Interaction of Different Types of Cells on Physicochemically Treated Poly(L-lactide-*co*-glycolide) Surfaces

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ABSTRACT: To improve the cell compatibility of poly(L-lactide-*co*-glycolide) (PLGA; 75/25 molar ratio of lactide to glycolide) surfaces, we experimented with physicochemical treatments. Chemical treatments employed 70% chloric acid, 50% sulfuric acid, and 0.5*N* sodium hydroxide solutions, and physical methods included corona and plasma treatments. The water contact angle of surface-treated PLGA decreased from 73 to 50–60°; that is, the hydrophilicity increased because of the introduction of oxygen-containing functional groups onto the PLGA backbone according to electron spectroscopy for chemical analysis. The physicochemically modified PLGA surfaces were used to investigate the interaction of four different types of cells—hepatoma (Hep G2), osteoblast (MG 63), bovine aortic endothelial (CPAE), and fibroblast (NIH/3T3) cells—in terms of the surface hydrophilicity and hydrophobicity of PLGA. The cells that adhered and grew on the physicochemically modified PLGA surfaces were counted and observed with scanning electron microscopy. The adhesion and growth of Hep G2, MG 63, CPAE, and NIH/3T3 cells on physicochemically treated PLGA surfaces, especially on chloric acid-treated PLGA surfaces, were more active than on the control. This result seems closely related to the serum protein adsorption on the surface; the serum proteins were also adsorbed more on the hydrophilic surface. Surface hydrophilicity apparently plays an important role in cell adhesion, spreading, and growth on PLGA surfaces. The surface modification technique used in this study may be applicable to tissue engineering for the improvement of tissue compatibility of film- and scaffold-type substrates.
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INTRODUCTION

Recently, poly(L-lactide-*co*-glycolide)s (PLGAs) have been extensively used and tested for a wide range of medical applications as bioerodible materials because of 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 hydrolytic scission of their ester bonds.[2] The hydrolysis of PLA yields lactic acid, which is a normal byproduct of anaerobic metabolism in

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the human body and is incorporated into 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 acid,[3] 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 with changes in the ratio of monomers and the processing conditions.[5] It might be a suitable biomaterial for use in tissue engineered repair systems[6–11] in which cells are cultured within PLGA films or scaffolds and in drug delivery systems[12–16] in which drugs are loaded within PLGA microspheres. However, it is more desirable to change the hydrophobic PLGA surface to be hydrophilic for biomedical applications.[17–19] Hydrophobic surfaces possess high interfacial free energy in aqueous solutions that tends to unfavorably influence their cell, tissue, and blood compatibility during the initial stage of contact.[20]

In our previous work, to impart new functionality to PLA, PGA, and PLGA, we investigated several modification methods involving plasma discharge treatment,[20] corona discharge treatment for wettability chemogradients,[21–32] solvent treatments for surface oxidation,[33–35] the blending of poly(ω-methacryloyloxyethyl phosphorylcholine-*co*-ethylhexylmethacrylate),[36] impregnation with demineralized bone particles and small intestine submucosa, and molecular blending with bioactive molecules and peptides for controlled release systems.[37] The cells adhered and grew more on the hydrophilic surfaces than the hydrophobic surfaces, and the maximum adhesion of cells appeared moderate on hydrophilic surfaces with, for example, 50–55° water contact angles because of the preferential adsorption of serum protein.[21–32] Also, it has been demonstrated that the control of cell adhesion can be achieved by the incorporation of phosphorylcholine groups,[36] and the control of cell activation can be achieved with bioactive molecules.[37] For chemical treatments, the effectiveness of surface treatments was, for the measurement of blue dye intrusion and the culturing of fibroblast cells, in the following order: perchloric acid > sulfuric acid > sodium hydroxide.[33–35]

In this study, systematic physicochemical modification methods for the PLGA surface were investigated with chemical methods such as 50% sulfuric acid, 70% perchloric acid, and 0.5*N* sodium hydroxide and physical methods such as plasma and corona discharge treatments on the basis of our previous work. The surface properties were characterized by measurements of the water contact angle and electron spectroscopy for chemical analysis (ESCA). Four different types of anchorage-dependent cells—hepatoma (Hep G2), osteoblast (MG 63), bovine aortic endothelial (CPAE), and fibroblast (NIH/3T3)—were cultured on PLGA surfaces with physicochemical treatment for an evaluation of cell attachment and proliferation behavior in terms of surface hydrophilicity and hydrophobicity and preferential adsorption of serum proteins.

