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The influence of triethylcitrate on the biological properties of poly (L-lactic-co-glycolic acid) membranes

L. Pietro · D. R. M. Silva · M. C. Alberto-Rincon · E. A. R Duek

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Abstract Biodegradable polymers have a variety of uses in basic and clinical research, as well as important therapeutic applications. The most commonly used are poly (lactic acid), poly (glycolic acid) and their copolymer, poly (L-lactic-co-glycolic acid) or PLGA. The incorporation of a plasticizer into a polymer can be used to obtain a product with specific properties. In this work, we examined the influence of a plasticizer (triethylcitrate) on the properties of PLGA membrane implants for human clinical uses. Membranes with and without plasticizer were dense and compact and contained no pores. The incorporation of 7% plasticizer enhanced the degradation the polymer when compared to polymer without plasticizer. In membranes without plasticizer, the initiation of degradation was very slow and was seen only 60 days after implantation, should allow the use of this material in the repair of damage tissue. In both cases, macroscopic analysis showed that there was no adhesion of the membrane to capsule fibrous, and this adversely affected preservation of the polymer. With time, the adherence of the polymer to surrounding tissue increased. Overall there was little degradation of membranes without plasticizer compared to those containing plasticizer.

L. Pietro · E. A. R Duek (✉)
Laboratory of Biomaterials, Faculty of Biology, University Catholic of São Paulo (PUC-SP), Dr. José Ermirio de Moraes Square, 290, 18030-230, Sorocaba, SP, Brazil

D. R. M. Silva · 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

Introduction

Synthetic bioreabsorbable polymers have been used as sutures and in the repair of bone fractures and skin lesions. These polymers can be used to form dense membranes that function as barriers against soft tissue invasion during bone recuperation (a technique known as "guided tissue regeneration") or as porous membranes that provide a support for cell growth, fixation and tissue transplants [1,2,3].

In recent years there have been several reports of the mechanical strength and degradation of completely biodegradable polymers *in vitro.*

Poly (L-lactic-co-glycolic acid) (PLGA) copolymers were among the few synthetic polymers approved for human clinical use in recent years, nearly 1966. PLGA copolymers can be easily processed into the desired configuration and their physical, chemical, mechanical, and degradative properties can be engineered to fit a particular need [4, 5].

The rate of polymer degradation may affect many cellular process, including cell growth, tissue regeneration, and host response [6]. Barbanti et al. in study of cytocompatibility and adhesion of osteoblasts cells culture on the same PLGA evaluated in this work and PLLA verified that osteoblasts showed low adhesion to PLLA compared to PLGA, and the cell morphology on the surface of these materials was highly dispersed, which indicated a good interaction on the cells with the polymer substrate [7] Previous work [8] showed that adding a plasticizer (triethylcitrate) to PLGA altered the flexibility of the material and allowed the formation of pores in the polymer. Depending on the quantity of the plasticizer used, membranes of different porosities can be produced, and this allows control of the degradation of the polymer and invasion of the membrane pores by tissue elements. The accurate addition of plasticizer thus allows the production of material for different applications [9,10,11].

The aim of this work was to examine the biological and morphological properties of a porous membrane of PLGA containing 7% triethylcitrate at 2, 30, 60 and 120 days after implantation in the subcutaneous tissue of rats. A knowledge of the interaction between this polymer and tissue should allow the use of this material in the repair of damaged tissue.

Materials and methods

Preparation of implants

PLGA was provided as pellets by PURAC (Gromingen, The Netherlands). Ten grams of polymer was dissolved in 100 ml of methylene chloride (CH_2Cl_2, Merck) containing 7% triethylcitrate (Aldrich) in a closed recipient at room temperature [8]. Other membranes were prepared without triethyltcitrate. The mixture was then poured onto a glass plate (100 cm^2) that was air dried (air flow of 1 L/min) at room temperature. After 15 h, the membrane was removed from the plate and vacuum dried for 24 h. Disks 5 mm in diameter and 620 μm thick were cut and used in the studies described below.

Atomic force microscopy (AFM)

The AFM analyses were performed using a Digital Instruments NanoScope IIIa Scanning Probe Microscope Controller. It was employed a Si cantilever with constant forces of 13-70 N/m. The images were obtained in the taping mode at room temperature, in the fundamental resonance frequency of Si of about 300 kHz. The scanning rate was 1 Hz, and the maximum scale for the scanning heads was 10×10 μm. All images were obtained with directional amplitude of $A_0 \approx 500$ nm.

Implantation

The membranes were immersed in 70% ethanol and then vacuum dried. Sixteen female Wistar rats 3 months old obtained from university's central animal house were used. The rats were housed at $22 \pm 2^{\circ}$C on a 12 h light/dark eycle with food and water *ad libitum.* Two membranes were implanted in the dorsal subcutaneous tissue of rats anaesthetized with ketamine and xylazine-HCl (16.6 and 3.33 mg/kg, i.p., respectively) (Virbac, Brazil). Tissue samples were obtained 2, 30, 60 and 120 days post-implantation, after the rats were anaesthetized and sacrificed.

Light microscopy

Fragments of skin were fixed in Bouin solution for 24 h, at 4°C and embedded in paraffin with pH 7.4 (Phosphate buffer solution 0.1 M). Sections 5 μm thick were stained with Masson's trichromic method and Sirius red. Membrane fragments that had adhered to adjacent tissue were fixed in 4% paraformaldehyde and embedded in glycol methacrylate. Sections 2 um 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 after 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 sputter-coated with 25 nm gold thickness (SCD 050, Balzers). The samples were examined in a Jeol JMS 5800 LV Scanning electron microscopy (Japan).

