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N-hydroxysuccinimidyl phenyl azide와 광반응을 이용한 펩타이드의 마이크로형태 고정화

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Micropatterning of Peptides to Solid Surface by Deep-UV Lithography using N-hydroxysuccinimidyl phenyl azide

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요 약 : 폴리머 표면에 생체물질의 고정화 방법은 생체적합성을 지닌 재료의 개발에있어서 중요한 방법중의 하나이다. 광반응을 이용한 photolithography방법을 사용하여 재료 표면의 원하는 부위에 단백질을 고정할 수 있다. 본 연구에서는 파이브로넥틴의 세포부착 리간드, GRGDS펩타이드를 N-hydroxysuccinimidyl phenyl azide를 이용하여 미세한 선의 형태로 표면에 광반응으로써 고정하였다. 광반응 유도체가 고정된 표면은 형광물질을 사용하여 확인하였다. 또한 혈관내피 세포는 GRGDS펩타이드가 고정화된 표면에서만 부착됨을 관찰하였다.

Abstract : Defined spatial localization of biomolecules on the polymer surface is potentially powerful method to generate biocompatible surface. Photolithography and photochemistry can be used to immobilize peptides only at a given region of the surface. In this study, peptide RGDS, one of the endothelial cells recognition sites of fibronectin, was covalently immobilized on the polystyrene coated surface with micropattern. It was performed by photochemical reactivity of a synthesized N-hydroxysuccinimidyl phenyl azide. The micropatterning was confirmed by staining with fluorescent dye, aminoacetamido fluorescein. Endothelial cell adhesion was observed only on the RGDS immobilized areas.
Key words : Photolithography, photochemistry, peptide, RGDS, micropatterning

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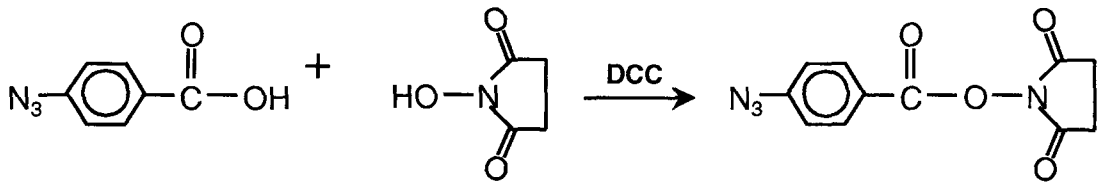
INTRODUCTION

Various surface modification by the introduction of functional groups has been the subject of intensive research toward the development of resist materials, biosensor, and biomaterials[1-4]. In the field of biochemical or medical



4-azidobenzoic acid **N-hydroxysuccinimide** **N-hydroxysuccinimidyl phenyl azide (NHS-PA)**

Fig. 1. Synthetic route of a photoreactive compound N-hydrosuccinimidyl phenyl azide (NHS-PA)

technology, protein fixation techniques have been extensively studied to develop devices for protein separation and diagnosis[5-7], biosensors[8], and biomaterials[1,9]. The controlled immobilization of biomolecules onto the material surfaces has important implications in many areas of biomedical technology.

Protein patterning[10], the defined spatial localization of molecules on a surface, is a potentially powerful approach to generating biological assays for analytical application and has been made possible photochemistry and photolithography.

Photochemistry[11,12], which induces surface reactions only at irradiated portions, seems to be a suitable method to accomplish this purpose. Precise surface designs enabling the control of protein adsorption and cell adhesion have been needed in the field of biomedical applications[13-15].

Matsuda et al.[11] described the use of an aryl azide to immobilize an octapeptide onto localized regions of a polyvinyl alcohol surface, in order to control cellular growth. Proteins were fixed by the photochemical reaction between the phenylazido derivatized polymer and amine groups of protein. This method allowed to immobilize peptides only at a given region of the photo-irradiated surface.

Yan et al.[17] reported the surface modification with N-hydroxysuccinimide-functionalized (NHS) perfluorophenyl azides (PFPAs) by a simple spin-coating technique and photolysis. To create patterned surface, a polystyrene film is created by spin casting a polystyrene solution onto a glass, and a solution of the NHS-PFPA is spun cast onto the polystyrene.

