Use of triethylcitrate plasticizer in the production of poly-L-lactic acid implants with different degradation times

S. M. N. SCAPIN, D. R. M. SILVA, P. P. JOAZEIRO, M. C. ALBERTO-RINCON*
Department of Histology and Embryology, Institute of Biology, State University of Campinas (UNICAMP), PO Box 6109, 13083-970, Campinas, SP, Brazil
E-mail: arincon@unicamp.br

R. M. LUCIANO, E. A. R. DUEK
Department of Material Engineering, Faculty of Mechanical Engineering, State University of Campinas (UNICAMP), PO Box 6109, 13083-970, Campinas, SP, Brazil

Bioabsorbable materials have been widely used in the repair of damaged tissue as well as in the controlled release of drugs and as a supports for cultured cells. The degradation time of poly-L-(lactic acid) (PLLA) may be controlled by altering the polymer porosity through the addition of the plasticizer triethylcitrate. This in turn influences the extent cellular infiltration. In this study, we examined the degradation of PLLA membranes containing different concentrations of plasticizer. PLLA discs were implanted subcutaneouly in rats and withdrawn 2, 14 and 60 days after implantation. The samples were processed for light microscopy and scanning electron microscopy (SEM). Polymer degradation was proportional to the concentration of plasticizer, indicating that triethylcitrate could affect the degradation time of the implants, without damaging the polymer biocompatibility.

Introduction

The increasing use of artificial materials to repair human tissues has led to development of new products to meet this demand. The availability of polymers that can be absorbed by the host organism has eliminated the need for a second surgery to remove the implanted material, and this in turn has reduced hospital expenses, surgical complications and the time needed for recuperation [1].

Poly-L-(lactic acid) (PLLA) is a semi-crystalline polyester that can be degraded through hydrolysis of its ester bonds, and has considerable strength retention capacity and excellent biocompatibility. Non-toxic degradation products are excreted by the kidneys or eliminated as carbon dioxide and water through the tricarboxylic acid cycle [2]. Although bioabsorbable materials are recognized by the organism as ''foreign bodies'' before their absorption, their finite exposure time and the mild inflammatory response they elicit are clearly advantageous.

Biomaterials have a variety of applications, including the construction of orthopedic protheses and guided tissue regeneration [3], controlled release of drugs [4, 5], as a type support for cultured cells [6] and tissue engineering [7, 8]. The extent of polymer degradation influences not only the type and intensity of the inflammatory response [9], but also its functional application, faster or slower degradation depending on the tissue to be repaired. Many factors may influence the kinetics of biomaterials degradation, including the molecules chemical structure, mass, superficial texture, porosity, pore dimensions, size and shape of the implant, site of implantation, and the microlocomotion of polymer fragments at the interface with adjacent tissue [10].

The inclusion of a plasticizer in polymer chains decreases the interaction among them but increases the flexibility, porosity and degradation of the polymer [11, 12]. In this work, we examined the biological properties and degradation process of PLLA membranes containing different concentrations (5, 9 and 11%) of the plasticizer triethylcitrate and assessed their interaction with adjacent tissue *in vivo*.

Materials and methods

Production of implants

PLLA (MW 300 000) was provided as pellets (Medsorb Technologies International L.P., Cincinnati, OH, USA). Ten grams of polymer were dissolved in 100 mL of methylene chloride (CH_2Cl_2, Merck KgaA, Darmstadt, Germany) containing 5%, 9% or 11% triethylcitrate (Aldrich, Milwaukee, WI, USA) in a closed recipient at room temperature. The mixture was then poured on to a glass plate (100 cm^2), which was air-dried (air flow of 1 L/min) at room temperature. After 15 h, the membranes were removed from the plates and vacuum dried for 24 h.

*Author to whom all correspondence should be addressed.

Discs 5 mm in diameter and 620 μm thick were cut and used in the studies described below.

Implantation

The membranes were immersed in 70% ethanol and then vacuum dried. Eighteen female Wistar rats 3 months old obtained from the university's central animal house (CEMIB) were used. The rats were housed at $22 \pm 2\,^{\circ}C$ on a 12 h light/dark cycle with food and water *ad libitum*. Two membranes of each plasticizer concentration were implanted in the dorsal subcutaneous tissue of each rat ($n = 18$) anesthetized with ketamine and xylazine-HCl (16.6 mg/kg and 3.33 mg/kg i.p., respectively) (Virbac, Roseira, São Paulo, Brazil). The health and behavior of the rats were assessed daily until sample collection at 2, 14 and 60 days post-implantation.

