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Effect of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Surface with Different Wettability on Fibroblast Behavior

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Abstract: Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) is a microbial storage polymer with biodegradable properties. In order to improve the cell compatibility of PHBV surfaces, the physicochemical treatments have been demonstrated. In this study, physical method was corona discharge treatment and chemical method was chloric acid mixture solution treatment. The physicochemically treated PHBV film surfaces were characterized by the measurement of water contact angle, electron spectroscopy for chemical analysis, and scanning electron microscopy (SEM). The water contact angle of the physicochemically treated PHBV surfaces decreased from 75 to 30~40 degree, increased hydrophilicity, due to the introduction of oxygen-based functional group onto the PHBV backbone chain. The mouse NIH/3T3 fibroblasts cultured onto the physicochemically treated PHBV film surfaces with different wettability. The effect of the PHBV surface with different wettability was determined by SEM as counts of cell number and [^{3}H]thymidine incorporation as measures of cell proliferation. As the surface wettability increased, the number of the cell adhered and proliferated on the surface was increased. The result seems closely related with the serum protein adsorption on the physicochemically treated PHBV surface. In conclusion, this study demonstrated that the surface wettability of biodegradable polymer as the PHBV plays an important role for cell adhesion and proliferation behavior for biomedical application.

Keywords : poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), corona discharge, chloric acid mixture solution, wettability, NIH/3T3 fibroblasts.

Introduction

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) is a microbial storage polymer with biodegradability and biocompatibility. These biodegradable and biocompatible polymers, because of their unique physicochemical properties, are regarded as specialty materials in various fields of biomedical application. One of the most significant candidates for the study of biodegradable polymer is the family of polyhydroxyalkanoates (PHA) due to its relatively good biocompatibility with no acute inflammation, no abscess formation, and no tissue necrosis.[1] Also, these polymers are entirely natural and obtained from micro-organisms, *Alicaligen eutrophus* as gram-negative bacteria. Poly(3-hydroxy-

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butyrate) (PHB) and its copolymers with hydroxyvalerate with varying HV ratios are the most widely existing members of this biopolymer group. These biodegradable and biocompatible polyesters, because of their unique physicochemical properties, such as piezoelectricity, are claimed to induce bone reformation in load-bearing sites.[2,3]

It is recognized that the behavior of the adhesion and proliferation of an anchorage-dependent cells and tissue on polymeric materials depend on the surface characteristics such as wettability (hydrophilicity/hydrophobicity or surface free energy), chemistry, charge, roughness, and rigidity.[4-6] A large number of research groups have studied the interactions of different types of cultured cells with various polymers with different wettabilities to correlate the relationship between surface wettability and blood-, cell-, or tissue-compatibility.[7-9] Some others have studied the interactions of different type cells with a range of copolymers with different wettabilities, such as hydroxyethyl methacrylate (HEMA, hydrophilic)/methyl methacrylate (MMA, hydrophobic),[10-12] HEMA/ethyl methacrylate (EMA, hydrophobic),[13-15] HEMA/styrene (hydrophobic) copolymers[15] with different compositions. One problem derived from the study using different kinds of polymer is that the surfaces are heterogeneous both chemically and physically (different surface chemistry, roughness, rigidity, crystallinity, and so on), which may result in considerable variation.

In our previous study, to improve the cell compatibility of the group of poly(α-hydroxy acid) such as polyglycolide (PGA), polylactide (PLA), and poly(lactide-*co*-glycolide) (PLGA), several modification methods have been investigated by means of physical treatment for wettability chemogradient[16-27] and chemical treatment for surface oxidation.[4,28-30] It has been demonstrated that the cells were more adhered and grown on the hydrophilic surface than those of hydrophobic, and the maximum adhesion of cells appeared moderate hydrophilic surface such as 50~55 degree of water contact angle due to the preferential adsorption of serum protein.[16-27] In addition, the PLGA films were treated with chemical treatments that were chloric acid mixture, sulfuric acid, and NaOH solution. It was observed that the adhesion and growth of various cells on the chemically treated PLGA surfaces, especially chloric acid treated PLGA surface, were more active than on the control.[31]