These studies demonstrated that the combination of surface modification using photoreactive linker with deep ultra violet (DUV) could be used to form closely defined region of functionalized polymer surface and to immobilize protein on the defined region.

Immobilization of adhesive peptides containing Arg-Gly-Asp (RGD) sequence on material surface has been investigated as a means of providing cell-adhesive surfaces for applications in various biomedical fields[14,16,18]. It has been demonstrated that patterning techniques on biomaterials can be used to determine the shape of a cell, as well as control the area of cell adhesion and growth[19-21].

In this study, synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS), one of the endothelial cells recognition sites of fibronectin, was covalently immobilized on the micro-patterned surface using a photolithographic technology. We also observed peptide patterning from the immobilized region visualized by fluorescence and the adhesion of endothelial cells

MATERIALS AND METHODS

Synthesis of N-hydroxysuccinimidyl phenyl azide (NHS-PA)

All the treatments for the preparation of photoreactive compound including N-hydroxysuccinimidyl phenyl azide (NHS-PA) were carried out in the dark. N-hydroxysuccinimide (64.6 mM, Tokyo Kasei Organic Chemicals Co. Japan) and 4-azidobenzoic acid (58.7 mM, Tokyo Kasei) were dissolved in 150 ml of tetrahydrofuran (THF, Tokyo Kasei) under cooling in an ice bath [11]. A solution of dicyclohexylcarbodiimide (DCC; 64.6 mM, Aldrich, Milwaukee, Wis.) was dissolved in 50 ml of THF and was added to the mixture under stirring and cooling in an ice bath. The reaction mixture was allowed to stand overnight under continuous stirring. A white solid was formed and filtered off and the solvent was removed under reduced pressure. The yellow residue obtained was crystallized from isopropyl alcohol. The synthetic route used to prepare NHS-PA and its chemical structure are shown in Figure 1.

Characterization of NHS-PA

Chemical analysis of synthesized NHS-PA was character-

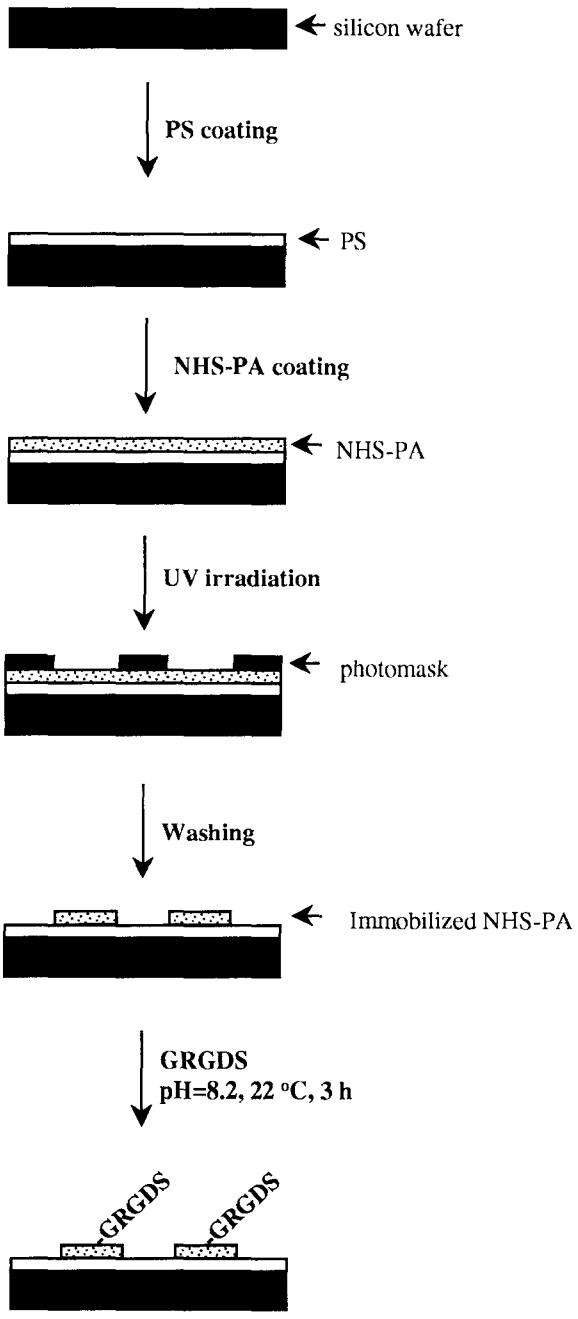


Fig. 2. Schematic diagram for the photochemical micro-fixation of peptide GRGDS; UV light passing through a mask irradiates an NHS-PA surface. The peptide was immobilized only in areas protected by UV light

ized by Proton nuclear magnetic resonance (^{1}H-NMR) spectrscopy, Electron-Impact(EI) mass spectrometry, and Fourier-Transform Infrared spectroscopy (FT-IR).

