

Enhancing the cell affinity of macroporous poly(L-lactide) cell scaffold by a convenient surface modification method

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Abstract: In this study, the macroporous poly(L-lactide) (PLLA) cell scaffold was modified for enhancing its cell affinity by an improved surface-treating medium, a mixture of aqueous 0.25 M NaOH/ethanol. Ethanol was applied as a co-treating medium to wet the polylactone and assist the hydroxide nucleophilic attack on the ester bond. Low concentration of NaOH could be applied, severe bulk degradation could be avoided and the residual alkali was easy to remove. Treating time could also be shortened. After treatment under optimal conditions, the surface hydrophilicity and surface energy of PLLA were improved significantly and the surface roughness was also changed. Modification of the spherulite structure on PLLA surface was observed with the treating time using a computer-assisted image analysis system (CAIAS). The results of gel permeation chromatography measurements indicated that only the outer layer of the PLLA was modified and the bulk properties were not altered. Mouse 3T3 fibroblasts culture results indicated that the improved surface-treating medium was effective and convenient for enhancing the cell affinity of PLLA cell scaffold.

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Keywords: PLLA; surface hydrolysis; cell affinity; surface modification; cell scaffold; tissue engineering

INTRODUCTION

Biodegradable polymers have been widely used in tissue engineering.^{1–6} Aliphatic poly lactones such as poly(L-lactide) (PLLA) and its copolymers have been extensively studied as cell scaffold in tissue engineering and drug carriers.⁷ Cell adhesion is an important factor to be concerned with when biodegradable polymeric material is used as cell scaffold in tissue engineering. Cell behaviour would be different when responding to different surface chemistry of a material. The reason is that the surface property of the materials plays an important role in determining the composition of the adsorbed protein layer which, in turn, regulates how cells responds to the material. Many studies have proved that hydrophilicity/hydrophobicity,⁸ surface energy,⁹ charge^{10,11} and roughness¹² of the material surface greatly influence the cell attachment and cell growth on the material.

Poly(L-lactide) is a versatile, well-characterized biodegradable polymer, which has been admitted in clinical application. However, cell affinity of PLLA is often compromised because of the hydrophobicity and the low surface energy of the polymer. Recently,

chemical co-polymerization modification was developed aiming at modifying PLLA surface for controlling ligand immobilization, but the bulk properties were also changed. Many surface modification techniques, such as plasma treatment,^{6,13} surface physical interpenetrating techniques,¹⁴ hybrid modification^{15,16} and surface alkali hydrolysis treatment,^{17,18} have been developed for improving the cell affinity of polymers. Surface alkali hydrolysis treatment is the most simple and convenient method. After surface hydrolysis of aliphatic polyester, the hydrophilic carboxyl and hydroxyl could be produced with cleavage of the ester bonds. The resulting groups could also be used to conjugate the bioactive molecule, such as L-lysine, collagen and Arg-Gly-Asp (RGD) peptide, that could be recognized by the cell adhesion receptors. However, strong alkali treatment is accompanied by extended bulk degradation of the polyester and the residual alkali is not easy by removed, only by rinsing, and it was shown that a mild alkali treatment could not break the ester bonds effectively in a short time. It has been reported that a mixture of NaOH and acetonitrile can be applied to modify the surface properties of poly(ethylene terephthalate) films and membranes

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by controlled wet chemistry, in which acetonitrile was used as a co-treating medium,¹⁹ but acetonitrile is expensive and toxic, and pollution of the environment cannot be neglected.

Considering that a low concentration of aqueous NaOH solution could not break the ester bond of the PLLA surface because the hydrophobicity of PLLA surface retarded the hydroxide nucleophilic attack on ester bonds, our strategy was to use a mixture of aqueous NaOH and ethanol to modify the surface properties of PLLA film, in which non-toxic and cheap ethanol was used as a co-treating medium. The changes of surface properties and morphology were confirmed by measurement of the water contact angle and surface energy, scanning electron microscopy (SEM) and optical microscopy. The cell affinity of the PLLA before and after surface modification was evaluated by mouse 3T3 fibroblasts culture.

