DOI: 10.1002/masy.200651372

Preparation and Characterization of Resorbable Hydrogel Membranes Useful for Tissue Engineering

Walker Soares Drumond,[1] *Walter Israel Rojas-Cabrera,*[2] *Lili Zhao,*[1]
Maria Leonora de Castro,[1] *Dalva Cruz Laganá,*[2] *Shu Hui Wang**[1]

Summary: New synthetic polymeric membranes of lactic acid were prepared with different chemical composition through copolymerization with ε-caprolactone and ethylene oxide segments. The prepared copolymers were characterized by hydrogen and carbon-13 nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) and presented wide range of mechanical properties demonstrated by dynamic mechanical thermal analysis (DMA). Porous membranes were prepared by dissolving the polymer in the presence of salts (NaCl or hydroxyapatite) and/or lower mass poly(ethylene glycol) ($Mn = 600$ or $1{,}000\ g \cdot mol^{-1}$). The membranes presented new mechanical and biological characteristics and were suitable for cell penetration, anchorage, differentiation and proliferation. Adherence of cultured fibroblasts on membranes indicates biocompatibility.

Keywords: biocompatibility; copolymers; lactide; mechanical properties; porous membrane

Introduction

The Guided Bone Regeneration (GBR) therapy praises the use of a membrane as physical barrier to re-cover the zone of bone defect. The membranes protect the injured area from invasion of connective and epithelial tissues, thus allowing the bone cell regeneration in this area.[1,2] These membranes also act protecting and preserving the blood clot also essential for bone process repair.[3]

Non-degradable membranes, as expanded polytetrafluoroethylene (e-PTFE) and polystyrene, have been used in GBR. However these materials require removal in a second chirurgic operation after bone regeneration. To avoid this problem, membranes from biodegradable materials, for example collagen and synthetic biodegradable polymers, have been investigated.[4]

Collagen membranes have been used due to their excellent biocompatibility, however, these membranes have an antigenic effect and carry the risk of bovine spongiform encephalopathy transmission. Moreover, pure collagen membranes are not osteoinductors and are quickly resorbed, jeopardizing the barrier function.[5]

Synthetic biodegradable poly(α-ester)s, e.g. poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(caprolactone)(PCL) and copolymers of them, have been manufactured because of their biodegradability since the 1970s and are natural candidates for this type of application. In living organism, PLA is degraded by hydrolysis into lactic acid, which under aerobic conditions is further metabolized into water and carbon dioxide and finally excreted by the organism.[6]

Commercial polylactides are found in two forms, semicrystalline poly(l,l-lactide) (PLLA) and amorphous poly(d,l-lactide) (PDLLA). Recent studies have demonstrated that poly(L-lactide) (PLLA) or poly(ε-caprolactone) are slowly degraded

---

[1] Departamento de Engenharia Metalúrgica e Materiais, Escola Politécnica da Universidade de São Paulo, Av. Prof. Mello Moraes 2463, 05508-900, São Paulo, SP, Brazil
E-mail: wangshui@usp.br

[2] Departamento de Prótese, Faculdade de Odontologia da Universidade de São Paulo, Av. Professor Lineu Prestes 2227, CEP 05508-900 São Paulo, SP, Brazil

due to their crystallinity and may take several years to be completely absorbed into tissues, and therefore may impart long-term potential for tumorigenicity.[7] On the other hand, amorphous PDLLA is readily degraded in short time and its products on the market are reported to disappear in two weeks, which is too fast for replacement by natural tissue produced from cells.

The copolymerization with chemical composition and crystallinity adjustments represents an interesting alternative to prepare polymer materials with fine-tuned mechanical properties and controlled hydrophilic-hydrophobic balance, along with designed biodegradation profile. The microstructure and architecture of copolymers, prepared by the ring opening copolymerization of cyclic esters, result from the kinetics and thermodynamic of the polymerization systems and are related to reaction medium, catalyst, nature of the co-initiator and temperature.[8–16]

Polymers of l,l-lactide (LL) and ε-caprolactone (CL) have been usually prepared in bulk or in solution, using an organometallic initiator either in the presence or absence of a co-initiator containing active hydrogen(s). The initiator tin(II) octoate has been widely used in polymeric biomaterials preparation.