^{1}H-NMR spectra were monitored from sample in chloroform solution using a Bruker AMX-500 NMR spectrometer at 500 MHz.

Electron Impact (EI) mass spectra of NHS-PA were obtained using a VG 70-VSEQ mass spectrometer. These spectra were obtained under the conditions of 70 eV of electron energy, 200 ℃ of source temperature, and 300 μA of trap current.

The composition of NHS-PA synthesized and decomposition of azide upon photolysis were monitored by FT-IR. FT-IR data for the composition of NHS-PA were obtained from KBr plates. The decomposition of azide in NHS-PA upon photolysis was obtained using a KBr disk coated with 5% PS in xylene(Aldrich) and successively 0.5% NHS-PA in nitromethane(Aldrich).

Preparation of micro-patterned peptide chip

A silicon wafer was spin-coated with a solution of 5% polystyrene (PS ; MW 250,000, Aldrich) in xylene at 1,000 rpm for 1 min to produce a PS film (less than 1 μm thick). A solution of 0.5 wt % NHS-PA in nitromethane was spin-coated on the top of the PS film at 1,000 rpm for 1 min. The film was baked in an oven at 60 ℃ for 20 min. A quartz photomask, which has a 250 μm stripe pattern or 5 μm stripe pattern with line width of 5, 10, 15 μm, was then placed on the surface. A photomask of 250 μm line width was used for cell culture, The surface was ultraviolet(UV)-irradiated at room temperature with a 254 nm UV lamp (UVGL-58, 115V, 60HZ, Upland, CA, USA) at a distance of 30 cm (intensity, 3.2 mW/cm^2) for 5 min to enable the azido group to react with a C-H group of PS. Subsequently, the surface was washed with nitromethane to remove unreacted NHS-PA. A solution of 100 μM Gly-Arg-Gly-Asp-Ser (GRGDS; Sigma, St. Louis, MO) in 0.1 M $NaHCO_3$ buffer (pH 8.2) was dropped on the pattern and incubated at 25 ℃ for 3 hrs. The GRGDS-immobilized surface was washed thoroughly with phosphate buffered saline (PBS, pH 7.4). Figure 2 describes peptide immobilization by photoreaction.

Measurement of peptide surface concentration

The surface concentration of GRGDS was determined using ^{3}H-labeled glycine (Amersham, Buckinghamshire, UK).

For the measurement of peptide concentration bound on the surface, the NHS-PA coated PS surface was irradiated with 254 nm UV light (3.2 mW/cm^2) for 5 min without a

mask. The prepared surfaces were incubated with 50 μM ^{3}H-labeled glycine in 0.1 M $NaHCO_3$ buffer (pH 8.2) for 3 hrs at room temperature. The surface was then rinsed with PBS containing 0.05%-Tween 20 (Sigma) at room temperature for 5 min and finally washed with PBS (pH 7.4) for 30 min. Surface concentration was determined by counting washed silicon sample in a beta-counter and calculating values according to the specific activity of the ^{3}H-labeled glycine.

Examination of peptide array

To examine the immobilization of NHS-PA in well-defined patterns, a fluorescent dye was immobilized on functionalized PS film[22]. The NHS-PA coated PS surface obtained above was immersed in a solution of 5-(aminoacetamido) fluorescein (Molecular Probe. Eugene, OR) in ethanol (4.0 mg/1.0 ml) at 25 ℃ for 1 hr[23]. After washing in pure nitromethane, patterns were observed through a fluorescent microscope (IMT-2, Olympus, Japan) equipped with a fluorescent filter set (excitation wavelength 450-490 nm, emission wavelength > 510 nm).

