Comparison of Fibroblast and Nerve Cells Response on Plasma Treated Poly (L-lactide) Surface

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ABSTRACT: Plasma technique can easily be used to introduce desired functional groups or chains onto the surface of materials, and so it has a special application to improve the cell affinity of polymers surfaces. The purpose of this study is to elucidate the interaction between the cells and the surface of crystalline poly (L-lactide) (PLLA) samples, which were modified using a low-temperature plasma treatment apparatus. The plasma treatments were carried out in the carbon dioxide (CO_2) gas. The results showed that the contact angle of the samples, which was plasma treated in CO_2 gas, decreased compared with that of the untreated samples. The hydrophilicity increased because of the introduction of oxygen-containing functional groups onto the PLLA surfaces according to the spectroscopy for chemical analysis. High quantities of —C—O groups, such as hydroxyl and carboxyl could be in corporate into the surface of PLLA. The surface wettability, topography, and chemistry of treated PLLA samples were characterized by contact angle measurement, scanning electron microscope (SEM), and ATR-FTIR spectroscopy. The origin and plasma-treated samples were used to investigate the interaction of two different types of cells namely, B65 glial nervous, and L929 fibroblast cells. The nervous cell response on the PLLA plasma treated in the CO_2 gas were significantly superior to that of the L929 fibroblast cells and untreated one. The surface modification technique used in this study may be applicable to tissue engineering for the improvement of nerve tissue compatibility of polymer and scaffold-type substrates. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 112: 3429–3435, 2009

Key words: PLLA; plasma treatment; nervous cell; fibroblast cell; wettability

INTRODUCTION

Polymer surface engineering may potentially be used to create materials that elicit controlled cellular adhesion and maintain differentiated phenotypic expression.[1] Surface modification of biomaterials is becoming an increasingly popular method to improve device multifunctionality, biological, and mechanical properties, as well as biocompatibility of artificial devices while obviating the needs for large expenses and long time to develop brand new materials.[2,3]

Various methods such as surface coating,[4] surface chemical modification,[5] radiation technique treatment, and so on were well performed.[6–9] For example, the anhydrous ammonia plasma treatment can improve the surface hydrophilicity of polymer. As pointed on published paper, the cell affinity and cell adhesion force of the scaffold had been greatly improved by such plasma treatment.[10–12] However, it had also been found that the plasma treatment condition could not only influence the modifying depth, but it also influences the degradation of the PLLA scaffold.[13] So, to control the parameters of the plasma treatment becomes important.

Hydrophobicity of poly (L-lactide) is a main drawback in obtaining a sufficient mass of seeded cells for satisfying the requirements of cell seeding. Therefore, plasma treatment is a useful technique to enhance the hydrophilicity of the polymers.

Although poly (L-lactide) possesses good biocompatibility, biodegradability, and mechanical property, a high hydrophobic polyester (PLLA) scaffold disables for penetrating of cell suspensions into insides of the scaffold. Furthermore, nutrient supplying, waste removal and ingrowths of cells into the scaffold are also influenced disadvantageously by the hydrophobicity of the scaffold. It is important to modify PLLA (films or scaffolds) for cell seeding applications.

Cell affinity includes two aspects: cell attachment and cell growth. The cell attachment belongs to the first phase of cell/materials interactions and the quality of this phase will influence the cell's capacity to proliferate and to differentiate itself in contact with the implant.[14] A tool of cell growth improvement on a plasma-treated technical material can be developed for various cell types, e.g., for skin cells (keratinocytes) or cornea cells of the eye.

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Comparison of cornea cells grown on plasma-treated polyester fabric and on nontreated fabric indicates that the modified sample surface enhances cell growth and the survival of these cells.[15]

When cells are cultured *in vitro*, their adhesion, proliferation, and differentiation depend on both chemical and topographical cues arising from the substrate and on cell culture media. The surface chemical functionalities densities, their spatial distribution, as well as their molecular conformation, surface charge, and presence of hydrophilic and hydrophobic domains have shown to be important cues in affecting cell behavior.[16–19]

