J Mater Sci: Mater Med (2008) 19:1617–1623
DOI 10.1007/s10856-008-3378-5

# A novel enzymatically-mediated drug delivery carrier for bone tissue engineering applications: combining biodegradable starch-based microparticles and differentiation agents

**Elizabeth Rosado Balmayor · Kadriye Tuzlakoglu · Alexandra P. Marques · Helena S. Azevedo · Rui L. Reis**

Received: 8 September 2007 / Accepted: 4 January 2008 / Published online: 24 January 2008


**Abstract** In many biomedical applications, the performance of biomaterials depends largely on their degradation behavior. For instance, in drug delivery applications, the polymeric carrier should degrade under physiological conditions slowly releasing the encapsulated drug. The aim of this work was, therefore, to develop an enzymatic-mediated degradation carrier system for the delivery of differentiation agents to be used in bone tissue engineering applications. For that, a polymeric blend of starch with polycaprolactone (SPCL) was used to produce a microparticle carrier for the controlled release of dexamethasone (DEX). In order to investigate the effect of enzymes on the degradation behavior of the developed system and release profile of the encapsulated osteogenic agent (DEX), the microparticles were incubated in phosphate buffer solution in the presence of α-amylase and/or lipase enzymes (at physiological concentrations), at 37°C for different periods of time. The degradation was followed by gravimetric measurements, scanning electron microscopy (SEM) and Fourier transformed infrared (FTIR) spectroscopy and the release of DEX was monitored by high performance liquid chromatography (HPLC). The developed microparticles were shown to be susceptible to enzymatic degradation, as observed by an increase in weight loss and porosity with degradation time when compared with control samples (incubation in buffer only). For longer degradation times, the diameter of the microparticles decreased significantly and a highly porous matrix was obtained. The in vitro release studies showed a sustained release pattern with 48% of the encapsulated drug being released for a period of 30 days. As the degradation proceeds, it is expected that the remaining encapsulated drug will be completely released as a consequence of an increasingly permeable matrix and faster diffusion of the drug. Cytocompatibility results indicated the possibility of the developed microparticles to be used as biomaterial due to their reduced cytotoxic effects.

E. R. Balmayor (✉) · K. Tuzlakoglu · A. P. Marques · H. S. Azevedo · R. L. Reis
3B's Research Group – Biomaterials, Biodegradables and Biomimetics, Department of Polymer Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
e-mail: erosado@dep.uminho.pt
URL: www.3bs.uminho.pt

E. R. Balmayor · K. Tuzlakoglu · A. P. Marques · H. S. Azevedo · R. L. Reis
IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

## 1 Introduction

Development of materials in the particulate form has been a subject of great interest for several decades. In fact, nowadays polymeric microparticles have been used in several applications, including chemical, biological and medical uses [1–4], and more recently in Tissue Engineering research [1, 5–7]. Since the early 80s, the possibility of incorporating different drugs and bioactive agents into small polymeric vehicles, was predicted as a promising approach for the administration of those agents [1, 8–10]. More recently, the use of biodegradable polymers to design controlled drug delivery carriers, having the additional advantage of minimizing the need for device removal after the release of the bioactive agent, has been reported [9].

The fact that the degradation process affects, and ultimately controls the rate of drug release from biodegradable carriers, controlling the degradation rate of polymeric materials has

been one of the critical issues in general biomaterials research [11]. A potential disadvantage of using biodegradable polymers in biomedical applications is the eventual toxicity of the degradation products, which are aimed at being non-toxic leachables and easy metabolized molecules [12].

Biodegradable starch-based polymers have been proposed for several biomedical applications such as drug delivery systems [13, 14], bone cements [15] and bone tissue engineering scaffolding [16, 17], due to their great processing versatility [17] and to their promising properties regarding biodegradability [18] and biocompatibility [12, 19].

