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Surface Characteristics and Fibroblast Adhesion Behavior of RGD-Immobilized Biodegradable PLLA Films

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Abstract: The interactions between the surface of scaffolds and specific cells play an important role in tissue engineering applications. Some cell adhesive ligand peptides including Arg-Gly-Asp (RGD) have been grafted into polymeric scaffolds to improve specific cell attachment. In order to make cell adhesive scaffolds for tissue regeneration, biodegradable nonporous poly(L-lactic acid) (PLLA) films were prepared by using a solvent casting technique with chloroform. The hydrophobic PLLA films were surface-modified by Argon plasma treatment and *in situ* direct acrylic acid (AA) grafting to get hydrophilic PLLA-*g*-PAA. The obtained carboxylic groups of PLLA-*g*-PAA were coupled with the amine groups of Gly-Arg-Asp-Gly (GRDG, control) and GRGD as a ligand peptide to get PLLA-*g*-GRDG and PLLA-*g*-GRGD, respectively. The surface properties of the modified PLLA films were examined by various surface analyses. The surface structures of the PLLA films were confirmed by ATR-FTIR and ESCA, whereas the immobilized amounts of the ligand peptides were 138-145 pmol/cm^2. The PLLA surfaces were more hydrophilic after AA and/or RGD grafting but their surface morphologies showed still relatively smoothness. Fibroblast adhesion to the PLLA surfaces was improved in the order of PLLA control < PLLA-*g*-PAA=PLLA-*g*-GRDG < PLLA-*g*-GRGD, indicating that PLLA-*g*-GRGD has the highest cell adhesive property.

Keywords: tissue engineering, PLLA film, plasma treatment, direct AA grafting, RGD immobilization, fibroblast adhesion.

Introduction

Tissue engineering which is one of the new fields opened with the development of the science and which is an applied study that utilizes the basic concept and technique of life science and engineering gives a clue to understand co-relationship between a structure and a function of body tissue and make a substitute of the body tissue for transplantation, thereby to maintain, improve or restore the function of human body.[1,2]

One of the typical tissue engineering techniques comprises taking out a required tissue from a patient body, followed by isolating cell from the tissue, proliferating the isolated cell, seeding the cell in the biodegradable porous polymer scaffolds, culturing the cell *in vitro* for a predetermined period, and then, transplanting the obtained hybrid-type cell/polymer structure into the human body. After transplantation is achieved, by virtue of diffusion of body fluids, oxygen and nutrients are provided to transplanted cells in biodegradable porous polymer until a blood vessel is newly formed. When a blood vessel is formed to which blood is supplied, the cells are cultivated and divided to form a new tissue and an organ. During new tissues and organs form, the polymer scaffolds are degraded and disappeared.[3,4]

Among many biodegradable polymers, poly(α-hydroxy acids) such as poly(glycolic acid) (PGA) and poly(L-lactic acid) (PLLA) have attained a unique position in the field of biomedical materials because of their excellent mechanical properties and biological affinity.[5–7] However, their highly crystalline and hydrophobic nature has interfered with modulation of their degradation rate and mechanical properties. It has also been difficult to impart functionality to these polymers by application of the ordinary chemical modification methods. Consequently, various attempts have been made to control their physicochemical properties by copolymerization of lactide monomers with other functional monomers, although few works have been successful in proper functionalization so far.[8,9]

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Although such synthetic biodegradable polymers are relatively biocompatible and appropriately biodegradable, they have insufficient initial cell binding sites and thus lack the ability to cell adhesion for *in vitro* and *in vivo* tissue culture because of their hydrophobic characteristics. To improve initial cell seeding efficiency, many researchers reported that the hydrophobic surface of porous biodegradable polymer scaffolds was modified into hydrophilic surface by various kinds of treatments. Those include surface hydrolysis with acid or alkaline (NaOH) solution,[10] the adsorption of cell attachment protein (collagen),[11] and surface modification.[12-16] The latter method comprises physical modifications using UV,[12] electron beam,[13] and plasma treatment.[14-16] In particular, acrylic acid (AA) was introduced indirectly by plasma treatment to endow hydrophilicity, functionality, and biocompatibility to polymer scaffolds.[14-16]

