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Patterning of Collagen on PTFE Surface Using Ion Irradiation-induced Surface Grafting for the Selective Cell Adhesion

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Abstract In this study, the preparation of patterned-fluoropolymer substrate using ion irradiation-induced surface grafting of poly(acrylic acid) (PAA) and collagen modification was investigated for the selective cell adhesion. First, the surface of a polytetrafluoroethylene (PTFE) film was selectively irradiated with proton (H^+) ions and was functionalized by graft polymerization of acrylic acid onto the irradiated regions. In turn, the collagen was immobilized covalently on the PAA regions using EDC/NHS coupling reaction. The results of the surface characterization (including peroxide concentration and grafting degree, chemical structure, and wettability), clearly revealed that PTFE surface was functionalized with PAA (fluence dependent manner) and collagen was successfully immobilized on the grafted PAA chains. Moreover, the result of the *in vitro* cell culture revealed that cells selectively adhered to the collagen patterns (but not on the control PTFE surfaces) and produced well-arranged 100 μm cell micropatterns.

Key words: Ion irradiation, Surface grafting, PTFE, PAA, Collagen, Cell micropatterns

1. INTRODUCTION

Positioning of cells within confined regions of polymer substrate is crucial in many *in vitro* biological and clinical research fields such as immunological assays, biosensors, biochips, and others [1,2]. Bio-functionalization with cell-adhesive proteins, including collagen, fibronectin, vitronectin has been extensively adopted for the induction of cell adhesion because the cells inevitably recognize them as adhesion site onto the polymer surface prior to adhesion [3,4]. Therefore, formation of patterned-immobilization of cell-adhesive proteins on polymer surface can be the most reasonable and powerful way to achieve desirable cell patterns [5]. However, modification of polymer surfaces was needed first to create adhesion sites with cell-adhesive proteins due to their natural chemical inertness [6].

Surface grafting has been a preferred method to functionalize a polymer surface because it can afford polymer chains with highly dense and stable functionality onto the surface without affecting the bulk properties [7,8]. Thus, a variety of surface grafting methods using chemical initiators, UV-radiation, plasma treatment, and high-energy radiation (γ-rays, electron beams, and ion beams) have been utilized to functionalize polymer surfaces for further introduction of biomolecules such as genes, proteins, enzymes [9,10]. The ion beam-induced surface grafting is among the surface modification methods most attractive for the patterned-functionalization owing to its several advantages. These include such as ease of selective irradiation and no use of harmful chemicals (e.g., chemical initiator or organic solvent) due to its greater linear energy transfer and a straighter penetration trajectory, and reliable and controllable pro-

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cess [11,12]. Therefore, this method can be efficient for the introduction of functionality providing stable sites to anchor cell-adhesive proteins on polymer surface [13].

Polytetrafluoroethlyene (PTFE) possessing excellent chemical inertness and mechanical stability is one of promising candidate as substrate for well-organized cell patterning because of its natural fluoropassivation effect preventing nonspecific binding of biomolecules [11,14]. In this study, we described a facile method for the fabrication of collagen-patterned PTFE substrate using ion irradiation-induced surface grafting and collagen immobilization to ensure selective cell adhesion (Fig. 1). The PTFE film surface was selectively irradiated by proton (H^+) ions and followed by graft polymerization of acrylic acid (AA) to form PAA patterns on the PTFE surface. The collagen was then covalently introduced to PAA patterns using EDC/NHS coupling reaction. Analyses of peroxide concentration, grafting density, chemical structure, and wettability were conducted to confirm surface modification by surface grafting and collagen immobilization. Finally, *in vitro* cell culture on a collagen-patterned substrate and cell proliferation assays demonstrated the formation of well-arranged cell micropatterns.

2. MATERIALS AND METHODS

2.1. Materials

Polytetrafluoroethlyene (PTFE) films (100 μm thickness, Asahi Glass Co., Ltd.) were cleaned by ultrasonication in acetone for 15 min and dried in a vacuum oven at room temperature overnight prior to use. Acrylic acid (AA), Mohr's salt ($(NH_4)_2Fe(SO_4)_2$), 1,1-dipheyl-2-picryhydrazyl (DPPH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS), and FITC-conjugated collagen (type I) were obtained from Sigma Aldrich. All chemicals were used without further purification. A pattern mask (100 μm spaces/300 μm pitches) was supplied by Youngjin Astech Co., Ltd.

