

Interaction of Different Types of Cells on Poly(L-lactide-co-glycolide) Surface with Wettability Chemogradient

Gilson Khang and John M. Rhee

*Department of Polymer Science and Technology, Chonbuk National University,
664-14 Dukjin Dong 1 Ga, Dokjin Ku, Chonju 561-756, Korea*

Jin Ho Lee

*Department of Polymer Science and Engineering, Hannam University,
133 Ojeong Dong, Daedeog Ku, Taejon 306-791, Korea*

Ilwoo Lee

*Department of Neurosurgery, Catholic University Medical College,
520-2 Deaheung Dong, Jung Ku, Taejon 301-723, Korea*

Hai Bang Lee*

*Biomaterials Laboratory, Korea Research Institute of Chemical Technology,
P.O. Box 107, Yusung, Taejon 305-606, Korea*

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Abstract : A wettability chemogradient on poly(L-lactide-co-glycolide) (PLGA) films was prepared by treating the films in air with corona from a knife-type electrode whose power increases gradually along the sample length. The PLGA surfaces oxidized gradually with the increasing corona power, and the wettability chemogradient was created on the surfaces as evidenced by the measurement of water contact angles and electron spectroscopy for chemical analysis. The wettability chemogradient PLGA surfaces were used to investigate the interaction of four different types of cells such as hepatoma (Hep G2), osteoblast (MG 63), bovine aortic endothelial (CPAE), and fibroblast (NIH/3T3) cells in terms of the surface hydrophilicity/hydrophobicity of PLGA. The cells adhered and grown on the chemogradient surface along the sample length were counted and observed by scanning electron microscopy. It was observed that the cells were adhered, spread, and grown more onto the positions with moderate hydrophilicity of the wettability chemogradient PLGA surface than the more hydrophobic or hydrophilic positions, regardless of the cell types used. The maximum adhesion and growth of the cells appeared at around water contact angles of 53~55°. This result seems closely related with the serum protein adsorption on the surface; the serum proteins were also adsorbed more onto the positions with moderate hydrophilicity of the wettability chemogradient surface. It seems that the wettability plays important roles for cell adhesion, spreading and growth on the PLGA surface. The surface modification technique used in this study may be applicable to the area of tissue engineering for the improvement of tissue compatibility of films- or scaffold-type substrates.

Introduction

Recently, poly(lactide-co-glycolide)s (PLGA) are extensively studied or tested for a wide range of

medical applications as bioerodible materials due to their good biocompatibility, controllable biodegradability, and relatively good processability.¹ PLGA is a bioresorbable polyester belonging to the group of poly(α -hydroxy acids). This polymer and its homopolymers (polylactide (PLA) and polyglycolide (PGA)) degrade by nonspecific

*e-mail : hblee@pado.kRICT.re.kr

hydrolytic scission of their ester bonds.² The hydrolysis of PLA yields lactic acid which is a normal byproduct of anaerobic metabolism in human body and is incorporated in the tricarboxylic acid (TCA) cycle to be finally excreted by the body as carbon dioxide and water. PGA degrades by a combination of hydrolytic scission and enzymatic (esterase) action producing glycolic acid³ which can either enter the TCA cycle or be excreted in urine and be eliminated as carbon dioxide and water.⁴ The degradation time of PLGA can be controlled from weeks to over a year by varying the ratio of monomers and the processing conditions.⁵ It might be a suitable biomaterial for use in tissue engineered repair systems⁶⁻¹¹ in which cells are cultured within PLGA films or scaffolds and in drug delivery systems¹²⁻¹⁶ in which drugs are loaded within PLGA microspheres. However, it is more desirable to change the hydrophobic PLGA surface to be hydrophilic for the biomedical applications.¹⁷⁻¹⁹ Hydrophobic surfaces possess high interfacial free energy in aqueous solutions, which tend to unfavorably influence their cell-, tissue- and blood-compatibility in initial stage of contact.²⁰

In our previous work, a wettability chemogradient was prepared on polymer surfaces using corona discharge treatment.²¹ The wettability chemogradient was produced by treating the polymer sheets with corona from a knife-type electrode whose power was changed gradually along the sample length. The polymer surfaces oxidized gradually with the increasing corona power and the wettability chemogradient was created on the sample surfaces. The wettability chemogradient prepared by corona discharge treatment was used as a tool to investigate cell or protein interactions continuously related to the surface wettability of polymeric materials.^{22,23}