The adhesion rate of human endothelial cells on PLLA is only 8% after 30 min and 10% after 1 h, compared with a corresponding 43 and 59% on tissue culture polystyrene (TCPS), whose static water contact angle is 35°.[20] Although the relationship between cell behaviors and surface properties of biomaterials is not completely understood, it has been widely accepted that cells prefer to attach to hydrophilic surfaces than hydrophobic surfaces.[20] It has been demonstrated that cells attach and spread more easily and effectively on hydrophilic surfaces modified with positively charged amine groups than on hydrophobic surfaces, both in the presence and absence of serum.[21] Other studies have demonstrated that maximum cell attachment was observed on materials with moderate wettability (water contact angle between 20 and 60°.[22]

This study was therefore carried out for the investigation and comparison of adhesion and proliferation levels of B65 neuroblastoma cell line and L929 fibroblast cell line grown onto the plasma-treated PLLA in terms of surface hydrophilicity and hydrophobicity, chemical composition, and morphology.

EXPERIMENTAL

Materials

Poly L-lactic acid (PLLA) or Resomer 210 (L) was purchased from Boehringer Ingelheim (Germany). 1, 4-Dioxane (Merk) was used as a solvent for the fabrication of biodegradable polymeric film using casting method. The polymers and solvents were used without further purification. The physical and mechanical properties of crystallizable polymers such as isotactic PLLA are largely dependent on their solid state morphology and level of crystallinity and this polymer is a semicrystalline and rigid at room temperature.

Preparation of films using casting method

About 1% wt of PLLA solutions in 1, 4-dioxane were separately poured into the plate form vessel (diameter of 10 cm) which was covered by Teflon. After solvent evaporation in air at room temperature the vessel put in vacuum oven at 30°C under vacuum condition for 48 h. Opaque uniform films with 0.3 mm thickness were obtained. Prepared films were kept in desiccators under vacuum in 4°C conditions.

Plasma surface treatment

The RF-plasma reactor employed for the plasma surface treatment of biodegradable polymer film (PLLA). Polymeric films were maintained on the sample stage in the plasma reactor chamber. Samples were placed inside a Pyrex tubular reactor of plasma EMITEC K 1050X instrument for treatment. Samples surfaces were placed above the inner electrode. The gas was CO_2 (>99%). The pressure inside the plasma chamber was kept at 6×10^{-1} (mbar). The electrode power was 30 W for a plasma treatment. The gas flow was chosen in such a way that the above-mentioned plasma pressure were realized.

Contact angle measurement

Hydrophilicity was evaluated by measuring the contact angle formed between water drops and the surface of the modified samples using contact angle measuring system G 10 (KRUSS). For this purpose, the drops of water were mounted on five different areas of the surface with a microsyring. Five independent determinations at different sites of one sample were averaged. Deionized water was used for the measurement.

Hydrophobic recovery test

To quantify the effect of the preserving conditions on the hydrophobic recovery, one group of samples were stored in air after plasma treatment. Water drop contact angles were measured in different time intervals after plasma treatment.

Scanning electron microscopy observation of samples

Scanning electron microscopy (SEM) was performed on gold-coated samples using a Polaron sputter coater. A Vega Tescan SEM operating typically at 15 kV employed for morphology study. Samples mounted onto the sample holder, sputter coated with gold, and studied with SEM.

Cell culture method

Cell culture reaction of the prepared films was evaluated by *in vitro* cell culture test. The nervous tissue B65 cell line was used in this study and purchased

from Pastor Institute of Iran. The cell suspension of: 95%, 1.2×10^6 cells/vial were prepared before seeding. The duplicate specimens of each sample were sterilized in 70% ethanol and washed in culture medium before the cell culture procedure. They were placed in a multiwell tissue culture polystyrene plate with 5-mL cell suspension, with one well kept as a negative control and then maintained for 48 ± 1 h in a CO_2-controlled incubator at 37°C. After incubation the samples were washed with phosphate buffered saline solution (PBS). The cells were fixed with 2.5% glutaraldehyde and dehydrated in graded ethanol (60, 70, 80, and 95%).

The mouse L929 fibroblast cells were used as a test model in this study. Cells, which have fibroblast-like morphology, grow only in mono-layer culture. In the cell culture studies, the culture medium was Dulbecco's modification of Eagle's MEM for L929 cells. This media was modified by using 10% fetal calf serum and 100-mg/mL gentamycin modified this media. The cell suspension of 1.8×10^5 cells/mL was prepared before seeding. The duplicate specimens of each sample were sterilized in 70% ethanol and washed in culture medium before the cell culture procedure. They were placed in a multiwell tissue culture polystyrene plate with 5 mL cell suspension, with one well kept as a negative control and then maintained for 48 ± 1 h in a CO_2-controlled incubator at 37°C. After incubation the samples were washed with PBS. The cells were fixed with 2.5% glutaraldehyde and dehydrated in graded ethanol (60, 70, 80, and 95%). The cells were observed with light microscopy (TE2000-U, Nikon ECLIPSE).