The biocompatibility of polymers depends on the adsorption of proteins to the polymer surface and on the interaction of the proteins with receptors on cells. Bioactive polymers offer a way to control cell material interactions. To modify polymer surfaces, bioactive ligands such as peptides and polysaccharides may be either adsorbed or covalently grafted to the surface or included in the bulk composition. Among them, it is well known that a tripeptide Arg–Gly–Asp (RGD) is the minimal common sequence involved in adhesive proteins such as fibronectin.[17] The cell attachment of various polymers immobilized with RGD and Arg–Gly–Asp–Ser (RGDS) oligopeptides has been studied.[18-20]

Recently, some synthetic cell adhesive oligopeptides such as RGD and Arg-Glu-Asp-Val (REDV) have been immobilized into polymeric scaffolds to improve specific cell attachment. Thus, cell adhesive peptides have been evaluated extensively for surface modification to enhance cell adhesion or to allow biospecific cell adhesion. Generally, however, the effects of these peptides on aspects of cell behavior other than adhesion are not well understood. The tripeptide RGD sequence, found in many extracellular matrix proteins, is the binding motif for cell surface integrin receptors and has been extensively studied. RGD peptide covalently bound to glass substrates has been found to increase adhesion and spreading of fibroblasts.[21,22]

In this study, nonporous PLLA films were first prepared by using a solvent casting technique with chloroform. The obtained PLLA films were surface-modified by Ar plasma treatment and *in situ* direct AA grafting and further RGD immobilization to get PLLA-*g*-GRGD. The surface properties of the modified PLLA films were examined by various surface analyses such as ATR-FTIR, ESCA, SEM, water contact angle, and amino acid analysis. In addition, the adhesion and spreading of the fibroblasts on PLLA-*g*-GRGD were evaluated and compared to PLLA control.

Experimental

Materials. Poly(L-lactic acid) (PLLA, M_w 110,000), that is a biodegradable polymer for making nonporous films, was purchased from Boehringer Ingelheim (Germany). Acrylic acid (AA) for a surface modifying monomer containing carboxylic acid was purchased from Aldrich (USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 2-*N*-morpholinoethane sulfonic acid (MES) were obtained from Aldrich (USA). Ligand peptides, Gly-Arg-Gly-Asp (GRGD) and Gly-Arg-Asp-Gly (GRDG), were purchased from AnyGen Co. (Korea). All other chemicals were used to a reagent grade.

Film Preparation. PLLA films were made using a solvent

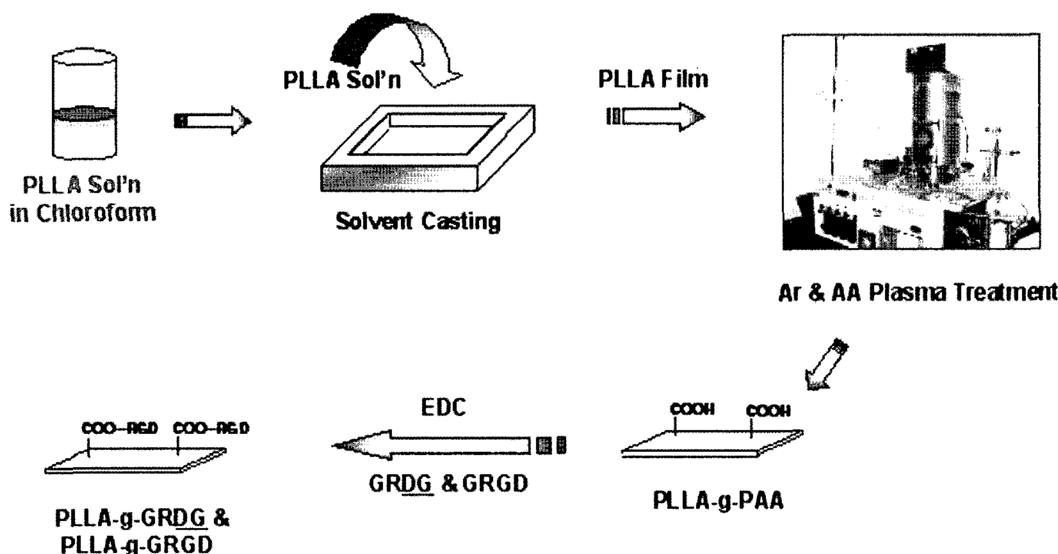

Figure 1. Preparation and surface modification of nonporous PLLA films.

casting technique. PLLA sample was dissolved in chloroform (8 wt%). After a Vortex mixer was used, PLLA solution became uniform mixture. This solution was transferred to a glass dish and dried over 48 h *in vacuo* to obtain PLLA film with 0.4~0.5 mm in thickness.