Results

Macroscopic analysis

The inflammatory response to biomaterial is determined by the composition and purity of the material, the shape and surface properties of the implant, the site of implantation, position stability at the implantation site, porosity, chemical stability, and so on [2]. Macroscopic observation of the samples with and without plasticizer showed that there was no adhesion of the tissue to the membranes during the first 30 days after implantation. At intervals beyond this period, the sample was incorporated into the host tissue such that it was often difficult to locate the implant macroscopically in the skin at the site implantation.

Microscopic analysis

- Membrane appearance after implantation in Light Microscopy

2 days post-implantation

Light microscopy of the samples with and without plasticizer removed on the 2nd day after implantation showed edema and a massive infiltration of polymorphonuclear cells embedded in a fibrin net. The membrane was separated from the adjacent tissue. There was no invasion of the membrane by tissue elements (Figs. 1,2).

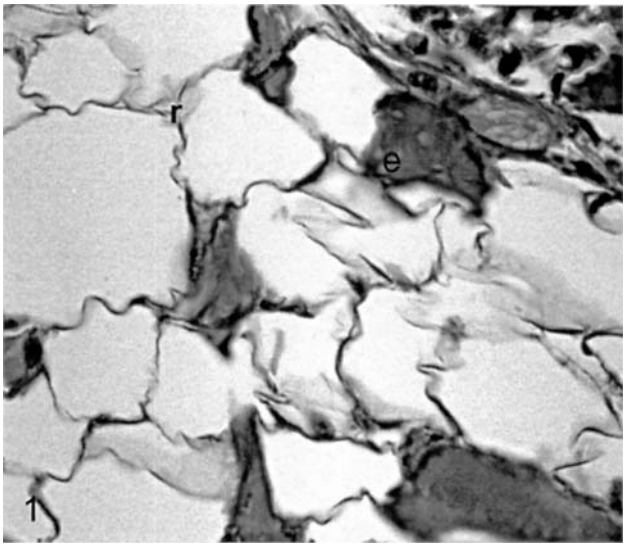

Fig. 1 PLGA membrane without plasticizer 2 days after implantation. Note the polymorphonuclear infiltrate, vascular edema (e) and network (r). Paraffin, HE, 400X.

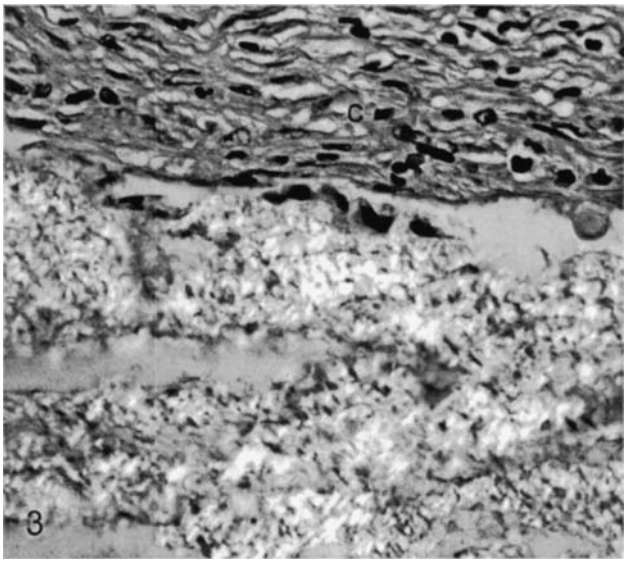

Fig. 3 PLGA membrane without plasticizer 30 days after implantation. Note the connective tissue fibers (c) formed around the membrane. Paraffin, HE, 400X.

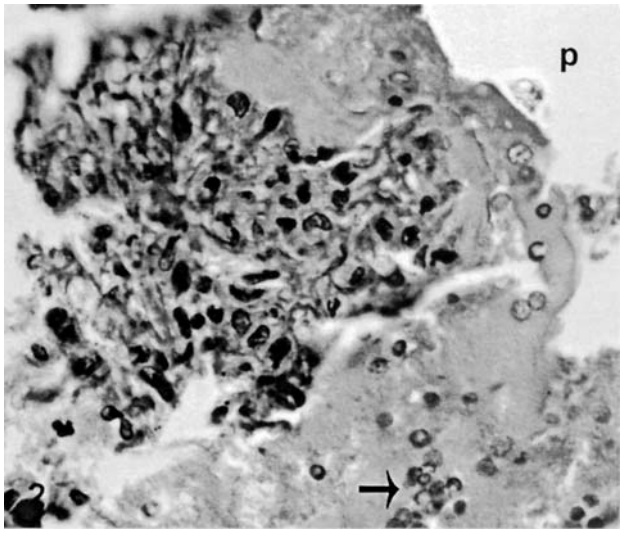

Fig. 2 PLGA membrane without plasticizer 2 days after implantation. Note the polymorphonuclear cells (arrow) within the polymer (p). Paraffin, HE, 400X.