Light microscopy

Fragments of skin were fixed in Bouin solution and embedded in paraffin (Merck). Sections 5 μm thick were stained with Masson's trichrome and sirius red. Membrane fragments that had adhered to adjacent tissue were fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) and embedded in glycol methacrylate resin (Kulzer, Zweigniederlassung, Wehrheim, Germany). Sections 2 μm thick were stained with toluidine blue. These samples were observed and photographed with a Nikon Eclipse E800 photomicroscope. The samples stained with sirius red were observed and photographed under polarized light to assess invasion by collagen fibers.

Scanning electron microscopy (SEM)

Samples from the different periods of implantation were fixed in 2.5% paraformaldehyde (Sigma) and 2.5% glutaraldehyde (Electron Microscopy Science, Washington, PA, USA) containing 0.5% tannic acid (Sigma) in 0.1 M phosphate buffer, pH 7.4, followed by post-fixation in 1% osmium tetroxide (Sigma) in the same buffer. After dehydration in a graded ethanol series, the samples were freeze-fractured in liquid nitrogen then critical point dried (CPD 030, Balzers) and sputtered with gold (SCD 050, Balzers). The samples were examined in a Jeol JMS 5800 LV scanning electron microscope (Japan).

Results

No signs of a strong inflammatory process, such as an increase in local blood flow, edema, blush or necrosis were seen during tissue sampling. The membranes with more plasticizer (9% and 11%) adhered more to the adjacent tissue, whereas those with 5% plasticizer easily detached from it. Scanning electron microscopy (SEM) analysis of membranes not implanted revealed different size pores delimited by globular units of the wrinkled surface. Examination of the polymer fracture surface showed that the membrane faces differed in porosity (Fig. 1). As the amount of plasticizer increased, the pores extended deeper and the porous area became thicker than

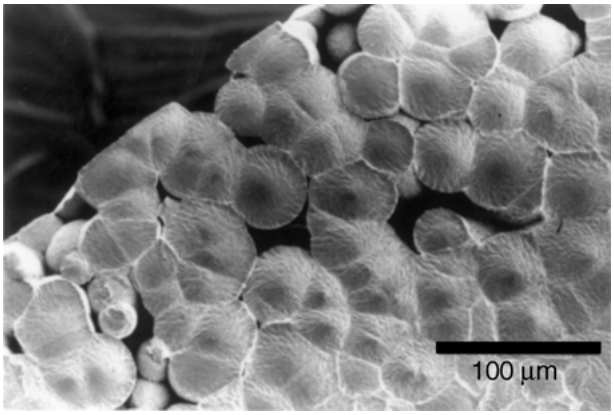

Figure 1 Electron micrograph of PLLA membrane with 5% plasticizer before implantation. Note that there is one porous face and another with a denser structure.

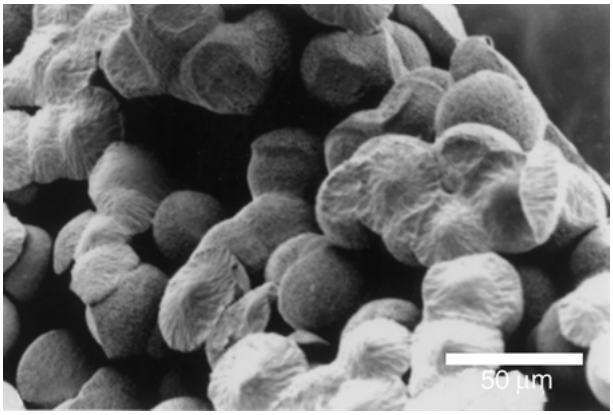

Figure 2 Electron micrograph of PLLA membrane with 11% plasticizer before implantation. Note the completely porous structure.

the dense area (of low porosity) so that in samples containing 11% plasticizer it was not possible to distinguish a non-porous face (Fig. 2).

Light microscopy

Independently of the percentage of plasticizer, two days after implantation the samples showed the typical features of acute phase inflammation, including neutrophil infiltration, fibrin nets and soft tissue edema (Figs. 3 and 4). In the chronic phase (14 and 60 days after implantation), fibrous capsule formation around the implant and invasion of the pores by vascular connective tissue and giant cells were observed (Figs. 5 and 6).

