outlined schematically in Figure 1. Conjunctival tissue over the cornea was surgically removed to re-expose transparent corneal stroma. Then, the bioengineered corneal epithelial sheet, fabricated in vitro from autologous corneal tissue excised at the initial surgery, was placed on the transparent stromal bed using PVDF membrane transfer. A few minutes after engrafting without suture, the PVDF membrane was simply removed, leaving the cell sheet on the stroma. Finally, the corneal surface was covered with a soft contact lens for healing protection. After surgery, topical antibiotics (0.3% ofloxacin) and steroids (0.1% betamethasone) were applied three times daily. For cell tracing, cultured corneal epithelial cells were stained with 10 nM of a fluorescent dye, PKH26 (Sigma, St. Louis, MO), in serum-free media for 30 minutes at 37°C and washed with serum-free media for 30 minutes at 37°C 1 day before transplantation. Rabbits were killed 10 days after transplantation. The cryosections of regenerated cornea were costained with a DNA-binding dye.

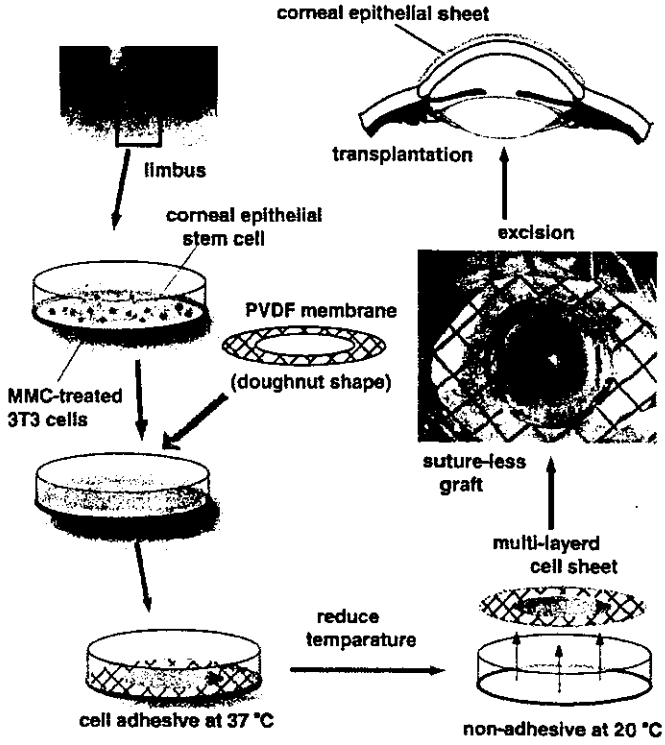

FIGURE 1. Corneal epithelial cell sheet transplantation. Limbal stem cells are collected and seeded on temperature-responsive culture surfaces. After 2 weeks in culture with a mitomycin C (MMC)-treated 3T3 feeder layer, all the multilayered cells are harvested as a cell sheet using a doughnut-shape poly(vinylidene difluoride) (PVDF) membrane after release by temperature reduction. The cell sheet is grafted onto the corneal stroma without sutures.

RESULTS

Corneal Epithelial Cell-Sheet Harvest from Limbal Stem Cell Culture on Temperature-Responsive Polymer-Grafted Culture Surfaces

To create functional bioengineered corneal epithelial sheets suitable for transplantation, we harvested human corneal limbal epithelial cells, containing corneal epithelial stem cells, from a USA eye-bank cornea (Fig. 1). These cells were seeded onto temperature-responsive culture surfaces on which MMC-treated 3T3 feeder cells had been plated 1 day previously. The outcome was highly reproducible (i.e., we always succeeded in making and harvesting cell sheets with similar morphologic characteristics). Corneal epithelial stem cells remain in the presence of feeder cell layers (*19–22*) and other specialized conditions (*23*). Under our culture conditions, epithelial cell colonies grew to reach confluency within 1 to 2 weeks. After a few additional days in culture, a multilayered corneal epithelial sheet forms spontaneously, with cells on the sheet surface exhibiting a cobblestone morphology characteristic of epithelial cells (Fig. 2a). Corneal epithelial cell phenotype was confirmed by immunohistochemistry with an anti-keratin 3 antibody specific for corneal epithelial cells (*1, 24*) (Fig. 2b). BrdU-uptake was observed in a small portion of cells (Fig. 2c). These multilayered corneal epithelial cells could be harvested as a transplantable cell sheet

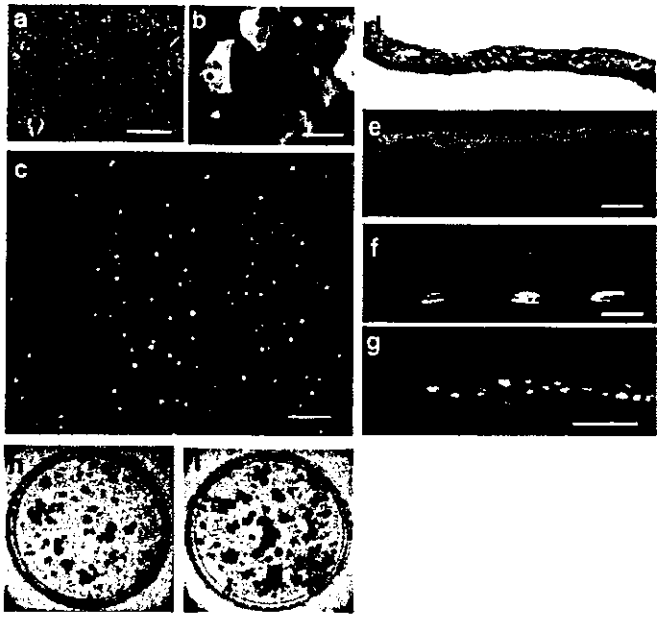

FIGURE 2. Cultured and harvested human corneal epithelial cell sheets. Phase contrast micrograph (a), anti-keratin 3 staining (b), and anti-BrdU staining (green in c) of human corneal epithelial cells cultured on temperature-responsive culture dishes. Hematoxylin eosin (d), anti-keratin 3 (green in e), anti-BrdU (green in f), and anti-p63 (green in g) staining of human corneal epithelial cell sheets harvested by reducing temperature treatment. Nuclei were costained with propidium iodide (red in c, e to g). Colony-forming assay of primary (h) and secondary culture (i). Scale bars: 50 μm in a, b and g; 100 μm in c; 20 μm in e and f.

simply by reducing culture temperature to 20°C, without use of proteases or EDTA. Harvested sheets, flattened on their basal and apical surfaces, comprised three to five cell layers with small basal cells, flattened middle cells, and polygonal flattened superficial cells (Fig. 2d). Such morphologic characteristics are similar to human corneal epithelium in vivo to some extent but are less mature and differ in that the multilayer characteristics seemed to vary depending upon location, and basal cells tended to be flattened, not cuboidal, especially in regions with fewer layers. Interestingly, only the flattened superficial cells expressed keratin 3 (Fig. 2e), whereas BrdU uptake was detected in some basal cells (Fig. 2f). p63, which has recently been proposed as a corneal epithelial stem cell marker (25), was expressed continuously in the basal cells of cell sheets (Fig. 2g). Immunostaining patterns of keratin 3 and p63 are highly similar to those in native limbal epithelium. These observations imply that epithelial stem cells were localized in the basal layer of harvested cell sheets, and differentiated epithelial cells migrated to surface layers as in vivo.

