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Preparation and characterisation of composites based on biodegradable polymers for "in vivo" application

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Abstract

Poly(L-lactic) acid (PLLA), polycaprolactone (PCL), three different copolymers based on poly(L-lactic) acid and polyglycolic acid (PLLA-*co*-PGA), and their composites with hydroxyapatite obtained from bovine bone (ossein), were tested in order to have information on the thermal, morphological, mechanical and biochemical properties in view of their use as biocompatible/biodegradable materials. Ossein, which is essentially a biological hydroxyapatite, was found to improve the modulus and increase the hydrophilicity of the polymeric substrate. In addition, the size of the ossein particles was found to be critical for the improvement of mechanical properties. Finally, preliminary results on the in vitro biocompatibility of selected blends carried out by using primary cultures of human osteoblasts showed that the presence of hydroxyapatite stimulates a more positive cellular response.

Keywords: Poly(L-lactic) acid (PLLA); Polycaprolactone (PCL); Poly(L-lactic)-*co*-polyglycolic acid copolymers (PLLA-*co*-PGA)

1. Introduction

Originally limited to the commercial sutures application, biodegradable and bioreabsorbable polymers are slowly, but progressively extending their use to different areas of surgery, including dental repair, fracture repair and ligament reconstruction [1,2]. It was in fact recognised that the controlled in vivo degradation of the element should avoid the need to remove it after recovery. Among the polymeric matrices so far proposed, polyhydroxyacids derived from lactic acid and ϵ-hydroxycaproic acid have proven to be very successful, particularly, as drug release matrices and in bone fracture fixation [3,4]. The main peculiarity of such polymers is their mechanism of biodegradation through hydrolysis of the ester linkage and the formation of decomposition products which are normal intermediates of cell metabolism [5]. The rate of decomposition can be varied through copolymerisation or formulation, according to the need of keeping the biological function for days or even months [6].

A critical aspect of the use of such polyesters is represented by their poor hydrophilic properties, which prevent cell adhesion. In the present paper we report the preparation and properties of polyhydroxyacid based composites containing ossein, a biological hydroxyapatite, which constitutes a natural substrate for osteoblasts growth and contributes to enhance the hydrophilic behaviour of the polymeric surface.

In our research we have tested many polymers either as such or in combination with ossein and compared their physical–chemical, morphological and mechanical behaviour as well as the cellular response that they induce in human osteoblasts. In this paper we focus our attention on the first set of characterisation and give some insight on the biological response.

2. Experimental

2.1. Materials

The employed polymers and fillers are reported in Table 1. All the polymers and fillers were stored in a desiccator over P_2O_5 before use.

Ossein is a biological hydroxyapatite and consists of elemental calcium and phosphorous, as configured by energy dispersive analysis X-ray (EDAX) reported in Fig. 1 in the natural ratio 2:1. Ossein particles are roughly spherical and are characterised by dimensions ranging from less than 1 μm to more than 70 μm.

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Table 1
List of investigated materials

Material	Code and supplier	Molecular weight (a.m.u.)
Poly(ϵ-caprolactone)	(PCL) CAPA 650 (Solvay)	55,000
Poly(L-lactic) acid	(PLLA) (SIGMA)	120,000
Poly(DL-lactic-*co*-glycolic) acid 50/50	(PDLLA-*co*-PGA 50/50 (SIGMA)	60,000
Poly(DL-lactic-*co*-glycolic) acid 75/25	(PDLLA-*co*-PGA 75/25 (SIGMA)	100,000
Poly(DL-lactic-*co*-glycolic) acid 85/15	(PDLLA-*co*-PGA 85/15 (SIGMA)	65,000
Ossein (biological hydroxyapatite)	Ossein PET (NBS Lane)	