The aim of this study was to develop an enzymatic-mediated degradation carrier system, based on a blend of starch with poly-ε-caprolactone in microparticulate form, for the controlled release of differentiation agents for bone tissue engineering applications. For this purpose, the degradation behavior of the developed system was investigated in presence of relevant enzymes and the release of dexamethasone was studied under these conditions. The cytotoxicity of the developed system was also assessed by culturing osteoblast-like cells in extracts of the materials after increasing incubation periods in cell culture medium.

## 2 Experimental

### 2.1 Materials

The studied material was a polymeric blend of corn starch with poly-ε-caprolactone (SPCL, 30–70 wt.%). The material was originally in a granular form. More details on this material can be found elsewhere [20]. Dexamethasone (DEX, HPLC grade, ≥98%, Sigma) was used as a bioactive molecule for the encapsulation studies. Solvents used in HPLC analysis were HPLC grade. Other chemicals were of reagent grade and used as received.

The enzymes selected for this study were α-amylase (EC 3.2.1.1) from *Aspergillus oryzae* and lipase (EC 3.1.1.3) from *Aspergillus oryzae*, both purchased from Sigma–Aldrich Chemical Co. (St. Louis, USA).

### 2.2 Methods

#### 2.2.1 *Preparation of starch-poly-ε-caprolactone (SPCL) microparticles*

The SPCL microparticles were prepared as previously described [21]. Briefly, SPCL was dissolved in methylene chloride under vigorous stirring. This solution was dropped into a 200 ml PVA solution, and emulsified under agitation (600 rpm) for 4 h. The microparticles were then collected by filtration, washed with distilled water and dried under vacuum. For encapsulation of DEX, the drug was directly added to the polymeric solution at 15% (wt.%, relatively to polymer weight).

#### 2.2.2 *In vitro enzymatic degradation study*

The enzymatic degradation of the developed carrier system was investigated by incubating the microparticles in a ratio 1:10 with phosphate buffered saline solution (PBS, 0.01 M, pH 7.4) containing α-amylase (150.5 U/l) and/or lipase (102 U/l), at 37°C and 60 rpm up to 12 weeks. To avoid microbial growth, sodium azide (0.02%) was added to the degradation solution. As a control, the samples were also incubated in PBS solution without enzymes. At the end of the pre-selected degradation times, the supernatants were removed and stored at −80°C until further analysis. The microparticles were then thoroughly washed with distilled water and allowed to dry at room temperature until constant weight. The final weight of the samples was used for the calculation of the weight loss [22]. The supernatant solutions were used to determine the concentration of reducing sugars released into the solution as a result of starch degradation (hydrolysis of glycosidic linkages in starch molecule with the consequent release of soluble oligosaccharides). The amount of reducing sugars was estimated by the dinitrosalicylic acid (DNS) method [23]. Experiments were performed in triplicate for all the conditions.

#### 2.2.3 *Physical and chemical characterization*

The changes on the surface morphology of the microparticles after degradation were analyzed by Scanning electron microscopy (SEM, Leica Cambridge S-360 model (Cambridge, UK)). The samples were fixed to the support using a carbon tape and gold sputter-coated (Fisons Instruments, Sputter Coater SC502, UK) prior to observation.

To assess eventual changes in the chemical composition caused by the degradation process, the microparticles were analyzed by Infrared spectroscopy in transmission mode (FTIR-IRPrestige-21 FRIT-8400S, Shimadzu, Japan). Preweighed microparticles (1 mg) were mixed with KBr (40 mg) and then formed into a disc in a press. Transmission spectra were recorded using at least 32 scans with 4 $cm^{-1}$ resolution, in the spectral range of 4,000–600 $cm^{-1}$.

#### 2.2.4 *In vitro release of DEX from SPCL microparticles*

The release of DEX encapsulated in the SPCL microparticles was assessed in PBS only and in PBS containing

α-amylase and lipase enzymes in order to investigate the effect of enzymatic degradation on the release kinetics of DEX. For that, the microparticles were suspended in the release solution (2.5 mg/ml). Samples were maintained at 37°C and 50 rpm up to 4 weeks. At pre-determined time periods, 1 ml aliquots were taken from the supernatant and replaced with the same volume of fresh medium solution. All the release experiments were carried out in triplicate.