Preservation of Colony-Forming Progenitor Cells in Bioengineered Cultivated Human Corneal Epithelial Sheet

The colony-forming efficiency of the primary cultures was 4.0±2.5% (mean±SD, n=8) for our tissues that had a mean storage time in Optisol of 5.6 days (Fig. 2h). Harvested progenitor cells retained a colony-forming capacity in cell sheets harvested by temperature reduction after 2 weeks in culture (191.2±163.5% (mean±SD) of the primary culture (n=8) (Fig. 2i). This result is significant for the tissue-engineering potential of the viable sheet constructs.

Preservation of Cell-Cell Junctional Structures and Adhesive ECM in Bioengineered Human Corneal Epithelial Sheet after Harvest by Reducing Temperature Treatment

TEM revealed numerous small microplicae and microvilli in the apical cell membranes of superficial cells (Fig. 3, a and b). Also, prominent filamentous glycocalyx was evident. This structure is important for proper visual function because it is intimately associated with the mucus of the tear film layer. Tight junctions were found between lateral membranes of the apical cells, but these appeared less numerous than in vivo. Prominent desmosomes were observed along cell-cell junctions of middle cells. Interestingly, basal cells retained a continuous basement membrane-like linear deposition, although the normal complement of hemidesmosomes was not evident. Immunohistochemical analyses indicate a continuous, thick deposition of type IV collagen in sheets recovered by low temperature harvest, but faint discontinuous deposition is observed in the cell sheets harvested by dispase treatment (Fig. 4, a and b). Immunoblotting revealed that dispase treatment degraded cell-cell junctional proteins including occludin, a tight junction component, E-cadherin, and desmocolin 3, desmosomal components (Fig. 4c). These were maintained during the alternative low temperature harvest.

Transparency of Bioengineered Human Corneal Epithelial Sheet

Optical transparency was compared among the carrier-free cell sheets (n=4) and two transplantable constructs using fibrin gels (n=4) and amniotic membranes (n=4) on which corneal epithelial multilayer cells were developed, as described previously (Fig. 5). Statistical analysis revealed significant differences in light transmission measured by spectrophotometry between the cell sheet architecture and the previously reported corneal epithelial constructs. No significant difference was observed between the fibrin gel and amniotic membrane-carrier constructs.

Corneal Cell Sheets Engrafted In Vivo

We performed autologous corneal transplantation (n=20) with a rabbit model commonly used for human ocular surface

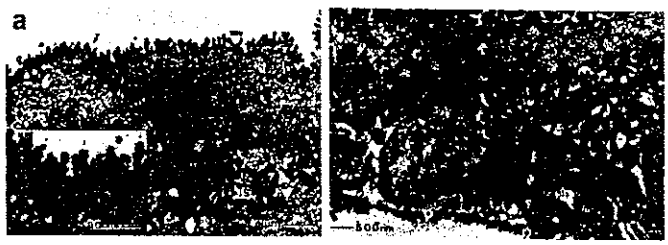

FIGURE 3. Harvested human corneal epithelial cell sheets. Apical (a) and basal surfaces with linear basement membrane-like materials (b) of harvested human corneal epithelial cell sheets. Tight junctions (*single arrow*), desmosomes (*double arrows*). Inlet shows the apical membrane with glycocalyx (*single arrows*) at higher magnification. gly, glycogen granules; mv, microvilli; IS, intercellular space; m, mitochondria; *, basement membrane-like material.

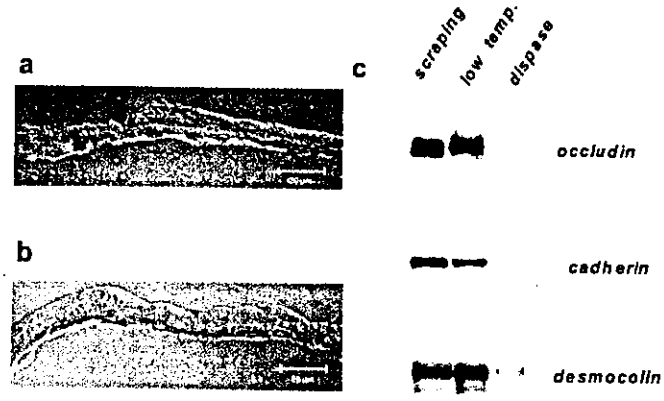

FIGURE 4. **Preservation of basement membrane and cell-cell junctional molecules. Anti-type IV collagen staining of corneal epithelial cell sheets harvested by the present method (a) and with dispase (b). (c) Western blotting with anti-occludin, anti-E-cadherin, and anti-desmocolin 3 antibodies of cell sheets recovered from temperature-responsive culture dishes by scraping, low temperature treatment, or dispase treatment.**

