Plasma-treated, collagen-anchored polylactone: Its cell affinity evaluation under shear or shear-free conditions

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Abstract: Poly(L-lactic acid)(PLLA) and poly(L-lactic-*co*-glycolic acid) (PLGA) (85/15) were modified by plasma treatment. Then they were collagen anchored (PT/CA), and the cell affinity was evaluated by cell culture under shear or shear-free conditions. A convenient and "intuitionistic" dyeing method has been proposed for measuring the modified depth when plasma treatment is applied for the treatment of porous scaffolds. A parallel plate flow chamber was developed in order to study the cell affinity of a material under shear stress. Our results show that a porous scaffold can be modified by plasma treatment and that a depth of about 4.0 mm for this modification can be reached with NH_3 plasma treatment (50 w, 20 Pa, 5 min). PT/CA modification is an effective surface modification method for facilitating cell transplantation and improving the cell affinity of three-dimensional porous cell scaffolds in tissue engineering. It can solve the problem of non-uniform cell distribution in most synthetic porous cell scaffolds. Using the flow chamber system, a series of quantitative data, including cell adherent fraction, cell area, and mean shape, were compared to evaluate the cell affinity of PLLA before and after PT/CA modification. The results indicate that the quality of cell attachment on PT/CA-modified PLLA apparently is better than that on unmodified PLLA. The flow chamber system potentially may be a tool for evaluating surface modification methods. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 67A: 1139–1147, 2003

Key words: polylactone; surface modification; flow chamber; cell affinity; tissue engineering

INTRODUCTION

Tissue engineering using selective cell transplantation now is being explored as alternative therapeutic strategies for restoring tissue and for organ failure.[1–6] A series of biodegradable polylactone materials, such as poly(L-lactide) (PLLA), polyglycolide (PGA), poly(L-lactide-*co*-glycolide) (PLGA), and polycaprolactone (PCL), have been approved by the Food and Drug Administration (FDA) for clinical application and have been studied extensively in tissue engineering research.[7,8] However, cellular function often is compromised since no natural recognition sites are available on the synthetic matrix surface when polylactone is used as the cell scaffold material.[9] None of these polylactones provides a chemically reactive pendent chain for the attachment of drugs, crosslinkers, or biologically active moieties.[10] Therefore it is not easy to modify a surface property by common chemical methods to promote the cell affinity of polylactone.

Plasma treatment is a convenient method for modifying surface properties of a material. It also easily can be used to introduce the desired groups or chains onto the surface of a material.[11–15] Thus it is a valuable method for designing the surface of a material. However, the disadvantage of using plasma treatment is that the modifying effects decline with time.[16]

In our previous work, an effective method of preserving sealed materials at a low temperature after plasma treatment was proposed to maintain the modifying effects,[17] but such a method is an added inconvenience when a plasma treatment is applied. In addition, the issue about the treatment depth still is unknown when the plasma technique is applied for treating three-dimensional porous scaffolds. This is very important but seldom discussed problem in the literature.

In recent years, many studies have focused on coating or grafting cell-recognized molecules, such as RGD (Arg-Gly-Asp) peptide,[18] collagen,[19] or L-lysine,[20] onto a material surface for improving its cell affinity. We herein report a unique method of combining plasma treatment with collagen anchorage (PT/CA) for improving the cell affinity of poly(D,L-lactide)

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film. PT/CA can overcome the disadvantages of using only plasma treatment or collagen coating.[21]

In general, cell adhesion can be studied in a static system. In order to investigate the effects of shear stress on cell adhesion, a device that can generate shear stress is necessary. Spinning discs,[22] cone-plate devices,[23] stagnation-point flow chambers,[24] capillary tubes,[25] micropipette suctioning, [26] and parallel plate flow chambers[27,28] all can generate shear stress. In this study we used a parallel plate flow chamber, which offers the possibility of monitoring in "real time" individual cells *in situ* during their exposure to shear.

In the present paper, the macroporous PLGA (85/15) scaffold was modified by PT/CA and then evaluated in cell culture to investigate whether or not PT/CA modification is suitable for modifying three-dimensional cell scaffolds in tissue engineering. In addition, a parallel plate flow chamber[29] was developed, based on our previous work,[28] and this flow system was utilized for a quantitative cell affinity evaluation of poly(L-lactic acid) (PLLA) films modified by PT/CA under shear stress.