2.2. Ion irradiation-induced surface grafting

Surfaces of well-cleaned PTFE films were irradiated with 150 keV H^+ ions at room temperature at various fluences ranging from 1×10^{14} to 1×10^{15} ions cm^{-2} according to our previous study [11]. The pressure in the implanter's target chamber was 1×10^{-5} to 1×10^{-6} Torr. The current density of ion beam maintained less than 1.0 μA cm^{-2} to avoid a thermal effect on the surface. The irradiated films were left in air for 24 h for further oxidation. The films were put into glass vials containing 20 wt% AA aqueous solution and purged with nitrogen gas for 30 min to remove oxygen. For surface grafting, the vials were placed in water bath at a constant temperature (65°C) for 12 h. After the grafting reaction, the resulting films were taken out of the vials and thoroughly washed with deionized water to remove any remaining monomers and homopolymers. The resulting PAA-grafted PTFE films (PTFE-*g*-PAA) were then dried under a vacuum at 50°C. For patterned surface grafting of PTFE, the same procedure mentioned above was performed by selective ion irradiation through a SUS pattern mask.

2.3. Immobilization of collagen proteins

Collagen was covalently immobilized on the PAA-grafted PTFE surface through the well-known EDC/NHS coupling reaction. In detail, PTFE-*g*-PAA films were immersed into a solution containing 45 mM EDC and 15 mM NHS at room temperature for 12 h to pre-activate the carboxyl groups in PAA to form reactive intermediates [15]. The activated surfaces were then washed slightly with PBS buffer and covered with FITC-conjugated collagen solution (100 μg mL^{-1}) for 3 h at 36°C, resulting in the covalent immobilization of collagen on PAA of PTFE the surface (PTFE-*g*-PAA-Col). The selective attachment of collagen was confirmed using fluorescence microscopy.

2.4. Surface characterization

The amount of peroxide groups generated on the PTFE surface after ion irradiation was determined by a well-known DPPH method [11]. In brief, the irradiated PTFE films were immersed in a DPPH solution (10^{-4} M) in toluene in a glass tube. After purging with nitrogen gas, the solution was incubated at 70°C for 5 h to decompose the peroxide groups formed on the surfaces. The density of grafted COOH was determined by a TBO staining method reported in the literature [11,12]. The grafted films (2×2 cm^2) were immersed in the 0.5 mM TBO solution at pH 10 for 6 h at room temperature until the grafted surface was stained a blue color. After that, the films were washed with an excess amount of

sodium hydroxide solution (pH 10) thoroughly to remove the free TBO molecules. The TBO molecules were detached from the PAA grafted surface by incubating in a 50% acetic acid solution, and the optical density (OD) of the resulting solution was then measured at 633 nm using a UV-Vis spectrophotometer (MQX 200 model, Bio-Tek Instruments). The density of COOH groups was calculated from a calibration plot of the optical density versus TBO concentration based on the occurrence of reaction at the stoichiometric ratio of 1 : 1 between TBO and carboxyl group. The chemical structures of the control PTFE, PP-*g*-PAA, and PP-*g*-PAA-Col surfaces were analyzed using an attenuated total reflectance Fourier transform infrared spectrometer (ATR-FTIR, 640-IR, Varian, USA). The static water contact angles and images were obtained using a sessile drop method with a Phoenix MT(T) bubble micro-water drop analyzer (Surface Electro Optics Co., Korea). A droplet (4 μL) of deionized water was applied to the surface at room temperature. The average value was obtained through five independent measurements.

2.5. *In vitro* cell culture

HEK 293 cells (human embryonic kidney 293) were cultured *in vitro* on the patterned surfaces. Cells with a seeding concentration of 2.5×10^5 cells mL^{-1} were seeded onto the patterned surface after it was sterilized with 70% ethanol for 10 min. The cells were cultured in DMEM medium under culture conditions of 37°C and 5% CO_2 for 72 h. Rhodamine-phalloidin (molecular probes, Life Technologies) was used to stain the F-actin for 30 min. After washing with PBS three times, cell adhesion to the patterned surface was analyzed using optical and fluorescence microscopy. Cell proliferation on the surfaces was measured with a CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan) [16]. HEK 293 cells with a density of 1×10^4 cells $well^{-1}$ were seeded on control PTFE, PTFE-*g*-PAA, and PTFE-*g*-PAA-Col surfaces with dimensions of 2×2 cm^2. Every 24 h, the used media were replaced with 1 mL of fresh medium containing 10% CCK-8 assay solution. After incubation for 30 min, the optical density (OD) of the resulting medium was measured at 450 nm using a UV-Vis spectrophotometer (MQX 200 model, Bio-Tek Instruments, USA). All experiments were performed in triplicate.