In this study, the PHBV film surfaces were treated with physicochemical treatment by corona discharge and chloric acid mixture solution treatment to increase surface wettability resulting in the improvement of cell compatibility. The PHBV film surfaces were characterized by the measurement of water contact angle, electron spectroscopy for chemical analysis (ESCA), and scanning electron microscope (SEM). NIH/3T3 fibroblasts were cultured on the surfaces of the physicochemically treated films with different wettability for the evaluation of cell adhesion and proliferation behavior. Also, the activities of DNA synthesis on the PHBV films with different wettability were determined.

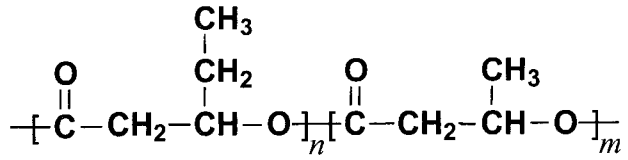

Figure 1. Chemical structure of the poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate).

Experimental

Materials. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (15% HV content), used for the preparation of films, were received as a gift from Monsanto Chem. Co. Ltd. (Delaware, USA) (Figure 1). Perchloric acid and potassium chlorate were purchased from Junsei Chem. Co. (Tokyo, Japan). Rosewell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), trypsin-EDTA, antibiotic-antimycotic (penicillin G sodium, streptomycin sulfate, and amphotercin B in saline), and phosphate buffered saline (PBS) were purchased from GIBCO BRL (Grand Island, USA).

Preparation of PHBV Films. In order to fabricate film, hot pressing method was used. The PHBV pellets were placed between two pieces of stainless plate that had been heated at ~170 °C for 5 min in a heating press (100 kg$_f$/cm^2) (31000-946, Carver Laboratory Press, Fred S. Carver Inc., USA). The PHBV films were allowed to cool at room temperature and then were cut into 6 × 2 cm rectangular. They were approximately 180 ± 20 μm in thickness. The films were ultrasonically washed in ethanol and were kept in a vacuum until use.

Physicochemical treatment of PHBV Films. The PHBV film was treated by physical treatment method with a radio-frequency (RF) corona discharge apparatus for the preparation of gradient surfaces, in a manner similar to that used in our previous study.[16,18,27,32] Briefly, a knife-type electrode was connected to the RF generator and the power increased gradually by a motorized drive. The cleaned PHBV film was placed on the sample bed and dry air was purged through the apparatus at a flow rate of 20 L/min. The electrode was 1.5 mm away from the sample bed was translated at a constant speed, 1.0 cm/s, the corona from the electrode was discharged onto the sample with gradually increasing power (from 10 to 50 W at 100 kHz). The sample film (6 × 2 cm) was treated for 5 s. By this treatment, the sample surface was continuously exposed to the corona with increasing power, resulting in the formation of wettability gradient on the surface.

The PHBV films were treated by chemical treatment method with the chloric acid mixture solution [70% perchloric acid ($HClO_4$)/potassium chlorate ($KClO_3$) in aq. saturated solution, ratio of 3 : 2] for up to 5 hrs. The PHBV films were

dried *in vacuo* overnight to remove residual solvent and then were totally cleaned with distilled water several times.

Characterizations of the Physicochemically Treated PHBV Films. The physicochemically treated PHBV film surfaces were characterized by the measurement of water contact angle and SEM. The water contact angle, an indicator of the wettability of surfaces, was measured by a sessile drop method at room temperature using an optical bench-type contact angle goniometer (Model 100-0, Rame-Hart, Inc.). Drops of purified water, 3 μL, were deposited onto the sample surfaces using a microsyringe attached on the goniometer. The visual changes of the physicochemically treated PHBV film surfaces were observed by SEM (S-2250N, Hitachi Co., Japan). The changes in the chemical structure of physicochemically treated PHBV film surfaces were analyzed by ESCA (ESCALAB MK II, V. G. Scientific Co., UK) equipped with an Al K_α radiation source at 1487 eV and 300 W power at the anode. Survey and C 1 *s* core level scan spectra were taken to analyze the section of the physicochemically treated PHBV film surfaces.

