

SURFACE OF GELATIN MODIFIED POLY(L-LACTIC ACID) FILM*

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Abstract In this paper, the surface structure of poly(L-lactic acid) (PLLA) film modified with gelatin was investigated. The PLLA film specimens were treated directly with aqueous alkali solution to provide their surfaces with carboxyl groups, so that these functional groups could become the reactive sites for gelatin immobilization. The functional groups of the PLLA films were identified by ATR-FTIR spectra and XPS spectra, the changes in surface morphology were observed by using environmental scanning electron microscopy (ESEM), and the hydrophilicity of modified PLLA films was examined by water contact angle measurement. Experimental results showed that the gelatin was immobilized with water-soluble carbodiimide (EDC) onto the PLLA film's surfaces, and the gelatin content on the polymer surface was related to carboxylic group formed in the controlled hydrolysis process. Rough surfaces caused by hydrolysis will predominantly favor the adhesion and growth of cell; and the hydrophilicity of these surfaces after the modification procedure is enhanced.

Keywords Gelatin, Poly(L-lactic acid), Surface modification

INTRODUCTION

Tissue engineering is an interdisciplinary field, which applies the principles and methods of engineering and life sciences to the maintenance, improvement, or restoration of tissue function in which a deficiency has arisen^[1]. One of the fundamental approaches to the engineering of new tissues needs a biomaterial scaffold to afford cell living space. Scaffold materials are required to possess several key features including: biocompatibility; surface properties that enable the adhesion, proliferation, and differentiation of cells; a controllable degradation rate in vivo; ability to be formed into 3-dimensional irregular structures, and sufficient mechanical properties to withstand stresses incurred during manufacture and usage^[2]. The surface properties of biomaterial have recently attracted much attention^[3].

As a result of their excellent biodegradability and tissue compatibility, poly(lactic acid) (PLA) and related poly(oxyacid)s have been used for scaffold fabrication in tissue engineering^[4]. But they lack the ability to interact biospecifically with cells. To overcome this problem surface engineering will provide benefits for cell adhesion. Shakesheff *et al.* entrapped poly(ethylene glycol) (PEG) and poly(L-lysine) (PLL) onto the PLA surface. The immobilized PLL surface offers functional amine groups with which biological ligands can be covalently coupled^[5]. Sittinger *et al.* dipped the PLA scaffolds in PLL or type II collagen to improve initial chondrocyte attachment^[6].

Gelatin is largely composed of denatured collagen, a cell extracellular matrix (ECM) protein particularly rich in proline and hydroxyproline. Gelatin's unique characteristics give it wide applicability in tissue matrixes^[7, 8].

Yamaoka *et al.*^[9] immobilized gelatin on a PLA surface via a direct alkaline treatment process. Entrapped

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gelatin molecules that have various functional groups can also be utilized on graft molecules through bioactive molecules, *e.g.* cell adherent peptide, growth factor and differentiation-inducing moiety can be immobilized. Grainger *et al.* found that fibroblasts in the proteinic culture environment exhibit attachment growth and spreading substantially better on a carboxyl terminated hydrophilic self-assembled monolayer (SAM) modified surface than on a methyl-terminated hydrophobic surface^[10].

Ratner and coworkers used engineering surfaces for biorecognition through imprinting biomaterial surfaces with protein-recognition sites by using fibronectin as a template. So one could prepare a surface that would have a natural affinity for the protein, which was particularly important for healing^[14].

A study on surface modification of PLLA film with gelatin reported out in this paper. The work focused on controlling the surface concentration and distribution pattern of gelatin on PLLA films, which were characterized by using XPS spectra, environmental scanning electron microscopy (ESEM), infrared spectra (IR), and water contact angle measurements. These surface characteristics can affect cell attachment and growth behavior.

EXPERIMENTAL

Materials and Methods

Materials

Gelatin ($M_w = 1.5 \times 10^5$, $M_n = 1.0 \times 10^5$) was obtained from Sigma Chemical Co. and PLLA ($M_n = 1.08 \times 10^4$) was kindly provided by the Department of Chemical Engineering, the University of Massachusetts USA. Other reagents were of chemically pure grade.

Preparation of PLLA film and immobilization of gelatin

Films were prepared by solvent casting with a 25% solution of PLLA in dichloromethane and dried in vacuum. The thickness of the film was *ca.* 100 μm .

The immobilization procedures described here are according to Ref. [9]. Briefly, the PLLA film was kept in 1 mol/L NaOH solution at 40°C for 60 min, then rinsed successively with 0.1 N HCl and distilled water at room temperature.

