J Mater Sci: Mater Med (2007) 18:1627–1632
DOI 10.1007/s10856-007-3038-1

# Effect of poly(L-lactide) surface topography on the morphology of in vitro cultured human articular chondrocytes

**E. Costa Martínez · J. L. Escobar Ivirico · I. Muñoz Criado · J. L. Gómez Ribelles · M. Monleón Pradas · M. Salmerón Sánchez**

Received: 3 January 2006 / Accepted: 5 May 2006 / Published online: 5 May 2007


**Abstract** Human articular chondrocytes were cultured in vitro on poly(L-lactic) acid, PLLA, substrates. Influence of the surface topography on cell morphology was found. Different surface microtopographies were obtained on PLLA by crystallizing at 120 °C after nucleation treatments that include isothermal stages at temperatures just below (55 °C) and just above (75 °C) the glass transition temperature ($T_g$ = 65 °C). Isothermal crystallization from the melt gave rise to big spherulites (approx. 50 μm diameter) with approx. 1 μm depth. Crystallization after nucleation treatments results in smaller (approx. 5 μm)—difficult to distinguish—spherulites. Cell viability was excellent and not affected by the surface roughness. Cell population on the nucleated samples resembles the result of culture on the reference tissue culture polystyrene (TCPS). However, cells cultured on big spherulites (PLLA isothermally crystallized without nucleation treatment) show a peculiar morphology, with a more isolated disposition and growth oriented in a characteristic direction.

E. Costa Martínez · J. L. Escobar Ivirico · J. L. Gómez Ribelles · M. Monleón Pradas · M. Salmerón Sánchez
Center for Biomaterials, Universidad Politécnica de Valencia, 46022 Valencia, Spain

J. L. Gómez Ribelles · M. Monleón Pradas · M. Salmerón Sánchez (✉)
Centro de Investigación Príncipe Felipe, Autopista del Saler 16, 46013 Valencia, Spain
e-mail: masalsan@fis.upv.es

I. Muñoz Criado
Casa de Salud Hospital, c/ Dr. Manuel Candela 41, 46021 Valencia, Spain

## Introduction

Cells are known to respond to three main categories of physicochemical stimuli: chemical, topographical and mechanical [1]. The topographical cell reactions include cell orientation, rates of movement and activation of the cells [2]. Several micropatterned surfaces have been prepared both in metals [2–6] and polymer materials [2, 7–9] seeking to understand the influence of topography on cellular behaviour. The width and depth of the surface topography can influence cell responses, since cells can orient themselves along the grooves and ridges, which is called *contact guidance* [10, 11].

Even though biodegradable materials such as (PLLA)-type polylactone systems are widely used in several applications including tissue engineering scaffolds, studies on the influence of surface topography on cell response have barely been carried out on these important biomedical polymers. The alignment of osteoblast on patterned polylactide hydrophobic surfaces obtained by a solvent-cast process using a silicon template was described in [9]. Different size hemispherical island structured PLLA surfaces were created by using polystyrene (PS) with hemispherical pits as template [12]. These regular arrays of pits and islands provided possibility for cells to have specific mechanical interactions with these substrates.

PLLA is a semi-crystalline polymer. Both crystallinity and crystal shape depend on the thermal history of the material. Besides, crystallinity and surface roughness are related to each other: the size of the spherulites mainly depends on the number of crystals growing simultaneously, i.e., the number of crystal germs. The crystallization rate of PLLA is quite slow, thus, it is possible to get a quasi-amorphous polymer at low temperatures by simply quenching from the melt [13–17]. Even if the crystal

growth is nearly null when the glass transition temperature region is reached, the number of crystal nuclei should be considerable, since the nucleation rate increases with the distance to the equilibrium melting temperature. Nucleation progresses with an isothermal annealing at temperatures just above the glass transition. If the temperature is then increased to the interval in which crystal growth is significant, a great number of spherulites grow simultaneously and yield a crystalline morphology very different from that obtained by isothermal crystallisation at high temperature after a temperature jump from the melt, or by slow cooling from the melt [18].

In this work we have prepared PLLA films with different surface roughness by means of different crystallization histories that include nucleation treatments before isothermal crystallization. Human chondrocyte response to the different surfaces was evaluated by in vitro cultures focusing on changes in cell morphology. This work is a first step in the fabrication process of PLLA polymer scaffolds with interconnected pores with application in cartilage tissue engineering. The surface roughness of the pore walls in the scaffold might influence tissue regeneration. The aims of this work in to understand the influence of PLLA roughness on cell morphology in a simple, 2D, situation.