Recently, we have shown the correlation among composition, microstructure and biodegradability for a series of poly(ester-urethane)s containing different polycaprolactone segments.[17,18]

The objectives of the present study are to prepare biodegradable copolyesters (Fig. 1) with a wide span of mechanical properties and their porous membranes and to evaluate the influence of the polymer structure in their biocompatibility.

Experimental

Materials

l,l-Lactide [(3S)-cis-3,6-dimethyl-1,4-dioxane-2 ,5-dione], 98%, and stannous 2-ethylhexanoate ($SnOct_2$), and ε-caprolactone were supplied by Aldrich. Poly(ethylene

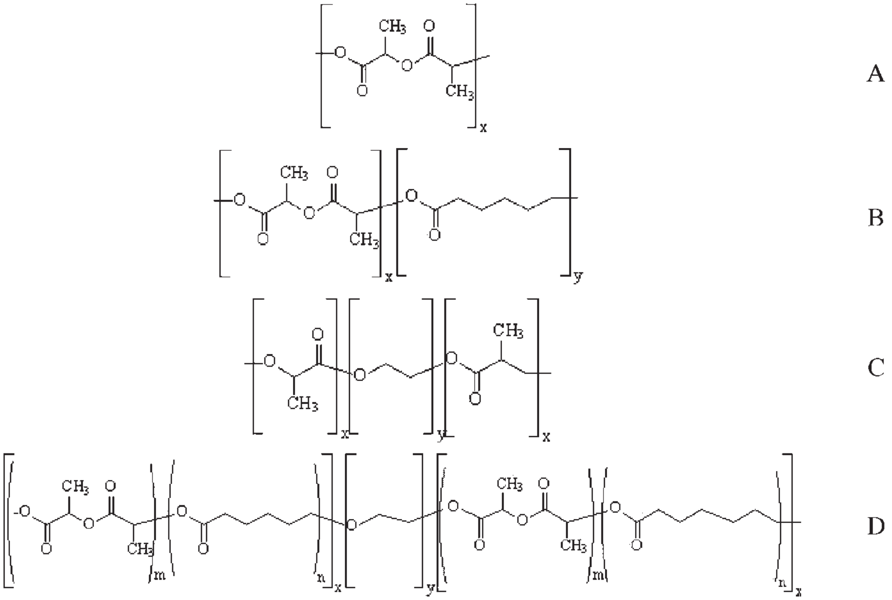

Figure 1.
General formula of (A) poly(l,l-lactide) (PLLA), (B) poly(l,l-lactide) – stat – poly(ε - caprolactone) (PLLA-PCL), (C) poly(l,l-lactide) – block – poly (ethylene oxide) – block – poly(l,l-lactide) (PLLA-b-PEO-b-PLLA) and (D) poly(l,l-lactide – stat – ε - caprolactone) – block – poly (ethylene oxide) – block – poly(l,l-lactide – stat – ε - caprolactone) (PLLA-PCL-b-PEO-b-PLLA-PCL).

oxide)s $M_n = 600, 1{,}000, 4{,}000\ g \cdot mol^{-1}$ from Oxiteno do Brasil, and $M_n = 20{,}000\ g \cdot mol^{-1}$ (Carbowax 20M) from Supelco, were dried in vaccum oven before use. Chloroform, methanol, hexane, toluene, tetrahydrofuran (THF) and acetone, analytical grade, were supplied by Synth (Brazil). All materials were used without further purification. Collagen membranes (20 × 30 mm sizes) were supplied by Consulmat (Brazil).