EXPERIMENTAL

Materials

Poly(L-lactide) ($M_w = 96\,800$, $M_w/M_n = 1.44$) was prepared by ring-opening polymerization of L-lactide at 140 °C for 10 h under vacuum in a sealed tube, where stannous octoate was used as catalyst and hexadecanol as molecular weight modulator. The PLLA was purified by dissolving the polymer in chloroform and then re-precipitated by ethanol. The PLLA sponge-like scaffold was fabricated by salt leaching techniques²⁰ (thickness: 1.48 mm, pore size: $194 \pm 44 \mu\text{m}$). PLLA film was prepared by standard solution casting techniques (thickness: 0.10 mm).

Surface hydrolysis treatment

The PLLA films and scaffolds were immersed into NaOH solution or the mixture of 0.25 M NaOH aqueous solution and ethanol (v/v = 1/1) for a predetermined incubation period at different temperatures. The treated films and scaffolds were rinsed with β -distilled water ($3 \times 10 \text{ min}$) and then dried under vacuum at room temperature.

Weight loss

The weight loss of the samples, after modification, was calculated through the following formula:

$$\text{Weight loss (\%)} = \frac{M_0 - M_1}{M_0} \times 100$$

where M_0 and M_1 are the weights of the dried untreated and treated samples, respectively.

Contact angles and surface energy

The contact angles of PLLA were measured on the air surface of the samples using the FACE CA-D type Contact Angle Meter (Kyowa Kaimenkagaku Co, Ltd). Ten independent determinations at different sites of the sample were averaged. Deionized water and di-iodomethane were used for the measurements.

The surface energy was calculated according to the harmonic mean equations by Matlab software and expressed as follows:

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

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

where the γ^d and γ^p are the dispersive component, and polar component, respectively; θ_1 and θ_2 are the contact angles to water and to di-iodomethane, respectively. 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 $\gamma_2^p = 6.7 \text{ mJ m}^{-2}$.

Gel permeation chromatography

Gel permeation chromatography (GPC) measurements were carried out on a Waters 510 apparatus equipped with a differential refractometer detector and Shodex GPC KF-800 columns thermostated at 35 °C. Chloroform was used as the eluent at a flow rate of 1.0 ml min^{-1} . Molecular weight and polydispersity of the polymers were calibrated according to polystyrene standards.

Scanning electron microscopy

Poly(L-lactide) films were dried, sputter-coated with gold and examined by scanning electron microscopy (SEM; Hitachi, S-530) before and after treatment.

Surface spherulite structure

The surface spherulite structure was observed by computer-assisted image analysis system (CAIAS). A CCD-camera (type WV-CP460, Panasonic, Japan) examined a field of 0.28 mm^2 through a light microscope (Olympus IMT-2, Phase Contrast, $10\times$ objective A10PL, Olympus photo-ocular NFK $2.5\times$ LD). The frame grabber was installed on a compatible personal computer, digitized the information to images with 768×576 pixel resolution and 24-bit intensities and which displayed them on a video screen. The image analysis software was provided by Yalien company (China).

Cell seeding and thiazolyl tetrazolium bromide (MTT) assay

Mouse 3T3 fibroblasts were cultured with Dulbecco's Modified Eagles Medium (Gibco) buffered with *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) and supplemented with 10% fetal bovine serum (Gibco) and 100 U ml^{-1} of penicillin and streptomycin according to standard culture procedures.⁶

The hydrolyzed sample groups (five samples/each group) and control were immersed in 75% ethanol for 2 h, and then ethanol was exchanged with an excess of phosphate buffered saline (PBS). The PBS in disks was removed by pipette. The disks were then

sterilized by ultraviolet light for 2 h. Cell suspension with a cell density of 4.32×10^6 cells ml^{-1} was placed on the scaffold with a micropipette and allowed to adhere for 3 h before adding the culture medium. The cell-cultured scaffolds were observed by SEM via sequential dehydration in 50, 70, 95, 100% ethanol and freeze-dried after cultured for two weeks.