RESULTS AND DISCUSSION

Effects of plasma treatment on contact angle

Water drop contact angles of the untreated and plasma-treated PLLA is shown in Figure 1. The hydrophilicity of the sample improved following plasma treatment. Water drop contact angle of untreated PLLA is about $86 \pm 4°$; therefore, it shows that the PLLA is a hydrophobic polymer. Water drop contact angle of CO_2 plasma-treated PLLA decreased (depends on treatment time), and as can be seen in Figure 1, different wettable surfaces obtained and 30 s treatment time give the better hydrophilicity, and other experiments were carried out on this sample. Plasma treatment shows drastic changes on the surface of PLLA and a moderate wettable and hydrophilic surfaces were obtained in contrast to untreated one. As noticed in Figure 1 by increasing treatment time causes in decreasing wettability, which influence the morphology structure (increasing porosity content) and crosslinking on the surface. As can be seen in this Figure, after passing time (1, 3, 5 h) contact angle increased slightly for CO_2-treated samples. It indicates that the chemical structure of PLLA during CO_2 plasma treatment changes to hydrophilic character, which influence of plenty of polar groups including hydroxyl, carboxyl, and other oxygen functional groups produced on the surface. Moreover, the hydrophobic recovery, namely the deterioration of the surface properties as indicated by an increase in the water drop contact angle over time, is due to the reorientation of polar groups toward the bulk of the material to reduce the interfacial energy in response to the adjacent environment. A number of procedures can be applied to the treated polymer surfaces to slow down hydrophobic recovery and to minimize the migration of polar groups and surface conformation.[23–25] PLLA 210 is a crystalline polymer in which after plasma treatment, reorientation of polar groups from surface to bulk is negligible. Therefore, PLLA 210 has a stable structure morphology, and dynamic of chains on to the surface is more conformational. Water drop contact angles results conclude that these polymers are hydrophobic and low wettable, which after plasma treatment changed to the hydrophilic surface.

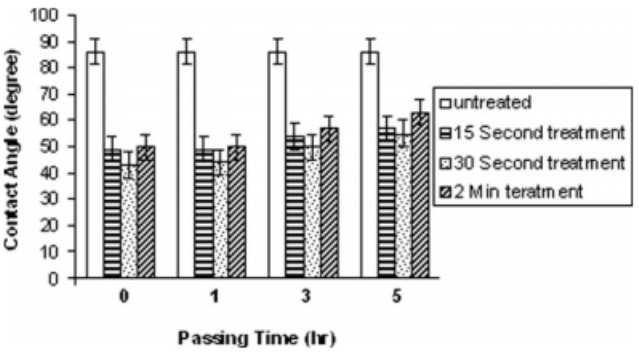

Figure 1 Water drop contact angles of plasma-treated and untreated PLLA samples versus passing time.

ATR-FTIR study

To detect the effect of plasma treatment on the surfaces of polymers and chemical functional groups after plasma treatment, ATR-FTIR spectroscopy has been performed on untreated and CO_2 plasma-treated PLLA. Figure 2(a–b) shows the ATR-FTIR spectra of untreated (a), and CO_2 plasma treated (b) PLLA210 films. As can be seen from Figure 2(a), untreated sample shows a peak at 1747 cm^{-1}, which exhibit acidic groups on the structure of PLLA 210. Peaks of methyl groups in 3000 cm^{-1} is negligible. Figure 2(b) shows the spectra of CO_2 plasma-treated PLLA. As noticed in this figure, peak of acidic group at 1742 cm^{-1} is bigger and broader when compared with