In this study, a wettability chemogradient onto the PLGA surfaces was prepared by corona discharge treatment to study the interaction between selected cells and PLGA surfaces. The surface properties of them were characterized by the measurements of water contact angle and electron spectroscopy for chemical analysis (ESCA). Hep G2 hepatoma, MG 63 osteoblast, CPEA bovine aortic endothelial, and NIH/3T3 fibroblast cells

were cultured on PLGA surfaces with wettability chemogradient to evaluate of cell attachment and proliferation behavior in terms of surface hydrophilicity/hydrophobicity.

Experimental

Materials. Monomers as L-lactide and glycolide were purchased from Boehringer Ingelheim, Germany. Stannous 2-ethylhexanoate as a catalyst was purchased from Wako Chemical Co., Japan. Toluene (Junsei Chem Co., Japan), methylene chloride (MC, Tedia Co. Inc., USA), and methyl alcohol (Junsei Chem Co.) were used as received. All other chemicals were a reagent grade.

PLGA Synthesis and Characterizations. A 30 g mixture of the L-lactide (75 mole%) and glycolide (25 mole%) was preheated in an evacuated flask at 60°C for 2 hrs to remove water trace. Stannous 2-ethylhexanoate in toluene (150 ppm) was added to polymerization reactor (ϕ 30 mm \times 35 cm length) with the agitation of 100 rpm. Dry nitrogen gas was flushed through whole processing. After adding the catalyst, the copolymerization reaction was carried out at 165°C for 4.5 hrs. The light brownish PLGA obtained was purified by dissolving in MC, followed by slow precipitation in excess methanol. The polymer was dried *in vacuo* at room temperature for 7 days and kept until use. Further detailed procedures for copolymerization were described in previous papers.^{15, 16, 20}

To characterize synthesized PLGA, gel permeation chromatography (GPC) was used. Measurement was carried out on a Waters Chromatograph 200 Series equipped with 6 μ m Styragel[®] columns in series with 10⁵, 10⁴, 10³, and 500 Å of pore size, respectively. Tetrahydrofuran was used as an eluent solvent. The temperature, the flow rate, and the injection volume were 30°C, 1 mL/min, and 15 μ L, respectively. The series of polystyrene monodisperse standard were used to calibrate the molecular weight. The average molecular weight (MW) and molecular weight distribution (MWD) of purified samples were in the range of 50,000~70,000 g/mole and 1.5~1.9, respectively, with good reproducibility.

Preparation of PLGA Films. Ten percent (by w/v) of PLGA (MW: 55,000 g/mole and MWD:

1.79) was dissolved in MC. Two grams of PLGA solution were cast onto Pyrex petri dishes (diameter: 100 mm) with a horizontal level in order to get 400~600 μm thickness of PLGA films. After evaporation of the MC at room temperature, the films were cut into 7 \times 5 cm rectangular. The PLGA films were dried in vacuo overnight to remove residual MC and were ultrasonically washed in ethanol. The PLGA films were kept in a vacuum until use.

Preparation and Characterization of Wettability Chemogradient PLGA Surfaces. The PLGA film was treated with a radio-frequency (RF) corona discharge apparatus designed for the preparation of gradient surfaces, in a manner similar to that used in our previous works.²¹⁻³² Briefly, a knife-type electrode was connected to the RF generator whose power gradually increases by a motorized drive (Figure 1). The cleaned PLGA 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 PLGA surface. At the same time as the sample bed was translated at a constant speed, 1.0 cm/sec, the corona from the electrode was discharged onto the sample with gradually increasing power (from 10 to 50 watt at 100 kHz). The sample sheet (7.0 \times 5.0 cm) was treated for 5 sec. By this treatment, the sample PLGA surface was continuously exposed to the corona with increasing power.

