Periodontal membranes from composites of hydroxyapatite and bioresorbable block copolymers

P. CERRAI, G. D. GUERRA, M. TRICOLI
CSMM-C.N.R., c/o Department of Chemical Engineering (University), Via Diotisalvi 2, I-56126 Pisa, Italy
E-mail: cerrai@ing.unipi.it

A. KRAJEWSKI, A. RAVAGLIOLI
IRTEC-CNR, Via Granarolo 64, I-48018 Faenza, Italy

R. MARTINETTI, L. DOLCINI
FIN-CERAMICA Faenza S.r.l., Via Ravegnana 102, I-48018 Faenza, Italy

M. FINI
Experimental Surgery, Orthopaedic Institutes ''Rizzoli'', Via di Barbiano 1, 40100 Bologna, Italy

A. SCARANO, A. PIATTELLI
Dentistry Disciplines School, University of Chieti, Via Dei Vestini 11, I-66013 Chieti Scalo, Italy

Biomembranes are frequently proposed as devices for ''guided bone regeneration.'' Such membranes consist generally of a thin sheet of polymeric material, mostly textured from polymeric yarns or clots, which all have a diffuse very fine winding porosity. The cross-section size of the holes of such porosity is nanometric (diameter < 0.1 μm); thus these holes can be indicated as nanoholes. Whatever the method of production, the surface density of nanoholes (number per square centimeter) has to be as high as possible. It is important also that no variation of this density occurs. The fine dimension of these microholes allows the crossing of small molecules (O_2, CO_2, H_2O, sugars, many nutritional organic compounds and even some simple proteins) but not other larger molecules and particulates, including cells of any kind. These biomembranes have, consequently, a semipermeable behavior, providing the functional role which is the interposition of a barrier for the cells, separating the bone from the surrounding soft tissues. The kinetic of proliferation of osteoblasts is lower than that of fibroblasts. Most membranes of this kind are not resorbable. The main problem for the resorbable ones is the speed of size increase of the holes during the time. Their diameter must not exceed a threshold value until the reconstruction of bone is complete, otherwise soft tissue cells will invade the growing bone tissue with formation of undesirable mixed tissue.

The present paper deals with a resorbable membrane made with a composite polymer/ceramic. A poly(ε-caprolactone)-*block*-poly(oxyethylene)-*block*-poly(ε-caprolactone) copolymer is the polymeric matrix which contains dispersed ceramic hydroxyapatite microgranules, a stiff filling additive. The main possible use is that of periodontal membranes. The copolymer, obtained by thermal polymerization of ε-caprolactone onto poly(ethylene-glycol), presents good biological tolerance, is resorbable under physiological conditions and can promote cell growth. Histological tests, performed 6 months after implantation, showed that the polymeric matrix is almost totally resorbed. New-formed bone colonizes even the innermost parts of the membrane, with bone trabeculae closely surrounding the hydroxyapatite granules.

1. Introduction

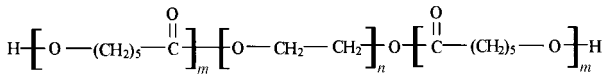
Polymeric membranes for ''guided bone regeneration'' are widely used in the biomedical field and several interesting applications occur in surgery [1, 2]. Periodontal membranes for the regeneration of bone in dentistry generally employ special textured polymeric cloths. All the components used must obviously be biocompatible and the whole membrane must have good biological tolerance and suitable bioresorbability, together with good elastoplastic performance. The latter property is particularly useful for the adaptation of the membrane to every different anatomical shape of the surgical site to be protected without breaking the membrane. In particular, to have a bioresorbable membrane the polymeric matrix must completely dissolve in a suitable time, so that the new formed bone can join tightly with the soft tissues.

The specific role of such biomembranes is to prevent non-osteoblastic cells invading the cavity in which new bone has to grow; therefore, the polymeric matrix must be semipermeable with a porosity suitable to prevent such undesired crossing of cells, but however to allow exchange of suitable molecules, even sufficiently large, that is they represent a biological barrier, but not completely a biochemical barrier. On the other hand, the process of dissolution must not give rise to formation of too large cavities across the membrane at least for a period of time (3 months) sufficient to have a quite complete reconstruction of the bone side.

