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# Improving Cell Affinity of Poly(D,L-lactide) Film Modified by Anhydrous Ammonia Plasma Treatment

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## ABSTRACT

*Anhydrous ammonia gaseous plasma treatment was used for improving cell affinity of the poly(D,L-lactide) film surface. The contact angle was measured and the surface energy was calculated through harmonic mean equations. The surface chemical composition of the sample was identified by X-ray photoelectron spectroscopy (XPS) analysis. It was demonstrated that the O-containing and N-containing groups such as hydroxyl and amine were incorporated onto the surface of films. The effect of preserving methods for maintaining the hydrophilicity was also investigated. The result indicated that preserving at low temperature (0–4°C) was sufficient to maintain the improvements. It could resolve the problem of hydrophilicity being partially lost with preserving time after plasma treatment. Mouse 3T3 fibroblast cells were used as model cells to compare the cell affinity of the modified films and control. The ammonia plasma treatment could promote the cells attachment and cells growth. After 4 days culture, the plasma treated films yielded almost two magnitude cells compared with the control. Copyright © 2002 John Wiley & Sons, Ltd.*

KEYWORDS: poly (D,L-lactide); ammonia plasma treatment; surface modification; cell affinity; tissue engineering

## INTRODUCTION

One approach in tissue engineering [1] is attaching mammalian cells to natural or synthetic matrices and implanting the resulting device to replace lost tissue function. Cell affinity of biomaterials plays an important role in tissue engineering which is related to the surface properties of biomaterials such as morphology, hydrophilicity, surface energy, surface charge, chemical composition and so on. Cell affinity includes two aspects: cell attachment and cell growth. The cell attachment belong 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 on contact with the implant [2]. The attachment of cells is mediated by adhesive glycoproteins such as fibronectin and vitronectin, which compete with other proteins for adsorption on to polymer surfaces. Much attention has been directed toward identifying what kind of surface chemical groups can benefit for adsorbing the glycoproteins.

Plasma treatment is a convenient and widely used method for modifying the surface of materials. It has been reported that the surface of cell culture devices (such as petri dishes, microcarriers and membrane) can be modified by plasma treatment to improve cell growth, protein binding

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and non-binding, and cell-specific attachment by controlling surface chemical structures, surface energies and/or surface charge states [3]. Functional groups such as $-NH_2$, $-OH$ and $-COOH$ are most frequently grafted by means of plasma treatment of non-deposition gas such as $NH_3$, $O_2$, $H_2O$, etc. [4]. Wang and coworkers [5] demonstrated that both cell attachment and growth on nylon meshes were improved significantly by ammonia gaseous plasma treatment. Sipehia [6] modified the surface of petri dishes by anhydrous ammonia plasma treatment and promoted the attachment and growth of endothelial cells. It has been demonstrated that plasma modification technique is a unique and powerful method for the modification of polymeric materials without altering their bulk properties. However, the problem is that the modified results will decline [7] with time after plasma treatment, which will affect its further application.

Poly(D,L-lactic acid) (PDLLA) is versatile, and one of the few biodegradable polymers, which has been approved by the Food and Drug Administration of the USA. Like other synthetic materials, there are no natural recognition sites on the surface of poly(lactic acid) (PLA) [8], and the surface hydrophobicity and surface energy of the PLA affect the cell attachment and growth on the polymer. In this paper, we aimed to improve cell affinity of the PDLLA, the surface of PDLLA was improved by $NH_3$ plasma treatment and the effects of plasma treatment on the attachment and growth of mouse 3T3 fibroblast cells were investigated. In addition, the effects on hydrophilicity of different preserving methods were also discussed.

## EXPERIMENTAL

### Materials

D,L-Lactic acid (80%, Shanghai Yierbao) was used as purchased. Stannous 2-ethyl-hexanoate (Sigma Chemical Co.) as a polymerization catalyst was used as received. Hexadecanol (C.P. grade) was supplied by Beijing Chemical Factory and freeze-dried before use.

### Methods

*Synthesis of PDLLA.* D,L-Lactide was synthesized from D,L-Lactic acid according to the literature method [9]. The resulting lactide was recrystallized three times before polymerization. PDLLA ($M_n = 50000$) was synthesized at 140 °C for 10 hr under vacuum in a sealed tube using stannous octoate as catalyst, hexadecanol as molecular weight modulator.

