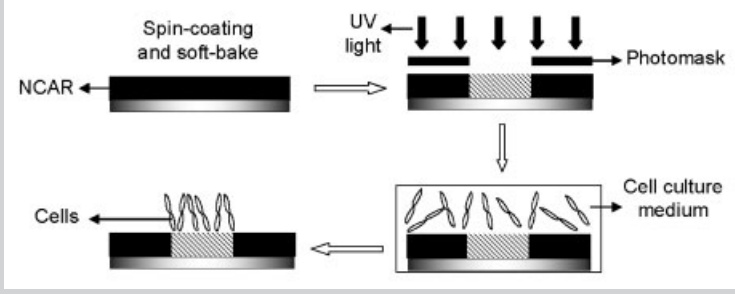
Summary: A simple lithographic process in conjunction with a novel biocompatible nonchemically amplified photoresist material was successfully used for cell patterning. UV light irradiation on selected regions of the nonchemically amplified resist film renders the exposed regions hydrophilic by the formation of carboxylic groups. Mouse fibroblast cells were found to be preferentially aligned and proliferated on the UV light exposed regions of the nonchemically amplified resist film where carboxylic groups were present.



Schematic representation of the simplified lithographic process used for cell patterning.

Simple Patterning of Cells on a Biocompatible Nonchemically Amplified Resist

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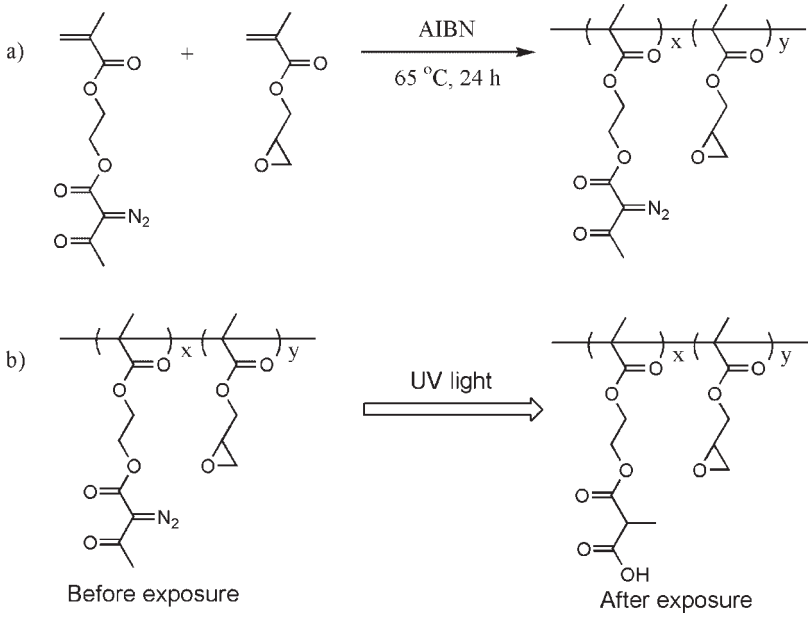
Introduction

Currently, there has been a great interest to generate patterns of biologically active molecules, such as DNA, proteins, cells, and spores on solid substrates for their applications in biosensors, biomaterials, and tissue engineering.[1] The spatial organization of cells is a crucial step in tissue engineering that requires precisely defined cellular architectures.[2] Such spatial organization of cells could be achieved through several techniques such as conventional photolithography and soft lithography.[3] These techniques, however, have several drawbacks to be applied in tissue engineering due to the use of toxic organic developers in the conventional photolithography and the limited size of poly(dimethyl siloxane) stamps in soft lithography.[4] Recently, an alternative method of using chemically amplified resist, which does not require the development step, was developed for patterning of cells.[5] However, this resist material requires photoacid generator (PAG) for the pattern generation, which makes the material and process not suitable for tissue engineering.

In this paper, we report a simple lithographic process for cell patterning in conjunction with a novel nonchemically amplified photoresist material. The major advantage of this process is that it does not require the addition of PAG to generate patterns thereby making the material and the process highly suitable for the immobilization of biomolecules and cells. Furthermore, the patterned resists can be used directly for the coupling of specific cell-adhesion



Scheme 1. (a) Synthetic scheme of poly(DOBEMA-*co*-GMA); (b) Photoreaction of the NCAR upon UV light exposure.

peptides or proteins for the alignment of cells, and therefore no further process of development is required after UV light exposure. Another unique feature of this process is that, unlike the chemically amplified resist approach, no post-exposure bake (PEB) step is required to create patterns.