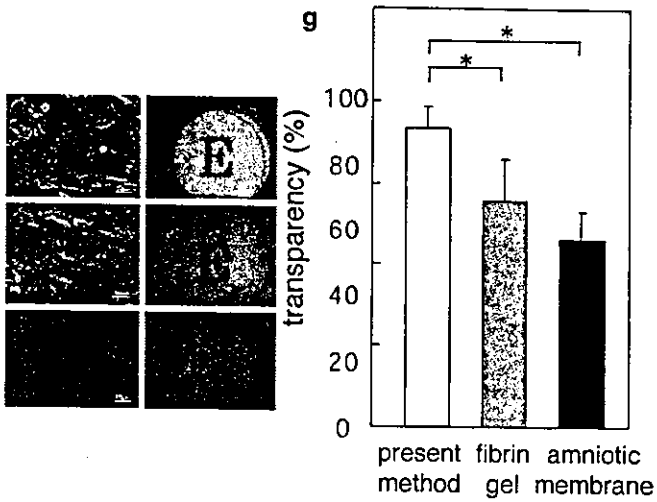

FIGURE 5. **Optical transparency of transplantable constructs. Phase contrast micrographs (a, c, and e) and macroscopic views (b, d, and f) of typescript beneath the present carrier-free construct (a and b), constructs using fibrin gel (c and d) and amniotic membrane (e and f). (g) Optical transparency (n=4, mean±SD) at 650 nm of the present carrier-free construct (*white column*), constructs using fibrin gel (*gray*), and amniotic membrane (*black*). *$P<0.01$.**

disease with limbal stem cell deficiency (*26, 27*). The procedure involves a keratectomy of the entire corneal surface, including the limbus, and excision of the entire conjunctival tissue within 5 mm of the limbus for complete loss of the corneal and limbal epithelium, including corneal stem cells. Three weeks after surgery, conjunctival scar tissue with vascularization from migrating conjunctival cells covered the entire corneal surface, leading to severe corneal opacity. This is the expected result without intervention. For grafting experiments, the conjunctivalized ocular surface was surgically modified again (Fig. 6a). First, conjunctival scar tissue on the cornea was surgically removed to re-expose the native, transparent corneal stroma. Then, cultured, autologous, multilayered corneal epithelial cell sheets were harvested by reducing temperature using a PVDF membrane with an outer diame-

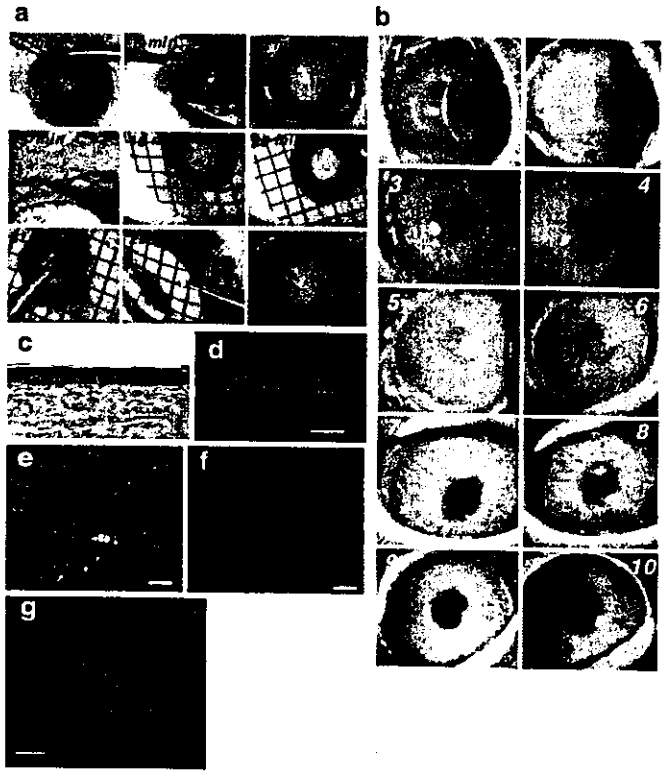

FIGURE 6. **Regeneration of rabbit corneas by the autologous transplantation of bioengineered corneal epithelial cell sheets. (a) Serial photographs of the transplantation procedures. Labeling indicates approximate time stage. Three weeks postsurgery of keratectomy, including the limbus, the entire corneal surface was covered by conjunctival tissue with neovascularization (photograph at 0 min). Conjunctival tissue over the cornea was surgically removed to re-expose transparent corneal stroma (5 min and 10 min). Then, the bioengineered corneal epithelial sheet was harvested from temperature-responsive culture dishes using a supporter (15 min) and placed on the stromal bed (16 min). The sheet readily adhered to corneal stroma in a few minutes without suture (20 min), and the PVDF membrane was simply removed (21 min and 25 min), leaving the cell sheet on the stroma (30 min). (b) Treated eyes were examined without (*1, 3, 5, 7, 9*) or with fluorescein (*2, 4, 6, 8, 10*) before transplantation (*1, 2*), immediately after transplantation (*3, 4*), and 10 days (*5, 6*), 30 days (*7, 8*), and 180 days (*9, 10*) after transplantation. Green fluorescence resulted from the loss of functional epithelial barrier. (c) Hematoxylin eosin stained section of the regenerated cornea 3 weeks after transplantation. (d) Double staining of keratin 3 (red) and nuclei (blue) of the regenerated cornea 3 weeks after transplantation. (e and f) Cultured corneal epithelial cells were stained with a cell-tracer (red) 1 day before harvest. (g) The regenerated cornea 10 days after transplantation of the sheet that had preoperatively been stained with a cell-tracer (red) was stained with a DNA-binding dye (blue) after sectioning. Scale bars: 50 μm in c, d and e, f; 20 μm in g.**

ter of 30 mm and 12 mm-diameter hole punched out of its center. This PVDF geometry permits sufficient support for cell sheet harvest while suspending a central portion of the epithelial sheet freely across the 12 mm-diameter central opening. This unique configuration conveniently allows application of a 12 mm-diameter corneal sheet construct directly onto the surgically prepared corneal surface. The intact multilayered corneal sheet was placed directly over the bare corneal stroma. After 5 minutes of contact after placement, the sheet had readily adhered to the underlying corneal stroma, self-stabilizing without any suturing. The PVDF membrane was then excised.

Immediately after surgery, fluorescein staining showed that the corneal epithelial sheet covered the entire corneal surface homogeneously, reflecting a successful procedure (Fig. 6b). The next day, the graft remained firmly attached to the corneal surface, with some ocular surface inflammation seen. During postoperative healing, ocular surface inflammation subsided, and corneal transparency was restored. During observation periods of up to 180 days, all bioengineered corneal sheet grafts remained stable at the initial placement site, covering the entire corneal surface. The corneal epithelium regenerated by corneal sheet graft healing exhibited a normal appearance, with slightly thin corneal epithelium, cuboidal basal cells, and flattened medial and superficial cells (Fig. 6c). All the epithelial cell layers expressed keratin 3 in each of two regenerated corneas examined by immunohistochemistry (Fig. 6d).

To follow the transplanted corneal epithelial cells after grafting, transplanted cells were stained with a cell-tracer red dye 1 day before surgery (Fig. 6, e and f). Later, the entire thickness of the regenerated corneal epithelium exhibited red fluorescence (Fig. 6g), indicating that the regeneration originated with the transplanted cells.

