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In vitro Biodegradability and Surface Properties of Block Copoly(ester-ether)s Consisting of Poly(L-lactide) and Polyether

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Abstract: Cell attachment and proliferation on the polymer films of triblock copolymer(ester-ether)s comprising poly(L-lactide)(PLLA) and poly(oxyethylene-*co*-oxypropylene)(PN) were investigated using 3T3 fibroblasts. It was found that on the tissue culture polystyrene(TCPS) and the PLLA control film the cells could spread well while on the copolymer films the cells showed a rounded morphology without spreading and proliferated weakly. Especially, little cells proliferated on the films of copolymer having a LN composition of 20 wt%. While the water absorption of the copolymer films increased with increasing PN content, the contact angle against water of copolymer films immersed in aqueous medium was almost identical, being slightly lower than that of the PLLA film. These properties were compatible with the results of cell attachment. The *in vitro* hydrolysis of the films of triblock and multiblock type copolymers was faster with increasing PN content. The increased hydrolyzability, the flexibility and the decreased cell attachment suggested that these copolymers may have high potential as biodegradable materials for medical use.

Keywords: cell attachment, proliferation, fibroblast, flexibility, biodegradable material.

Introduction

In the decades there has been growing demand for the polymeric materials which can temporarily substitute a partial function and morphology of the living tissue and organ. Such materials for surgery and devices like dialyzer and artificial blood vessel that have been employed to maintain the functions lost tissues to support human health, and to survive patients are all called biomaterials. For application of artificial materials to biomedical field, control of surface and mechanical properties is one of the most important issues because their contact with body fluid and tissue should incur various bio-reaction and physical distorting. However, most of the polymeric materials developed thus far have been based on so-called hand-in-hand polymers that for different purposes. Therefore molecular and material designs are necessary for future biomaterials having better biocompatibility and higher reliability. Bioresorbable polyesters in the poly(L-lactic acid) (PLLA) and poly(glycolic acid) (PGA) family are attractive materials for tissue regeneration scaffolds because they are safe and tissue compatible, and offer a wide range of physical properties and degradation rates.[1-4] The resorbable suture, drug carriers, the screws, and plates for the internal bone fixation made from these resorbable materials are in various stage of investigation and development as clinical products.[5-7] Very recently, we have reported[8,9] it is found that the triblock copolymer and the multiblock copolymer of oligo(L-lactic acid) and PN have excellent and they are good candidates as biomaterials applied for soft tissues. These materials must fulfill a lot of the material requirement such as biocompatibility and biodegradability in adapting as scaffolds in soft tissues. For instance, the polymer scaffold implanted to the defect sites must maintain its shape strength throughout the degradation process while tissue regeneration. In addition, the adhesion, migration, differentiation, and growth of cells on the material is also important. These issues are inextricably intertwined. In the present study *in vitro* biodegradability of the PLLA/poly (oxyethylene-*co*-oxypropylene) (PN; the common trade name is Pluronic^{TM}) block copolymer films and cell attachment on them were examined.

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Experimental

Materials. PLLA homopolymer, triblock type, and multiblock type PLLA/PN(EO/PO = 80/20; M_n = 8,400) copolymers with different composition were synthesized as described in reported theses.[8,9] By changing the PN ratio to L-lactide in feed, a series of copolymers with different composition were prepared. Their name is abbreviated as LN(t)-a, where L, N, (t) and a denote PLLA, PN (the constituent segments), triblock type (copolymer type), and PN content, respectively. By a similar method, a series of copolymers with different composition were prepared by changing the ratio of PN to oligo(L-lactic acid) in feed. The name of the products is abbreviated as LN(m)-a, where L, N, (m) and a denote PLLA, PN (the constituent segments), multiblock type (copolymer type), and PN content, respectively. The other reagents and solvents were used without further purification.

