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Fibronectin Immobilization using Water-soluble Carbodiimide on Poly-L-lactic Acid for Enhancing Initial Fibroblast Attachment

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ABSTRACT: The aim of this study is to evaluate the influence of fibronectin immobilization on poly-L-lactic acid (PLA) films on the initial attachment of human gingival fibroblasts. Carboxylic acid groups are chemically introduced on the PLA films' surface by surface hydrolysis with 0.5 M NaOH. The contact angle of PLA surface with respect to double-distilled water decreases significantly after NaOH hydrolysis. X-ray photoelectron spectroscopy (XPS) also reveals significantly higher intensities of C_{C=O}/C_{C-O} after NaOH hydrolysis. Fibronectin is immobilized onto the hydrolyzed PLA surface through a condensation reaction between the carboxylic acid groups on the hydrolyzed PLA surface and the amino groups of fibronectin using water-soluble carbodiimide. XPS analysis shows that the fibronectin-immobilized PLA surface is enriched with nitrogen atoms. The immobilization of fibronectin significantly enhances the number of initially attached human gingival fibroblasts on the PLA surface. No obvious

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differences in morphology are noted between fibroblasts cultured on native PLA and on fibronectin-immobilized PLA. Fibronectin can be immobilized onto the PLA surface after NaOH hydrolysis and this is effective in enhancing the initial attachment of human gingival fibroblasts.

KEY WORDS: polylactic acid, fibronectin, fibroblast, initial cell attachment.

INTRODUCTION

Nowadays biodegradable polymers, such as polylactic acid (PLA), poly(glycolic acid) (PLGA), and poly(lactic acid–glycolic acid) are widely used as scaffolds in tissue engineering [1–3], carriers of cytokine or protein in drug delivery system [4,5], osteofixation devices in craniomaxillofacial surgery, and membrane for guided tissue regeneration (GTR) or guided bone regeneration (GBR), for reconstruction of gingival recession defects [6–9].

As scaffolds for regenerating cells, biodegradable polymers, such as PLA require not only suitable mechanical properties closely matched to the target tissues, but also good biological interaction with cells and/or the host when implanted. An adsorption of body fluid including extracellular matrix components occurs at the implant–tissue interface as the first biological reaction after implantation of biomaterials [10]. To make biomaterial surfaces more conducive to cell attachment and spreading, immobilization of proteins, such as cell adhesive protein or peptide on the polymer surface is a promising approach. The coating of PLGA film with type I collagen significantly enhanced keratinocyte migration [11]. The coating of PLA films with extracellular matrix proteins, such as fibronectin or laminin significantly increased the attachment of human skeletal muscle cells on the coated surface compared to noncoated PLA [12].

Covalent binding of proteins to polymers requires the presence of functional groups on the polymer surfaces. Since most biodegradable polymers intrinsically do not possess such groups on their surfaces, these functional groups must be introduced. Carboxylic acid groups are introduced on the PLA surface by graft-induced polymerization of polyacrylic acid, and the amino groups of protein or peptides are subsequently coupled to the carboxylic groups of the graft polymer [13]. Fibronectin, albumin, RGD-peptide, and laminin-peptides are also immobilized using the above-mentioned technique. The immobilization of gelatin, collagen, or chitosan on the PLA surface by photooxidation, and the subsequent graft copolymerization of methacrylic acid using UV irradiation have been reported [14]. Furthermore, Shu et al. [15] grafted type I collagen on ozone-oxidized PLA membrane. The *in vitro*

attachment, growth, and collagenous protein synthesis of rat calvaria osteoblasts are improved on modified PLA membrane.

