Enhanced cell affinity of poly (D,L-lactide) by combining plasma treatment with collagen anchorage

Jian Yang, Jianzhong Bei, Shenguo Wang*

Center for Molecular Sciences, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, China

Received 27 February 2001; accepted 17 November 2001

Abstract

Surface properties of poly (D,L-lactide) (PDLLA) were modified by combining plasma treatment and collagen modification. The changes of surface properties were characterized by contact angles, surface energy, X-ray photoelectron spectra and scanning electron microscopy. The mouse 3T3 fibroblasts were used as model cells to evaluate the cell affinity of PDLLA before and after modification. Effects of different modification methods including plasma treatment, collagen coating and combining plasma treatment with collagen anchorage were investigated and compared. The results showed that the hydrophilicity and surface-free energy were improved and reduced, respectively, after each modification. Plasma pre-treatment could improve the roughness as it incorporated the polar groups and positively charged groups onto the sample surface; so the plasma pre-treated surface would benefit in anchoring more collagen tightly. As a result, cell affinity of PDLLA modified by combining plasma treatment with collagen anchorage was greatly improved. The modified materials could endure rinsing by PBS, which would facilitate further application when the modified materials were used as cells scaffold in tissue engineering. © 2002 Published by Elsevier Science Ltd.

Keywords: Cell affinity; Poly (D,L-lactide); Plasma treatment; Collagen; Cells scaffold; Tissue engineering

1. Introduction

Cell affinity is the most important factor to be concerned with when biodegradable polymeric materials are utilized as cell scaffold in tissue engineering [1,2]. Commonly, cell affinity includes two important factors: 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 on contact with the material [3]. The interaction of cells with the material is a result of specific recognition among cell surface adhesion receptors, which are integrins and extracellular matrix (ECM) proteins (fibronectin, vitronectin, collagen) that have a cell-binding domain containing the Arg-Gly-Asp (RGD) sequence [4]. Many studies have proved that hydrophilicity/hydrophobicity [5], surface energy [6] and charge [7,8] of the material surface greatly influence the cell attachment and cell growth on the material. In addition, surface roughness would benefit in adsorbing the proteins in culture medium to form biofilms [9], which could mediate the cell adhesion.

Poly (lactic acid) (PLA) is one of the biodegradable polymers, which has been approved to implant in the body by Food and Drug Administration of USA. However, like other synthetic materials, there are no natural recognition sites of cells on the surface of PLA [10], and the hydrophobicity and the low surface energy of the PLA affect the cell attachment and growth on the PLA. Since the functional groups are absent in the backbone of the PLA, it is difficult to modify the surface property by common chemical methods such as by attaching cell-recognizing ligands to promote cell adhesive property of the PLA. Plasma technique is a convenient method for modifying surface properties of a material. It has an advantage of treating surfaces of complex shape because the plasma treatment is conducted in a vacuum and it tends to be pervasive [11]. This advantage can benefit in treating cells scaffold with interpenetrating porous structure in tissue engineering. Plasma technique can be easily used to induce the desired groups or chains onto the surface of a material [12–17], so it has a special application to improve the cell affinity of the cells scaffold. On the other hand, it has
been demonstrated that plasma treatment is a unique and powerful method for modifying polymeric materials without altering their bulk properties. However, the problem is that the modified effects will decline with preserving time [13] and it will affect its further practical application. In recent years, many studies have focused on coating or grafting bioactive molecular on the polymeric surface in order to mediate the cell attachment and cell growth [10,18–22]. Collagen coating is often utilized to modify the cell affinity of polymeric materials, but the coated collagen molecules may be removed from the surface when exposed to culture medium for a period of time.

The objective of the present study is to enhance the cell affinity of poly (d,l-lactide) (PDLLA) by combining plasma treatment with collagen anchorage in order to enhance its cell affinity. The effect of the improved surface treating method was characterized by means of surface contact angles, surface energy, scanning electron microscopy (SEM) and X-ray photoelectron spectra (XPS). The cell affinity of the PDLLA before and after the surface modification was also evaluated and compared by 3T3 fibroblasts culture.