The corona-treated PLGA surfaces were characterized by the measurements of water contact angle and ESCA. The water contact angle, an indicator of the wettability of surfaces, was measured by a sessile drop method at room temperature

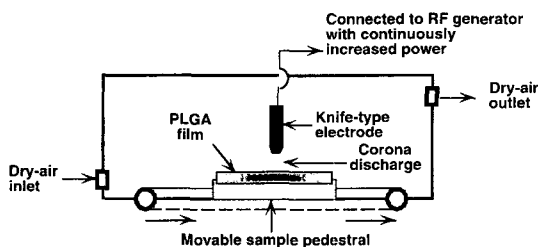


Figure 1. Schematic diagram of the corona discharge apparatus for the preparation of wettability chemogradient.

using an optical bench-type contact angle goniometer (model 100-0, Rame-Hart, Inc., U. S. A.). Drops of purified water, 3 μL , were deposited onto corona-treated PLGA surface along the sample length using a micro-syringe attached on the goniometer. The direct microscopic measurement of the contact angles was done with the goniometer. More than three different films were used for this. To identify the functional groups introduced on the PLGA films, the corona-treated PLGA surfaces were analyzed by ESCA (ESCALAB MK II, V. G. Scientific Co., UK) with $\text{AlK}\alpha$ at 1487 eV and 300 W power anode (incidence angle 30 $^\circ$). Survey scan and carbon 1S core level scan spectra were taken to analyze each section (analysis area, $\sim 5 \text{ mm}^2$) of the chemogradient along the sample length. More details in the corona discharge apparatus and the characterization of wettability chemogradient surfaces were described in previous papers.²¹⁻³²

Cell Culture on Wettability Chemogradient PLGA Surfaces. Hep G2 hepatoma (Korean Cell Line Bank (KCLB) 58065), MG 63 osteoblast (KCLB 21427), CPEA bovine aortic endothelial (KCLB 10209), and NIH/3T3 fibroblast cells (KCLB 21658) were obtained from Korean Cell Line Bank and used to study the effects of surface wettability of PLGA on the behavior of cultured cells. The cells routinely cultured in tissue culture polystyrene flasks (Corning, USA) at 37 $^\circ\text{C}$ under 5% CO_2 atmosphere were harvested after the treatment with 0.25% trypsin (Gibco Laboratories, USA). The PLGA surfaces with wettability chemogradient (size: 1.0 \times 5.0 cm) were placed in custom-made cell culture chamber. The PLGA film surfaces placed on the culture plate were equilibrated with Dulbecco's phosphate buffered saline (PBS, pH 7.3~7.4; Sigma, USA) free of Ca^{2+} and Mg^{2+} for 30 min. After removing the PBS solution from the chambers by pipetting, the cells ($4 \times 10^4/\text{cm}^2$) were seeded to the surfaces. The culture medium used was RPMI 1640 nutrient mixture (Gibco Laboratories) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ gentamycin sulfate.

The cell culture on the PLGA surfaces with wettability chemogradient was carried out for up to 2 days. The culture medium was changed once

after 1 day. After incubation at 37°C under 5% CO₂ atmosphere, the surfaces were washed with PBS and the cells attached on the surfaces were fixed with 2.5% glutaraldehyde (Gibco Laboratories) 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. The cell-attached PLGA surfaces were gold deposited in vacuum 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 the sections along the sample length with different wettability. Different fields for each section were randomly counted and the results were expressed in terms of the number of cells attached per cm². Further detailed procedures for the cell culture on the chemogradient surfaces were described in previous papers.^{23,26-28,32}

Results and Discussion

Characterization of Wettability Chemogradient PLGA Surfaces. In order to prepare PLGA surfaces with wettability chemogradient, the corona discharge apparatus with increasing power output was used. This is a simple and convenient tool to incorporate carboxyl and other oxygen-containing functional groups onto polymeric surfaces. Wettability or hydrophilicity can be adjusted by controlling power output of RF generator.