## Materials and methods

### Material

PLLA was synthesised by classical polycondensation procedures. The polymerization reactions were carried out as described elsewhere [19]. Briefly, a glass polymerization reactor equipped with a nitrogen flow-through inlet and a vacuum connection, was placed in a temperature-controlled bath containing silicone oil. Polymerization was performed in a nitrogen atmosphere at a temperature range of 100–150 °C for 12–48 h. In order to remove residual monomers, chloroform and methanol were used as solvent and precipitant, respectively. The molecular weights of the polymer, $M_n$ and $M_w$, were 58,000 and 13,2000, respectively, evaluated by gel permeation chromatography (Shimadzu, LC 10A, Japan) using PS as standard and chloroform as solvent. Samples for AFM and cell culture were casted from a 1 wt% solution in chloroform on circular microscopy slides (∅ = 15 mm). The thickness of the polymer layer was around 5 μm, estimated from the weight of the sample and the PLLA density.

### Thermal treatments

The thermal treatments started with annealing for 2 min at 200 °C, it was then cooled to 55 or 75 °C, maintained at this temperature for 12 h and subsequently crystallized at 120 °C for 2 h. A set of samples were made without nucleation treatment, i.e. crystallizing at 120 °C for 2 h from the melt. Surfaces between samples are highly reproducible (for the same thermal treatment) as checked by Atomic Force Microscopy.

### Atomic force microscopy, AFM

AFM micrographs were recorded with a Nanoscope III from Digital Instruments. The microscope was placed on a vibration-protected table. A SSS-NCH Nanoworld cantilever, with a constant force of 42 N/m and a tip radius with a 5 nm curvature was used. All the samples were characterised using a set-point amplitude ratio of around 0.7. The samples were placed in the AFM device and scanned at ambient conditions.

### Chondrocyte isolation

Human articular cartilage from the knee of a patient undergoing total knee arthroplasty was processed for chondrocyte isolation. Briefly, the cartilage tissue was aseptically dissected from the joint, minced, and washed with Dulbecco's modified Eagle's medium (DMEM; Life Technologies). Then, the cartilage was incubated for 30 min with a 0.5 mg/mL hyaluronidase (Sigma-Aldrich) solution and for 1 h with a 1 mg/mL pronase (Merck) solution in a shaking water bath at 37 °C. After that, the cartilage fragments were washed with DMEM and incubated with a 0.5 mg/mL collagenase-IA (Sigma-Aldrich) solution in a shaking water bath at 37 °C overnight. The resulting cell suspension was filtered with a 70 μm cell strainer (BD Biosciences) to remove any undigested tissue and collagenase was rinsed off with DMEM containing 10% FBS (Invitrogen SA). Finally, the cell suspension obtained was transferred in 15 mL DMEM supplemented with 10% FBS and 50 μg/mL ascorbic acid (Sigma-Aldrich) to a 75 $cm^2$ tissue culture flask (Nunc) and maintained at 37 °C, in a humidified atmosphere under 5% $CO_2$. The culture medium was replaced every 2 days and cells were allowed to grow until subconfluence. Then, the cells were harvested by trypsinisation and counted with a hemacytometer for the experiment.

### Cell culture

PLLA films pre-sterilized with 25 kGy gamma radiation were placed in a 24-well tissue culture plate and were soaked in culture medium for 72 h before cell seeding. Then, 40 $\mu$L ($5 \times 10^3$ cells) of the chondrocyte dispersion were placed onto the polymer films and were incubated at 37 °C under 5% $CO_2$ condition for 1 h. After this time,

600 $\mu$L of fresh medium were added to each well. Samples were maintained at 37 °C, in a humidified atmosphere under 5% $CO_2$ for 11 days. The culture medium used was DMEM supplemented with 10% FBS and 50 μg/mL ascorbic acid and it was renewed every 2–3 days. Cell viability was accomplished by a dye exclusion stain. Both contrast phase light microscopy and scanning electron microscopy (SEM) were carried out to observe morphological changes in cell shape. Each experiment was performed in triplicate per topography and TCPS served as the control substrate.

### Cell viability

After 7 and 11 days of culture, cell viability was measured by means of trypan blue dye exclusion. In order to test only the cells adhered onto the polymer films, samples were changed to another tissue culture well before performing the assay. Cells were observed by contrast phase light microscopy (Nikon Eclipse TS100, ×100 magnification) after being stained and the viability percentage was calculated using a counting chamber of a hemacytometer.

