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The use of electron beam lithographic graft-polymerization on thermoresponsive polymers for regulating the directionality of cell attachment and detachment

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ABSTRACT

A simple process for nano-patterned cell culture substrates by direct graft-polymerization has been developed using an electron beam (EB) lithography system requiring no photo-masks or EB-sensitive resists. The compound *N*-isopropylacrylamide (IPAAm) was locally polymerized and grafted directly by EB lithographic exposure onto hydrophilic polyacrylamide (PAAm)-grafted glass surfaces. The size of the surface grafted polymers was controlled by varying the area of EB dose, and a minimal stripe pattern with a 200 nm line-width could be fabricated onto the surface. On the stripe-patterned surfaces, above the lower critical solution temperature (LCST), the cells initially adhered and spread with an orientation along the pattern direction. The magnitude of the spreading angle and elongation of adhered cells depended on the pattern intervals of the grafted PIPAAm. When culture temperature was lower than the LCST, cultured cells detached from the surfaces with strong shrinkage along the pattern direction, and sometimes folded and became parallel with the stripe pattern. This patterned cell recovery technique may be useful for the construction of muscle cell sheets with efficient shrinkage/relaxation in a specific direction and spheroidal 3D cell structures, with application to tissue engineering and microfluidic cellular devices.

1. Introduction

Microfabrication based on surface chemistry offers the possibility of improving the chemical and structural properties of material interfaces [1–3]. These patterning technologies have been used routinely for cellular assays that spatially and temporally control cell adhesion [4,5], due to the ability to promote dense and well-defined cell alignments. Chemical and topographic features on patterned surfaces can provide external cues for various aspects of cellular development, such as adhesive morphology [6,7], proliferation [8,9], differentiation [10], and gene expression [11]. Cells *in vivo* are immobilized within tissue *via* extracellular matrix (ECM) proteins, and receive biophysical cues from micrometer- and nanometer-scale ECM structural components. Advances in cell patterning methods hold promise for mimicking the *in vivo* cellular microenvironment, and may be applied to biological assays dealing with cell functions and to the field of regenerative medicine [12,13].

Functional cell recovery systems for *in vitro* tissue reconstructions have been developed using thermoresponsive polymers [14,15]. Thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm) responds to external temperature changes by discontinuous changes in water solubility [16]. Surfaces grafted with this compound, which exhibit significant hydrophilic/hydrophobic property changes with temperature changes, are used in biomedical applications [17,18]. Various proteins and cells can be bound onto PIPAAm-grafted surfaces above the lower critical solution temperature (LCST), which spontaneously release when the incubation temperature is lowered below the LCST, *via* dynamic hydration of graft PIPAAm layers. In these cell recovery systems, all of the cells can be harvested as a single continuous cell sheet with intact cell–cell junctions and ECM upon a decrease in culture temperature after the cells reach confluency on PIPAAm-grafted surfaces [19]. The harvested cell sheets maintain their function and membrane proteins, so that they can be transferred for stratification with other cell sheets *in vitro* [20] and for direct transplant *in vivo* [21]. Recently, two-dimensional functional cell sheets, such as patterned co-cultures of heterotypic cells [22] and vascularized tissues consisting of endothelial cells [23,24], have been successfully recovered from PIPAAm-grafted surfaces improved by

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micro-patterning methods. These sheets were on a micrometer scale for construction of a macro-ordered tissue structure. For the next generation, nanometer-scale patterning is needed to obtain cell sheets to allow more precise control of cellular alignment and functionality of surface chemical and topographic features. This patterning approach shows promise for the construction of functional tissue mimicking cell sheet materials for clinical applications.

Here, we present a simple graft-polymerization method using an electron beam (EB) lithography system to fabricate micrometer- and nanometer-patterned PIPAAm layers. Conventional preparation of nano-patterned grafted polymer layers requires multiple processes, such as polymerization after nanofabrication [25–27], and pre-polymerization for EB-lithographed patterned cross-linking [28,29]. However, for surface modification of patterned polymers, it is desirable to perform polymerization, surface modification, and pattern formation in a single process. The simplest fabrication of nano-patterned polymer-grafted surfaces simultaneously controls both patterning and graft-polymerization from the monomer solution without any photo-sensitive resists. A previous study has reported the covalent grafting and polymerization of an IPAAm monomer onto silanized glass surfaces during EB irradiation [30]. To take this study a step further, here the simultaneous local polymerization and direct surface grafting of IPAAm monomers are examined by irradiation with a focused EB lithography system. This EB lithographic polymerization of IPAAm on glass substrates permits easy fabrication of patterning surfaces, as well as the ability to harvest cell sheets with cellular morphologies and functions controlled by micrometer- and nanometer-scale surface features. A discussion then is presented on whether designed surface patterning can regulate cellular adhesive orientation and detachment behavior from these PIPAAm-grafted surfaces.

2. Experimental

2.1. Materials

IPAAm was obtained form Wako Pure Chemicals, Industries, Co. Ltd. (Osaka, Japan), purified by recrystallization from *n*-hexane, and thoroughly dried under vacuum at 25 °C. The following reagents were used as received: 2-propanol from Kanto Chemical Co., Inc. (Tokyo, Japan), Acrylamide (AAm) and Ammonium peroxodisulfate (APS) from Wako Pure Chemicals, and 3-methacryloxypropyltrimethoxysilane (MPTMS) from ShinEtsu Chemical Industry (Tokyo, Japan). Espacer 300, a conductive water-soluble polymer, was obtained from Showa Denko (Tokyo, Japan). Trypsin–EDTA solution and penicillin/streptomycin solution were obtained from Gibco BRL (Grand Island, NY). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Chemical Co. (St Louis, MO).

