Available online at www.sciencedirect.com
ScienceDirect
Journal of the European Ceramic Society 27 (2007) 1589–1594
www.elsevier.com/locate/jeurceramsoc

Biphasic calcium phosphate coated with poly-D,L-lactide-*co*-glycolide biomaterial as a bone substitute

Nenad Ignjatovic [a], Petar Ninkov [b], Zorica Ajdukovic [c], Dana Vasiljevic-Radovic [d], Dragan Uskokovic [a,*]

[a] *Institute of Technical Sciences of SASA, Belgrade, Serbia and Montenegro*
[b] *Faculty of Medicine, Clinic of Stomatology, University of Novi Sad, Novi Sad, Serbia and Montenegro*
[c] *Faculty of Medicine, Clinic of Stomatology, University of Niš, Niš, Serbia and Montenegro*
[d] *IHTM, Institute of Microelectronic Technologies and Single Crystals, Belgrade, Serbia and Montenegro*

Available online 5 June 2006

Abstract

A calcium phosphate/poly-D,L-lactide-*co*-glycolide (BCP/DLPLG) composite biomaterial was synthesized in the shape of spherical granules (150–200 μm) and nanostructured particles (30–40 nm) by an emulsification/evaporation procedure. Calcium phosphate components were coated with amorphous polymer phase. The influence of the processing technique on the structure and characteristics of the BCP/DLPLG composite biomaterial was studied by X-ray diffraction analysis (XRD), differential scanning calorimetry (DSC), scanning electronic microscopy (SEM), and atomic force microscopy (AFM). In vitro cytotoxicity research was conducted on cellular cultures of fibroblasts of animals. The possibility of BCP/DLPLG application was examined by its use in reparation of lost bone tissue of patients.

Keywords: Powders-chemical preparation; Composites; Interfaces; Chemical properties; Biomedical applications

1. Introduction

The success of a biomaterial in the body depends on many factors such as properties, design, and biocompatibility of that material, as well as other factors not under control of the engineer, including the technique used by the surgeon, health, condition and activities of the patient.[1] Of particular interest is the central position that biomaterials (especially composites) have taken in the development of novel treatments over the last 10 years.[2] The most used calcium phosphate in implant fabrication is hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, since it is the most similar material to the mineral component of bones. It exhibits good properties of biomaterials, such as biocompatibility, bioactivity, osteoconductivity, direct bonding to bone, etc.[3,4] Lately, great attention has been paid to biphasic calcium phosphate (BCP) consisting of a mixture of hydroxyapatite (HAp) and β-tricalcium phosphate (TCP).[5] Biodegradable polymers produce biocompatible, toxicologically safe by-products that are further eliminated by normal metabolic pathways. Poly(D,L-lactide-*co*-glycolide) (DLPLG) has been widely used as a carrier in controlled release/delivery systems due to its biodegradability and relatively good biocompatibility.[6] Copolymers of glycolide with L-lactide and D,L-lactide have been developed for both device and drug delivery applications.[7] DLPLG has been used in tissue engineering, drug and gene therapy, preparation of nanoparticles for anticarcinogenic drugs, etc.[8–10]

The production of inorganic–organic hybrids is a subject of many researches, as well as the manufacturing of calcium phosphate/bioresorbable polymer[11–17] composite biomaterials. Calcium phosphate mixed with DLPLG polymer intensifies the activity of alkaline phosphatase, which is important for the differentiation of osteoblasts that dictate regeneration process within the organism.[18,19] Calcium phosphate/DLPLG composite biomaterials were obtained by hydrolysis of α-tricalcium phosphate-DLPLG precomposite at nearly physiological temperatures.[20] Highly porous calcium phosphate/DLPLG composite was used in tissue engineering. Short calcium phosphate fibers were incorporated into polymer foams to improve their mechanical properties.[21] The size of calcium phosphate particles was found to be critical for the improvement of their mechanical properties.[22] In our recent study, we reported

* Corresponding author at: Institute of Technical Sciences of the Serbian Academy of Sciences and Arts, K. Mihajlova 35/IV, 11000 Belgrade, Serbia and Montenegro. Tel.: +381 11 2636994; fax: +381 11 185263.
E-mail address: uskok@itn.sanu.ac.yu (D. Uskokovic).