*Preparation of PDLLA Film.* PDLLA film was prepared by a solution casting method using 8% wt of PDLLA chloroform solution into a poly-(tetrafluoroethylene) mould. After solvent evaporation in air at room temperature, the film was removed from the mould and followed by drying *in vacuum* at room temperature for 48 hr. The produced transparent film was then cut into a certain shape and sterilized before use.

*Plasma Treatment.* The plasma treatment was carried out on a Samco Plasma Deposition (Model PD-2, 13.56 MHz) with anhydrous ammonia gas. The chamber was evacuated to less than 10 Pa before filling with anhydrous ammonia. After the pressure of the chamber had stabilized to a proper value, a glow discharge plasma was created by controlling the electrical power at a radio frequency of 13.56 MHz for a predetermined time. Finally the plasma-treated films were further exposed to ammonia gas for another 10 min after turning off the power.

*Cell Culture.* Mouse 3T3 fibroblast cells were grown in 50 ml cell culture flask with Dulbecco's Modified Eagles Medium (Gibco) supplemented with 15% calf serum (Gibco) and 100 $U/cm^3$ each of penicillin and streptomycin. Cell culture was maintained in a 37 °C gas-jacket incubator equilibrated with 5% $CO_2$. When the cells had grown to confluence, the cells were digested by 1 ml 0.25% trypsin for 1–2 min, then 3 ml culture medium were added to stop digestion and the culture medium was aspirated to obtain cell dispersion which was used after counting the cells.

*Morphology Observation of Cell Attachment.* PDLLA films were cut into small pieces (1 cm × 2.5 cm) and located (modified side up) in culture dishes with 30 mm of diameter. Cell dispersion of 200 µl (about $3 \times 10^4$ cells) was placed on the film samples and cultured for 4 hr before 3 ml of culture medium were added into the culture dish. Morphology of cell attachment was observed and photographed by invert light microscopy (Olympus Optical Co., Ltd.) after culturing for a period of 4, 9 and 24 hr, respectively.

*Cell Growth.* Circular disks of 15 mm diameter were cut from PDLLA film. The plasma modified disks (modified side up) and the control disks were placed in 24 well cell culture cluster (Costar, USA). Each group included 16 samples and cells were seeded at a density of $3–5 \times 10^4$ per well in 2 ml culture medium. The cell medium was replenished every second day. Cell numbers were counted per day for up to 4 days after trypsin digestion and expressed by average value measured from four samples.

*Contact Angle Measurement.* The contact angle of the samples with water was measured in air using a FACE CA-D type Contact Angle Meter (Kyowa Kaimenkagaku Co., Ltd). Four independent determinations at different sites were averaged. Deionized water and di-iodomethane was used for the measurement.

*X-Ray Photoelectron Spectroscopy (XPS) Analysis.* XPS spectra of the plasma modified samples and the

**TABLE 1.** Dependence of Contact Angle on Parameters of Plasma Treatment

| *Sample* | *Condition* | | | *Contact angles to $H_2O$* |
|---|---|---|---|---|
| | *Power (W)* | *Time (sec)* | *Pressure (Pa)* | *(deg)* |
| 1 | 50 | 10 | 20 | 60.5 |
| 2 | 50 | 20 | 20 | 47.5 |
| 3 | 50 | 30 | 20 | 37.0 |
| 4 | 50 | 60 | 20 | 25.0 |
| 5 | 50 | 120 | 20 | 21.5 |
| 6 | 30 | 120 | 60 | 49.5 |
| 7 | 30 | 120 | 40 | 44.0 |
| 8 | 90 | 120 | 20 | 35.0 |
| 9 | 70 | 120 | 20 | 27.5 |
| 10 | 50 | 120 | 80 | 40.5 |
| Control | — | — | — | 78.5 |

control samples were acquired on a VG Escalab 220i-xl spectrometer using Al-$K_\alpha$ radiation at a power of 300 W. A take-off angle of 90° with respect to the samples surface was used.

## RESULTS AND DISCUSSION

### Effects of $NH_3$ Plasma Treatment on Contact Angles and Surface Energy of PDLLA Films

Contact angle measurement is a simple method for evaluating the hydrophobicity or hydrophilicity of a sample surface. The contact angle of a typical hydrophobic surface to water is approximately 65–95° [10].

Table 1 showed the change of contact angles to water upon exposing the PDLLA film to anhydrous ammonia gaseous plasma at different plasma treatment parameters. From Table 1, we could know that the hydrophilicity of PDLLA film had been improved after the plasma treatment. A series of PDLLA films with different hydrophilicity had been achieved. The results showed that the contact angle of the sample depended strongly on the plasma parameters including power, pressure, and treatment time.