Yamaoka et al. [16] reacted gelatin and PLA with NaOH solution, and then immobilized gelatin on the PLA surface. They did not use any condensation reagent for the immobilization of gelatin. NaOH solution produced carboxylic acid groups on the PLA surface, and the amino groups of gelatin then reacted with the carboxylic acid groups of PLA. The gelatin immobilized PLA surface improved cell attachment properties for 3T3 fibroblasts *in vitro*. On the contrary, Cai et al. [17] and Cui et al. [18] modified the PLA surface with silk fibroin or gelatin after NaOH surface hydrolysis of PLA film using water-soluble carbodiimide. The proteins were immobilized by a condensation reaction between the carboxylic acid groups on the alkali-hydrolyzed PLA film and the amino groups of the proteins. Cell activities, such as adhesion and proliferation were improved by the surface modification of PLA film with proteins. The advantage of the above-mentioned method of protein immobilization onto PLA is its simplicity.

Fibronectin is a well-known cell-adhesive protein and is effective in promoting cellular adhesion and spreading [19–21]. Li et al. [22] found that the adhesion of CHO-T cells on PMMA was accelerated by fibronectin immobilization. We hypothesize that fibronectin immobilization onto PLA film enhances cell activities, such as adhesion and spreading.

In the present study, we immobilized fibronectin on PLA surface after NaOH surface hydrolysis and investigated the initial attachment of human gingival fibroblasts on the fibronectin-immobilized PLA surface. The effect of fibronectin immobilization on the morphology of human gingival fibroblast was also evaluated by scanning electron microscopy.

MATERIALS AND METHODS

Surface Hydrolysis of PLA Films

The surface hydrolysis of PLA films and the immobilization of fibronectin onto the PLA surface were performed according to the method reported by Cai et al. [17], as shown in Figure 1. First, a carboxylic acid group was introduced on the PLA surface by NaOH hydrolysis. Then, fibronectin was immobilized through a condensation reaction between the amino group of fibronectin and the carboxylic acid group on the PLA surface.

Approximately 1 g of PLA (Purasorb[®], inherent viscosity: 2.53 dL/g, PURAC Biochem, Gorinchem, The Netherlands) was dissolved in 20 mL

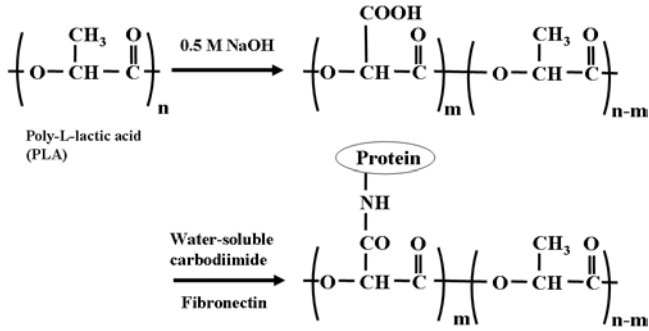


Figure 1. Scheme of activation of PLA surface by NaOH treatment followed by fibronectin immobilization.

chloroform. This solution was transferred to a 15-mm-diameter glass plate (Matsunami, Glass Inc, Ltd. Osaka, Japan) and evaporated to form a film. PLA films were delaminated from the glass plates. Then, PLA films were immersed in 0.5 M NaOH solution at 37°C for 0.5, 1, 3, and 5 h for surface hydrolysis. They were rinsed successively with double distilled water, 0.1 N HCl, and double distilled water, dried and stored in a desiccator.

Fibronectin Immobilization onto Hydrolyzed PLA Surface

The PLA films hydrolyzed with NaOH for 3 h (PLA-COOH) were used for fibronectin immobilization. The films were immersed in 0.1% solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (WSC, Peptide Institute Inc, Osaka, Japan) in 2-*N*-(morpholino)ethane-sulfonic acid (MES, Sigma, St. Louis, MO, USA) buffer at 4°C for 24 h. Then, the films were rinsed with MES buffer for 10 min followed by double distilled water.

The activated PLA-COOH films were immersed in 5% fibronectin (Human cellular fibronectin, 270 kDa, Upstate Biotechnology, Lake Placid, NY, USA) solution in MES buffer solution for 48 h at 4°C. After the immobilization reaction, the fibronectin-immobilized PLA (FN-PLA) films were washed with phosphate-buffered saline (PBS) solution at pH 7.4, and then dried and stored. The prepared FN-PLA membranes were sterilized by ethylene oxide before cell assay.