The corona discharge treatment did not show any visible changes on the PLGA surface, but the water contact angles of the PLGA surface gradually decreased (from 75° to 47°) along the sample length with increasing corona power (Figure 2). The decrease in the contact angles (and thus the increase in wettability) along the sample length may be due to the oxygen-based polar functionalities incorporated on the surface by the corona discharge treatment.²¹⁻²³

To examine the changes in chemical composition on the PLGA surfaces by the corona discharge treatment, ESCA analysis was performed. The typical PLGA surface showed carbon peak (C1S) at the binding energy of 285 eV, and oxygen

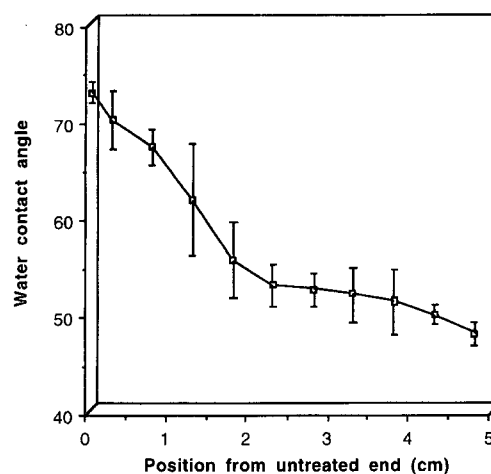


Figure 2. Changes in the water contact angle of corona-treated PLGA surface along the sample length. Sample numbers, $n = 3$.

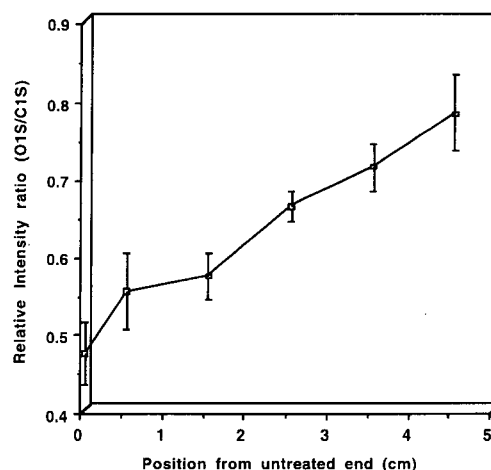


Figure 3. The relative intensity ratio (O1S / C1S) from ESCA survey scan spectra of corona-treated PLGA surface along the sample length. $n = 3$.

peak (O1S) at 532 eV. The C1S peak decreased with increasing corona power, while the oxygen O1S peak increased. The increased oxygen peaks on the corona-treated surface indicate that the PLGA surface was oxidized by the corona discharge treatment, resulting in the increased wettability or hydrophilicity. Figure 3 shows the oxygen to carbon ratio of the PLGA surface along the sample length which was calculated from the peak areas of ESCA spectra. The ratio increased gradually along the sample length with the

increasing corona power as shown in the figure.

The possible mechanism of oxidation on the surface may be involved the decomposition and the formation of free radicals of the PLGA molecular chain backbone by corona discharge. When the corona-treated PLGA film is subsequently exposed to oxygen in air, the radicals formed on the surface react with atmospheric oxygen and form peroxides which may be further decomposed to produce a variety of oxidation-containing functionalities ranging from alcohols to carboxylic acids.^{21,22}

Cell Adhesion and Growth on Wettability Chemogradient PLGA Surfaces. Four different types of the cells which are anchorage-dependent

were cultured on the wettability chemogradient PLGA surfaces for 1 and 2 days to investigate the effect of cell adhesion and growth on surface wettability. The culture media were changed after 1 day. As the surface wettability increased along the sample length, the cells adhered on the surface increased and then decreased, regardless of the cell types used, as shown in Figure 4. The cells were adhered more on the positions with moderate hydrophilicity of the wettability chemogradient PLGA surface than more hydrophobic or hydrophilic positions. The maximum adhesion of the cells appeared at around the position, 2.5~3.5 cm (water contact angle, around 53° ~55°: see Figure 2). The cell morphology was

