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Technical note

Photolithographic patterning of polyethylene glycol hydrogels

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Abstract

A simple, inexpensive photolithographic method for surface patterning deformable, solvated substrates is demonstrated using photoactive poly(ethylene glycol) (PEG)-diacrylate hydrogels as model substrates. Photolithographic masks were prepared by printing the desired patterns onto transparencies using a laser jet printer. Precursor solutions containing monoacryloyl-PEG-peptide and photoinitiator were layered onto hydrogel surfaces. The acrylated moieties in the precursor solution were then conjugated in monolayers to specific hydrogel regions by exposure to UV light through the transparency mask. The effects of UV irradiation time and precursor solution concentration on the levels of immobilized peptide were characterized, demonstrating that bound peptide concentration can be controlled by tuning these parameters. Multiple peptides can be immobilized to a single hydrogel surface in distinct patterns by sequential application of this technique, opening up its potential use in co-cultures. In addition, 3D structures can be generated by incorporating PEG-diacrylate into the precursor solution. To evaluate the feasibility of using these patterned surfaces for guiding cell behavior, human dermal fibroblast adhesion on hydrogel surfaces patterned with acryloyl–PEG–RGDS was investigated. This patterning method may find use in tissue engineering, the elucidation of fundamental structure–function relationships, and the formation of immobilized cell and protein arrays for biotechnology.

Keywords: Photolithography; Biomimetic; Patterning; Polyethylene glycol

1. Introduction

Controlling cell–microenvironment interactions is important in generating tissue engineered constructs that mimic native tissue architecture and for guiding cellular differentiation and organization. Recent advancements in patterning technologies have significantly enhanced our ability to spatially control surface chemistry and topography at the micrometer level and thus our ability to tailor cell microenvironment. Common patterning methods include including photolithography [1,2] and soft lithographic approaches such as microcontact printing [3], microfluidic patterning [4,5], and micromolding [6,7]. These techniques have been widely used for the high fidelity patterning of rigid substrates, such as modified silicon or glass [8,9]. However, the surface patterning of deformable, solvated, biocompatible platforms relevant to tissue engineering, such as hydrogels, has not received a similar degree of attention.

Given the importance of deformable, hydrated substrates as scaffolds for soft tissue engineering applications, development of methods for direct, high fidelity patterning of these platforms is desirable. This work develops transparency-based photolithography as a simple, versatile, and inexpensive technique for surface patterning bioactive peptides and 3D structures onto hydrated, photoactive poly(ethylene glycol) (PEG)-based hydrogel substrates. PEG-diacrylate (PEGDA) hydrogels are biocompatible and intrinsically resistant to protein adsorption and cell adhesion. In addition, acrylate-terminated PEG macromers undergo rapid polymerization upon exposure to UV light when in the presence of appropriate photoinitiators [10,11]. Thus, the material properties and photoactivity

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of PEGDA hydrogels can be exploited to tailor in desired bioactivity via light-based patterning [12]. In transparency-based photolithography, masks are prepared by printing the desired patterns onto transparencies using a standard laser jet printer, obviating the need for expensive equipment and clean room use for mask fabrication. The range of feature sizes and shapes that can be generated using this methodology are investigated, and the effects of UV irradiation time and precursor solution concentration on the patterning outcome are characterized. Furthermore, the feasibility of using these patterned hydrogels to control cell behavior is evaluated by examining human dermal fibroblast (HDF) adhesion onto ACRL–PEG–RGDS patterned hydrogels.

2. Materials and methods

2.1. Cell maintenance

HDFs (Cambrex) were maintained in MEM (ATCC) supplemented with 10% FBS, 100 U/L penicillin, and 100 mg/L streptomycin at 37 °C/5% CO_2. Cells were used at passages 6–9. All cell culture reagents were obtained from Sigma unless otherwise noted.