2.2. Preparation of composites

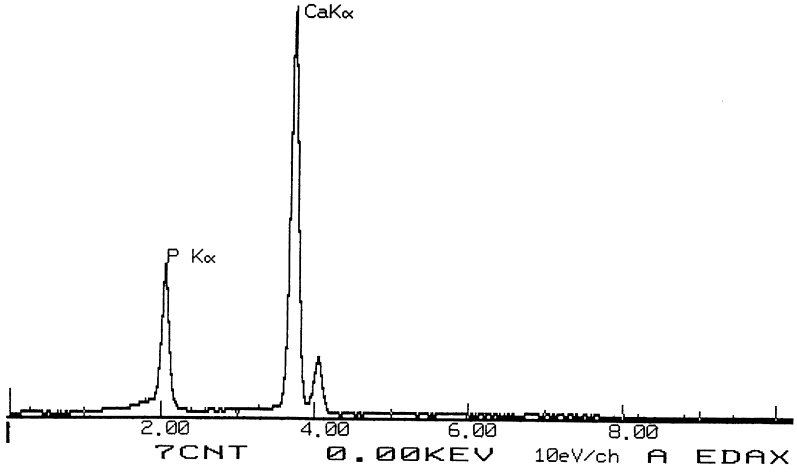
9.5 g of the chosen polymer in powder form was melt mixed with 0.5 g of biological ossein for 10 min in a home-made static extruder. This consists of a stainless-steel screw with a screw profile specifically designed for efficient mixing (see Scheme 1), mounted on the axis of a variable speed powerful motor and immersed in a glass vial. The speed was adjusted at 40 rpm, while the glass vial was warmed by a heating tape above the melting temperature of the polymer (100°C for PCL and 190°C for PLLA and PLLA–PGA copolymers). The glass vial was fitted with N_2 inlet to carry out the mixing in inert atmosphere. At the end of the preparation, the molten mixture was cooled under N_2 and then grounded in liquid N_2. To have homogeneous controls, neat polymers also were subjected to the same treatment before testing.

2.3. Techniques

Physical–chemical characterisation was carried out by using a Mettler TA-3000 differential scanning calorimeter. The samples were heated from −60 to 250°C at 20°C/min, quenched down to 30°C/min and re-heated to 250°C at 20°C/min.

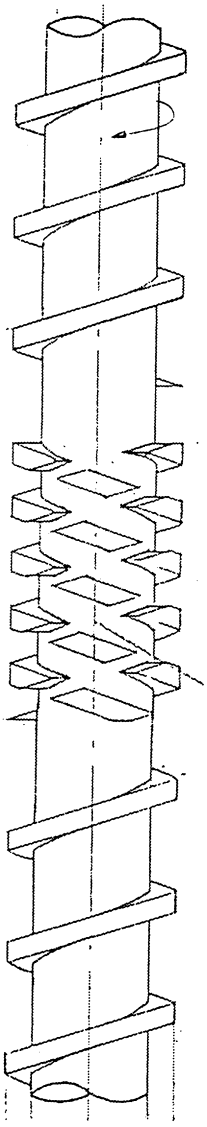
Scanning electron microscopy (SEM) was effected on the cryo-fractured surface of compression moulded specimens coated with Au/Pd alloy by means of a Philips XL20 SEM apparatus.

Dynamometric tensile measurements were carried out on an Instron Apparatus, model 1122, at room temperature at a cross-head speed of 10 mm/min. Dumbbell samples 5.0 cm long, 3.0 mm wide and 1 mm thickness were used. The specimens were obtained by compression moulding in a common heated press at temperatures above the DSC melting peak.



ELEM	CPS	AT% ELEM
P K	268.0950	33.40
CA K	805.9100	66.60
TOTAL		100.00

Fig. 1. Atomic emission spectrum of biological hydroxyapatite.



Scheme 1.

The morphological observation of the surface of films after exposure to osteoblast cultures, was carried out as follows: after the removal of the medium, the specimens were fixed with 0.5% glutaraldehyde in 0.01 M phosphatase buffer, pH 7.0, for 20 min at room temperature. After being rinsed several times in the same buffer, the cells were dehydrated in an ethanol–water series of 30, 50, 70, 95% ethanol. The samples were submitted to critical point drying and then coated with a thin layer of an alloy of gold and palladium. Successively, the samples were examined by using a Philips XL 20 electron microscope.

Table 2
DSC thermal data of homopolymers and composites

Codes	% Ossein	Tf_1 (°C)	ΔHf_1 (J/g)
PCL	–	62.4	32.6
PCL/OSS 95/5	5	66.5	27.0
PLLA	–	176	41.3
PLLA/OSS 95/5	5	172	41.0
PLLA/OSS 95/5 (particle size <63 μm)	5	179	46.0
PLA–PGA 85/15	–	55.5	8.1
(PLA–PGA 85/15)/OSS 95/5	5	55	10.4
PLA–PGA 75-25	–	51.6	6.8
(PLA–PGA 75/25)/OSS 95/5	5	58	10

2.4. Evaluation of synthesised proteins

Human osteoblasts were obtained by migration and proliferation of cells from an adult membranous bone on glass fragments. Osteoblasts grown in multilayers produced a thick extracellular matrix, mainly composed of type I collagen. Confluent cultures of osteoblasts at different passages (4th and 8th) were washed with tris-buffered saline (TBS) and the cell layer was resuspended in 0.5 M acetic acid containing 250 μg/ml pepsin [7].