### Cell morphology

The attached cells onto the PLLA surfaces were observed daily by contrast phase light microscopy (CPLM). Samples cultured for 11 days were also prepared to be examined by SEM. Before SEM, cells were fixed with ethanol/acetone (1:1). Afterwards, samples were dehydrated using series of ethanol solutions (30%, 50%, 70%, 90%) for 15 min with final dehydration in absolute ethanol for 30 min. Samples were allowed to dry at room temperature in vacuo. Finally, polymer films were coated with gold and cells were observed by scanning electron microscope (Jeol JSM-6400).

## Results and discussion

The change in the material surface topography (roughness) due to the different thermal treatments was studied by atomic force microscopy, AFM. Figure 1 shows the topography pictures corresponding to samples crystallised at 120 °C after different thermal treatments. The isothermal crystallisation (2 h at 120 °C) after a temperature jump from the melt produces large spherulites (approx. 30–50 μm diameter). The roughness of the surface can be seen from the depth of the colour scale generated in Fig. 1a. The height difference between the black and bright-yellow zones is about 1 μm. To emphasize this feature, the cross section along the straight line shown in Fig. 1 has been drawn in Fig. 2.

Figure 1b shows the topography of the sample crystallized for 2 h at 120 °C after cooling from the melt to 75 °C and nucleating for 12 h. Spherulites are much smaller than those shown in Fig. 1a. The decrease in the size of the spherulites demonstrates the increase in the number of crystallization nuclei at the start of the isothermal crystallization at 120 °C.

Figure 1c shows the effect of cooling the sample from the melt to 55 °C and nucleating for 12 h. The picture corresponds to a surface of 50 × 50 μm (as in Fig. 1a, b). The cross section along the horizontal line in Fig. 1 c is shown in Fig. 2. This topography is characteristic of the structure in which the growth of the spherulites is not complete.

The different PLLA crystalline morphology, which gives rise to different surface topographies, obtained as a consequence of the different thermal treatments was recently explained and quantified as a consequence of the increase in the number of nuclei attained in the sample before the crystallization process starts [18].

Samples were seeded with human articular chondrocytes and the culture was analyzed after 7 and 11 days. Cell viability was evaluated by trypan blue uptake. Trypan blue stains dead cells in blue, while live cells exclude the dye. The results revealed that there was no significant difference in cell viability among the three surfaces and the TCPS. The cell viability remained without significant differences between day 7 and 11 of culture among the different polymer surfaces and the TCPS control.

Cell morphology was observed by contrast phase light microscopy and SEM (Fig. 3, 4). It can be seen that the

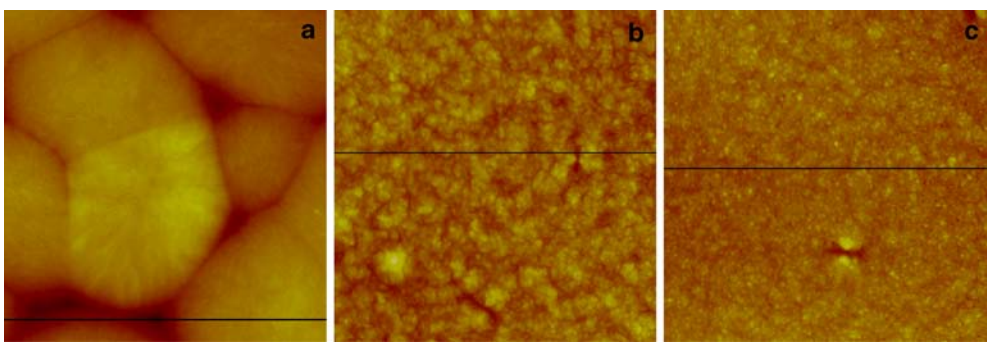


**Fig. 1** AFM images of the topography of samples crystallized for 2 h at 120 °C after different nucleation treatments. The picture dimensions are 50 × 50 μm (**a**) Temperature jump from 200 °C to 120 °C, (**b**) Sample nucleated for 12 h at 75 °C, (**c**) Sample nucleated for 12 h at 55 °C