Samples containing 5% plasticizer retained a dense aspect 60 days after implantation, which made cellular invasion and adhesion to adjacent tissue difficult. Examination under polarized light revealed break-up of the implant, with the fracture area being invaded by collagen fibers proceeding of the capsule (Fig. 7). In samples containing 9% plasticizer there was an intense penetration of blood vessels through the membrane pores (Fig. 8). Polarized light showed extensive invasion by collagen fibers from the capsule (Fig. 9). Within 14 days of implantation, samples containing 11% plasticizer were invaded by large amounts of connective tissue and were surrounded by a capsule rich in blood vessels (Fig. 10).

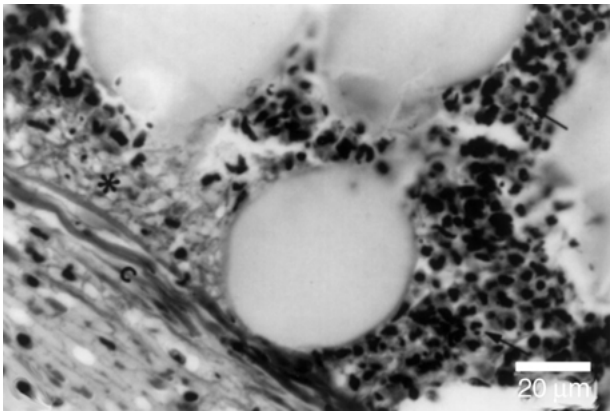

Figure 3 Photomicrograph of PLLA membrane with 9% plasticizer two days after implantation. Observe the presence of fibrin nets (*) and the large number of neutrophils (arrows) inside the polymer pores. The capsule (c) is in the initial stages of formation.

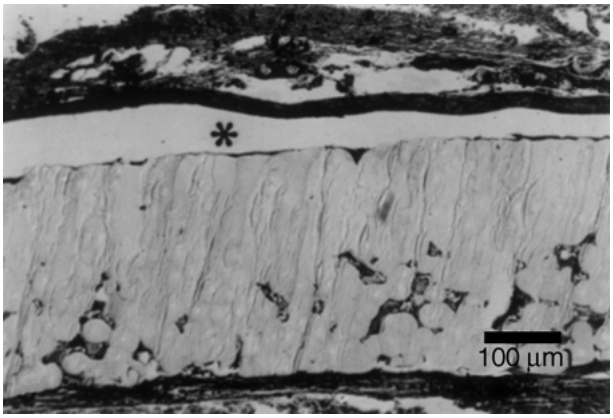

Figure 6 Photomicrograph of PLLA membrane with 5% plasticizer 60 days after implantation. The membrane is separated from the adjacent tissue (*) and is still dense, with low porosity.

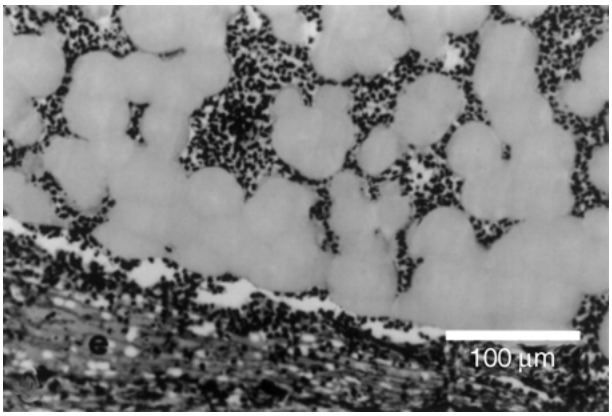

Figure 4 Photomicrograph of PLLA membrane with 11% plasticizer two days after implantation. Note the vascular edema (**e**) and neutrophils (*) inside the polymer.

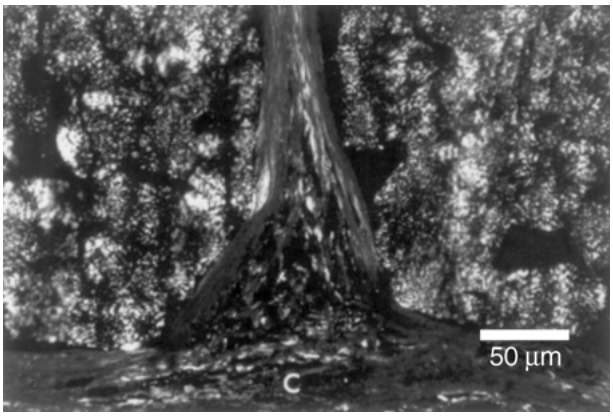

Figure 7 Photomicrograph of PLLA membrane with 5% plasticizer 60 days after implantation. Note the rupture region of membrane being invaded for many collagen fibers from the capsule (c).