After 16 h of gentle shaking at 4°C, the insoluble residue was removed by centrifugation at 27,000*g* for 1 h.

Pepsin solubilised collagen was quantified by the Lowry et al. method [8] employing commercial collagen as the standard and then blotted onto a nitrocellular filter. Further the proteins were immunolabeled with rabbit antibodies against human type I or type III collagen. Finally the collagen content was evaluated by means of a scanner.

3. Results and discussion

The use of hydroxyapatite coatings to improve the biological response of implant materials has already been reported [9–11]. To the best of our knowledge, the use of natural ossein has been proposed in microfibers reinforced ceramic composites, but not in biodegradable polymers [12]. The latter approach may improve the response of biodegradable matrices since the presence of such a filler will positively influence the adhesion of growing tissues on the prosthesis element during its degradation. In order to verify the influence of the surface/volume ratios of the filler on the characteristics of the composites, we prepared composites containing a fixed amount of ossein, on a weight basis, but of different granulometry, obtained by passing the commercial ossein powder through a 60 μm mesh. For the preparation of composites, we used a melt mixer, which closely mimes a single screw extruder (see Section 2 for details). The processing temperatures were chosen on the basis of the thermal behaviour of the polymer component, as given by the DSC data. The amount of added ossein (5% (w/w)) was chosen as a compromise between the need of influencing physical–chemical and morphological characteristics of polymers without depressing the mechanical performance.

3.1. Thermal properties

Table 2 summarises thermal data of homopolymers and

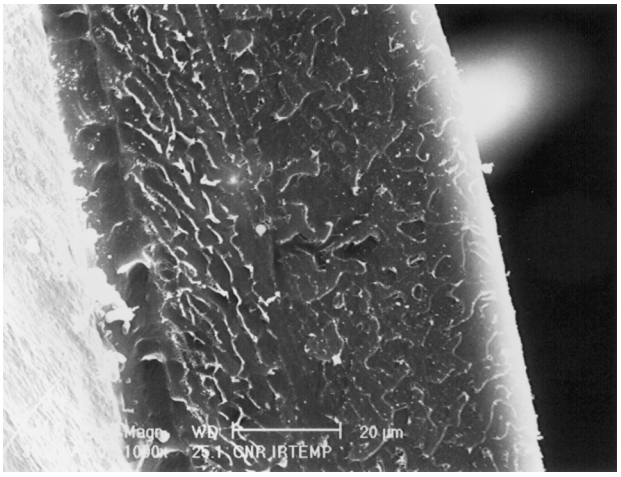


Fig. 2. SEM Micrograph of fracture surface of pure PLLA (magnification 1000 ×).

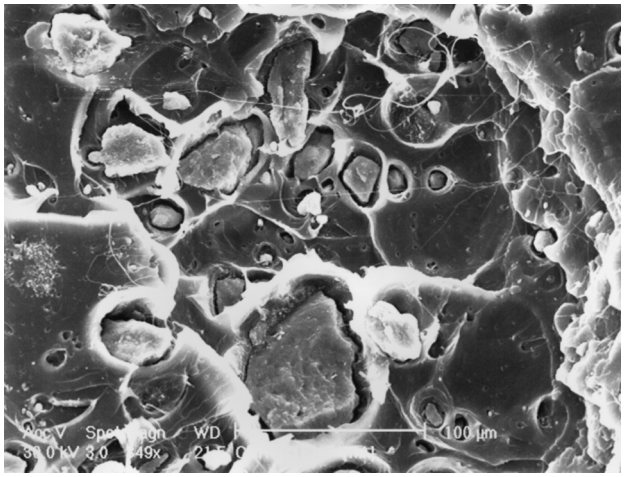


Fig. 4. SEM Micrograph of fracture surface of composites PLLA/Ossein 95/5 (as received ossein) (magnification 49 ×).

composites. Values are referred to the II Run after quenching from the melt.

