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THE EFFECT OF TRIETHYLCITRATE ON THE POROSITY AND BIOCOMPATIBILITY OF POLY(LACTIC ACID) MEMBRANES

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ABSTRACT

The aim of this study was to investigate stability and tissue response to poly (L-lactic acid) (PLLA) membranes implanted in sub-dermal tissue of rats. Membranes with and without plasticizer (triethylcitrate) were compared. Membranes without plasticizer were denser and more compact than those with triethylcitrate. Fifteen days and 30 days after implantation, the membranes with tissue adhered were removed and processed for light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). By 15 days post-implantation, membranes lacking plasticizer showed invasion of the pores by connective tissue. Thirty days after implantation, the pores of membranes with plasticizer were invaded by blood vessels, and multi-nucleated giant cells surrounded by globular units of the membranes. Membrane debris was also detected in the cytoplasm of multi-nucleated giant cells. These data show that the addition of plasticizer to PLLA results in a more porous membrane, therefore enabling them more suitable in tissue repair (than membranes without plasticizer).

Key words: Biocompatibility, connective tissue, implants, poly (lactic acid), porosity, triethylcitrate

INTRODUCTION

Interest in the medical application of polymeric materials has increased since Kulkarni *et al.* [3] introduced the concept of bioabsorbable materials that could be used as sutures and in the repair of bone fractures and skin lesions.

The main advantage of these polymers is their degradation by the simple hydrolysis of ester bonds in an aqueous environment. The products of degradation (carbon dioxide and water) are metabolized by the organism, thus avoiding the need for a second surgery to remove the implant. The earliest and most commonly used bioabsorbable polymers included poly-L-lactic acid (PLLA), polyglycolic acid (PGA), their copolymers (PLLA-PGA) and poly(ortho esters). Numerous other materials have been developed and used experimentally in recent years [7].

Bioabsorbable implants have been produced by a variety of techniques ranging from direct machining of orthopedic plates and screws from polymer blocks to the extrusion of polymer melts. Membranes obtained by solution processing of poly(α-hydroxy acids) have found applications as supports for fixation and cell growth, as well as drug delivery systems, vascular grafts, biodegradable skin substitutes and wound coverings. For these applications, the membranes should be porous, with interconnections among the pores. The size of the pores should take into consideration the size of the invading cells and the mechanical resistance desired [15,9].

Porous membranes can be obtained by several methods, depending on the desired characteristics [6], whereas dense membranes can be produced by casting from a solution of polymer or by molding melted polymer.

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The technique most used for producing porous membranes is phase separation, which consists of immersing a polymer solution in a bath with no solvent. This technique is widely used to obtain membranes for nanofiltration. Although these membranes are porous and homogeneous, their pore size of 10 μm generally precludes the entry of cells [12,13].

van Sliedregt *et al*. [14] synthesized porous membranes of poly (lactic acid) by adding known concentrations of salt using controlled granulometry. After solvent evaporation, the membranes contained salt particles, which could be extracted by washing with water to produce porous membranes with pores of 100-500 μm. However, the size of the pores was not uniform throughout the membrane and the membranes themselves had poor mechanical properties [14].

Typical reactions between polymeric material and host tissue may involve various cell types, including fibroblasts, hystyocytes, lymphocytes, eosinophils, fibrocytes, macrophages, foreign body giant cells, polymorphonuclear cells, eosinophils and lymphoid cells. The type of cells present at the site of implantation may vary depending on the purity of the implant (i.e. the presence of catalyst and monomer residues, particulate material and solvents), the implant mass and geometry, the positional stability at the implantation site, and ability of the polymer to crystallize. The *in vivo* resorption of polylactides is determined largely by their chemical composition, the degree of chain orientation, surface properties, and morphology [5].

Freed *et al*. [2] studied the growth of chondrocytes cultivated on fibrous poly(glycolic acid) for 6 weeks. The density of cells obtained was similar to that for cells grown on collagen. In contrast, chondrocytes attacked the pores of poly(lactic acid) when this was used as substrate, and the number of cells obtained was only one-half of that with normal cartilage. These differences were attributed to polymer geometry and the rate of biodegradation. These authors concluded that chondrocytes grown on these polymeric substrates, maintained their original properties and could form cartilage [2].

van Sliedregt *et al*. [14] and Wald *et al*. [15] showed that porous devices provide an appropriate space for the growth of cells and for the production of extracellular matrix. An uniformly arranged and interconnected pore structure is important so that cells can easily distribute throughout the device, and be organized into a network of tissue constituents, especially for the reconstruction of structural tissues like bone and cartilage.