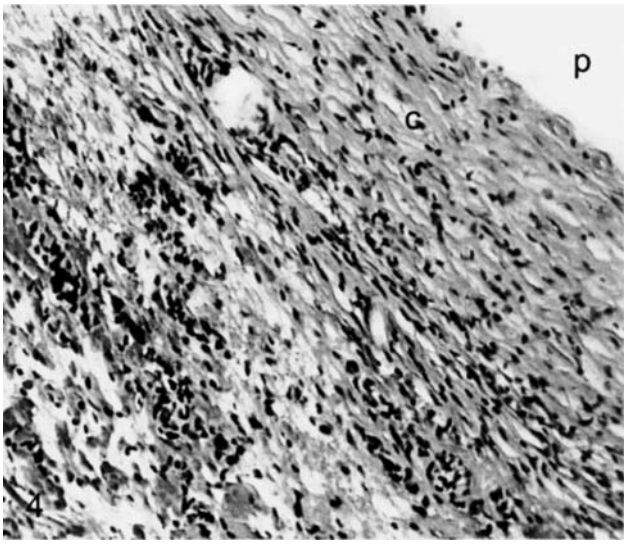

Fig. 4 PLGA membrane with 7% plasticizer 30 days after implantation. Note the capsule with collagen tissue (c) and the degraded polymer (p). Paraffin, HE, 200X.

30 days post-implantation

In samples with and without plasticizer there was a fibrous capsule with a large number of thin collagen fibers on the surface of the implant, as well as numerous fibroblasts and macrophages. A fibrous capsule covered the implant surface and there was no cellular invasion of the membrane (Figs. 3,4).

60 days post-implantation

In membranes without plasticizer there was no preservation, no invasion of cells, and no inflammatory reaction (Fig. 5). Membranes with plasticizer contained numerous macrophages, but no marked degradation (although a lightly greater than in previous intervals) and no pores. A capsule of conjunctive tissue was present (Fig. 6).

120 days post-implantation

Extensive tissue invasion and degradation were seen in membranes. Degradation resulted in the release of globular units from the membrane. The polymer was broken into small

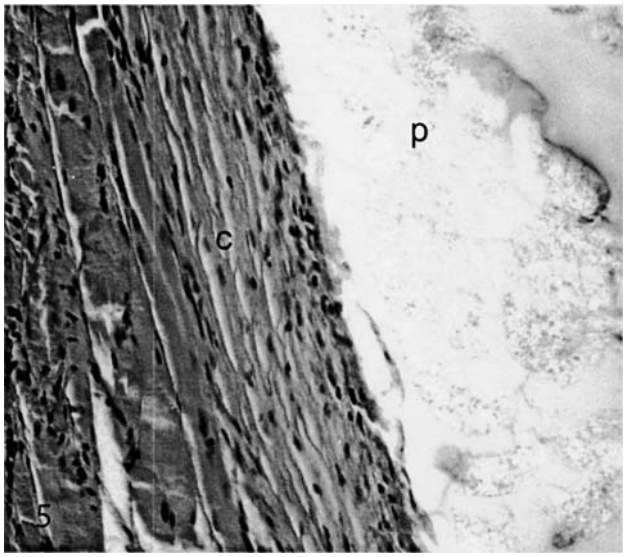

Fig. 5 PLGA membrane without plasticizer 60 days after implantation. Note the capsule (c) surrounding the degraded polymer (p). Paraffin, HE, 200X.

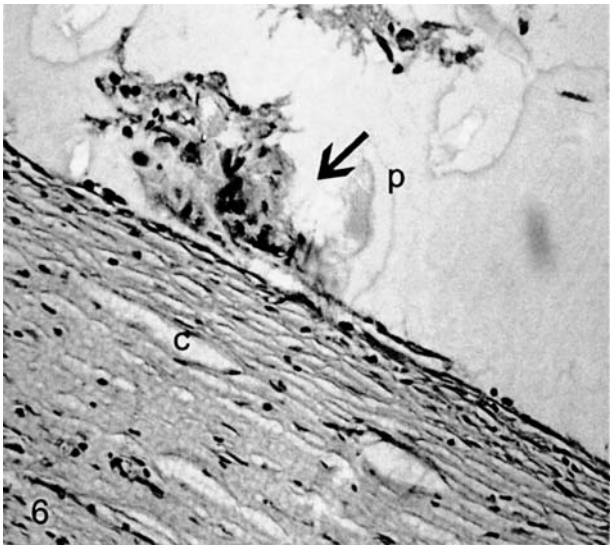

Fig. 6 PLGA membrane with 7% plasticizer 60 days after implantation. Note the capsule surrounding the membrane (c) and the cellular infiltration with giant cells (arrow) around the polymer fragment (p). Paraffin, HE, 200X.

fragments surrounded by a delicate network of connective tissue, with giant cells invading the small fragments (Fig. 7). In membranes with plasticizer the invasion was less marked and there was less fragmentation. Cells were seen adhered to the membrane and the capsule contained a large number of collagen fibers (Fig. 8).