In the case of PLLA, a comparison is shown between the thermal behaviour of the composite with normal ossein and with meshed ossein. While normal ossein does not influence the thermal properties of PLLA, the meshed one leads to an increase in temperature and enthalpy of melting. Similar increases are found more or less for all the investigated polymers in the presence of meshed ossein. Moreover, for PLLA and its copolymers, the addition of meshed ossein causes a complete crystallisation when quenching from the melt, while none of the said polymers is able to crystallise without ossein. It is clear that, at a certain size, ossein can act as a nucleating agent for the employed polymers. No evidences of the effect of ossein on the T_g of polymers is observed, even though a change in the shape of T_g is recorded. Particularly, the phenomenon of enthalpy relaxation above T_g, typical of PLLA, is no more observed when using meshed ossein. While this effect is probably related to a reduction in the available free volume to amorphous chains, it needs further experiments as, for example, the thermal mechanical analysis for a complete clarification.

3.2. Morphological analysis

The SEM analysis has been performed in order to investigate the mode and the state of dispersion of the ossein particles in the polymeric matrices.

Micrographs have been taken on compression moulded samples fractured in liquid nitrogen. Figs. 2, 5, 7 and 9 report each homopolymer, while Figs. 3, 6, 8 and 10 show the analogous composite with meshed ossein. In the case of PLLA, the comparison has been extended to the composite with normal ossein. It is evident for all the investigated polymers that a high level of interpenetration is achieved with the use of meshed ossein. The homogeneity of the fractured surfaces is high, and individual ossein particles are seldom seen. On the contrary, when normal ossein is

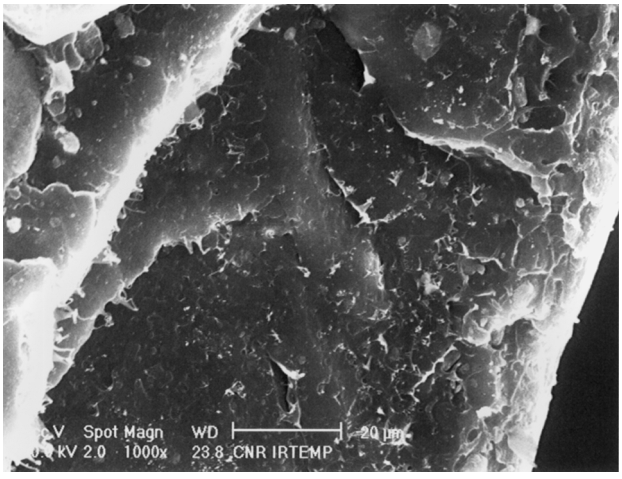


Fig. 3. SEM Micrograph of fracture surface of composites PLLA/Ossein 95/5 (magnification 1000 ×).

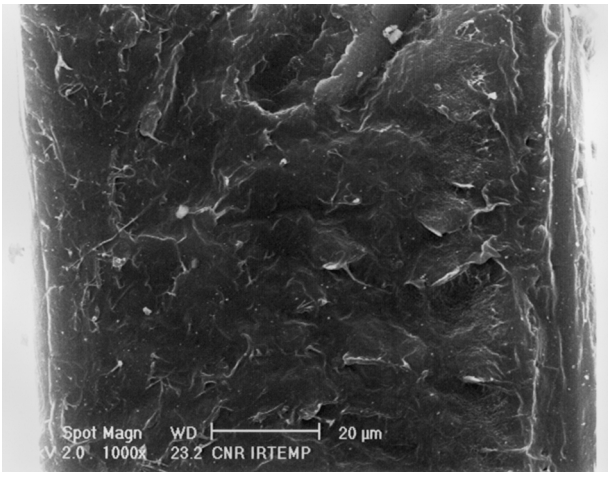


Fig. 5. SEM Micrograph of fracture surface of pure PCL (magnification 1000 ×).