2.2. Polymer synthesis

PEGDA was prepared by combining 0.1 mmol/mL dry PEG (3400 MW, Fluka), 0.4 mmol/mL acryloyl chloride, and 0.2 mmol/mL triethylamine in anhydrous dichloromethane (DCM) and stirring under argon overnight. The resulting solution was washed with 2 M K_2CO_3 and separated into aqueous and DCM phases to remove HCl. The DCM phase

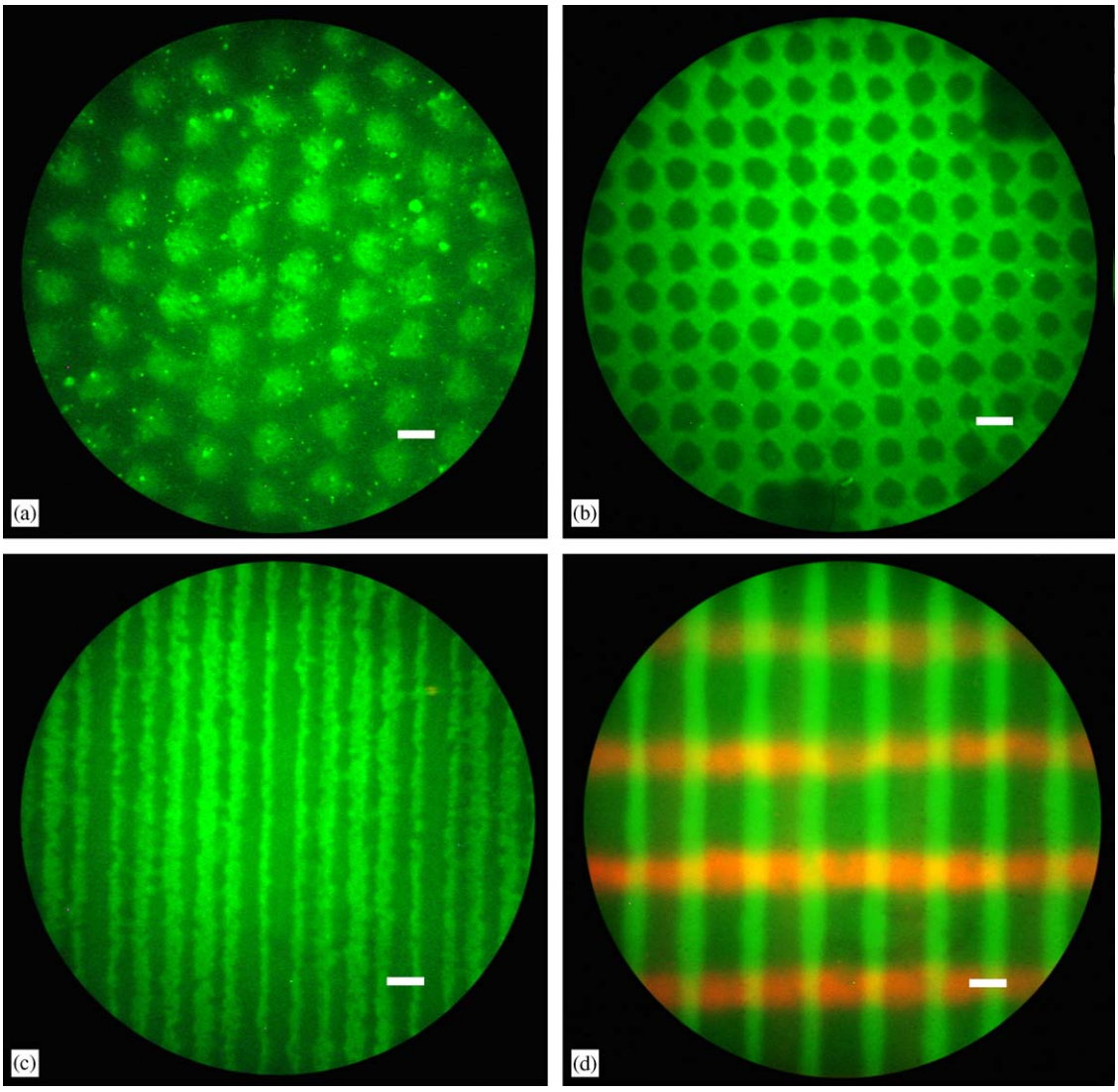

Fig. 1. (a–c) Fluorescence images of PEGDA hydrogels patterned with ACRL–PEG–RGDS which demonstrate the pattern types and feature sizes that can be readily created using transparency-based photolithography. Both exclusion and inclusion patterns can be formed (a, b) as can gradient patterns based on spatial variations in pattern spacing and thickness (c). (d) A fluorescence image of a PEGDA hydrogel patterned with two different cell adhesion peptides. Patterned ACRL–PEG–RGDS is shown in green and ACRL–PEG–REDV in red. Scale bars = 250 μm.