- Membrane appearance after implantation in Scanning electron microscopy (SEM)

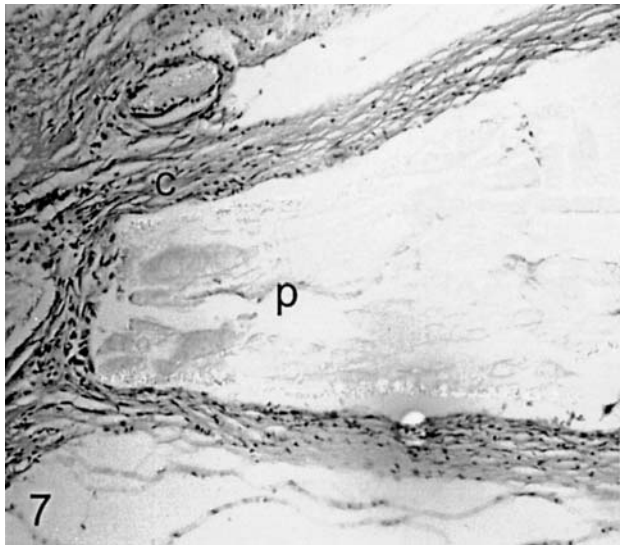

Fig. 7 PLGA membrane without plasticizer 120 days after implantation. Note the connective tissue infiltrate (c) within the polymer particles (p). Paraffin, HE, 100X.

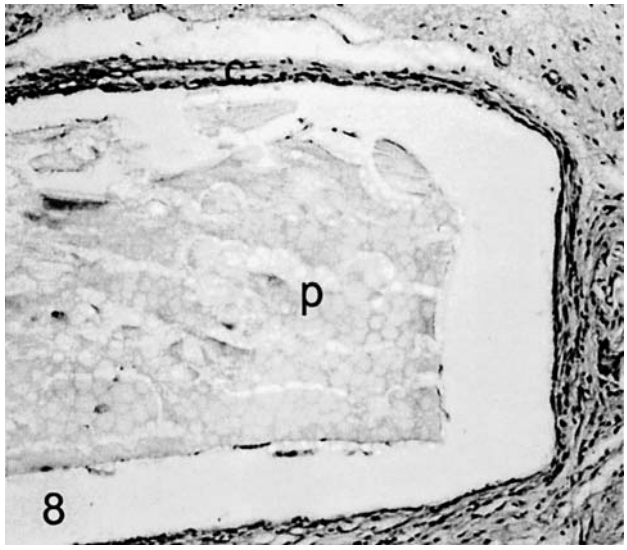

Fig. 8 PLGA membrane with 7% plasticizer 120 days after implantation. Note the collagen fibers of the capsule (c) within the polymer (p). Paraffin, HE, 100X.

Without implantation

SEM showed that before implantation the membranes with and without plasticizer had a dense morphology and smooth structure. Heterogeneous globule formation was seen in some places and there were no pores in the membranes because of the amorphous nature of this polymer (Figs. 9,10).

2 days post-implantation

SEM of membranes without plasticizer revealed only a few cells adhered to the membrane surface but no invasion of

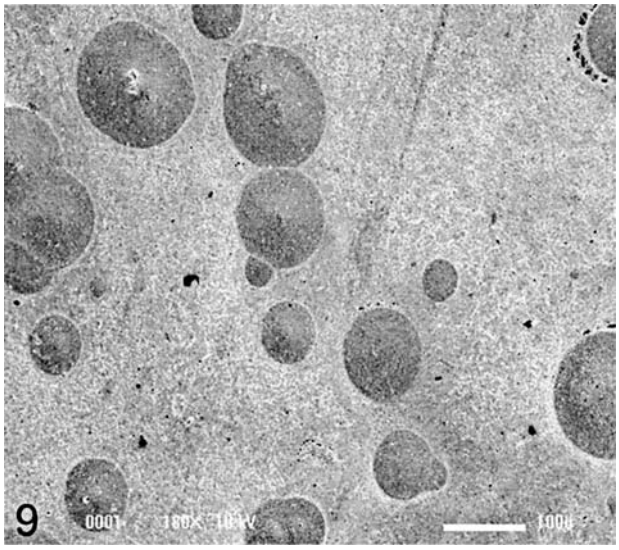

Fig. 9 SEM of PLGA membrane without plasticizer, before implantation. Note the face of evaporation of the membrane.

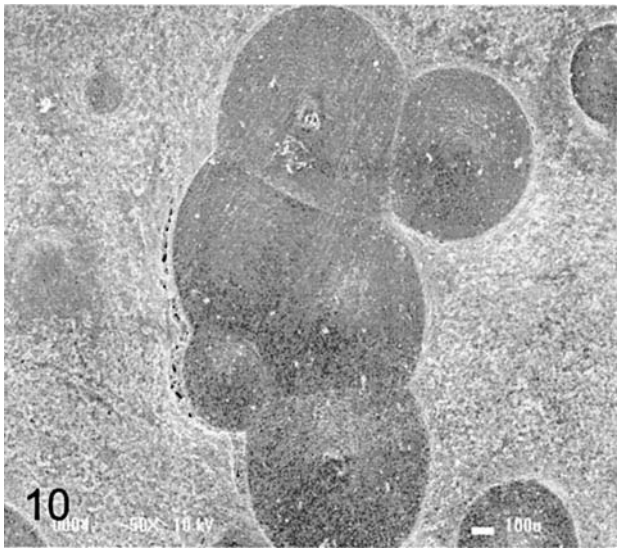

Fig. 10 SEM of PLGA membrane with 7% plasticizer, before implantation. Note the densely compact morphology of the membrane.

the polymer by cells and or components of the extracellular matrix (Fig. 11). In membranes with plasticizer there was considerable interaction of the polymer with the tissue. Fibrin was observed and the polymer showed extensive roughness that facilitated cell adhesion (Fig. 12).