The present paper proposes a resorbable membrane made with a new polymer/ceramic composite in which ceramic particles are dispersed inside a polymeric matrix. Bioactive ceramic materials (hydroxyapatite or tricalcium phosphate) can be used as suitable filler, and homo- or copolymers of different lactones as polymeric matrix.

In this case ceramic granules of hydroxyapatite (HA), $Ca_5(PO_4)_3OH$, constitute the ceramic component in question. The role of hydroxyapatite (HA) is not simply to confer suitable stiffness to the resulting material (because the stiffness is not sufficient if the polymer is utilized alone), but its addition was suggested also for biological activity. At the same time, the ceramic particles must act to nucleate new bone growth and, depending on their chemical nature, remain in the reconstructed bone or be resorbed.

The polymeric matrix of the membrane object of this communication is the poly(ε-caprolactone)-*block*-poly (oxyethylene)-*block*-poly(ε-caprolactone) (PCL-POE-PCL) copolymer



(PCL-POE-PCL)

This copolymer presents good biological tolerance, is resorbable under physiological conditions and can promote cell growth [3]. A polymer/ceramic membrane, based on poly(ε-caprolactone) homopolymer (PCL) alone, was proposed previously [1]. However, it has been shown in clinical trials to reach a complete resorption too slowly, not synchronized with respect to the complete bone regeneration. This new proposal of polymeric-ceramic membrane has shown in clinical applications to be able to reach a complete resorption in less times.

2. Experimental procedure

2.1. Materials

The following products were utilized to produce the membrane:

- ε-caprolactone monomer (CL): ''purum'' Fluka product ($\geq$ 99% by GC) was distilled under vacuum over CaH_2. The fraction collected at 96–98 °C (5 mmHg) was used in all the syntheses; its purity, checked by GC after distillation, was $\geq$ 99.8%.
- Poly(ethylene glycol) (PEG): Merck-Schuchardt product, hydroxyl bi-terminated, with a molecular mass of 35 000 Da, was extracted with boiling ethyl ether, dried and used.
- Hydroxyapatite (HA): powder produced by precipitation method and selected on the strength of its purity and suitability for use in medical applications. The powder conforms to ISO/DIS 13779 Implants for surgery-ceramic materials based on hydroxyapatite. The granules were produced by granulation method (patent no. BO93A000435) starting from the powder described above. The granules produced are in the granulometric range of 40–150 μm. The microstructures analyzed by scanning electron microscopy (SEM) showed a homogeneous grain size in the range 0.5–1.0 μm, while the pore size results between 0.5–30 μm [3] with a whole porosity in the range 25–30% (vol%) and a mean apparent density of the granule of $2.30 \pm 0.08\ g\,cm^{-3}$ (theoretical of HA monocrystal is $3.155\ g\,cm^{-3}$).

2.2. PCL-POE-PCL copolymer synthesis

The chemical synthesis of PCL-POE-PCL triblock copolymers was carried out according to the general scheme in Fig. 1 [4]. The reaction mixture was prepared by introducing under N_2 atmosphere a known volume of purified liquid CL monomer into a Pyrex phial containing a pre-weighed amount of PEG-35 000. The phial was connected to a high vacuum line, evacuated, sealed off and placed in an oven at 185 °C, under continuous stirring. After quantitative conversion of the CL–PEG mixture to copolymer, the latter was recovered in the form of plastic material. The progress of the synthesis as well as the completion of CL monomer addition can be followed by gel permeation chromatography (GPC). The specific gravity, measured with a density bottle, is $1.13 \pm 0.01\ g\,cm^{-3}$.

2.3. PCL-POE-PCL copolymer characterization

PCL-POE-PCL copolymer was characterized by means of different techniques in order to define physicochemical characteristics, as well as to evaluate biocompatibility and biodegradability.