To confirm the effectiveness of the chemical immobilization of fibronectin onto PLA in cell assay, fibronectin precoating PLA film was prepared. The native PLA film was immersed in 5% fibronectin solution in MES buffer solution for 48 h at 4°C. Afterwards, the fibronectin

precoating PLA (FN precoating PLA) film was dried, stored, and sterilized by ethylene oxide before cell assay.

Surface Characterization of PLA Surface

The contact angles of PLA films before and after NaOH hydrolysis with respect to the double-distilled water were measured using a contact angle meter (CA-D, Kyowa Interface Science Co. Ltd., Tokyo, Japan). Five measurements of 15 s each were made for each surface type, and all analyses were performed at the same temperature and humidity.

Surface analyses of PLA film after NaOH hydrolysis (PLA-COOH) and after fibronectin immobilization (FN-PLA) were performed using X-ray photoelectron spectroscopy (XPS, ESCA-750, Shimadzu, Kyoto, Japan) by evaluating the intensity of C1s, O1s, and N1s. Argon-ion sputtering was performed at 2 kV and 15 $\mu\text{A}/\text{cm}^2$. The binding energies of each spectrum were calibrated for C1s at 284.6 eV.

Cell Isolation and Cultures

Human gingival fibroblasts used in *in vitro* experiments were isolated from pieces of gingival tissue excised during routing periodontal surgical procedures at the dental hospitals affiliated with Nihon University School of Dentistry at Matsudo (No:EC02-002). The study was explained to the patients and their informed consent was obtained. Small pieces of tissue were washed several times with Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Rockville, MD, USA) containing 100 U/mL penicillin G (Gibco BRL, Rockville, MD, USA), 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco BRL, Rockville, MD, USA), and 0.2 $\mu\text{g}/\text{mL}$ amphotericin B (Gibco BRL, Rockville, MD, USA). The epithelial layer was then mechanically separated from the connective tissue with sterile forceps. Primary cultures were incubated in an atmosphere of 5% CO_2 , 95% air at 37°C in DMEM supplemented with 10% fetal calf serum, 100 $\mu\text{g}/\text{mL}$ streptomycin, 100 U/mL penicillin G, and 0.2 $\mu\text{g}/\text{mL}$ amphotericin B. The cells were routinely passaged using 0.25% trypsin and 0.02% EDTA in Dulbecco's phosphate-buffered saline (PBS) solution. The cells were fed twice per week and subcultured using 0.25% trypsin. Cells between passages 15–20 were used in the experiments.

Cell Attachment Assay

When the outgrown fibroblasts from the gingival tissue became confluent after 15–20 passages, the cells were trypsinized and

resuspended in DMEM. The suspension of human gingival fibroblasts was plated on native PLA films, FN precoating PLA films, or FN-PLA films in 24-well tissue culture polystyrene dishes (TCPS, Falcon, Becton Dickinson Labware, NJ, USA) at a cell density of 1×10^5 cells/mL. The cell suspension was also plated in TCPS as a control. The cell suspension was incubated for 1.5 h under standard culture conditions, and washed three times with PBS to remove nonadherent cells. The adherent cells on the native PLA films, FN precoating PLA films, FN-PLA films, and TCPS were trypsinized and the number of adherent cells were counted using a Coulter counter (Nikkaki, Tokyo, Japan).

Scanning Electron Microscopy (SEM) Observation of Cell Morphology

After incubation of cells on the test materials as described above, the wells containing test materials were rinsed three times with PBS. Attached cells were fixed with 1% glutaraldehyde for 15 min, rinsed three times with PBS, and then fixed with 2% osmium tetroxide for 15 min. Fixed cells were dehydrated in a series of ethanol (50, 70, 80, 90, 95, 99.9, and 100%) for 5 min each, dried using critical drying apparatus, and ion-coated with gold and palladium. The morphology of cells was observed with a scanning electron microscope (S-2150, Hitachi, Tokyo, Japan).