Argon Plasma Treatment and AA Grafting. The surface of the nonporous PLLA films (1 × 2 cm) was modified through *in situ* direct graft polymerization of a hydrophilic monomer such as AA onto the surface of the plasma-treated film with radio frequency glow discharge (RFGD) device (Model PTS-003IDT, I.D.T. Eng., Inc., Korea). In brief, the obtained film was fixed between radio frequency generating electrodes located in a chamber of a plasma-discharging device, all valves of the device were closed, and the pressure was then set up to be 10^{-3} torr with a vacuum pump. Under these conditions, consequently argon (Ar) and AA was directly injected into the chamber in a gas phase. Ar and AA injection valves were adjusted such that the pressure was 0.2 torr, respectively. When the desired pressure was maintained, the RF power and the pulse type negative voltage were applied in order to generate plasma. The RF power was 50 Watt, and the discharging was performed for 2 min to obtain PLLA-*g*-PAA having some carboxylic acids on the film surfaces.

Ligand Peptide Immobilization. The PLLA-*g*-PAA films containing carboxylic groups were immersed in a solution of EDC 50 mg in 0.1 M MES buffer (pH=4.6) for 4 h at room temperature under shaking. These activated films were immersed in a 10^{-3} M solution of GRDG and GRGD in phosphate buffered saline (PBS, pH=7.4) for 2 h at room temperature under shaking, respectively.[23] The PLLA films were treated individually in small test tubes containing 1 mL of the GRDG and GRGD solution. At the end of the immobilization, the films were washed under shaking with PBS (15 min), distilled water (5 min), 0.1 M MES buffer (15 min), and distilled water (15 min), and then dried at room temperature to get finally PLLA-*g*-GRDG and PLLA-*g*-GRGD.

Surface Analysis. Attenuated total reflection-Fourier transform infrared (ATR-FTIR) measurements of PLLA film surfaces were performed using IFS 66 spectrometer (Bruker, Germany) in the frequency range of 400-4000 cm^{-1} at 4 cm^{-1} resolution. The chemical composition of PLLA film surfaces was investigated by electron spectroscopy for chemical analysis (ESCA; S-Probe Surface Science, USA). The ESCA was equipped with AlK_α radiation source at 1,487 eV, 300 W at the anode and take-off angle of 55 degree with respect to the film surface. The morphology of the PLLA films was observed by scanning electron microscopy (SEM; Hitachi, Japan). The PLLA films were gold coated using a sputter coater (Eiko IB3, Japan) of 0.1~0.05 torr and ion current was 6~7 mA for 5 min, and the microscope was operated at 15 kV to image the samples. Water contact angle of PLLA film surfaces was measured by contact angle machine (VCA Optima XE Video Contact Angle System, Crest Technology Ltd., Singapore). A distilled water (3 μL) drop is put on five different sites of diverse PLLA films (1 × 1 cm) and the measured angles were averaged. The ligand peptide molecules (GRDG and GRGD) immobilized onto the PLLA films were determined using an amino acid analysis (AAA). AAA was performed after hydrolysis of the films in 500 μL 6 N HCl at 110 °C for 24 h in a sealed glass tube. After evaporation of the hydrochloric acid, the residue was dissolved in 200 μL amino acid analysis starting solvent. After microcentrifuge, up-layer solution was measured by Hewlett Packard 1100 Series. The presence of degradation products of the PLLA, poly(acrylic acid) and grafting regents may lead to additional peaks. However, in the most cases, they do not interfere with amino acids and do not have any effect on the analysis.