Schugens *et al*. [10] synthesized a biodegradable implant of poly(lactic acid) with a controlled porosity which consisted of an aggregate of poly(L-D lactic acid) (PDLLA) spheres of known size. In addition, triethylcitrate, a biocompatible plasticizer was used. The plasticizer acted on the polymer chains to reduce the interaction among the chains, thus favoring a flexible membrane. This arrangement resulted in a decrease in the glass transition temperature. Since poly(lactic acid) is biodegradable, it is possible to control the degradation time by altering the molecule's physicochemical characteristics [R.M. Luciano, Master dissertation, FEM, State University of Campinas, 1977].

In this work, we compared the interaction of host tissue with PLLA membranes in the presence or absence of 15% plasticizer.

MATERIAL AND METHODS

Production of implants

PLLA (MW 300,000) was provided as pellets by Medsorb Technologies International L.P., (Cincinnati, OH, USA). Fifteen grams of polymer were dissolved in 100 mL of methylene chloride (CH_2Cl_2, Merck) containing 10% triethylcitrate (Aldrich) in a closed recipient at room temperature [R.M. Luciano, Master dissertation, FEM, State University of Campinas, 1977,12]. Other membranes were prepared without triethylcitrate. The mixture was then poured onto 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 plates and vacuum dried for 24 h. Disks 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. Eight female Wistar rats 3 months old obtained from university's central animal house (CEMIB) were used. The rats were housed at 22 ± 2°C on a 12 h light/dark cycle with food and water *ad libitum*. Two membranes were implanted in the dorsal subcutaneous tissue of each rat (n = 8) anesthetized with ketamine and xylazine-HCl (16.6 mg/kg and 3.33 mg/kg. i.p., respectively) (Virbac, Brazil). The health and behavior of the rats were assessed daily until sample collection. After rat cervical dislocation, the

membranes were collected with surrounding tissue at 15 and 30 days post-implantation.

Light microscopy

Fragments of skin were fixed in Bouin solution and embedded in paraffin. Sections 5 µm thick were stained with Masson's trichrome. Membrane fragments that had adhered to adjacent tissue were fixed in 4% paraformaldehyde and embedded in glycol methacrylate. Sections 2 µm thick were stained with toluidine blue. These samples were observed and photographed with a Nikon Eclipse E 800 photomicroscope.

Scanning electron microscopy (SEM)

Samples from the different periods of implantation were fixed in 2.5% paraformaldehyde and 2.5% glutaraldehyde containing 0.5% tannic acid in 0.1 M phosphate buffer, pH 7.4, followed by post-fixation in 1% osmium tetroxide 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).

Transmission electron microscopy (TEM)

Samples were fixed in 2% (w/v) glutaraldehyde, 2% paraformaldehyde and 0.5% tannic acid in 0.1 M phosphate buffer pH 7.4, for 3 h at 4°C, followed by fixation in 1% OsO_4 for 1 h at room temperature. The samples were then embedded in Epon resin and uranyl acetate/lead citrate stained sections 60 nm thick were examined in a LEO Zeiss electron microscope.

RESULTS

Normal membrane appearance

Morphologically, membranes without plasticizer had a dense, smooth structure, whereas those with plasticizer had a completely porous structure (Figs. 1, 2, respectively). The pores of the membrane corresponded to spaces delimited by juxtaposed globular units (Fig. 2). Examination of the fracture surface revealed differences in the porosity of the membrane faces.

As shown in Fig. 2, the distribution and diameter of the pores were not uniform along the membrane. The surface of the globular units had a rough appearance (Fig. 3).

Membrane appearance after implantation
15 days post-implantation

The light microscopy of the samples without plasticizer removed on the 15th day after implantation showed the presence of a fibrous capsule with a large number of thin collagen fibers around of the implant (Fig. 4). These results were confirmed by SEM (Fig. 5).

The examination of samples with plasticizer on the 15th day after implantation showed the presence of a capsule around the implant, together with a large number of collagen fibers. Invasion of the membrane pores by tissue elements was also seen.

The globular units of the membrane showed some degradation and separation from each other. In addition, particles of different sizes were also present (Fig. 6).

SEM revealed cell adhesion to the membrane surface (Fig. 7) as well as invasion of the polymer by cells and components of the extracellular matrix. TEM showed the presence of giant cells and fibrillar elements of the extracellular matrix, with intimate contact between the tissue elements and the polymer surface (Fig. 8).