Released DEX was quantified by reverse phase high-performance liquid chromatography (RP-HPLC). Before chromatographic analysis, samples were extracted three times with a mixture of hexane and ethyl acetate in the same proportions. HPLC was performed on a Jasco PU-2080 Plus system using a RP-18 column (LiChrospher, 5 μm, Merck, Germany) and acetonitrile-water (50:50 v/v) as mobile phase (0.5 ml/min). Absorbance was monitored at 254 nm (UV detector, Jasco 870-UV). The column was eluted in isocratic conditions over 20 min. Data acquisition and peak areas were determined with the Shimadzu C-R6A Chromatopac software. The concentration of DEX was calculated from a calibration curve of known DEX concentrations and using triamcinolone as internal standard.

### 2.2.5 *Cytotoxicity evaluation on materials extract*

The cytotoxicity evaluation of the SPCL microparticles, was conducted by the use of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) test, which is extensively used to quantify the number of viable cells [24, 25]. SPCL microparticles were sterilized by ethylene oxide (EtO) using pre-optimized conditions [26]. Each sample was incubated in Dulbecco's Modified Eagle's Medium without phenol red (DMEM, Sigma, USA) (0.5 mg/ml) supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Biochrom AG, Germany) and 1% of antibiotic (Gibco, USA), for 24, 48, and 72 h at 37°C and 60 rpm, in order to obtain the extract of the materials containing potential toxic leachables and degradation products. Latex rubber extract and fresh complete culture medium were used respectively as negative and positive controls for viability [25]. A Human Osteoblast-like cell (SaOs-2, ECACC, UK) suspension was plated in 96-well plates, at density of $6.6 \times 10^4$ cell/ml, 0.2 ml/well, and incubated at 37°C in a humidified atmosphere containing 5% of $CO_2$ for 24 h to allow about 80% of confluence. After this period, the culture medium was replaced by the material extract and plates were incubated for further 24 h. The MTS test was performed according to the CellTiter 96 One Solution Proliferation Assay Kit (Promega, USA) manufactures instructions and as described previously [25]. The absorbance was determined in a microplate reader (EL 312e Biokinetics reader, Biotek Instruments) at 490 nm. The results are expressed as the percentage (%) of cell viability in comparison with the 100% proliferation attributed to the positive control. The samples were tested in five replicates, and three independent experiments.

### 2.2.6 *Statistical analysis*

In order to confirm the difference in the rate of release of DEX in PBS only and in PBS containing α-amylase and lipase enzymes, the release data was analysed for statistical significant differences. The statistical analysis was performed with the OriginPro® 7.0 (Microcal software, USA), fixing the level of significance at $P < 0.05$. The normality of the data was checked by applying the Shapiro–Wilk test implemented by Origin for this purpose. The statistical analysis (Student's *t*-test for two independent samples) was used to test the two different data (DEX release in PBS, and DEX release in enzymatic environment) for the hypotheses of the release rate in the presence of enzymes to be significant higher than the release rate in PBS alone.

## 3 Results

The microcarriers used in this work are shown in Fig. 3a where microparticles with round shape, porous surface and diameter of about 400 μm can be observed.