was subsequently dried with anhydrous $MgSO_4$, and PEGDA was precipitated in diethyl ether, filtered, and dried under vacuum.

2.3. Fluorescently labeled monoacryloyl PEG-peptide synthesis

Peptides RGDS (American Peptide) and REDV (American Peptide) were conjugated to PEG (3400 MW) by reaction with acryloyl-PEG-*N*-hydroxysuccinimide (ACRL–PEG–NHS, Nektar) at a 1:1 M ratio for 2 h in 50 mM sodium bicarbonate buffer, pH 8.5. Alexa Fluor 488 carboxylic acid, tetrafluorophenyl (TFP) ester (Molecular Probes) was then added to the ACRL–PEG–RGDS reaction mixture at approximately 10 mole dye per mole ACRL–PEG–RGDS and allowed to react for 1 h at room temperature. Alexa Fluor 594 carboxylic acid, succinimidyl ester (Molecular Probes) was similarly added to the ACRL–PEG–REDV and allowed to react overnight at room temperature. The desired products were purified by dialysis and then lyophilized.

2.4. Photolithographic method

The desired patterns were prepared using Photoshop and printed onto transparencies using a standard laser jet printer (LaserWriter 16/600 PS). A solution of 10% (w/v) PEGDA in HEPES-buffered saline (HBS, pH 7.4) was prepared. Ten μL/mL of 300 mg/mL 2,2-dimethoxy-2-phenyl-acetophenone (DMAP) in *N*-vinylpyrrolidone (NVP) was added to the solution, which was then polymerized between two clamped glass plates separated by 0.5 mm spacers via exposure to UV light (B-200SP UV lamp, UVP, 365 nm, 10 mW/cm^2) for 1 min.

The upper glass plate was removed, the surface of the hydrogel was rinsed with sterile PBS, and the spacers were altered as needed to match desired feature height (in the case of 3D patterning). A thin layer of precursor solution of either 30 μmol/mL ACRL–PEG–peptide (for monolayer patterns) or 10% (w/v) PEGDA (for 3D patterns) dissolved in HBS and containing 1% (v/v) 300 mg/mL DMAP in NVP was applied to the gel surface. The patterned transparency mask was layered over the precursor solution with the printed side facing the hydrogel surface, and the upper glass plate was replaced and clamped. The acrylate-derivatized moieties in the precursor solution were then conjugated to specific regions of the hydrogel surface by applying UV light through the transparency mask for 45 s (monolayer patterns) or 1 min (for 3D patterns). The mask was removed and the gel rinsed with sterile PBS to remove unbound precursor. To immobilize multiple peptides in distinct patterns on the same hydrogel surface, sequential patterning processes were carried out with intermediate PBS wash steps.

2.5. Quantification of bound peptide concentration

The effects of UV irradiation time and precursor solution concentration on the degree of precursor immobilization were characterized by using the ninhydrin assay to quantify the amount of ACRL–PEG–RGDS conjugated to PEGDA gel surfaces under various patterning conditions [13]. All hydrogel substrates to be patterned were prepared with a uniform UV exposure time of 45 s, resulting in the surface density of free acrylate groups being approximately constant across gel substrates. To investigate the relationship between bound surface peptide and precursor solution concentration, ACRL–PEG–RGDS precursor solutions were prepared and spread onto hydrogel surfaces such that the initial peptide surface concentrations of 0.144, 0.25, 0.718, and 1.85 mg/cm^2 were achieved. These solutions were then exposed to UV light through a blank transparency mask for 45 s in the presence of photoinitiator. Similarly, to examine the dependence of bound surface peptide concentration on UV exposure time, a precursor solution of ACRL–PEG–RGDS was layered onto hydrogel surfaces such that the initial surface peptide concentration on each was 0.718 mg/cm^2. These gels were then exposed to UV light through a blank transparency mask for 9, 45, and 180 s.