Fibroblasts Adhesion and Proliferation. NIH/3T3 mouse embryo fibroblasts (KCLB 21658, Korean Cell Line Bank, Seoul, Korea) were cultured in RPMI 1640, supplemented 10% FBS and antibiotic-antimycotic. The cells were used to study the effects of surface wettability on the behavior of cultured cells. The cell culture on the physicochemically treated PHBV films was carried out for up to 2 days. After incubation at 37 °C under 5% CO_2 atmosphere, the surfaces were washed with PBS and the cells attached on the surfaces were fixed with 2.5% glutaraldehyde in PBS for 24 hrs at room temperature. After thorough washing with PBS, the cells on the surfaces were dehydrated in ethanol graded series (50, 60, 70, 80, 90, and 100%) for 10 min each and allowed to dry in a clean bench at room temperature.

SEM Observation. The cell-attached surfaces were gold deposited with plasma sputter (Emscope, Model SC 500 K, UK) and examined by SEM with a tilt angle of 45 degree. The cell density on the surfaces was estimated by counting the number of attached cells on. Four fields for each sample were randomly counted whose actual counting area was 1,127,607.5 μm^2 and the results were expressed in terms of the number of cells attached per cm^2. Further detailed procedures for the cell culture on the polymer films were described in previous papers.[27,33]

[^{3}H]thymidine Incorporation. To determine the DNA synthesis, 2 μCi [^{3}H]thymidine (Amersham Life Science, Buckinghamshire, UK) was added 2 hrs before harvesting. At 2, 4, and 8 days after seeding, the cell layers were rinsed with cold PBS and treated with 5% trichloroacetic acid (TCA, Sigma Co., USA). The pellets were dissolved with 0.2 N sodium hydroxide (Junsei Chem. Co., Japan), and the solution was neutralized with 0.2 N hydrochloride (Junsei Chem. Co., Japan). The solution was then assayed for radioacitivity using a liquid scintillation spectrometer (Beckman, Palo Alto, CA, USA).

Statistical Analysis. Measured data were subjected to statistical analysis using Student's *t*-tests (independent-difference) for independent samples with unequal of equal variances were used to test equality of the mean values at a 95% confidence interval ($P < 0.05$).

Results and Discussion

Characterizations of the Physicochemically Treated PHBV Films. The physicochemically treated PHBV films did not show any visible changes (date not shown). However, the water contact angles of the corona discharge treated and the chloric acid treated PHBV film surfaces gradually decreased from 75.3 to 34.6 degree and from 75.3 to 29.5 degree, respectively (Figure 2). In corona discharge treatment, surface wettabilities of the PHBV film surfaces gradually decreased along the sample length with increasing corona power and the chloric acid treated PHBV film surfaces were gradually increased with increase of treatment time. It was also confirmed by FTIR-ATR and ESCA in this study. The