Degradability of PCL-POE-PCL copolymer has been

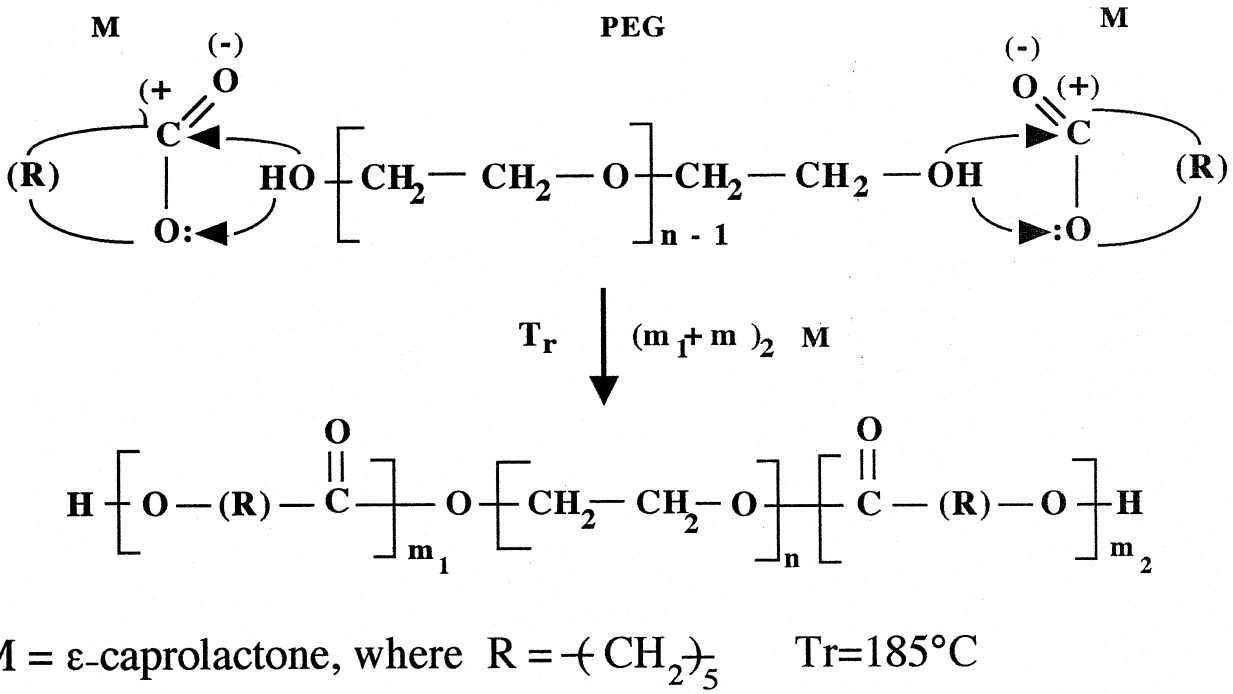


Figure 1 General scheme of reaction for the chemical synthesis of the PCL-POE-PCL copolymer.

previously ascertained through *in vitro* tests of dissolution rate in phosphate-buffered saline (PBS), aimed to determine the nature and the amount of degradation products, as well as the interaction of the same products with the cellular metabolism [5]. The 6-hydroxyhexanoic acid, HO-$(CH_2)_5$-COOH, as the ultimate hydrolytic product from copolymers, has been proved to be active in endothelial cell metabolism [6].

All the characterization results obtained indicate that PCL-POE-PCL copolymer is a good candidate for producing biomembranes.

3. Methods and results

3.1. Manufacturing the membranes

The membranes were prepared by dissolving a known weight of PCL-POE-PCL copolymer in $CHCl_3$, by adding 50% by weight of fine porous granules of HA and then by evaporating the solvent (final vol % of HA was $32.9 \pm 0.7\%$, in practice 1/3). The solid dispersion obtained was laminated to obtain membranes 200 μm thick. The obtained layer was left in air flux to remove all $CHCl_3$ residuals and cut through a piercing die to obtain samples of the membrane. Every sample was enveloped in a polymer bag, then sterilized with γ irradiation.