MATERIALS AND METHODS

Materials

L-lactide (LA) and glycolide (GA) were synthesized from L-lactic acid (Yierbao lactic acid factory, Shanghai) and glycolic acid (Xizhong chemical factory, Beijing). PLLA (M_w = 130,000, M_w/M_n = 1.57) was measured by gel permeation chromatography (Waters 510 with Shodex KF-800 columns, polystyrene standard), and PLGA (85/15) (mole ratio: LA/GA = 85/15, M_w = 126267, M_w/M_n = 1.96) was synthesized under high vacuum in the presence of stannous octoate as a catalyst (0.05 wt %) at 140°C for 12 h and 180°C for 20 h, respectively.

The PLLA and PLGA were purified by dissolution of the polymer in chloroform and precipitation in ethanol, followed by drying in vacuum at room temperature for 48 h. PLGA (85/15) porous scaffolds were prepared by a solvent casting/particulate leaching technique using NaCl as the particulate porogen.[30,31] The technique used for characterization of the scaffold (pore size:194 ± 44 μm; porosity: 91.4%; thickness: 1.48 mm) can be seen in detail in our previous work.[32]

Surface modification

Anhydrous ammonia plasma treatment

The detailed experimental procedure of plasma treatment is described in an earlier publication.[17] In brief, the plasma treatment was carried out using Samco plasma deposition (Model PD-2, 13.56 MHz) with anhydrous ammonia gas. The polymeric scaffold or film was located on the lower electrode. 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 (20 Pa), a glow discharge plasma was created by controlling the electrical power (50 W) for 5 min. Finally, after turning off the power, the plasma-treated samples were exposed further to ammonia gas for another 10 min.

PT/CA modification[21]

The ammonia plasma pretreated sample was put into a 0.1-mg/mL collagen (Type I, sterilized, Sigma) solution immediately for at least 2 h, then the sample was rinsed in PBS thee times and sterilized by ultraviolet for 30 min. Finally, the PT/CA-modified sample was dried and preserved in sterilized culture dishes.

Depth measurement following plasma modification

PLLA scaffolds of different thicknesses (1.3, 2.0, 3.0, and 4.4 mm) were modified by ammonia plasma treatment under the conditions described above and then immediately put into blue ink (Beijing ink factory, Beijing) for 5 min. The dyed scaffolds then were taken out of the blue ink, and the ink in the scaffold pores was removed with filter paper. After having been dried under vacuum, the scaffolds were broken in half and frozen in liquid N_2. The results the dyeing process were recorded by taking pictures of the scaffold surfaces and cross sections. The ink-dyeing depth was measured using a micrometer.

Parallel plate flow chamber

The parallel plate flow chamber used is shown in Figure 1. The chamber consisted of a nickel-coated stainless steel bottom part and a top part that enclosed two glass plates measuring 7.6 × 5.0 × 0.2 cm (l × w × h) separated from each other by two spacers. The effective chamber dimension was 7.6 × 3.8 × 0.2 cm (l × w × h). The power resistor served as the heating unit for the flow chamber. The O-ring ensured that there was no liquid leakage. The temperature of the flow chamber was detected by a Pt thermocouple.

Flow system

The parallel plate flow chamber was fixed on the microscope stage via a fixation plate. The inlet and outlet of the chamber were connected with vessels that could accommodate serum-free culture medium and were located at an appropriate height. A peristaltic pump (LanGe-pump, Type YZ1515, Beijing Baoding Lange Peristaltic Pump Co. Ltd.)

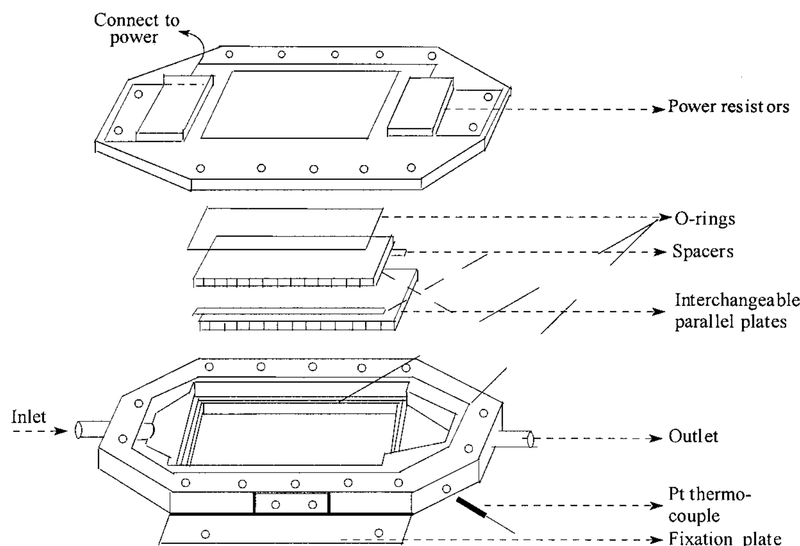

Figure 1. Sketch map of the parallel plate flow chamber.

connected with the two vessels ensured a steady flow in this loop. The vessel connected with the outlet was double-walled. The medium circulating in this flow system was kept at 37°C by heating the double-walled vessel though a thermostat water bath. The computer-assisted image analysis system (CAIAS), applied to record the changes in cell adhesion, included a CCD camera (type WV-CP460, Panasonic, Japan) that grabbed a field of 0.28 mm^2 though a light microscope (Olympus IMT-2, phase contrast, 10× objective A10PL, Olympus photo-ocular NFK 2.5× LD). The image analysis software was provided by Yalien Company (China).