EXPERIMENTAL

Materials

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

PLGA Synthesis and Characterization

A 30-g mixture of L-lactide (75 mol %) and glycolide (25 mol %) was preheated in an evacuated flask at 60°C for 2 h for the removal of water traces. Stannous 2-ethylhexanoate in toluene (150 ppm) was added to a polymerization reactor (ϕ = 30 mm × 35 cm long) with an agitation of 100 rpm. Dry nitrogen gas was flushed throughout the processing. After the catalyst was added, the copolymerization reaction was carried out at 165°C for 4.5 h. The light brownish PLGA obtained was purified by dissolution in MC followed by slow precipitation in excess methanol. The polymer was dried *in vacuo* at room temperature for 7 days and kept until it was used. Further detailed procedures for copolymerization have been described in previous articles.[15,16,20]

To characterize synthesized PLGA, we performed gel permeation chromatography. Measurements were made with a Waters (Milford, United States) series 200 chromatograph equipped with 6-μm Styragel columns in series with 10^5-, 10^4-, 10^3-, and 500-Å pore sizes. Tetrahydrofuran was used as an eluent solvent. The

temperature, flow rate, and injection volume were 30°C, 1 mL/min, and 15 μL, respectively. A series of polystyrene monodisperse standards were used for calibration of the molecular weight. The average molecular weight and molecular weight distribution of purified samples were 50,000–70,000 g/mol and 1.5–1.9, respectively, with good reproducibility.

Preparation of PLGA Films

Ten percent (w/v) PLGA (molecular weight = 55,000 g/mol, molecular weight distribution = 1.79) was dissolved in MC. Two grams of a PLGA solution was cast onto Pyrex petri dishes (100 mm in diameter) with a horizontal level for 400–600-μm-thick PLGA films. After the evaporation of MC at room temperature, the films were cut into 2 cm $\times$ 2 cm rectangles. The PLGA films were dried *in vacuo* overnight for the removal of residual MC and were ultrasonically washed in ethanol. The PLGA films were kept *in vacuo* until use.

Preparation and Characterization of Physicochemically Modified PLGA Surfaces

The physical methods were plasma and corona discharge treatments. The PLGA film was treated with a custom-designed radio-frequency glow-discharge (RFGD) plasma-generating apparatus consisting of a Pyrex camber (ca. 6-L volume) with an upper stainless steel electrode and a lower brass band electrode in dry air. The power supply of the RFGD generator was 200 V and 20 mA at 100 kHz. The plasma treatment was performed in a reactor at 0.1 Torr. The cleaned PLGA film was located in the lower part of the reactor chamber. The samples in the reactor chamber were exposed to the plasma for 15 s; this was the best treatment condition.[20] After the chamber was degassed for 10 min, the surface-treated PLGA sample was carried out of the Pyrex chamber and used for surface characterization and observation of cell adhesion.

The PLGA film was treated with an RFGD corona discharge apparatus designed for the preparation of gradient surfaces in a manner similar to that in our previous works.[21–32] Briefly, a knife-type electrode was connected to the radio-frequency generator. 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 the sample bed was translated at a constant speed (1.0 cm/s), the corona from the electrode was discharged onto the sample with 20 W of power at 100 kHz.

The chemical treatments were a 50% sulfuric acid, 0.5*N* sodium hydroxide, and 70% chloric acid mixture composed of a 3/2 $HClO_4/KClO_3$ saturated solution in an aqueous medium with an orbital shaker at a 5° angle at 30 rpm for 10 and 30 min. Chemically treated PLGA surfaces were washed five times with distilled water for the elimination of residual chemicals and then dried with a freeze drier at −55°C and 30 mTorr for more than 2 days.

The physicochemically modified PLGA surfaces were characterized by the measurement of the 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 with an optical bench-type contact-angle goniometer (model 100-0, Rame-Hart, Inc., Mountain Lakes, United States). Drops of purified water (3 μL) were deposited onto physicochemically modified PLGA surfaces with a microsyringe attached to the goniometer. The direct microscopic measurement of the contact angles was performed with the goniometer. More than three different films were used for this. To identify the functional groups introduced onto the PLGA films, we analyzed the physicochemically modified PLGA surfaces by ESCA (Escalab MK II, V. G. Scientific Co., London, United Kingdom) with Al Kα at 1487 eV with a 300-W anode (incidence angle = 30°). Survey scan and carbon 1S core-level scan spectra were taken for the analysis of each physicochemically modified PLGA sample. More details in the characterization of physicochemically modified surfaces are given in previous articles.[21–32]