The changes of surface energy of the plasma treated PDLLA film and control film had also been determined by means of measuring the contact angles to two kinds of reference liquid ($H_2O$ and $CH_2I_2$) and the surface energy was calculated through Harmonic mean equations as below:

$$(1+\cos\theta_1)\gamma_1 = 4((\gamma_1^d\gamma_s^d/(\gamma_1^d+\gamma_s^d)+ \gamma_1^p\gamma_s^p/(\gamma_1^p+\gamma_s^p)) \qquad (1)$$

$$(1+\cos\theta_2)\gamma_2 = 4((\gamma_2^d\gamma_s^d/(\gamma_2^d+\gamma_s^d)+ \gamma_2^p\gamma_s^p/(\gamma_2^p+\gamma_s^p)) \qquad (2)$$

Where the $\gamma^d$ is dispersive components; $\gamma^p$ is polar components; $\theta_1$ is contact angle to water, $\theta_2$ is contact angle to di-iodomethane. For water, $\gamma_1 = 72.8\,mJ/m^2$, $\gamma_1{}^d = 22.1\,mJ/m^2$, $\gamma_1{}^p = 50.7\,mJ/m^2$. For di-iodomethane, $\gamma_2 = 50.8 mJ/m^2$, $\gamma_2^d = 44.1 mJ/m^2, \gamma_2^p = 6.7 mJ/m^2$. From the data in Table 2, it can be seen that after plasma treatment the surface energy had increased from 42.2 to 69.1 mJ/m², and the contribution of polar components in surface energy of plasma treated film had increased greatly than that of control. It could be inferred that the polar groups had been incorporated into the surface of the plasma-modified PDLLA film.

### Effect of $NH_3$ Plasma Treatment on Composition of Sample Surface

XPS survey spectra of samples were used to compare composition change of sample surface

**TABLE 2.** The surface energy[a] of plasma-modified film[b] and control film (mJ/m²)

| *Surface* | *$\theta$ (deg) to $H_2O$* | *$\theta$ (deg) to $CH_2I_2$* | $\gamma_s$ | $\gamma_s^d$ | $\gamma_s^p$ | $X^p$ |
|---|---|---|---|---|---|---|
| Control | 78.0 | 37.0 | 43.2 | 32.5 | 10.7 | 0.25 |
| $NH_3$ plasma | 21.5 | 40.0 | 69.1 | 26.7 | 42.4 | 0.67 |

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[a] $\gamma_s$, surface energy; $\gamma_s^d$, dispersive components; $\gamma_s^p$, polar components; $X^p = \gamma_s^p/\gamma_s$.

[b] Plasma treatment parameters were 50 W, 20 Pa, and 120 sec.

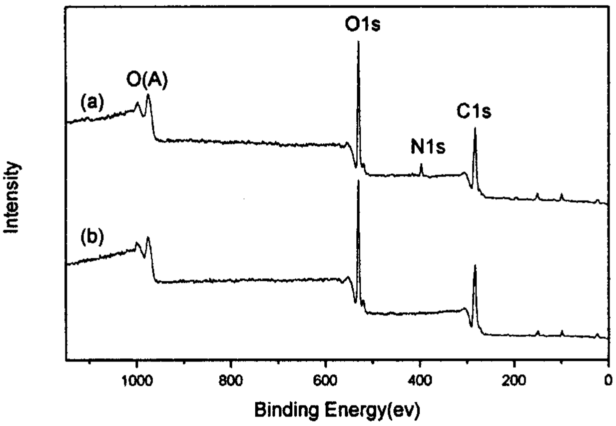

**FIGURE 1.** XPS survey spectra of (a) plasma modified and (b) control surface of PDLLA film.

before and after plasma treatment (50 W/20 Pa/120 sec) as shown in Fig. 1. It could be seen that a new $N_{1s}$ peak could be observed after $NH_3$ plasma treatment. Three peaks corresponding to $C_{1s}$ (285 eV), $N_{1s}$ (400 eV) and $O_{1s}$ (532 eV) [11] implied that N-containing groups, for example $-NH_2$, might be incorporated into the plasma-treated surface of PDLLA. Typical chemical compositions calculated from the XPS survey spectra were shown in Table 3.