Polymer Synthesis

Three copolymers and one homopolymer (PLLA) was synthesized in bulk. For copolymers a monomer weight ratio, LL:CL, of 2:3 in feed was used. Stannous 2-ethyl-hexanoate was added as initiator. Poly(ethylene oxide) was used as co-initiator in block copolymer synthesis. The polymerization was carried out according to reference [19] for homopolymers and statistical copolymer preparation and to references [20,21] for block copolymers preparation. In a glove box purged by dry nitrogen, pre-weighed amounts of monomer(s), l,l-lactide (and ε-caprolactone) and stannous 2-ethyl-hexanoate, at a proportion of 0.5 % per total monomers, were introduced in a dry tube. The tube was sealed and immersed into silicon oil bath, at 120 °C for 3 hrs. The copolymer thus obtained was purified by dissolution in chloroform and precipitation in methanol (three times). The white solid copolymer was filtered and dried under reduced pressure at room temperature for 24 hours. For block copolymers, before the addition of monomer(s), a pre-weighed amount of poly(ethylene oxide) and stannous 2-ethyl-hexanoate were introduced in a dry tube, sealed and into silicon oil bath at 120 °C for ten minutes, for co-initiator activation, and the polymerization time ranged from 168 hrs to 336 hrs.

Membrane Synthesis

Polymer membranes were prepared by solution casting followed by evaporation to dryness. The chloroform solutions were prepared by dissolving the polymer in the presence of salts (NaCl or hydroxyapatite) and/or lower mass poly(ethylene glycol) ($M_n = 600$ or $1{,}000\ g \cdot mol^{-1}$). Porous membrane were formed after selective dissolution by immersion of films in water at room temperature for 48 hrs and vacuum drying at room temperature.

Characterization of Copolymers

NMR Spectrometry

^{1}H-NMR spectra were measured at 200 MHz in deuterium chloroform ($CDCl_3$) solution using a Bruker AC-200 spectrometer and tetramethylsilane (TMS) was used as the internal reference. The carbon-13 NMR spectra were obtained using a Bruker AC 200 spectrometer at 50 MHz. The composition, the assignment of microstructure sequences and sequence lengths were performed based in the triad composition

Thermal Analyses

DSC runs were performed in a Mettler Toledo 822 equipment under nitrogen flux and samples were sealed in aluminum pans. DSC equipment was previously calibrated with indium ($T_m = 156.6\,°C$ and $\Delta H_f = 3.26$ $kJ \cdot mol^{-1}$). Each sample of about 10–15 mg was pre-heated to 180 °C and kept at this temperature for 5 min., cooled down at a rate of $20\,°C \cdot min^{-1}$ to −65 °C and, after remaining at this temperature during 5 min., was heated up to 250 °C at a rate of $10\,°C \cdot min^{-1}$. A Rheometrics Scientific DMTA V was used to perform tension tests. The samples were cut from the bulk pressed sheets and had the following dimensions: length of 23 mm, width of 8mm and thickness of 2 mm. A strain limit of 0.1% and a frequency of 1 Hz were applied in the temperature range from −100 to 100 °C at a heating rate of $2\,°C \cdot min^{-1}$.

Scanning Electron Microscopy

Philips XL-30 Scanning Electron Microscope was used for examining the morphology (porosity) of membranes (PLLA/PEG/

HA) and the cells growth. Preparation of the samples for imaging and analysis by SEM was straightforward. The polymer films were deposited on a conductive tape and submitted to gold sputtering prior to the analyses.

Polymer Biocompatibility

Membranes were sterilized by γ-ray irradiation prior to tests. The biocompatibility was determined "in vitro", by culturing fibroblasts on the membranes. A FMM1 cell line was used, which was established from a human gingival fragment and obtained after a gingivectomy for prosthetic reason. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co), supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Cells were incubated at 37 °C under humidified 5% CO_2 and 95% air atmosphere.[22]

Results and Discussion

PLLA and three copolymers of l,l-lactide (two triblock copolymers) were obtained by variation of the relative mass of l-lactide in feed. Poly(ethylene oxide) ($M_n = 4000$ or 20,000 $g \cdot mol^{-1}$) was used as the central segment of block copolymers (Table 1).

The prepared polymers presented molecular mass around 25,000 $g \cdot mol^{-1}$, except PLLA-PCL-b-PEO-b-PLLA-PCL, which initial prepolymer already had 20,000 $g \cdot mol^{-1}$ (Table 1). For composition characterization of the prepared polymers, NMR spectra were measured in deuterium chloroform (Figure 2).