Cell Cultures on Physicochemically Modified PLGA Surfaces

Hep G2 [Korean Cell Line Bank (KCLB) 58065], MG 63 (KCLB 21427), CPAE (KCLB 10209), and NIH/3T3 cells (KCLB 21658) were obtained from KCLB and used to study the effects of the surface wettability of PLGA on the behavior of cultured cells. The cells, routinely cultured in tissue culture polystyrene flasks (Corning, Rochester, United States) at 37°C under a 5% CO_2 atmosphere, were harvested after treatment with 0.25% trypsin (Gibco Laboratories, Grand Island, United States). The physicochemically modified PLGA surfaces (2.0 cm $\times$ 2.0 cm) were placed in

custom-made cell culture chambers. The PLGA film surfaces placed on the culture plate were equilibrated for 30 min with Dulbecco's phosphate-buffered saline (PBS; pH 7.3–7.4; Sigma, St. Louis, United States) free of Ca^{2+} and Mg^{2+}. After removal of the PBS solution from the chambers through pipetting, the cells ($4 \times 10^4/cm^2$) were seeded to the surfaces ($n = 3$). The culture medium used was the Rosewell Park Memorial Institute (RPMI) 1640 nutrient mixture (Gibco Laboratories) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL gentamycin sulfate.

The cell culture on the physicochemically modified PLGA surfaces was carried out for up to 2 days. The culture medium was changed once after 1 day. After incubation at 37°C under a 5% CO_2 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 h 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 vacuo* with plasma sputtering (SC 500K, Emscope, London, United Kingdom) and examined with scanning electron microscopy (SEM; model S-2250N, Hitachi Co., Ltd., Tokyo, Japan) at a tilt angle of 45°. The cell density on the surfaces was estimated from the number of attached cells. Four fields for each sample were randomly counted, the actual counting area of which was 1,127,607.5 μm^2, and the results were expressed in terms of the number of cells attached per centimeter squared. Further detailed procedures for the cell culture on the physicochemically modified PLGA surfaces have been described in previous articles.[23,26–28,32]

Serum Protein Adsorption

The same FBS added to the cell culture media was used to study the relationship between the behaviors of cell adhesion and serum protein adsorption on the physicochemically modified PLGA surfaces. FBS was diluted with PBS to make a 10% solution (the same concentration used for the cell culture media). The physicochemically modified PLGA surfaces were mounted in the same test chambers used for the cell adhesion tests. The surfaces mounted in the test chambers were equilibrated with PBS for 30 min. After the removal of a PBS solution from the chambers, the FBS solution was introduced to the chambers. After 1 h of incubation at 37°C, the surfaces were washed with PBS and then washed with purified water for the removal of unadsorbed serum proteins. After vacuum drying, the protein-adsorbed physicochemically modified PLGA surfaces were analyzed by ESCA. The nitrogen 1S peaks from the survey scan spectra were used for the analysis of proteins adsorbed on the physicochemically modified PLGA surfaces.[32]

Table I Changes in the Water Contact Angle ($n = 3$) and the O1s/C1s Ratio of ESCA Survey Scan Spectra ($n = 2$) for Physicochemically Treated PLGA Surfaces

	Water Contact Angle	O1s/C1s
Control	73.2 ± 4.5	0.46 ± 0.10
Sulfuric acid	61.3 ± 2.8	0.54 ± 0.08
Chloric acid	60.2 ± 4.5	0.72 ± 0.13
Sodium hydroxide	61.2 ± 5.0	0.60 ± 0.15
Corona	56.1 ± 4.1	0.62 ± 0.09
Plasma	52.3 ± 3.0	0.65 ± 0.12

RESULTS AND DISCUSSION

Characterization of Physicochemically Modified PLGA Surfaces

To investigate the effects of the surface hydrophilicity and hydrophobicity of PLGA surfaces on the adhesion and growth of four different types of anchorage-dependent cells such as hepatoma, osteoblatoma, fibroblast, and endothelial cells, we applied physicochemical treatments. Physical treatments were plasma and corona glow-discharge treatments, and chemical treatments were sulfuric acid, sodium hydroxide, and chloric acid.