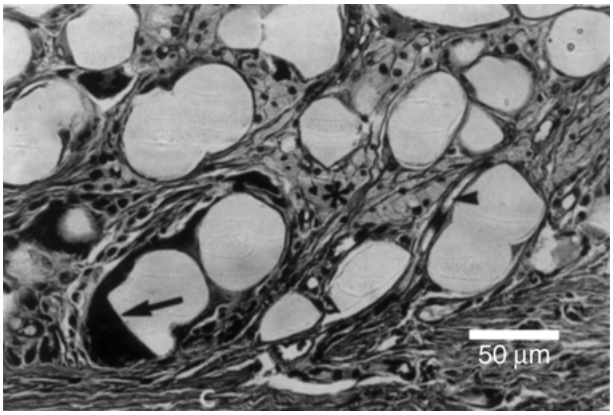

Figure 5 Photomicrograph of PLLA membrane with 9% plasticizer 60 days after implantation. Note the presence of the fibrous capsule (c) and giant cells (arrow). There is also an infiltration of blood vessels (arrowhead) and connective tissue (*).

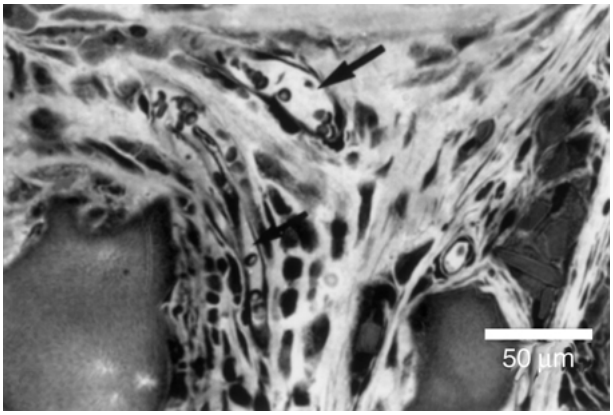

Figure 8 Photomicrograph of PLLA membrane with 9% plasticizer 60 days after implantation. There is an extensive infiltration of blood vessels (arrows) through the polymer pores.

After 60 days, those implants were covered with tissue (Fig. 11).

Scanning electron microscopy

SEM provided little information about the tissue component since with this technique it is not to possible distinguish among different types of cells. However, SEM provided greater insight into the pores of polymer degradation.

Two days after implantation, when the degradation process had not yet started, acute phase cells and fibrin nets were seen through the membrane pores (Fig. 12), independently of the amount of plasticizer used. Fourteen days after implantation, the polymer units of membranes containing 5% and 9% plasticizer were still intact.

Figure 9 Photomicrograph of PLLA membrane with 9% plasticizer 60 days after implantation. Polarized light shows pore invasion by collagen fibrils (arrows) from the capsule (c).

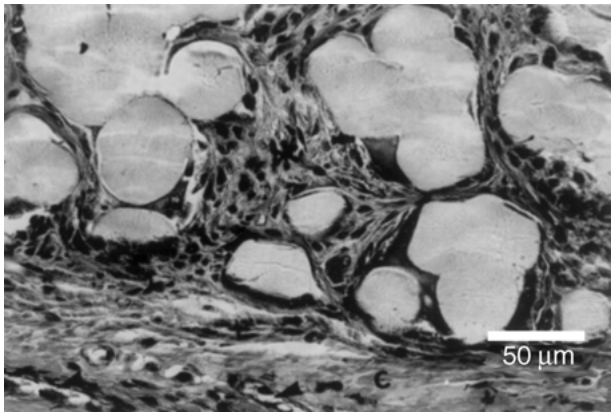

Figure 10 Photomicrograph of PLLA membrane with 11% plasticizer 14 days after implantation. Note the fibrous capsule (c) with many blood vessels (arrowheads) surrounding the implant and the intense connective tissue infiltration (*).