The PLLA spectrum is shown in Fig. 2a and the perfect isotactic configuration of the polymer is demonstrated by its clear ^{13}C-NMR spectrum without resonance signal multiplicity due to microstructural stereo arrangement. PLLA and PLLA-b-PEO-b-PLLA have shown the same ^{13}C-NMR spectrum in the carbonyl region. The copolymers' relative molar compositions in terms of lactate and caproate units in PLLA-PCL and PLLA-PCL-b-PEO-b-PLLA-PCL were determined by ^{13}C-NMR spectrometry, using the carbonyl signals. Figures 2b and 2c show the ^{13}C-NMR spectra in the carbonyl region of the copolymers presenting segments of poly(l,l-lactide-stat-ε-caprolactone) and the assignments of the peaks according to references [10,14,15,19] (Table 2).

^{1}H-NMR spectra in $CDCl_3$ (Figure 3) were employed to estimate the EO molar fraction in the block copolymers and to identify the specific ester bond due to the copolymer formation. PLLA segments presented the following absorptions: 5.23–5.15 δ multiplet, inner methine units; 4.2–4.4 δ multiplet, end chain methine units; 1.4–1.6 δ inner methyl units. PEG segments presented the following absorptions: 3.65 δ singlet, inner methylene units; 4.2–4.4 δ multiplet, methylene protons of the acylated PEG end unit.

Figure 4 presents the DSC curves of the synthesized polymers. DSC analyses have shown the gradual change of the morphology in copolymers. PLLA presented T_g at 55 °C, a cold crystallization peak at $T_c = 108$ °C and melting at $T_m = 167$ °C. On the other hand, PPLA-PCL, which is a statistical copolymer with soft elastomer

Table 1.
Characteristics of polymers.

Sample	Composition, wt.%			Molar mass, $g \cdot mol^{-1}$	
	PLLA	PCL	PEO	PEO segment	Copolymer
PLLA	100	–	–	–	23,700
PLLA-PCL	63	27	–	–	24,700
PLLA-b-PEO-b-PLLA	84	–	16	4,000	25,000
PLLA-PCL-b-PEO-b-PLLA-PCL	45	29	26	20,000	78,300

Molar mass by GPC, using monodisperse polystyrene calibration.

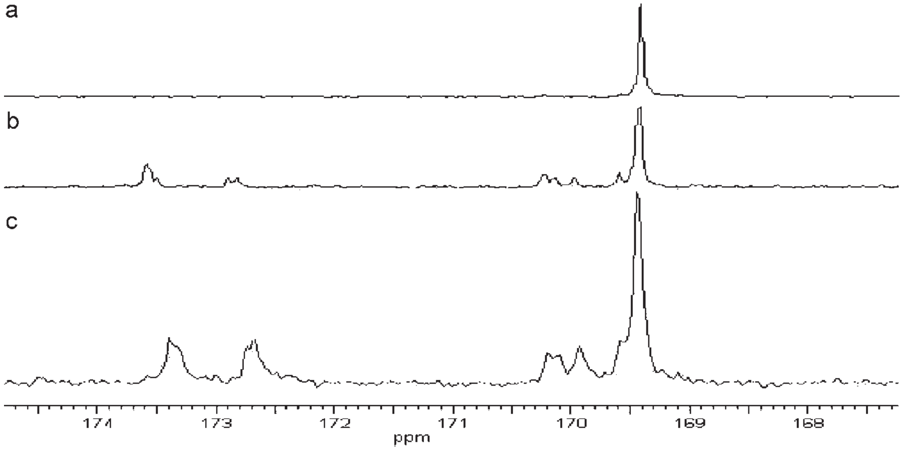

Figure 2.
^{13}C-NMR spectra of (a) PLLA, (b) PLLA-PCL and (c) PLLA-PCL-b-PEO-b-PLLA-PCL in the carbonyl region.

Table 2.
Carbonyl ^{13}C-NMR signals.

Carbonyl type	Carbonyl chemical shift ppm
Caproate	173.5 to 172.8
Lactate	170.3 to 169.6

characteristics, presented $T_g = -28\,°C$ and a broad melting peak at 108 °C. The only observed T_g suggests complete miscibility of the statistical copolymer segments in the amorphous phase, which along with the low crystallinity impart to the copolymer a rubbery nature.