The degradation behaviour of the developed microparticles was studied by incubating them in different degradation solutions containing relevant enzymes and their weight loss was followed over time (Fig. 1). It is well known that starch is degraded by α-amylase and that PCL

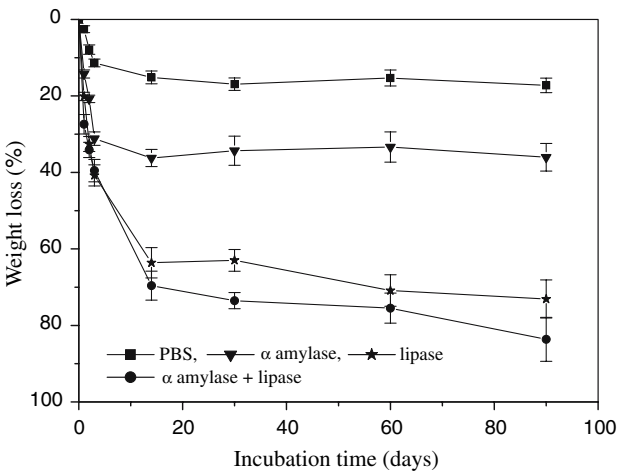


**Fig. 1** Weight loss of SPCL microparticles after incubation in different degradation solutions at 37°C. Error bars are standard deviations ($n = 3$)

hydrolysis can be catalysed by lipase enzymes [11]. An increase in weight loss was observed at initial stages of degradation for all conditions, which then remains constant for the samples incubated in PBS and α-amylase solution. In the same figure, significant differences in the percentage of weight can be observed, depending on the degradation media. The samples incubated in presence of enzymes show higher percentages of weight loss, when compared with the control (PBS), being this effect more notorious for the degradation solutions containing lipase enzyme. At the end of the degradation period, the SPCL microparticles lost approximately 15% of their original weight when incubated in PBS, 45% in presence of α-amylase and 70% in the presence of lipase. These results clearly indicate a high degree of degradation of the SPCL microparticles in the presence of the enzymes.

In order to estimate the degradation of the starch component of the blend in the various degradation conditions, the amount of reducing sugars released in the solutions, as result of starch hydrolysis, was measured (Fig. 2). The supernatants where the SPCL microparticles were incubated in the presence of α-amylase showed the highest concentration of reducing sugars, whereas a negligible amount of sugars was observed in the control. This result confirms the susceptibility of the starch present in the blend to be hydrolysed by α-amylase enzyme at physiological concentrations. In the presence of lipase, the amount of detected reducing sugars was very low but a decrease on the solution pH was observed over time (from pH 7.4 at day 0 to pH 5.2 at the end of the degradation period). This drop in the solution pH is due to the release of carboxylic end groups to the solution as consequence of the ester hydrolysis in the PCL chain catalyzed by lipase.

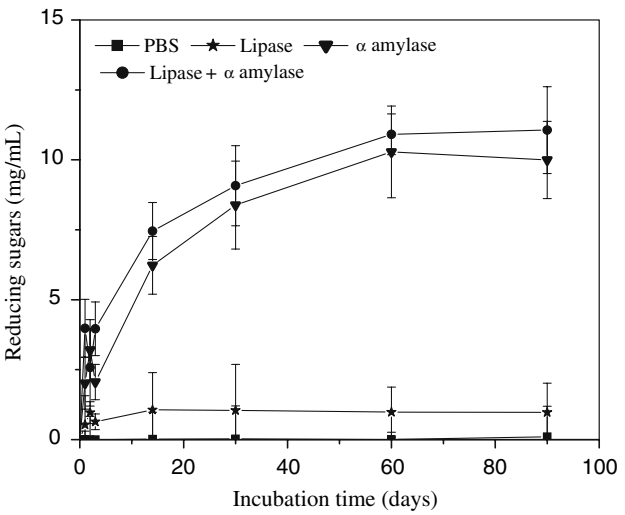


**Fig. 2** Concentration of reducing sugars released from SPCL microparticles when incubated in different degradation solutions at 37°C. Error bars are standard deviations ($n = 3$)

The extent of the enzymatic degradation is well illustrated in the SEM micrographs shown in Fig. 3. After degradation in lipase-containing solution, the diameter of the SPCL microparticles decreased significantly and a highly porous matrix was obtained.