3.2. Physicochemical and biocompatibility tests

Membranes were finally submitted to specific characterization tests, both *in vitro* and *in vivo* conditions. The response of membranes to the applied conditions was followed with time by optical and SEM.

Physicochemical tests were carried out by GPC, nuclear magnetic resonance (^{1}H-NMR) and Fourier transform infrared (FT-IR) spectroscopies, differential scanning calorimetry (DSC), X-ray diffractometry (XRD) and stress–strain tensile analysis. GPC analysis excluded the undesired presence of PCL homopolymer in the matrix and simultaneously estimated the dispersivity degree of the PCL-POE-PCL copolymer to be 2. ^{1}H-NMR and FT-IR spectroscopies proved the ether–ester chemical nature of the copolymers and allowed the calculation of both the molar composition of copolymers (66 mol % oxycaproyl units and 34 mol % oxyethylene units) and their mean molecular mass (about 200 000 Da). DSC and XRD ascertained the crystallization pattern of the copolymer, with particular regard to the separate crystallization modes of PCL and POE blocks, indicating a pronounced clear crystallization of the PCL blocks alone.

Finally, stress–strain measurements gave information on the typical behavior of ductile material. The yield stress was 2.40 ± 0.12 MPa and the ultimate tensile stress (UTS) was 2.56 ± 0.15 MPa.

Biocompatibility was checked by cytotoxicity, mutagenicity and haemocompatibility tests. Absence of cytotoxicity of the polymeric fraction was already tested previously by Neutral Red, MTT, Kenacid Blue assays and by cell adhesion and proliferation tests on copolymer films [4]. Biocompatibility tests were carried out also on the membrane.

Haemocompatibility was checked by contact activation tests both of plasma prekallikrein and thrombocytes. All these tests gave excellent results.

Cytotoxycity testing was performed also with the membrane using coloration with propidium iodide. This did not exhibit a cytotoxic effect on cell line L-929.

Mutagenicity and genotoxic analyses, carried out on extracts of the membrane according to the UNI-CEI EN-45001 standard, showed no effects when submitted to: (a) the Ames's test; (b) the evaluation of exchanges among brother chromatides; and (c) the evaluation of chromosomic aberrations.

3.3. *In vitro* degradation tests

In vitro testing of membranes has been carried out using Dulbecco's PBS (D5527 lot no. 34H2371–Sigma Chemical Co., St Louis, MO, USA). The tests were performed in a device which gave 0.0032 $cm^3 s^{-1}$ steady continuous flux rate of the solution at constant 37 °C. PBS solution was renewed every 7 or more days. The samples, drawn out from the solution at prefixed dates (15 days and multiples), were washed with distilled water, dried, weighed and examined by SEM (Leica Cambridge Stereoscan), so collecting kinetic dissolution rate data and images of the micromorphological rearrangement of the membrane microstructure (Figs 2 to 5). During *in vitro* tests, the membrane produced random homogeneous diffused distribution of elongated micropores, the size of which increases with time in PBS. In particular, Fig. 5 shows the microstructure of the membrane after 180 days in solution; it is possible to observe on the left side a detail of HA microstructure completely uncovered by the polymeric matrix. An important observation from microphotographs is that both types of membranes in contact with the solution auto-produce a homogeneous diffused random distribution of elongated micropores, the maximum size of which is 5 μm at 30 days, 8 μm after 60 days, 14 μm after 90 days. The mean size exceeds 25 μm after 180 days, but at that time the thickness of the membrane is so thin to be evanescent (practically insubstantial or disappeared). This range of microporosity assures intercommunication to liquids and large molecules, but does not allow cells of any kind to cross through the membrane for at least 3 months from implantation, so assuring for a long time the guided reconstruction of bone (such as the best in the market).