2. Experimental

2.1. Materials

d,l-lactide was synthesized from d,l-lactic acid (80%, Shanghai Yierbao) and the resulting d,l-lactide was recrystallized three times by dried toluene, ethyl acetate and toluene, respectively. PDLLA (Mn = 50,000) was synthesized by ring-opening polymerization using stannous 2-ethyl-hexanoate (sigma chemical co.) as a catalyst under vacuum at 140°C for 10 h in the presence of hexadecanol (CP grade), which was used as molecular modulator. The transparent PDLLA film was prepared by casting 8wt% of PDLLA chloroform solution into a poly (tetrafluoroethylene) mold. After solvent evaporation in air at room temperature, the produced film was removed from the mold and then dried in vacuum at room temperature for 48 h. The thickness of the produced film was 0.10 mm. Finally, the PDLLA film was cut into a certain size.

2.2. Surface modification

2.2.1. Plasma treatment

The oxygen and ammonia plasma treatment was carried out on Samco Plasma Deposition (Model PD-2, 13.56 MHz). PDLLA film was placed over the electrode in the plasma chamber. The chamber was evacuated to <10 Pa before filling with the desired gas. 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. The plasma-treated sample was further exposed to the desired gas for another 10 min before the sample was taken out from the chamber.

2.2.2. Collagen coating and collagen anchorage

The purchased collagen (sterilized, BM) was diluted by phosphate buffer saline (PBS) to form a 0.1 mg/ml of solution. Collagen-anchored sample was obtained by immersing the NH3 plasma-treated sample in the collagen solution for at least 2 h, then the sample was rinsed in PBS for three times and sterilized by ultraviolet radiation for 30 min. The collagen-coated sample was obtained by immersing the untreated PDLLA sample in the collagen solution. All procedures were carried out in ultra-cleaning workstation.

2.3. Characterization of surface property

2.3.1. Determination of contact angles and calculation of surface energy

The Contact angles of PDLLA samples were measured on the air surface of the samples using the FACE CA-D-type Contact Angle Meter (Kyowa Kaimen- gaku Co., Ltd). Four independent determinations at different sites were averaged. Deionized water and di-iodomethane were used for the measurement. The surface free energy was calculated according to the Harmonic mean equations and expressed as follows:

\[
(1 + \cos \theta_1) \gamma_1 = 4\left(\gamma_{1d}^{1d} + \gamma_{1s}^{1s} + \gamma_{1p}^{1p} / (\gamma_{1}^{1d} + \gamma_{1}^{1s} + \gamma_{1}^{1p})\right),
\]

(1 + \cos \theta_2) \gamma_2 = 4\left(\gamma_{2d}^{2d} + \gamma_{2s}^{2s} + \gamma_{2p}^{2p} / (\gamma_{2}^{2d} + \gamma_{2}^{2s} + \gamma_{2}^{2p})\right).

where \( \gamma^{d} \) is the dispersive component; \( \gamma^{p} \) is the polar component; \( \theta \) is the contact angle to water, and \( \theta \) is the contact angle to di-iodomethane. For water, \( \gamma_{1} = 72.8 \text{ mJ/m}^2, \gamma_{1}^{d} = 22.1 \text{ mJ/m}^2, \) and \( \gamma_{1}^{p} = 50.7 \text{ mJ/m}^2. \) For di-iodomethane, \( \gamma_{2} = 50.8 \text{ mJ/m}^2, \gamma_{2}^{d} = 44.1 \text{ mJ/m}^2, \) and \( \gamma_{2}^{p} = 6.7 \text{ mJ/m}^2. \)

2.3.2. Observation of scanning electron microscopy (SEM)

The PDLLA samples were dried and sputter-coated with gold and examined under an SEM (Hitachi, S-530).