30 days post-implantation

The morphological characteristic of the dermal tissue response to PLLA membrane implants without plasticizer and obtained 30 days after implantation was similar to those for 15 days. The implant surface was surrounded by a fibrous capsule and there was no cellular invasion of the membrane.

Samples with plasticizer removed on the 30 days after implantation showed the presence of a fibrous capsule composed of collagen fibers surrounding the implant and an intense tissue invasion observed inside the membrane including the presence of giant multinucleated cells (Fig. 9). Analysis of the capsule revealed the presence of polymeric particles of different diameters as well as numerous blood vessels (Fig. 10).

TEM examination showed connective tissue invasion throughout the pores. Various cell types (fibroblasts, macrophages and giant cells), small caliber blood vessels and extracellular matrix were present in the membrane (Fig. 11).

SEM analysis revealed that degradation of the membrane occurred simultaneously with the disintegration of the membrane units and with invasion by cells and extracellular matrix components. (Fig.12).

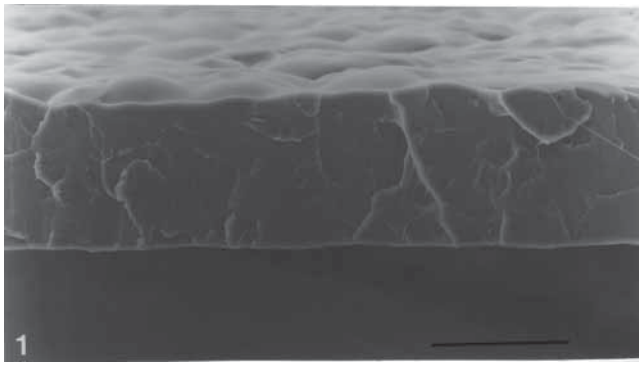

Figure 1. Scanning electron micrograph of PLLA membrane without plasticizer, before implantation. Note the densely compact morphology of the membrane interior. Bar = 100 μm.

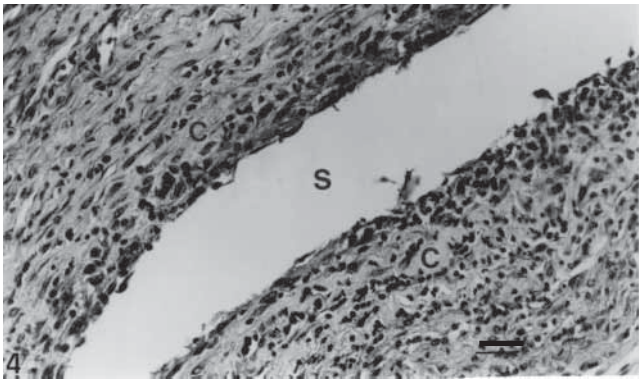

Figure 4. Photomicrograph of PLLA membrane without plasticizer on the 15th day post-implantation. Note the capsule of connective tissue (C) and the previously occupied space (S) by the membrane. HE. Bar = 10 μm.

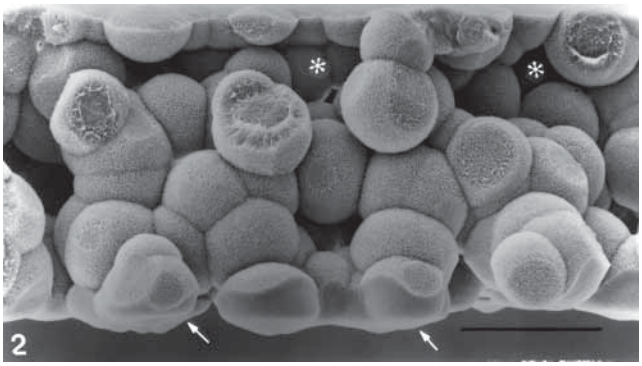

Figure 2. Scanning electron micrograph of PLLA membrane with plasticizer, before implantation. Note the porous morphology of the interior (*) and surface (arrows) of membrane. Bar = 100 μm.