The viability and proliferation of fibroblast cells was determined by MTT assay. The porous scaffolds were cut into small disks (7 mm in diameter) with the aid of a cork borer in order to locate the disks into the 96-well tissue culture plate. Cell suspension (40 μl) with a cell density of 5×10^5 cells ml^{-1} was seeded evenly into the scaffolds with a micropipette. The cell-seeded scaffolds were maintained at 37 °C under 5% CO_2 for 3 h and then 150 μl of culture medium was added to the wells. The disks were rinsed by PBS three times after being cultured for every 24 h. Then 200 μl of culture medium was added to each well. Five millilitres of MTT solution (5 mg ml^{-1}) was added to the culture well incubated at 37 °C and 5% CO_2 for 4 h. The upper medium was removed carefully and the intracellular formazan was solubilized by adding 200 μl of 0.04 mol l^{-1} HCl/isopropanol to each well. The absorbance of formazan produced was measured at 570 nm with a microplate reader. The statistical significance between two sets of data was calculated using Student's *t*-test.⁵ Data were taken to be significant, when a *P*-value of 0.05 or less was obtained (showing a 95% confidence limit).

RESULTS AND DISCUSSION

The purpose of this study was to modify the PLLA outer surface without altering the bulk properties for improving its cell affinity. Since PLLA is a hydrophobic material, water molecules cannot easily diffuse in the bulk of the polymer. Alkali treatment is often used to modify the surface property of polyester materials. However, severe degradation or dissolution could influence other properties, such as mechanical properties, of the polymer when strong alkali is used. Therefore, mild saponification could be adopted, which would not result in visible degradation or dissolution. Cleavage of ester bonds take place because of the hydroxide nucleophilic attack, but the hydrophobicity of PLLA would restrict. Acetonitrile was never used as co-treating medium in our experiments this side reaction, as it could cause rapid polymer gelation at the surface, just like treating the PLLA surface with 2,2,2-trifluoroethanol/water as a solvent/non-solvent mixture,²¹ and then it would enhance the surface alkali hydrolysis. However, acetonitrile is not easily removed completely after treatment and its toxicity might harm the cells cultured on the sample surface. Pollution to environment is also inevitable. Hence, it was preferable to wet the PLLA surface by a non-toxic medium. It is well known that ethanol is a good wetting medium for polyester.²² Ethanol is also non-toxic and it is easy to be removed

completely by rinsing. Therefore, our strategy was to choose ethanol as a co-treating medium to assist the hydroxide nucleophilic attack on the ester bond.

PLLA films were used for evaluating the modifying effects. The dependence of weight loss on different conditions is shown in Fig 1. When the PLLA sample was placed in 0.5 M NaOH solution at 20 °C, the weight loss was nearly 30% after 24 h. However, when the concentration of NaOH was lowered to 0.25 M, there were no apparent changes in weight loss after 24 h of treatment. In addition, the treating temperature greatly influences the weight loss which can be inferred from curves A and D in Fig 1. Therefore, the weight loss could be well controlled by adjusting the concentration of treating medium, treating time and treating temperature. The treating medium of 0.25 M NaOH/ethanol does not result in severe weight loss at room temperature.

It is well known that the ester bond can be broken by chemical hydrolysis, which results in the generation of hydrophilic polar hydroxyl and carboxylic acid terminal groups. Consequently, the water contact angles and surface energy of PLLA before and after surface treating will differ. Figure 2 show the changes of water contact angles with treating time. The contact angles were changed from 78° to 68° after treating for 6 h using 0.25 M NaOH. Thereafter there was no apparent changes until 48 h. However, when ethanol was applied, an obvious difference occurred. The contact angles were lowered to 39° after treating for 6 h. Thereafter the contact angles to increased to some extent. Eventually the contact angles decline swiftly again. Therefore, the improved treating medium of NaOH/ethanol mixture was applied to modify the surface properties in the current study. The surface energy analysis in Table 1 also shows that, after treating by the mixture, the surface energy of the samples were all increased compared with the control. Treating for 4 to 6 h could improve the surface