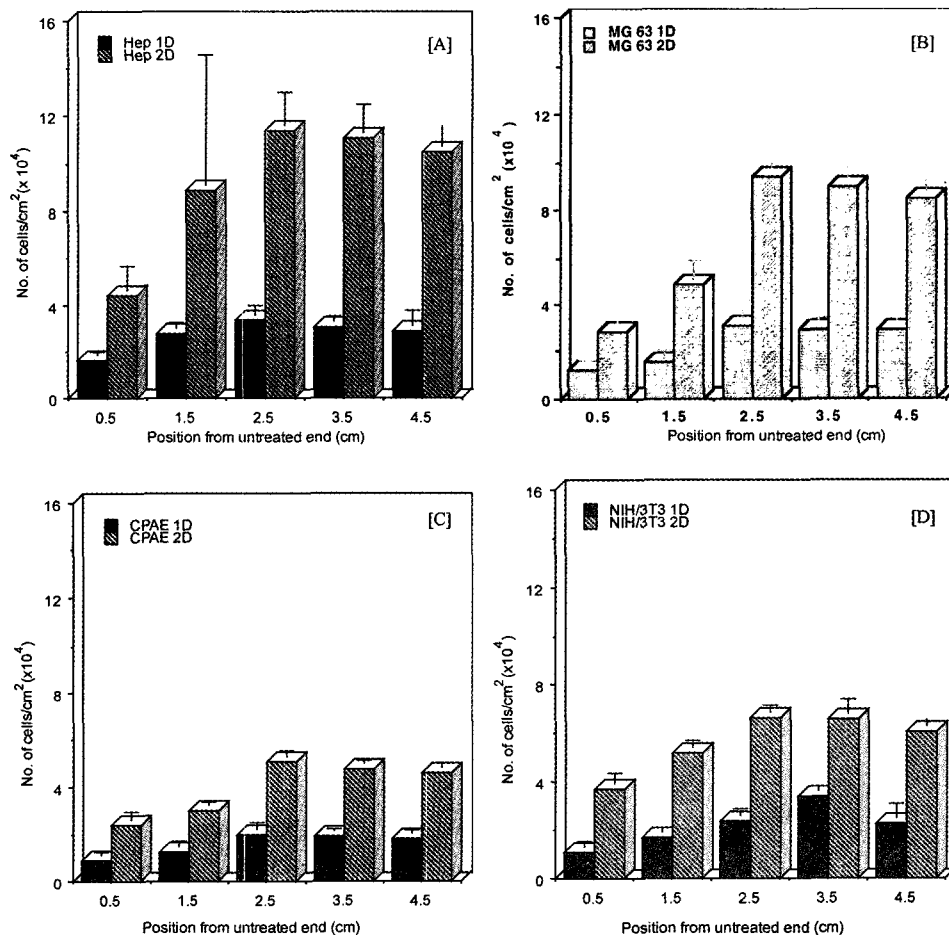


Figure 4. Hep G2 [A], MG 63 [B], CPAE [C] and NIH/3T3 [D] cell adhesion (after 1 day) and growth (after 2 days) on corona-treated PLGA surfaces with wettability chemogradient along the sample length (number of seeded cells; $4 \times 10^4/\text{cm}^2$). $n = 3$.

Interaction of Cells on PLGA Chemogradient Surface

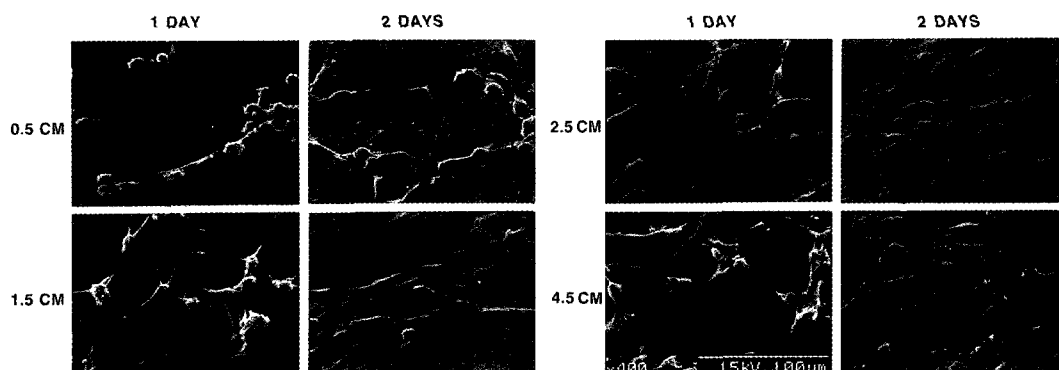


Figure 5. SEM microphotographs of fibroblast cells attached on corona-treated PLGA surfaces with wettability chemogradient along the sample length after 1 and 2 day culture (original magnification, $\times 400$).

also changed with the wettability gradient, as observed by SEM, as shown in Figures 5 and 6. The cells, regardless of the cell types used, were spread and flattened more on the position with moderate hydrophilicity of the wettability chemogradient PLGA surface than more hydrophobic or even hydrophilic ones after a 1 day culture. The cells after a 2 days culture were almost flattened on all positions of the gradient except the hydro-

phobic one (position, 0.5 cm) as shown in the figure.