None of the treatment methods showed any visible changes on the PLGA surfaces because each method was the best condition. For example, PLGA films were changed into an opaque state after 40 min of chemical treatment. The water contact angles of the physicochemically modified PLGA surfaces gradually decreased from 73 to 50–60°, as listed in Table I. The decrease in the contact angles (and, therefore, the increase in wettability) may be due to the oxygen-based polar

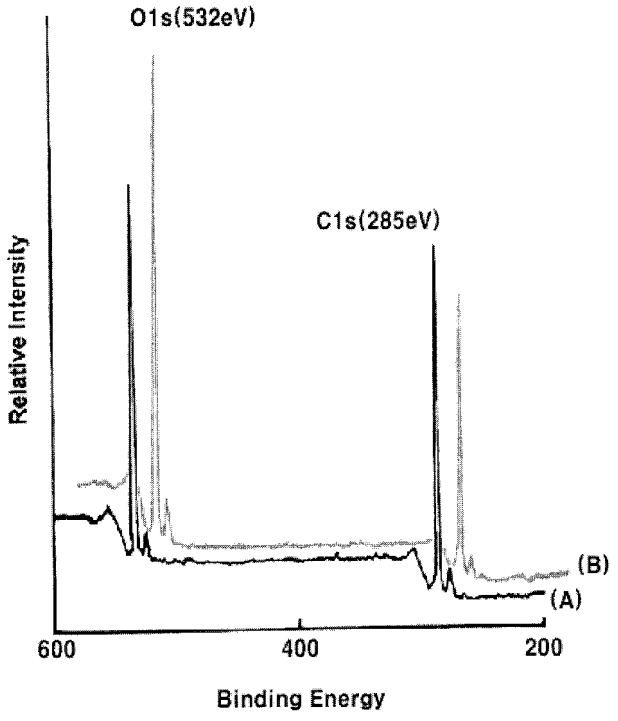

Figure 1 Typical ESCA survey scan spectra of (A) control and (B) plasma-treated PLGA surfaces for 15 s.

functionalities incorporated onto the surfaces by the physicochemically modified treatment, that is, the oxidation process.[21–23]

To examine the changes in chemical composition on the PLGA surfaces from the physicochemically modified treatment, we performed ESCA analysis. The typical PLGA surface showed a carbon peak (C1S) at a binding energy of 285 eV and an oxygen peak (O1S) at 532 eV, as shown in Figure 1 for the plasma treatment. The C1S peak decreased with plasma treatment, whereas O1S peak increased. The increased oxygen peaks on the physicochemically modified surfaces indicate that the PLGA surface was oxidized by the oxidation treatment, resulting in increased wettability or hydrophilicity. Table I lists the oxygen/carbon (O1s/C1s) ratio of the control and physicochemically modified PLGA surfaces as calculated from the peak areas of ESCA spectra. The ratio increased from 0.46 to 0.55–0.77. The chloric acid treatment among the chemical treatments and the plasma treatment among the physical treatments were the most effective. Figure 2 shows the changes in carbon 1s core-level spectra for control, chloric acid, sodium hydroxide, and plasma treatments. The control PLGA surfaces showed an alkyl carbon (—*C*—C—) peak at a binding energy of about 285 eV. The PLGA surface after physicochemical treatment showed bigger peaks at higher binding energies, indicating the formation of carbon–oxygen functionalities. The peaks of the high binding energy region correspond to —*C*—O— at about 286.6 eV (e.g., hydroxyl or ether groups), to —*C*═O at about 287.9 eV (e.g., ketone or aldehyde groups), and to O═*C*—O— at about 289.1 eV (e.g., carboxylic acid or ester groups).[35] The intensities of the oxygen-based functional groups were different in the following order: chloric acid > plasma treatment = corona treatment > sodium hydroxide > sulfuric acid. It might be assumed that the oxidation pathway of each treatment was different.

The possible mechanism of oxidation on the surface may be involved in the decomposition and formation of free radicals of the PLGA molecular chain backbone by physicochemical oxidation. When the physicochemically modified PLGA film was subsequently exposed to oxygen in air, the radicals formed on the surface reacted with atmospheric oxygen and formed peroxides that further decomposed to produce a variety of oxidation-containing functionalities ranging from alcohols to carboxylic acids.[21,22]

Cell Adhesion and Growth on Physicochemically Modified PLGA Surfaces

In the field of biomaterials, the nature of the biomaterial surface, including the wettability (hydrophilicity and hydrophobicity or surface free energy), chemistry, charge, and roughness, has been shown to be critical for biocompatibility. A large number of research groups have extensively studied the effect of surface wettability on the interactions of biological species with solid substrates because wettability is one of the most important parameters when biomaterials or implant devices are designed. In this study, four different types of anchorage-dependent cells were cultured on physicochemically modified PLGA surfaces to investigate the effect of cell adhesion (for 1 day) and growth (for 2 days) in terms of the surface hydrophilicity and hydrophobicity. The culture media were changed after 1 day. After surface modification, that is, as the surface wettability increased, the cells adhered more on the surface-modified PLGA surfaces than the control surface, regardless of the cell types, as shown in Figures 3–9. The cell morphology was also changed after