0955-2219/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jeurceramsoc.2006.04.104

on the production of BCP/DLPLG composite biomaterials in the form of granules suitable for filling up different bone defects.[23] Spherical granules of BCP covered with DLPLG were the basis of composite biomaterials. Application of BCP/DLPLG facilitated overgrowth of new-formed vascular tissue, fibroblasts and intensified the activity and adherence of osteoblasts.[24] Injectable biomaterials, based on BCP and bioresorbable polymer, are of especial interest in bone tissue engineering. Their advanced properties are good mechanical characteristics, biocompatibility and facile defect regeneration.[25] Nano-sized spherical particles of BCP/DLPLG composite biomaterial can be an ideal basis for injectable biomaterials with designed hydro-dynamic properties.

In this paper we report on a new biphasic calcium phosphate/poly(D,L-lactide-*co*-glycolide) (BCP/DLPLG) composite biomaterial prepared in the form of micro- or nanospheres of calcium phosphate coated with polymer. Cytotoxicity was studied by in vitro test on cellular cultures of mice (L-929). In a preliminary study, the possibility to substitute bone tissue with BCP/DLPLG was examined by repairing alveolar bones and monitoring alkaline phosphatase of patients.

2. Materials and methods

A calcium phosphate gel was prepared by precipitation of calcium nitrate and ammonium phosphate in an alkaline medium.[11,12] Poly-D,L-lactide-*co*-glicolide (DLPLG) (50:50) (Sigma Chemical Company, USA) was used as a polymer component. PVA was of 98%-hydrolization degree.

2.1. Preparation of composite biomaterials

2.1.1. Procedure 1

Gel of calcium phosphate was added into completely dissolved polymer, in amount of 80 mass%. The suspension was mixed at a velocity of 1200 rpm, and then methanol was added. Afterwards, PVA (0.02%) was added into the suspension (DLPLG/PVA = 10/1). Addition of methanol into the three-component system of solvent-polymer-calciumphosphate caused its thermodynamic destabilization. This induced sedimentation of polymer onto calciumphosphate particles and their covering with polymer. After the solvent evaporation, the particles were dried at the room temperature for 24 h. The particles of calcium phosphate/DLPLG composite biomaterial were sterilized by γ rays (25 kGy) before use.

2.1.2. Procedure 2

The gel was dried, granulated at room temperature and calcined at 1100 °C for 6 h. Granules of calcium phosphate were added into completely dissolved polymer, in amount of 80 mass%. The suspension was mixed at a velocity of 30 rpm, and then methanol was added. Afterwards PVA (0.02%) was added into the suspension (DLPLG/PVA = 10/1). After the solvent evaporation, the granules were dried at room temperature for 24 h. The granules of calcium phosphate/DLPLG composite biomaterial, sizes of 150–200 μm, were sieved[23,24] and sterilized by γ rays (25 kGy) before use.

X-ray structural (XRD) analyses were made by using an *Enraf Nonius FR590* diffractometer. Microstructural characterization was done by AFM (*Thermo Microscopes, Autoprobe CP Research*) and SEM (*JSM 5300*). Infrared spectra were obtained at room temperature using an Avatar 370 FTIR Thermo Nicolet Fourier-transform infrared spectrometer in the spectral range between 400 and 4000 cm^{-1}.

Cytotoxicity investigations were performed by cell line, mouse fibroblasts (L929). Only living (so-called viable) cells were used. Petri dishes with seeded cells were left in a thermostat at 37 °C, in air comprising 5% of CO_2, for 24 h. After the incubation, the cells were separated from discs by tripsization and then counted on a light microscope. Discs of implanting material were after 24 h-incubation soaked into a medium of L929 cell cultures and fixed on sample carriers by a carbon tape. After cooling, they were soaked into liquid nitrogen until reaching the freezing point and then observed by low-vacuum SEM (JEOL-JSM 6460LV).