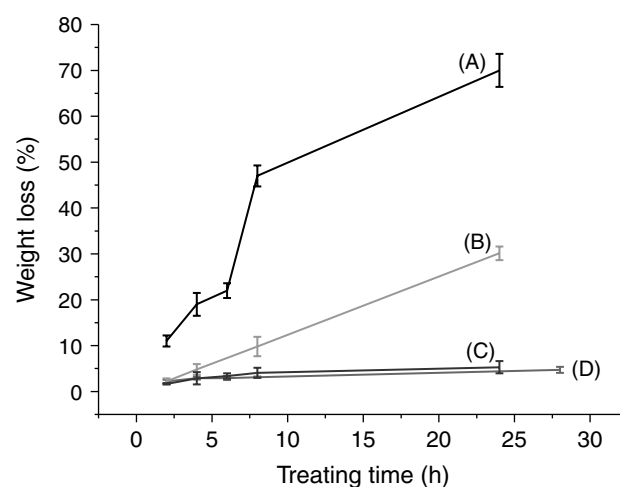


Figure 1. Dependence of the weight loss of PLLA films on the treating time in different treating media. (A) 0.25 M NaOH/ethanol ($v/v = 1/1$), 37 °C; (B) 0.5 M NaOH solution, 20 °C; (C) 0.25 M NaOH solution, 20 °C; (D) 0.25 M NaOH/ethanol ($v/v = 1/1$), 20 °C.

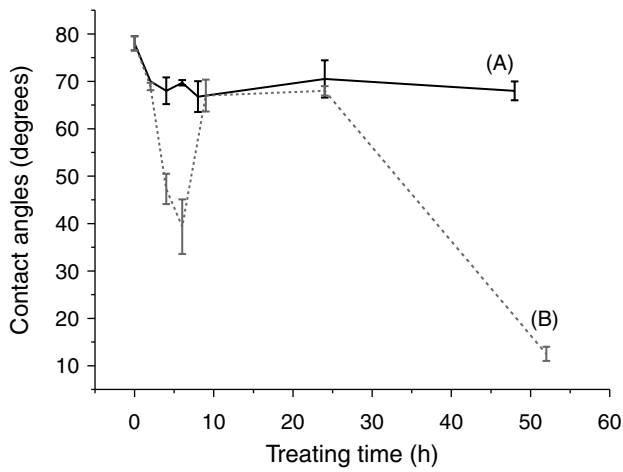


Figure 2. Changes of water contact angle with treating time. (A) 0.25 M NaOH solution, 20 °C; (B) 0.25 M NaOH/ethanol (v/v = 1/1), 20 °C.

Table 1. Changes of water contact angles and surface energy with treating time of PLLA in NaOH/CH₃CH₂OH solution (v/v = 1:1) at 20 °C

Treating time (h)	γ_s (mJ m ⁻²)	γ_s^d (mJ m ⁻²)	γ_s^p (mJ m ⁻²)	X^p (mJ m ⁻²)
0	42.2	32.5	10.7	0.25
2	46.7	29.3	17.5	0.37
4	56.3	30.2	26.1	0.46
6	63.2	32.4	30.8	0.49
8	49.9	33.3	16.6	0.33
12	51.4	34.5	16.9	0.33
25	56.6	33.5	23.2	0.40
52	73.1	30.5	42.6	0.58

γ_s : surface energy, γ_s^d : dispersive component, γ_s^p : polar component, $X^p = \gamma_s^p / \gamma_s$.

energy efficiently. Although the surface energy could be increased to 73.1 mJ m⁻² after treating 52 h, the weight loss (15.4%) could not be neglected. Thereby,