The activated PLLA-COOH specimens were treated with a 0.1% (*W/V*) solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, Sigma) in 2-(*N*-morpholino)-ethane-sulfonic acid (MES) buffer at 4°C for 24 h. The samples were rinsed with MES buffer for 10 min, and then with distilled water twice for 10 min. The films were immersed in a 1% gelatin solution for 48 h at 37°C. After the immobilization reaction, the polymer films were washed with phosphate-buffered saline (PBS) for a predetermined time, washed with distilled water several times at 37°C, and then kept dry.

Characterization

ATR-FTIR spectra of the surface-modified films were recorded using a FTS6000 FTIR spectrometer (Bio-Rad. Co.), IR spectra were collected at a resolution of 8 cm^{-1} and 128 scans over a range of 400–4000 cm^{-1} .

XPS spectra of the blend films were determined by using the PHI-5600 ESCA system. The residual pressure in the spectrometer was 1.1×10^{-8} Pa. An Mg anode (1253.6 eV) powered at 200 W, was used as X-ray source. The constant pass energy was 23 eV, and analyzed area was 0.8 mm^2 . Surface elemental compositions relating to carbon were calculated from peak heights with a correction for atomic sensitivity.

As an indication of surface energy of the modified polymer surface, water-contact angles were measured at 25°C using the sessile drop method and an image analysis system for the drop profile. The water droplet volume was about 0.5 μL to prevent gravitational distortion of the spherical profile. Each determination was obtained by averaging the results of at least four measurements.

Preparation of Cell Culture

Chondrocytes were isolated from transparent knuckle cartilage taken from a 4-week-old rabbit by aseptic operation, washed with PBS 3 times, and then chopped into 1 cm^3 nubs. Cells were digested in 25% trypsin (Sigma) at 37°C for 30 min to remove pancreatin. After rinsing with PBS three times, cells were suspended in

Modified Eagle Medium (DMEM; Gibico) containing 2 mg/mL clostridiopeptidase II at 37°C under 5% CO₂ conditions overnight. Then the cells were waved and came to loose, and filtered. The cell soliquoid was centrifuged at 1000 r/min, then washed with 15% fetal calf serum (FCS, Hyclone) DMEM twice. The cells were diluted at 2.0×10^5 cell/mL with 15% FCS DMEM, and planted in a 25 cm² cell culture bottle (Nunc) at 7.0×10^5 cell/flask and cultivated at 37°C, under 5% CO₂, and 95% relative humidity. The medium was changed every 3 days.

MTT Assay

The second passage chondrocytes were seeded onto the modified PLLA films and the control ones with 5.0×10^4 cell/well in 24-well plate. After chondrocytes had been cultured for 7 days, to every well was added 50 μL MTT (5 mg/mL, Sigma) with PBS, and left for 4 h at 37°C. In order to dissolve the blue formazan reaction product, 200 μL DMSO was added to every well. The solutions were transferred to a 96 well plate. The absorbance was measured at 570 nm using a Tecan Spectra III microplate reader.

RESULTS AND DISCUSSION

PLLA surface is difficult to be modified because of the lack of functional groups. Therefore, the PLLA film specimens were treated directly with aqueous alkali solution to induce partially hydrolytic degradation. The degradation could provide the surface of PLLA film with carboxyl groups to be coupled with gelatin. Samples 1[#]–5[#] of modified poly (L-lactic acid) film with gelatin were prepared according to different erosion times (cf. Table 1).

Table 1. Sample number of different erosion time					
Sample number	1 [#]	2 [#]	3 [#]	4 [#]	5 [#]
Erosion time (min)	10	20	30	60	120

Surface Characteristics of Gelatin-Modified PLLA Film

From the ATR-FTIR spectra (Fig. 1), it was found that in the spectra of modified PLLA film 4[#] and 5[#], as compared with that of the PLLA control, there were an amino band at 1540 cm⁻¹ and a carbonyl peak at 1651 cm⁻¹ which characterize the presence of gelatin on the modified films.