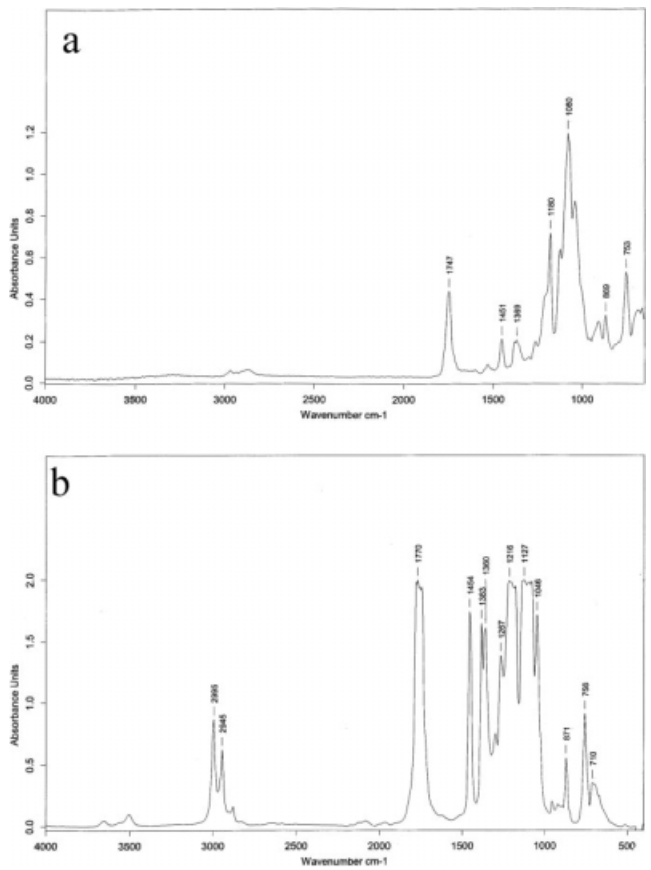

Figure 2 ATR-FTIR spectra of (a) untreated PLLA210, (b) CO_2 plasma-treated PLLA210.

the untreated one. It confirmed that CO_2 plasma treatment produced more oxygen functional groups on to the surface during reaction of radicals with oxygen. When the modified PLLA film was subsequently exposed to oxygen in air, the produced radicals on the surface reacted with atmospheric oxygen and peroxides obtained. Further decompositions produce a variety of oxidation-containing functionalities ranging from alcohols to carboxylic acids.[26,27] Peaks at 2915 and 2995 cm^{-1} depends on methyl groups which exhibit chain scission of molecular chain of polymer. ATR-FTIR spectra confirmed the wettability results.

Surface morphology

Surface morphology has an important effect on the cell attachment and growth of the biomaterials. It has been reported that surface morphology plays a critical role in the adhesion process of adjacent cells and that increase of surface roughness promotes more cell adhesion.[28] The surface morphology of the PLLA was observed by SEM.

The SEM photomicrographs of untreated and plasma-treated PLLA 210 was shown in Figure 3(a–d), respectively. It could be seen that the surface of the control was almost smooth and there is no crack or porosity on its surface because the films were prepared at room temperature and the solvent evaporated very slowly. Figure 3(b) shows the SEM photomicrographs of plasma-treated PLLA 210 (15 s treatment time) and as can be seen there are microspherulite on the treated surface. We suppose that the origin of spherulite and roughness on the treated surface attributed to rapid heating-induced desorption in which the solid substrate on which the molecules are adsorbed simply acts as a chromospheres taking up the energy provided by the short, focused, plasma radiation. Thermal diffusivity in most solids is sufficiently low that considerable local heating occurs during treatment. We assume that the adsorbate molecule does not receive energy directly from the plasma radiation and concentrate over attention on the energy flow from the hot surface to the cold adsorbate species. There is clearly a high degree of order in polymer chains by plasma irradiation, and this orderly leads to a considerable reduction in entropy and causes spherulite crystal on the surface. Figure 3(c-d) shows the SEM photomicrographs of samples that treatment times are 30 s and 2 min, respectively. As can be seen by increasing treatment time, spherulite, and roughness increased.