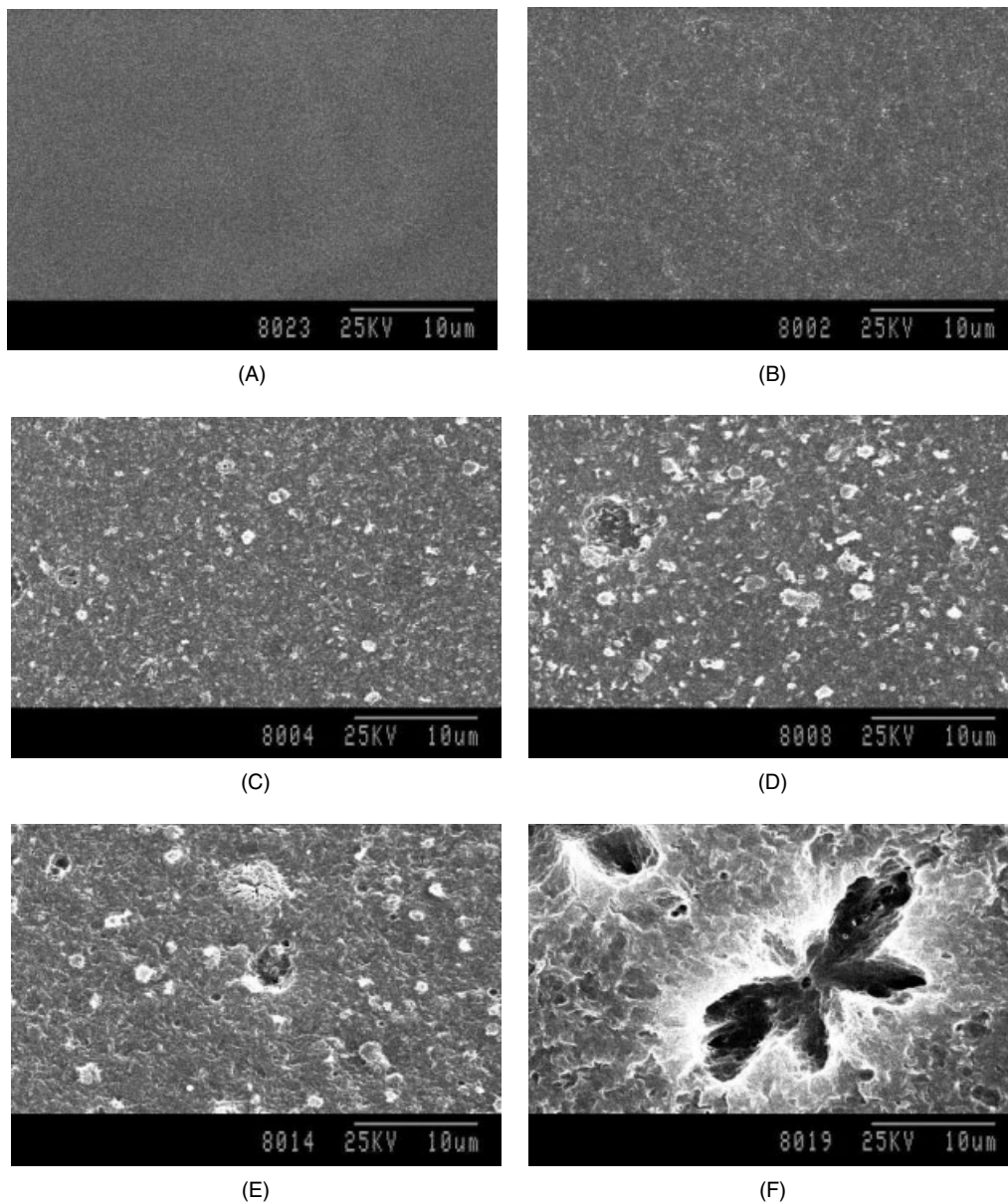


Figure 3. SEM observations of the PLLA surface morphology after different treating times. (A) Control; (B) 2 h; (C) 4 h; (D) 6 h; (E) 24 h; (F) 52 h.

the optimum treating time for improving the surface hydrophilicity and surface energy was 4–6 h. The fractions of polar components in surface energy (X^P) were also increased compared with the control. It may prove that the surface was enriched polar groups.

In addition, the improvement of surface hydrophilicity and surface energy may be attributed in part to the increase of the surface roughness. The surface morphology of the PLLA films was characterized by SEM (Fig 3). After treatment, the surface of PLLA film samples tended to be rough. After treating 52 h, the surface of PLLA was etched severely and the weight loss could not be neglected anymore. Besides, an obvious spherulite area could be seen in the picture after 52 h treatment. It could also be observed by computer-assistant image analysis.