In all cases a weight loss with time was observed as a consequence of the release of polymeric material to the solution, the rate of which was closely correlated with the frequency of renewal of the solution. The difference between the actual weight of every sample and the one it had at the beginning of the test was calculated, obtaining a value for each sample; the mean value of such differences and the related standard deviation were calculated. Table I reports the experimental weight loss values.

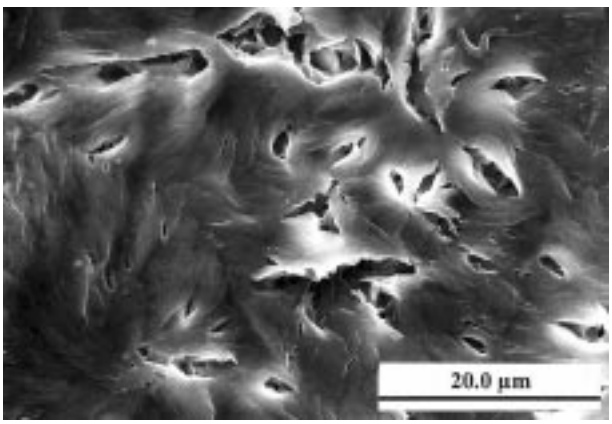


Figure 2 In vitro test after 30 days.

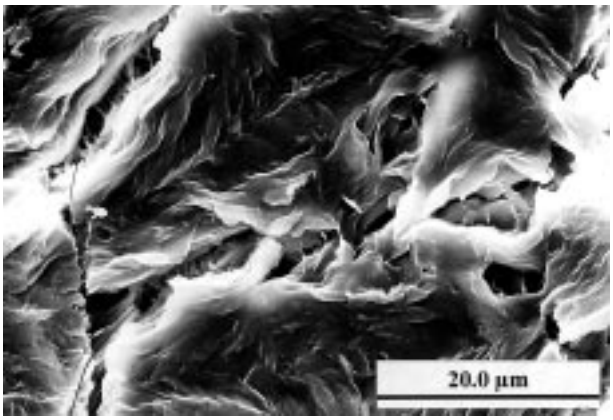


Figure 3 In vitro test after 60 days.

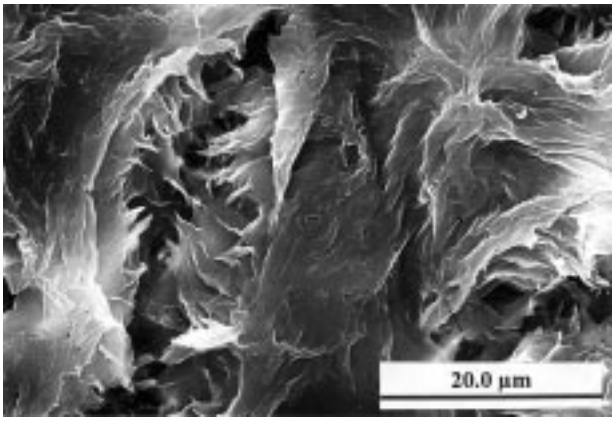


Figure 4 In vitro test after 90 days.

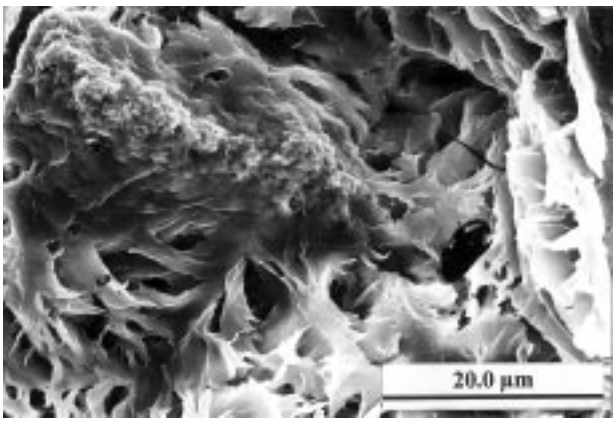


Figure 5 In vitro test after 180 days.