Endothelial Cell Culture

Endothelial cells were isolated from human umbilical cord veins (HUVECs) according to the modified method of Jaffe et al.[24] The veins were rinsed with cord buffer (0.14 M NaCl, 0.004 M KCl, 0.001 M phosphate buffer, 0.011 M glucose, pH 7.4) filled with 0.2% collagenase CLS I (Worthington Biochemical Corp., Freehold, NJ) in cord buffer and incubated for 10 min at 37℃. After the cells were collected and centrifuged at 800 g, the pellets were resuspended in endothelial cell basal medium(EBM; Clonetics Co., Walkersville, ML) containing 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY), 2 mM glutamine (GIBCO), 10 ng/㎖ human recombinant Epidermal Growth Factor (hEGF, Sigma), 1 ㎍/ml Hydrocortizone(Sigma), 50 ㎍/㎖ Gentamicine(Sigma), 50 ng/㎖ Amphotericin-B (Sigma), 12 ㎍/㎖ Bovine Brain Extract (BBE, Clonetics). HUVECs were cultured in 25T culture flask (Corning, Cambridge, MA) and harvested by tripsinization. Subcultured cells from passages of 2-3 were used in this study.

Evaluation of endothelialization on the micro-patterned GRGDS array

HUVECs were seeded on the patterned GRGDS array in the growth medium at a seeding density of 1.5×10^5 cells/cm^2 and allowed to attach and spread at 37℃ under 5% CO_2 in air. A stereo-microscope (SZH10, Olympus) and a CCD camera(Sanyo, Japan) were used to visualize cell spreading. Images were acquired with a CCD camera and digitized with an image processing system (BiPS, Biomedlab, Korea).

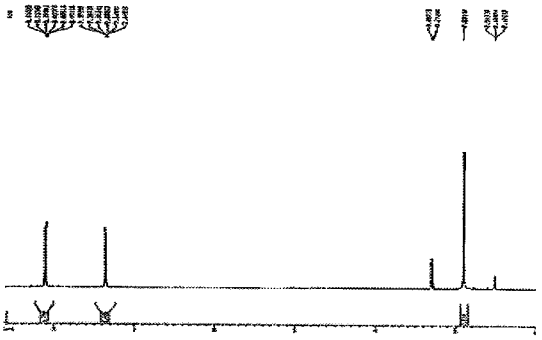


Fig. 3. 500 MHz ^{1}H NMR spectrum of NHS-PA; Assignments : (a) phenyl protons δ(4H, phenyl)=7-8 ppm; (b) succinimidyl protons, δ(4H, CH_2)=2.9 ppm

RESULTS

Characterization of NHS-PA

The N-hydroxysuccinimide and 4-azidobenzoic acid with dicyclohexylcarbodiimide (DCC) as a catalyst were reacted to produce NHS-PA. NHS-PA has two functional groups. One is an N-acyl succinimide group for an amide bond formation with free amino group of biomolecules and the other is a photoreactive phenyl azido group. For chemical structure analysis, ^{1}H-NMR spectroscopy and EI mass spectrometry were used. Figure 3 shows the ^{1}H-NMR spectrum of NHS-PA at 500 MHZ. This spectrum consists of three sharp peaks corresponding to the phenyl group and the succinimidyl group. The phenyl protons appear to down field compared to succinimidyl protons, δ(4H, CH_2)=2.9 ppm δ(4H, phenyl)=7-8 ppm. Figure 4 shows the EI mass spectrum of synthesized NHS-PA. FT-IR spectrum of NHS-PA is shown in Figure 5. The FT-IR spectrum showed strong absorption at 2140.91 cm^{-1} due to the phenyl azide group in NHS-PA. A salient feature of the photochemistry of phenyl azido group is the facile elimination of molecular nitrogen from the azido group upon UV irradiation and the formation of phenyl nitrine[12]. From these results, this compound was proved to be NHS-PA.