Preliminary clinical research was done on women aged from 46 to 62 years. Alkaline phosphatase concentrations in blood of 50 menopausal women were determined. In the experimental group of 25 patients (A), teeth of the premolar or molar region were extracted and the missing tissue replaced with BCP/DLPLG, while in the control group also of 25 patients (B), only extraction of teeth was performed.

3. Results and discussion

The XRD patterns of DLPLG and prepared BCP/DLPLG composite biomaterials are showed in Fig. 1. The most intense peaks at $2\theta = 31.8$, 32.9, 25.9 and 46.7° originate from calcium hydroxyapatite (HAp) and those at $2\theta = 31$, 34.3 and 27.8° from β-tricalcium phosphate (β-TCP). Based on earlier described methodology, mass contents of HAp and β-TCP of 80 and 20%, respectively, were calculated.[26] Thereby, this calcium phosphate is also called biphasic calcium phosphate (BCP) and was used to produce BCP/DLPLG composite biomaterial. DLPLG is a com-

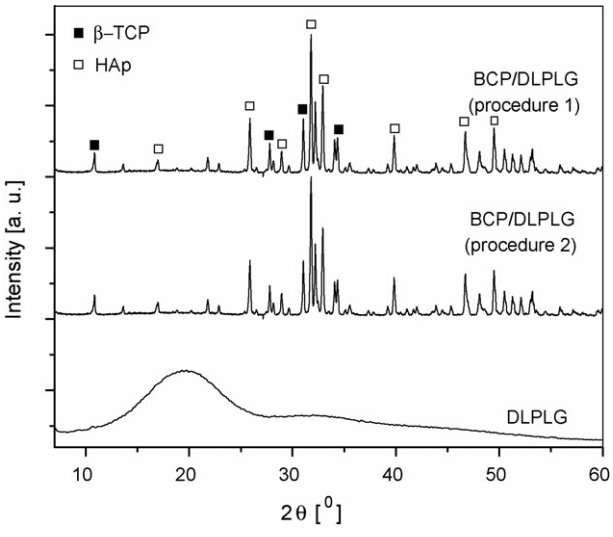

Fig. 1. XRD patterns of DLPLG and BCP/DLPLG composite biomaterials.

