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Osteointegration of poly(L-lactic acid)PLLA and poly(L-lactic acid)PLLA/poly(ethylene oxide)PEO implants in rat tibiae

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Abstract Natural or synthetic materials may be used to aid tissue repair of fracture or pathologies where there has been a loss of bone mass. Polymeric materials have been widely studied, aiming at their use in orthopaedics and aesthetic plastic surgery. Polymeric biodegradable blends formed from two or more kinds of polymers could present faster degradation rate than homopolymers. The purpose of this work was to compare the biological response of two biomaterials: poly(L-lactic acid)PLLA and poly(L-lactic acid)PLLA/poly(ethylene oxide)PEO blend. Forty four-week-old rats were divided into two groups of 20 animals, of which one group received PLLA and the other PLLA/PEO implants. In each of the animals, one of the biomaterials was implanted in the proximal epiphysis of the right tibia. Each group was divided into subgroups of 5 animals, and sacrificed 2, 4, 8 and 16 weeks after surgery, respectively. Samples were then processed for analysis by light microscopy. Newly formed bone was found around both PLLA and PLLA/PEO implants. PLLA/PEO blends had a porous morphology after immersion in a buffer solution and in vivo implantation. The proportion 50/50 PLLA/PEO blend was adequate to promote this porous morphology, which resulted in gradual bone tissue growth into the implant.

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1 Introduction

The skeleton is subject to several injuries, such as abnormal development or osseous defects, where the bone loss has been caused by tumor resection. Moreover, mechanical traumas to the skeleton due to both automobile and occupational accidents tend to affect young people at the most productive period of their working life [1–3]. The material of first choice in the treatment of bone defects is autogenous bone graft. In spite of being an important resource for bone reconstruction, its utilization is limited to small quantities. In addition, complications such as donor-site morbidity and postoperative reabsorption are likely to occur [4]. In face of the challenge of finding a good substitute material for bone grafting, metals and ceramics have been clinically used to reinforce or replace bone grafts. Although the use of these alternate materials in maxillofacial and orthopaedical surgery had reported initially positive results, it was later recommended with reservation, given the possibility of bone reabsorption at the implant site and fracture resulting from stress transfer. Moreover, the need for subsequent surgery to remove the material would lead to additional expenses and traumas [5]. Hydroxyapatite ceramics (HA) has shown to be biocompatible, nontoxic and osteointegrable. Nevertheless, in spite of several applications in dentistry and cranial facial surgery, this porous ceramics presents low mechanical resistance, what restricts its usage [6]. Yet, researchers have reported cytokine and protease expression and production by fibroblasts, in response to particles released by

HA implants in vitro. These proteins act on the development of osteolysis, possibly leading to loss of material when implanted [7]. In order to substituting both metals and ceramics for more biointegrable and resistant materials, several polymers have been studied and indicated for medical applications. Poly(methylmethacrylate) PMMA [8], as well as some biodegradable polymers, such as poly(dioxanone) PDS [9] and poly(caprolactone) PCL [10], are some of the polymers which have been researched. Apart from the utilization of polymers in pure form, researchers have prepared polymeric mixtures, denominated blends, with a view to controlling its mechanical resistance and degradation speed. In order to facilitate osseointegration with host tissue and even allow association with osteoinductive drugs, different polymers might be combined [11, 12]. The poly(L-lactic acid) PLLA/poly(ethylene oxide)PEO blend has been investigated in vitro tests [13] where PLLA/PEO membranes have been submerged in a phosphate buffer solution pH 7.4 at 37°C, so as to simulate in vivo implant conditions. The objective of this work was to assess in vitro and in vivo tissue response to poly(L-lactic acid) PLLA and 50/50 poly(L-lactic acid) PLLA/poly(ethylene oxide)PEO blend when implanted in defects surgically produced in the tibia of rats.

2 Materials and Methods

2.1 Production of blend discs

The blend was prepared by mixing PLLA (Medisorb; MW = 300,000 g/mol) and PEO (Aldrich; MW = 200,000 g/mol) in a mini injector LMM-2017 Mini Max Molder. Sticks of PLLA and 50/50 PLLA/PEO were prepared through the melting of homopolymers at 190°C, using a 2.0 mm diameter and 9.3 cm high (internal dimensions) mold, which remained at 120°C during the processing. The heating of the homopolymer mixture was carried out for 1 min followed by 2 min of shearing and mold injection. The mold was cooled at room temperature for 20 min.