The crystallizing behaviour of PLLA had already been studied by some investigators.²³ In commonly, the spherulite structure appeared the delustering image of Maltese crossing or ring shape when observed by optical microscopy. The pictures in Fig 4 were obtained by CAIAS. PLLA is a partially crystalline polymer. Generally, the small crystal area would be dispersed in an amorphous area even if the PLLA films were treated 6 h by the mixture (Fig 4A). With the increase of the treating time, the amorphous area and faulty crystals could be etched first by alkali treatment. Then, the complete crystals appeared clearly. In the pictures after 24 h and 52 h treatment, the complete

spherulite structure could be observed. It could be inferred that the surface roughness varied with etching of the amorphous area and faulty crystals and exposure of complete crystals. This result could explain why water contact angles would decline, then increase to some extent with treating time. In the first stage, the enriched hydrophilic polar hydroxyl and carboxylic acid terminal groups, together with the increase of surface roughness, contribute to the improvement of water contact angles and surface energy. However, with longer treating time, the PLLA surface tended to be come hydrophobic to some degree because of exposure of complete crystals, thus giving rise to a decrease of surface roughness. In the last stage, the surface of the samples was etched severely and a large number of complete crystallized macromoleculular chains were broken, and the sample surface would tend to become hydrophilic again.

Gel permeation chromatography measurement (Table 2) indicates that there were no apparent changes after 52 h of treatment. The data for M_n , M_w and polydispersity obtained by GPC showed also no apparent changes. Therefore, it could be inferred that only the surface layer had been modified while the bulk properties were not altered. Thus, treating 4–6 h in 0.25 M NaOH/ethanol are optimal conditions for improving the surface hydrophilicity, surface energy and surface roughness of PLLA without altering its bulk properties.

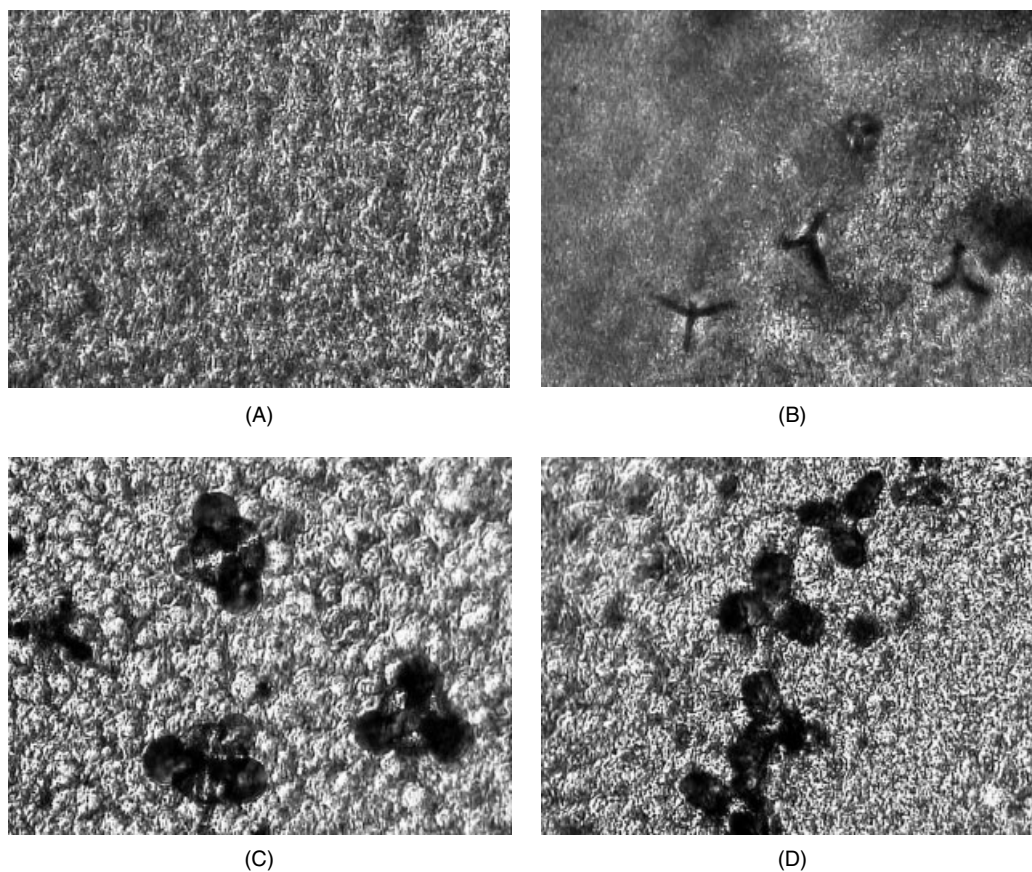


Figure 4. Observation of surface spherulite structure by CAIAS on the surface of modified PLLA films at different treating times. ($\times 1000$): (A) 6 h; (B) 12 h; (C) 24 h; (D) 52 h.