The fact that cells are more adhered, spread, and grown on the moderately hydrophilic surfaces was also observed by other research groups³³⁻³⁷ as they cultured endothelial cells, HeLa S₃, or fibroblasts onto various polymer substrates with different surface wettability. In our previous works,^{23,26,32} it was observed that Chinese hamster

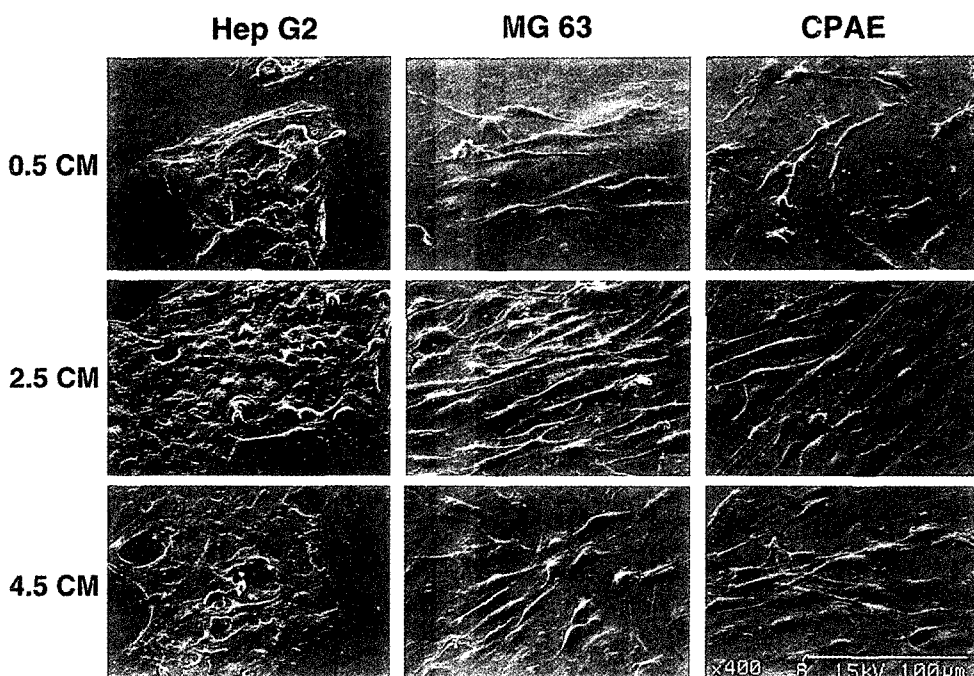


Figure 6. SEM microphotographs of Hep G2, MG 63, and CPAE cells attached on corona-treated PLGA surfaces with wettability chemogradient along the sample length after 2 days culture (original magnification, $\times 400$).

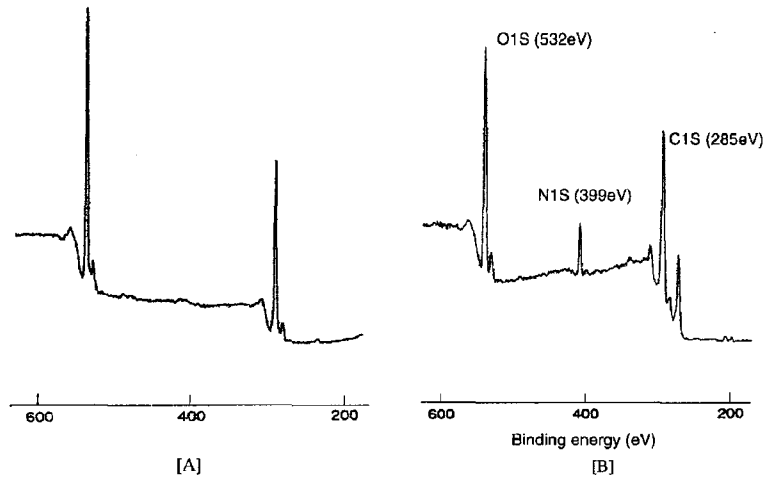


Figure 7. ESCA survey scan spectra of corona-treated PLGA surface at position 2.5 cm (A) before and (B) after serum protein adsorption (1 hr adsorption in 10% FBS RPMI 1640 nutrient media).