PLLA-b-PEO-b-PLLA is also a brittle polymer, however due to the block structure with a short segment of poly(ethylene glycol) in the middle of the copolymer chain, extensive crystallization of PLLA was complicated, leading to $T_m = 100\,°C$ and PEG segment crystallization was forbidden. PLLA-PCL-b-PEO-b-PLLA-PCL is also a block copolymer, however the PEO central segment is much longer ($M_n = 20{,}000\ g \cdot mol^{-1}$), while the long lateral blocks are comprised by a statistical distribution of lactate and caproate units, which turned to be soft and amorphous. The thermal transitions thus observed,

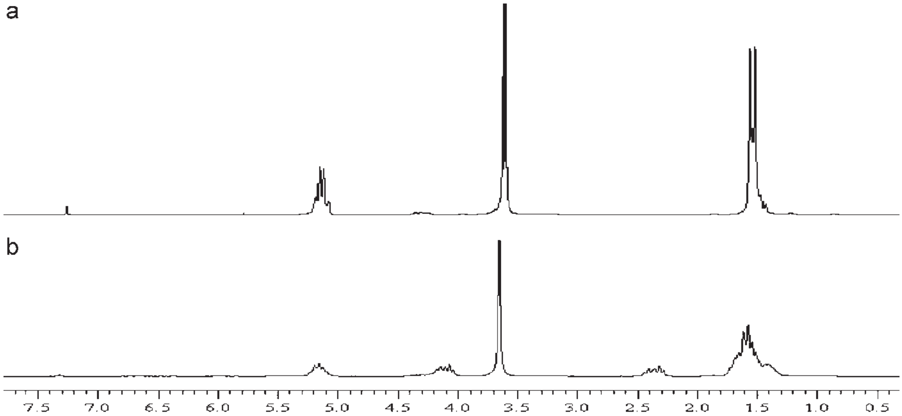

Figure 3.
^{1}H-NMR spectra of (a) PLLA-b-PEO-b-PLLA and (b) PLLA-PCL-b-PEO-b-PLLA-PCL.

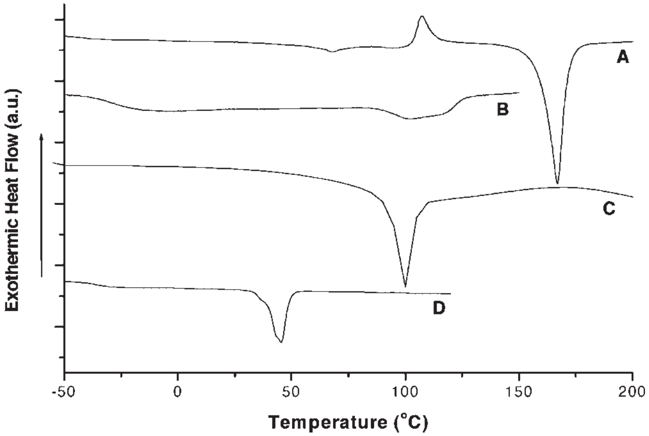

Figure 4.
DSC curves of (A) PLLA, (B) PLLA-PCL, (C) PLLA-b-PEG-b-PLLA and (D) PLLA-PCL-b-PEO-b-PLLA-PCL.

$T_g = -36\,^\circ C$ and $T_m = 45\,^\circ C$, are attributed to the amorphous phase formed by the mixing of the PEO and PLLA-PCL segments and to the melting of the PEO crystallites, respectively.

DMTA analyses allowed the measurement of the storage (E′) and loss (E″, not shown in this work) modulus and tan δ (Figs. 5A–5D), leading to the confirmation of the diversified chemical structures of the polymers and the resulting dissimilarity in mechanical and thermal properties. PLLA and PLLA-b-PEG-b-PLLA are rigid plastics at room temperature and the observed storage modulus E′, 1 GPa (Fig. 5A) and 186 MPa (Fig. 5C) at 25 °C, respectively, attested their mechanic characteristics. PLLA-PCL and PLLA-PCL-b-PEO-b-PLLA-PCL are elastomers, and as expected E′ were found to be 54 MPa and 60 MPa at 25 °C.