Effects of plasma treatment on cells activity

In the field of biomaterials, the nature of the biomaterial surface, including the wettability (hydrophilicity and hydrophobicity or surface free energy), chemistry, surface charge, and roughness has been shown to be critical for biocompatibility.[29] A large number of research groups have extensively studied the effect of surface wettability on the interactions of biological species with solid substrates because the wettability is one of the most important parameters when biomaterials or implant devices are designed.[30] In this study, two different types of cells were cultured on plasma-treated PLLA surfaces to investigate the effect of cell adhesion and growth in terms of the surface hydrophilicity and hydrophobicity. Figure 4(a,b) shows the attachment and growth of glial nervous B65 cells on the untreated and CO_2 plasma-treated PLLA, respectively. Figure 4(b) shows the improved attachment and growth of cells on plasma-treated PLLA and maximum cells are in webbing and flattening state. It is noticed that plasma treatment improved attachment and growth of the B65 cells on to the PLLA surface drastically. After surface modification the wettability of PLLA increased and the B65 cells adhered more on the modified PLLA surfaces than the control. As shown in this figure, the cell morphology was also changed after the treatment. The cell mass culturing of the surface of the sample, which was plasma treated in

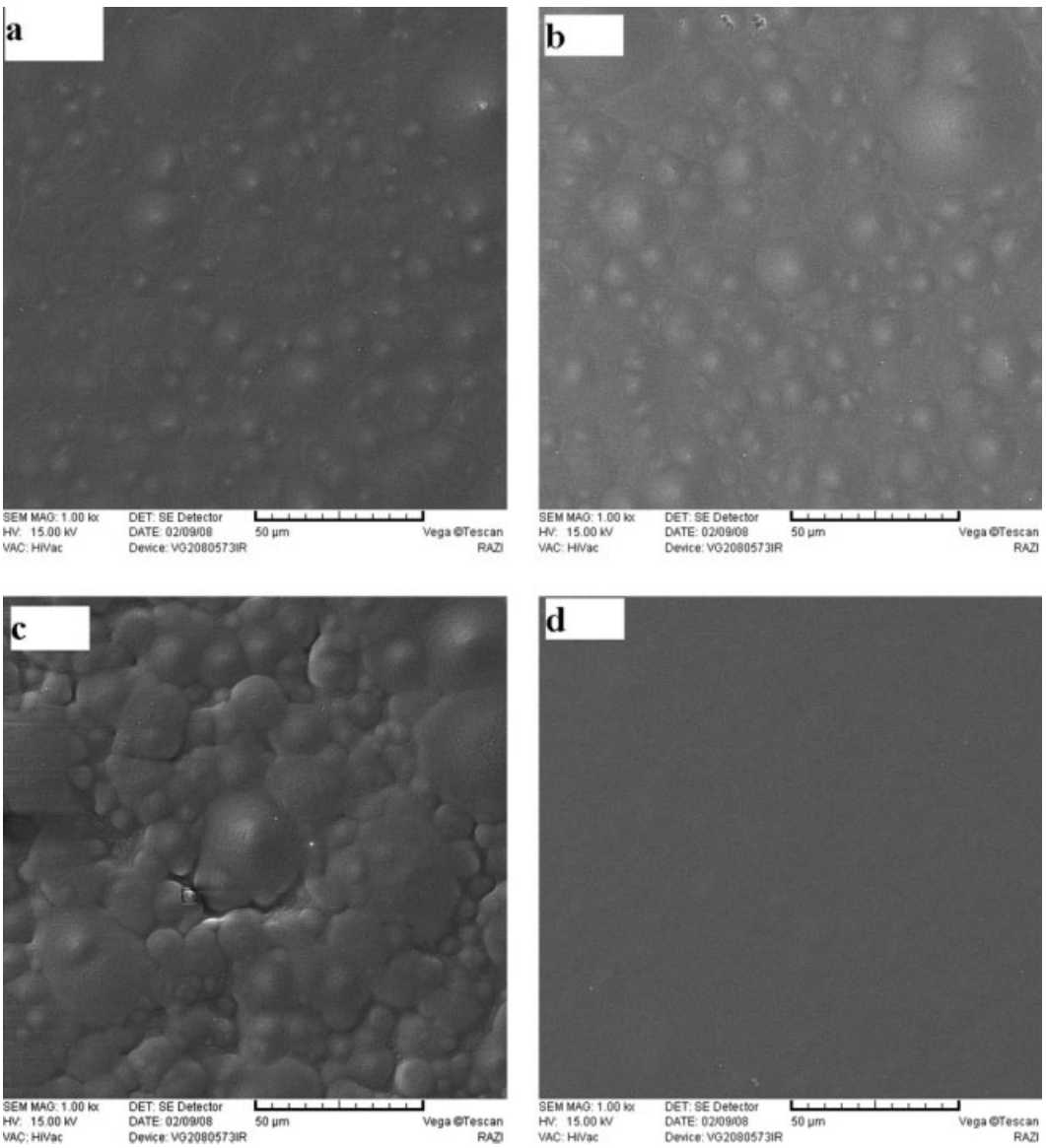

Figure 3 SEM photomicrographs of untreated and CO_2 plasma-treated PLLA210; (a) 15 s treatment time; (b) 30 s treatment time; (c) 2 min treatment time; (d) untreated sample (magnification; ×1000).