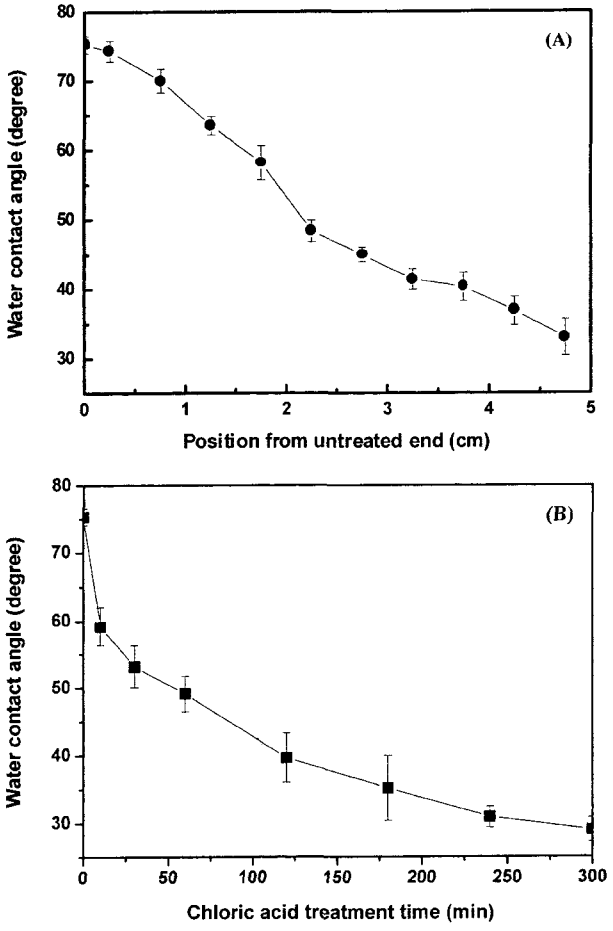

Figure 2. Water contact angles of (A) the corona discharge treated and (B) the chloric acid treated PHBV films.

Table I. ESCA Results of Physicochemically Treated PHBV Film Surfaces

Method	Condition	Atomic %[a]		Ratios of	-C-O-/-C-C-[b]	-C=O/-C-C-[b]	O=C-O-/-C-C-[b]
		C	O	O1s/C1s	(~286.6 eV)	(~287.1 eV)	(~289.1 eV)
Corona Discharge	Control	72.5	27.5	0.38	0.37	0.19	0.36
	0.5 cm	69.4	30.6	0.44	0.56	0.17	0.86
	1.5 cm	62.5	37.5	0.6	0.83	0.21	1.6
	2.5 cm	50.9	49.1	0.96	1.6	0.21	2.8
	3.5 cm	50.1	49.9	0.99	1.8	0.23	3.9
	4.5 cm	47.3	52.7	1.11	2.7	0.23	4.5
Chloric acid Mixture	Control	72.5	27.5	0.38	0.37	0.19	0.36
	10 min	61.7	38.3	0.62	0.98	0.22	1.9
	60 min	52.8	47.2	0.89	2.1	0.21	2.9
	180 min	49.8	50.2	1.01	2.9	0.25	3.8

[a]Analyzed from survey scan spectra.
[b]Analyzed from carbon 1s core-level spectra.

changes in chemical structure of the film surfaces were further analyzed by ESCA carbon 1*s* spectra. The physicochemically treated PHBV film surfaces showed carbon (binding energy, ~285 eV) and oxygen (~532 eV) peaks, as expected. On the other hand, the control showed alkyl carbon (-C-C-, ~285.0 eV), ether carbon (-C-O-, ~286.6 eV), carboxylic carbon (O=C-O-, ~289.1 eV), and carbonyl carbon (-C=O, ~287.1 eV) peaks (Table I). The decrease in the contact angles (and thus the increase in wettability) and the increase in the amount of the peroxide concentration may be due to the oxygen-based polar functionalities incorporated on the PHBV surface by the physicochemical treatment.

A schematic diagram is the possible mechanism of surface oxidation which occurs on the PHBV surface by physicochemical treatment (Figure 3). The physicochemical treatment of a polymer surface produces carbon radicals from the hydrocarbon backbone, followed by the formation of unstable hydroperoxides to produce various oxygen-based functionalities (hydroxyl group, ether, ketone, aldehyde, carboxylic acid, carboxylic ester, and so on) by reaction with additional oxygen.[21,23,25,26]

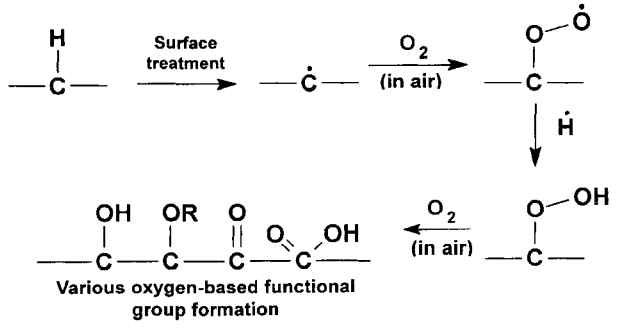

Figure 3. Schematic diagram of the formation of oxygen-based functional groups on the PHBV surface by the physicochemical treatment.