2.2 In vitro study

In vitro degradation tests were carried out using 50/50 PLLA/PEO blends. Samples were immersed in a buffer solution (KH_2PO_4–NaOH; pH = 7.4) at 37°C, which was changed every day. Tests were performed during a 2-week period. These conditions have often been referred to in literature as degradation tests [14]. Blends were immersed for different periods (7 or 14 days). After each period, samples were dried at 50°C until they reached a constant mass. The mass loss percentage was calculated by comparing mass values of the samples before and after submitting them to degradation tests. Samples are denoted here as a function of the degradation time. PLLA/PEO $t = 0$ was used for blends that were not immersed in the buffer solution, whereas PLLA/PEO $t = 7$ or $t = 14$ days were used for blends that went through the degradation process. Samples were fractured after immersion into liquid nitrogen. Surface fracture was covered with gold by sputtering and observed in a JEOL JXA 840 scanning electron microscope.

2.3 In vivo study

A total of 44-week-old male albino Wistar rats (Rattus norvegicus) were used. The animals were anesthetized with a solution of 1:1 Chlorhydrate of Xylazine (Virbaxyl 2%®) + Ketamine (Francotar®) administered intramuscularly at the dose of 1.5 ml/kg body weight. A longitudinal incision was made through the skin on the medial surface of the right posterior limb below the knee. The muscular tissue and the periosteum were removed to expose the cortical bone of the medial face of the proximal epiphysis of the tibia. After that, a bone defect was produced with a 2 mm diameter drill attached to a low-speed dental engine. During the procedure, the wound was flushed with isotonic saline to reduce heat generation and limit the temperature to which the bone was exposed. The bone defect was filled with a 2 mm long and 2 mm in diameter cylinder-shaped implant. The animals were divided into two groups, 20 animals received the implant of poly(L-lactic acid) PLLA/poly(ethylene oxide)PEO blend and the remaining 20 animals received the poly(L-lactic acid) PLLA implant. After the 2, 4, 8 and 16-week post-surgical periods the animals were sacrificed with a lethal dose of the anesthetic and the right tibia were dissected. The segment of the bone contained the implant was fixed in a 10% formaline buffered solution 0.1 M pH 7.3 for 72 h. Right after that, the samples were immersed in EDTA solution for decalcification. The samples were paraffin-embedded and transversal sections (7 μm) were obtained and stained with Hematoxylin and Eosin (HE).

The experimental protocol used in this work is in agreement with both the standards of The American Society for Testing and Materials (ASTM F-981-93) and the Ethical Principles for Animal Experimentation adopted by the Brazilian Animal Experimentation Board (COBEA). It was also approved by the Internal Ethics in Animal Experimentation Commission (CEEA) from the Biology Institute of The State University of Campinas IB-UNICAMP under protocol number 097-02.

3 Morphometry and statistical analysis

The volume of newly formed bone closed to implants was obtained using a 100-point quadrilateral grid system coupled to the ocular (10×) of a Carl Zeiss light microscope. Five areas of the implantation site of each animal were used for quantification. The volume (%) of bone was calculated using the formula $Vv = Pp/Pt$, where Vv is the volume, Pp is the number of points on the newly formed bone, and Pt the total number of the system points (100), according to the Delesse Principle [15].

Statistical analysis was conducted for two variables: material implanted (PLLA and PLLA/PEO) and experimental periods. Nonparametric variance was carried out, coupled with the respective multiple-comparison tests [16]. Analyses of statistic test were made using significance level of 0.05.

4 Results

4.1 In vitro study

The mass loss percentage for PLLA/PEO blend occurred during the period of 14 days of in vitro degradation. The highest mass loss percentage occurred during the first week of in vitro degradation (47.0% in $t = 7$ days and 46.0% in $t = 14$ days). This process is related to water diffusion in the blend followed by dissolution of the PEO fraction. Since the PLLA fraction presents a low degradation rate, mass loss practically did not change during the period from 7 to 14 days of degradation. The Fig. 1 shows the surface fracture of PLLA/PEO blend observed by scanning electron microscopy as a function of the degradation time. For samples, which were not immersed into the buffer solution ($t = 0$), the occurrence of phase separation was not clear. However, after a period in a buffer, all the channels could be observed. The morphology of the blends at the end of the first week ($t = 7$ days) was similar to that shown in blends at the end of the second week ($t = 14$ days). For 50/50 PLLA/PEO blends, an intermediate situation was found, where the dense structure became cracked and surrounded by channels with pores in them.