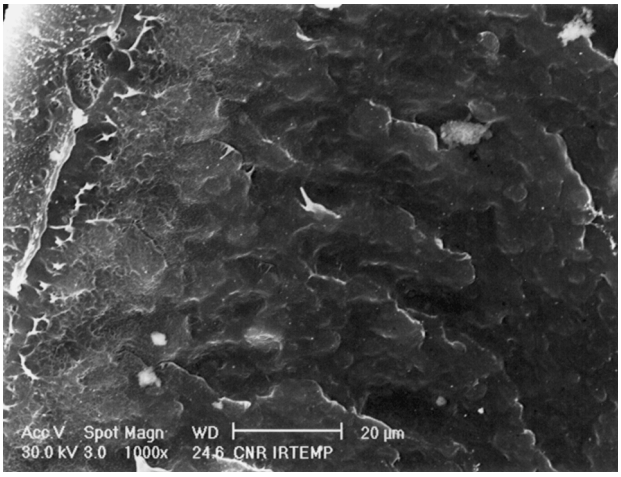


Fig. 6. SEM Micrograph of fracture surface of composites PCL/Ossein 95/5 (magnification 1000×).

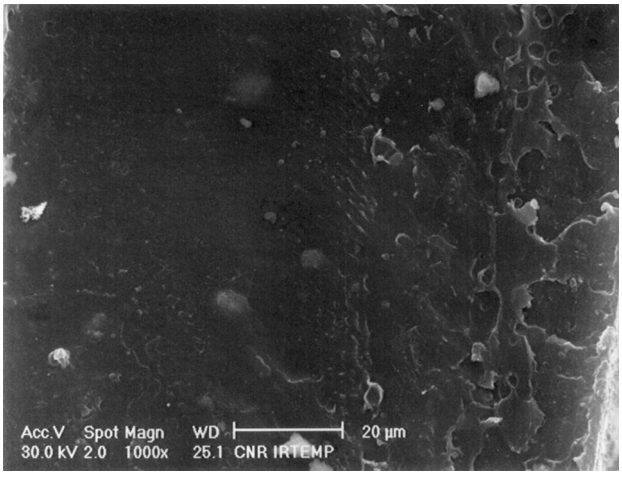


Fig. 8. SEM Micrograph of fracture surface of composites (PLA–PGA 85/15)/Ossein 95/5 (magnification 1000×).

used (see Fig. 4) the fracture surface is very irregular, particles are debonded from the matrix, especially those of larger size. Moreover, particularly in the case of PLLA, the addition of ossein changes significantly the appearance of the fracture surface, leading to patterns characterised by multiple cracks instead of large fracture lines.

3.3. Mechanical properties

The samples used for tensile tests were prepared by compression moulding. Results of tensile test results related to PLLA, PCL and their composites are reported in Table 3. The most relevant indication is that the addition of 5% ossein to homopolymers does not change the mechanical parameters of the material dramatically.

As expected, the rigid filler increases the modulus and decreases the strain- and stress-at-break of both polymers. The strain and the stress values at the yielding point decrease, too, except that of the yielding stress of PCL, which increases. This last result can be an indication of the good interfacial adhesion between PCL and ossein. In such a case, in fact, the small particles of ossein increase the energy necessary for the chains of PCL to flow and orient in the direction of the applied stress.

3.4. Biocompatibility test

In order to be proposed as biomaterials, the composites prepared and their corresponding homopolymers were subjected to the biocompatibility test using primary cultures of human osteoblastic cells as the model system [13,14].

Several features such as plating efficiency, adhesion, morphology of the cells as well as the specific biochemical parameters of osteoblastic phenotype, namely alkaline phosphatase activity and osteocalcin levels were studied. In addition, the colonisation of materials by osteoblastic cells was verified by means of SEM. Finally, the osteogenic

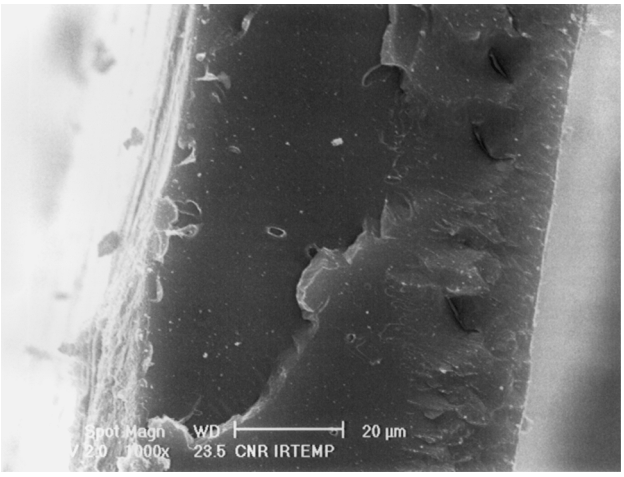


Fig. 7. SEM Micrograph of fracture surfaces of pure copolymer PLA–PGA 85/15 (magnification 1000×).