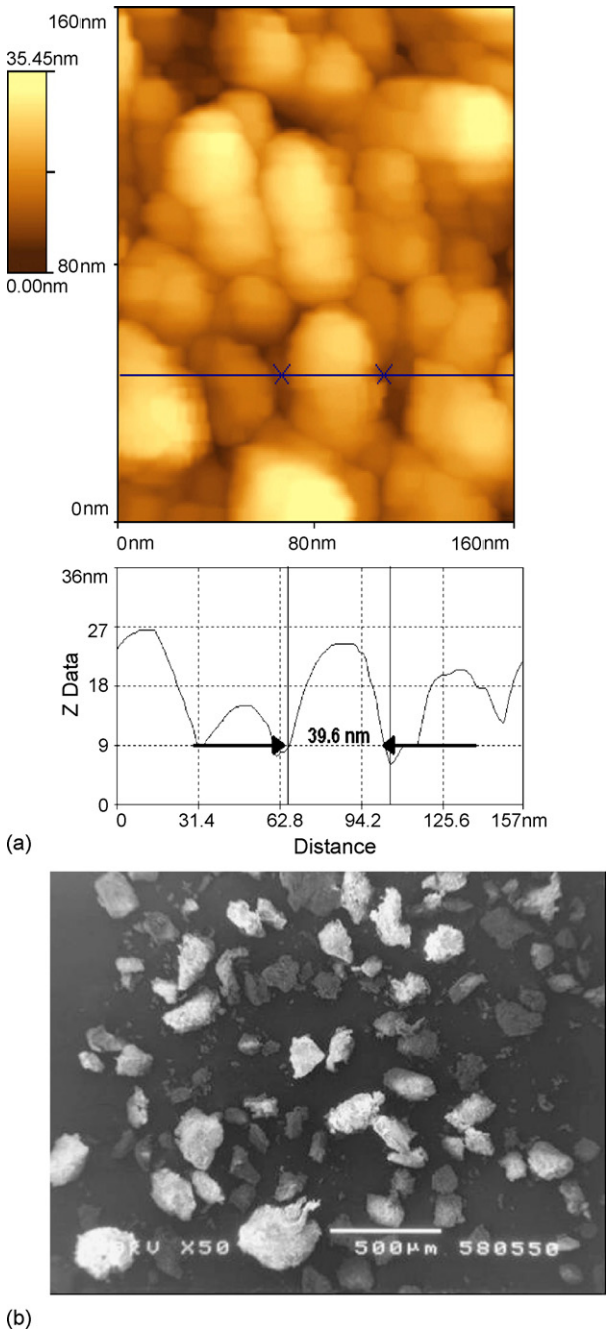

Fig. 2. (a). AFM topology of the surface of BCP/DLPLG composite biomaterial obtained by procedure 1; (b). SEM topology of the surface of BCP/DLPLG composite biomaterial obtained by procedure 2.

pletely amorphous polymer as confirmed by a pattern without peaks, Fig. 1. The XRD result on DLPLG corresponds to the XRD results obtained by other authors.[27]

According to DSC analysis, all transformations occurring in the temperature range from 305 to 207 °C are connected with DLPLG. The only transformation common to both composites (procedure 1 and 2) is characterized by a peak at the glass transition temperature of 51 °C. These results indicate that DLPLG is a completely amorphous polymer.

The microstructure of BCP/DLPLG composite biomaterial was studied by AFM and SEM. Fig. 2 shows the topology of the surface of BCP/DLPLG composite biomaterial prepared according to both procedures (1 and 2). Fig. 2a shows an AFM image of BCP/DLPLG composite. Spherical forms are discernable in Fig. 2a. BCP particles are coated with the polymer and the average diameter is 40 nm. Composite production via emulsification/evaporation procedure provides covering of BCP with DLPLG polymer.[23,24] Using BCP gel (procedure 1), we obtained a nanospherical form of BCP/DLPLG composite biomaterial. The nanospherical form of BCP/DLPLG enabled synthesis of an injectable paste, which can be successfully used in reconstruction of small bone damages. Nanoparticles may have advantages in favor of adhesion, adsorption and interaction with cells.[10] To our knowledge, none of the papers of other authors have shown the possibility of obtaining nanospherical particles of calcium phosphate coated with bioresorbable polymers. Fig. 2b shows an SEM image of BCP/DLPLG composite biomaterial obtained according to the procedure 2. BCP granules are coated with the polymer and their average diameter is 150–200 µm.