the CO_2 gas, significantly increased compared with the control when cultured for 1 day. We used B65 nervous tissue cells for interaction of biodegradable polymers (plasma treated and untreated) with this cell. There are many reports, which used this cell in central nervous system research.[31,32] B65 cell has sensitive cell membrane, which in contact with environment media interact fast and sensitive. Therefore, by modification of PLLA with CO_2 plasma causes better attachment and growing of nervous cells, which will be applied for nerve tissue engineering purpose. Figure 5(a,b) shows the results of adhesion of L929 fibroblast cells containing culture medium to untreated and plasma treated PLLA 210 films. Optical Photomicrographs of cell attachment show that there are not significant differences between the L929 cells adhesion and growth on to the treated and untreated PLLA 210 surfaces. This was most evident for PLLA210 films with contact angles about 40°. In contrast to L929 cells, plasma-treated PLLA210 notice a high number of attached B65 cells on its surface [Figure 4(b)]. The B65 cell, regardless of the L-929 cell, were protruded fillodopia and lamelliopodia that spread out and flattened more on the PLLA surfaces than controls after 1 day of culturing. Plasma treatments especially showed better activated cell morphology than controls. When the surface of the base material is moderately hydrophilic, the cell is liable to stably stick. It is known that the highest amount of proteins is adsorbed and the cell adheres well and proliferates on the material when its contact angle with water is about 50–70°.[33] That is, the biomaterial adheres well to the surface, which is not extremely hydrophilic or extremely