DISCUSSION

Clinical applications of cultivated corneal epithelial cell transplants are of increasing interest because of burgeoning therapeutic needs. Several trials have been reported to date (*8–10*, *12*, *28*), but several compelling appear to remain. Pellegrini et al. (*28*) initially reported the possibility of the clinical use of corneal epithelial cell sheets. At that time, these were harvested by dispase treatment from culture dishes. Results from the present study show that such proteolytic treatment upon harvest causes marked degradation of cell-cell junctional and ECM proteins, and cell sheets recovered by proteolytic treatment appear to be suboptimal in their adherence to integration with the cornea. In later work, Pellegrini's group switched their method to fibrin gel carriers in an attempt to overcome this problem (*12*).

After Pellegrini's initial report, several investigators reported transplantation of cultivated corneal epithelial cells using carriers such as amniotic membrane (*8–11*) and fibrin gel (*12*) without the use of dispase. In these methods, limbal epithelial cells are cultivated on the carrier, and the resultant constructs, composed of both epithelium and carrier, are transplanted onto the cornea with suturing. Although these methods provide easy handling of the constructs, posttransplant effects from the carrier are expected to influence the clinical outcome, namely corneal transparency and graft survival, postoperatively. Postoperative observations in many cases showed that the amniotic membrane carriers permanently stay between grafted epithelial layers and the corneal stroma, with only loose attachment to corneal stroma. Alternatively, fibrin gel is an attractive carrier for keratinocyte-sheet transplantation, but unlike its application to skin, the gel on the cornea must be resolved completely without any scarring. Even microtrauma or subtle inflammation often leads to corneal opacity with fibrin. Suboptimal performance leading to poor transplant integration and healing is therefore expected to cause some corneal opacification.

In the present study, we show that viable, intact, multilayered corneal epithelial cell sheets can be conveniently produced from limbal stem cells expanded ex vivo on polymer-grafted temperature-responsive cell culture surfaces. Histologic and biochemical analyses clearly demonstrate a well-structured, compact multilayered cell sheet architecture, with the expected native cell microstructures such as microvilli, tight junctions, desmosomes, and basement membrane similar to those in native corneal tissue. Neither destructive proteolytic enzymes nor EDTA are required for cell sheet harvest, and thus cell-cell junctions and basal ECM critical to sheet integrity and function remain intact in our harvested cell sheets. As a result, the sheets are easily manipulated and readily adhesive to tissue and material substrates using endogenous, intact ECM on the basal cell surface. In addition, cell sheets generated on the temperature-responsive culture surfaces retain viable corneal epithelial progenitor cells and are successfully transplantable, producing corneal surface reconstruction in a rabbit model.

Clearly, donor cell survival is a key issue for the long-term success of reconstructive surgeries using bioengineered materials and warrants further investigation. Colony-forming assay revealed a 4.0% primary colony-forming efficiency. This is lower than the 16% to 27% efficiency found by Pellegrini and associates (*29*), possibly because our cells were collected from eye bank eyes that had various death-to-preservation times (range 2.5–13 hours) with subsequent storage in Optisol for an average of 5.6 days. Other distinctions were that Pellegrini's team seeded cells from the limbus only, whereas we used those from the limbus and the peripheral cornea because of partial loss of limbal epithelium at the eye bank, and that our system used an NIH/3T3 feeder layer rather than the J2–3T3 feeder layer that is thought to promote better colonization. Preliminary work (data not shown) indicated that the use of the PIPAAm culture surface is not a factor in the primary colony-forming efficiency because cells grow as well on this as on the plastic plates normally used. A key finding from this study is the fact that cultured cell sheets retain progenitor cell populations close in number to those originally seeded. This result is of particular importance for clinical application of such sheets in transplants.

The rabbit studies showed that convenient and robust tissue transfer to the corneal surface was feasible, facilitating graft placement, stabilization, and adhesion without the trauma of sutures. Our approach also allows corneal regeneration without graft removal. The fact that transplanted sheets adhere strongly to the corneal stroma within a few minutes without sutures is likely attributable to intact ECM on the basal side of the sheet that promotes sheet-stroma integration. Analogously, we previously demonstrated that vascular endothelial cell sheets (*16*) and cardiomyocyte sheets (*17*) harvested from temperature-responsive surfaces easily adhere onto other surfaces by way of this same mech-

anism. An interesting observation here is the differential immunofluorescence of keratin 3 in the cell sheet in vitro compared with the sheet in situ. In the isolated cell sheet, only superficial keratin 3 immunolocalization was seen, whereas keratin 3 was found throughout the whole epithelium 4 weeks after grafting. We suspect that this might be caused by environmental cues in much the same way as Espana et al. (*30*) demonstrated with their identification of keratin 3 in limbal cells when grown in organ culture in the center of a debrided cornea. Further investigation of this mater is planned.

On the basis of the above results from animal experiments, we have recently initiated clinical application of our cell sheet transplantation approach to regenerate damaged ocular surface. So far, we performed the same surgical techniques as those used in the animal experiments in four patients with limbal stem cell deficiencies. Two grafts were allogenic, one autologous and one living-related (mother donor). For the autologous and living-related surgeries, cell sheets were generated from 5×10^3 cells/cm^2 obtained from a 1 to 2 mm^2 limbal biopsy. But because stored tissues were being used for the allogenic surgeries, we seeded twice as many cells (1×10^4 cells/cm^2). Despite the short-term observation periods (6 months, 5.5 months, 3 months, and 2 months), bioengineered corneal sheet grafts remained stable, covering the entire corneal surface. In all cases, corneal transparency was recovered and visual acuity restored.

In summary, cell sheet engineering techniques using temperature-responsive culture surfaces permit the rapid attachment of an intact corneal epithelial sheet without any carriers or sutures. With the present method, the entire corneal surface remains completely covered by the grafted epithelial cell sheet when surgery is finished. This would minimize corneal opacification. Because no biologic materials except epithelial cells and their deposited matrix are grafted, host immunologic reaction can also be minimized. The results shown here suggest promising clinical capabilities for a bioengineered corneal sheet that is ideal for transplantation. The approach overcomes a number of problems associated with other related techniques. Furthermore, the present grafting method without any carriers or sutures could, in principal, be applied to corneal endothelial cells as well as retinal pigmented epithelial cells. Such cell sheets harvested from temperature-responsive culture dishes should enable further functional and histologic tissue replacement by way of integration with host tissues.