The chemical changes resulting from the enzymatic degradation of the particles were detected by FTIR (Fig. 4). A decrease of the intensity of the band at 1,040–1,100 $cm^{-1}$ was detected when samples were incubated with α-amylase, indicating the action of the enzyme in cleaving the glycosidic linkages of starch. A reduction on the intensity of the PCL ester band at 1,700–1,740 $cm^{-1}$ was observed for the samples incubated with lipase, confirming the lipase activity in hydrolyzing ester bonds. The differences on the bands of starch and PCL demonstrated the degradation of the two components of the material as a consequence of the combination of α-amylase and lipase activities.

The release profile of DEX from SPCL microparticles for a period of 4 weeks, in PBS and in presence of enzymes (α-amylase + lipase) is illustrated in Fig. 5. As a general observation, the release profiles are characterized by two main stages: the initial burst, mainly during the first day where more than 20% of the drug was released, and a sustained release of almost 45% of the DEX at the end of a 4 weeks period. The release profile obtained in an enzymatic environment showed a higher amount of the released DEX into the medium. The statistical analysis of the data reveals no significant differences between the two release conditions (PBS and enzymatic environment) for the first 3 h ($P$ values $(0.70) > 0.05$). For time periods up to this time (more than 3 h) and until the end of the release study, the statistical calculations confirm the hypothesis, at the 0.05 level, the release rate of DEX in the presence of enzymes is significantly greater than the release in PBS alone ($P$ values $(0.018) < 0.05$). A confidence interval of 95% was fixed for all the calculations.

The MTS results depicted in Fig. 6 showed that 48% of the cells remained viable when incubated with the extracts from the SPCL microparticles and the extraction time did not show any influence on the cytotoxicity results. The percentage of cell viability may be related with the presence of residual solvent, used during the preparation of the microparticles, that might be released to the extract solution and causing cell death. Additional washing steps will be included in the microparticle's preparation to ensure complete removal of solvents or other toxic reagents.

## 4 Discussion

Polymeric biomaterials when exposed to body fluids or placed in contact with tissues, may undergo several