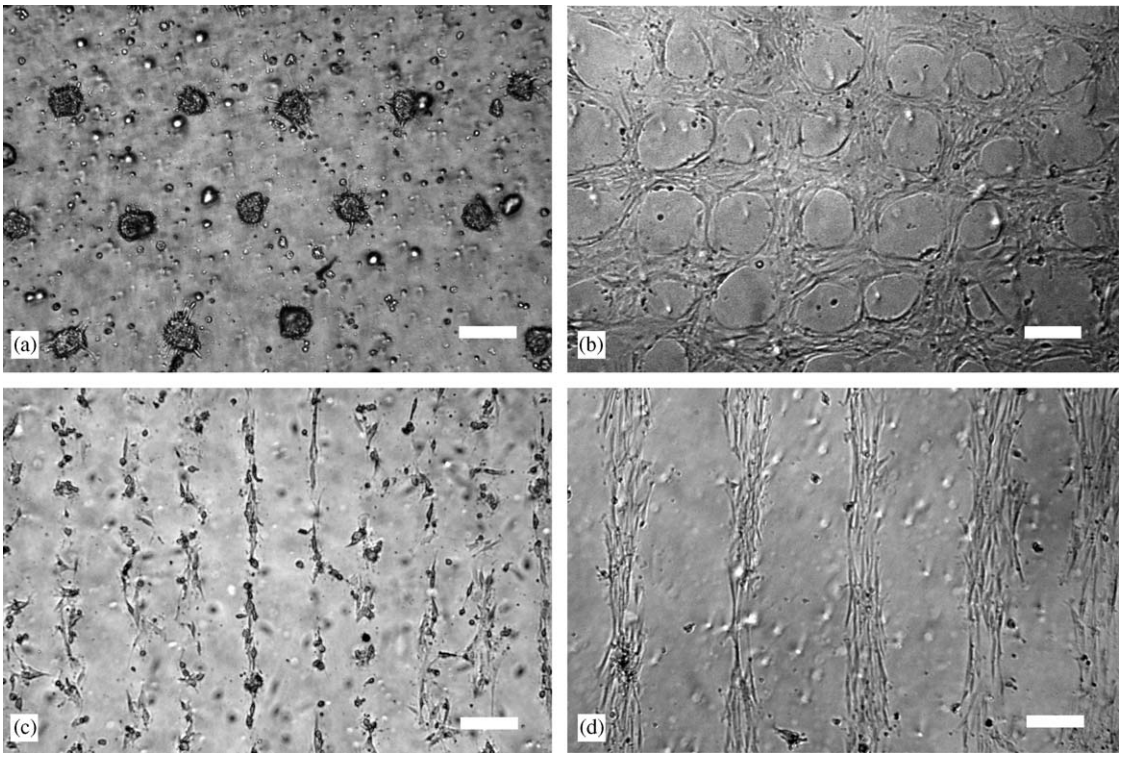

Fig. 2. Phase contrast images of HDFs attached to the surface of the ACRL–PEG–RGDS patterned hydrogels shown in Fig. 1. Note that in (d), HDFs have bound to RGDS patterned regions but not to REDV patterned regions, as expected. Scale bars = 200 μm.

Each patterned hydrogel was cut into three 0.7 cm diameter discs and washed extensively on an orbital shaker for 3 days in PBS containing 0.2 mg/mL sodium azide. The discs were then hydrolyzed in 150 μL of 6 N HCl at 150 °C for 100 min, and the resulting solutions were dehydrated and their acid removed using a rotavap. The free amino acids were re-dissolved in 100 μL of 0.1 M sodium citrate buffer (pH 5.0), followed by the addition of 100 μL of ninhydrin reagent (Spectrum). The samples were boiled for 15 min, cooled, and their absorbance at 570 nm read. PEGDA hydrogels containing known amounts of ACRL–PEG–RGDS were used as controls.