2.3.3. Analysis of X-ray photoelectron spectra (XPS)

XPS spectra of the modified samples and control were acquired on a VG Escalab 220i-xl spectrometer using Al Kα radiation at a power of 300 W. A take-off angle of 90° with respect to the sample surface was used. The high-resolution spectra C1s and N1s were deconvoluted and curve-fit to analyze the chemical bonding state. All measurements were taken under vacuum (2 × 10⁻³ Pa).
2.4. Cell culture

Mouse 3T3 fibroblasts were cultured in 50ml cell culture flask with Dulbecco’s Modified Eagles Medium (Gibco) buffered with N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), supplemented with 15% calf serum (Gibco) and 100 U/cm² each of penicillin and streptomycin. Cell culture was maintained in a gas-jacket incubator equilibrated with 5% CO₂ at 37°C. When the cells had grown to confluence, the cells were digested by 1ml 0.25% trypsin (Sigma) for 1–2 min, then 3ml of culture medium was added to stop digestion and the culture medium was aspirated to get cell dispersion, which was used after counting the cells.

PDLLA film was cut into small pieces (1 × 2.5 cm²) and the samples, modified side up, were placed in culture dishes with 30 mm in diameter. 200 µl (about 3–5 × 10⁴ cells) of cell dispersion was placed on the samples and cultured for 4 h before 3ml culture medium was added into the culture dish. The morphology of cell attachment was observed and photographed by inverted light microscope (Olympus Optical Co., Ltd.) after culturing for a predetermined time.

Circular disks with 15 mm in diameter were cut from PDLLA film with the aid of a cork borer. The modified disks, modified side up, and the control disks were placed in a 24-well cell culture plate (Costar, USA). Cells were seeded at a density of 3–5 × 10⁴ per well in 2ml of culture medium. The cell medium was replenished every second day. Cells numbers were counted per day for up to 4 days after rinsing by PBS in order to remove the unattached cells and digestion by trypsin. The statistical significance between two sets of data was calculated using Student’s t-test. Data were taken to be significant, when a P-value of 0.05 or less was obtained (showing a 95% confidence limit).

3. Results and discussion

3.1. Effects of plasma treatment on surface hydrophilicity and surface energy

Oxygen and anhydrous ammonia gas were used in the plasma treatment of PDLLA film. Contact angles were obtained by sessile drop techniques. A comparison of surface contact angles to water between the untreated and plasma-treated PDLLA samples were listed in Table 1. It could be seen that the surface hydrophilicity of the PDLLA films was improved by plasma treatment and the improvement was closely related to the kind of gas as well as the treating parameters such as power, pressure and treating time. Therefore, plasma treatment was a convenient method to improve the hydrophilicity of the polymer.

The surface energy of PDLLA samples before and after modification was calculated from the contact angle and shown in Table 2. It showed that the surface energy had increased after plasma treatment. The larger increment of surface free energy from 43.2 to 69.1 mJ/m² and the higher contribution of polar components to surface energy in the case of NH₃ plasma were obtained than for other gas plasmas. It revealed that the polar groups might be easier to be incorporated into the surface of PDLLA film by ammonia plasma treatment, which resulted in the improvement of hydrophilicity. However, there had some defects that plasma treatment was utilized as the sole method to modify the materials because the hydrophilicity of the material would be decline with preserving time owing to the surface mobility [13,23,24]. Therefore, in the present research the ammonia plasma was chosen for pre-treatment to anchor the collagen. The data showed that the surface energy of collagen-anchored films was higher than that of control and the contact angle to water of collagen anchored samples were less than that of control. Hence, combination of ammonia plasma treatment with collagen anchorage would do help to improve the hydrophilicity and surface energy of PDLLA.