It could be seen that the O/C content ratio of the PDLLA surface was significantly increased from 0.40 to 0.58 after plasma treatment. Based on the peak-fitted $C_{1s}$ spectra, peak at 284.6 represents $-C-H-$ or $-C-C-$, the peak at 286.4 represents $-C-O-$, the peak at 288.6 represents $>C=O$ or $-COOH$, the fractions of different carbon functional groups were analyzed in Table 4. The fraction of carbon with a single bond to oxygen in plasma treated samples was more than that of the control. It indicated that a greater number of alcohol or ether had formed on the surface of the PDLLA film. It meant that the hydrophilic groups such as alcohol, ether and N-containing groups such as $-NH_2$ were incorporated into the surface of the film, which resulted in the improvement of hydrophilicity and an increase in the fraction of polar components on the surface of PDLLA.

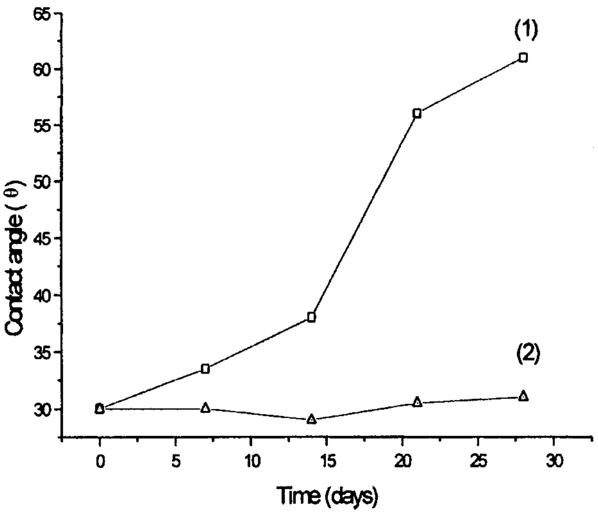

**FIGURE 2.** Change in the contact angle (using water) of the plasma modified film versus preserving time in different preserving conditions: (1) at room temperature; (2) at 0–4 °C.

### Effect of Preserving Condition on Results of $NH_3$ Plasma Treatment

It had been reported that a disadvantage of treatment in ammonia plasma was that the modified surfaces would develop surface restructuring with time, which resulted in a partial loss of the treatment effects and would affect further application [12]. The changes of the contact angles to water with preserving time in different preserving conditions had been studied. One group of samples was kept from moisture and preserved at room temperature after plasma treatment, another group of samples was kept from moisture and preserved at low temperature (0–4 °C) after plasma treatment. The contact angles were measured immediately and after 7 days preserving intervals after plasma treatment. The change in contact angle of samples to water in a month are shown in Fig. 2.

**TABLE 3.** XPS Analysis of Surface Compositions of PDLLA Films

| *Surface of PDLLA films* | *O1s (%)* | *N1s (%)* | *C1s (%)* | *O/C* | *N/C* |
|---|---|---|---|---|---|
| Plasma modified | 34.67 | 5.04 | 60.29 | 0.58 | 0.08 |
| Control | 28.61 | — | 71.39 | 0.40 | — |

**TABLE 4.** Fraction of Carbon Functional Groups from High-resolution C1*s* XPS Peaks of PDLLA Surface

| *Sample* | *284.6 eV —C—H, —C—C— (%)* | *286.4 eV —C—O— (%)* | *288.6 eV >C=O or —COOH (%)* |
|---|---|---|---|
| Plasma modified | 44.77 | 31.18 | 24.05 |
| Control | 46.32 | 24.45 | 29.23 |

It could be seen from Fig. 2 that the apparent difference had been achieved by different preserving conditions. For the plasma treated film preserved at room temperature, the contact angle increased from 30° to 61° within 4 weeks. However, for the film preserved at low temperature, there were almost no change in contact angle. It had been reported that polymer surfaces were highly mobile and surface restruction had often been observed leading to a considerable decrease in surface density of polar groups attached by non-deposition surface treatment [12, 13]. From the results in Fig. 2, we thought that the preserving temperature (4 °C) was far lower than the glass transition temperature ($T_g$) of PDLLA, the mobility of surface molecular chains was nearly frozen, which meant that the hydrophilicity could be kept unchanged. Therefore, it was suggested that a convenient method to maintain the hydrophilicity of plasma treated samples was to preserve the modified film at lower temperature.