Cell proliferation

Mouse 3T3 fibroblasts were grown in 50-mL cell culture flasks with Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), 0.05 mg/mL of ascorbic acid, 0.3 mg/mL of L-glutamine (Gibco), 3.7 mg/mL of $NaHCO_3$ (AR grade, Beijing chemical factory), and 100 Units/mL each of penicillin and streptomycin. Cell culture was maintained in a gas-jacket incubator equilibrated with 5% CO_2 at 37°C.

Static cell culture on porous scaffolds

The porous PLGA (85/15) scaffold was cut into small pieces (1.5 × 3.5 cm) and placed in culture dishes (6 cm). One group was set aside for PT/CA modification. The control group was prewetted in 75% ethanol for 2 h, and then the ethanol was exchanged with an excess amount of phosphate-buffered saline (PBS).[33] A cell suspension with a cell density of 4.32×10^6 cells/mL 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 10 mL of new culture medium were added into the culture dishes. After culturing for 2 or 4 weeks, the culture was stopped for SEM observation or H&E staining.

MTT assays

The porous PLGA (85/15) scaffolds were cut into small disks (7 mm in diameter) with the aid of a cork borer in order to place the disks into a 96-well tissue culture plate. One group was used for PT/CA modification. The control group was prewetted, as above. The cell suspension (40 μL), with a cell density of 5 $\times 10^5$ cells/disk, 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 160 μL of culture medium were added to the wells.

After cell culturing for 1, 3, or 5 days, the viability and proliferation of fibroblast cells were determinate by MTT assay. The disks were rinsed with PBS three times, and then 200 μL of culture medium were added to each well. Five μL of fresh MTT solution (5 mg/mL) were added to the culture wells and 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 of HCl/iso-propanol to each well.

The absorbance of the formazan produced was measured at 570 nm with a microplate reader (Tecan, Australia). Five disks were applied for PT/CA-modified samples and the control. The statistical significance between two sets of data was calculated using the Student's *t* test. Data were considered to be significant when a p value of 0.05 or less was obtained (showing a 95% confidence limit).

Dynamic cell culture on PLLA films

The PLLA film was prepared by casting 4% PLLA solution in $CHCl_3$ on the lower glass plate of the parallel plate flow

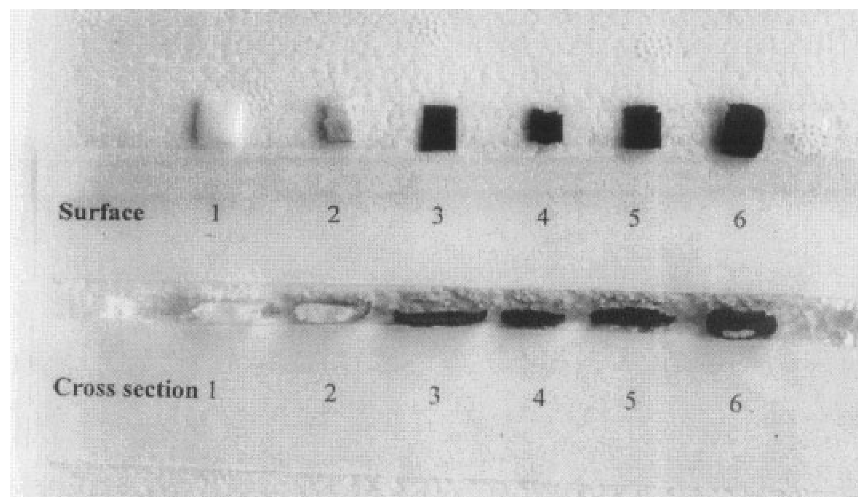

Figure 2. Result of ink dyeing of PLLA scaffold cross section (pore size, 194 ± 44 μm) of different thicknesses: (1) control, 3.0 mm; (2) ink-dyed control, 3.0 mm; (3–6) NH_3 plasma (50 w, 20 Pa, 5 min)-modified and ink-dyed scaffolds with thicknesses of 1.3, 2.0, 3.0, and 4.4 mm, respectively. Original magnification × 1.