3.2. Effects of modification on surface compositions

The surface composition of PDLLA before and after modification was identified by XPS analysis. Figs. 1 and 2 showed the deconvoluted C₁s spectra and N₁s spectra of PDLLA. The C₁s spectra were deconvoluted into

### Table 1
Effect of plasma treatments by various gases on contact angle of PDLLA

<table>
<thead>
<tr>
<th>Plasma Condition</th>
<th>Contact angle θ H₂O (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power (W)</td>
<td>Pressure (Pa)</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
</tr>
<tr>
<td>O₂</td>
<td>50</td>
</tr>
<tr>
<td>O₂</td>
<td>100</td>
</tr>
<tr>
<td>N₂</td>
<td>50</td>
</tr>
<tr>
<td>N₂</td>
<td>50</td>
</tr>
<tr>
<td>NH₃</td>
<td>50</td>
</tr>
<tr>
<td>NH₃</td>
<td>50</td>
</tr>
<tr>
<td>NH₃</td>
<td>50</td>
</tr>
<tr>
<td>NH₃</td>
<td>50</td>
</tr>
<tr>
<td>NH₃</td>
<td>30</td>
</tr>
<tr>
<td>NH₃</td>
<td>50</td>
</tr>
<tr>
<td>NH₃</td>
<td>50</td>
</tr>
<tr>
<td>NH₃</td>
<td>30</td>
</tr>
<tr>
<td>O₂ + NH₃</td>
<td>50</td>
</tr>
</tbody>
</table>

*60 s for O₂ and then 60 s for NH₃, respectively.
three peaks: 284.6 eV (–C–H–), 286.6 eV (–C–O–) and 288.6 eV (–COO–). The peak located at 399.7 eV was assigned to –N–H–, and the charged nitrogen species at 401.1 eV was assigned to polaron C–N+. The fraction of various carbon functional groups or nitrogen functional groups on the surface of control and modified PDLLA films was shown in Table 3. It indicated that the plasma-modified samples exhibited more enriched –C–O– groups than that on control. For plasma pre-treated collagen-anchored sample, the higher content of –C–H–, and the lower content of –C–O– and –COO– was detected. It was considered that more collagen was anchored on the plasma pre-treated surface. Nitrogen-containing groups could be incorporated onto the modified PDLLA surface. The positively charged components in NH3 plasma resulted in the appearance of C–N+ on plasma-treated sample. The positive charged groups also appeared on the plasma pre-treated collagen-anchored sample surface. It might be originated from NH3 plasma or the protonation of amine, which existed in collagen molecule. The high content of –N–H– in collagen-anchored sample was because of the high content of –N–H– that existed in the amino acid sequence of collagen molecule.

From the XPS analysis, it can be understood that the polar O-containing groups and N-containing groups as well as the positively charged groups could be incorporated into the modified sample surface, while the collagen was anchored on the sample surface.

### 3.3. Effects of modification on surface morphology of PDLLA

Fig. 3 shows the morphologies of the PDLLA by various modification methods. It could be seen that the surface of control was smooth (Fig. 3A). After plasma treatments, the surface turned rough (Fig. 3B), as was the sign for plasma etching or removal of the surface layer. The increase in roughness was another reason for the improvement of hydrophilicity of the plasma-modified PDLLA films since the surface wettability was the effect of surface roughness [25]. The protruding islands were observed in the pictures of collagen-coated and the collagen-anchored PDLLA films (Figs. 3C and D). These islands were collagens. It can be seen that there were more collagens in Fig. 3(D) than in Fig. 3(C). The distribution of collagen in Fig. 3(D) was also more even than that in Fig. 3(C). The surfaces of control were smooth and lacking in polar groups. But after plasma pre-treatments, more collagen could be easily anchored on the surface of materials. We assumed that the reasons were that hydrophilic surface could accelerate to adsorb the collagen. The enriched polar groups on the surface (such as –OH, –COO–, –NH–) could provide many sites to obtain the collagen by polar interaction and hydrogen bonding, just like many arms to scratch the collagen. In addition, surface roughness would help to adsorb the collagen. These were the reasons why we used the word “anchorage” which was different from the word “coating”. Likewise, the enriched polar groups originated from plasma treatment would not move to the inner side of the polymer because of the effective interaction between the collagen molecule and the polar groups. Thus, the improved method could avoid the faults of plasma treatment and collagen coating when they were used singly.