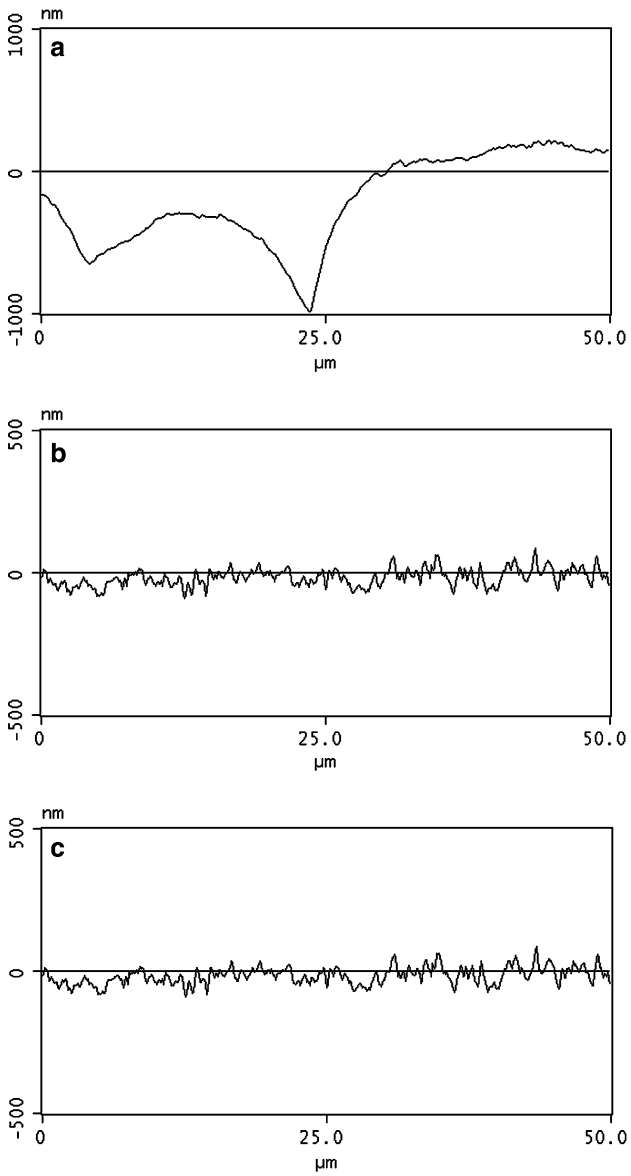


**Fig. 2** Cross-section of the pictures shown in Figure 1a, 1b and 1c, (**a**), (**b**), and (**c**) respectively, along the straight lines shown in the Figures

width of the cells on the surfaces nucleated at 55 °C and 75 °C is larger than that of cells on the sample with the biggest spherulites, i.e. those directly crystallized at 120 °C from the melt. It is suggested that the cells are able to adhere onto the small, relative to their own dimension, crystallites, grow along the surface and adopt a spread-stellate-like morphology (insets in Fig. 4). As a result, the contact area of cells on more uniform surfaces (those obtained with nucleation treatments) was larger than that of cells on a countrified surface, i.e. those crystallized at 120 °C directly from the melt, with bigger spherulites.

Figure 4 shows a higher magnification of cell morphology on the different surfaces as observed by SEM. It can be seen that the cells show different features depending on the characteristics of the surface. Cells are able to stride over the sub-micrometer irregularities of samples nucleated at 55 °C and 75 °C (Fig. 4b–c). Cell population on the nucleated samples, either at 55 °C or 75 °C, resembles the culture on the reference PS, TCPS (Fig. 3). However, cells cultured on bigger spherulites (Fig. 4a), show a peculiar morphology, with a more isolated disposition and growth oriented in a characteristic direction. This phenomenon is quite similar to the so-called *contact guidance* described in literature as to be caused by specifically designed groove-ridge topography [10, 11]. However, this is not the situation in our surfaces, since they were obtained without any template, just by crystallizing the material in different conditions.

These results suggest that more attention is deserved on the procedure the substrate is obtained when PLLA is employed in tissue engineering applications since surface topography/roughness depends on the thermal treatment and it has a consequence on cell morphology and orientation. The cell morphology did not change either all along the experiment, although a generalized increase in cellular density was observed.