Interaction of Cells with the Physicochemically Treated PHBV Films. The physicochemically treated PHBV films were used to study the effect of surface wettability on cell adhesion and proliferation behavior. In this study, fibroblasts with anchorage-dependent were cultured on the physicochemically treated PHBV film surfaces to investigate the effect of cell adhesion (after 1 day) and proliferation (after 2 days) in terms of the surface hydrophilicity/hydrophobicity for cell-polymeric material interaction. After equilibrating the physicochemically treated PHBV films mounted in test chambers with PBS for 30 min, fibroblasts (4×10^4/cm^2) were seeded the surfaces. The fibroblasts were cultured for up to 2 days on the physicochemically treated PHBV films. The culture medium was changed after 1 day.

The fibroblasts cultured on the wettability gradient PHBV surfaces for up to 2 days. The cell adhesion and proliferation behavior on the wettability gradient PHBV surfaces showed a similar trend as shown in Figure 4; as surface wettability increased, the cells attached on the surface increased. As the surface wettability increased along the sample length, the cell adhesion on the surface increased and then decreased. The cells were adhered more on the positions with moderate hydrophilicity of the wettability gradient surface than more hydrophobic or hydrophilic positions even though the difference in the number of cells attached was not significant on the hydrophilic positions.[27] The maximum adhesion of the cells appeared at around position 2.5 cm (water contact angle, about 50 degree).

Another result is that the fibroblasts cultured on the chloric acid treated PHBV surfaces for up to 2 days. The cell adhesion and proliferation behavior on the chloric acid PHBV surfaces showed a similar trend as shown in Figure 5; as surface wettability increased, the cells attached on the surface increased. As the surface wettability increased along the treatment time, the cell adhesion on the surface increased and then decreased. In the same result, the maximum adhesion