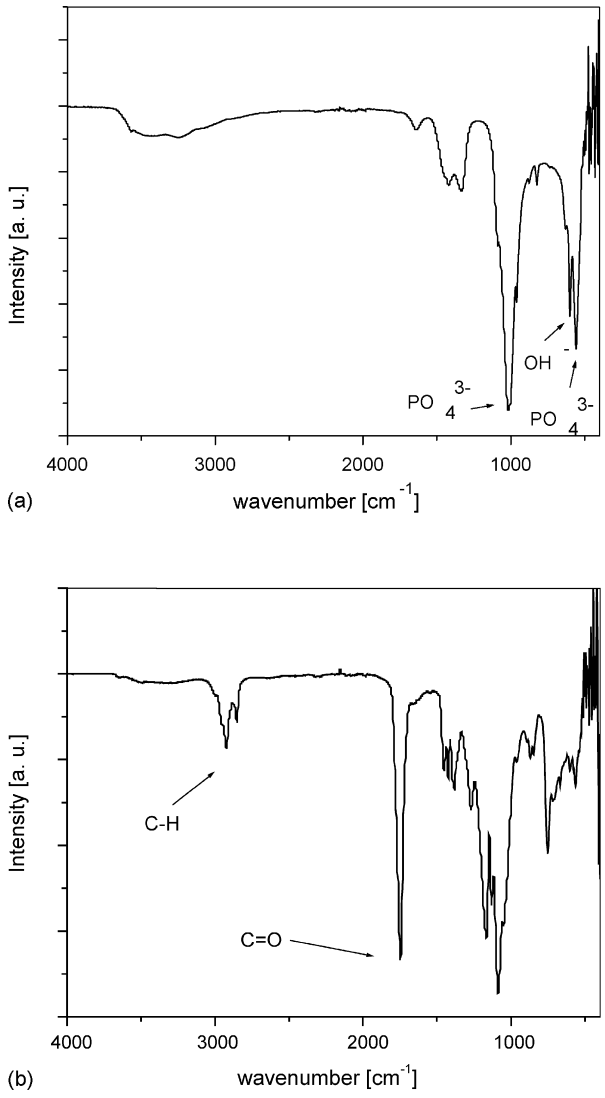

Fig. 3. FT-IR spectra of BCP/DLPLG composite biomaterials obtained by procedure 1; (a) BCP from BCP/DLPLG (b) DLPLG from BCP/DLPLG.

The composite biomaterial, whose topology is shown in Fig. 2a, was analyzed by FT-IR spectroscopy to verify indirectly whether each BCP particle was coated with DLPLG. Analysis of the obtained FT-IR spectrum was impossible since it was not made of clear bands. This was expected since it is the spectrum of very small particles and thin polymer layers (far below 50 nm). Therefore, the composite was dissolved in chloroform, centrifuged, vacuum vaporized and dried. The final goal was to separate calcium phosphate particles from the polymer, i.e. take off the polymer layer from the calcium phosphate particle surface. Components were separated and their spectra shown in Fig. 3a and b. The spectrum shown in Fig. 3a corresponds to calcium phosphate since it contains groups characteristic of BCP,[23] while the spectrum shown in Fig. 3b corresponds to the polymer since it contains groups characteristic of DLPLG.[24] BCP is identified within the spectrum by a doublet with maxima at 1052 and 1087 cm^{-1}, which are the most intense and originate from phosphate groups, and by a triplet with maxima at somewhat lower frequencies of 571 and 602 cm^{-1}, arising from the PO_4^{3-} group vibrations, and at 632 cm^{-1}, assigned to hydroxyl group vibrations appearing also at 3567 cm^{-1}.[23] DLPLG is characterized by an absorption band at 1756 cm^{-1} corresponding to C=O group vibrations and two lower maxima at 2996 and 2944 cm^{-1} ascribed to C–H group vibrations. Absorption maximum at 1449 cm^{-1} originates from the CH_3 group.[24]

A detail from the spherical granule surface in Fig. 2b shows a BCP granule coated with DLPLG polymer, which was clearly confirmed in our recent research by SEM and EDX analysis.[24] This methodology could not have been used on nanoparticles, so the proof was sought in indirect FT-IR analysis.

All further in vitro and in vivo researches were done with BCP/DLPLG composite biomaterial granules, made by procedure 2. In vitro and in vivo research on nano-sized particles of the same composite is on the way and will be published separately, due to specific preliminary results.

In Fig. 4a, the fibroblasts from the cell culture L929, developed on the BCP/DLPLG substrate for 24 h, can be seen. After rinsing, the number of survived cells was followed up for 24, 48 and 72 h, and the results are shown in Fig. 4b. The increase in cell number indicates successful and complete recovery of fibroblasts, stimulation of metabolic activities and the absence of cytotoxicity.

Alkaline phosphatase is a biochemical indicator of bone synthesis, i.e., intensity of newly formed bone-matrix synthesis. Fig. 5a shows the place of extraction of teeth (groups A and B) and replacement of the lost bone tissue with the composite (group A). Fig. 5b illustrates alkaline phosphatase concentra-