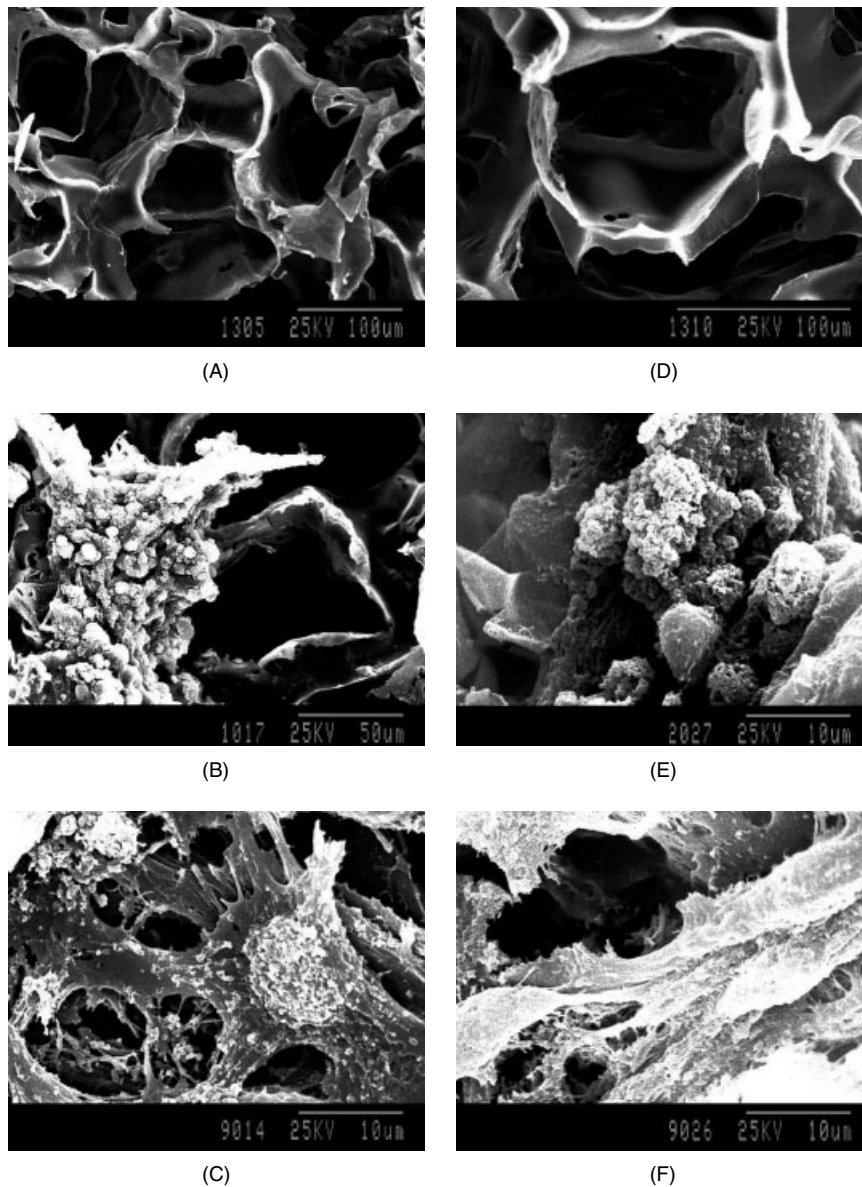


Figure 5. Cell morphology observation of mouse 3T3 fibroblasts on the PLLA scaffold (two weeks): (A) control surface; (B) cells on control surface; (C) cells on modified sample surface; (D) control cross-section; (E) cells on control cross-section; (F) cells on modified sample cross-section.