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Cell micropatterning using photopolymerization with a liquid crystal device commercial projector

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Abstract

Photopolymerization has been widely used for surface micropatterning. The technique often requires photomasks and light sources with appropriate energies or filters. For rapid prototyping of surface photo-micropatterning, we have developed a novel device by modifying a commercially available liquid crystal device projector. In place of the image expansion unit of the projector, we attached an image reduction unit, an adjustable stage, and an optical monitoring unit. The device projected computer-generated images onto surfaces and subjected these patterns to photopolymerization. Micropatterned images can be easily prepared with various software run on personal computers. With the developed photopolymerization device, micropatterning of poly(ethylene glycol) (PEG) was achieved with PEG-diacrylate and a visible light photopolymerization initiator, camphorquinone. Selective cell adhesion control was also achieved on the micropatterned surfaces.

Keywords: PEG; LCD projector; Cell patterning; Cell culture; Photopolymerization

1. Introduction

Surface micropatterning has been widely investigated for a wide range of biomedical applications from single cell manipulations in basic cell biology [1,2] to complex multi-patterned biochips [3]. Various methods have been exploited to produce reliable patterns, including micro-contact printing [4,5], laser ablation [6,7], photolithography [8–11], inkjet printing [12–14], and microscale self-assembly [15]. Except for inkjet printing and self-assembly, patterning has generally utilized photomasks and energy sources such as light and electron beams [16]. Preparation of photomasks is often time-consuming and expensive. Qin et al. showed the utilization of high quality laser printers and commercial transparency sheets for over-head projectors to prepare quality photomasks [17]. However, high resolution requires an expensive high-end laser printer. In addition, adequate light sources and optics are requisite for precise micropatterning.

In order to overcome these shortcomings, we developed a novel device by modifying a commercially available liquid crystal device projector (LCDP) for rapid photo-prototyping of micropatterned surfaces. Recent innovations have realized large-scale liquid crystal panels with a high density and micrometer scale resolution. A typical pixel size is approximately 20 μm. Such resolution can be useful for micropatterning in several biomedical fields, particularly those associated with cell patterning and arraying technologies. Furthermore, LCDP has a well-controlled light source. Projection images can be easily generated by a personal computer (PC) without any special software. Here we describe cell patterning on surfaces micropatterned with this new device.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) (PEG)-diacrylate (MW = 1000) was kindly provided by NOF Co. (Tokyo, Japan).

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3-methacryloxypropyltrimethoxysilane (MPTMS, Shin-Etsu Chemical Co., Tokyo, Japan), anhydrous methanol (Kanto Chemical Co., Tokyo, Japan), (±)-camphorquinone, *N,N*-dimethyl-*p*-toluidine, anhydrous 1,4-dioxane (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and all other chemicals were used as received. An LCDP (LP-SG7) was kindly provided by SANYO Electric Co. (Osaka, Japan). A objective lens having long working distance (M Plan Apo, 2×, N.A. 0.055) and a tube lens (MT-40) were purchased from Mitutoyo Co. (Kanagawa, Japan).

2.2. Functionalization of glass surfaces

Both sides of cover glass slips (24 × 50 mm, 0.2 mm in thickness, Matsunami Glass Inc., Japan) were treated by oxygen plasma (irradiation intensity: 400 W, oxygen pressure: 0.1 mmHg) for 180 s in a plasma dry cleaner (PX-1000, SAMCO International, Kyoto, Japan) to clean the surfaces. Plasma-treated coverslips were installed in a separable flask, and dried under vacuum for 30 min. Ten milliliters of MPTMS and 500 ml of anhydrous methanol were poured into the flask under nitrogen gas. The coupling reaction of MPTMS with clean, dry coverslip surfaces proceeded under reflux for 24 h at 60°C. The modified coverslips were rinsed repeatedly with methanol and distilled water, and dried for 24 h at 70°C.

2.3. Surface patterning of PEG derivatives by photopolymerization

Surface patterning of PEG derivatives on the coverslip was performed by irradiation of visible light through patterned images on the liquid crystal panel. A typical preparation procedure follows: PEG-diacrylate (0.60 g), camphorquinone (20 mg) as a photopolymerization initiator [18,19], and *N,N*-dimethyl-*p*-toluidine (2.0 μl) as photosensitizer were dissolved in 0.80 g of 1,4-dioxane. The solution (4.0 μl) was dropped on the MPTMS-immobilized coverslip. This solution was then covered with an untreated coverslip (24 × 24 mm), creating a liquid film spread uniformly between the coverslips. The approximate thickness of the solution between coverslips is ~7 μm. The set of coverslips was placed in the LCDP apparatus where the MPTMS-immobilized coverslip was the front side toward the light source. Visible light irradiation was performed directly through the top of the MPTMS-immobilized coverslip for 20 min. After irradiation, the untreated coverslip was stripped off from the microfabricated PEG immobilized on the MPTMS-immobilized coverslip. The resulting PEG-micropatterned coverslip was repeatedly washed with acetone and further characterized.

2.4. Characterization of LCDP photo-patterned surfaces

Micropatterned surfaces were examined under a phase contrast microscope (ET-300, Nikon, Tokyo, Japan) and a scanning electron microscope (S-800, Hitachi, Tokyo, Japan). For electron microscopy, micropatterned surfaces were deposited by a thin conducting gold sputtered layer. Three-dimensional profiles of the surfaces were obtained by reflective confocal laser scanning microscopy (ICM-1000, Leica Microsystems, Wetzlar, Germany) and Tapping mode® atomic force microscopy (AFM) (Nano Scope IIIa, Digital Instruments, Santa Barbara, CA). Micropatterned surfaces were stained with a hydrophobic fluorescent dye, DiIC18 (Molecular Probes, Eugene, OR), at concentration of 25 μg/ml for 20 min, and washed with distilled water. Fluorescence images on DiIC18-stained micropatterned surfaces were obtained with a microscope equipped with an epifluorescence unit.

2.5. Cell culture

Bovine aortic endothelial cells (JCRB0099) were provided from Japan Health Science Foundation and cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Before cell seeding, micropatterned surfaces were incubated with fibronectin solution (50 μg/ml in Dulbecco's phosphate buffered saline, pH 7.4) at 37°C overnight and then washed. Endothelial cells (5.0×10^4 cells/ml) were seeded on these treated glass coverslips. Cell morphology in culture was monitored under a phase contrast microscope (ET300, Nikon, Japan).