2.6. Cell patterning

The bioactivity of patterned cell adhesion peptide RGDS was confirmed by seeding HDFs onto the surface of ACRL–PEG–RGDS patterned hydrogels. Prior to cell seeding, patterned hydrogels were exposed to media for 1 h at 37 °C. After 24 h, unbound cells were rinsed away. Cell adhesion, localization, and morphology were examined via phase contrast microscopy (Zeiss) at days 1 and 4.

3. Results and discussion

Figs. 1a–c demonstrate the range of 2D pattern types and feature sizes that can be created on PEGDA hydrogel surfaces using transparency-based photolithography. The minimum feature size that has been obtained with the current printer and UV lamp light source is ~40 μm (Fig. 1c). This relatively large minimum feature size should not generally be a limitation for tissue engineering applications, since mammalian cells have repeatedly been shown to apoptose for 2D feature sizes under ~20 μm × 20 μm [3]. However, further reduction in minimum feature size can potentially be achieved by using a higher resolution printer in mask fabrication and/or a collimated light source. Patterns composed of repeating spatial gradients in stripe spacing and thickness are also rapidly generated using this technique (Fig. 1c). Spatial concentration gradients prepared by modulating the spacing/thickness of patterned stripes of bioactive moieties have been shown to influence cell alignment, morphology, and locomotion [14,15]. Thus, transparency-based photolithography can be applied to the investigation of the effects of spatial variations in peptide density on cell behavior.

Multiple peptides can be immobilized in distinct patterns on the same hydrogel surface by sequential application of the patterning technique, as demonstrated in Fig. 1d, which shows a hydrogel patterned with the generic cell adhesion peptide RGDS and the endothelial cell specific adhesion peptide REDV [16]. This feature opens the possible extension of this technique to preparation of co-cultures and multiple cell arrays. The bioactivity of immobilized cell adhesion peptide RGDS was confirmed by seeding HDFs onto the surface of the ACRL–PEG–RDGS patterned hydrogels shown in Fig. 1. HDFs adhered to and spread on only the RGDS patterned regions of these hydrogels and did not bind to unpatterned or REDV patterned regions, as expected (Fig. 2). In addition, this HDF spatial localization was retained throughout the observed culture period.

As previously mentioned, the amount of precursor immobilized to the hydrogel surface during patterning is dependent on several variables, including the surface density of free acrylate groups, the precursor solution concentration, and the UV light intensity and exposure time used in patterning. Fig. 3a illustrates the dependence of bound ACRL–PEG–RGDS concentration on the initial surface concentration provided by the precursor solution with patterning exposure time held constant. Initially, as the precursor surface concentration increases, the concentration of conjugated peptide increases rapidly. However, at higher precursor surface concentrations, the efficiency of the immobilization reaction decreases, and eventually the amount of bound peptide begins to saturate the free acrylate groups available on the gel surface.