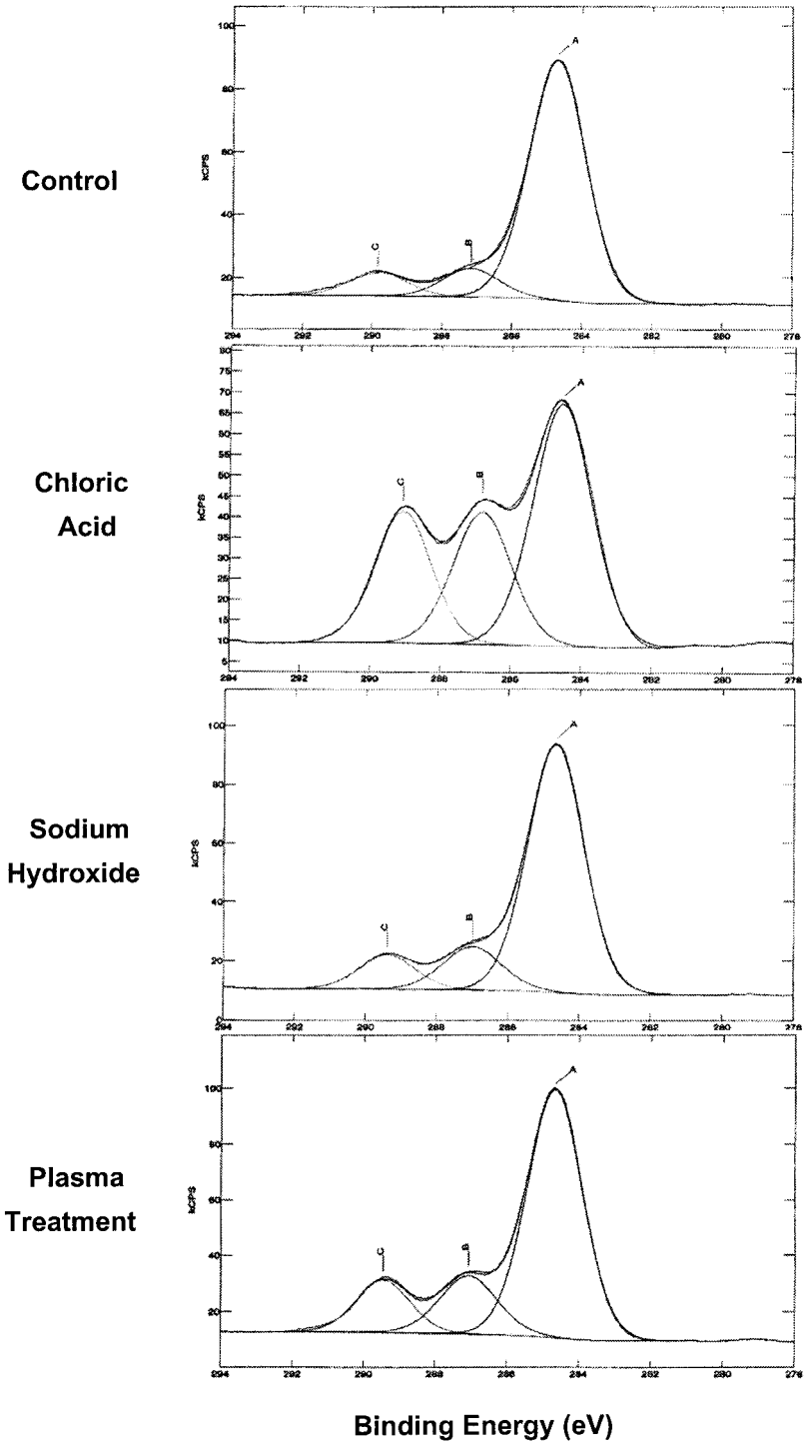

Figure 2 ESCA carbon 1s core-level spectra of control, chloric acid-treated, sodium hydroxide-treated, and plasma-treated PLGA surfaces.

the treatment, as shown in the figures. The cells, regardless of the cell types used, were protruded fillodopia and lamelliopodia that spread out and flattened more on the hydrophilic PLGA surfaces than controls after 1 day of culturing; chloric acid and plasma glow-discharge treatments especially showed better activated cell morphology than controls. The cells after 2 days of culturing were