Our results indicate that the micro-topography/roughness of the PLLA films can induce cell orientation and promote changes in cell shape. When a material is placed in a culture medium a protein layer will adsorb on its

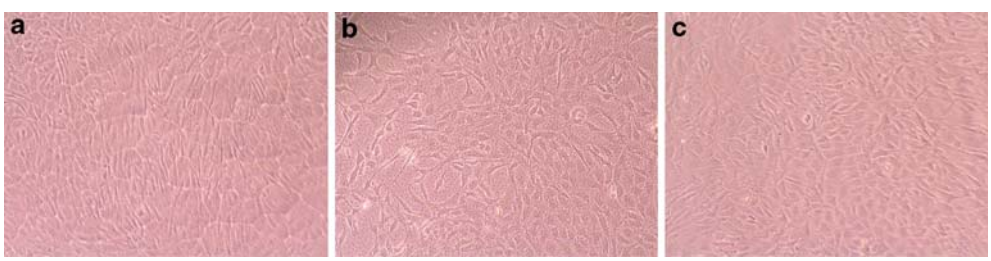


**Fig. 3** Phase light contrast microscopy images of human articular chondrocytes on PLLA films day 7 (100×). (**a**) on samples crystallized at 120 °C from the melt; (**b**) on samples crystallized at 120 °C after nucleating at 75 °C for 12 h; (**c**) on samples crystallized at 120 °C after nucleating at 55 °C for 12 h

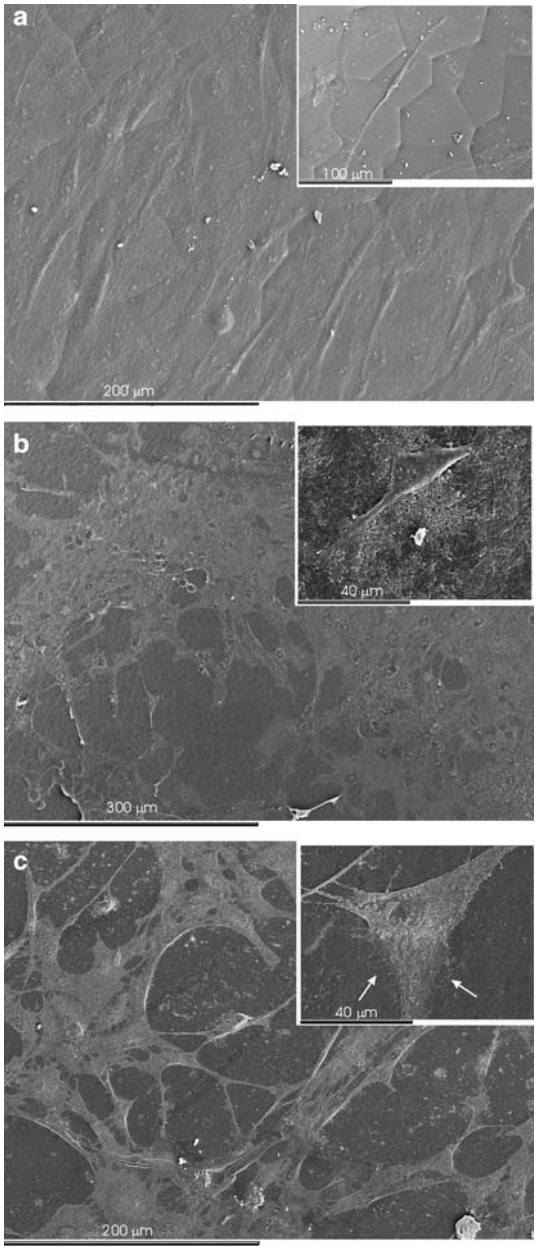


**Fig. 4** SEM pictures of human articular chondrocytes grown on different PLLA sufaces after 11 days of culture. (**a**) on samples crystallized at 120 °C from the melt; (**b**) on samples crystallized at 120 °C after nucleating at 75 °C for 12 h (the production of extracelular matrix is clearly shown in the inset); (**c**) on samples crystallized at 120 °C after nucleating at 55 °C for 12 h (the arrows in the inset shows filopodial interaction with the material)

surface, then the amount of adsorbed proteins and their distribution will mediate the interaction of the material with the cells. The adhesion molecules involved in the cellular adhesion process are the integrins that, after the binding to a ligand, will cluster together into focal contacts [20]. Cells on smooth surfaces tend to have a better organized ECM and the focal contacts are evenly distributed, while on rough surfaces focal contacts are situated at the extremities of the cells, where the cell is in contact with the substrate [21]. This might have also influenced cell behaviour through the effect on cell morphology: differences in topography might result in differences in the distribution of forces on a cell, resulting in a change in cell behaviour [22]. Further studies are needed in order to evaluate the cell adhesion process in detail as well as to investigate if this surface-dependent adhesion behaviour will modulate cell phenotype characteristics.