30 days post-implantation

SEM of membranes without plasticizer revealed extensive adhesion of the tissue to the surface of the membrane (Fig. 13). In membranes with plasticizer, there was degradation of the polymer with the appearance of fractures in the membrane, as well as cellular invasion (Fig. 14).

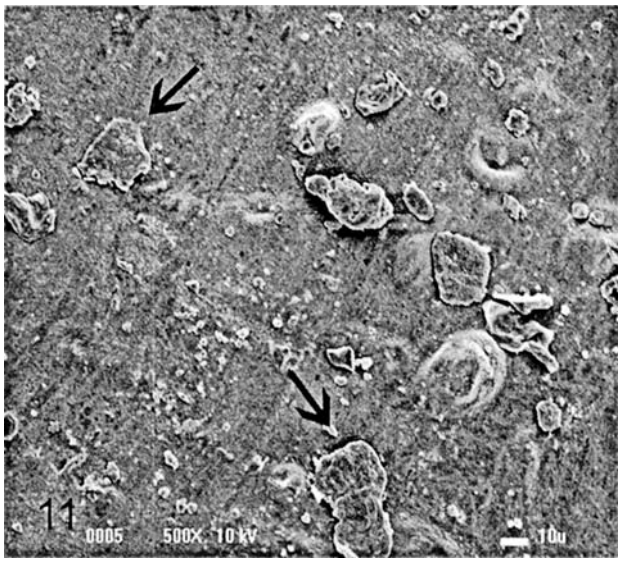

Fig. 11 SEM of PLGA membrane without plasticizer 2 days after implantation. Note the adhesion of few cells to the membrane surface (arrows).

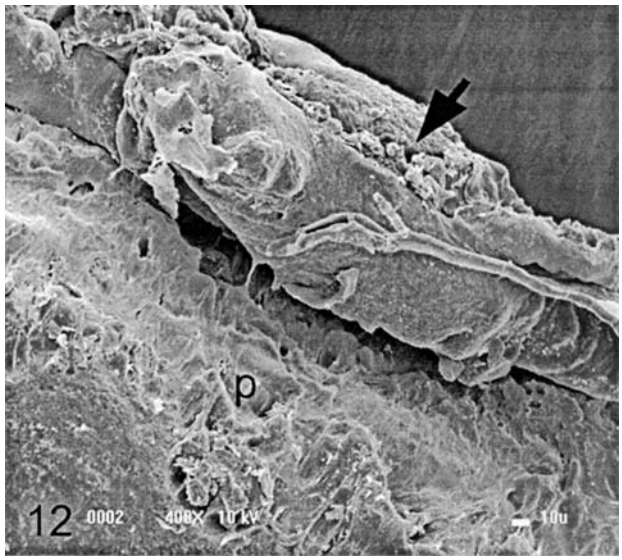

Fig. 12 SEM of PLGA membrane with 7% plasticizer 2 days after implantation. Note the adhesion cells to the membrane surface (arrow) and extensive rugosities to the polymer (p).

60 days post-implantation

SEM of membranes without plasticizer showed a fibrous capsule and a few cells adhered to the polymer, with no uniform degradation (Fig. 15). In membranes with plasticizer, tissue was seen adhering to the surface of the membrane and there were fractures in the membrane surface (Fig. 16).

120 days post-implantation

SEM of membranes without plasticizer showed that most of the units were fragmented, indicating marked degradation

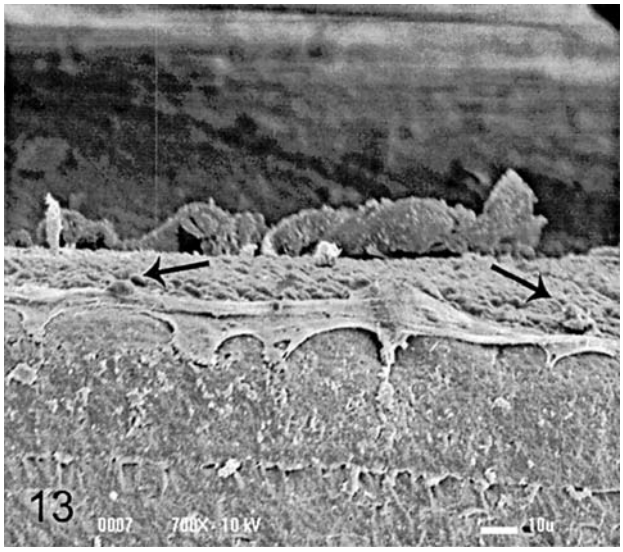

Fig. 13 SEM of PLGA without plasticizer 30 days after implantation. Note the adhesion to the cells and tissue to surface of the membrane (arrows).