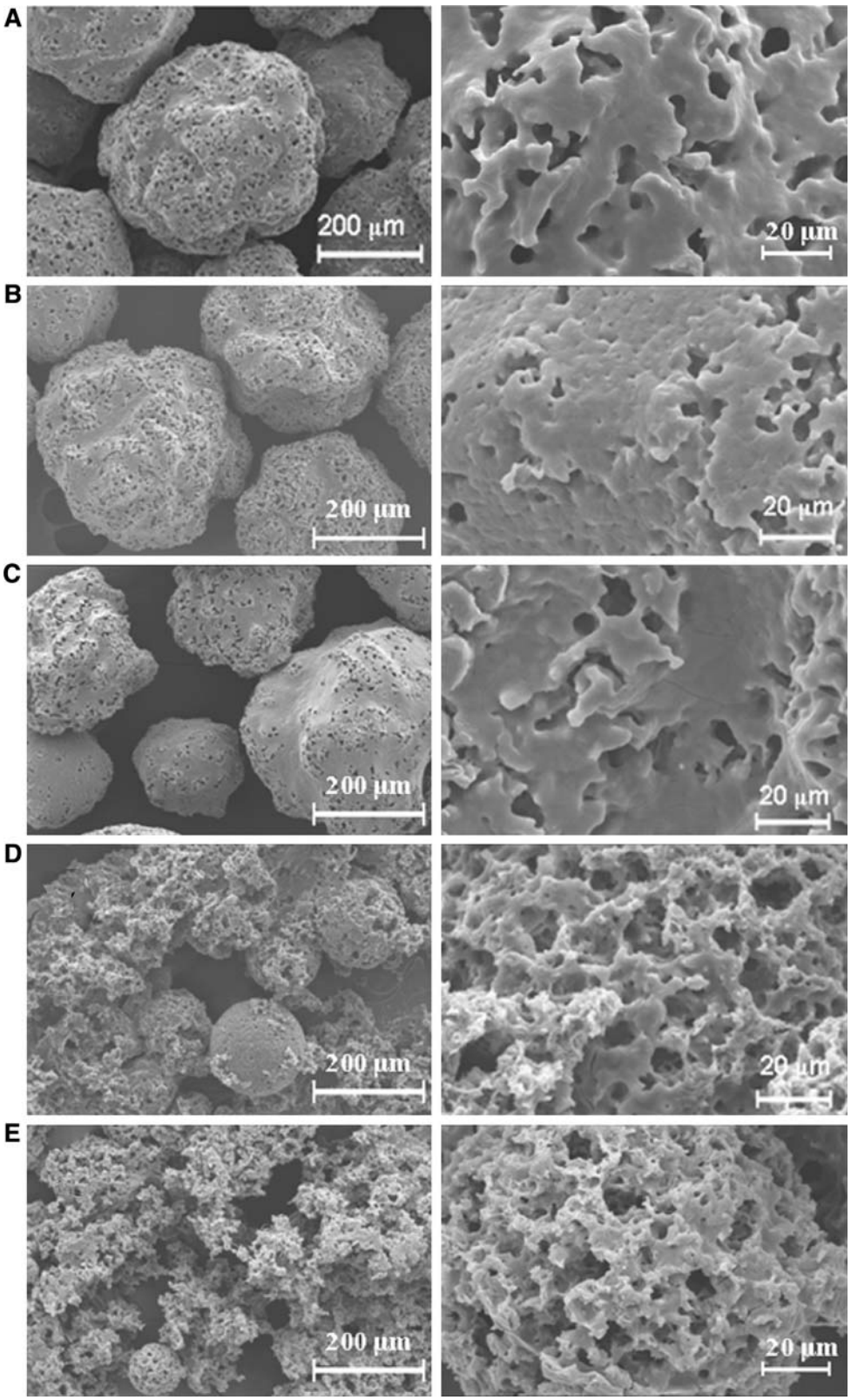


**Fig. 3** SEM micrographs of SPCL microparticles before (**a**) and after degradation for 90 days in PBS (**b**), α-amylase (**c**), lipase (**d**), α-amylase and lipase (**e**) solutions

physicochemical changes as result of the most diverse interactions with the surrounding environment [1, 2, 11]. The degradation tests performed in this study showed a higher degradation rate when α-amylase and lipase are present in the degradation solution. The samples incubated with lipase enzyme completely lost their spherical shape at

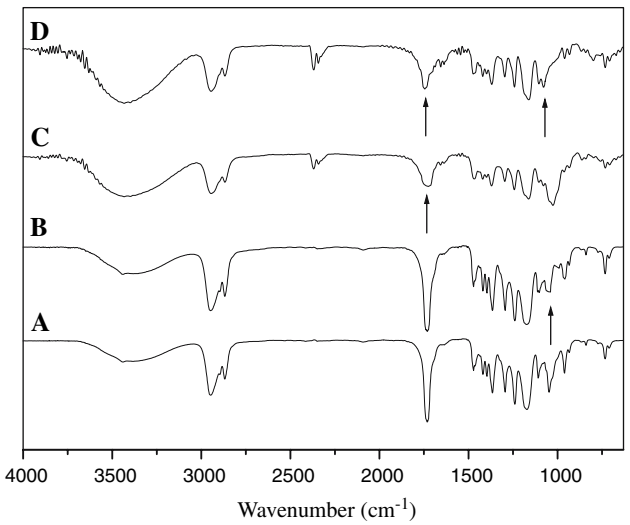


**Fig. 4** FTIR spectra of SPCL microparticles before (**a**) and after degradation in presence of α-amylase (**b**), lipase (**c**), and α-amylase and lipase (**d**) solutions