Experimental Part

Materials

Triethylamine, 2-(methacryloyloxy)ethyl acetoacetate, and glycidyl methacrylate (GMA) were purchased from Aldrich Chemical Company and used without further purification. 2, 2′-Azoisobutyronitrile (AIBN) was purchased from Junsei Chemical Company and purified by recrystallization in methanol. *p*-Toluenesulfonyl azide was prepared from *p*-toluenesulfonyl chloride and sodium azide according to the literature.[6] The monomer 2-(2-diazo-3-oxo-butyryloxy)ethyl methacrylate (DOBEMA) was synthesized according to the reported procedure.[7] All cell culture reagents were obtained from GibcoBRL (Rockville, Maryland).

Instrumentation

Chemical structures were determined by IR spectroscopy (Bio-Rad FTS-165 FT-IR spectrometer) and ^{1}H NMR and ^{13}C NMR spectroscopy (Bruker AM-300 FT-NMR-spectrometer). UV light irradiation was carried out using a deep UV exposure system (Oriel corporation model 82531) with a filter transmitting light between 220 and 260 nm. The optical images of cells were taken by an Olympus optical microscope. Using an hematocytometer, cells were counted (dead cells were excluded by Trypan Blue staining). A contact angle instrument (Phoenix 300, Surface Electro Optics Co. Ltd., Korea) was used to determine the water contact angle of the polymer before and after the UV light irradiation. The water contact angles were measured at 22 °C and below 30% humidity.

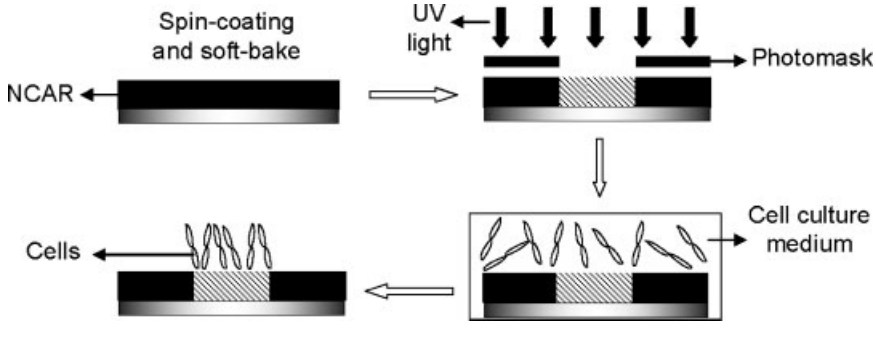


Figure 1. Schematic representation of the simplified lithographic process used for cell patterning.

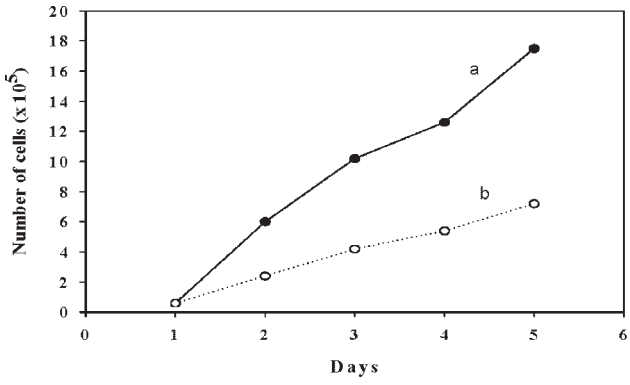


Figure 2. Proliferation of fibroblast cells on (a) UV-exposed, and (b) UV-unexposed films of the NCAR.

Synthesis of Poly(DOBEMA$_{0.84}$-co-GMA$_{0.16}$)

A solution of 5 g of DOBEMA and 0.59 g of GMA in 30 g of tetrahydrofuran containing 0.123 g of AIBN was heated to 65 °C in a sealed ampoule under nitrogen atmosphere. After 24 h, the polymer was precipitated into diethyl ether and subsequently redissolved in tetrahydrofuran and reprecipitated to remove the unreacted monomers completely. The precipitated polymer was collected by filtration, washed with the precipitating solvent and dried in vacuum for 24 h to obtain a white powdery material (4.02 g, 73% yield). The number-average molecular weight of the polymer was found to be 11 750 with a polydispersity index of 2.37.

Sample Preparation for Cell Culture

A 10 wt.-% solution of the above-mentioned polymer in cyclohexanone was spin-coated onto 25×25 mm^2 glass slides at a speed of 1 100 rpm. The samples thus prepared were soft-baked at 90 °C for 90 s and then subsequently used for UV light exposure (dose = 50 mJ · cm^{-2}) under a photomask, using a high pressure Hg/Xe lamp in a contact printing mode. The samples were used for cell culture without further development.