Measurements. The number-average molecular weight (M_n) and the molecular distribution (M_w/M_n) were determined by gel permeation chromatography (GPC). The analyzer was composed of a Shimadzu LC-10A pump, a Shodex RI SE-31 RI detecter, a Shimadzu C-R7A Chromatopac data processor, a Shodex DEGAS KT-16 degassor, and a Sugai U-620 column oven. A combination of two polystyrene gel columns of Toso TSK gel G4000H and G2500H (7.5 mm i.d.× 300 mm, each) was used with tetrahydrofuran (THF) as the eluent at 35 °C. The molecular weight was calibrated according to polystyrene standards. Contact angle of polymer films against water was measured by a Kyowakaimenkagaku surface tension meter CDVD-A1 in the room set up to maintain at 20 °C. The phase-contrast microscope was used a Orimpus microscope IMT-2.

Film Preparation. 6 mL of a concentrated solutions (ca. 10 wt%) of PLLA homopolymer, A-B-A triblock type copolymer and multiblock type copolymer in chloroform were cast in a glass petridishes with the diameter of 10 cm at 4 °C for 3 h, and the polymer films were carefully taken off, and dried *in vacuo*. They were transparent films with the thickness of 80 μm.

Cell Proliferation Studies for the Triblock Type Copolymer. The polymer disks with the diameter of 14 mm were sterilized by exposing to UV light at the distance of 60 cm for 30 min at each side before use, and put at the bottom of 24-well tissue culture dishes (IWAKI, Japan). 3T3 fibroblasts (4.42×10^3 cells/cm^2) were plated on the polymer films and cultured in Eagles minimum essential medium (MEM) containing 10% FCS (Dainippon Pharmaceutical, Japan) at 37 °C under the atmosphere of 5% CO_2-95% air. The cells were washed with PBS(-) twice, and removed by incubating in 1 mL 0.2% trypsin/0.02% EDTA for 5 min, and then the number of cells was counted on the hemocytometer. Also, the morphology of the cells attached on the samples was observed under the phase-contrast microscope.

Contact Angle Measurements. Contact angle of the copolymer film against water were measured according to Wilhelmy method.[10,11] An example of the result obtained for the silicone film is shown in Figure 1. At first, the sample is hung separately from the water surface. As the stage arises, the sample sheet contact with the water, and then water crawls up and pull the sheet downward point **A** in Figure 1. As the sample film enters the water, the surface tension of the film remains constant, but the buoyancy linearly increases, resulting in a constant slope on the X-Y recorder. After scanning up to some immersion distance, the movement of the water container is reserved to lower the surface of water and the force exerted by the film is measured by the electric balance to determine the receding contact angle. The advancing (θ_a) and receding (θ_γ) contact angles would be calculated according to equation (1) and (2)

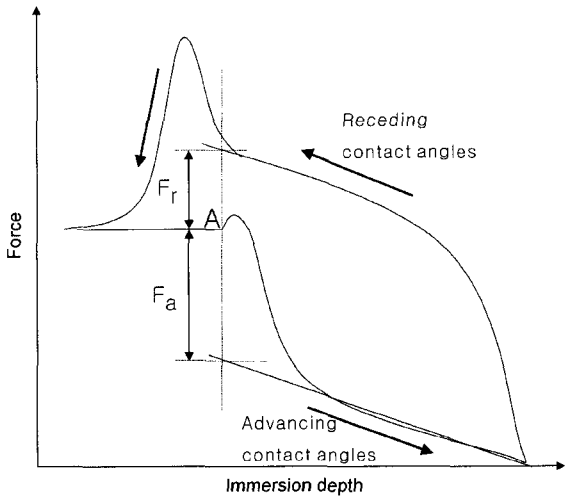

Figure 1. Hysteresis loop observed for a silicone film by the Wilhelmy plate technique.

$$F_a = \gamma L\cos\theta_a \quad (1)$$

$$F_\gamma = \gamma L\cos\theta_\gamma \quad (2)$$

where F_a and F_γ are the force at zero depth of immersion of the sample film at advancing and receding scanning as shown in Figure 1, respectively. γ is the surface tension of the tested liquid and L is the peripheral of the sample film.