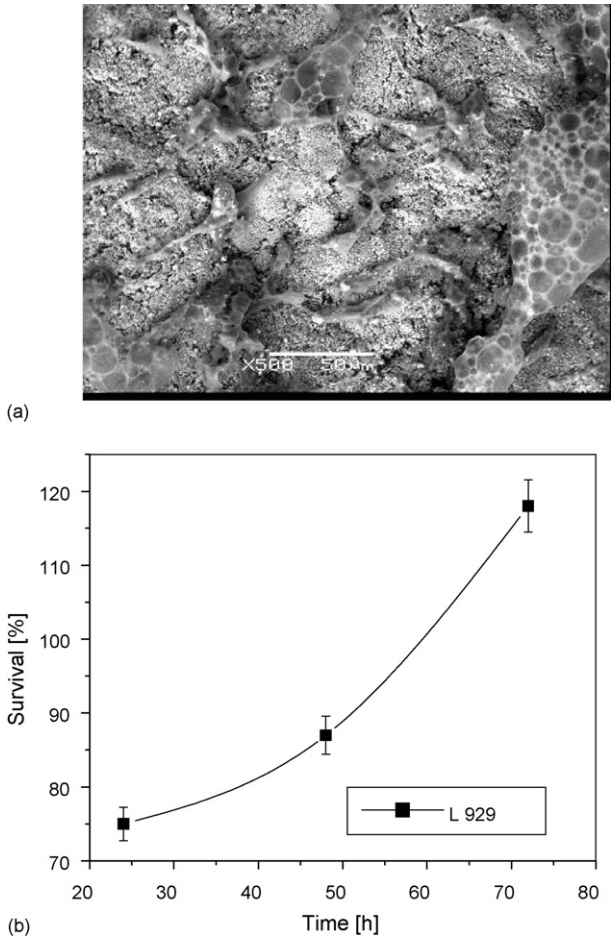

Fig. 4. (a). SEM image of fibroblasts from the cell culture L929 on the surface of BCP/PLGA after 24 h; (b). Dependence of the cell culture L929 survival on time after treatment with BCP/DLPLG.

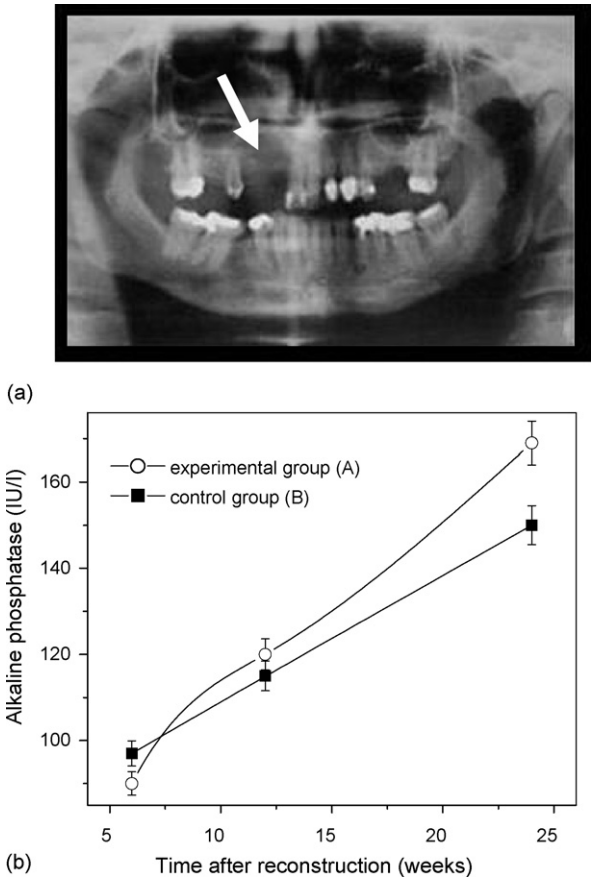

Fig. 5. (a). X-ray image of the place of extraction and reconstruction of alveolar bone; (b). Concentration of alkaline phosphatase after reconstruction.

tions after 6, 12 and 24 weeks after extraction of teeth and implantation of the composite.

Six weeks after the reconstruction, a decrease in alkaline phosphatase in the experimental group of patients (A) contrary to the control one (B) was observed. A difference between the alkaline phosphatase of the experimental (A) and the control group (B) becomes obvious after 12 weeks. After the sixth week, alkaline phosphatase in the experimental group (A) slowly increases, but the difference is not that significant when compared with the control group (B). Increased alkaline phosphatase in the experimental group (A) in the 12th week indicates active deposition of the bone matrix and more intense osteogenesis in the alveolar bone. Significantly higher alkaline phosphatase levels in blood serums of patients of group A than those of the control one (B), suggesting rapid osteogenesis, were registered in the 24th week. These higher levels of alkaline phosphatase in blood serum of group (A) compared with those of the sixth and 12th week are a sign of regenerative and reparative processes taking place in bones, induced most probably by BCP/DLPLG composite. Increase in alkaline phosphatese is significant, because the osteoblasts secrete more alkaline phosphatase while actively deposit bone matrix.