3. RESULTS AND DISCUSSION

The amounts of peroxide groups formed on the irradiated PTFE surfaces at various ion fluences were quantified using a DPPH method. As shown in Fig. 2(a), the peroxide concentration formed on the PTFE surface was increased with an increase of ion fluence to 1×10^{15} ions cm^{-2}. This result indicates that the peroxide groups were formed effectively on the PTFE surface through ion irradiation-induced formation of radicals and oxidation in air [17]. As shown in Fig. 2(b), the density of carboxyl groups grafted on PTFE was gradually increased from 0.08 to an average of 0.15 nmol cm^{-2} with increase of ion fluence, which trend is similar to that of the peroxide

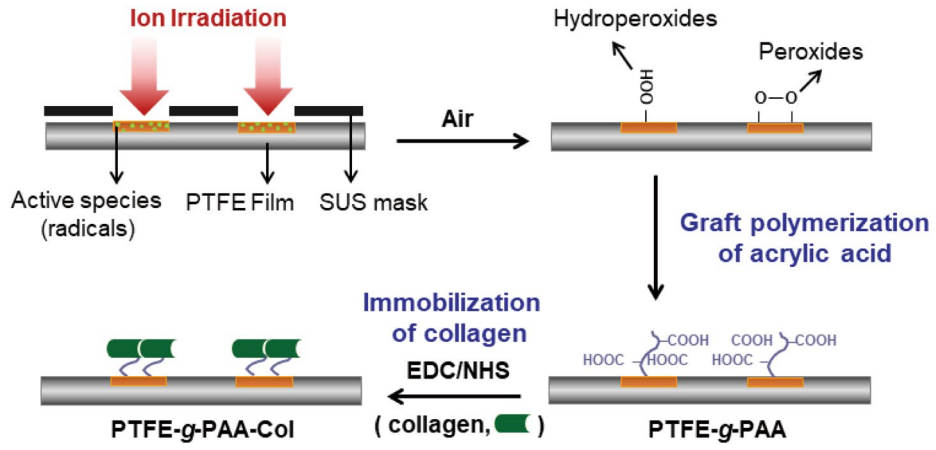

Fig. 1. Schematic illustration for the preparation of collagen-patterned PTFE surface by ion irradiation-induced surface grafting and collagen immobilization.

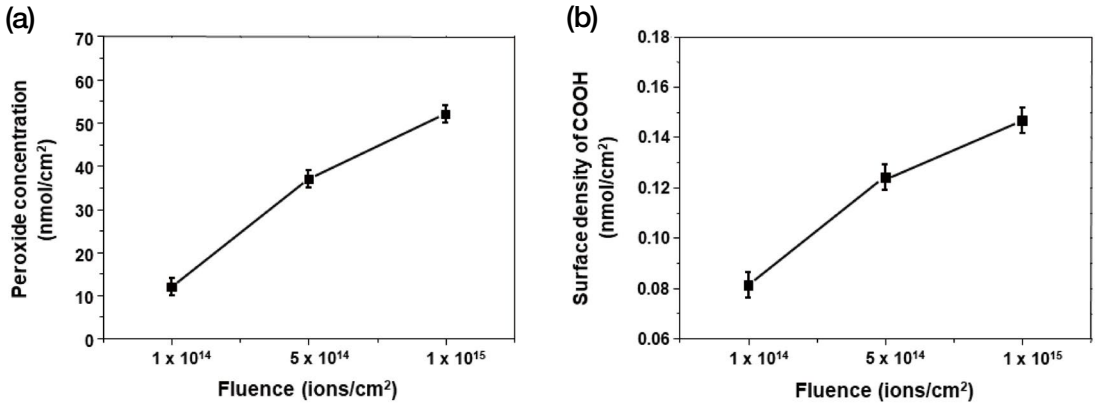

Fig. 2. Peroxide concentration (a) and surface density of carboxyl groups (b) at different ion fluences.