4.2 Microscopic observations

4.2.1 PLLA implants

An immature bone layer was formed around the PLLA implant 2 weeks after the implantation (Fig. 2a). The defect in the cortical layer of the bone was filled with blood vessels, connective tissue and inflammatory cells. After 4 weeks, the defect was partially filled by bone tissue. Some areas of the bone layer formed around the implant presented mature aspect, in which the matrix showed small lacunae. After 8 weeks, the defect in the cortical layer was filled with secondary bone and connective tissue cells. The bone layer around the implant was thicker and uniform than in other periods. A thin layer of fibroblast-like cells was formed on the surface of the implant (Fig. 2b). After 16 weeks, the defect of the cortical layer was filled with bone tissue. The newly formed bone layer around the implant presented mature aspect. In two animals, there were some little spaces formed on the surface of the implants, which were filled with bone tissue.

4.2.2 PLLA/PEO blend implants

Bone formation occurred around the implant two weeks after the implantation of the blend. The defect was occupied mainly by connective tissue with indifferentiated cells, which had also invaded spaces formed on surface of the implant in the blend (Fig. 2c). Four weeks after implantation, the layer of newly formed bone surrounding the implant was thicker. Bone formation and connective tissue were observed in the defect of the cortical region. After 8 weeks the layer of newly formed bone surrounding the implant was evidentially thicker than in the previous periods. Several spaces were present in the implants, which were filled by bone tissue (Fig. 2d). With 16 weeks, the defect of the cortical layer of the tibia was completely filled by mature bone tissue. In two animals, greater amount of

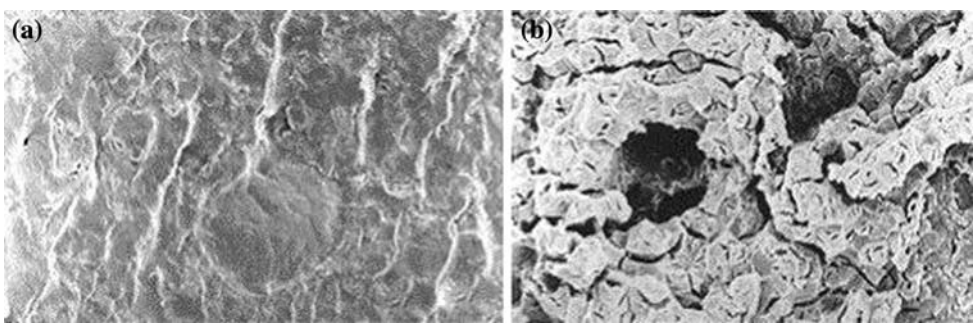

Fig. 1 Scan photomicroscopy of 50/50 PLLA/PEO blend. (**a**) Before immersion in buffer solution ($t = 0$), (**b**) after immersion in buffer solution ($t = 14$). 500×