4. Conclusion

A calcium phosphate/poly-D,L-lactide-*co*-glycolide (BCP/DLPLG) composite biomaterial was synthesized in the shape of spherical granules (150–200 μm in diameter) and nanoparticles (30–40 nm in diameter). Each BCP granule or nanoparticle was coated with amorphous DLPLG polymer.

Calcium phosphate present in the composite was in the form of biphasic calcium phosphate consisting of 80% calcium hydroxyapatite and 20% tricalciumphosphate.

In vitro research on cellular cultures of mice (L-929) showed good adherence of fibroblast cells to the composite biomaterial surface. The granules of BCP/DLPLG composite biomaterial did not inhibit the fibroblast growth in cellular cultures. Survival of cells was high and, therefore, non-essential cytotoxic effects were present.

The use of granules of BCP/DLPLG composite biomaterials in treatment of alveolar bone defects showed high level of osseous regeneration. After the extraction of teeth and reconstruction of lost bone tissue with the composite, increase in alkaline phosphatase in patients' blood indicate highly active deposition of the bone matrix.

Acknowledgments

This work was supported by the Ministry of Science, Technology and Development of the Republic of Serbia, under Project No. 142006: Synthesis of functional materials with controlled structure on molecular and nano level. The authors thank Drs. M. Mitric and Lj. Kandic for their help in X-ray measurements, and J. Vukelic and M. Miljkovic for FT-IR and SEM analysis.