Fibroblast Culture. Fibroblasts were obtained from L929 (mouse fibroblast subcutaneous connective tissue). The cells were maintained in Dulbeccos Modified Eagles Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco). The cell culture was performed by incubation at 37 °C in a humidified atmosphere containing 5% carbon dioxide. To seed the fibroblasts onto the PLLA films, the modified PLLA films were placed on the bottom of the 96-well tissue culture plates and cultured for 6, 12, 24, and 48 h. The cells were seeded with the density of 5×10^3/mL. For SEM observation, the PLLA films were completely dehydrated by a series of ethanol solution after fibroblasts fixation of 2.5% glutaraldehyde for 24 h at 4 °C. Cell WST-1 viability was measured after adding 1/10 μL WST-1 solution/Media to every well and incubating for 3 h. To a 96-well ELISA plate 200 μL pigment solution was added and the absorbance was measured at 460 nm.[24,25]

Results and Discussion

Surface Modification of PLLA Films. Polymers play an important role in tissue engineering. Polymer scaffolds for tissue regeneration should have nontoxicity, good biocompatibility minimizing the interactions with blood/tissue cells and proteins, suitable biodegradability which degrades at the proper periods of time, and finally the interaction with desired specific cells. Many nonbiodegradable or biodegradable scaffolds have been used in tissue engineering field. PGA, PLLA, and their copolymers are only being used as biodegradable scaffolds because they are nontoxic and approved from FDA. However, they have insufficient initial cell binding sites and hydrophobic characteristics and thus lack the ability to cell adhesion. To improve initial cell adhesion, a number of studies were performed in terms of hydrophilization and ligand immobilization onto the polymers by various surface modifications including plasma treatment.[14-16]

Although many physical and chemical methods are app-

lied, these are very complicated and decreasing mechanical properties. Among them, plasma treatment is very simple and reliable methods as well as unchangeable bulk properties of polymeric materials.[26,27] The indirect AA grafting to the polymer scaffolds was achieved by plasma treatments to make hydrophilic, biocompatible, and functional surfaces until now.[28,29] In this paper, the nonporous PLLA films were treated with Ar plasma treatment and *in situ* direct AA grafting in plasma chamber for introducing carboxylic groups and hydrophilic property and then GRGD immobilization. Such surface modifications of the PLLA films were confirmed from various surface analyses and adhesion behaviors of fibroblasts.

Surface Characterization of Modified PLLA Films. ATR-FTIR, ESCA, SEM, water contact angle, and AAA were utilized for evaluating the surface properties of the modified PLLA films.

Figure 2 shows ATR-FTIR spectra of various modified PLLA films. PLLA control displayed a typical ester peak by lactide at 1750 cm^{-1}. PLLA-*g*-PAA also revealed nearly one peak which is similar to PLLA control because of overlapping carboxylic acid and ester peaks.[30,31] Generally, the ligand peptide immobilized on PLLA film shows small absorption bands of amides I (1640 cm^{-1}) and II (1555 cm^{-1}), but in the case of PLLA-*g*-GRDG and PLLA-*g*-GRGD, their amide peaks couldn't be detected due to the existence of small amount of peptide on the surface. The detail atomic compositions of the PLLA surfaces were determined by ESCA. Figure 3 shows ESCA wide scan spectra of various modified PLLA films. While the surfaces of PLLA control and PLLA-*g*-PAA demonstrated C1s and O1s peaks, the ones of PLLA-*g*-GRDG and PLLA-*g*-GRGD did C1s, O1s, and N1s by virtue of the immobilized ligand peptides. Table I lists ESCA data for various modified PLLA films. After the AA grafting on PLLA control, the oxygen atomic percent of PLLA-*g*-PAA increased at the expense of a decrease in the carbon atomic percent. In addition, the nitrogen atoms of PLLA-*g*-GRDG and PLLA-*g*-GRGD films appeared by the immobilization of ligand peptide having nitrogen atoms and their atomic percents were 2.1 and 2.0%, respectively. These values coincided with other ESCA data regarding RGD immobilization surfaces.[32]

The SEM morphologies of various modified PLLA film surfaces were shown in Figure 4. The PLLA control surface was considerably smooth and the morphologies of other modified PLLA surfaces were very similar to PLLA control,