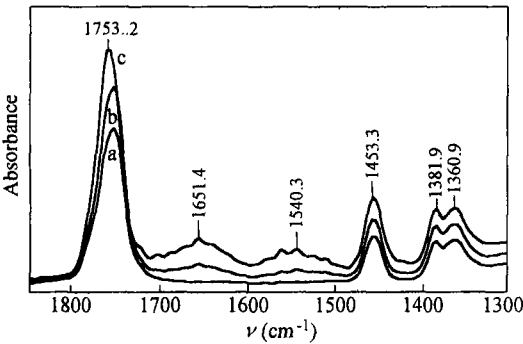


Fig. 1 ATR-FTIR spectra of (a) PLLA film, (b) gelatin-modified PLLA film 4[#], (c) gelatin-modified PLLA film 5[#]

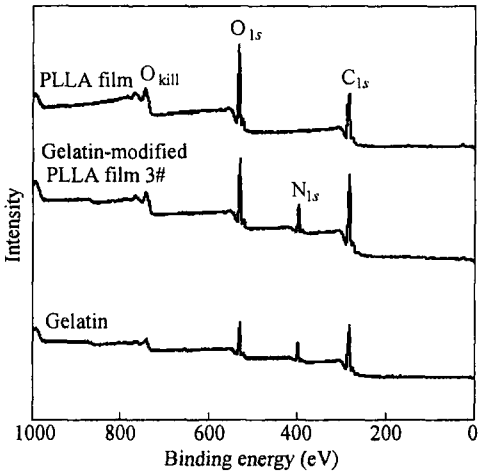


Fig. 2 Wide-scan XPS spectra of PLLA film, gelatin and gelatin-modified PLLA film

The XPS spectra of PLLA film, gelatin and gelatin-modified PLLA film 3[#] are shown in Fig. 2. The modified PLLA film was evidenced by the high percentage nitrogen content, stemming from gelatin. Figure 3 shows the high-resolution XPS C1s spectra of PLLA film, gelatin and gelatin-modified PLLA film. Three carbon

peaks can be observed in PLLA film, while four carbon peaks can be observed in gelatin-modified PLLA film because of the amide carbon ($-\text{O}=\text{C}-\text{NH}_2$) peak (288.27 eV) in gelatin.

These results from ATR-FTIR and XPS analysis confirmed that gelatin was immobilized by EDC onto the PLLA film's surfaces.

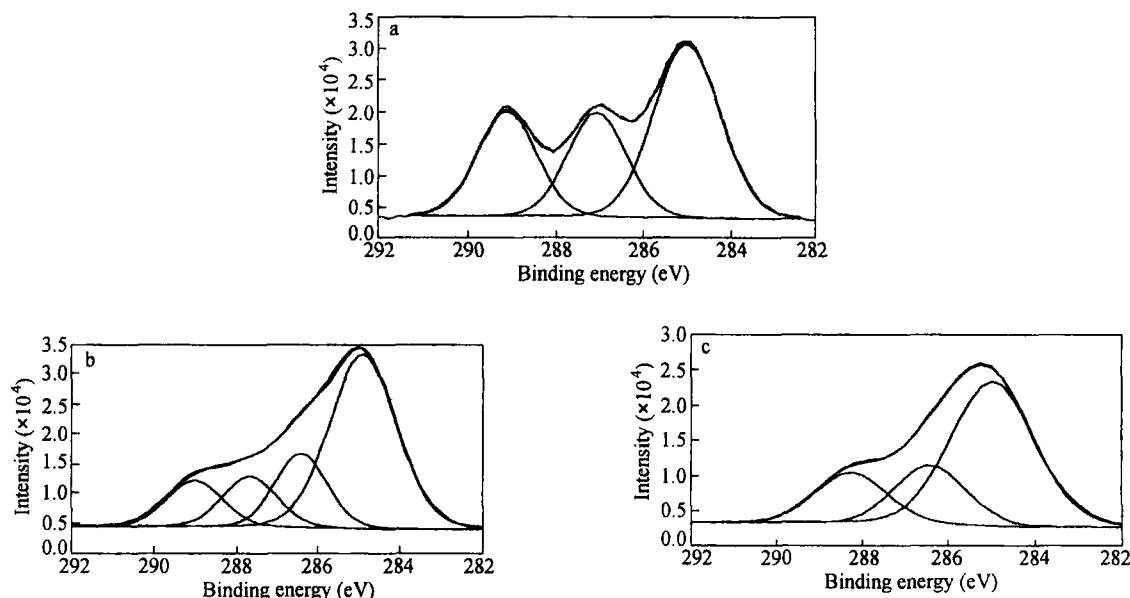


Fig. 3 High-resolution XPS C1s spectra of (a) PLLA film, (b) gelatin-modified PLLA film and (c) gelatin

The elemental composition of PLLA film, gelatin and gelatin-modified PLLA films 1[#]–5[#] are compared in Table 2. The atomic nitrogen content derived from the gelatin content on the PLLA surface relates nonlinearly to erosion time. The reason is that the gelatin content on polymer surfaces corresponds to the carboxylic group formed during the hydrolysis. On extending the hydrolysis time, disruption of PLLA chain occurs on the surface, and its fragments are dissolved in alkaline solution, so carboxylic group decreases gradually. In our case, erosion of PLLA film is stepwise in aqueous alkaline solution. The immobilized content of gelatin on PLLA surface can be obtained by controlling the aqueous alkaline solution concentration and reaction time.