chamber. After the solvent was completely removed, the glass plate with PLLA film was sterilized by ethanol (75%) immersion for 30 min and ultraviolet for 1 h. A mouse 3T3 fibroblast suspension with a density of $1.2{\sim}1.5 \times 10^5$ cell/mL was seeded onto the PLLA film and cultured in an incubator for 6 h in order to allow cell adhesion and spreading. Then the cell-seeded glass plate was assembled, together with the upper glass plate, in the flow chamber. All bubbles in the whole pipeline, including the flow chamber, were carefully removed. As the starting point, an image arbitrarily was grabbed in the center of the flow chamber. Then the flow was initiated by turning on the peristaltic pump to control a proper flow rate. The changes in cell adhesion were recorded by taking photos every 2 min. PLLA films on a glass plate modified by PT/CA also were applied for the above experiment.

Data processing

The shear stress was calculated from Equation (1).[34]

$$Tw = \mu \cdot (6Q/wh^2) = 27.63Q\ (\text{dyn/cm}^2) = 2.763Q\ (\text{N/m}^2)$$

with μ the viscosity of the flow fluid (0.007 g/cm/s, at 37°C), w the chamber width (3.8 cm), h the chamber height (0.02 cm), and Q the flow rate (mL/s).

The recorded images were analyzed by image analysis software. The border of the individual cell could be traced with a computer mouse, and then the produced cell image could be processed with the computer. Thee parameters are determined for each cell in each recorded image: Cell area (A), perimeter (P), and two-dimensional, projected shape factor (S). Shape factor is a function of the perimeter. When a cell is round, the projected cell shape also is round (S = 1). When a cell spreads, S deviates from 1.

$$S = P/2(\pi A)^{1/2}$$

The fraction of adherent cells with time under shear stress was obtained by calculating the cell numbers at different times using image analysis software.

RESULTS

Depth of plasma-modified PLLA porous scaffolds

The NH_3 plasma-modified and unmodified PLLA scaffold were dyed in ink, and the result is showed in Figure 2. For the unmodified scaffold, only a trace of the ink could be found on the surface of the scaffold; the cross section of the scaffold had not been dyed. However, for the plasma-modified scaffolds, the surfaces and the cross sections were all clearly dyed. Only a small part (about 0.4 mm of thickness) in the middle of the scaffold had not been dyed when the thickness of the scaffold reached to 4.4 mm. Therefore, it can be inferred that the plasma modifying depth should be about 4.0 mm for a three-dimensional PLLA scaffold under the present modifying condition.

Cell culture under shear-free conditions

The morphology of cells cultured on PLGA (85/15) scaffold under static cell culture was observed by SEM, as shown in Figure 3. It could be seen that for the unmodified scaffold [Fig. 3(B)], there were only a few cells in the scaffold pores and the morphology of the cells was not satisfactory. However, for the PT/CA-modified scaffold [Fig. 3(C)], a large number of cells could be found in the scaffold cross section. The cells adhered to the pore walls through filopodial exten-

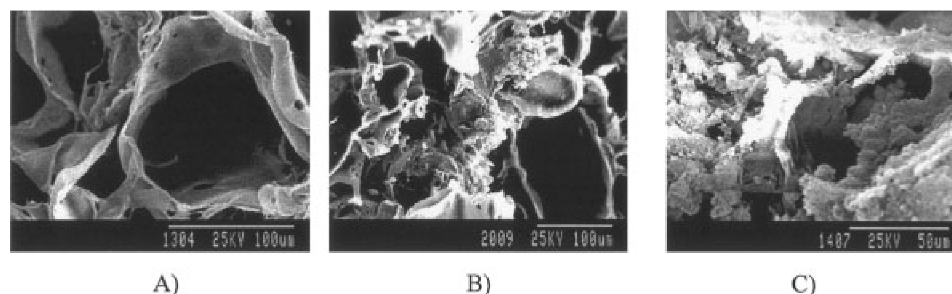

Figure 3. SEM images of the PLGA (85/15) scaffolds before and after the culturing of 3T3 fibroblasts for 2 weeks: (A) cross section of scaffold before cell culture (original magnification ×500); (B) cross section of control after cell culture (original magnification ×300); and (C) cross section of PT/CA-modified scaffold after cell culture (original magnification ×400).

sions, the metabolism of the cells was vigorous, and a great deal of extracellular matrix (ECM) had been secreted.