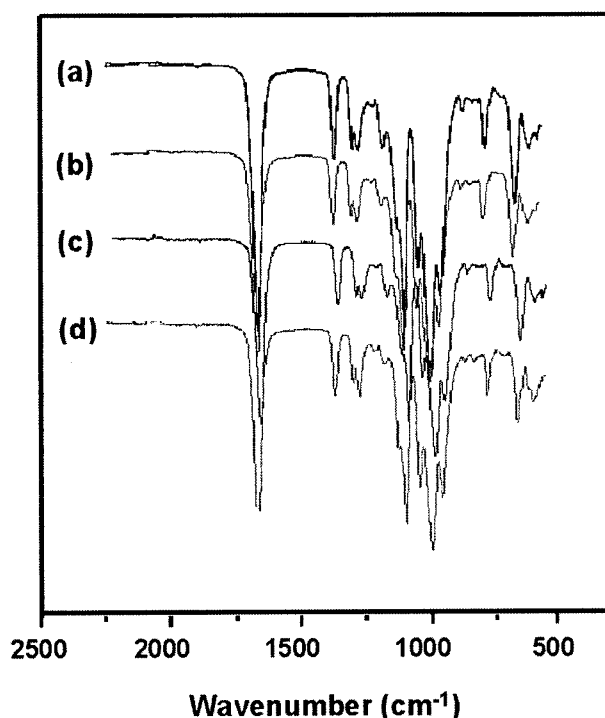

Figure 2. ATR-FTIR spectra of various modified PLLA films : (a) PLLA control, (b) PLLA-*g*-PAA, (c) PLLA-*g*-GRDG, and (d) PLLA-*g*-GRGD.

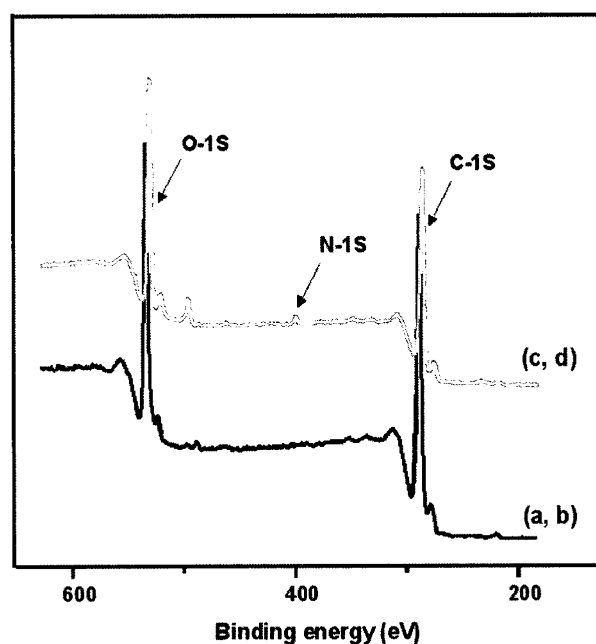

Figure 3. ESCA wide scan spectra on various PLLA films : (a, b) PLLA control and PLLA-*g*-PAA and (c, d) PLLA-*g*-GRDG and PLLA-*g*-GRGD.

Table I. ESCA Data for Various PLLA Films

Material	Atomic %			N/C
	C	O	N	
PLLA Control	79.2	20.8	-	-
PLLA-g-PAA	54.9	45.1	-	-
PLLA-g-GRDG	65.1	32.8	2.1	0.032
PLLA-g-GRGD	65.3	32.7	2.0	0.031

irrespective of AA grafting and ligand peptide immobilization. It implies that no significant change in surface morphology of the modified PLLA films was observed after surface modification. Figure 5 shows the water contact angles of various modified PLLA films. The contact angle of PLLA control was 74 degree, which means the typical hydrophobic surface, whereas PLLA-*g*-PAA exhibited lower contact angle of 45 degree due to the grafting of hydrophilic carboxylic groups.[33] The PLLA-*g*-GRGD displayed somewhat higher contact angle of 50 degree than PLLA-*g*-PAA, indicating still relatively hydrophilic property. The contact angle of PLLA-*g*-GRDG was nearly similar to that of PLLA-*g*-GRGD. Table II lists the amounts of ligand peptide immobilized on PLLA films measured by AAA. The peptide values in PLLA-*g*-GRDG and PLLA-*g*-GRGD were 145 and 138 pmol/cm^2, respectively, and those are corresponded well with the existing results reported by other investigators.[18,34]