### Effect of $NH_3$ Plasma Treatment on Cell Affinity of PDLLA Surface

The effect of $NH_3$ plasma treatment on cell affinity of PDLLA surface was identified by observing the morphology through the culture of mouse 3T3 fibroblast cells. Most of the mammalian cells were characteristic of anchorage on culture matrix. It was a key step that would affect the following growth, proliferation and migration. The morphology of cell attachment at different periods on modified films and control was observed by photomicrography as shown in Figs 3 and 4. For the control film, as shown in Fig. 3, the cells were round and only a few cells have attached to the surface of the film after 4 hr of culture. However, a great number of cells had attached to the surface of the modified film and appeared as characteristic spindle shape. After 9 hr of culture, some cells attached to the control film but many cells were still round and the distribution of cells was sparse. The cells cobblestone started to appear. However for modified film, many cells attached to the surface of the film and the cells stretched very well. No cells cobblestone appeared on the surface. After 24 hr, there were many cells attached to the control film but many cells cobblestone appeared and there were still a number of round cells. On the modified film, the cells attached tightly and appeared spindle shape, no cells cobblestone appeared and the cells were distributed evenly and stretched very well. Based on the results, it could be proved that the ammonia plasma modified surface facilitated the attachment of 3T3 fibroblast cells.

The relative rate of growth of fibroblast on modified films and control over a period of 4 days were compared in Fig. 5. From the first day, more cells attached to the modified films. It meant that improvement of cell attachment had been achieved after plasma treatment. It could be suggested that the growth rate of cells on modified films was faster than that on control films. On control films, the cells that attached could not proliferate adequately. After 4 days of culture, plasma modified surfaces yielded an order of almost two magnitude cells compared to those on the control surfaces.

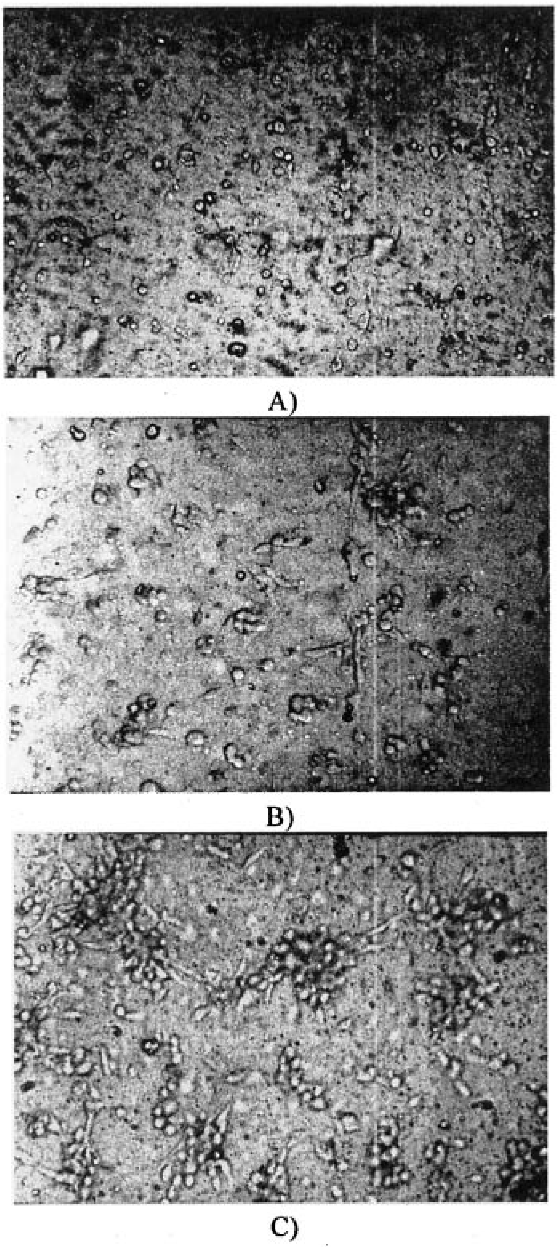

**FIGURE 3.** Photomicrograph (×100) of 3T3 fibroblast cells on the control surface of PDLLA at different culture times: (A) 4, (B) 9, (C) 24 hr.