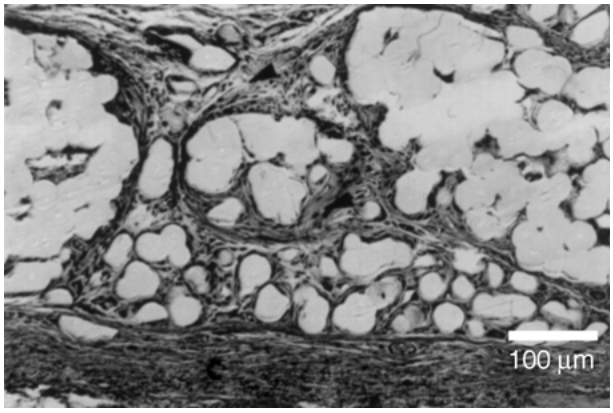

Figure 11 Photomicrograph of PLLA membrane with 11% plasticizer 60 days after implantation. Note the thick capsule (c) and the tissue invasion (arrowheads) that eventually leads to separation of the polymer units.

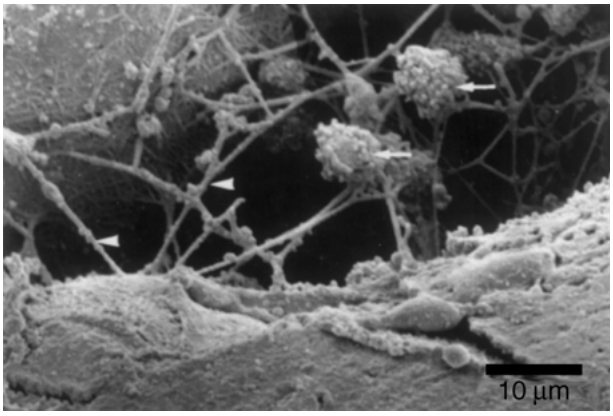

Figure 12 Electron micrograph of PLLA membrane with 5% plasticizer two days after implantation. Fibrin nets (arrowheads) and inflammatory cells are present (arrows).

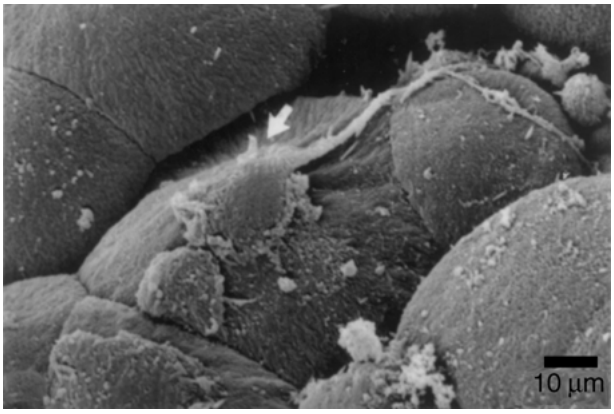

Figure 13 Electron micrograph of PLLA membrane with 9% plasticizer 14 days after implantation. Note the presence of a cell firmly adhered to the polymer surface (arrow). The membrane units do not show fractures at this stage.

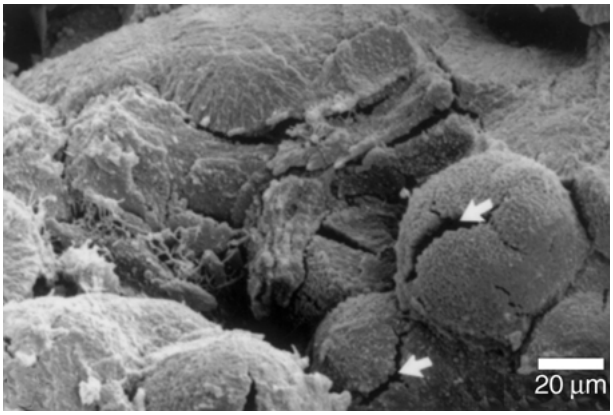

Figure 14 Electron micrograph of PLLA membrane with 11% plasticizer 14 days after implantation. The globular units of the membrane show the typical centrifugal fractures (arrows) of the degradation process.

Although in the 9% samples there was greater cell infiltration, with some of the cells adhering firmly to the polymer surface (Fig. 13). In samples containing 11% plasticizer, the initiation of polymer degradation was observed after 14 days, with the appearance of centrifugal fractures in the membrane units (Fig. 14).