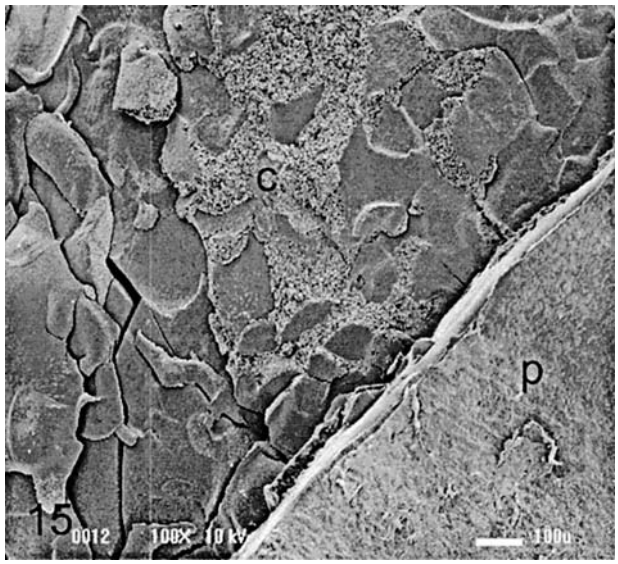

Fig. 15 SEM of PLGA without plasticizer 60 days after implantation. Note the capsule of connective tissue (c) with few cells adhered, and the polymer (p) with non uniform degradation.

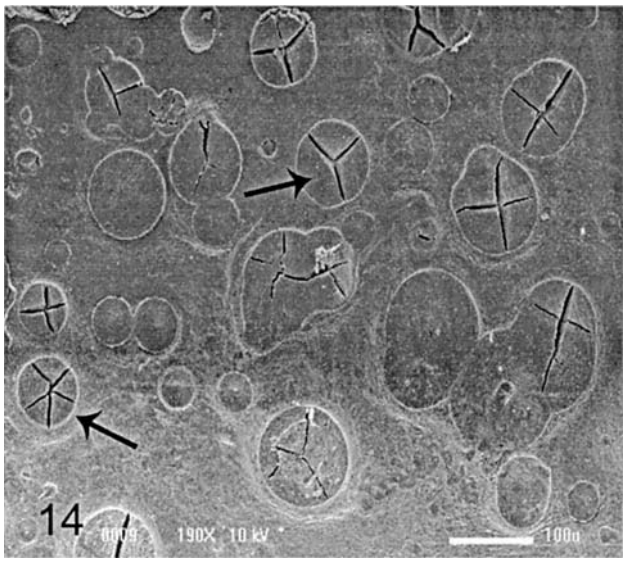

Fig. 14 SEM of PLGA with 7% plasticizer 30 days after implantation. Note the degradation process with the appearance of fractures (arrows).

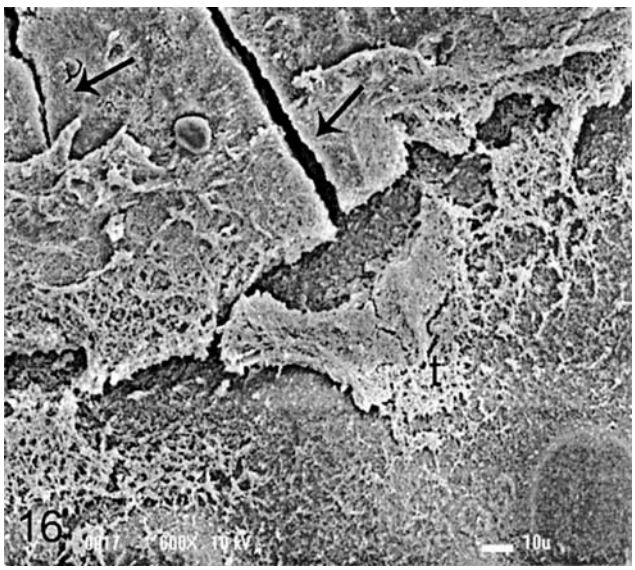

Fig. 16 SEM of PLGA with 7% plasticizer 60 days after implantation. Note the fractures (arrows) in the membrane surface with tissue adhered (t).

and tissue invasion (Fig. 17). In membranes with plasticizer there was intense degradation with fragments of polymer embedded in conjunctive tissue, as well as a large number of cells and collagen fibers (Fig. 18).

The surface chemical composition and topography affect the interaction force that acts between the biomaterial and biological medium (for instance, water and ion sorption, protein adsorption, adhesion, cellular expansion and proliferation). The surface roughness is one of the most important parameter to be considered in the design of devices to be used in tissue engineering. The surface roughness can influence the adsorption of protein layers, inflammatory response, and cellular adhesion [13].

Data of AFM showed that for pure PLGA the mean roughness was 1.4 nm (Ra) with a rough and homogeneous surface (Fig. 19). On the other hand, the plasticizer had strongly altered the PLGA surface becoming more heterogeneous with a flat surface but with irregular elevations, so his roughness was elevated to 21.3 nm (Ra) (Fig. 20).

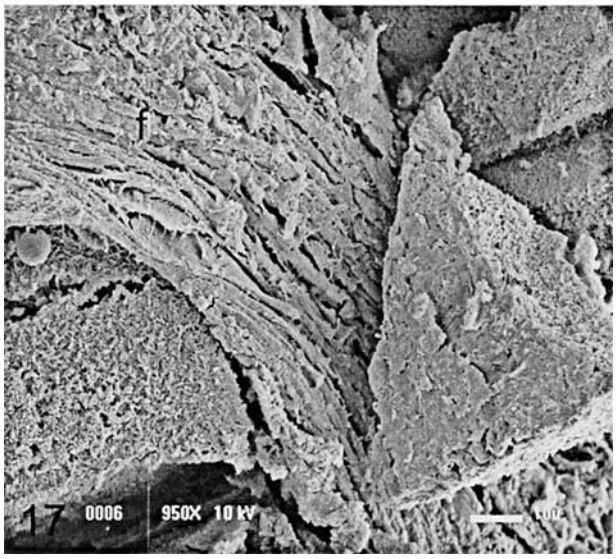

Fig. 17 SEM of PLGA without plasticizer 120 days after implantation. Note the invasion of cells and collagen fibers thought the fractures in the membrane (f).