3. Results

3.1. PC-controlled photopolymerization system

Typically a commercial LCDP connected to computers generating images will automatically expand the images to project them onto a large screen. Attached adjustable lenses permit focus of the projected image. Fig. 1a shows the optical diagram of typical projectors. Light irradiated from the lamp (120 W) is decomposed though the dichroic mirror to three colors: red, green and blue. After passing through each liquid crystal panel, the three-color light beams are re-integrated again by the second dichroic mirror. Then, the integrated light is expanded with a projection lens and projected onto a surface external to the projector. For our purposes, the optical path was modified as follows (see Figs. 1b and c). In the modified LCDP unit, light illuminated from the

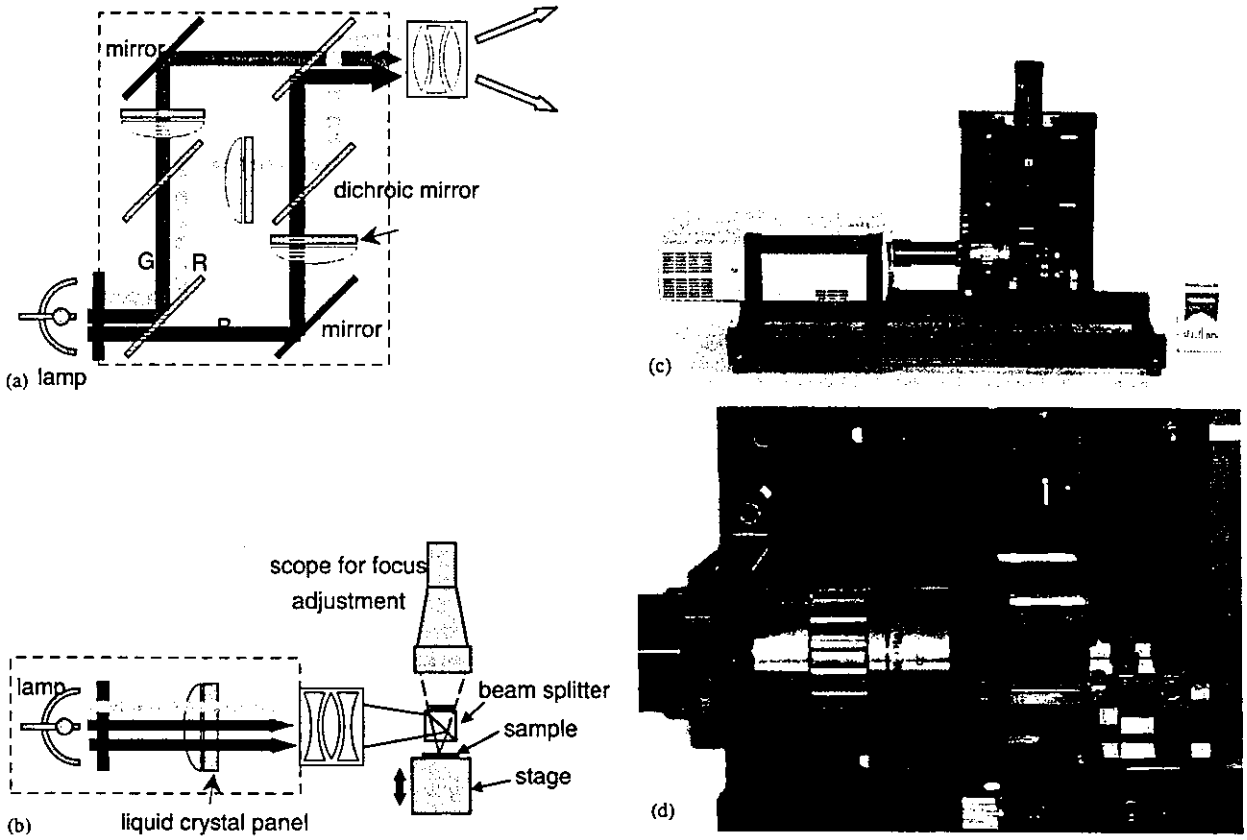

Fig. 1. Photopolymerization device. Schematic optical diagrams of LCD (a) and the modified device (b). The overall view of the device (c) and the magnified view of additional optics (d).

lamp passed through only one liquid crystal panel without light decomposition. The emitted light was down-sized by the projection lens, turned downward by the beam splitter, then irradiated onto the sample stage. For focus adjustment, the position of the sample stage was monitored and controlled (Fig. 1d). The LCDP used here has three liquid crystal panels each of 1.78 cm in diagonal consisting of 480,000 pixels (800 × 600). Each square pixel has sides of 18 μm on the liquid crystal panel, but was reduced through the projection optics. In the present study, the reduced individual pixel size was fixed to 10 μm, so that the final projection area was 8 × 6 mm. Mask patterns were generated on a typical interfaced PC with commercially available software such as Microsoft Word® and Microsoft Power Point®. The same images appearing on the PC monitor were projected onto the sample stage in a reduced size by the re-arranged internal LCDP optical path.

3.2. Micropatterned PEG graft on glass surfaces

PEG was covalently grafted onto silanated glass surfaces in order to confirm the fidelity of the surface micropatterning using the novel device. An image comprising black and white domains was used as the projected mask, although the device could also project images in an eight-bit gray scale. After photopolymerization, the resultant micropattern was easily observed under a phase contrast microscope, with generally high fidelity with the mask image generated by the PC (Fig. 2a). Each micropattern comprised small square dots having 10 μm sides. Because of the shading from embedded electronic wires aligned between pixels on the liquid crystal panel to switch each pixel on and off, small gaps were created among the square dots (arrow in Fig. 2c). Scanning electron microscopy revealed that the micro-patterned surfaces had micrometer-scale three-dimensional structures (Fig. 3). Three-dimensional profiles of the micropatterned surfaces were obtained by means of reflective confocal laser scanning microscopy and AFM (Fig. 4). The height difference was around 0.9 μm between the convex and concave domains, corresponding to white and black domains in PC-generated images, respectively.