Sixty days after implantation, the samples containing 5% plasticizer remained dense, with little invasion by tissue elements, although some globular units showed signs of degradation (centrifugal fractures) (Fig. 15). After 60 days, implants containing 9% and 11% plasticizer were in an advanced state of degradation in which the polymer units were extensively disrupted and immersed in a large amount of connective tissue (Fig. 16).

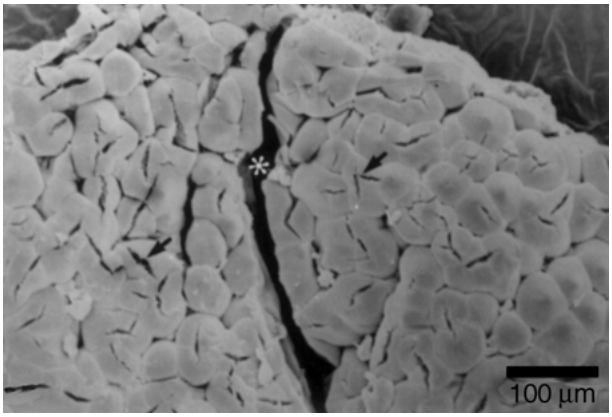

Figure 15 Electron micrograph of PLLA membrane with 5% plasticizer 60 days after implantation. The polymer shows some signs of degradation (arrows). Although the membrane is still dense, some breaks are present (*).

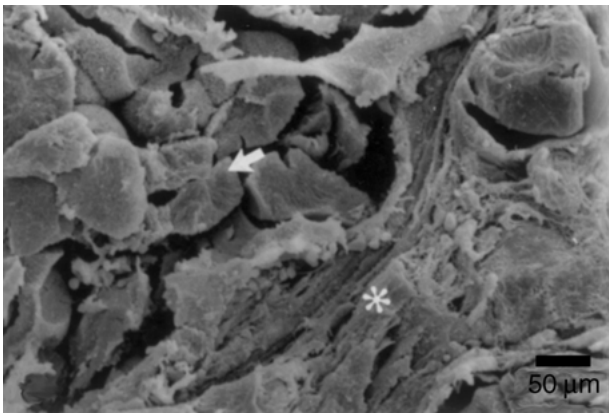

Figure 16 Electron micrograph of PLLA membrane with 9% plasticizer 60 days after implantation. Note the extensively degraded membrane units (arrow) and the abundant tissue invasion (*).

Discussion

According to Mainil-Varlet *et al*. [13], the inflammatory response to biomaterials is determined by the composition, purity, conformation, superficial properties, implantation site, porosity, chemical structure, and other properties of the material. In the case of absorbable polymers, these factors also influence the extent of degradation by tissue. The tissue reaction to PLLA was reflected by the presence of fibroblasts, macrophages, giant cells and polymorphonuclear cells.

The type of inflammatory response to PLLA implants depended on the time since implantation time rather than on the concentration of plasticizer, although implants with a containing higher of concentration plasticizer, had greater porosity and a more intense cell response. An acute inflammatory reaction was seen in all the samples obtained two days after implantation and reflected the local surgical trauma. Subsequently, a chronic reaction was observed in response to the implant. In this chronic phase, the formation of a fibrous capsule around the membrane and the presence of giant cells suggested that the organism was trying to isolate and absorb the implant. The invasion of the membrane by vascular connective tissue indicated that, in addition to stimulating the inflammatory reaction, the membrane also enhanced regeneration of the damaged tissue.

PLLA implants devoid of plasticizer do not have pores [12] and, consequently, do not allow cellular invasion. The addition of plasticizer provides malleability and porosity, and modifies the tissue reaction to the implant. As shown here, the greater amount of plasticizer added, the greater implant porosity, cellular invasion and speed of degradation.

According to Luciano *et al*. [14], the plasticizer changes the morphology of membranes drastically. This can be explained by the interaction of plasticizer with amorphous regions of polymer provoking the separation of the polymeric chains. Moreover, the process of the formation is facilitated and results in the formation of spherulitic structures. The agglomeration of these spherulitic structures causes the formation of an interconnected porous surface due to the addition of plasticizer. The authors verified that the elongation until rupture increases with the addition of plasticizer, which indicates a higher flexibility of the membranes. This data was confirmed by thermal analysis showing that the glass transition temperature decreases with addition of plasticizer [14].

The membranes containing 5% plasticizer had a very dense structure, which hindered the tissue invasion and vascularization of the implant. These membranes were unable to accommodate the natural malleability of subcutaneous tissue, and in some samples they even tore. Such membranes may be useful in guided tissue regeneration or other techniques of recuperation that require tissue isolation through a thick membrane with a long degradation time.