3.4. *In vivo* histological tests

In vivo tests were performed up to 12 weeks after implantation in tibia of New Zealand rabbits. In every hind leg a hole (3.5-mm diameter defect) was made across the femur thickness through a coring-tool extractor machine. A screw (3.75 mm diameter, 8.5 mm length, titanium MAC SYSTEM dental implant, medical grade 2, flat head-end, SE-SI Srl., Milano, Italy) was inserted into every cortical hole of the tibia (the surface of the screw-head was sunk about 100–200 μm under the cortical surface) and each implant was covered with a square membrane of 1 cm side. Histological examination

TABLE I Average weight loss percent (AWtL%) and standard deviation (ΔWtL% ±) of the membrane samples in the time (days) under PBS (calculated on 20 different samples at every time)

Days	0	15	30	45	60	75	90	120	180
AWtL%	0	10	16	22	28	45	52	57	60
ΔWtL% (±)	(0)	(1)	(1)	(2)	(3)	(6)	(10)	(12)	(20)

was carried out on samples extracted from rabbits sacrificed at different times (1, 4, 8, and 12 weeks) to check the condition of the membranes and of the surrounding tissues, in particular connected with the hole side. The samples were fixed and dehydrated in steps of immersion in a series of alcoholic solutions with increasing concentration, ranging from 70 to 100%. Each step was for 24 h. The dried samples were then infiltrated with monomer Technovit 7200 VLC resin (Kulzer, Wehrheim, Germany) and polymerized. The obtained solid blocks were sliced by a disc cutting system (''Precise I'', Assying, Milan, Italy).

Histological examination of the samples showed that the polymeric matrix of the membrane was almost totally resorbed (85% volume) in 12 weeks. The new-formed bone colonized even the innermost parts of the membranes, with bone trabeculae closely surrounding the hydroxyapatite granules. Figs 6 and 7 show typical optical micrographs collected after 4 and 12 weeks.

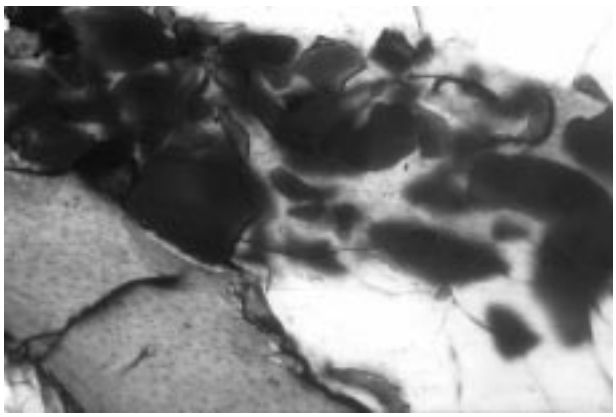


Figure 6 Histological result after 4 weeks implantation. Presence of cells into the membrane. The membrane is colonized by neoformed bone in many points. Horizontal side length corresponds to 360 μm.

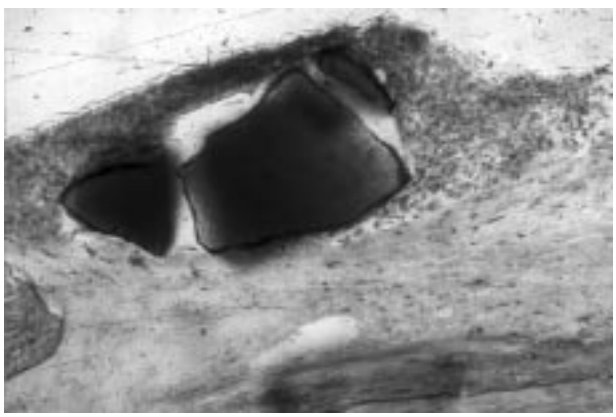


Figure 7 Histological result after 12 weeks implantation. Note the complete resorption of the polymeric component. Some connective cells are present among residual parts of membrane. No inflammatory reaction is detected. Horizontal side length corresponds to 180 μm.