The DMTA analysis of a composite membrane of PLLA showed a storage modulus of 77.80 MPa (25 °C) (Fig. 5E), higher than that observed for the commercial porous collagen membrane, 0.88 MPa (25 °C) (Fig. 5F). At physiological temperature (37 °C) the storage moduli for collagen and PLLA composite membranes were 1.23 and 3.31 MPa, respectively, now quite similar.

These DMTA results let us envisage the possibility of reach biodegradable hydrophilic polyesters with useful properties as different kinds of biomaterial, by a careful choice of compositions of the polymers presented in this work.

Additionally, porous polymeric membranes were prepared in order to mimetize collagen membranes. The porous structure of membranes contributes to the biological cells fixation and colonization, which is the most important step for the synthetic polymer substitution by the living tissue.

The porous structures were prepared by polymer, salt (sodium chloride or hydroxyapatite) and/or low molar mass PEG ($M_n = 600$ or $1{,}000\ g \cdot mol^{-1}$) dispersion in chloroform, followed by casting and evaporation to dryness in vacuum oven under reduced pressure. The dry films thus prepared were submitted to extraction by water at room temperature for 24 hrs and, again, evaporated to dryness in vacuum oven, forming dry porous films.

The SEM pictures (Figure 6) show a uniform distribution of homogeneous porous sizes for PLLA/PEG/HA membrane in its whole extension, with pores diameter of 8.5 mm, essential for nutrient supply and surface cell adaptation.[22]

The porosity degree of membrane affected the magnitude of storage modulus of materials (collagen and PLLA/PEG/HA