Table II. Amounts of Ligand Peptide Immobilized on PLLA Films

Film	pmole/cm^2
PLLA-*g*-GRDG	145
PLLA-*g*-GRGD	138

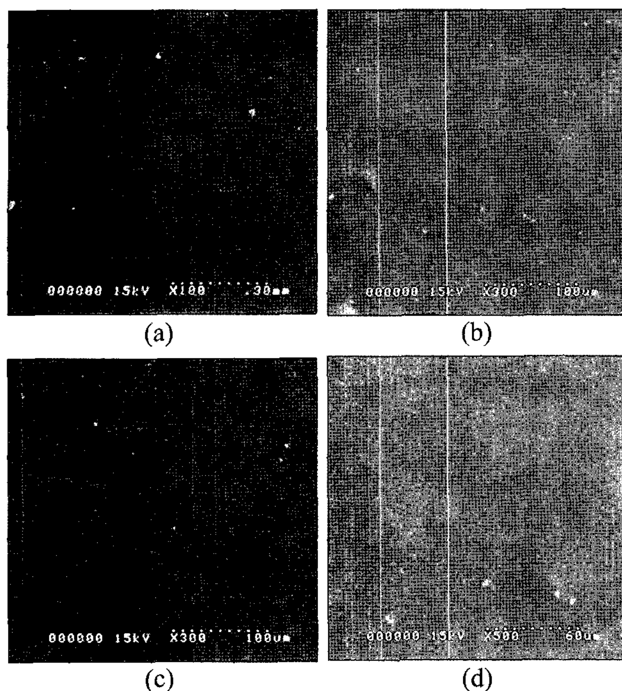

Figure 4. SEM morphologies of various modified PLLA films : (a) PLLA control, (b) PLLA-*g*-PAA, (c) PLLA-*g*-GRDG, and (d) PLLA-*g*-GRGD.

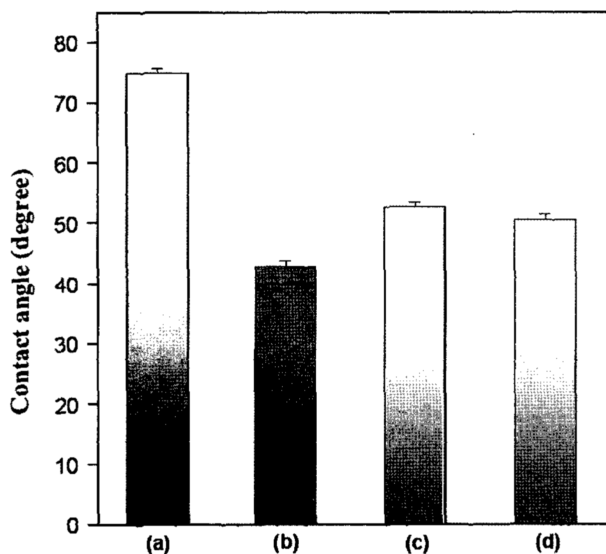

Figure 5. Water contact angles of various modified PLLA films : (a) PLLA control, (b) PLLA-*g*-PAA, (c) PLLA-*g*-GRDG, and (d) PLLA-*g*-GRGD.

Cell Adhesion of Modified PLLA Films. Fibroblasts were cultured upon various modified PLLA films to assess their cell adhesive properties. Figure 6 demonstrates SEM morphologies of fibroblast adhesion from 6 to 48 h on various modified PLLA films. All modified PLLA films showed more adhesion and spreading of fibroblasts than did PLLA control. Furthermore, fibroblast adhesion and spreading increased with increasing culture time to 48 h in all PLLA films. Among them, PLLA-*g*-GRGD displayed the most cell adhesion and spreading as compared to other PLLA films.