### 3.4. Effects of modification on cell affinity of PDLLA

The morphology of 3T3 fibroblasts cultured on control and modified sample was observed by inverted light microscope, shown in Fig. 4. It could be seen that after being cultured for 9 h, the cells stretched very well on the collagen-anchored sample, which was even better than the plasma-modified or collagen-coated samples. It suggested that the collagen-anchored PDLLA samples were most beneficial for the attachment of 3T3 fibroblasts.
The proliferation of 3T3 fibroblasts on collagen-modified PDLLA sample and control for 4 days culturing was compared in Fig. 5. It could be seen that the number of cells on collagen-modified sample was obviously more than that on control ($P > 0.05$). It meant that the cell growth had been faster due to collagen modification. However, there was no statistical significance between the number of cells on both kinds of collagen-modified samples (Figs. 5B and D) ($P > 0.05$). It showed that 2 h of immersion could be effective to adsorb collagen molecules onto the PDLLA surface for improving the cell growth. Although the number of cells were all decreased when the cells were cultured on the rinsed surface by PBS ($3 \times 10$ min), there was no statistical difference between the cell number on the collagen-anchored sample (Fig. 5D) and the rinsed sample (Fig. 5E) ($P > 0.05$), but the difference between the number of cells on both collagen-coated samples (Fig. 5B) and rinsed samples (Fig. 5C) was statistically significant ($P < 0.05$). The results showed that plasma pre-treated collagen-anchored surface was more beneficial for cell growth. We considered the reason that...
Table 3
The fractions of various carbon functional groups or nitrogen functional groups from the deconvoluted C₁s or N₁s XPS spectra

<table>
<thead>
<tr>
<th>Sample</th>
<th>C</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>46.3</td>
<td>24.5</td>
</tr>
<tr>
<td>II</td>
<td>37.3</td>
<td>36.7</td>
</tr>
<tr>
<td>III</td>
<td>61.8</td>
<td>20.9</td>
</tr>
</tbody>
</table>

I: Control; II: modified by NH₃ plasma treatment (50 W, 20 Pa, 300 s); and III: modified by NH₃ plasma pre-treatment (50 W, 20 Pa, 300 s) collagen-anchorage.

Fig. 3. SEM observation of surface morphologies of PDLLA samples. (A) Control (× 2000); (B) modified by NH₃ plasma (50 W, 20 Pa, 300 s) (× 10000); (C) modified by collagen coating (× 2000); and (D) modified by ammonia plasma pre-treatment collagen anchorage (× 2000).

Fig. 4. Photomicrograph (× 150) of 3T3 fibroblasts cultured on various PDLLA samples for 9 h. (A) Control; (B) modified by NH₃ plasma (50 W, 20 Pa, 300 s); (C) modified by collagen coating; and (D) modified by ammonia plasma pre-treatment collagen anchorage.
more collagen existed on the plasma pre-treated collagen-anchored surface and the ability of resisting the rinsing by PBS was improved because the collagens were anchored tightly on the sample surface by polar interaction or hydrogen bonding.

4. Conclusions

In the present study, an improved surface modification method combining the plasma treatment with collagen anchorage was proposed. It could be easy to modify the polymeric materials to meet the needs of tissue engineering. Ammonia plasma pre-treatment contributed to inducing the hydrophilic O-containing and N-containing groups and increased the roughness of the surface of PDLLA sample. Thereby the plasma pre-treated surface could easily anchor collagen. For plasma pre-treated collagen-anchored sample, the surface energy had also been improved more than that of collagen-coated sample. The positively charged groups had also been incorporated in the sample surface after plasma pre-treatment of collagen anchorage, as it could enhance the interaction between the PLLA surface and the negatively charged cells. The improved methods could avoid the faults of plasma treatment and collagen coating when they were used singly. Mouse 3T3 fibroblasts were used as the model cells to evaluate the cell affinity of the PDLLA samples. The results showed that the improved modification method could enhance the cell affinity of PDLLA obviously. The ability of resisting the rinsing by PBS was also improved which would facilitate further application when the modified PDLLA was used as cells scaffold in tissue engineering.

Acknowledgements

This research was performed with support from the National Basic Science Research and Development Grants (973, G1999054305 and G1999054306).

References