2.2. Silane immobilization onto glass surfaces

Glass substrates were treated by O_2 plasma ashing (irradiation intensity: 500 W, oxygen pressure: 0.1 Pa) for 300 s using a plasma-etching system (NE-550; ULVAC, Chigasaki, Japan) to activate silanol groups on the surface. These plasma-treated glass substrates were placed in a Teflon vessel together with a glass bottle containing 1 mL MPTMS. The vessel was sealed with a cap followed by heating in an oven. Silanization of the substrate surfaces by vaporized MPTMS was conducted for 2 h at 70 °C. After the coupling reaction, the MPTMS-immobilized glass was immediately dried at 70 °C for 1 h.

2.3. Surface modification with PAAm by free radical polymerization

Hydrophilic PAAm species were covalently grafted onto MPTMS-modified glass surfaces for preparation of protein-repelling adsorption surfaces. AAm (5 wt%) and APS (50 mg), acting as a polymerization initiator, were dissolved in 50 mL of distilled water, and this solution was degassed gently by bubbling with argon gas for 1 h in an ice bath. After degassing, 0.1 mL of TEMED was added into the AAm monomer solution. Then, the reaction solution was immediately poured into a plastic tube, into which an MPTMS-immobilized glass was inserted, and the polymerization reaction conducted for 2 h at 4 °C. The PAAm-grafted glass substrates then were rinsed repeatedly with distilled water, and dried at 25 °C under vacuum.

2.4. EB lithographic graft-polymerization of IPAAm onto glass surfaces

A patterned thermoresponsive PIPAAm was prepared by exposure to a focused EB using an EB lithography system (ELS-7500; Elionix, Hachioji, Japan) on PAAm-grafted glass substrates. IPAAm (55 wt%) was dissolved in 2-propanol, followed by filtration through a 0.25 μm pore filter. The PAAm-grafted glass substrates were sequentially covered with the IPAAm monomer solution and Espacer 300 using a spin coater. This coated substrate then was placed in the vacuum chamber of an EB lithography system, and a computer-controlled EB scanner was used to draw a nanometer- or micrometer-sized pattern. Only the IPAAm monomers in EB-exposed areas were directly polymerized and covalently grafted onto the PAAm-grafted surfaces. After the exposure, unreacted IPAAm monomer and the conductive polymer layer were removed with pure water, and the substrates were dried at 25 °C under reduced pressure. For surface characterization, MPTMS-immobilized surfaces with patterned PIPAAm also were prepared using the same protocol.

2.5. Surface characterization

The sessile drop method was used for measuring temperature-dependent contact angles with a contact angle meter (DropMaster500; Kyowa Interface Science, Saitama, Japan). A drop of de-ionized water from a syringe was placed on the surface of the plate in air. Sample temperature was regulated with a Thermo Plate® (TOKAI HIT Co. Ltd., Shizuoka, Japan). To estimate the grafted polymer morphologies, the patterned PNIPAAm-grafted surfaces were observed using atomic force microscopy (AFM: SPA-400; SII NanoTechnology, Tokyo, Japan) in non-contact mode in air. The pattern size and thickness of PNIPAAm layers were determined from the three-dimensional surface profiles.

2.6. Cell culture

Bovine carotid aortic endothelial cells (BAECs) and NIH-3T3 mouse fibroblast cells were purchased from the Japan Health Science Foundation (Osaka, Japan). The cells were cultured on commercial tissue culture polystyrene (TCPS) dishes with DMEM supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. The cells were harvested from the TCPS dishes with 0.25% trypsin–EDTA in phosphate-buffered saline. For the cell adhesion and spreading assay, cells were seeded to the patterned PIPAAm-grafted glass substrates at a density of 1.0×10^4 cells cm^{-2} followed by culturing at 37 °C. Cellular morphology was monitored at intervals and photographed using a phase-contrast microscope (Eclipse TS100; Nikon, Tokyo, Japan).

3. Results and discussion

3.1. Surface characterization of patterned surfaces by EB lithographic graft-polymerization

One of the key features of patterned cell cultures is the ability to spatially control cell–substrate interactions. Fundamentally, cellular responses, such as adhesion, proliferation, and differentiation, are triggered by an interaction between the cell transmembrane and the extracellular proteins, which are adsorbed onto the surface of the substrates. Thus, surface modifications are needed that can facilitate site-selection of protein adsorption. To achieve these surface properties, protein-repulsive surfaces were prepared by redox polymerization to modify MPTMS-immobilized substrates with a layer of PAAm [31]. The wettability of these surfaces then was investigated by measurement of static contact angles. The contact angles were $74.7 \pm 0.7°$ for MPTMS-immobilized surfaces and $18.1 \pm 1.0°$ for PAAm-grafted surfaces. We hypothesized that the methacryloyl groups in the surface-introduced MPTMS would react with the growing PAAm chains during polymerization, resulting in a hydrophilic surface. Fibroblast cells were cultured on the surfaces to confirm that cellular adhesion was consistent with the surface properties. While the fibroblast cells adhered, spread, proliferated, and reached confluency on the MPTMS-immobilized surfaces, exactly as observed on the commercial TCPS dishes, cell adhesion onto the PAAm-grafted surfaces was completely inhibited (data not shown). This was probably because hydration of the grafted PAAm prevents adsorption of the serum protein in the culture medium, inhibiting cellular adhesion.

As patterned cell-adhesive surface domains, thermoresponsive PIPAAm was locally bound onto the cell-repulsive PAAm-grafted