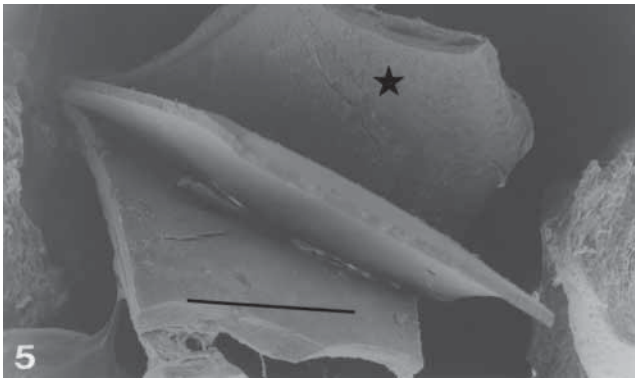

Figure 5. Scanning electron micrograph of PLLA membrane without plasticizer on the 15th day post-implantation. Note the capsule of connective tissue (star). Bar = 1 mm.

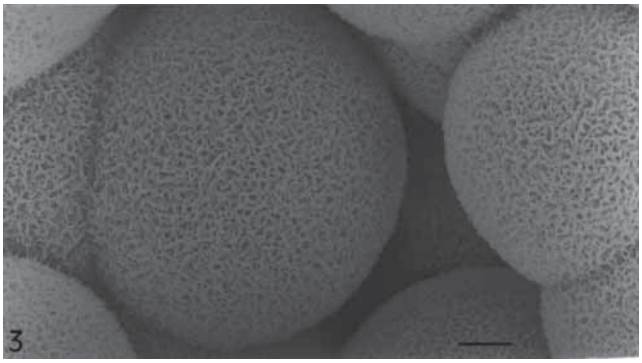

Figure 3. Scanning electron micrograph of PLLA membrane with plasticizer, before implantation. Note the rough microvilli-like surface of the globular units. Bar = 10 μm.

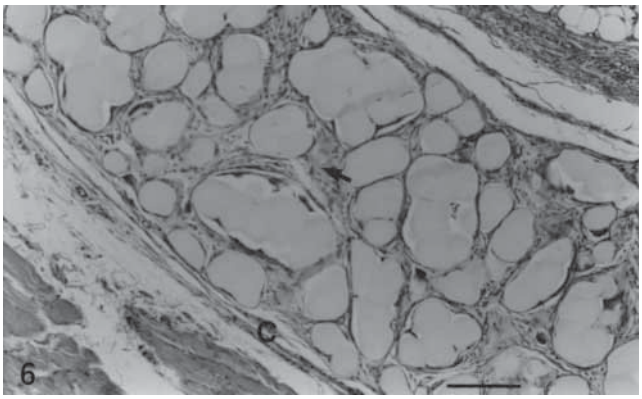

Figure 6. Photomicrograph of PLLA membrane with plasticizer on the 15th day post- implantation. Note the invasion of cells and collagen fibers through the interstices (arrow) of the globular units of the PLLA. Capsule of fibrous connective tissue (C) and fragments of membrane is also present in the implant. HE. Bar = 50 μm.

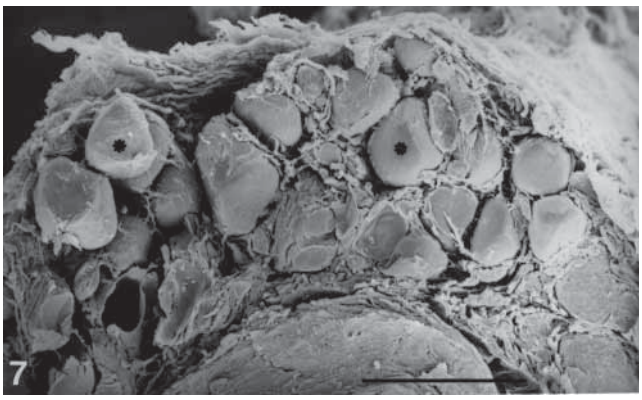

Figure 7. Scanning electron micrograph of PLLA membrane with plasticizer on the 15th day post-implantation. Note the fragments of membrane (*) surrounded by connective tissue. Bar = 100 μm.

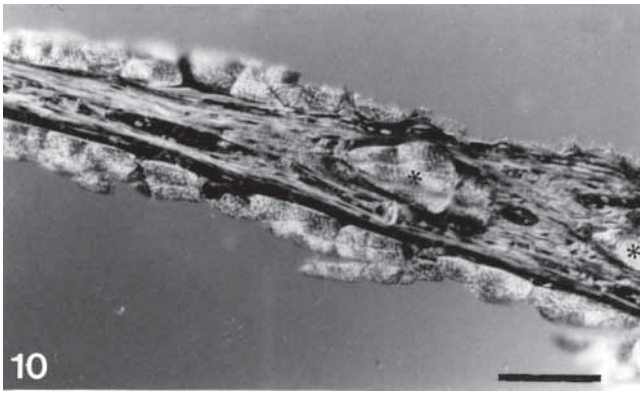

Figure 10. Photomicrograph of PLLA membrane with plasticizer on the 30th day post-implantation showing fragments of polymer surrounded by a vascularized capsule (*). Polarized light. Bar: 50 μm.