## Conclusions

Qualitatively different topographies (with different roughness) have been developed on PLLA by means of different thermal treatments. Cell viability has not been affected by topography and remains similar to that of TCPS. However, cell morphology strongly depends on the characteristic of the surfaces employed. Chondrocytes show the characteristic monolayer pattern distribution and morphology when cultured on films with small spehrulites (or crystallites), i.e. samples nucleated at 75 °C and 55 °C). However, on PLLA crystallized from the melt, with bigger spherulites, cells show a more elongated morphology, a more isolated disposition and growing in a characteristic direction.

**Acknowledgements** The authors are grateful to Kadriye Tuzlakoglu for providing the PLLA used in this work, the support of CICYT through the MAT2004–04980–C02–01 Project, and the support to their research group by the Generalitat Valenciana through the project GRUPOS03/018. AFM and SEM was conducted by the authors in the Microscopy Service of the Universidad Politécnica de Valencia, whose advice was greatly appreciated.

## References

1. J. Y. WONG, J. B. LEACH and X. Q. BROWN, *Surf. Sci.* **570** (2004) 119
2. A. CURTIS and C. WILKINSON, *Biomaterials* **18** (1997) 1573
3. O. ZINGER, G. ZHAO, Z. SCHWARTZ, J. SIMPSON, M. WIELAND, D. LANDOLT and B. BOYAN, *Biomaterials* **26** (2005)1837
4. G. RIZZI, A. SCRIVANI, M. FINI and R. GIARDINO, *Int. J. Art. Organs* **27** (2004) 649
5. X.L. ZHU, J. CHEN, L. SCHEIDELER, R. REICHL and J. GREIS-GERSTORFER, *Biomaterials* **25** (2004) 4087
6. T. M. LEE, R. S. TSAI, E. CHANG, C. Y. YANG and M. R. YANG, *J. Mater Sci: Mat. Med.* **13** (2002) 341
7. A. BRUINIK, J. P. KAISER and D. MEYER, *Adv. Eng. Mater.* **7** (2005) 411
8. M. LAMPIN, R. WAROCQUIER-CLEROUT, M. DEGRANGE and M. F. SIGOT-LUIZARD, *J. Biomed. Mater. Res.* **36** (1997) 99
9. H. KENAR, G. TORUN KÖSE and V. HASIRCI, *Biomaterials* **27** (2006) 885
10. D. M. BRUNETTE, *Exp. Cell Res.* **164** (1986) 11

11. P. T. OHARA and R. C. BUCK, *Exp. Cell Res*. **121** (1979) 235
12. Y. WANG, Z. LIU, X. QU, B. HAN, J. BEI and S. WANG, *Biomaterials* **26** (2005) 4453
13. S. IANNACE and L. NICOLAIS, *J Appl. Polym. Sci.* **64** (1997) 911
14. T. MIYATA and T. MASUKO, *Polymer* **39** (1998) 5515
15. M. L. DI LORENZO, *Polymer* **42** (2001) 9441
16. M. SALMERÓN SÁNCHEZ, J. L. GÓMEZ RIBELLES, F. HERNÁNDEZ SÁNCHEZ and J. F. MANO, *Thermochimica Acta* **430** (2005) 201
17. Y. WANG, J. L. GÓMEZ RIBELLES, M. SALMERÓN SÁNCHEZ and J. F. MANO, *Macromolecules* **38** (2005) 4712
18. F. HERNÁNDEZ SÁNCHEZ, J. MOLINA MATEO, F. J. ROMERO COLOMER, M. SALMERÓN SÁNCHEZ, J. L. GÓMEZ RIBELLES and J. F. MANO, *Biomacromolecules* **6** (2005) 3283
19. H. YAVUZ, C. BABAC, K. TUZLAKOGLU and E. PISKIN, *Polym. Degrad. Stabil.* **75** (2002) 431
20. A. J. GARCÍA, *Biomaterials* **26** (2005) 7525
21. K. ANSELME, M. BIGERELLE, B. NOEL, E. DUFRESNE, D. JUDAS, A. IOST and P. HARDOUIN, *Biomed. Mater. Res.* **49A** (2000) 155
22. M. C. SIEBERS, P. J. TER BRUGGE, X. F. WALBOOMERS and J. A. JANSEN *Biomaterials* **26** (2005) 137