Table 2. XPS Analysis of PLLA film and gelatin-modified PLLA film

Sample	Atomic percent (%)		
	C _{1s}	O _{1s}	N _{1s}
PLLA film	64.8	34.3	-
Gelatin-modified PLLA film 1 [#]	63.1	35.8	0.6
Gelatin-modified PLLA film 2 [#]	66.1	29.7	4.2
Gelatin-modified PLLA film 3 [#]	66.5	25.8	7.8
Gelatin-modified PLLA film 4 [#]	65.0	28.8	6.2
Gelatin-modified PLLA film 5 [#]	66.9	27.1	6.0
Gelatin	65.8	19.8	8.7

Morphology of Modified PLLA Film with Gelatin

The topological structure of a biomaterial surface can influence its cytocompatibility. To compare SEM micrographs of PLLA film with that of modified-PLLA film (Fig. 4), the surface of PLLA film becomes rougher after surface modification. Figure 4(a) shows that the surface of original PLA film is smooth and even. After it was modified by gelatin, some small cavities and cracks appeared on the surface of sample 1[#]. With increasing erosion time, round cavities became more apparent, and began to link together. Gradually, the links between cavities became thinner, and disrupted. That is to say, round cavities are interpenetrable. The membranes among

cavities turned into reticular form.

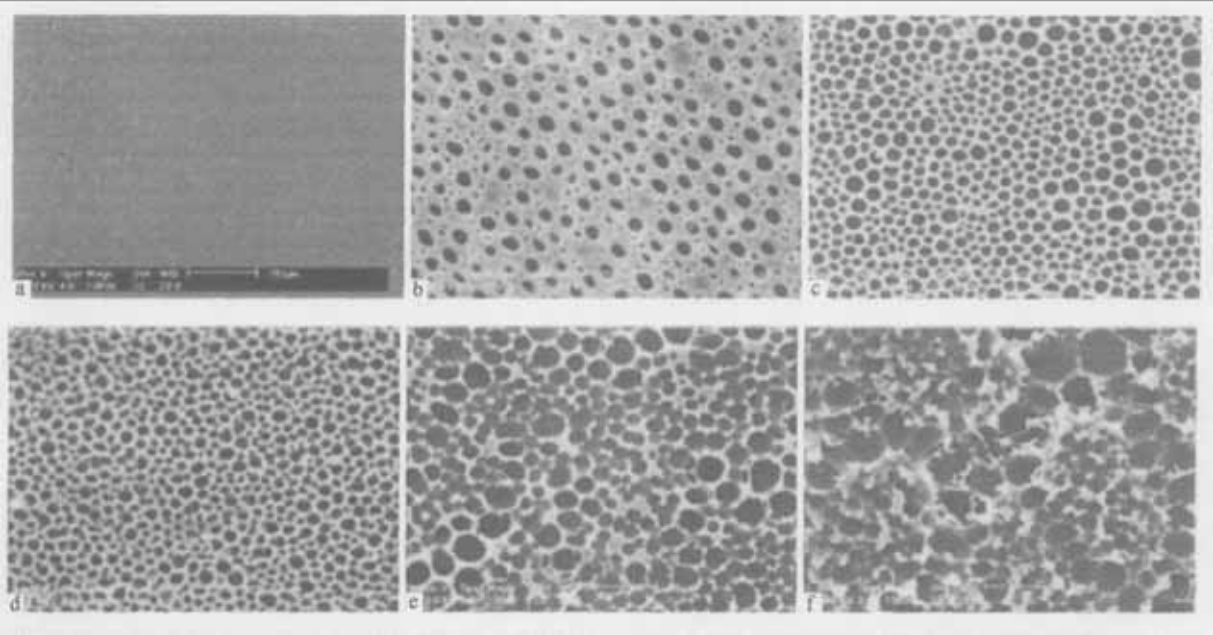


Fig. 4 SEM micrographs (1000×) of PLLA films (a) pristine PLLA film (b–f) gelatin-modified PLLA film1[#]–5[#]
The scale bars are 20 μm.