The results of H&E staining (Fig. 4) demonstrate that cells were distributed evenly throughout the whole PT/CA-modified scaffold [Fig. 4(C,D)] and had secreted a great deal of ECM. However, for the unmodified scaffold [Fig. 4(A,B)], most of the cells were distributed just on the scaffold surface and only a small number of cells were found in the scaffold cross section. MTT assays also showed that higher absorbance could be obtained when the scaffolds were modified by PT/CA (Fig. 5). The data were observed every day, and each day a statistical difference ($p < 0.05$) between the two sets of data was determined.

Cell culture under shear conditions

The percentages of fibroblasts adhering to PLLA, glass, and PT/CA-modified PLLA films at $29.5N/m^2$ of shear stress were compared and are shown in Figure 6. It is clear that the cells were almost completely removed from unmodified PLLA within 2 min. However, for PT/CA-modified PLLA, an apparent differ-

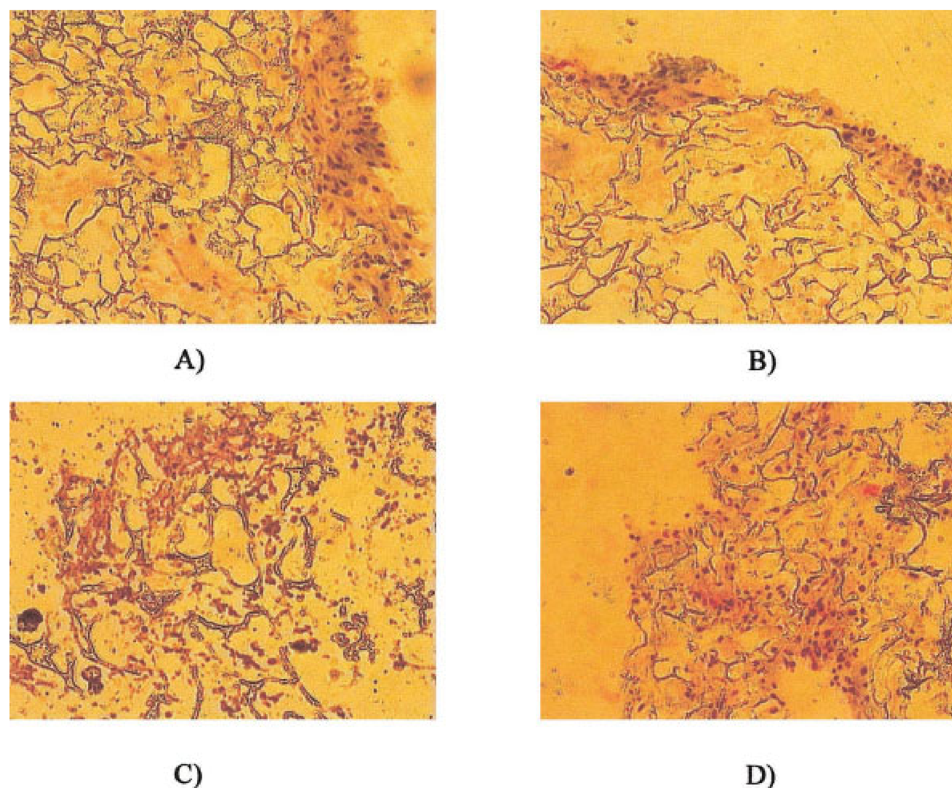

Figure 4. H&E-stained sections of PLGA (85/15) scaffolds after the culturing of 3T3 fibroblasts for 4 weeks. The light gray is the scaffold. Original magnification ×200. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

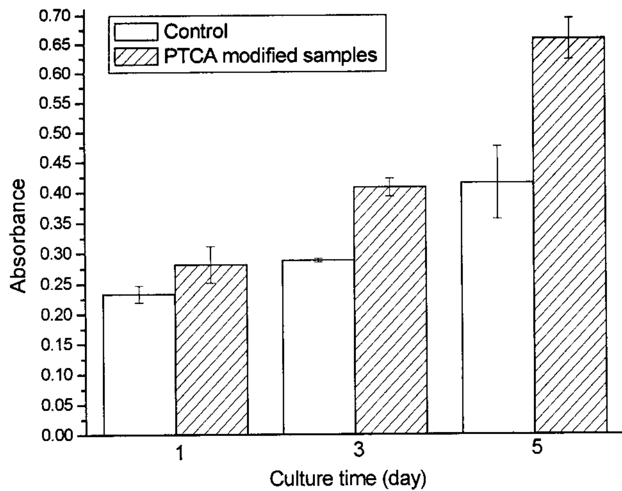

Figure 5. MTT–tetrazolium assay after the mouse 3T3 fibroblasts were cultured on PLGA (85/15) scaffolds. Formazan absorbance is expressed as a function of culture time. The statistical difference between the two sets of data was determined every day ($p < 0.05$).

ence was found. The cells detached slowly from the PT/CA-modified PLLA. Thirty six percent of the cells still remained on the PLLA films after 90 min under $29.5N/m^2$ of shear stress, indicating that cells can adhere tightly to PT/CA-modified PLLA. The quality of cell attachment on PT/CA-modified PLLA appeared to be better than that on unmodified PLLA, and even better than that on glass.