Figure 7 shows WST-1 protein contents of fibroblast adhesion on various modified PLLA films. As expected from the results of SEM, protein contents from fibroblast adhered on the PLLA films increased as the culture time increases from 6 to 48 h in all samples. PLLA-*g*-PAA exhibited more fibroblast adhesion than PLLA control. This is attributed to increased hydrophilicity by the grafting of carboxylic groups on PLLA surface. On the whole, fibroblast adhesion to the PLLA surfaces was improved in the order of PLLA control < PLLA-*g*-PAA ≈ PLLA-*g*-GRDG < PLLA-*g*-GRGD, indicating that PLLA-*g*-GRGD has the highest cell adhesive property due to RGD immobilization.[13,25,30] It is interesting to note that fibroblast adhesion to PLLA-*g*-GRDG demonstrated similar trend to PLLA-*g*-PAA, although GRDG as a negative ligand peptide was immobilized on PLLA-*g*-PAA. It suggests that only GRGD ligand peptide may induce the adhesion and spreading of fibroblasts.

In cell culture onto polymers, the differences in the response of cells to biomimetic surfaces were found to depend on cell type,[22] peptide concentration/distribution,[35] and method used for attachment of sequences.[18] Especially, the number of attached cells is clearly related to RGD surface density. A higher RGD surface density is furthermore related to cell spreading, cell survival, focal contact formation, and to