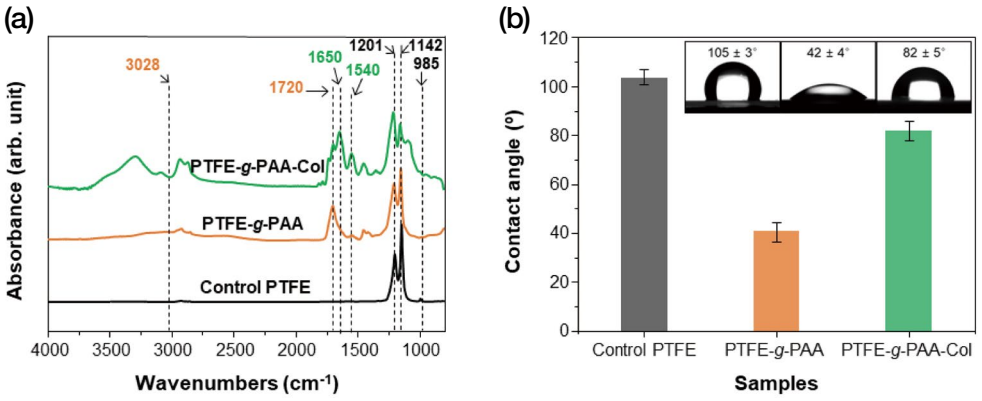

Fig. 3. FT-IR spectra (a) and water contact angles (b) of the control PTFE, PTFE-*g*-PAA, and PTFE-*g*-PAA-Col surfaces. The insets of (b) present images of water-drops for the respective surfaces.

concentration. This result can be ascribed to the fact that the grafting density was significantly dependent on the amounts of peroxide groups formed on the PTFE surface by ion irradiation because peroxide groups were used as initiation sites for the graft polymerization of AA [11].

To confirm the graft polymerization of AA and collagen immobilization on a PTFE surface, the chemical structures and wettability of the control PTFE, PTFE-*g*-PAA, and PTFE-*g*-PAA-Col surfaces were investigated. The PTFE-*g*-PAA and PTFE-*g*-PAA-Col samples prepared at a fluence of 1×10^{15} ions cm^{-2} were selectively used for the FT-IR and water contact angle analyses. As shown in FT-IR spectra for the chemical structure (Fig. 3(a)), the control PTFE spectrum exhibits characteristic peaks corresponding to the C–F in CF_2 at 1201, 1142, and in CF_3 at 985 cm^{-1} [11]. In the spectrum of PTFE-*g*-PAA, the new peaks present at 1710 and 3028 cm^{-1} corresponding to the C=O and –OH in carboxylic acid groups, confirming the presence of PAA on the PTFE surface [12,15]. In addition, in the PTFE-*g*-PAA-Col spectrum, the peaks at 1650 and 1540 cm^{-1} correspond to the peptide bonds (stretching of –C=O, and bending of N–H) for amide type I and amide type II [6,18,19]. This result indicates that collagen was successfully immobilized on the PAA chains grafted to the PTFE surface.

The change in the wettability was also investigated to verify successful surface modification by graft polymerization and collagen immobilization. As shown in Fig. 3(b), control PTFE surface exhibited a WCA of 105 ± 3° and PTFE-*g*-PAA surface was reduced to 42 ± 4°, indicating the presence of hydrophilic PAA chains on the PTFE [11]. On the PTFE-*g*-PAA-Col surface, WCA increased to 82 ± 5° again due to collagen immobilization [6,11]. This result suggests that immobilization of collagen improved the wettability of hydrophobic PTFE

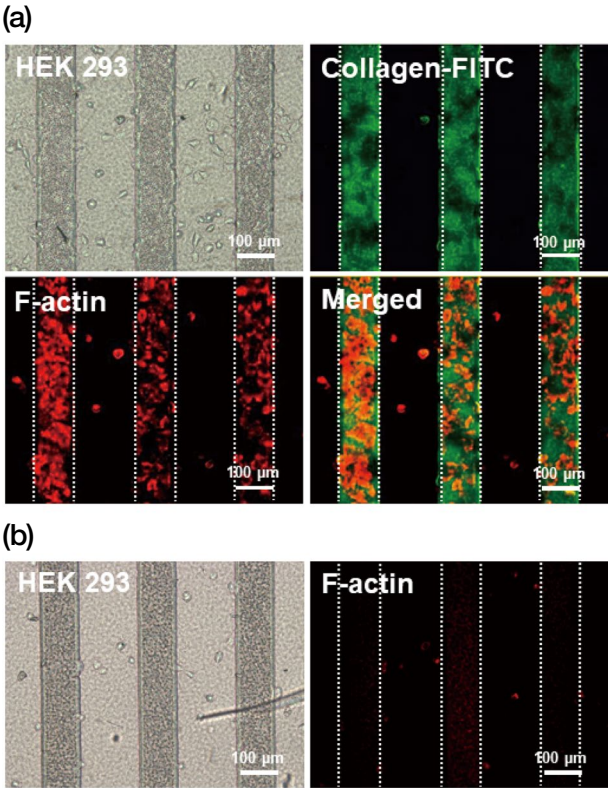

Fig. 4. Optical and fluorescence microscopy of HEK 293 cell adhesion on the patterned PTFE-*g*-PAA-Col (a) and patterned PTFE-*g*-PAA surface (b). The green and red pattens show immobilized collagen-FITC and F-actin of the cells, respectively.