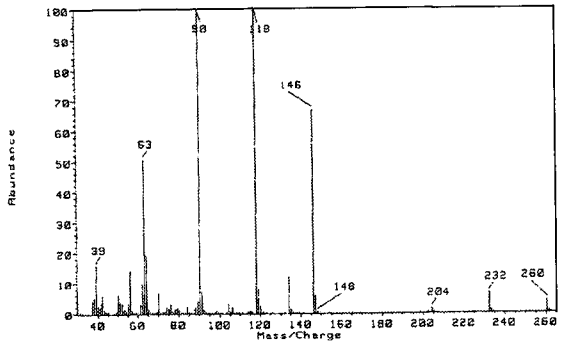


Fig. 4. Mass spectra of N-hydrosuccinimidyl phenyl azide (NHS-PA) ; 260(M^+), 232(M^+-N2), 146(M^+-NHS), 118(M^+-NHS-N_2), 114($ONC_4O_3H_4$), 90(NC_6H_4)

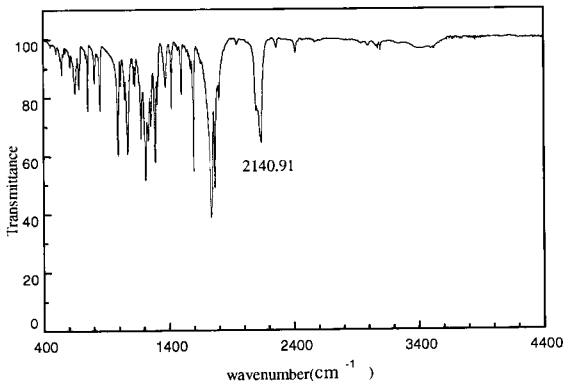


Fig. 5. FT-IR spectra of N-hydrosuccinimidyl phenyl azide (NHS-PA)

To examine the photoreactivity of NHS-PA, the decomposition of azide group upon photolysis was monitored by FT-IR using a KBr disk as the support. The FT-IR spectrum (Figure 6) showed strong absorption at 2121.7 cm^{-1} due to the azide group of NHS-PA spin-coated on the PS film. This absorption peak completely disappeared after the film was irradiated with a 254 nm lamp for 5 min. This result indicates that synthesized NHS-PA produces photoreactive phenyl nitrene upon UV irradiation.

Determination of GRGDS surface concentration

GRGDS was covalently immobilized to the NHS-PA coated surface by N-terminal glycine. Since this served as a spacer, ^{3}H-labeled glycine was used as a site for glycine. The number of reactive sites and the corresponding peptide concentration on the substrate surface were determined by titration with the radiolabeled glycine. Figure 7 shows a linear relationship between radioactivity and the concentration of ^{3}H-labeled glycine in solution. On the assumption that

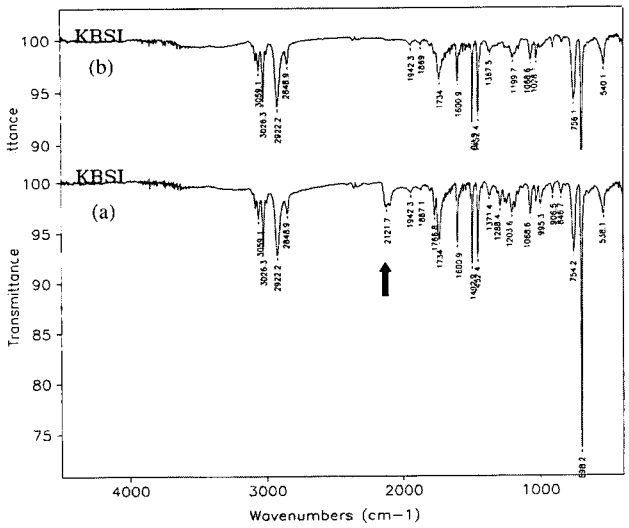


Fig. 6. FT-IR spectra of (a) PS coated with 0.5 % NHS-PA and (b) PS irradiated by UV light (3.2 mW/cm^2 at 254 nm) for 5 min