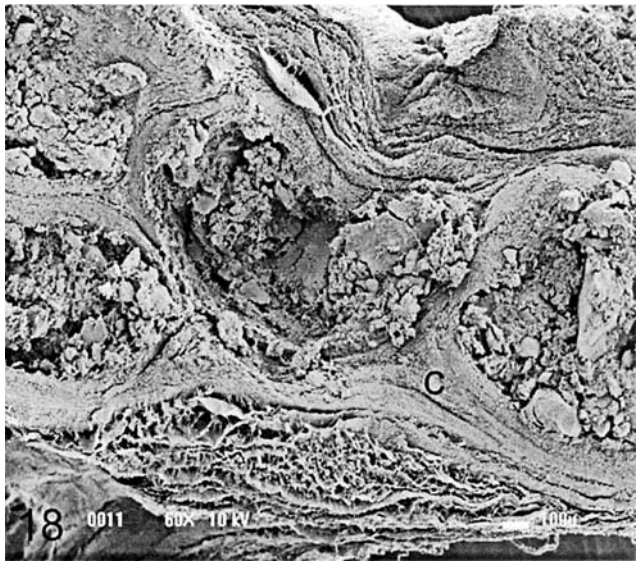

Fig. 18 SEM of PLGA with 7% plasticizer 120 days after implantation. Note the capsule of fibrous connective tissue (c) within the fragments of membrane.

Discussion

As observed by LUCIANO [8,9], the addition of triethylcitrate, a biocompatible plasticizer, resulted in porous membranes and conferred flexibility in PLLA polymers, that allowed the membranes to adapt to the movement of soft tissue. Membranes without plasticizer showed less tissue adhesion to the implanted material and degraded more slowly. Whereas low porosity membranes are suitable for guided tissue regeneration, high porosity membranes are useful for tissue reconstruction since they provide better cell adhesion and migration.

Porous matrices provide an appropriate environment for cell growth and extracellular matrix synthesis. The uniform distribution and interconnection of the pores is important for facilitating the formation of tissue in an organized network, as in the case of bone and cartilaginous tissues [9].

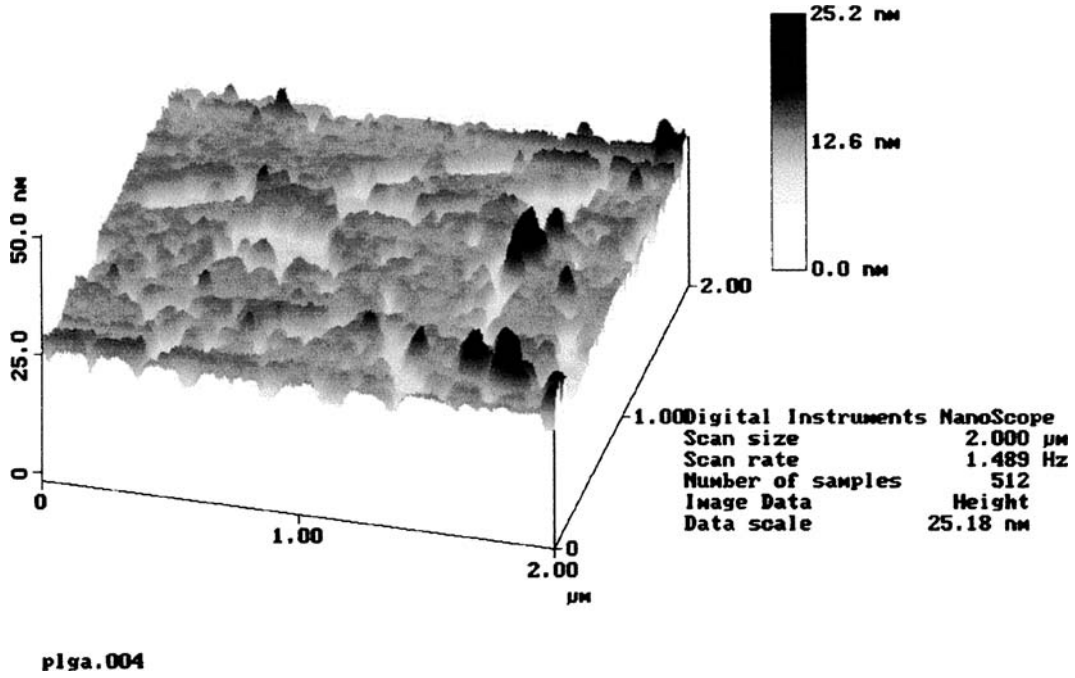

Fig. 19 AFM of PLGA without plasticizer.