Water Absorption Measurements. The dried copolymer disks with the size of diameter of 14 mm were weighed and immersed in phosphate buffered saline (PBS) of pH = 7.4 at 37 °C. At preset time intervals, hydrated samples were taken wiped out the surface water slightly, and weighed. Percent water absorption was calculated by dividing difference between the weights of immersed disks and original copolymer disks, by the original weight of the films shown as following equation.

$$W_{\%} = \frac{W_w - W_d}{W_d}$$

Where $W_{\%}$ denotes the percent of water absorption, W_w and W_d denote the weight of wet copolymer film, and that of dried copolymer film, respectively.

***In Vitro* Hydrolysis Test.** The polymer films with the size of 1× 5 cm were immersed in phosphate buffered saline of pH 7.4 in the test tubes which were gently shaked in the water bath at 37 °C± 0.2 °C. The specimens were recovered at different intervals, rinsed with distilled water, dried *in vacuo*, and were weighed in order to calculate the percentage of the weight change during the hydrolysis. The number and weight molecular weights of the sample after immersion were determined also by GPC. The percentage of the remaining molecular weight was evaluated relative to the original molecular weight.

Results and Discussion

Cell Proliferation on the Triblock PLLA-PN-PLLA Copolymer. Biodegradable polymers applied to various temporary implanted biomaterials such as surgical sutures and drug carriers directly contact with body fluid, cells tissues. Therefore, the adhesion, growth, and retention of function of cells cultured on materials surface are essential for their applications. Figure 2 shows the growth curves of 3T3 cells on the triblock type copolymers, PLLA homopolymer and tissue culture polystyrene (TCPS). After 24 h incubation, all the cells attached to the TCPS and PLLA surface but not to the copolymer films, and spread only onto the TCPS surface. On the copolymer surface, no cells adhesion has observed, and they were in a rounded morphology. By 48 h in culture, the cells spread and grew on the TCPS surface, and spread and slightly grew on the PLLA surfaces. By 84 h in culture, the number of cells on the TCPS and PLLA were about 5 times more than those at 48 h in culture. LN(t)-10 surface at 48 h culture, cells both in spread morphology and in spheroidal morphology could be observe. The cells grew slightly on LN(t)-10, LN(t)-15 and LN(t)-20 surface. After 110 h in culture, cell counts were slightly almost confluency numbers (about 1.0× 10^5 cells/cm^2) in mono layer culture on the TCPS and PLLA. As shown above, the growth of fibroblasts attached on triblock copolymer has been suppressed with an increasing PN composition, may be caused by the surface properties of the films.

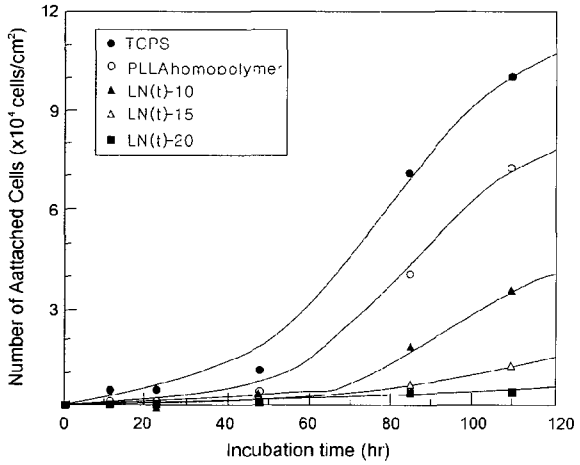

Figure 2. Time cources of the cell growth on (●) : TCPS; (○) : PLLA; (▲) : LN(t)-10; (△) : LN(t)-15; (■) : LN(t)-20.