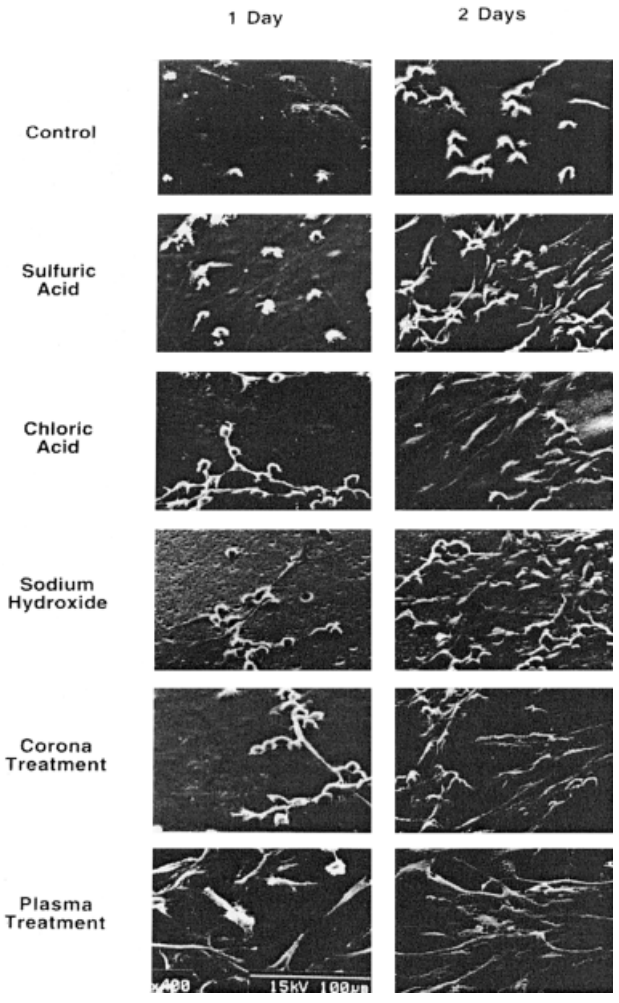

Figure 3 SEM microphotographs of fibroblast cells attached to physicochemically treated PLGA surfaces after 1 and 2 days of culturing (original magnification, ×400).

almost flattened on all samples, especially for chloric acid treatment among the five treatment methods, except that the control still showed round cell morphology.

The fact that cells adhered, spread, and grew more on the hydrophilic surfaces was also observed by other research groups[38–42] when they cultured endothelial cells, HeLa S_3, or fibroblasts onto various polymer substrates with different surface wettabilities. In our previous works,[23,26,32] we observed that Chinese hamster ovary, fibroblast, osteoblast, hepatoma, and bovine aortic endothelial cells adhered and grew more onto hydrophilic positions of polyethylene, polycarbonate, poly(methyl methacrylate), and PLGA surfaces. An inconsistency was observed between the cell culture trend and water contact angle and the O1s/C1s ratio because the

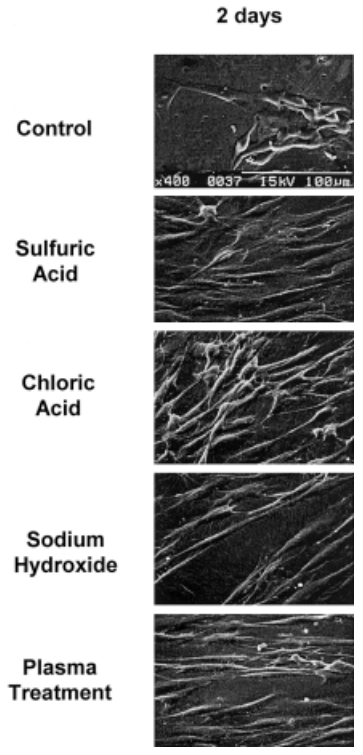

Figure 4 SEM microphotographs of MG 63 attached to physicochemically treated PLGA surfaces after 2 days of culturing (original magnification, ×400).

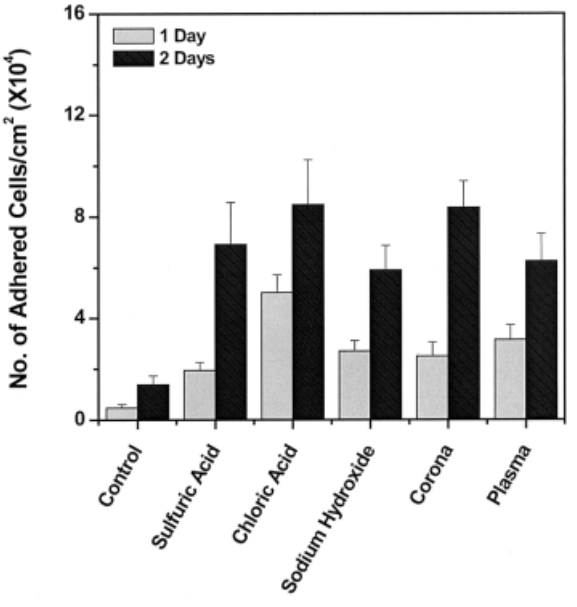

Figure 5 Fibroblast cell adhesion and growth of physicochemically treated PLGA surfaces after 1 and 2 days (number of seeded cells = $4 \times 10^4/cm^2$). $n = 3$.