surface that was not cell adhesive. Therefore, motivated by aforementioned results of surface characterization, patterned PTFE-*g*-PAA-Col was prepared by selective ion irradiation-induced graft polymerization of AA and collagen immobilization, to create cell micropatterns.

To investigate cell adhesion on the patterned surface, HEK 293 cells were cultured on the surface of PTFE-*g*-PAA-Col. As shown in Fig. 4(a), it was found that HEK 293 cells adhered to the collagen regions of the patterned PTFE-*g*-PAA-Col based on the patterned immobilization of collagen-FITC (green-stained), and then well-arranged 100 μm cell micorpatterns was formed. The image of collagen-FITC merged with F-actin (red-stained) also confirms that cell adhesion easily occurred on the collagen regions, while few cells adhere to the PTFE background regions. However, no cell micropatterns was formed on the patterned PTFE-*g*-PAA despite the presence of hydrophilic PAA, as shown in Fig. 4(b). This cell behavior can be ascribed to the steric repulsion effect of highly dense hydrophilic PAA chains [20].

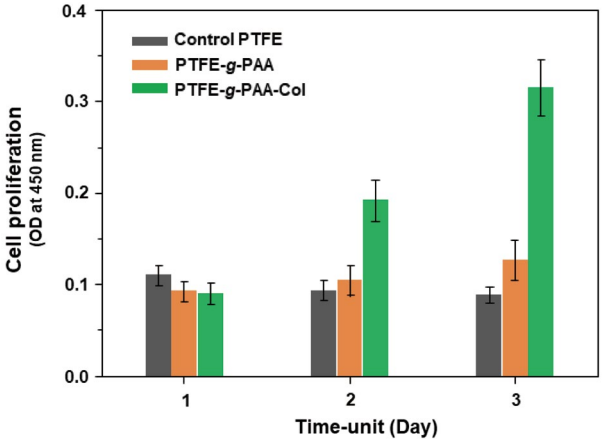

Fig. 5. Proliferation rate of HEK 293 cells on the control PTFE, PTFE-*g*-PAA, and PTFE-*g*-PAA-Col surfaces.

To elucidate further the difference in cell behaviors of PTFE-*g*-PAA and PTFE-*g*-PAA-Col, cell proliferation on the surfaces of PTFE, PAA, and collagen was investigated using CCK-8 assay. As shown in Fig. 5, PTFE-*g*-PAA-Col surface exhibited greater cell proliferation, while control PTFE and PAA surfaces exhibited very poor cell proliferation. This result indicates that patterned PTFE-*g*-PAA-Col can be a desirable substrate for achieving well-arranged cell micropatterns because of the coexistence of clearly distinguished cell-repellent PTFE and cell-adhesive collagen regions. Therefore, surface patterning by ion irradiation-based surface functionalization can be meaningful to the feasibility of patterned substrate for reliable cell micropatterning when applied to materials with extreme chemical inertness, such as fluoropolymer films.

4. CONCLUSION

In this study, a collagen-patterned PTFE surface was successfully prepared through ion irradiation-induced surface grafting of PAA and subsequent immobilization of cell-adhesive collagen for selective cell adhesion. The results of the peroxide concentration and grafting density measurements revealed that the peroxide groups (as initiators) were effectively created on the PTFE surface by ion irradiation and that grafting density was highly dependent on the ion fluence. The result of the chemical structure and wettability showed that the collagen was successfully immobilized on the PAA chains that were

grafted onto the irradiated regions of PTFE surface. Moreover, the results of cell culture confirmed that the 100 μm cell micropatterns were easily formed on the PTFE surface because the cells selectively adhered to the collagen regions. These findings on the controlled-surface functionalization illustrate the importance of ion irradiation-induced surface patterning as a promising strategy for spatial positioning of cell-adhesive proteins on the chemically inert fluoropolymer surfaces.

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