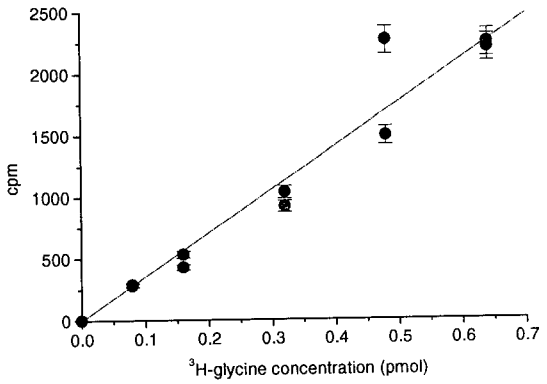


Fig. 7. Relationship between radioactivity and ^{3}H-glycine concentration; The least squares fit gives cpm Y = 3594.576χ, where χ is the concentration of ^{3}H-glycine (p value < 0.0001)

the activity of immobilized ^{3}H-labeled glycine is the same as that of native ^{3}H-labeled glycine, the concentration of immobilized GRGDS was calculated. The concentration of immobilized peptide on the NHS-PA coated surface was 0.6 pmol/cm^2.

Detection of micro-patterned array by fluorescent dye

To study the immobilization of primary amine-containing reagents in well-defined patterns, a fluorescent dye, 5-(aminoacetamido) fluorescein, was immobilized on functionalized PS film and patterns were visualized under a fluorescence miroscope. As a control experiment, a non-functionalized PS-coated microstructure surface was treated with the fluorescent dye. Figure 8 shows the micro-pat-

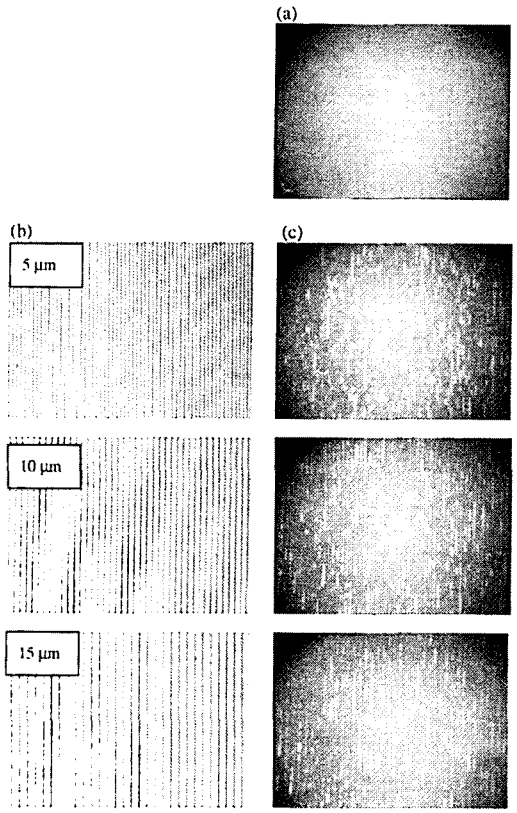


Fig. 8. Fluorescence image of micro-size pattern: (a) Nontreated PS surface;
(b) Micrographs of photomask pattern (line width : 5 ㎛, 10 ㎛, 15 ㎛); (b) Fluorescence images of micro-patterned array (line width : 5 ㎛, 10 ㎛, 15 ㎛)

terned array of NHS-PA coated surface as seen under the fluorescence microscope. The difference in white light intensity between the patterned and non-patterned regions of NHS-PA coated PS film is seen. 5-(aminoacetamido) fluorescein immobilized on the NHS-PA coated PS surface showed UV mask patterns, 5 ㎛ stripe pattern with line width of 5, 10, 15 ㎛, while a control sample without NHS-PA showed no fluorescent patterns.

HUVEC adhesion to the GRGDS pattern

Figure 9 shows HUVECs cultured on the GRGDS immobilized surface. The cells were observed to adhere on the GRGDS immiobilized surface during 4 hrs incubation. There was no cell attachment on the non-peptide immobilized surface. Cell spreading was only observed on the immobilized GRGDS area. These findings indicated that the peptide