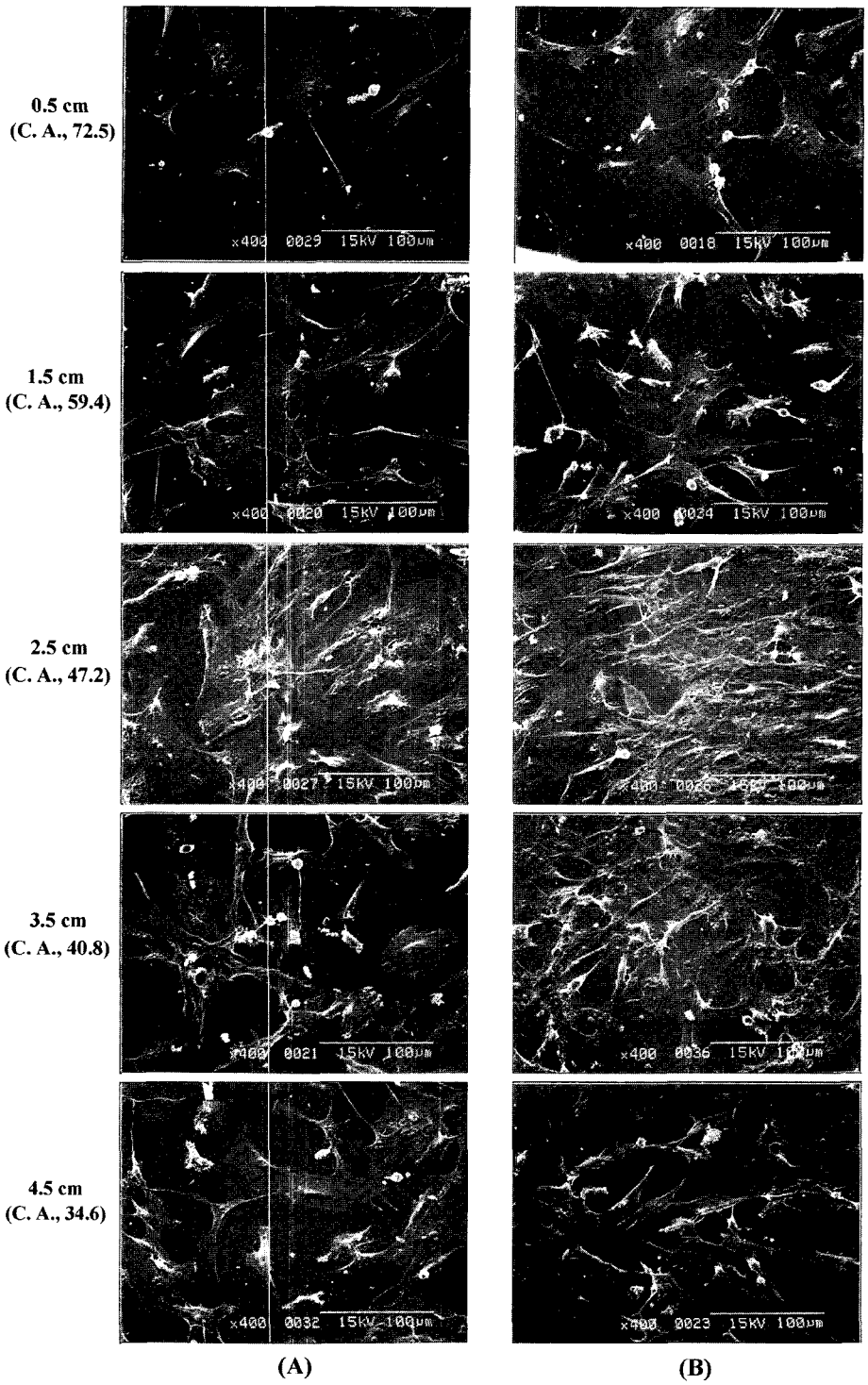

Figure 4. SEM microphotographs of the fibroblasts adhered on wettability gradient PHBV surfaces after (A) 1 and (B) 2 days culture (original magnification, ×400). C. A. means water contact angle (degrees).

of the cells appeared at the 180 min-treated position (water contact angle, about 50 degree). The cell morphology was also changed after the physicochemical treatment. The cells were protruded fillodopia and lamelliopodia resulting in more spread and flattened on the physicochemically treated PHBV films than control (untreated films) ones after a 1 day culture. The cells after 2 days culture were almost flattened on the physicochemically treated PHBV films. In contrast,