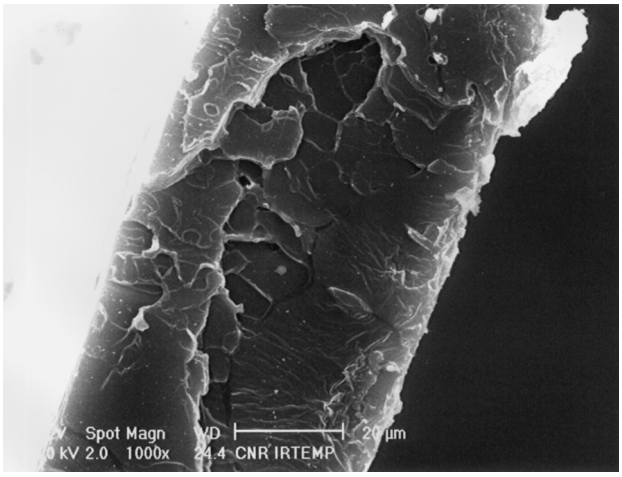


Fig. 9. SEM Micrograph of fracture surfaces of pure copolymer PLA–PGA 75/25 (magnification 1000×).

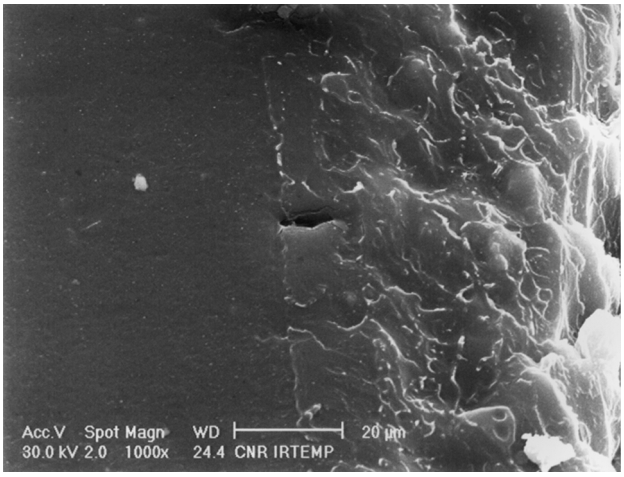


Fig. 10. SEM Micrograph of fracture surfaces of pure copolymer (PLA–PGA 75/25)/Ossein 95/5 (magnification 1000×).

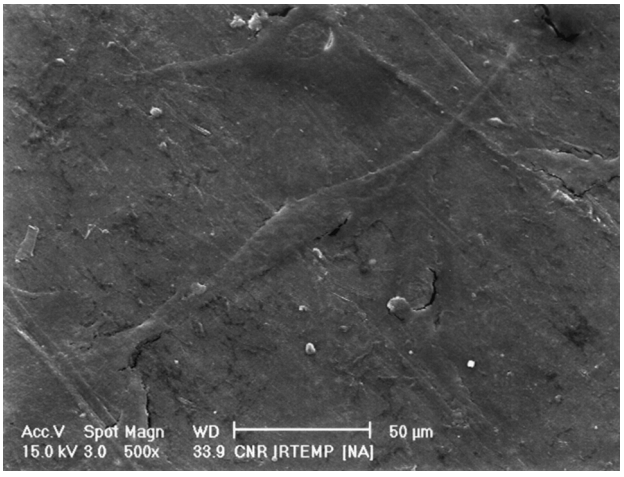


Fig. 11. SEM Micrograph of osteoblasts growth on pure PLLA (500×).

capacity, expressed by in vitro mineralization of the extracellular matrix, was verified [13,14].

While a more detailed report on the results of the above tests are beyond the target of the present paper and will be the matter of a forthcoming publication, we anticipate some positive results of the morphological investigation by SEM and of the synthesised proteins in the near future.