The change in the cell morphology on PT/CA-modified PLLA with time under shear stress is shown in Figure 7. The cells gradually withdrew from their borders, and although the process of the change was slow, with time under shear stress, cell extrusion was retracted to the cell body. However, for nonmodified PLLA films, we could not find this phenomenon. Almost without any steps of morphologic changes, the cells were removed from PLLA films within 2≈3 min, suggesting that the quality of cell attachment had been greatly improved by PT/CA modification.

The areas of the individual cells of a representative experiment are plotted in Figure 8 as a function of time. The cells were omitted from the histogram as soon as they detached. It was found that from the outset, the area of the cells on PT/CA-modified PLLA gradually reduced slowly with time. It also indicates that the quality of cell attachment on PT/CA-modified PLLA is very satisfactory. The cell morphology changes on unmodified PLLA could not be determined because they detached so quickly.

Figure 9 shows the mean shape factor of cells averaged for all the cells that remained on PLLA film at various times. From the start point (time = 0), the mean shape factors of cells on both PLLA films were almost the same, suggesting that cells on both PLLA films could attach and spread. But under the action of shear stress, the cells on PLLA films detached quickly. It was difficult to monitor the changes of cell morphology; however, for the cells on PT/CA-modified PLLA films, Figure 9 suggests that the deviation from the circular shape (S = 1) is largest at 20 min. It can be seen that the changing tendency of mean shape was decreasing although the differences are not significant, indicating that the cells gradually return to a more circular shape.

DISCUSSION

Porous biodegradable polylactone scaffolds have been tested extensively as supports for cell transplantation in tissue engineering. Cell affinity is the primary requirement for the scaffolds. The seeding of cells into the polymeric scaffold also is of critical importance in cell transplantation. This step must be atraumatic for cells and favor their homogeneous distribution throughout the scaffold.[35] However, the hydrophobicity of the polylactone scaffold will affect its cell affinity and inhibit the cells from penetrating the pores. The cells just migrate to the outmost portion of the scaffold. The cell distributions may not be uniform throughout the scaffold because of random motility and limitations in the diffusion of nutrients.[36] Recently, a biodegradable composite scaffold was proposed for facilitating cell transplantation.[37]

In our previous work, a surface modification method of combining plasma treatment with collagen anchorage (PT/CA) was proposed and it was demonstrated that the collagen was anchored onto the polymer surface. These experiments demonstrated that this method effectively could improve cell affinity of polylactone films.[21] In this study, the same methodol-

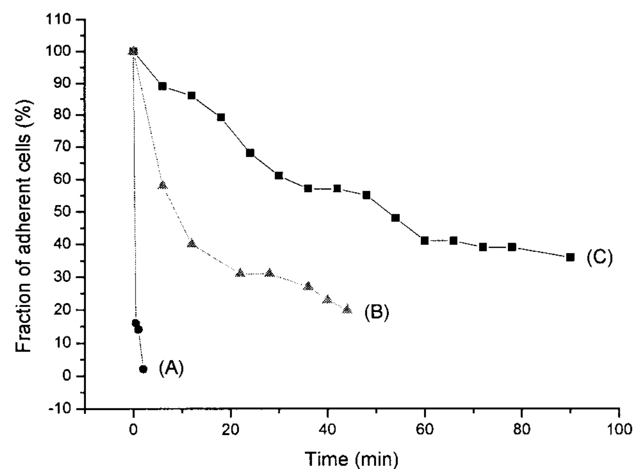

Figure 6. Fraction of 3T3 fibroblasts adhered onto (A) PLLA; (B) glass; and (C) PT/CA-modified PLLA as a function of time at $29.5N/m^2$ of shear stress.