Table 2. GPC measurements of the PLLA films after different treating time

Treating time (h)	M_n	M_w	Polydispersity
0	67 366	96 779	1.44
2	70 230	1 05 369	1.50
4	68 609	1 03 680	1.51
8	72 489	1 08 487	1.49
12	67 637	98 606	1.46
24	75 947	1 10 846	1.46
52	74 699	1 09 523	1.47

Poly(L-lactide) scaffolds were modified according to the above study, and applied for fibroblasts culture. After two weeks of cell culture, the SEM pictures (Fig 5) confirmed the presence of cells in confluent (multi-) layer on the surface of the scaffold. These cells had spread extensively and exhibited

filopodia (Fig 5C). The cross-section of the modified scaffold (Fig 5F) was filled with ECM produced by the fibroblasts. Cell metabolism was extremely vigorous. It means that the cells could migrate into the pores and proliferate promptly. However, the cells on control scaffold (Fig 5B, E) aggregated and the cells morphology was not good. MTT assays showed (Fig 6) that the higher absorbance could be obtained when the scaffolds were modified. There was a statistical difference ($P < 0.05$) between the two sets of data determined every 2 days. The cells attachment was mediated by adhesive glycoproteins, such as fibronectin and vitronectin, which competed with other proteins for adsorption on to polymer surface. The composition of the protein layer was influenced by the properties of the polymer surface.²⁴ Cells were very obviously sensitive to chemistry and to surface energy. In addition, the surface energy may play an important role in attracting particular proteins

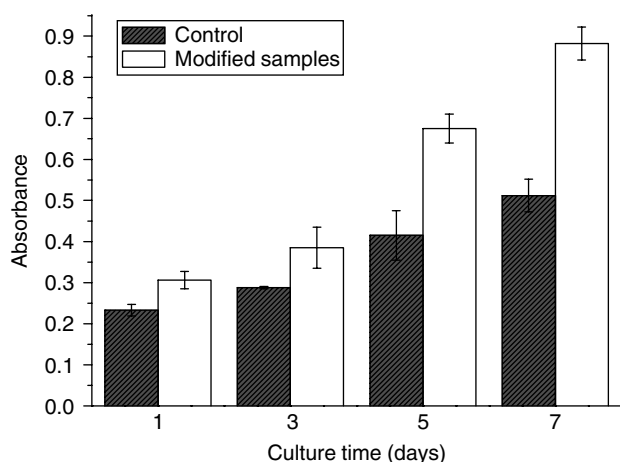


Figure 6. MTT-tetrazolium assay after mouse 3T3 fibroblasts cultured on PLLA scaffolds. Formazan absorbance expressed as a function of culture time. There is statistical difference between the two sets of data determined ($P < 0.05$).

to the surface of the materials and, in turn, this would affect the cell affinity to the materials.²⁵ Previously, studies using other types of polymer surfaces showed that the hydrophilicity of surface played a role.^{24,26} In our experiments, the surface hydrophilicity and surface energy have been improved significantly. The hydrophilic polar hydroxide and carboxyl groups originating from the cleavage of the surface ester bonds might be beneficial for cell attachment and cell growth. The surface roughness was also increased¹² and also does help the cell attachment and growth. This indicates that the cell affinity of PLLA scaffolds was enhanced by using the mixture of 0.25 M NaOH and ethanol (1:1, v/v) as the surface treating medium.

CONCLUSIONS

Alkali treatment is a convenient method to modify the surface of polylactone. The introduction of ethanol helps in the application of this convenient method. Non-toxic and cheap ethanol acts as a co-treating medium to assist the hydroxide nucleophilic attack on the ester bonds. A low concentration of alkali solution could be applied, therefore avoiding severe bulk degradation, and the residual alkali was easy to remove after surface treatment. Under our optimal treating conditions, the surface hydrophilicity and surface energy were improved significantly and the surface roughness was also changed. Only the outer layer of PLLA was modified and the bulk was not altered. The surface spherulite structure of modified PLLA was observed by CAIAS at different treating times. Cell culture experiments showed that the modified PLLA scaffolds were beneficial for fibroblasts attachment and growth. This convenient method is also suitable to treat other polylactone scaffolds, such as poly(lactide-co-glycolide), and poly(lactide-co-caprolactone). It is also a potential surface treatment medium for three-dimensional scaffolds in tissue engineering.

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