ovary (CHO), fibroblast, and bovine aortic endothelial cells were adhered and grown more onto moderately hydrophilic positions of wettability chemogradient polyethylene surface.

To observe the effect of serum proteins in cell culture media on the cell adhesion and growth behaviors, FBS was adsorbed onto the wettability chemogradient PLGA surface for 1 hr at 37°C. The FBS was diluted with PBS to make a 10% solution which is the same concentration as that in the cell culture media. The serum protein-adsorbed che-mogradient PLGA surface was analyzed by ESCA. Although ESCA is not a suitable method for the study of protein adsorption, it is simple and easy method to obtain semi-quantitative information on protein adsorption. As we investigated protein adsorption on polymer surfaces by ESCA and by using ¹²⁵I-labeled proteins in our previous studies,³⁸ we observed that the protein adsorption on the surfaces analyzed by both methods show almost same trend. For ESCA analysis, the nitrogen peak (binding energy, ~399 eV) from the survey scan spectrum was used as an indicator of the protein adsorption on the surface since it was observed that little nitrogen is incorporated onto the surface by corona treatment in air (Figure 7). It is mainly derived from peptide bonds of the adsorbed proteins. The ESCA spectra also showed increased oxygen to carbon ratio after serum protein adsorption on

the surface. Figure 8 shows the relative adsorbed amount of serum proteins on the wettability chemogradient PLGA surface. As the surface wettability increased along the sample length, the proteins adsorbed on the surface increased and then decreased; the proteins were adsorbed more on the positions with moderate hydrophilicity of the wettability chemogradient surface than more hydrophobic or hydrophilic ones. The maximum

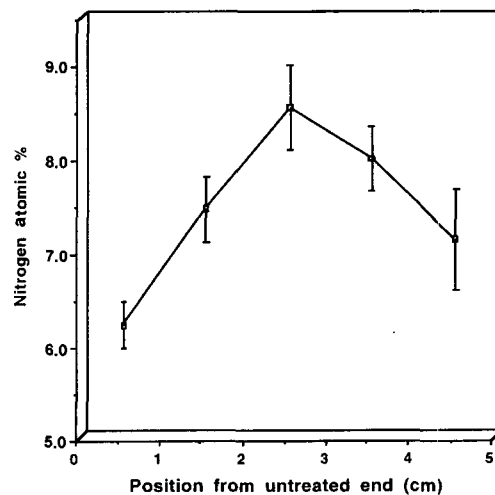


Figure 8. Serum protein adsorption on wettability chemo-gradient PLGA surface. Nitrogen atomic % represents the relative amount of the proteins adsorbed on the surface (1 hr adsorption in 10% FBS RPMI 1640 nutrient media). n = 3.

adsorption of the proteins appeared at around position 2.5 cm, which is the same trend as the cell adhesion and growth behaviors (see Figures 4, 5 and 6). Among the serum proteins, some proteins like fibronectin and vitronectin are known as cell-adhesive.^{39,45} The preferential adsorption of these cell-adhesive proteins from culture medium onto the moderately hydrophilic PLGA surface may be a reason for better cell adhesion, spreading, and growth.^{39,40,44,45}

The wettability chemogradient prepared on the PLGA surfaces by the corona discharge treatment method discussed in this study can be a simple and effective tool to systematically investigate the interactions of the different types of biological species in term of the surface hydrophilicity/hydrophobicity. Also, this surface modification technique can be used for the improvement of the adhesion and growth of cell and tissue onto PLGA film and scaffolds and can be applicable to the area of the tissue engineering. Studies on the application of this surface treatment method for the tissue engineering area and an animal experiment are in progress.

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