This result was similar to the reported literature in the case of using other types of polymer where the hydrophilicity of the surfaces played a role for cell attachment and a high polarity of the surface was evidently of most benefit for cell attachment [12]. Proper surface energy would also benefit cell attachment [14]. It could be suggested that O-containing and N-containing groups had been incorporated on the surface of PDLLA films, the hydrophilicity of films was improved and the surface energy was increased after treatment. N-Containing groups may play important roles for

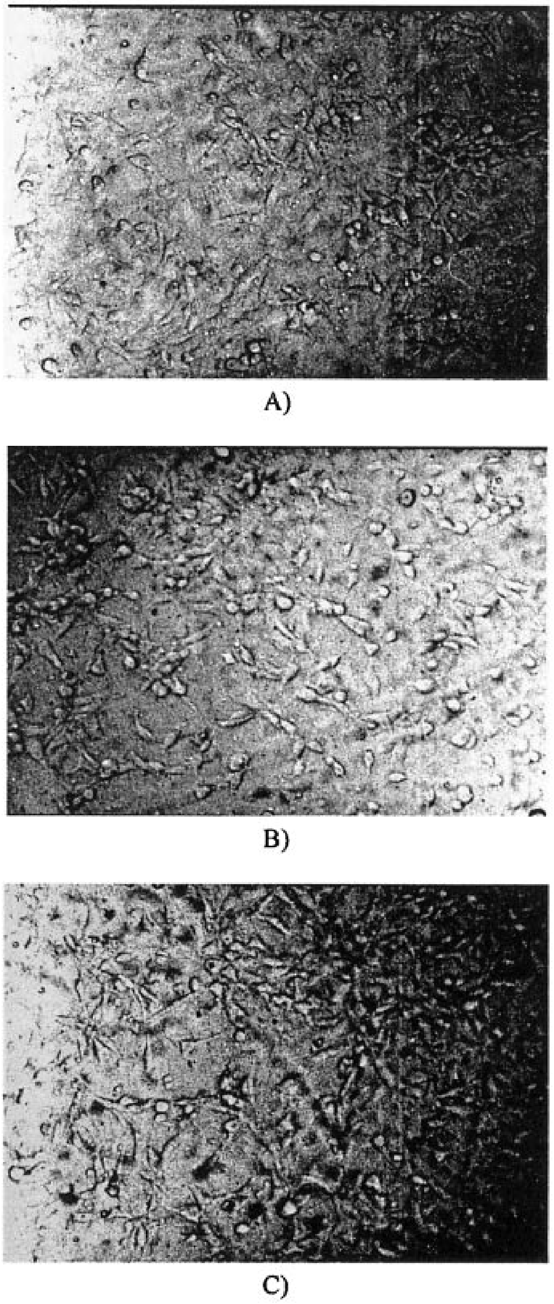

**FIGURE 4.** Photomicrography (×150) of 3T3 fibroblast cells on plasma treated surface of PDLLA at different culture times: (A) 4, (B) 9, (C) 24 hr.

cell attachment and growth. On the one hand, a possible advantage of placing N-containing groups such as amine on the surface was that a fraction of them may be positively charged at physiological pH because of the protonation in culture medium, which would enhance the interaction between the surface of materials and the cells which carried negative charge. On the other hand, since the adhesive glycoprotein such as fibronectin and vitronectin played important roles in the initial cell attachment [15, 16], the N-containing groups were capable of efficient interaction with proteins by hydrogen bonding, which could affect the adsorption of serum adhesive glycoproteins.

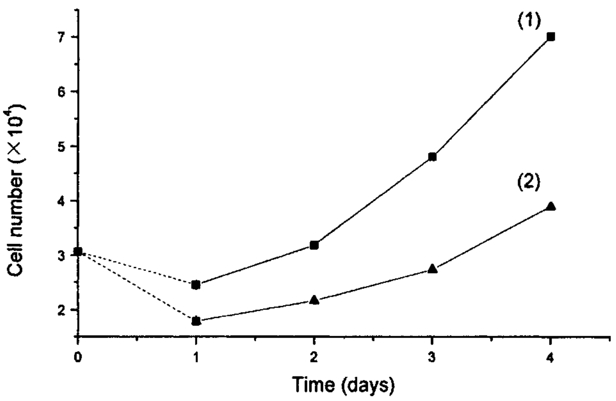

**FIGURE 5.** Comparison of 3T3 fibroblast cells cultured on (1) anhydrous ammonia gas plasma modified surface of PDLLA and (2) control.

## CONCLUSIONS

The hydrophilicity of PDLLA surface could be improved by anhydrous ammonia gaseous plasma treatment and the surface energy of PDLLA films could be increased after treatment. The hydrophilicity could be maintained well at low temperature. Based on the XPS analysis, it could be proved that the O-containing and N-containing groups were incorporated onto the surface of films resulting in the improvement of hydrophilicity and the increase in surface energy. Mouse 3T3 fibroblast cells culture experiment indicated that the anhydrous ammonia gaseous plasma could promote effectively cell attachment and cell growth on the surface of PDLLA films.

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