Cell Culture

The mouse fibroblast cells (NIH3T3) were seeded onto the samples at a density of 5×10^4 cells · mL^{-1}, maintained in a medium consisting of RPMI1640, 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cells were grown to near-confluence in T25 flasks and then subcultured every forth day by dissociating with 0.1% (w/v) trypsin/0.02% ethylenediaminetetraacetic acid in phosphate-buffered saline.

Results and Discussion

A novel copolymer of DOBEMA and GMA, poly(DOBEMA$_{0.84}$-*co*-GMA$_{0.16}$), was the nonchemically amplified resist material used in this study (hereafter the copolymer is referred to as NCAR). GMA was incorporated in the polymer to avoid peeling off of the resist film from the glass substrate. The synthetic scheme and the photoreaction of the NCAR are shown in Scheme 1. The polymer has diazoketo functional groups, which upon UV light irradiation undergo Wolff rearrangement to generate carboxylic groups, thereby making only the exposed regions hydrophilic.[7] The water contact angles of the NCAR film, before and after UV light irradiation, were found to be $57.3 \pm 0.85°$ and $51.4 \pm 0.72°$ respectively, which proves that after UV light irradiation, the hydrophilicity of the film was increased due to the formation of carboxylic groups.

Figure 1 shows the schematic representation of the simplified lithographic process that involves three simple steps: forming a thin film of the NCAR, UV light irradiation, and cell culture on the patterned substrate. Briefly, the NCAR was coated on a glass slide from a solution of the resist material in cyclohexanone and soft-baked to remove the solvent. UV light was then irradiated on selected regions to create patterns. The substrate was then subsequently placed in the cell culture medium for cell patterning.

Initial attachment of cells on a surface is a crucial step for subsequent cell proliferation. The *in vitro* growth of fibroblast cells on the exposed and unexposed films of NCAR

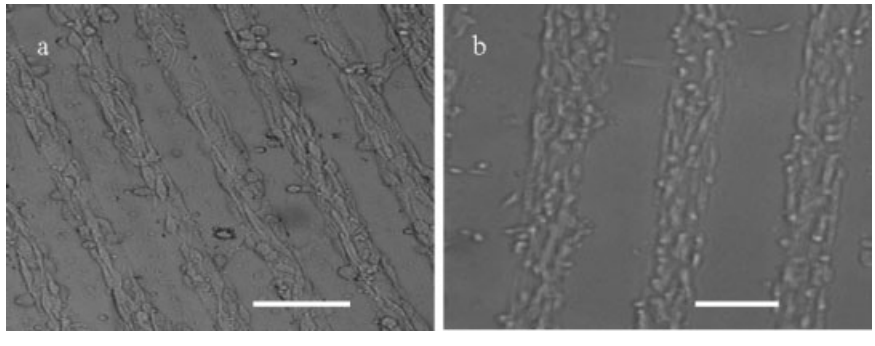


Figure 3. Optical micrographs of fibroblast cells cultured on (a) 25 μm/50 μm, and (b) 50 μm/100 μm line and space patterned surfaces of the NCAR after 7 d (100× magnification, scale bar = 100 μm).

were studied up to 5 d (Figure 2). Comparing the exposed and unexposed films of NCAR, there was a better cell growth observed on the exposed surface, indicating that the surface supports cell growth. On the other hand, the unexposed surface has less number of cells. Cell population on the exposed surface is nearly 200% more than that on the unexposed surface, indicating that the unexposed surface does not sufficiently support cell adhesion, which follows the trend similar to the previous report.[5] These results clearly indicate that the exposed surfaces, where carboxylic groups were present, were the more preferable surfaces for the cells. The difference in cell adhesion on these surfaces could be due to the different surface chemistries of the material.

Figure 3 shows the optical micrograph images of the cell culture results on patterned NCAR surfaces. Fibroblast cells were found to be preferentially aligned and proliferated on the UV light exposed regions of the patterned NCAR film. On the other hand, cells were randomly distributed and spindle shaped on the fully exposed nonpatterned NCAR film (data not shown). The preferential adsorption of serum proteins, that are present in the cell culture medium, to the carboxylic groups of the hydrophilic regions seems to be the driving force for the selective alignment and proliferation of cells. The patterned surfaces used in this study were essentially coplanar and thus the topographical cues such as contact guidance[8] could be ruled out. It is noteworthy that the cell alignment on the exposed regions was maintained during cell proliferation. These results suggest the potential application of this technique in combination with the 3-D constructs formed with the NCAR to produce an oriented tissue-like structure for tissue engineering.[9] The carboxylic groups on the exposed regions can be used to immobilize other bioactive agents such as cell-adhesive Arg-Gly-Asp (RGD) peptide, which would facilitate the cell adhesion and proliferation, to be used as biosensors.[10]