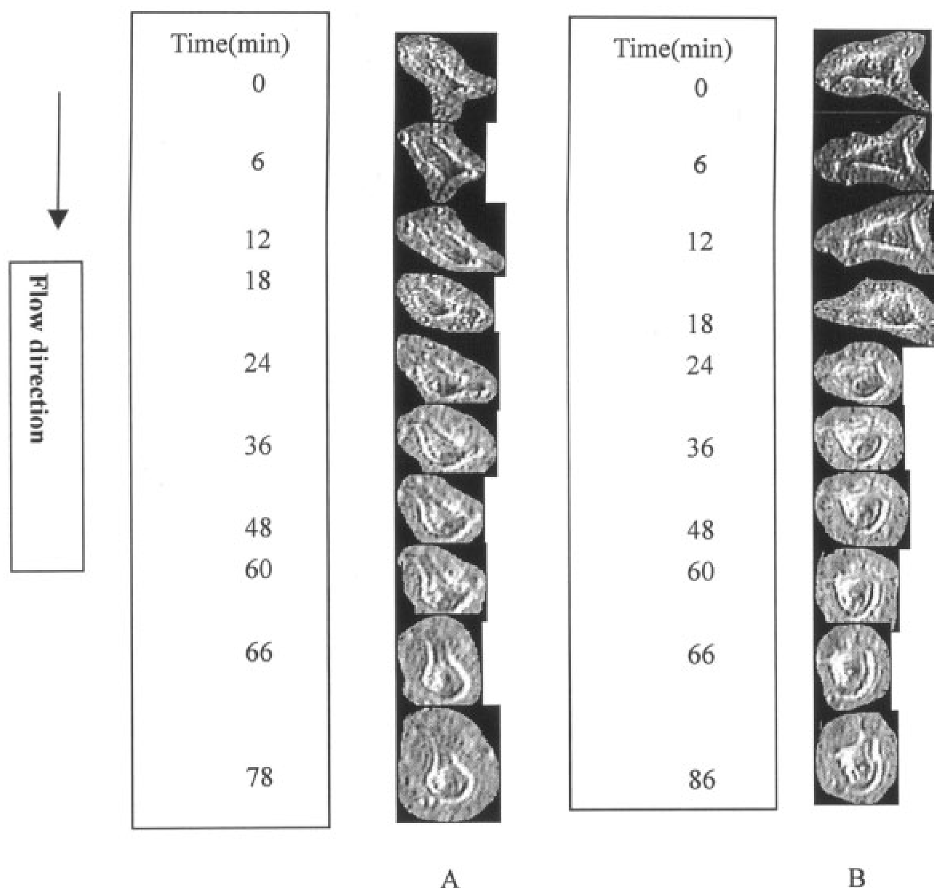

Figure 7. Light micrographs of mouse 3T3 fibroblasts adhered to PT/CA-modified PLLA films and exposed to $29.5N/m^2$ of shear stress. (A) and (B) represent different cells, separately monitored during detachment. Original magnification ×150.

ogy was used for the modification of three-dimensional polylactone scaffolds.

It is well known that plasma treatment is a very useful method for modifying the surface properties of a material. It has the advantage of treating a surface of complex shape because the plasma treatment is conducted in a vacuum and tends to be pervasive.[38] However, it is very important to determine the depth of the modification when a plasma treatment is used to modify three-dimensional cell scaffolds in tissue engineering.

In this study, a convenient and effective dyeing method is proposed for evaluating the modification depth of plasma treatment on three-dimensional scaffolds. After NH_3 plasma treatment, ink was used to infiltrate the scaffold quickly, and the inner portion of the scaffold was dyed. In contrast, for the untreated scaffold, only a weak trace of ink was found on the surface of the scaffold, and no ink was found inside the scaffold. This suggests that PT/CA modification also can be applied to three-dimensional scaffolds. Collagen can be anchored to the surface of the inner pores via this method. The cell affinity of PT/CA-modified PLGA (85/15) scaffold was evaluated by cell culture under shear or shear-free conditions.

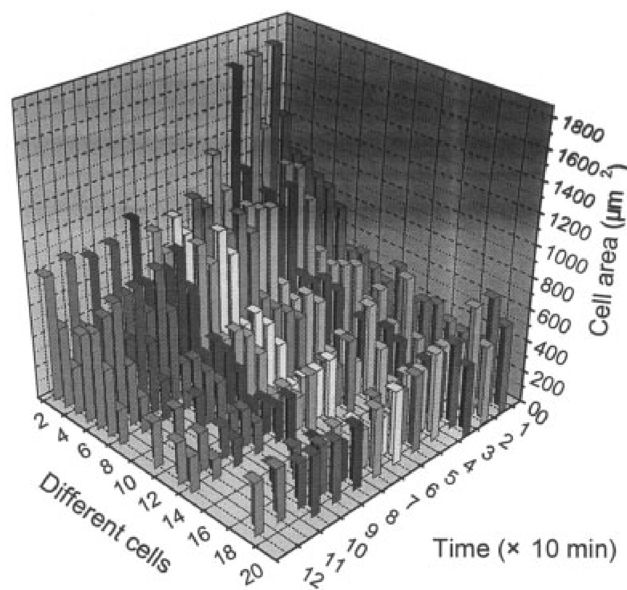

Figure 8. Area of spread cells on PT/CA-modified PLLA as a function of time for a representative experiment. Each block represents an individual cell. The cells are omitted from the histogram after detachment.