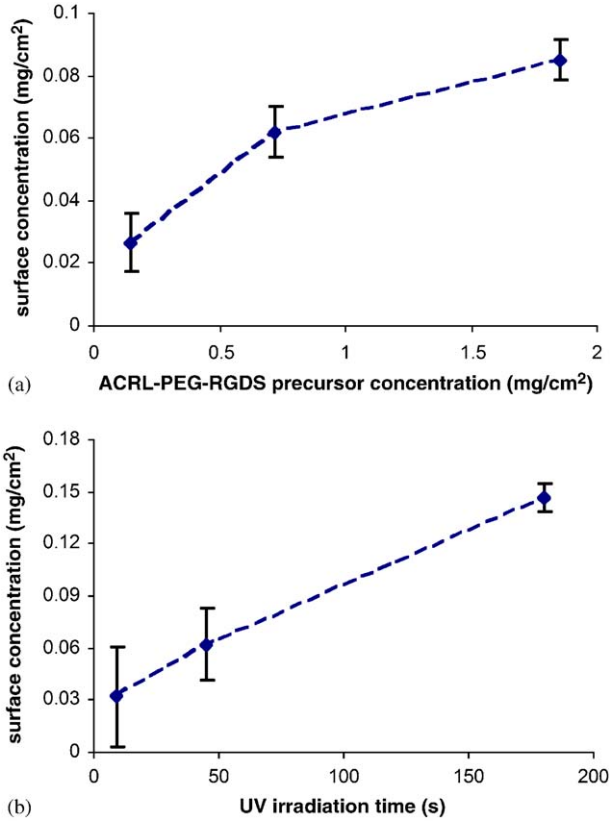

Fig. 3. (a) Demonstration of the effect of initial precursor surface concentration on the levels of immobilized ACRL–PEG–RGDS assuming constant UV exposure time. Note that the slope of the curve decreases within increasing precursor concentration, indicating decreasing immobilization efficiency with increasing precursor concentration. (b) Demonstration of the effects of UV exposure time on the levels of immobilized ACRL–PEG–RGDS with an initial precursor surface concentration of 0.718 mg/cm^2. Surface concentration depends linearly on UV irradiation time over a wide range of exposure times.

Fig. 3b shows the dependence of immobilized precursor levels on UV irradiation time at a fixed initial precursor surface concentration. The levels of bound ACRL–PEG–RGDS increase approximately linearly over a wide range of exposure times for the selected precursor surface concentration, although this relationship will likely become non-linear with longer exposure times and/or higher precursor surface concentrations [2]. Thus, by designating patterning exposure time and precursor surface