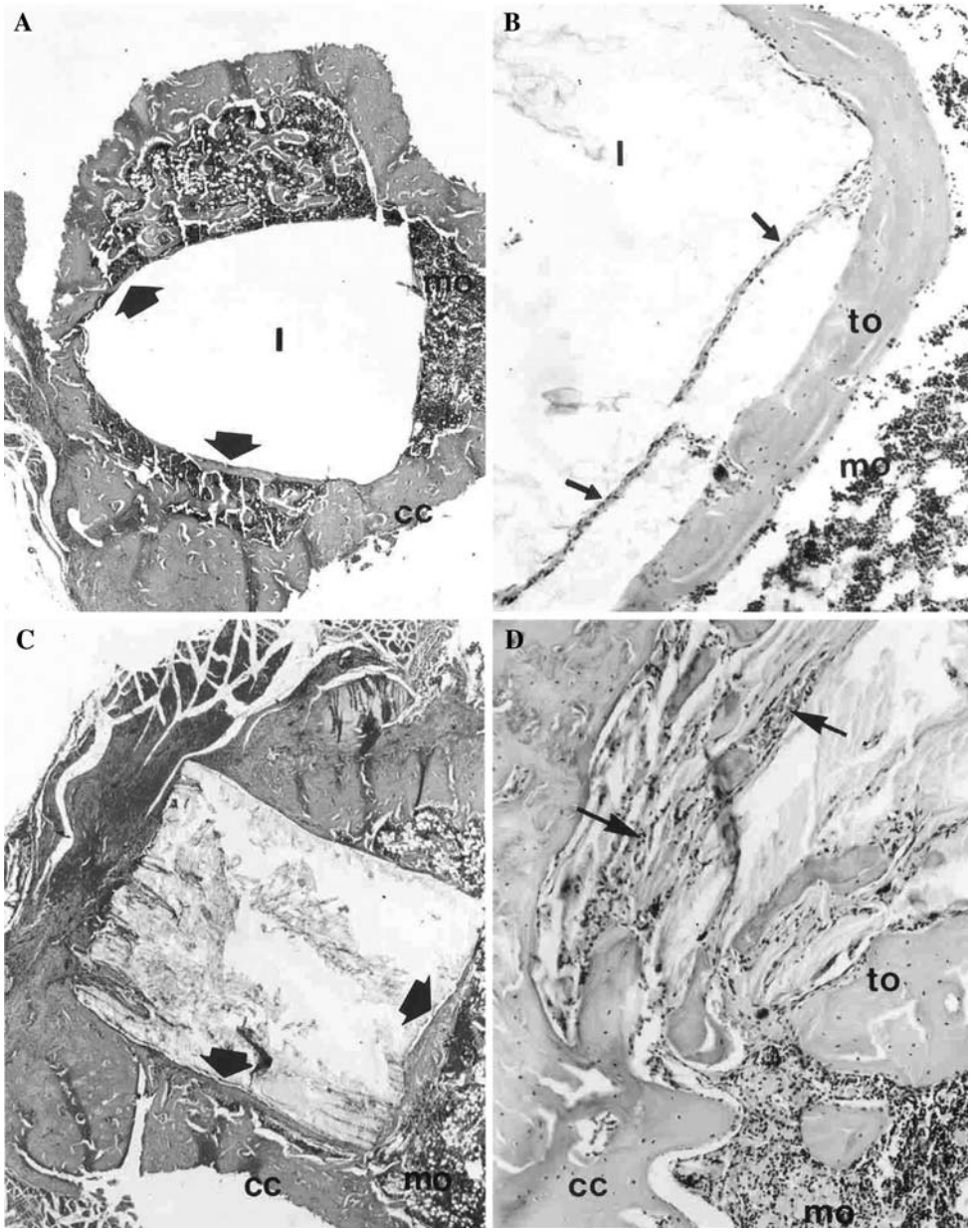

Fig. 2 (**a**) PLLA 2 weeks after surgery: bone tissue (arrows) around the implant (I); Cortical (cc). 23× (**b**) PLLA 8 weeks after surgery: bone tissue (to) around the implant; fibroblast-like cells (arrows) close to implant (I); bone marrow (mo). 113× (**c**) PLLA/PEO 2 weeks after implant showing tissue grown into the blend; bone (arrows) formed around the implant; cortical (cc); bone marrow (mo). 23× (**d**) PLLA/PEO implant 8 weeks after surgery: cell proliferation (arrows) and bone tissue (to) grown into implant; cortical bone (cc); bone marrow (mo). 113× H&E

spaces was filled by bone than previous periods. Other microscopic aspects were similar to the animals of 8 weeks.

5 Quantitative analysis

According to Fig. 3 and Table 1, the animals with PLLA/PEO blend showed no significant difference as to the volume of newly formed bone between 2 and 4 weeks of implantation. However, the values obtained for bone volume in animals with PLLA/PEO after 8 weeks were significantly bigger and remained constant until the sixteenth week. After 8 and 16 weeks the volume of newly formed closed to PLLA and PLLA/PEO bone increased.