In conclusion, a novel nonchemically amplified resist material was successfully used for the cell patterning in combination with a simplified lithographic process that does not require PEB and development steps. The NCAR has diazoketo functional groups, which undergo Wolff rearrangement upon UV light irradiation to generate carboxylic groups. This chemistry was used to create patterns of alternative hydrophilic and hydrophobic regions on the surface of the NCAR. The *in vitro* cell culture on the patterned surfaces showed good alignment of cells on the UV light exposed regions where carboxylic groups were predominantly present. Furthermore, cells were found to maintain their alignment during proliferation. This simple strategy of generating carboxylic groups on the UV light exposed regions by the simplified lithographic process opens up the possibility of immobilizing various biomolecules such as DNA, proteins, and cells.[11]

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[1] [1a] A. Bernard, D. Fitzli, P. Sonderegger, E. Delemarche, B. Michel, H. R. Bosshard, H. Biebuyck, *Nature Biotechnol.* **2001**, *19*, 866; [1b] G. J. Zhang, T. Tanii, T. Funatsu, I. Ohdomari, *Chem. Commun.* **2004**, *7*, 786; [1c] R. M. Crooks, A. J. Ricco, *Acc. Chem. Res.* **1998**, *31*, 219; [1d] M. Mrksich, G. M. Whitesides, *Trends Biotechnol.* **1995**, *12*, 228; [1e] M. A. Nandkumar, M. Yamato, A. Kushida, C. Konno, M. Hirose, A. Kikuchi, T. Okano, *Biomaterials* **2002**, *23*, 1121; [1f] K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, N. Maeda, H. Watanabe, K. Yamamoto, S. Nagai, A. Kikuchi, Y. Tano, T. Okano, *Transplantation* **2004**, *77*, 379.

[2] N. Patel, R. Padera, G. H. W. Sanders, S. M. Cannizzaro, M. C. Davies, R. Langer, C. J. Roberts, S. J. Tendler, P. M. Williams, K. M. Shakesheff, *FASEB* **1998**, *12*, 1447.

[3] [3a] T. H. Park, M. L. Shuler, *Biotechnol. Prog.* **2003**, *19*, 243; [3b] A. Douvas, P. Argitis, K. Misiakos, D. Dimotikali, P. S. Petrou, S. E. Kakabakos, *Biosens. Bioelectron.* **2002**, *17*, 269; [3c] K. E. Healy, C. H. Thomas, A. Rezania, J. E. Kim, P. J. McKeown, B. Lom, P. E. Hockberge, *Biomaterials* **1996**, *17*, 195.

[4] [4a] J. Nakanishi, Y. Kikuchi, T. Takarada, H. Nakayama, K. Yamaguchi, M. Maeda, *J. Am. Chem. Soc.* **2004**, *126*, 16314; [4b] D. V. Nicolau, T. Taguchi, H. Tanigawa, S. Yoshikawa, *Biosens. Bioelectron.* **1996**, *11*, 1237; [4c] Y. Xia, G. M. Whitesides, *Angew. Chem. Int. Ed.* **1998**, *37*, 2363; [4d] D. Falconnet, A. Koenig, F. Assi, M. Textor, *Adv. Funct. Mater.* **2004**, *14*, 749.

[5] [5a] W. He, C. R. Halberstadt, K. E. Gonsalves, *Biomaterials* **2004**, *25*, 2055; [5b] W. He, K. E. Gonsalves, J. H. Pickett, C. Halberstadt, *Biomacromolecules* **2003**, *4*, 75.

[6] M. Regitz, J. Hocker, "*Organic Syntheses*", J. Wiley & Sons, New York 1973, Vol. V, p. 179.

[7] [7a] J. B. Kim, K. S. Kim, *Macromol. Rapid Commun.* **2005**, *26*, 1412; [7b] J. B. Kim, K. S. Kim, *Proc. SPIE* **2003**, *5039*, 655.

[8] A. Curtis, C. Wilkinson, *Biomaterials* **1998**, *9*, 1313.

[9] V. C. Mudera, R. Pleass, M. Eastwood, R. Tarnuzzer, G. Schultz, P. Khaw, D. A. McGrouther, R. A. Brown, *Cell Motil. Cytoskeleton* **2000**, *45*, 1.

[10] E. Huang, F. Zhou, L. Deng, *Langmuir* **2000**, *16*, 3272.

[11] [11a] T. J. Park, K. B. Lee, S. J. Lee, J. P. Park, Z. W. Lee, S. Y. Lee, I. S. Choi, *J. Am. Chem. Soc.* **2004**, *126*, 10512; [11b] J. P. Park, K. B. Lee, S. J. Lee, T. J. Park, M. G. Kim, B. H. Chung, Z. W. Lee, I. S. Choi, S. Y. Lee, *Biotechnol. Bioeng.* **2005**, *92*, 160.