Statistical Analysis

The data were analyzed by analysis of variance (ANOVA) and Scheffe's test for the multiple comparison among the means. A *p* value less than 0.05 was regarded as significant.

RESULTS

Fibronectin Immobilization onto PLA surface

Table 1 lists the contact angles of PLA films before and after NaOH hydrolysis with respect to double-distilled water. The contact angles of PLA films decreased significantly after NaOH surface hydrolysis compared to before hydrolysis. No significant differences in contact angle were detected among the PLA films treated with NaOH hydrolysis for 0.5, 1, 3, and 5 h.

Figure 2 shows the C1s and O1s spectra of native PLA surface analyzed by XPS measurement. Three types of C1s peaks were observed.

Table 1. Contact angle of PLA films before and after NaOH hydrolysis with respect to double-distilled water.

| Hydrolysis time (h) | Contact angle (°) |
|---------------------|-------------------------|
| 0 | 79.3 (8.7) ^a |
| 0.5 | 57.9 (7.2) ^b |
| 1 | 53.1 (9.8) ^b |
| 3 | 44.9 (7.8) ^b |
| 5 | 49.2 (3.1) ^b |

^aValues in brackets show standard deviation.

^bMean values with different superscripts are significantly different at $P < 0.05$.

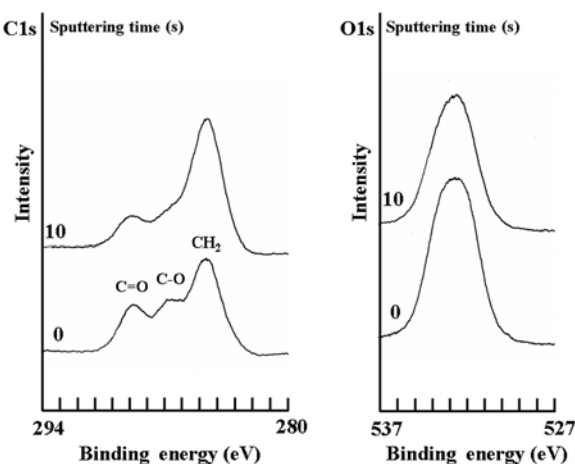


Figure 2. X-ray photoelectron spectra of C1s and O1s of a PLA film before NaOH hydrolysis.

The binding energies are 284.6 eV for C_{C-H} , 286.5 eV for C_{C-O} , and 289.0 eV for $C_{C=O}$, respectively [13,23]. After 10 s of argon-ion sputtering, the peak derived from C_{C-O} was observed as a shoulder of the C_{C-H} peak. The O1s peak derived from the oxygen of ester bond was observed at 532.9 eV.

Figure 3 shows the C1s and O1s spectra of PLA-COOH surface analyzed by XPS measurement. The PLA-COOH film was prepared by NaOH hydrolysis for 3 h. Three types of C1s peaks were also observed. The binding energies are 284.6 eV for C_{C-H} , 286.5 eV for C_{C-O} and 288.4 eV for $C_{C=O}$, respectively. After 10 s of argon-ion sputtering, the peak derived from C_{C-O} was observed as a shoulder of the C_{C-H} peak. The O1s peak was also observed at 532.9 eV.