Contact Angle to Water of the A-B-A Triblock Copolymer Films. In general, cell attachment on the materials is affected by the wettability against water, and electric charge of material surface. It is well known that cell attachment is dependent on a hydrophilic/hydrophobic balance of material surface, and the degree of the cell attachment is the highest at the surface with the contact angle of the 70°.[12] In this study the PLLA/PN copolymers with large PN content showed lower cell attachment, threrfore, the water contact angles of the triblock PLLA-PN-PLLA copolymers with different compositions were measured to investigate the reason for the tendency. Table I summarizes the contact angles of the copolymer films. The advancing water contact angles were about 87° for all the polymers examined. However, the receding contact angles of the copolymer films after immersion to the water were 52.6~58.4°, and were different around 15° as compared with the PLLA film (71.5± 0.6°). However the difference in the receding contact angles between the copolymer films is not large enough to result in the big difference in the cell attachment, suggesting that the other factor may affect on the cell attachment. The copolymer film surface may charge in their structures by swelling when they were immersed in the water. Then, the cells seems to be unable to adhere or proliferate for the swelled as shown in Figure 2.

Water Absorption of the Triblock and Multiblock PLLA/PN Copolymer Films. Figures 3 and 4 show the time courses of the water absorption by the triblock and multiblock PLLA/PN copolymer films, respectively. All the copolymer films started to swell immediately after immersion in the water, and the percentage of the water absorption leveled off after two days, reached at the equilibrium water absorption. In the case of LN(m)-87 film, the amount of water

Table I. Advancing and Receding Contact Angle Against Water of Triblock Type PLLA-PN-PLLA Films with Different Compositions

L-lactide/PN	Adv(degree)	Rec(degree)	Contact Angle (degree)[a]
PLLA	88.1± 1.7	71.5± 0.6	79.9± 0.6
LN(t)-10	88.1± 1.7	56.3± 1.1	72.9± 0.4
LN(t)-15	87.3± 2.4	58.4± 2.3	73.4± 1.9
LN(t)-20	88.4 ± 1.1	52.6± 1.4	71.4± 1.2

[a] $\cos\theta = \frac{\cos\theta_a + \cos\theta_\gamma}{2}$.

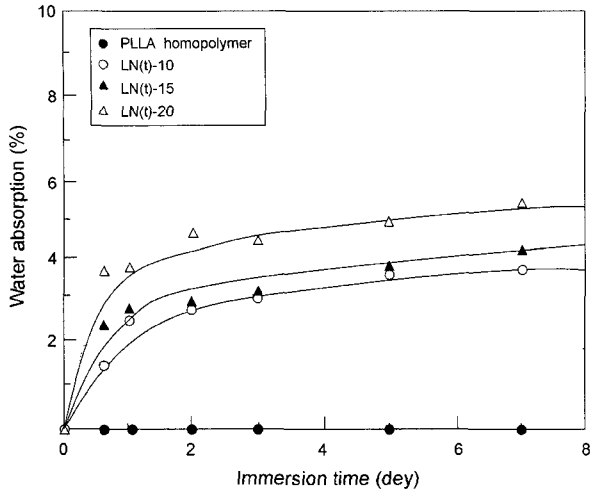

Figure 3. Water absorption of the triblock type PLLA-PN-PLLA copolymer films with different compositions. (●) : PLLA; (○) : LN(t)-10; (▲) : LN(t)-15; (△) : LN(t)-20.

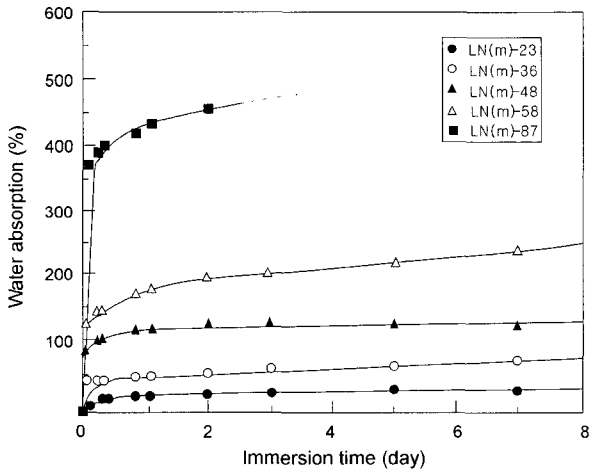

Figure 4. Water absorption of the multiblock type PLLA-PN copolymer films with different compositions. (●) : LN(m)-23; (○) : LN(m)-36; (▲) : LN(m)-48; (△) : LN(m)-58; (■) : LN(m)-87.