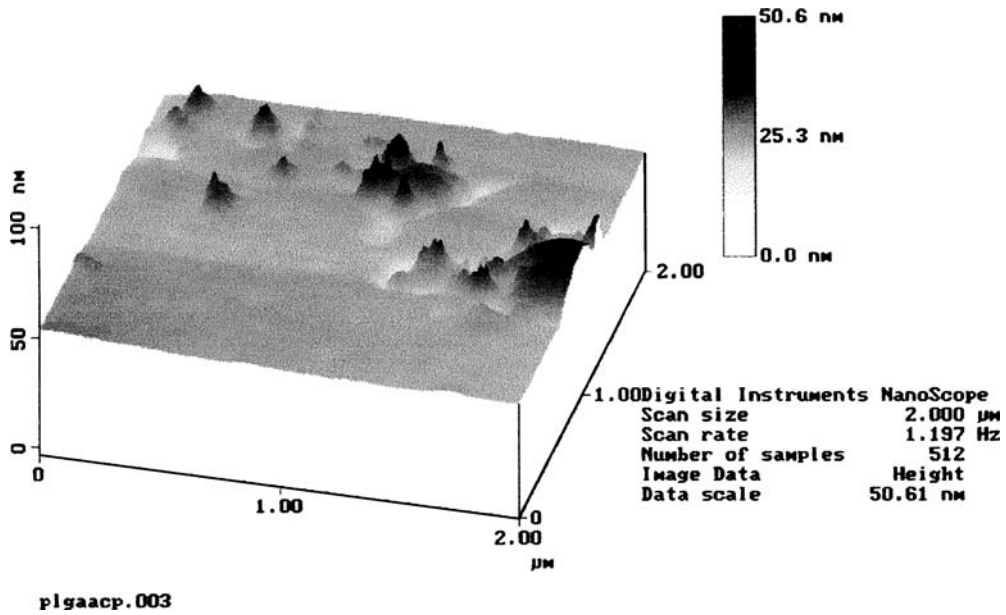

Fig. 20 AFM of PLGA with plasticizer.

Previous studies by our group [2,8,11] showed that the formation of pores in membranes is related to the semicrystalline structure of the polymer. In the present study, the addition of 7% plasticizer to PLGA, a completely amorphous polymer, prevented the appearance of pores, as shown by SEM. Light microscopy revealed less tissue invasion in membranes without plasticizer when compared the PLLA membranes with plasticizer.

Although the plasticizer prevented the formation of pores, as verified by SEM, AFM analysis showed that the plasticizer became the surface of membranes smooth with some point of roughness while, membrane without plasticizer presented a homogeneous roughness for all its extension. However, in case of PLGA, there is a competition between the effect of plasticizer and the short degradation time of polymer in the invasion cellular. In this case, the short degradation time resulting of addition of plasticizer, prevails over the roughness of surface and consequently allow a more invasion cellular.

In samples obtained early after implantation (2 and 30 days), the membrane was separated from its adjacent tissue. However, after longer periods of implantation (60 and 120 days), the polymer was surrounded by adjacent tissue in the subcutaneous compartment. Increasing vascularization of the implant area was seen during degradation of the samples, but there were no macroscopic signs of an inflammatory response. In all of the samples, except for that obtained after two days, there was a fibrous capsule around of the membrane similar to that described by Spector et al. [13].

Analysis of the sample obtained two days after implantation revealed an inflammatory reaction with many neutrophils, macrophages and, more rarely, eosinophils. Local trauma following surgical procedures provoked a strong inflammatory reaction for up to 7 days after the implantation. After this period, the strong inflammatory reaction was replaced by a reaction against the implant [14,15].

The presence of giant cells in all implants, except for the second day, has been noted by many authors. According to Lam et al. [16], the giant cells observed in foreign body reactions originate from the fusion of macrophages, a process induced by cytokines such as interleukin 4 and interferon gamma. These cells showed a large number of mitochondria and this may be associated with the elimination (degradation) of PLGA via the tricarboxylic acid cycle to generate carbon dioxide.

The degradation of membranes containing 7% plasticizer, was quicker than for membranes without plasticizer. Membranes with 7% plasticizer showed accentuated degradation about 60 days after implant action while in membranes without plasticizer this degradation occurred later.

SEM showed that prior to implantation the membranes has little porosity, whereas 60 days after implantation there was much more degradation than after 30 days. In this analysis its no possible observe different kinds of cells, but only cells morphology characteristics. Through the pictures, was possible observe the no interaction of the polymer with the capsule because the short period of implantation or because the polymer density, without pores, creating a space between the polymer and the capsule.

No neoplasms were observed in the 120 days following implantation. The addition of the plasticizer triethylcitrate minimized the rigidity of the polymer and reduced its degradation time, then decreasing the chances of a neoplasm formation in the area of the implant [9,11]. In addition to reducing the time needed for degradation

differentiated the presence of a plasticizer provides membranes with a porosity and malleableness that does not alter their biocompatibility. By reducing the time needed for polymer degradation plasticizer can decrease the mechanical shock between the implant and the tissue and, consequently the risk of malignant tumors [9].

The principal difference between the normal process of tissue repair and the response to implants of PLGA was the presence of a capsule of conjunctive tissue around the implant from the 30th day onwards. This capsule is part of a characteristic response to a foreign body, and is an attempt to isolate this body from the organism. The giant multinucleated cells may have an important role in the removal of foreign elements, as extensively discussed in the literature.

Conclusions

The addition of plasticizer greatly improved the tissue-membrane interaction, but that the membrane quickly lost its properties. In contrast, membranes without plasticizer retained their properties for much longer periods [2,9,11]. The plasticizer acted by reducing the interaction among the chains, thus favoring a flexible membrane. This finding limits the use of plasticizer to techniques that included recover from lesions or cell culture in which there is fast cell migration and invasion by extracellular matrix to substitute the degraded membrane.

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