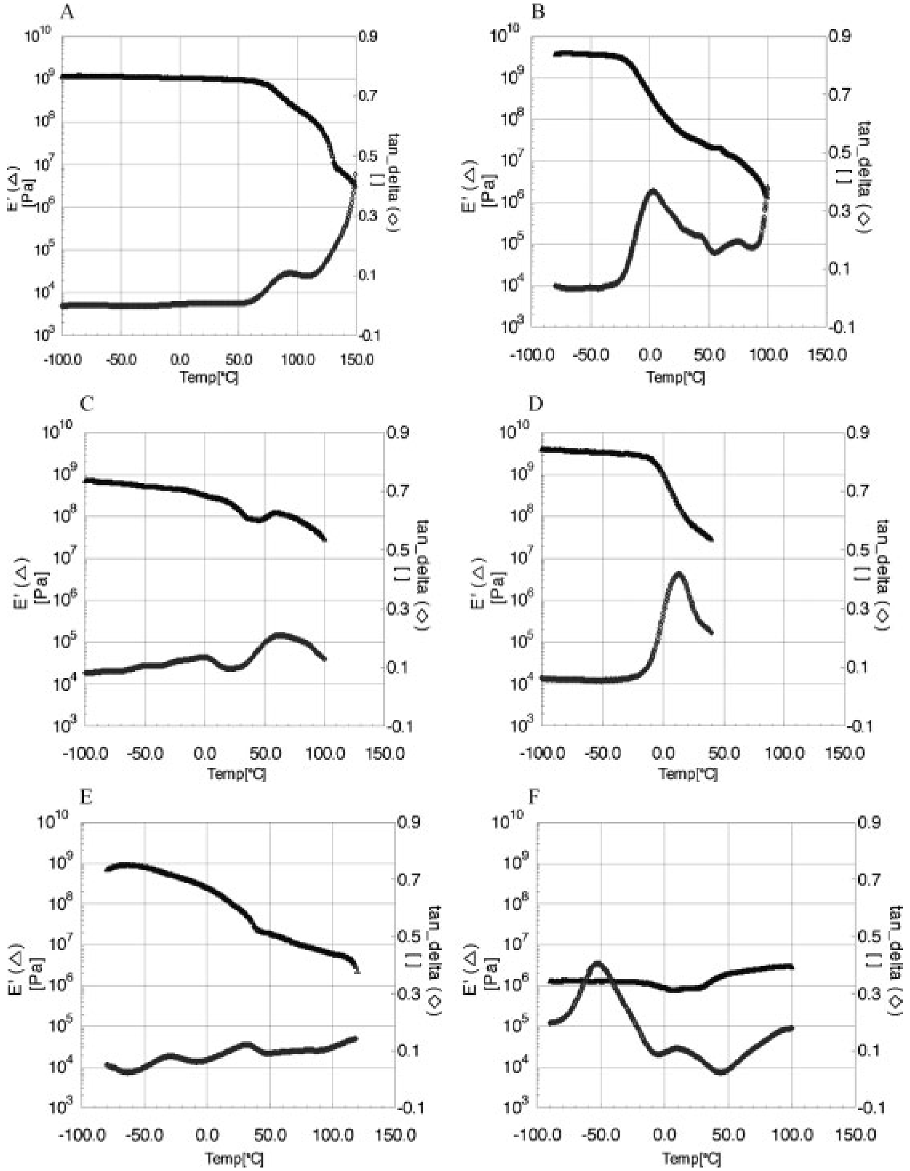

Figure 5.
DMTA curves of solid films, showing storage modulus (E′) and tan δ in function of temperature, for (A) PLLA, (B) PLLA-PCL, (C) PLLA-b-PEG-b-PLLA, (D) PLLA-PCL-b-PEO-b-PLLA-PCL, (E) PLLA composite and (F) a dry commercial collagen membrane.

dry membranes). The high porosity along with the incorporation of ceramics and cold crystallization of PLLA, confers the membrane, with elapsing of the time (seven to nine weeks), a fragile behavior. The blending with other rubbery polyester (PLLA-PCL or PLLA-PCL-b-PEO-b-PLLA-PCL) migth confer to the porous membrane better mechanical and aging properties.

SEM analysis also allowed the observation of the adherence of fibroblasts on the membranes (PLLA/PEG/HA and collagen), demonstrating biocompatible characters of both membranes (Figure 6).

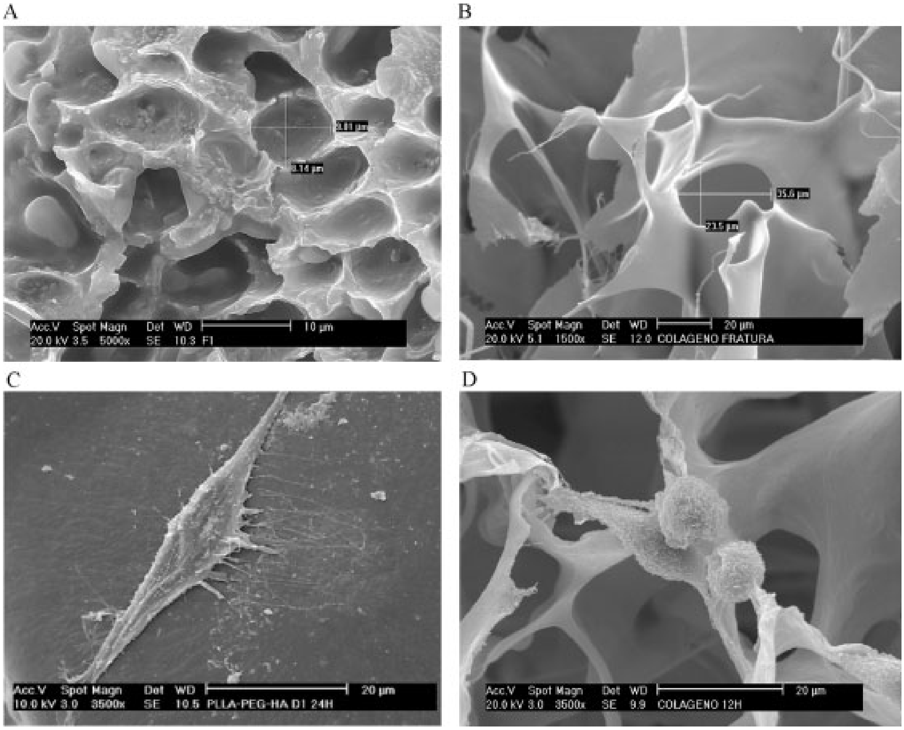

Figure 6.
Scanning electron micrographs of: transversal areas of (A) porous PLLA composite and (B) collagen membranes; and of fibroblasts on (C) porous PLLA composite and (D) collagen membranes.