absorbed could not be measured after 2 days immersion because of the fragmentation of the film by hydrolysis. Figure 5 shows the relationship between swelling and PN content of triblock and multiblock copolymer films after 2 days immersion. The percentage of the water absorbed rapidly increased with increasing PN composition and they are on a same curve although they have different molecular weights. The degree of the water swelling seems to be affected only be the PN content but not by the molecular weight of the copolymers. Moreover, the surface layer of the copolymers is considered to be fully swelled even for that with low PN content which absorbed against water are similar in all

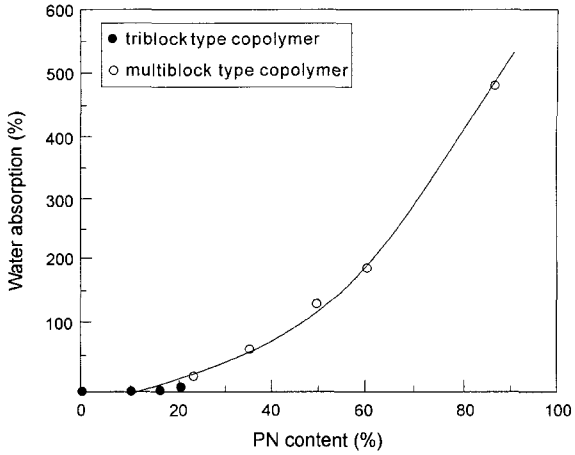

Figure 5. Relationship between swelling characteristics and PN content of PLLA/PN copolymer films after 2 days immersion in phosphate buffered saline of pH = 7.4 at 37 °C.

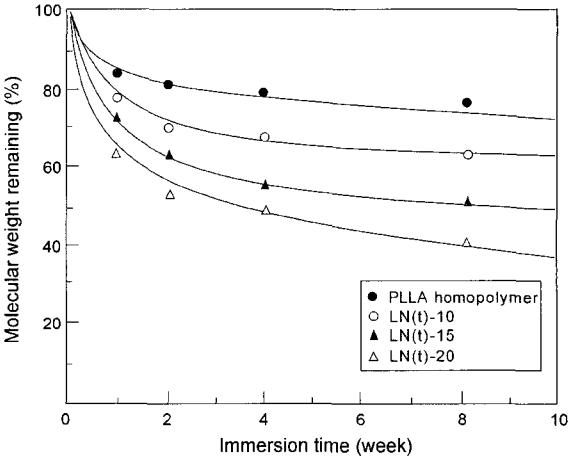

Figure 6. Changes in molecular weight of the triblock PLLA-PN-PLLA copolymer films with different compositions in phosphate buffered saline of pH = 7.4 at 37 °C. (●) : PLLA; (○) : LN(t)-10; (▲) : LN(t)-15; (△) : LN(t)-20.

copolymers irrespective of the PN content.

***In Vitro* Biodegradability of the PLLA/PN Copolymer Films.** The degradability by hydrolysis of the triblock and multiblock copolymer films were evaluated in a phosphate buffered saline of pH = 7.4 at 37 °C. Figure 6 shows the changes in molecular weight of the triblock type copolymer films with different PN contents as a function of immersion time. The molecular weight of all the samples rapidly decreased at early 2 weeks immersion, and then slowly decreased by 8 weeks. The degradation rate of the PLLA-PN-PLLA copolymer increased with increasing PN composition. It is considered LN(t)-20 film degraded very rapidly because it has lower crystallinity shown by DSC measurement[8,9] and higher hydrophilicity than the PLLA. Figure 7