Histological sections exhibit also the presence of some polynucleated cells in the samples 12 weeks after implantation. The only possible explanation is the need to obtain an ester-hydrolysis process of depolymerization, with a H^+ capture by the ester groups of the copolymer. This process probably interferes with the normal activity of the macrophages and induces them to try to phagocytize the long copolymer molecule or the ester-hydrolysis products.

However, the membrane shows a good course and it is well tolerated by tissues up to final complete dissolution of the polymeric matrix, with integration in the new-formed bone tissue, remodeling and partial dissolution of the HA grains.

4. Discussion and conclusions

The copolymer-ceramic membrane proposed here gives resorption times well synchronized with the bone regeneration and faster than those found with PCL homopolymer alone.

The presence of hydrophilic polyoxyethylene blocks in the copolymers enhances their solubility and consequently their complete resorption. On the other hand, the presence of HA ceramic fraction in the membranes enables the new-formed bone to consolidate and to join rapidly. HA particles produce the expected role of diffused points of nucleation of neoforming bone, factor which favor the maturation of grown compact bone and its consequent densification. On the other hand, the presence of a consistent amount of HA material in the volume of the membrane implies a lower metabolic endeavor in the resorption process of the remaining polymeric part. No toxic and inflammatory courses were identified in the neighbor tissues interested by the site of surgical implantation. Only sporadic and modest numbers of polynucleated cells were detected. However, their presence was observed only towards 12 weeks of implantation time. This late occurrence indicates that they may have a role in the elimination of the residuals of the membrane when it has lost its characteristic as a biological barrier.

On the basis of speed of the porosity formed by dissolution during *in vitro* testing this kind of membrane warrants its role as biological barrier for at least 4 months. The tendency to slow the dissolution rate in PBS with time depends closely on the frequency of change of the solution. Considering that PCL blocks are crystalline and that PCL is less soluble than POE segments, the probability of dissolution of the latter decreases with time. The variability of release rate may depend also on dimension and roughness of HA ceramic particles, on possible defects or micro-cracks in the matrix, etc. Biological resorption in the *in vivo* test shows that a time of 4 months is, in effect, reliable; on the other hand, the

biological turnover kinetic of rabbits is certainly faster than that of humans. Therefore, the biological resorption has to be expected to be slower in humans.

The good results obtained by the *in vitro* and *in vivo* testing of new membranes encourage their use as periodontal membranes in dentistry.

Finally, the low costs of the starting materials together with the ease of the chemical synthesis of PCL-POE-PCL copolymers, represent a further incentive to develop the applicative aspects of this work.

References

1. R. MARTINETTI, A. RAVAGLIOLI, A. KRAJEWSKI and C. MANGANO, *Biomateriali* **9** (1995) 43.
2. M. PIATTELLI, A. SCARANO, C. MANGANO, R. MARTINETTI and A. PIATTELLI, in Proceedings of the 4th Ceramics, Cells and Tissues Meeting Seminar series on Ceramic-Polymer Composites, edited by A Ravaglioli and A. Krajewski, Faenza, Italy (IRTEC-CNR, 1998) pp. 127–133.
3. A. RAVAGLIOLI, A. KRAJEWSKI, A. PIATTELLI, C. MANGANO and R. MARTINETTI, *Bioceramics* **9** (1996) 185.
4. P. CERRAI, G. D. GUERRA, L. LELLI, M. TRICOLI, R. SBARBATI-DEL-GUERRA, M. G. CASCONE and P. GIUSTI, *J. Mater. Sci.: Mater. Med.* **5** (1994) 33.
5. R. SBARBATI-DEL-GUERRA, C. CRISTALLINI, N. RIZZI, R. BARSACCHI, G. D. GUERRA, M. TRICOLI and P. CERRAI, *ibid.* **5** (1994) 891.
6. R. SBARBATI-DEL-GUERRA, P. GAZZETTI, G. LAZZERINI, P. CERRAI, G. D. GUERRA, M. TRICOLI and C. CRISTALLINI, *ibid.* **6** (1995) 824.

Received 1 December 1998
and accepted 9 April 1999