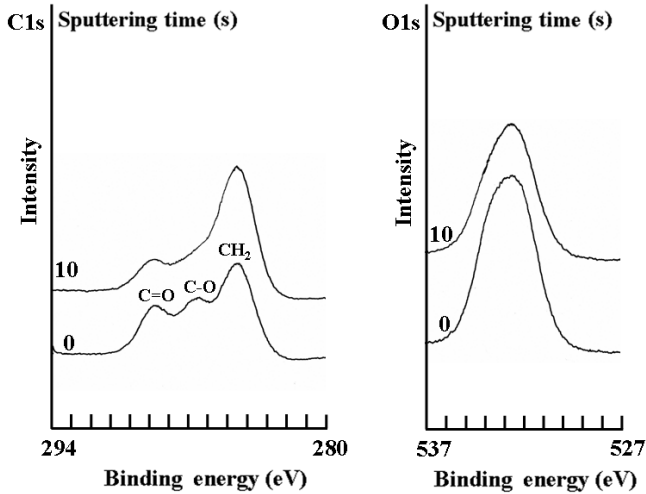


Figure 3. X-ray photoelectron spectra of C1s and O1s of PLA-COOH. PLA films were surface hydrolyzed for 3 h by 0.5 M NaOH.

Table 2. The intensity ratios of $C_{C=O}/C_{C-O}$ obtained from XPS in native PLA and PLA-COOH.

| | $C_{C=O}/C_{C-O}$ |
|------------|--------------------------|
| Native PLA | 0.49 (0.02) ^a |
| PLA-COOH | 0.54 (0.02) ^b |

^aValues in brackets show standard deviation.

^bMean values with different superscripts are significantly different at $P < 0.05$.

The intensity ratios of $C_{C=O}/C_{C-O}$ obtained from XPS for native PLA and PLA-COOH films are listed in Table 2. A significant difference in intensity ratio of $C_{C=O}/C_{C-O}$ was detected between native PLA and PLA-COOH.

Figure 4 shows the C1s, O1s, and N1s spectra produced by XPS analysis of the FN-PLA film. The peak derived from C_{C-H} was clearly observed at 284.6 eV. However, the peak derived from C_{C-O} appeared around 286.4 eV as a shoulder of the C_{C-H} peak, and this peak almost disappeared after 30 s of argon-ion sputtering. An O1s peak at 532 eV and an N1s peak at 399.6 eV are derived from the amide groups of the fibronectin [24,25]. This derivation was confirmed by observation of the original fibronectin based on XPS. Both peaks remained on the argon-ion sputtered surface after 60 s.

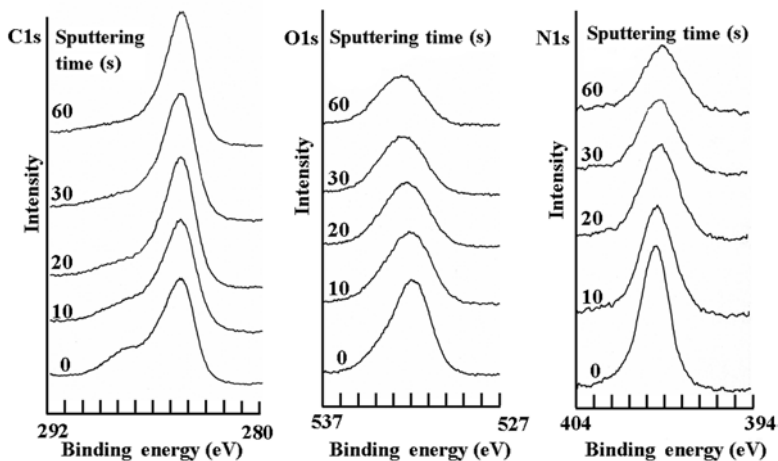


Figure 4. X-ray photoelectron spectra of C1s, O1s, and N1s of FN-PLA film. Fibronectin was immobilized to PLA after NaOH hydrolysis.

Table 3. Number of initially attached fibroblasts cultured on the various substrate.

| Substrate | Number of cells |
|-------------------|------------------------------|
| PLA | 6526 (547) ^a |
| FN precoating PLA | 22,291 (8424) ^a |
| FN-PLA | 45,851 (17,741) ^b |
| TCPS | 15,738 (6059) ^a |

^aValues in brackets show standard deviation.

^bMean values with different superscripts are significantly different at $P < 0.05$.