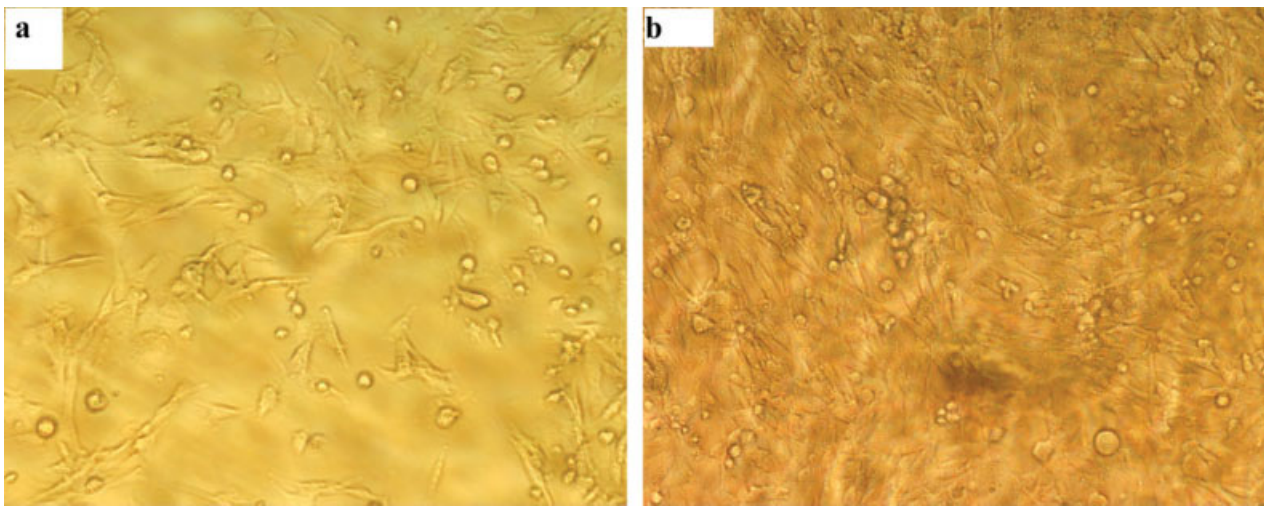

Figure 4 B-65 cell attachment on (a) untreated PLLA210; (b) CO_2 plasma-treated sample (magnification; ×200). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hydrophobic, and if the hydrophilic property or the hydrophobic property increases more than that, the amount of bonding decreases. We assume that the adhesion process is not only exclusively governed by this wettability of the surfaces, but also by chemical characteristics; subsequently surface charge. As reported[18] low adhesion of L929 cells on the plasma-treated polyurethane depends on decreasing of zeta potential of treated PU compared with virgin PU. We suppose that different cells have different response to polymer surface and consequently depend on wettability, surface charge, and morphology. In this experiment, it was clarified that the B65 cell adhesion capability and the cell mass culturing on the surface of the sample, which was plasma treated in the CO_2 gas, increased because of the influence of the increasing wettability compared with control (contact angle 46°) whereas L-929 cell adhesion capability do not influence significantly because of the moderately hydrophilic surface. This implies that it is difficult to evaluate the cell adhesion capability only by the contact angle with water, and that the adhesion capability is significantly affected by the physicochemical surface condition of the sample. Other published results indicated that the cells adhered, spread, and grew more on the hydrophilic surfaces than the hydrophobic surface.[34] They cultured endothelial cells, HeLa S_3, or fibroblasts onto various polymer substrates with different surface wettabilities. An inconsistency was observed between the cell culture trend and water contact angle and the oxygen functional groups ratio. However, we observed maximum cell adhesion at some point; for example, the maximum adhesion of the cells appeared around 45° water contact angles for B65 on plasma-treated PLLA surfaces.

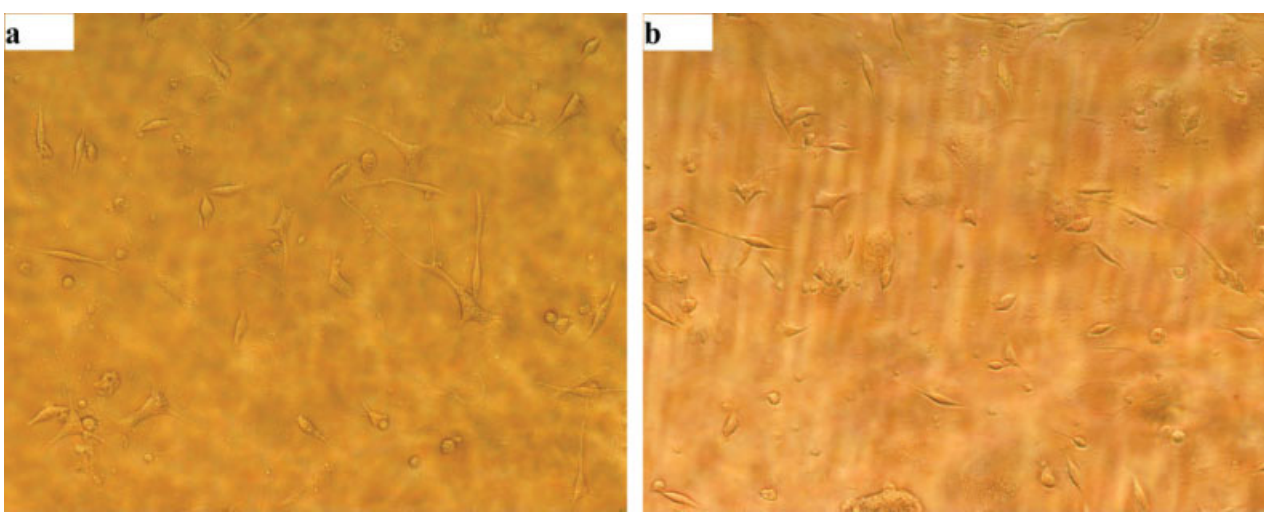

Figure 5 L929 cell attachment on (a) untreated PLLA210; (b) CO_2 plasma-treated (magnification; ×200). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSIONS

The results obtained in this study strongly support the idea that the plasma-modified PLLA represent a very powerful tool to study and modulate in detail fine and complex cell behaviors, including nerve cell and fibroblast cell adhesion and activation in terms of the surface hydrophilicity and hydrophobicity. Moreover, this study has shown that the synergic action of other factors, such as morphology and substrate chemistry, is likely to help elucidate the complex mechanisms underlying nervous system development and plasticity. Also, this surface modification technique can be used for the improvement of the adhesion and growth of nervous cells and tissues onto PLLA films and scaffolds and can be applicable to nerve tissue engineering.

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