In the present method, glass surfaces were at first treated with a surface-reactive cleaning plasma and introduced with silane-coupled double bonds prior to photopolymerization. After plasma treatment, glass surfaces were highly hydrophilic but changed to hydrophobic by the reactive silane coupling. After

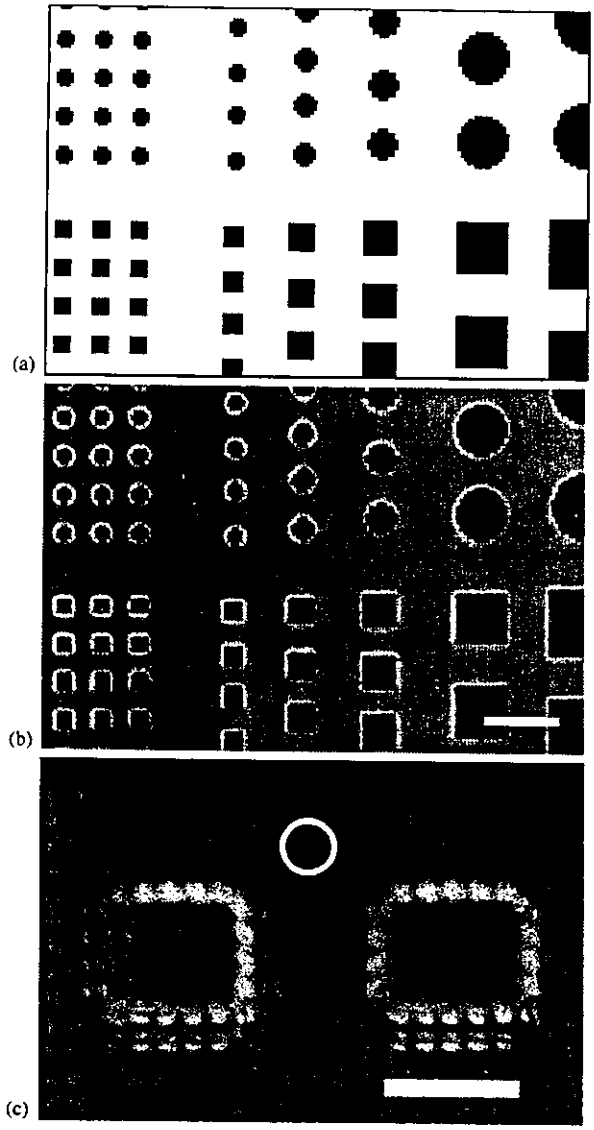

Fig. 2. Mask image generated by the PC (a), and phase contrast microscopy of micropatterned surfaces (b, c). Note small gaps among square dots (circle in (c)) Scale bar = 200 μm (b), 50 μm (c).

hydrophobic fluorescent dye (DiIC18) staining, convex patterned polymer domains corresponding to white domains in mask images showed a weak red fluorescence derived from the dye, but the concave domains corresponding to black domains in mask images did not exhibit fluorescence derived from the dye (Fig. 5). This observation strongly suggested that convex domains were slightly hydrophobic, while the concave domains were highly hydrophilic.

3.3. Micropatterned cell culture

Cell culture and in vitro adhesion experiments clearly confirmed that the surfaces formed PEG micropatterning according to exactly the original images designed

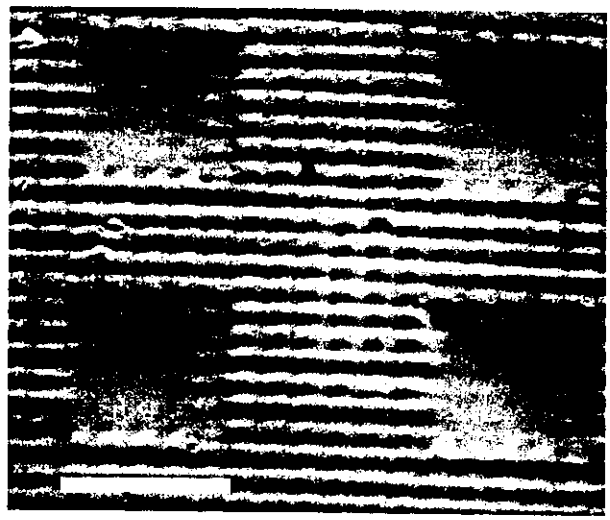

Fig. 3. Scanning electron microscopy of micropatterned surfaces. Scale bar = 50 μm.

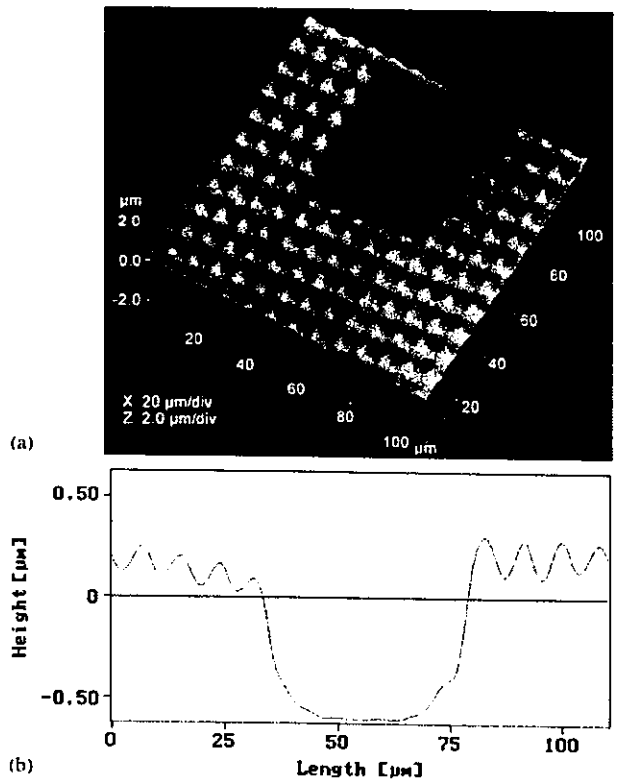

Fig. 4. Atomic force microscopy of micropatterned surface. Three-dimensional (a) and two-dimensional profiles (b).

with PC. On the silane-introduced glass surfaces, cell adhesion was hardly observed, even in the presence of serum or fibronectin (data not shown). On the micropatterned PEG surfaces, endothelial cells specifically adhered to the convex domains (Fig. 5). On the concave domains, cell adhesion was completely inhibited. Thus, micropattened cell seeding was achieved with a relatively