In contrast, membranes containing 9% and 11% plasticizer were much more porous and malleable. The presence of blood vessels in these samples suggested a first step to neovascularization, with regeneration the damaged tissue and perfect polymer integration into the site of implantation. These membranes would be more indicated for soft tissue regeneration or as a support for cultured cells, where the tissue reposition is faster. These membranes may be beneficial in tissue engineering because it would allow a higher number of cell to be cultivated in the polymer mold, it would facilitate the diffusion of factors among transplanted cells and adjacent tissue and would contribute to tissue vascularization [15]. In a comparative study of polymers with different degradation features, relevant to cartilage reconstruction, Rother *et al*. [16] concluded that polymer supports with a shorter degradation time were more appropriate for formation of the cartilage matrix, while polymers that were more stable apparently inhibited matrix production.

SEM confirmed that membranes with more pores degraded much quicker, probably because of the greater cellular infiltration and consequently greater participation of cells in the polymer degradation process.

Based on these results, we conclude that the addition of the plasticizer triethylcitrate to PLLA membranes can be useful for producing implants with different degradation time for various applications. The concentration of plasticizer affects the porosity and malleability of the implants without compromising biocompatibility of the polymer.

Acknowledgment

This work was supported by FAPESP (98/13339-4, 98/15373-1 99/10918-6 and 99/09730-2). The authors thank the Electron Microscopy Laboratory (I.B., UNICAMP) for use of their facilities and Mr. Baltazar Pereira de Paula for technical assistance.

References

1. J. C. MIDDLETON and A. J. TIPTON, *Biomaterials* **21** (2000) 2335.
2. D. BENDIX, *Pol. Degrad. Stab.* **59** (1998) 129.
3. L. M. PINEDA, M. BÜSING, R. P. MEINIG and S. GOGOLEWSKI, *J. Biomed. Mat. Res.* **31** (1996) 385.
4. M. VALLET-REGÍ, D. A. GRANADO, M. GORDO, M. V. CABAÑAS, C. V. RAGEL, A. J. SALINAS, A. L. DOADRIL and J. SAN ROMÁN, *ibid.* **39** (1998) 423.
5. K. KAWAI, S. SUZUKI, Y. TABATA, Y. IKADA and Y. NISHIMURA, *Biomaterials* **21** (2000) 489.
6. L. E. FREED and G. VUNJAK-NOVAKOVIC, *Adv. Drug. Deliv. Rev.* **33** (1998) 15.
7. R. LANGER and J. P. VACANTI, *Science* **260** (1993) 920.
8. B.-S. KIM and D. J. MOONEY, *Trends Biotechnol.* **16** (1998) 224.
9. K. H. LAM, J. M. SCHAKENRAAD, H. ESSELBRUGGE, J. FEIJEN and P. NIEUWENHUIS, *J. Biomed. Mat. Res.* **27** (1993) 1569.
10. G. J. BEUMER, C. A. VAN BLITTERSWIJK and M. PONEC, *Biomaterials* **15** (1994) 551.
11. C. SCHUGENS, C. GRANDFILS, R. JEROMES, P. H. TEYSSIE, P. DELREE, D. MARTIN, B. MALGRANGE and G. MOONEN, *J. Biomed. Mat. Res.* **29** (1995) 1349.
12. R. M. LUCIANO in ''Síntese, Caracterização e Degradação de Membranas de Poli(ácido lático), um polímero bioabsorvível'', UNICAMP, Masters dissertation, 1997.
13. P. MAINIL-VARLET, S. GOGOLEWSKI and P. NIEUWENHUIS, *J. Mat. Sci. Mat. Med.* **7** (1996) 713.
14. R. M. LUCIANO in ''Efeitos da Concentração de Plastificante em Membranas de Poli(ácido lático) para Implantes'', UNICAMP, Thesis, 2001.
15. D. J. MOONEY, D. F. BALDWIN, N. P. SUH, J. P. VACANTI and R. LANGER, *Biomaterials* **17** (1996) 1417.
16. N. ROTTER, J. AIGNER, A. NAUMANN, H. PLANCK, C. HAMMER, G. BURMESTER and M. SITTINGER, *J. Biomed. Mat. Res.* **42** (1998) 347.

Received 13 March
and accepted 17 October 2002