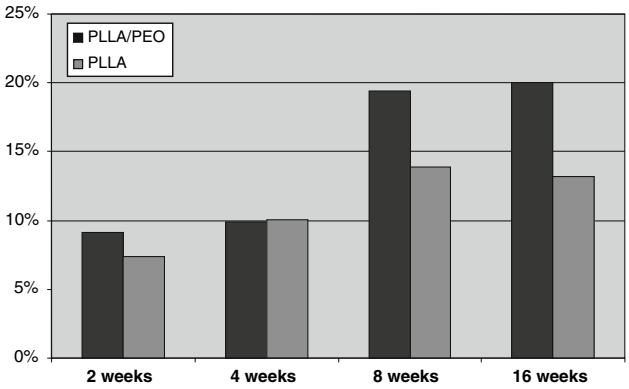

Fig. 3 Volume percent of newly formed bone close to PLLA and PLLA/PEO implant

Table 1 Percentage of newly formed bone and respective results of statistical test

Material	Experimental periods (weeks)				Results of statistical analysis
	2	4	8	16	
PLLA/PEO	9.11 ± 2.56[a]	9.91 ± 1.79[a]	19.40 ± 4.88[a]	20.00 ± 5.23[a]	12.33 ($P < 0.01$)
PLLA	7.38 ± 2.19[a]	10.03 ± 1.94[a]	13.88 ± 3.50[b]	13.20 ± 1.45[b]	13.23 ($P < 0.01$)

Fixed the time, different letters indicate significant differences

However, the volume of bone formed around the PLLA/PEO blend implants was significantly greater.

6 Discussion

Bone tissue was formed in all implantation sites of both PLLA and PLLA/PEO groups. In PLLA implants, bone tissue surrounded the whole implant surface, whereas in the PLLA/PEO group, besides the newly formed bone tissue around the implant, bone tissue was also found in grooves formed along the surface of the implant. According to Refs. [17] and [18], this bone layer that surrounded the implants is a tissue response to surgical implantation procedure. Osteogenic cells from the cortical layer, bone trabeculae and bone marrow were stimulated. After 4 weeks, there was a noticeable increase in the spaces formed inside PLLA/PEO implants, which were gradually filled by bone tissue. This result suggests that the action of the extra cellular fluid and the tissue response at the implantation site induced the degradation of the bend, leading a continuous increase of the spaces formed into the implant.

The PLLA/PEO implant degradation is due the PEO fraction of the blend. PEO is a polymer that quickly dissolves when in contact with tissular liquids. This assumption may be confirmed in vitro and by the formation of spaces inside the blend from the fourth week of implantation, which confer porosity to implant. Because PEO degradation provides adequate porosity for tissue ingrown to implant, the proportions between the polymers utilized in the preparation of the blend should be considered. The proportion 50/50 PLLA/PEO blend, utilized in this work, was found to be adequate for allowing bone tissue ingrown to implant. On the other hand, PLLA pure implants did not dissolve in the same way to allow bone tissue ingrown to implant. PLLA is a polymer that dissolves slowly, what, however, does not reduce its biocompatibility [18, 19]. Meikle et al. [20], studying 50/50 poly(DL-lactic acid)/poly(glycolic acid) associated with bone growing factors (BMP) in rabbit cranial defect repair, concluded that this blend allowed bone repair and the degradation of the polymer contributed for the release of BMP.

Regarding the volume of newly formed bone closed to implants, between 2 and 4 weeks following implantation, the amount it obtained for both PLLA and PLLA/PEO implants were equivalent. However, from the eighth week on, there was a significant increase of bone volume in the PLLA/PEO blend compared that PLLA implants, what was observed until the end of the experiment. This bone volume difference found between the two types of implants from eighth week is due the time of degradation of them, which was less for the PLLA/PEO blend. Thus, the PLLA/PEO blend became gradually more porous and allowed bone tissue ingrown closed to implants.

The results obtained in this work showed no evidence of incompatibility for both PLLA and PLLA/PEO implants after implantation. However, the PLLA/PEO blends promoted better osseointegration with host tissue than PLLA. In addition, PLLA/PEO blend might offer other alternatives for bone reaper treatment, such its association with antibiotic drugs to prevent bone infections [21–23], or even its association to osteoinductive proteins, such as BMP—Bone Morphogenetic Protein [24–26].

7 Conclusions

Based on our results, we conclude that the PLLA/PEO blends had a porous morphology after immersion in a buffer solution and in vivo implantation. The proportion 50/50 PLLA/PEO blend was adequate to promote this porous morphology and consequent gradual bone tissue growth into implant. Finally, neither PLLA nor PLLA/PEO implants showed evidence of incompatibility.

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