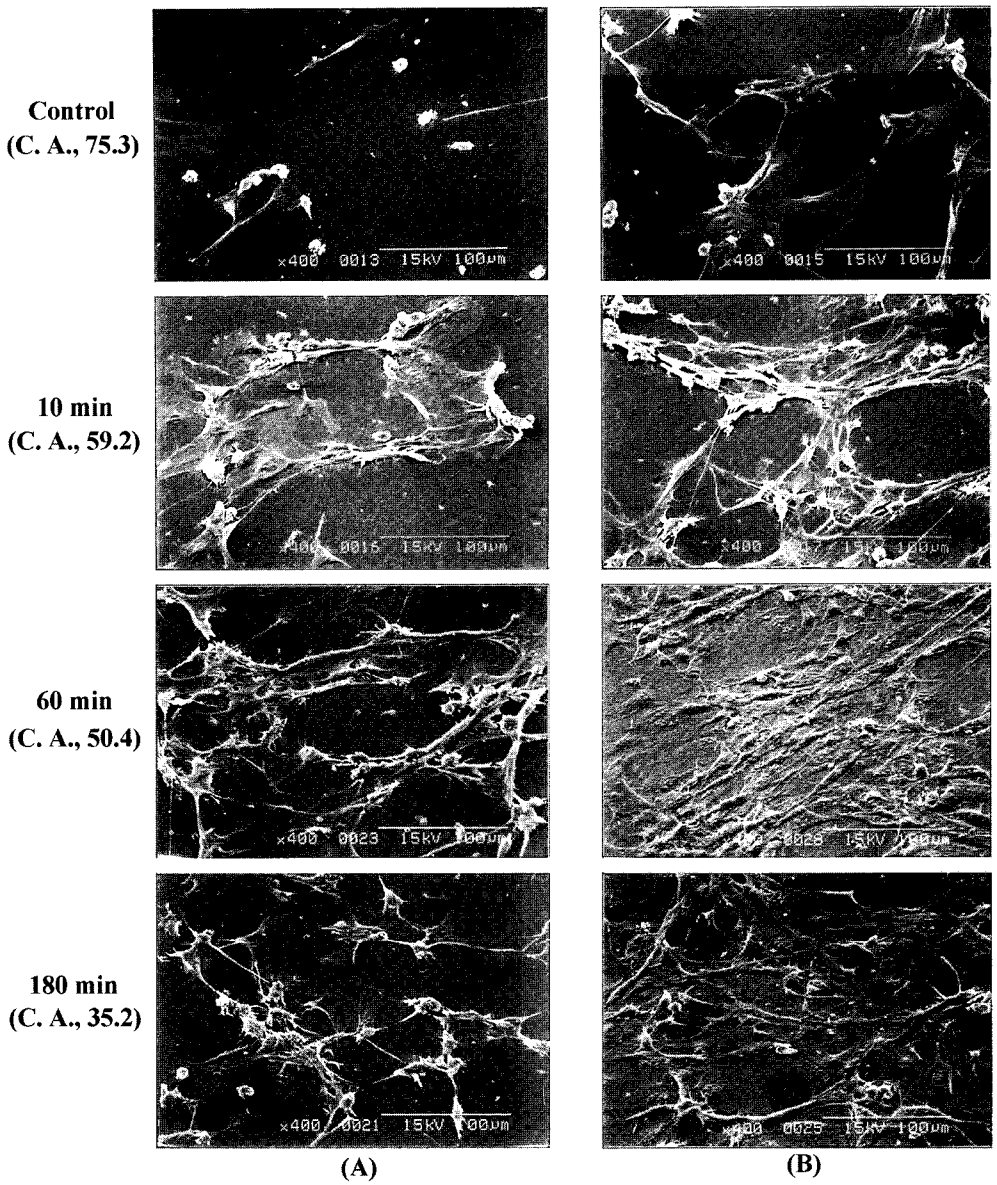

Figure 5. SEM microphotographs of the fibroblasts adhered on chloric acid treated PHBV surfaces after (A) 1 and (B) 2 days culture (original magnification, ×400). C. A. means water contact angle (degrees).

the control still showed round cell morphology.

As shown in Figure 6, substantially more fibroblasts were adhered and proliferated on the physicochemically treated PHBV films than those of the control. The cell proliferation behavior on the film surfaces after 2 days culture was almost similar to that of cell adhesion after 1 day, more attachment of the fibroblasts on the higher wettable PHBV film surfaces. As the surface wettability increased, the fibroblasts adhered on the surface were increased. Accordingly, NIH/3T3 fibroblasts are more adhered, spread, and proliferated on the higher wettable polymer surface. It was found from the result that the position of maximum adhesion and proliferation was the chloric acid-treated position for 180 min. These data lead us to the protocol that the effect of surface wettability on DNA synthesis by the fibroblasts was evaluated. [^{3}H]thymidine incorporation was increased substantially in the PHBV film with hydrophilic surface by physicochemical treatment in comparison with control (untreated film) as shown in Figure 7. The fact that the cells are more adhered, spread, and proliferated on the hydrophilic surfaces was also observed by other groups as they cultured various cells onto polymer materials with different surface wettability.[34-38] This phenomenon also seems closely related to the serum protein adsorption on the surfaces, as discussed earlier; the preferential adsorption of some serum proteins (or cell-adhesive proteins) such as fibronectin and vitronectin from