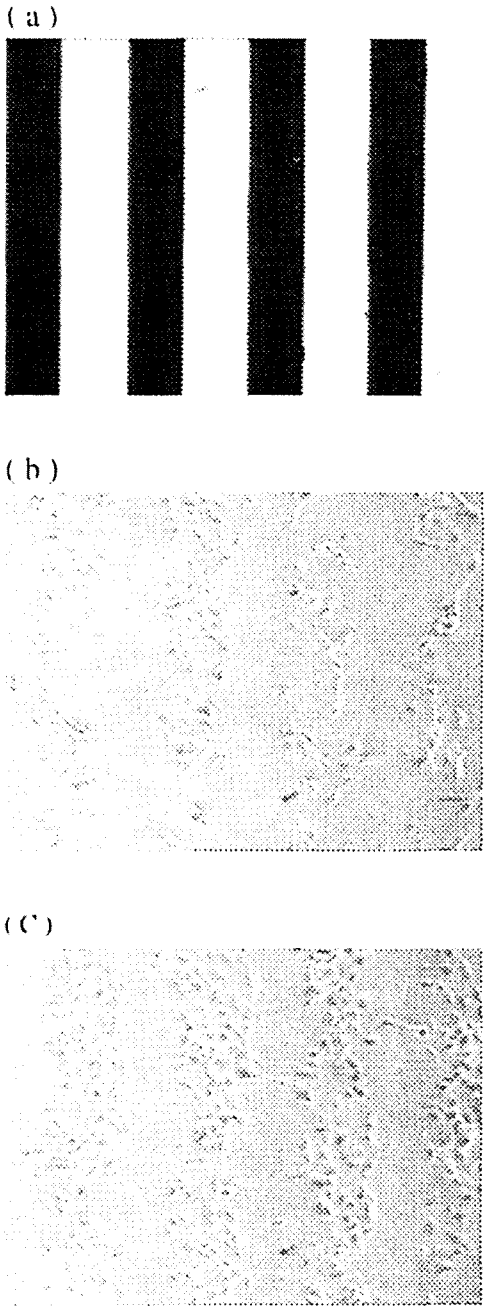


Fig. 9. Microscope image of micropatterned endothelial cells on GRGDS array; (a) Photomask of striped pattern(line width: 250 ㎛) (b) 4 hrs and (c) 1 day after seeding

GRGDS was pattern-immobilized according to the mask width and it enhanced cell adhesion by cell receptor-ligand interaction.

DISCUSSION

Photolithography is a method for the construction of

well-defined micrometer sized pattern using a photochemical reaction. It has been extensively used to direct synthesis of a matrix of biological ligands, which serves as a well-defined substrate to immobilize biological macromolecules such as proteins[7,13,16].

In photolithography, the UV light with a lithographic mask is used to spatially direct the simultaneous formation of chemical reactions[25]. These are based on UV energy in which photo irradiation area, photo intensity, and irradiation time can determine micro-order dimensional control, degree of surface modification or surface density, and the extent of grafting in principle [23,26]. The pattern of exposure to light or other forms of energy through a mask determines the regions of the support that are activated for chemical coupling[11,27].

Photochemical protein patterning methods are used for binding of target molecules to chemically labile species which can be activated upon UV irradiation. Arylazide photochemistry[16,17] is one of the most commonly used photochemical methods containing nitrobenzyl chemistry[28], and diazirine chemistry[29]. It is used to selectively immobilize proteins via an azide substituted aromatic group. Upon photolysis at the approciate UV wavelength, an arylazide results in a reactive nitrene which can insert into C-H bonds. The use of arylazide chemistry to immobilize peptide was reported by Masuda et al.[11]. Perfluorophenylazide (PFPA) photochemistry was described by Yan et. al.[17]. It is analogus to arylazide chemistry and improves the nitrene insertion efficiently into C-H bonds. These methods allowed to immobilize proteins only at a given region of the photo-irradiated surface. The various surface functionalization technique should be of general use for the delineation of a variety of biomolecular microstructures and may find applications in constructing novel microbiosensors and nucleotide screening assays[28,30].

In this study, we reported the use of arylazide chemistry with a photolithographic techniques to direct the covalent attachment of peptides to a functionalized surface. It was demonstrated that proteins could be immobilized on the surfaces in defined arrays by the use of photochemistry and photolithography. In order to examine the peptide patterning on surface, fluorescent dye was used and cellular adhesion to the substrate was also observed. Fluorescent method has shown to be a convenient means of visualizing these patterns(Figure 8). From the apporach of cellular adhesion onto the defined array, it was also conformed that peptide immobilized substrate was produced with micro-patterned width.

It has been demonstrated that patterning techniques could be used to develop a biosensor with multiple sensing regions and to engineer tissue and organ grafts as well as improve cellular adhesion to a substrate.

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