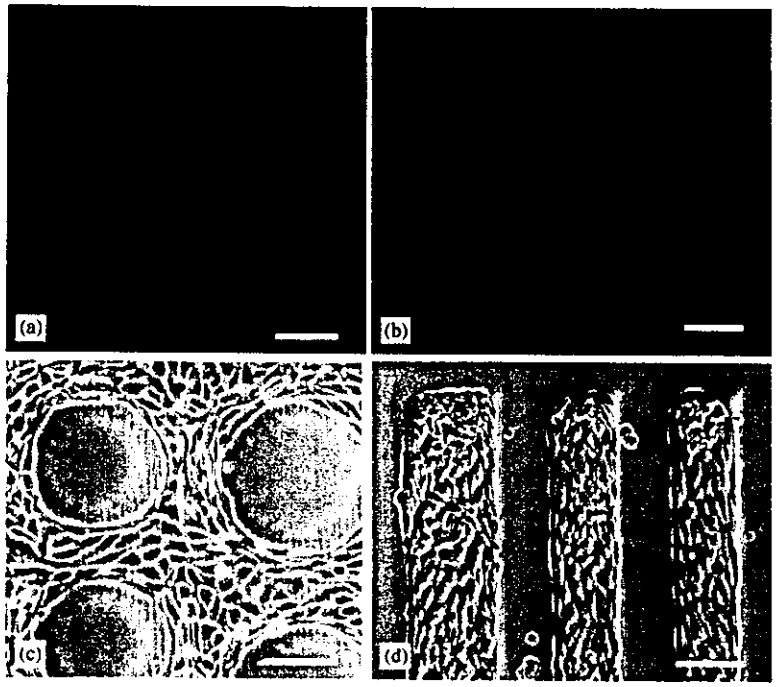

Fig. 5. Hydrophobic dye staining of micropatterned surfaces and cell culture. Micropatterned surfaces were subjected to either fluorescent staining with DiIC18 (a, b) or cells seeding (c, d). Micropatterned cell culture was microphotographed after one-day culture. Scale bar = 100 μm.

simple fabrication technique. The cell micropattern fidelity was maintained intact more than one month in the presence of serum culture. No cytotoxicity was observed during the culture (data not shown).

4. Discussion

Micro-patterned surfaces were fabricated easily and quickly with the present LCDP device without a need for expensive pattern masks, an additional light source, or other optical accessories. Even with the present typical LCDP, pattern fidelity and resolution is relevant to several biomedical applications in diagnostic arraying and microfluidics. Recent progress in high resolution and high density LCDP devices will achieve much finer micro-patterning required for other more advanced applications.

Results of the photopatterning prompt several questions about the spatial photochemistry occurring on the silane-coupled glass surfaces. From the following three lines of observation, we concluded that PEG was photo-grafted on both patterned (e.g., white and black) domains but at different degrees of polymerization under the present conditions. First, three-dimensional profiles were obtained with micropatterned surfaces. These profiles reflected the PC-generated images with high fidelity, where the white (photo-exposed) domains produced convex surface-grafted domains (Figs. 2–4) within a matrix of concave domains (non-photo-exposed regions). Vertical line topological resolution between each domain at their interface was not square, indicating some interfacial zone of continually changing topology that never reached to the glass-silane primed surface. This suggests that concave domains are covered by some photochemistry. Second, a hydrophobic fluorescent dye, DiIC18, specifically bound to the convex domains (Fig. 5). Such hydrophobic dye partitioning has been observed in many interfacial chemistry situations and is indicative of a preferred dye binding environment. Third, seeded cells selectively adhered the convex domains (Fig. 5). The concave domains repelled cells for a long time in serum-containing culture. Taken together, we assert that PEG was photo-grafted even onto the concave domains, which were masked with black domains in PC-generated images. While liquid crystal panels equipped inside the projector can completely shield emitted light to achieve black color, the irradiated light can be readily diffracted or scattered at the glass surface on both faces, within the glass substrate and between the glass sandwiching plates. Such effects could compromise the masking fidelity, permitting some light access to black-masked regions and permitting photochemistry here as well, but to a limited extend. Therefore, PEG is likely also grafted to some limited extent onto the black domains where directly irradiated light was otherwise completely shielded. Under these conditions, grafted PEG in these regions was much thinner (i.e., in the concave domains) than in the convex domains where emitted light was

irradiated directly through the liquid crystal panel at substantially higher intensity and photochemical efficiency. Molecular weight of the PEG-diacrylate used in the present study was 1000. In previous studies, protein and/or cell adhesion on PEG-grafted materials were reduced with increasing PEG molecular weight [20–22]. However, adhesion behavior on PEG-grafted surfaces has not been solely explained by PEG molecular weight effects [23]. Typical hydrogels prepared by chemical or radiation-induced procedures have cross-linking points distributed throughout the hydrogel where distances between relatively hydrophobic (e.g., acrylate or acrylamide linkages) in the gel network are spanned by relatively hydrophilic ethylene oxide chains of different lengths. By sharp contrast to previous work with longer PEG acrylates, the network structure of current grafted PEG hydrogels is considered to comprise shorter oligo-PEG acrylates where the crosslink points (hydrophobic domains) are more chemically dominant over the shorter spanning PEG chains (hydrophilic domains). Hydrophobic oligoacrylate sequences with lower PEG molecular weights would therefore be predictably exposed to adsorbing serum proteins [24,25], resulting in possibly selective adsorption of fibronectin on the convex domains. In support of this claim, hydrophobic fluorescent dye (DiIC18) was observed to selectively adsorb onto or within the convex domains. These correlative data imply that convex domains subject to intense direct LCDP irradiation are highly polymerized acrylate domains with less PEG mobility, reduced water content and generally increased local hydrophobicity capable of serum protein and dye interactions. It is plausible that the majority of the PEG was photo-immobilized at the both terminal ends to allow sufficient serum protein adsorption and subsequent cell adhesion on the convex domains. By contrast, our contention is that indirectly irradiated, less reacted PEG immobilized on the masked black regions (concave domains) maintains less crosslinking, more free terminal PEG ends, and more single-point grafting with hydrated mobility that reduces protein adsorption, and repels cells in culture.

As shown in Fig. 5, seeded cells were confined within the convex cell adhesive domains. Cells in the vicinity of edges of cell adhesive domains showed highly elongated shapes, and the cell long axis was completely parallel to the edge. To the contrary, cells in the center of cell adhesive domains did not show such a cell orientation. These findings imply that the micropattern fidelity achieved by the present method is sufficient for biomedical applications utilizing biomolecules and cells. The present LCDP device has both a liquid crystal panel imaging PC-generated patterns and a strong light source. Because of availability, cost and ease of modification to accommodate photo-patterning, such an all-in-one device should prove useful for the preparation of micro-patterned surfaces for biomedical applications in a rapid prototyping manner.

5. Conclusion

We developed an all-in-one device for photopolymerization-based surface micropatterning by modifying a commercially available liquid crystal device projector. Micropatterned surfaces were fabricated from the images prepared with various softwares run on personal computers. With PEG-diacrylate and a visible light photopolymerization initiator, camphorquinone, 10-μm resolution, which seems sufficient for biomedical applications, was obtained. On the micropatterned surfaces, selective cell adhesion control was also achieved.

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