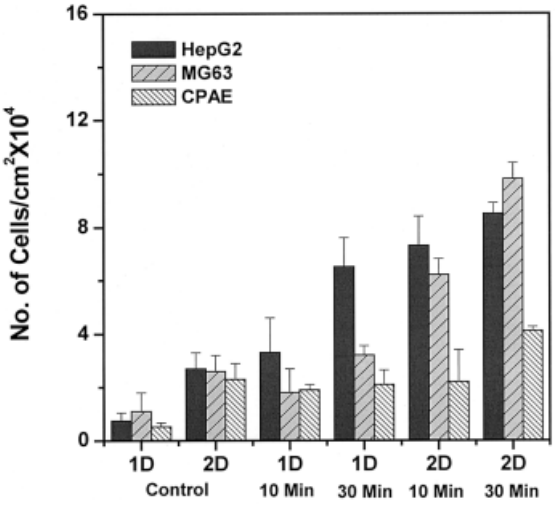

Figure 6 Hep G2, MG 63, and CPAE cell adhesion and growth of NaOH-treated PLGA surfaces for 10 and 30 min after 1 and 2 days (number of seeded cells = $4 \times 10^4/cm^2$). $n = 3$.

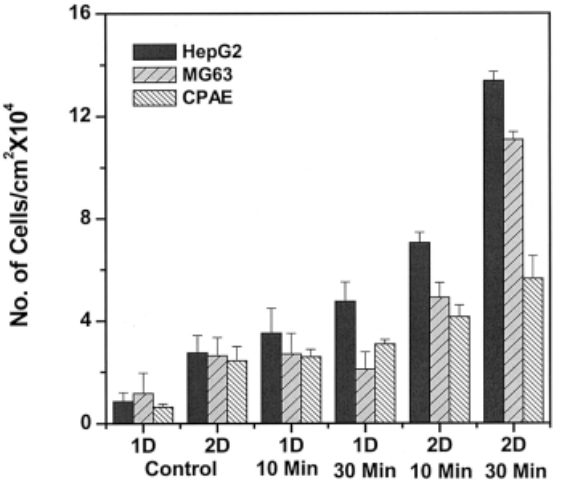

Figure 8 Hep G2, MG 63, and CPAE cell adhesion and growth of sulfuric acid-treated PLGA surfaces for 10 and 30 min after 1 and 2 days (number of seeded cells = $4 \times 10^4/cm^2$). $n = 3$.

suggested modification methods in this study were different, resulting in different amounts of functional groups, as shown in the changes in the carbon 1s core-level spectra of ESCA in Figure 2. However, we observed maximum cell adhesion at some point; for example, the maximum adhesion of the cells appeared around 53–55° water contact angles for corona-treated PLGA surfaces with different wettabilities. More detailed experiments on the effect of chemical species on cell adhesion and growth on PLGA surfaces are in progress.

To determined the effect of serum proteins in cell culture media on cell adhesion and growth behaviors, we adsorbed FBS onto physicochemically modified PLGA surfaces for 1 h at 37°C. FBS was diluted with PBS to make a 10% solution (the same concentration used for the cell culture media). The serum protein adsorbed on surface-treated PLGA was analyzed by ESCA. Although ESCA is not a very good method for the study of

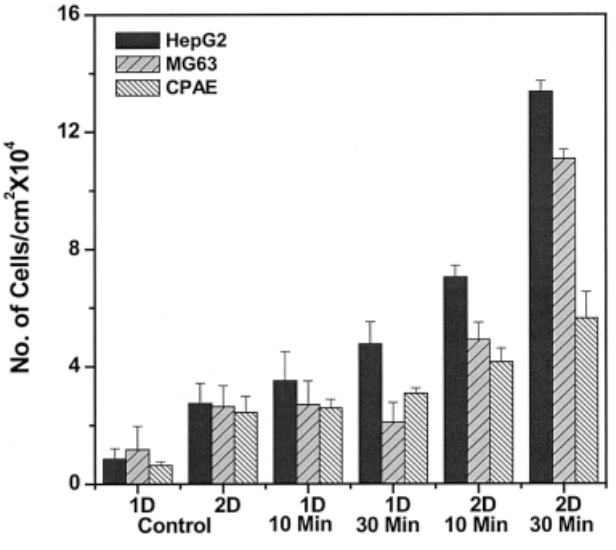

Figure 7 Hep G2, MG 63, and CPAE cell adhesion and growth of chloric acid-treated PLGA surfaces for 10 and 30 min after 1 and 2 days (number of seeded cells = $4 \times 10^4/cm^2$). $n = 3$.