Conclusions

Biodegradable polyesters were prepared with different mechanical properties by (block) polymerization of lactide, caprolactone and prepolymers of ethylene oxide. A correlation among polymer structure, mechanical and thermal behavior was confirmed. Biodegradable porous membranes were prepared and characterized, which have shown to be biocompatible by ''in vitro'' cell culturing.

Acknowledgements: The authors thank FAPESP and CNPq for financial support.

[1] N. Claffey, S. Motsinger, J. Ambruster, J. Egelberg, *J. Clin. Periodonto.* **1989**, *16*, 12.
[2] G. Greenstein, J. Caton, *J. Periodontol* **2000** 1993, *1*, 36.
[3] S. Pitaru, H. Tai, M. Soldinger, O. Azar-Avidam, M. Noff, *J. Periodontol. Res.* **1987**, *22*, 331.
[4] I. Magnusson, C. Batich, B. Collins, *J. Periodontol.* **1988**, *59*, 1.
[5] A. Linde, C. Thoren, C. Dahlin, E. Sandberg, *J. Oral Max. Surg.* **1993**, *51*, 897.
[6] P. A. MayesGlucolysis and Piruvate Oxidation, In: *Harper's Biochemistry*, Appleton and Lange, **1996**, p. 176.
[7] H.-J. Sung, C. Meredith, C. Johnson, Z. S. Galis, *Biomaterials* **2004**, *25*, 5735.
[8] E. Chiellini, R. Solaro, *Adv. Mater.* **1996**, *8*, 305.
[9] K. E. Uhrich, S. M. Cannizzaro, R. S. Langer, K. M . Shakesheff, *Chem. Rev.* **1999**, *99*, 3181.
[10] P. Vanhoorne, P. Dubois, R. Jerome, P. Teyssie, *Macromolecules* **1992**, *25*, 37.
[11] J. M. Vion, R. Jerome, P. Teyssie, M. Aubin, R. E. Prudhomme, *Macromolecules* **1986**, *19*, 1828.
[12] E. J. Choi, J. Park, H. N. Chang, *J. Polym. Sci. Polym. Phys.* **1994**, *32*, 2481.
[13] P. J. A. In't Veld, E. M. Velner, P. van de Witte, J. Hamhuis, P. J. Dijkstra, J. Feijen, *J. Polym. Sci. Polym. Chem.* **1997**, *35*, 219.
[14] Y. Shen, K. J. Zhu, Z. Shen, K.-M. Yao, *J. Polym. Sci. Polym. Chem.* **1996**, *34*, 1799.
[15] H. R. Kricheldorf, M. Sumbei, *Makromol. Chem.* **1988**, *189*, 317.

[16] H. R. Kricheldorf, K. Bornhorst, H. Hachmann-Thiessen, *Macromolecules* **2005**, *38*, 5017.

[17] S. H. Wang, L. F. Silva, J. Kloss, M. Munaro, G. P. Souza, M. A. Wada, J. G. C. Gomez, S. Zawadzki, L. Akcelrud, *Macromol. Symp.* **2003**, *197*, 255.

[18] J. Kloss, M. Munaro, G. P. Souza, J. V. Gulmine, S. H. Wang, S. Zawadzki, L. Akcelrud, *J. Polym. Sci. Polym. Chem.* **2002**, *40*, 4117.

[19] M. L. Castro, S. H. Wang, *Polym. Bul.* **2003**, *51*, 151.

[20] C. G. Mothé, W. S. Drumond, S. H. Wang, *Thermochimica Acta*, **2006**, *445*, 61.

[21] W. S. Drumond, C. G. Mothé, S. H. Wang, *J. Therm. Anal. Cal.* **2006**, *85*, 173.

[22] T. Sigurdsson, R. Hardwick, G. Bogle, U. Wikesjo, *J. Periodontol.* **1994**, *65*, 356.