Fibroblast Attachment

Table 3 lists the number of initially attached human gingival fibroblasts on native PLA, FN precoating PLA, FN-PLA, and TCPS. The number of cells adhered onto the FN-PLA films were significantly greater than that on PLA, FN precoating PLA, and on TCPS. The number of attached cells increased almost three times by fibronectin immobilization. There were no significant differences in the number of attached cells between PLA, FN precoating PLA, and TCPS. FN precoating PLA did not produce significant increase in the number of attached cells.

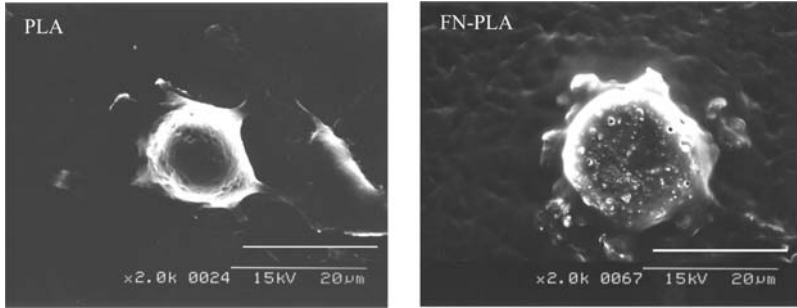


Figure 5. Scanning electron micrographs of human gingival fibroblasts on PLA or FN-PLA film (bar = 20 μm).

Figure 5 shows the scanning electron micrographs of human gingival cells adhered on native PLA films and FN-PLA films after 1.5 h in culture. A small degree of floppodia and lamellipodia formation was observed on the native PLA surface. Cells attached on the FN-PLA surface showed a roughened cell surface. No obvious differences in morphology were noted between fibroblasts cultured on native PLA and FN-PLA films. Both showed almost the same morphological appearances as fibroblasts cultured on TCPS.

DISCUSSION

In this study, we immobilized fibronectin onto PLA surface using NaOH hydrolysis and condensation reaction, and evaluated the efficacy of fibronectin immobilization of PLA in enhancing human fibroblasts activity, such as initial cell attachment.

Water-soluble carbodiimide is commonly used in coupling reactions between proteins and functional polymers, such as polyethylene glycol or acrylic acid-grafted polypropylene. To couple proteins onto polymer materials using water-soluble carbodiimide, hydroxyl group or carboxylic acid groups are required on the polymer surface. In the present study, surface hydrolysis by NaOH solution was used to introduce a carboxylic acid group onto the PLA surface.

The surface hydrolysis of the PLA film was confirmed by contact angle measurement with respect to double-distilled water. After 0.5 h NaOH hydrolysis, contact angles were significantly decreased compared to before NaOH treatment. This was due to the surface hydrolysis of PLA, i.e., introduction of carboxylic acid groups onto the PLA surface as shown in Figure 1. The difference in the duration of NaOH

hydrolysis (0.5, 1, 3, and 5 h) did not affect the hydrophilicity of the PLA surface.

The PLA film after 3 h of NaOH hydrolysis had the lowest contact angles with respect to double-distilled water (although there were no significant differences among the various durations of hydrolysis), and was therefore used in surface characterization by XPS and in subsequent immobilization of fibronectin. In the XPS spectra of native and hydrolyzed PLA, three types of C1s peaks, C_{C-H}, C_{C-O}, and C_{C=O}, were detected. There was no difference in the binding energy of C_{C-O} before and after NaOH hydrolysis. On the contrary, a significant increase was detected in the intensity ratio of C_{C=O}/C_{C-H} after NaOH hydrolysis. This was due to the introduction of carboxylic acid group onto the PLA surface after NaOH hydrolysis.