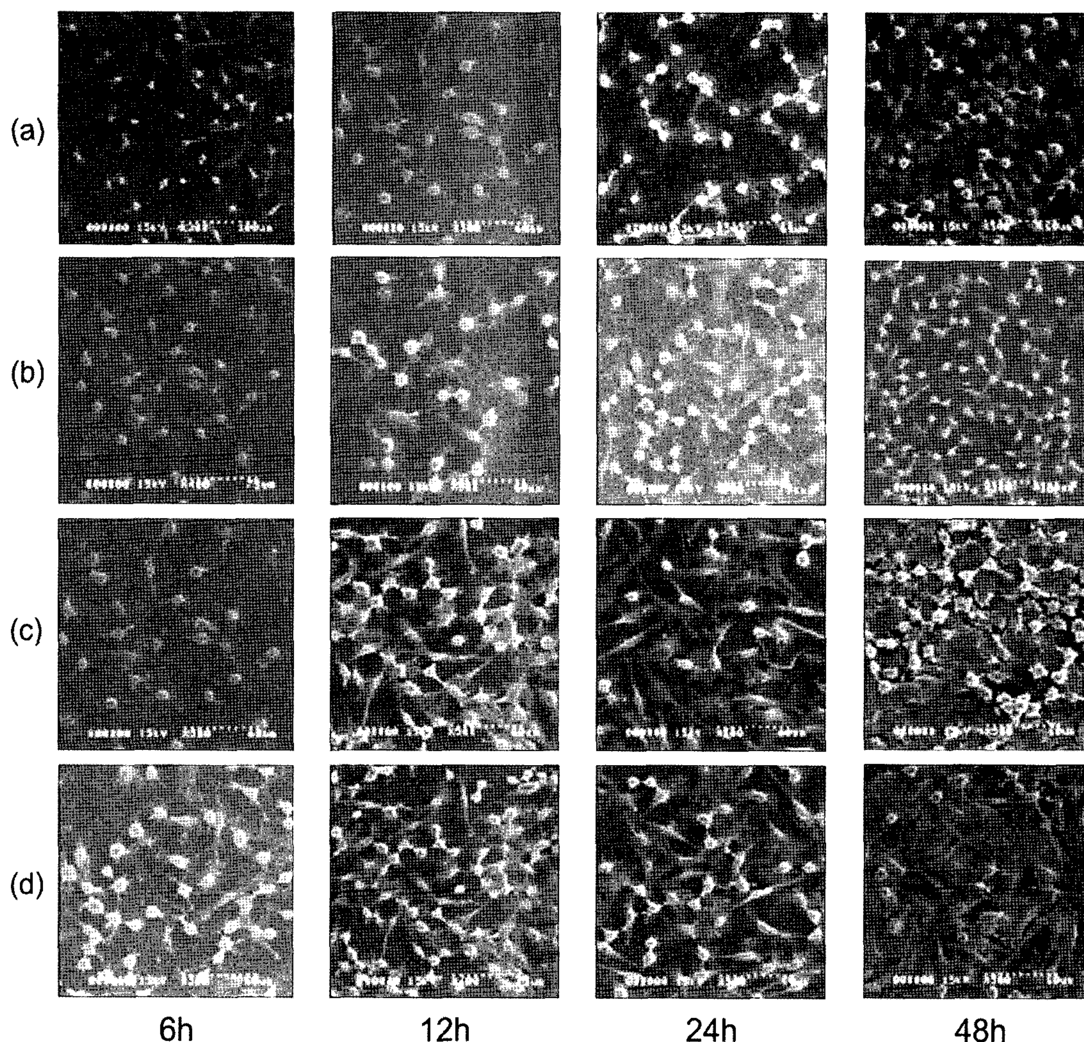

Figure 6. SEM morphologies of fibroblast adhesion from 6 to 48 h culture on various modified PLLA films : (a) PLLA control, (b) PLLA-*g*-PAA, (c) PLLA-*g*-GRDG, and (d) PLLA-*g*-GRGD.

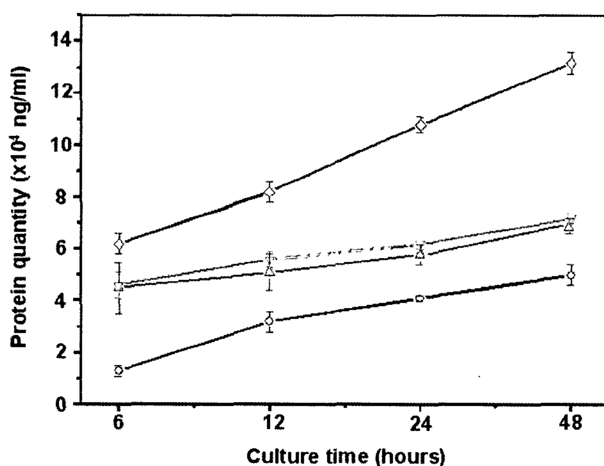

Figure 7. WST-1 protein contents of fibroblast adhesion on various modified PLLA films : (○) PLLA control, (△) PLLA-*g*-PAA, (▽) PLLA-*g*-GRDG, and (◇) PLLA-*g*-GRGD.

some extent proliferation. From the previous results of Hubbell and coworker,[22,36,37] a minimal amount of as low as 1 fmol RGD peptide/cm^2 needs for cell spreading and as low as 10 fmol/cm^2 for formation of focal contacts and stress fibers on RGD grafted glass surfaces. Therefore, our PLLA-*g*-GRGD, in which the amounts of the immobilized RGD peptide were 138 pmol/cm^2, may be sufficient to induce the adhesion, spreading, and focal contact of fibroblast to the surface.

Conclusions

Biodegradable PLLA film immobilized with cell adhesive ligand peptide (RGD), PLLA-*g*-GRGD, was prepared by surface modification including *in situ* direct AA grafting following Ar plasma treatment and further RGD immobilization. The surface properties of the modified PLLA films were examined by various surface analyses and cell adhe-

sion property was evaluated using fibroblast. The grafting of AA and ligand peptides, RDG and RGD, to the PLLA films was confirmed by ATR-FTIR and ESCA, whereas the immobilized amounts of the peptides were ca. 140 pmol/ cm^2. As AA and RGD were grafted to the PLLA surface, the hydrophilicity of the modified PLLA surfaces increased to some extent, but their surface morphologies showed still relatively smoothness. Fibroblast adhesion to the PLLA surfaces increased with increasing to 48 h and was improved in the order of PLLA control < PLLA-*g*-PAA=PLLA-*g*-GRDG < PLLA-*g*-GRGD. It suggests that PLLA-*g*-GRGD exhibits the highest adhesion and spreading of fibroblasts due to the immobilization of cell adhesive RGD peptide. Accordingly, such PLLA-*g*-GRGD, which has cell adhesive ligand peptide, may be useful as functional biodegradable scaffolds for tissue engineering.

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