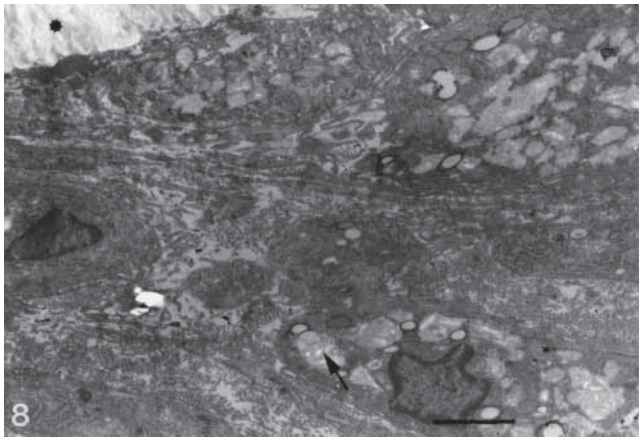

Figure 8. Transmission electron micrograph of PLLA membrane with plasticizer on the 15th day of implantation. Note the close contact between the dermal tissue and the synthetic membrane, as well as the fragments of polymer inside the cells (arrow). Bar = 2 μm.

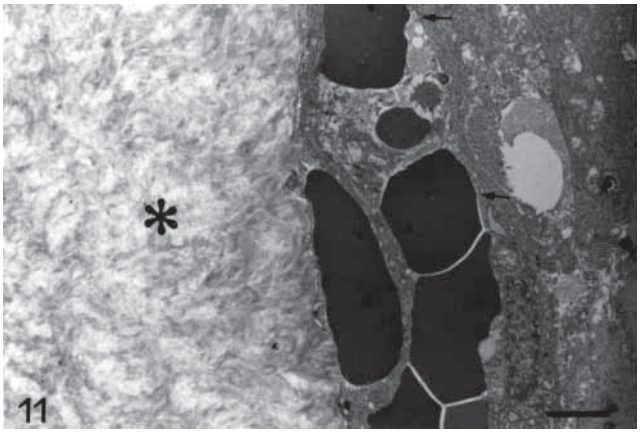

Figure 11. Transmission electron micrograph of PLLA membrane with plasticizer 30 days after implantation. Note the blood vessels (arrows) surrounding the PLLA membrane (*). Bar = 2 μm.

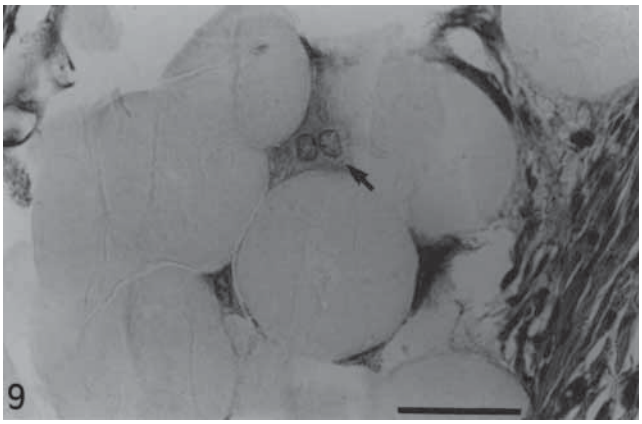

Figure 9. Photomicrograph of PLLA membrane with plasticizer on the 30th day post-implantation showing multinucleated giant cells surrounding the polymeric units (arrow). Bar: = 20 μm.

DISCUSSION

PLLA membranes containing plasticizer have a porous structure formed by globular units 60-100 μm in diameter [1].

Luciano [R.M. Luciano, Masters dissertation, FEM, State University of Campinas, 1997] synthesized and characterized poly(L-lactide acid) membranes with possible use as a support for cells in culture, for skin regeneration and for guided tissue regeneration. Different membranes were produced by varying the concentration of polymer.