The Hydrophilicity of Modified PLLA Film

Measurement of contact angles on the modified surfaces gave an indication of the relative hydrophilicity of these surfaces after the modification procedure. The water contact angles of modified PLLA film and control film are listed in Table 3. It was found that the contact angel of modified PLLA film was significantly lower (more hydrophilic) than that of control. This might be related to the water-soluble nature of gelatin that presented onto the modified surfaces and rough surface of the modified film.

Table 3. water contact angle on PLLA film and gelatin-modified PLLA film

Sample	Contact angle
PLLA film	75 ± 3
Gelatin-modified PLLA film 1 [#]	61 ± 2
Gelatin-modified PLLA film 2 [#]	54 ± 6
Gelatin-modified PLLA film 3 [#]	49 ± 4
Gelatin-modified PLLA film 4 [#]	52 ± 3
Gelatin-modified PLLA film 5 [#]	50 ± 5

* Average ±standard deviation (n = 4)

Cell Viability

Sample 5[#] was used in MTT assay to examine the relative cell viability of the modified films. In MTT assay, the chondrocytes were planted normally in polygonal or circular fashion on the material wall.

MTT reagent is a pale yellow substrate that produces a blue formazan derivative when incubated with viable cells. Therefore, the level of production of formazan can reflect the level of cell viability. As shown in Fig. 5, the result demonstrates that the cell viability of rabbit transparent chondrocytes cultured on gelatin modified PLLA film (1.0982 ± 0.1420) is significantly greater than that of a control (0.6335 ± 0.0738), but also a little higher than that found on poly-lysine (PLL) finished PLLA film (1.0735 ± 0.2966). Here gelatin may spatially mediate cellular response that can promote cell attachment and growth, indicating gelatin surface modification should be a useful surface engineering method for PLLA in tissue engineering^[6].

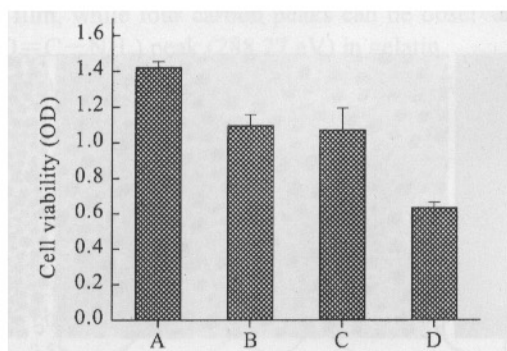


Fig. 5 MTT assay, formazan absorbance expressed as a measure of cell viability from rabbit transparent gristle cells cultured on A. The cell culture board (TCPS) B. The PLLA film processed 2 h by NaOH, then modified with gelatin C. The PLLA film finishing by PLL D. The PLLA film not modified (compared with plank film). Initial seeding density was 2.5×10^3 cells/cm². Error bars represent means $\pm$ SD ($n = 6$), * $P < 0.05$; ** $P < 0.01$ (compared to control)

The biocompatibility of biomaterial has a certain relation with the surface chemical conformation of the material. Besides that it has a close relation with the surface appearance of material. This subcapsular organism is formed by parallel fibroblastic, and it is easy to lead inflammation and tumour. Material with rough surface impels cell and organism to tightly combine with material. A rough surface on cell and organism not only increases the contact area but also increases the preferential adherence of osteogenetic organitissue and epithelia. In addition, a rough surface conformation has a contact guidance effect on cell growth. It has been reported that the material with rough surface will prominently hasten the adhesion and growth of cells on material surfaces and decrease subcapsular organism thickness, while rougher and smoother surfaces do not exert this effect^[12, 13]. By controlling the aqueous alkaline solution concentration and reaction time, in our case, one can make materials favoring cell adhesion by changing the topological structure on modified- PLLA film surface.

CONCLUSION

Gelatin was used to modify PLLA film. The PLLA film specimens were treated directly with aqueous alkali solution to induce partially hydrolytic degradation. The degradation could provide surface of PLLA film with carboxyl groups so that these functional groups could provide sites for gelatin immobilization via EDC. The gelatin content on polymer surface was related to carboxylic group formation in the hydrolysis process. Rough surfaces caused by hydrolysis will prominently favor the adhesion and growth of cells; hydrophilicity of these surfaces was enhanced after the modification procedure. The modified surfaces of PLLA film adsorb proteins similarly to the interface of biological organization and are beneficial to cell attachment and growth. Biomimetic surface engineering using gelatin to modify PLLA proves very useful in tissue engineering.

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