In Figs. 11 and 12 are reported the micrographs of pure polymers and the corresponding composites with ossein (95/5 (w/w)) after treatment with plated osteoblasts. It is evident

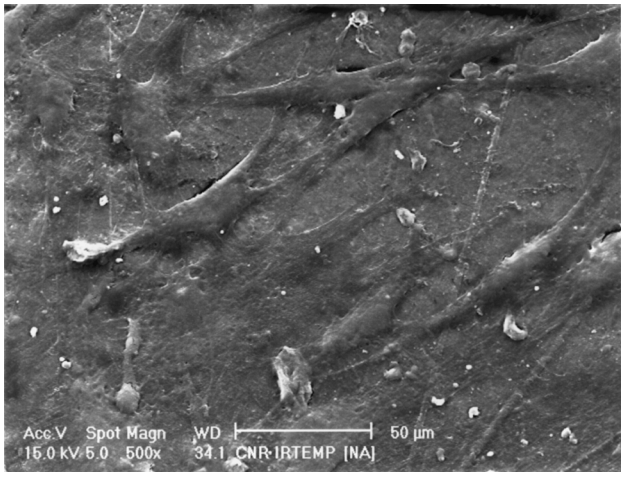


Fig. 12. SEM Micrograph of osteoblasts growth on pure PLLA/Ossein 95/5 (500×).

that the osteoblasts attached and colonised all the samples forming a dense network with ruffles and filapodia tightly anchoring the cells to biomaterials.

This phenomenon is more pronounced on the surfaces of composites, and it is possible to attribute the effect to the presence of the hydrophilic component (ossein) enhancing the cell adhesion on the otherwise hydrophobic polyesters. Similar results were observed for the other investigated polymers and corresponding composites.

The results of the investigation on the synthesised proteins are summarised in Fig. 13. We can see that for each couple, represented by the polymer and its corresponding composite, an increase of the synthesis of proteins is evident. Accordingly, we can suppose that there is also a bigger proliferative capacity of osteoblasts in the samples that are added with ossein.

4. Conclusion

The results of our research indicate that the properties of composites made of biodegradable polyesters and hydroxyapatite filler are influenced by the particle dimensions. In particular, we have demonstrated that when the size of the filler particles are reduced to microscopic levels (we tested sizes of 60 μm or less) their addition is efficacious even at low percentage (5%). Indeed, under these conditions even though the influence on the mechanical properties is

Table 3
Tensile tests

Sample	Strain-at-peak (%)	Stress-at-peak (MPa)	Strain-at-break (%)	Stress-at-break (MPa)	Young modulus (MPa)
PLLA	3.2	59.0	4.6	27.3	2330
PLLA/OSSEIN 95/5	2.6	42.0	3.0	22.7	2770
PCL	12	15.0	1085	28.4	328
PCL/OSSEIN 95/5	10	22.0	850	22.0	344

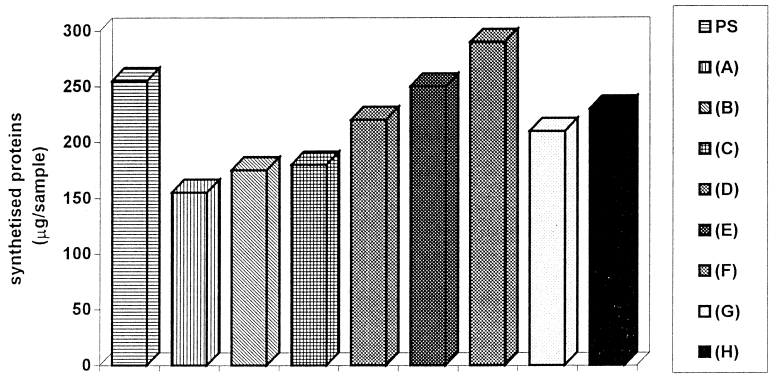


Fig. 13. Proteins synthesised by osteoblasts on each sample (medium value of 3 determinations), where PS (Polystirene); (A) PLLA; (B) PLLA/Ossein 95/5; (C) PCL; (D) PCL/Ossein 95/5; (E) PLA–PGA 75/25; (F) (PLA–PGA 75/25)/Ossein 95/5; (G) PLA–PGA 85/15; (H) (PLA–PGA 85/15)/Ossein 95/5.

rather limited, some parameters, such as the modulus, appear to be improved. Moreover, the added hydroxyapatite improves the biological response of primary cultures of osteoblasts. This effect was attributed to the improvement of the surface adhesion of growing cells due to a more pronounced hydrophilic nature of the artificial implant.

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