The addition of triethylcitrate, a biocompatible plasticizer, resulted in porous membranes and conferred flexibility which allowed the membranes to adapt to the movement and flexibility of soft tissue. Membranes without plasticizer showed less

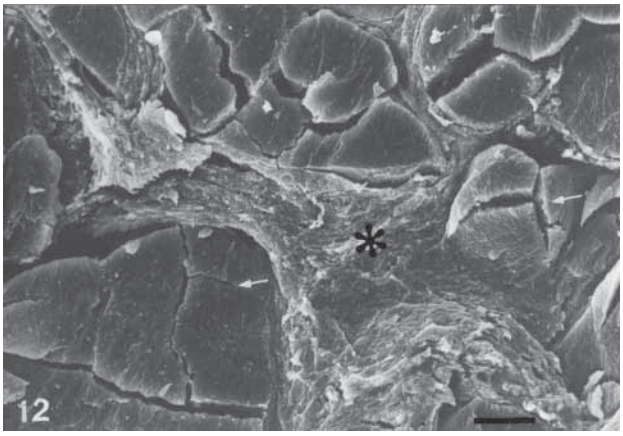

Figure 12. Scanning electron micrograph of PLLA membrane with plasticizer on the 30th day post-implantation. Note the fractures in the polymeric units (arrows) as a result of degradation, and also the fibrous tissue surrounding the fragments (*). Bar = 10 µm.

adhesion of the host tissue to the implanted material and degraded more slowly. Whereas low porosity membranes were suitable for guided tissue regeneration, high porosity were useful for tissue reconstruction since they allowed better cell adhesion and migration. Silva *et al*. [11] used light microscopy to show that membranes containing 10% polymer and 10% plasticizer had great cellular invasion by the 30th day post-implantation.

As shown here, membranes with and without plasticizer were covered with a capsule of connective tissue [5]. The surface of membranes without plasticizer remained smooth since it did not allow cell invasion, while the membrane containing plasticizer had a rough morphology that allowed cell growth on its surface, and was totally invaded by macrophages, fibroblasts and fibrillar elements of connective tissue.

Beumer *et al.* [1] studied synthetic degradable implants of poly(ethylene oxide)-co-(butylene terephthalate), Polyactive™ 14, in two different compositions, 55:45 and 40:60. These bilayered matrices are used as a dermal regeneration template for large surface area full-thickness skin defects. The porous underlayer consisted of copolymer or PLLA designed to allow the ingrowth of dermal components, while the dense top layer consisted of copolymers that served as a substrate for keratinocytes. At both subcutaneous and intramuscular implantation sites, the PLLA underlayer tended to elicit a more pronounced cellular response than copolymer layer. TEM examination of these membranes showed the presence of macrophages and mono and multinucleated phagocytic cells (foreign body giant cells) at the tissue interface by the 13th week of implantation, indicating intracellular degradation of the implants. Histological analysis of the implant area on the 26th week post-implantation showed a vascularized fibrous tissue with collagen deposition. These results confirmed the reconstitution of damaged tissue in addition to a foreign body cellular response. Implant degradation was related to *in vivo* biocompatibility, with no systemic effects being observed as a result of implant degradation or the accumulation of polymeric fragments during the first year post-implantation. The membrane surface area decreased by less than 20% in this period.

As shown here, 30 days after implantation the membranes containing plasticizer were totally invaded, indicating that degradation is faster and cellular growth was more accentuated than in these membranes used by Beumer *et al.* [1]. The presence of blood vessels in the samples on the 30th day after implantation indicated the onset of neovascularization, which facilitated the regeneration of damaged tissue.

Porous matrices promote an appropriate environment for cell growth and extracelular matrix synthesis. The uniform distribution and interconnection of the pores is important in order to facilitate the formation of the tissue in an organized network, which is important in the reconstruction of bone, and cartilaginous tissues [14].

Lam *et al.* [4] evaluated the influence of surface morphology and hydrophilicity of absorbable (PLLA, porous and dense) and non-absorbable (PTFE (Teflon) and expanded PTFE) membranes in relation to the inflammatory response after sub-dermal implantation in mice. There was no cellular invasion in membranes of expanded PTFE while in porous PLLA membranes there was extensive invasion. Six weeks after implantation, the inflammatory response to the porous PLLA membranes was greater than with other membranes. These authors concluded that the porosity increased the inflammatory response only when the hydrophilicity allowed cellular invasion.

In conclusion, the presence of plasticizer influences the porosity of PLLA, making it possible

to control the degradation time and the extent of cellular invasion, which are important factors in implant maintenance in host tissue.

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