References

1. Park, J. and Lakes, R., *Biomaterials: An Introduction* (*2nd ed.*). Plenum Press, New York, 1992, pp 1–6.
2. Langer, R. and Peppas, N., Advances in biomaterials, drug delivery and bionano-technology, Bioengineering. *Food and Natural Products*, 2003, **19**, 2990–3006.
3. Vallet-Regi, M. and Gonzalez-Calbet, J., Calcium phosphates as substitution of bone tissue. *Progress in Solid State Chemistry*, 2004, **32**, 1–31.
4. Bauer, T. and Smith, S., Bioactive materials in orthopaedics surgery: overview and regulatory considerations. *Clinical Orthopaedics and Related Research*, 2002, **395**, 11–22.
5. Legeros, R., Lin, S., Rohanizadeh, R., Mijares, D. and Legeros, J., Biphasic calcium phosphate bioceramics: preparation, properties and application. *Journal of Materials Science: Materials in Medicine*, 2003, **14**, 201–209.
6. Seo, S., Choi, H., Khang, G., Rhee, J. and Lee, H., A local delivery system for fentanyl based on biodegradable poly(L-lactide-*co*-glycolide) oligomer. *International Journal of Pharmaceutics*, 2002, **239**, 93–101.
7. Middleton, J. and Tipton, A., Synthetic biodegradable polymers as orthopedic devices. *Biomaterials*, 2000, **21**, 2335–2346.
8. Tosi, G., Rivasi, F., Gandolfi, F., Costantino, L., Vandelle, M. and Forni, F., Conjugated poly(D,L,-lactide-*co*-glycolode) for preparation of in vivo detectable nanoparticles. *Biomaterials*, 2005, **26**, 4189–4195.
9. Ravi Kumar, M., Bakowski, U. and Lehr, C., Preparation and characterization of cationic PLGA nanospheres as DNA carriers. *Biomaterials*, 2004, **25**, 1771–1777.
10. Si-Sheng, E., Li, M., Khin, W. and Guofeng, H., Nanoparticles of biodegradable polymers for clinical administration of paclitaxel. *Current Medicinal Chemistry*, 2004, **11**, 413–424.
11. Ignjatovic, N., Tomic, S., Dakic, M., Miljkovic, M., Plavsic, M. and Uskokovic, D., Synthesis and properties of hydroxyapatite/poly-L-lactide composite biomaterials. *Biomaterials*, 1999, **20**, 809–816.
12. Ignjatovic, N., Plavsic, M., Miljkovic, M., Zivkovic, Lj. and Uskokovic, D., Microstructural characteristic of Ca-hydroxyapatite/poly-L-lactide based composites. *Journals of Microscopy*, 1999, **196**, 243–248.
13. Kikuchi, M., Suetsugu, Y., Tanaka, J. and Akao, M., Preparation and mechanical properties of calcium phosphate/copoly-L-lactide composites. *Journal of Materials Sciences: Materials in Medicine*, 1997, **8**, 361–364.
14. Oliveira, J., Miyazaki, T., Lopes, M., Ohtsuki, C. and Santos, J., Bonelike®/PLGA hybrid materials for bone regeneration: preparation route and physicochemical characterization. *Journal of Materials Sciences: Materials in Medicine*, 2005, **16**, 253–259.
15. Suchanek, W. and Yoshimura, M., Processing and properties of hydroxyapatite—based biomaterials for use as hard tissue replacement implants. *Journal of Materials Research*, 1998, **13**, 94–117.
16. Liu, Q., de Wijn, J. and van Blitterswijk, C., Nanoapatite/polymer composites: mechanical and physicochemical characteristics. *Biomaterials*, 1997, **18**, 1263–1270.
17. Shikinami, Y. and Okuno, M., Bioresorbable devices made of forged composites of hydroxyapatite (HA) particles and poly-L-lactide (PLLA): part I. Basic characteristics. *Biomaterials*, 1999, **20**, 859–877.
18. Elgendy, H., Norman, M., Keaton, A. and Laurencin, C., Osteoblast-like cell (MC3T3-E1) proliferation on bioerodible polymers: an approach towards the development of a bone-bioerodible polymers composite materials. *Biomaterials*, 1993, **14**, 263–269.
19. Ara, M., Watanabe, M. and Imai, Y., Effect of blending calcium compounds on hydrolytic degradation of poly(D,L-lactic acid-*co*-glycolid acid). *Biomaterials*, 2002, **23**, 2479–2483.
20. Durucan, C. and Brown, P., Calcium deficient hydroxyapatite-PLGA composites: mechanical and microstructural investigation. *Journal of Biomedical Materials Research*, 2000, **51**, 726–734.
21. Lu, L. and Micos, A., The importance of new processing techniques in tisse engineering. *MRS Bull.*, 1996, **21**, 28–31.
22. Calandrelli, L., Immirizi, B., Malincowico, M., Volpe, M., Oliva, A. and Ragione, F., Preparation and characterization of composites based on biodegradable polymers for in vivo application. *Polymer*, 2000, **41**, 8027–8033.

23. Ignjatovic, N., Ninkov, P., Ajdukovic, Z., Konstantinovic, V. and Uskokovic, D., Synthesis, characterization and application of composite biomaterials biphasic calcium phosphate/poly-DL-lactide-*co*-glycolide as filler and blocks for reparation hard bone tissue. *Mater Science Forum*, 2005, **494**, 519–524.
24. Ignjatovic, N., Ajdukovic, Z. and Uskokovic, D., New biocomposite calciumphosphate/poly-DL-lactide-*co*-glycolide/biostimulatite agents filler for reconstruction of bone tissue changed by osteoporosis. *Journal of Materials Sciences: Materials in Medicine*, 2005, **16**, 624–626.
25. Temeno, J. and Mikos, A., Injectable biodegradable materials for orthopedic tissue engineering. *Biomaterials*, 2000, **21**, 2405–2412.
26. Raynaud, S., Champion, E., Assollant, D. and Laval, J., Determination of calcium/phosphorus atomic ratio of calcium phosphate apatites using X-ray diffractometry. *Journal of the American Ceramic Society*, 2001, **84**, 359–366.
27. Jeon, H. J., Jeong, Y. I., Jang, M. K., Park, Y. H. and Nah, J. W., Effect of solvent on the preparation of surfactant-free poly(D,L-lactide-*co*-glycolide) nanoparticles and norfloxacin release characteristics. *International Journal of Pharmaceutics*, 2000, **207**, 99–108.