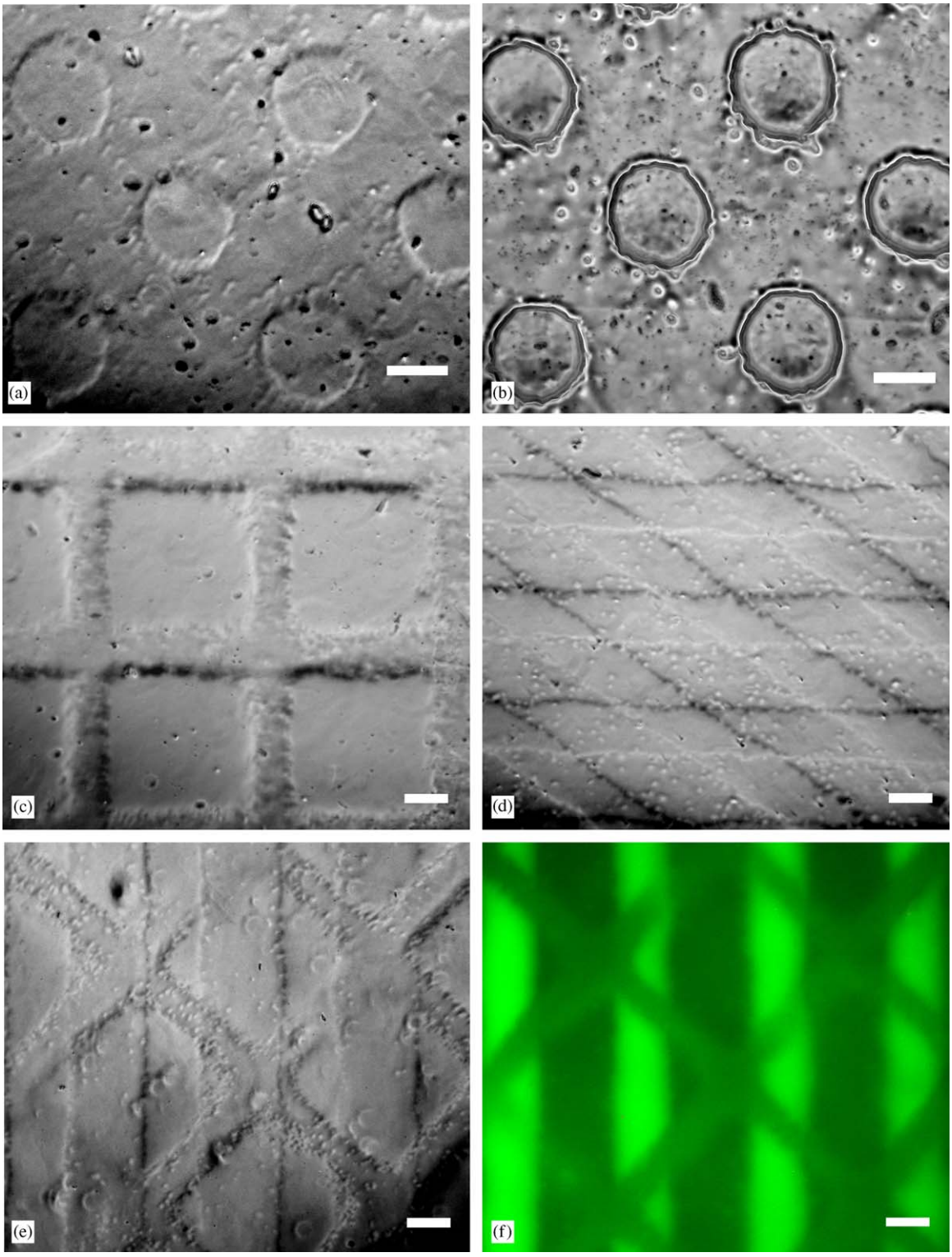

Fig. 4. (a, b) Phase contrast images of PEGDA hydrogels patterned with 3D structures using the same exclusion pattern but with different spacing between the hydrogel surface and the transparency mask. These images demonstrate that a range of feature heights can be created while maintaining lateral and axial feature fidelity. (c) A phase contrast image of a patterned PEGDA hydrogel which illustrates that 3D structures with sharp edges can be created with high fidelity. (d, e) Phase contrast images of multilayer 3D patterns generated by sequential patterning of a PEGDA precursor solution on a pure PEGDA hydrogel base (d) and on a PEGDA hydrogel base containing fluorescently labeled ACRL–PEG–RGDS (e). (f) The corresponding fluorescence image for (e) demonstrating the cell-adhesive wells created by multilayer patterning. Scale bars = 200 μm.

concentration, the amount of immobilized peptide can be controlled by the user. The ability to specify the levels of bound peptide is highly desirable in applications where defined surface modification is required.

When diacrylate-derivatized PEG macromers are incorporated into the precursor solution, a range of 3D structures can be surface patterned onto PEGDA hydrogels (Figs. 4a–d). The axial dimensions of the 3D structures can be controlled by altering the spacer thickness separating the glass plates used in patterning (Figs. 4a and b). Three-dimensional microstructures have been used to provide topographical cues to control cellular organization [17,18]. Since both monolayer and 3D features can be formed using transparency-based photolithography, this technique should allow for the controlled study of the interplay between feature 2D geometry and topography on cell behavior.

Multilayered 3D structures can also be generated via sequential application of this patterning method (Figs. 4d–f). The multilayered PEGDA patterns in Figs. 4e and f were created on a hydrogel substrate containing fluorescently labeled ACRL–PEG–RGDS, with the patterned PEGDA structures containing no cell-adhesive peptide. Thus, cell adhesion should occur only in the exposed/unpatterned regions of the hydrogel base, resulting in isolated wells of adherent cells. The ability to generate cell clusters separated by topographical barriers is useful in applications where cell outgrowth from the patterned region is unfavorable [7] or where cell guidance via barriers is desired [19]. Multilayer patterning also opens up the potential for the creation of spatially complex 3D scaffolds for tissue engineering applications.

4. Conclusions

This study demonstrates a simple inexpensive technique for patterning monolayers and 3D structures onto solvated, deformable substrates with high fidelity. Although it has been applied specifically to hydrated PEGDA gel substrates, this method for covalently immobilizing biomolecules in 2D and 3D can be expanded to a variety of other photoactive substrates, either solvated or non-solvated. This technique also has a number of advantages over other patterning methods, the foremost being its simplicity and low expense. The flexibility of this method in terms of feature topography, control over bound peptide concentration, and substrate type should prove useful in a range of biotechnology and tissue engineering applications.

Acknowledgements

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