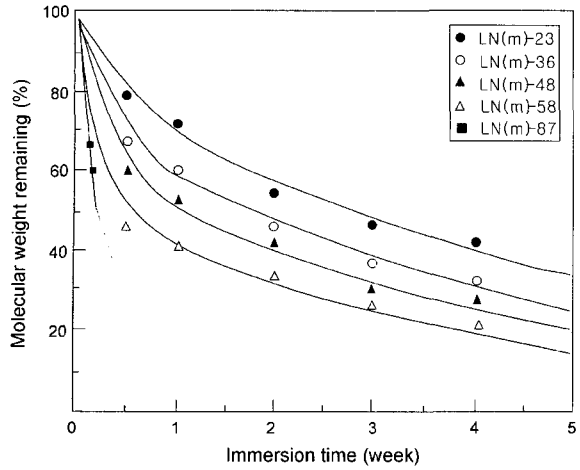

Figure 7. Changes in molecular weight of the multiblock type PLLA/PN copolymer films with different compositions in phosphate buffered saline of pH = 7.4 at 37 °C. (●) : LN(m)-23; (○) : LN(m)-36; (▲) : LN(m)-48; (△) : LN(m)-58; (■) : LN(m)-87.

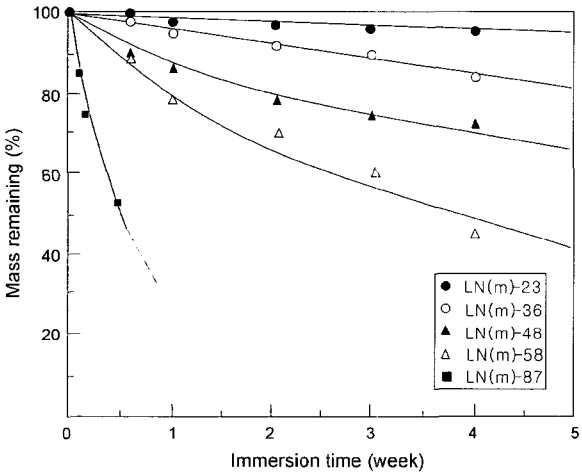

Figure 8. Changes in mass of the multiblock type PLLA/PN films with different compositions in phosphate buffered saline of pH = 7.4 at 37 °C. (●) : LN(m)-23; (○) : LN(m)-36; (▲) : LN(m)-48; (△) : LN(m)-58; (■) : LN(m)-87.

shows the results for multiblock PLLA/PN copolymer films with different contents of PN. The decrement of the molecular weight in the multiblock copolymer was much faster than the triblock type copolymer films, especially LN(m)-87 film didn't maintain the shape in 1 week immersion. It is clear that the degradation of these films was accerated by incorporation of PN segments.

The weight loss of the multiblock PLLA/PN copolymers is shown in Figure 8. The mass losses was much slower than the decrease in the molecular weight, but also increase with an increasing PN composition. The rate of mass loss was faster with increasing of PN composition.

The degradation of the biodegradable polymer is affected the molecular weight of the polymer, and polymers with high molecular weights degrade slowly than those with low molecular weight. In this study, the triblock type copolymers with high PN showed the high degradability. Their molecular weight may affect their biodegradability as well as the crystallinity. In the case of multiblock type copolymers with a same molecular weight, hydrolyzed depending on the PN contents indicating that the degradation of the PLLA/PN copolymers was suggested to be strongly affected by their crystallinity or hydrophilicity rather than their molecular weight.

Conclusions

3T3 fibroblasts were cultured on the PLLA/PN copolymers. The fibroblasts showed weaker attachment to the copolymer, and the proliferation rate of the cells decreased with increasing PN content. The copolymer surfaces were more hydrophilic as compared with the PLLA homopolymer surface, as indicated by the results in the contact angle and water absorption measurements. The *in vitro* hydrolysis of the copolymer films were more strongly affected by PN content. These results suggested that the biodegradability, the flexibility and the cell attachment of PLLA can be regulated by introduction of the soft PN segment.

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