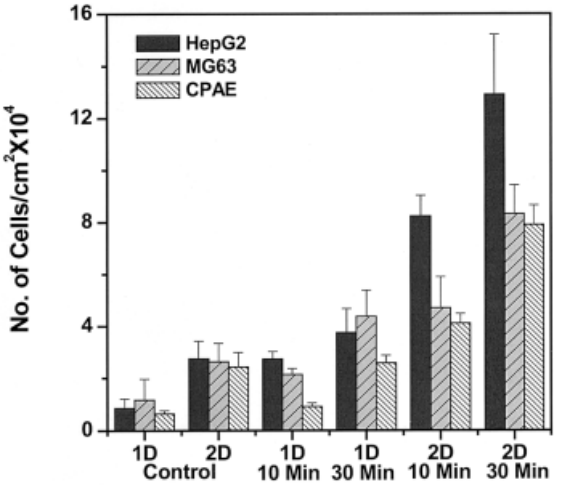

Figure 9 Hep G2, MG 63, and CPAE cell adhesion and growth of plasma-treated PLGA surfaces for 10 and 30 s after 1 and 2 days (number of seeded cells = $4 \times 10^4/cm^2$). $n = 3$.

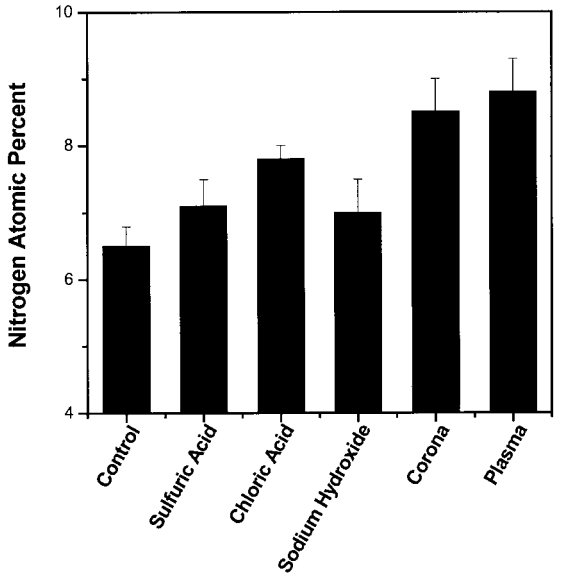

Figure 10 Serum protein adsorption on physicochemically treated PLGA surfaces. The nitrogen atomic percentage represents the relative amount of the proteins adsorbed on the surface (1 h of adsorption in 10% FBS/RPMI 1640 nutrient media). $n = 3$.

protein adsorption, it is a simple and easy method for obtaining semiquantitative information on protein adsorption. As we investigated protein adsorption on polymer surfaces with ESCA and ^{125}I-labeled proteins,[43] the protein adsorption on the surfaces analyzed by both methods showed almost the 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 because it was observed that little nitrogen was incorporated onto the surface by physicochemical treatments. It was mainly derived from peptide bonds of the adsorbed proteins. The ESCA spectra also showed an increased oxygen/carbon ratio after serum protein adsorption on the surface. Figure 10 shows the relative adsorbed amount of serum proteins on the physicochemically modified PLGA surfaces. The proteins were adsorbed more on the surface-modified PLGA; that is, there was more hydrophilic surface than on the control. Among the serum proteins, some proteins such as fibronectin and vitronectin are known to be cell adhesives.[44–50] The preferential adsorption of these cell adhesive proteins from a culture medium onto the hydrophilic PLGA surface may be the reason for better cell adhesion, spreading, and growth.[44,45,49,50]

The PLGA surfaces physicochemically modified by the corona discharge treatment, plasma discharge treatment, chloric acid treatment, sulfuric acid treatment, and sodium hydroxide treatment suggested in this study can be a simple and effective method for systematically investigating the interactions of different types of biological species in terms of the surface hydrophilicity and hydrophobicity. Also, this surface modification technique can be used for the improvement of the adhesion and growth of cells and tissues onto PLGA films and scaffolds and can be applicable to tissue engineering. Studies on the application of this surface-treatment method for three-dimensional scaffolds for tissue engineering and an animal experiment are in progress.

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