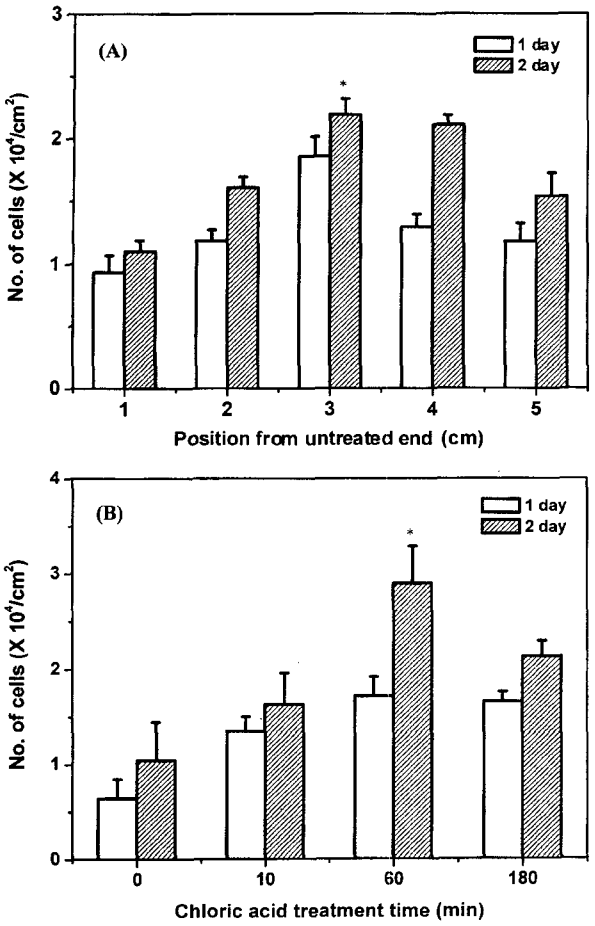

Figure 6. Changes of numbers of fibroblasts on (A) the corona discharge treated and (B) the chloric acid treatd PHBV surfaces for 2 and 4 days culture. $*P < 0.05$.

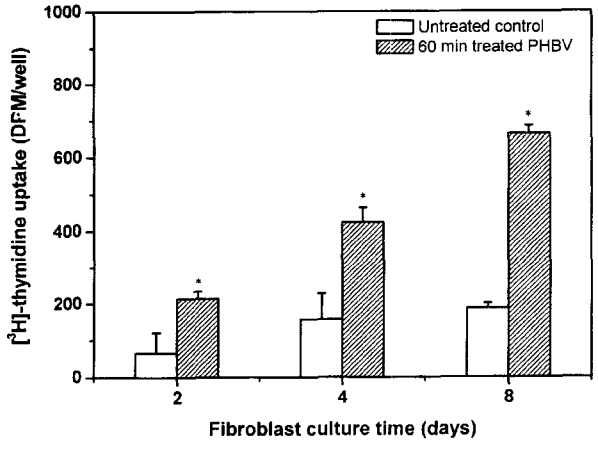

Figure 7. Effect of surface wettability on [^{3}H]thymidine incorporation. $*P < 0.05$ compared with control.

culture medium onto higher wettable polymer surfaces maybe a reason for better cell adhesion, spreading, and proliferation.[27,39,40]

In our previous studies, adhesion and proliferation of cells may be accomplished by various mechanisms. First of all, a cell contacting the surface coated with physiological adhesion proteins such as laminin, fibronectin, vitronectin, and so on will promote attachment, cell adhesion may be promoted by cell contact with specific synthetic peptides derived from extracellular matrix molecules, and others.[27,41] Adhesive proteins are well known to play an important role in cell attachment onto substrates. In conclusion, this study demonstrated that the surface wettability of polymer plays an important role for cell adhesion and proliferation behavior. The studies of biochemical analysis to assess state of cells are in progress.

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