Fibronectin was chemically immobilized on PLA-COOH through the condensation reaction between the amino groups of fibronectin and the carboxylic acid groups on the hydrolyzed PLA using water-soluble carbodiimide. Hayakawa et al. [24,25] immobilized fibronectin onto titanium using tresyl chloride technique and found N1s peaks at 399.9 eV and O1s peaks at 532 eV in XPS measurement. The present XPS measurement showed the same binding energies of N1s and O1s, and both peaks remained after argon-ion sputtering for 60 s. The O1s peaks derived from the oxygen of PLA and the C_{C=O} peak of PLA, which should appear at 532.9 eV, were not clearly distinguished because of the overlapping of the O1s peaks of immobilized fibronectin. Our finding indicates that the present immobilization technique using water-soluble carbodiimide is useful for immobilizing fibronectin onto PLA. Yamaoka et al. [16] reported that the amount of gelatin immobilized on PLA films after NaOH hydrolysis reached 2 μg/cm², which was 10 times greater than that in the case of physical adsorption. Ito et al. also [26] reported that the amount of protein attached to the matrix, such as polymethyl methacrylate was greater than that by physicochemical adsorption when using the same concentration of proteins. Quantitative analysis of fibronectin immobilized on the NaOH-hydrolyzed PLA films by our methods should be further investigated.

Cai et al. [17] immobilized silk fibroin on PLA surface and found no significant differences in cell adhesion between silk fibroin-modified PLA and the control sample (no protein on the surface) during the first 2 and 4 h of osteoblast culture, but detected significant differences in the number of attached cells at the time points of 6 and 8 h. However, in the present study, the FN-PLA surface showed a significantly larger number of attached fibroblasts at the time interval of 1.5 h compared with native PLA and TCPS. The difference between the present findings

and the results of Cai et al. [17] is due to the difference of immobilized protein. Fibronectin is a well-known cell-adhesive protein and is effective in promoting cellular adhesion and spreading [19–21]. The difference of cells between two experiments is also another explanation. Generally, osteoblasts populate a surface at a slower rate than fibroblasts. Thus, in the present study, the enhanced initial cell attachment at 1.5 h was a result of fibronectin immobilization.

The present study also revealed the effectiveness of chemically immobilized fibronectin onto PLA for initial cell attachment compared with precoating of fibronectin onto PLA. Although the clear reason is not still revealed, it is hypothesized that the conformation change after precoating onto PLA was not effective or surface concentration of precoated fibronectin was not enough for enhancing initial fibroblast attachment compared with immobilized fibronectin. The conformation change after precoating or immobilization should be further analyzed besides the quantitative analysis of fibronectin immobilized on PLA film.

Nagai et al. [27] observed the formation of lamellipodia and filopodia in human gingival fibroblasts cultured on titanium after collagen coating. However, in the present study, fibronectin immobilization did not cause any significant change in cell morphologies. Cai et al. [17] also reported no obvious differences in the morphology of cultured cells on silk fibroin-modified PLA surface and native PLA surface. Detailed observation of cell morphology, such as actin fiber alignment should be further investigated.

The binding stability of fibronectin and degradation properties of FN-PLA in a biological environment is important when FN-PLA is implanted in living tissues. There was no degradation of FN-PLA in the medium during the 1.5-h cell assay. The influence of long-term immersion in biological conditions, pH and/or ionic strength on the immobilization of fibronectin and degradation of FN-PLA should be investigated as the next series of our experiment.

Parker et al. [28] reported no noticeable effects of precoating PLA with fibronectin in their implantation study. They inserted implants into subcutaneous pockets on the flanks of goats, and claimed that cell attachment to a substrate in cell culture is inherently different to tissue attachment to a substrate in living tissue. *In vivo* mechanical compression may contribute to tissue attachment of wound bed to the substrate. The implantation study using the present FN-PLA will be a next step of our research.

In conclusion, the present study demonstrates that fibronectin immobilization onto PLA by condensation reaction using water-soluble carbodiimide enhances initial cell attachment of human gingival

fibroblasts. The present technique will be applied to various forms of PLA, such as porous materials, which will be useful as scaffolds in tissue engineering. The present FN-PLA film may be applied as capping materials for dental pulp, wound-dressing material, or biomedical adhesive for the skin.

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