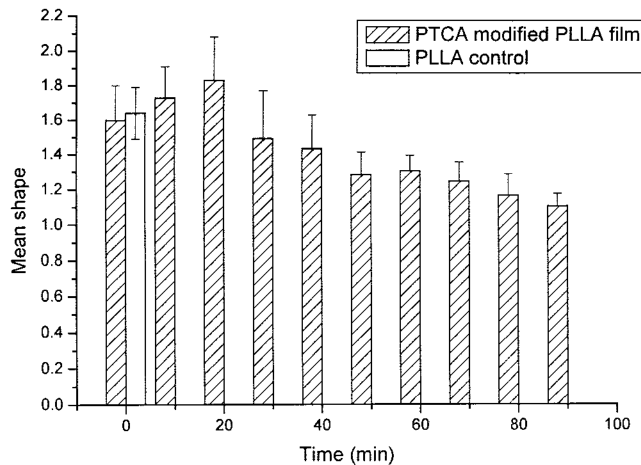

Figure 9. The mean shape (S) of the cells. Each picture was recorded by an image analysis system at $29.5 N/m^2$ of shear stress. It is important to note that once a cell has detached, it no longer is included in the calculation of the mean shape.

The results of SEM observation and MTT assays confirmed that the cell affinity of PLGA scaffolds were improved greatly after PT/CA modification. From H&E staining pictures of different magnifications, it was observed that cell distribution was uniform throughout the whole scaffold. This result confirmed that the PT/CA modification method could resolve the problem of non-uniform cell distribution for synthetic polymeric scaffolds. We know that plasma treatment can be used for sterilization of medical devices. Now it is apparent that PT/CA scaffolds can be used for cell seeding as long as all processes after the step of plasma treatment are kept aseptic.

The hydrophilicity of the scaffold improved greatly after PT/CA modification. Therefore cells could be seeded directly onto the PT/CA-modified scaffolds and maintained in a dry state; they did not need to be prewetted with ethanol. Thus the problem of toxicity from trace ethanol[39] also can be avoided. The dry state of the modified scaffold facilitates absorbance and accommodation of the cell suspension. It also facilitates uniform cell distribution.

It has been reported that cell attachment belongs to the first phase of cell/materials interactions and that the quality of this phase influences a cell's capacity to proliferate and to differentiate itself upon contact with materials.[40] We all know that polyester materials can support cell attachment and growth. We also found that there are no apparent differences between the morphology of the cells cultured on PT/CA-modified PLLA films and the morphology of those cultured on unmodified PLLLA films for 6 h (not shown). The cells on both PLLA films were all spread and spindle shaped.

However, when both PLLA films were evaluated by cell culture under shear stress, differences were apparent. The results show that the quality of cell attachment was improved greatly after PT/CA modification, indicating that PT/CA modification is an effective method for improving the cell affinity of polymeric materials. It also is suitable for modifying three-dimensional porous scaffolds in tissue engineering.

By using the shear-generating flow chamber system, a series of quantitative data, such as the fraction of adherent cells, cell area, and mean shape factor, are obtained. Thus different materials can be compared using this parallel plate flow chamber system. Of equal importance, by way of these quantitative data, the modifying effects on materials modified by many different surface modification methods can be evaluated using this flow chamber system. Nowadays many surface modification methods are proposed in the literature, but which method is the best for a specific material? We suggest that the flow chamber system can be utilized as a potential tool for comparing these various surface modification methods.

CONCLUSIONS

A convenient and effective dyeing method has been proposed to measure the modification depth of ammonia plasma treatment on three-dimensional PLLA scaffolds. The results show that modifying depth reaches about 4.0 mm under proper plasma-treatment conditions. A new method of plasma treatment followed by collagen-anchorage modification was used to modify the three-dimensional PLGA (85/15) scaffold. The results of static cell culture demonstrated that PT/CA modification effectively can facilitate cell transplantation and that cells can be distributed evenly throughout the scaffold. The cell affinity of PLGA (85/15) scaffold was enhanced greatly after PT/CA modification. A parallel plate flow chamber system was developed and utilized to evaluate the cell affinity of PLLA films under shear stress condition. It indicated that the quality of cell attachment on PLLA films was improved greatly after PT/CA modification. The flow chamber system could be utilized as a potential tool for comparing the various surface modification methods.

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