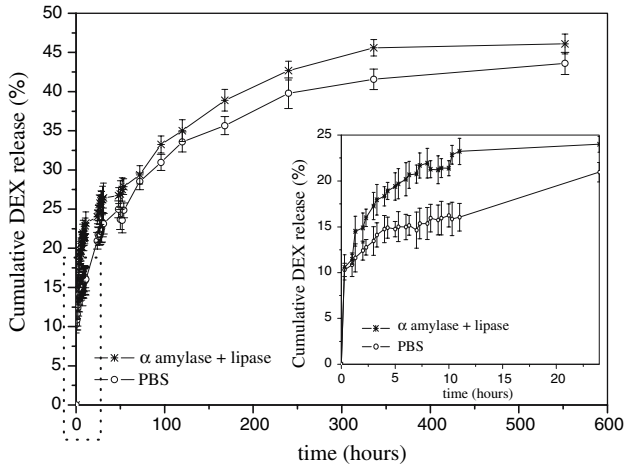


**Fig. 5** Release profiles of dexamethasone from SPCL microparticles in PBS and in presence of enzymatic activity at 37°C for a period of 4 weeks. The insert graph shows the DEX release for a period of 24 h. The release rate of DEX in the presence of enzymes is significantly greater than the release in PBS alone, for time periods longer than 3 h ($P < 0.05$). Error bars are standard deviations ($n = 3$)

the end of degradation period. The degradation products in the presence of α-amylase are mainly sugars resulting from the cleavage of the glycosidic bonds in the starch structure. On the other hand, lipase acts in the polyester component in the blend, at a specific position on the ester bond of the macromolecule. As result of the degradation process, carboxylic acid units are released to the solution, which explain the observed decrease of the pH. The combination of the two enzymes resulted in higher values of weight loss and reducing sugars in solution, indicating the degradation of both components of the blend.

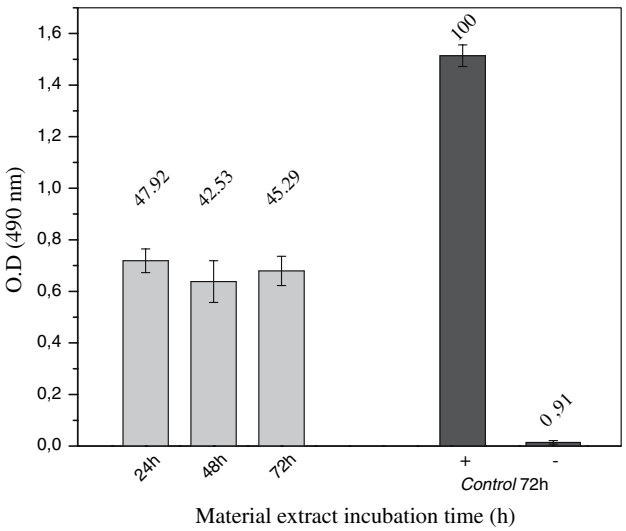


**Fig. 6** Optical density of the MTS solutions after incubation with SaOs-2 grown for 24 h in the presence of SPCL microparticles extracts obtained in culture medium for 24, 48 and 72 h. Percentages of viable cells, compared to positive control, are presented

The SEM analysis confirmed the results discussed above. The appearance of porosity, cavities and fractures, and the decrease on the diameter of the SPCL microparticles as result of degradation process, was more notorious in the presence of lipase and of both enzymes.

There are two main ways for a drug to be released from a polymeric carrier [27]: (i) diffusion through the polymer, which depend on the ability of the aqueous environment to penetrate the drug-containing polymer, inducing the swelling of the matrix and consequently the release of the entrapped drug; (ii) degradation of the material containing the drug, by water, enzymes and acidic or basic conditions. In this case, the drug starts to be released by diffusion until a gradual degradation of the polymer takes place thus controlling the release process. The remaining drug in the polymeric matrix is released as the degradation process becomes more significant. For the developed DEX loaded SPCL microparticles, the initial burst effect was clearly observed in both studied environments, associated with the fast release of the drug located close to the surface of the particles, mainly by the diffusion phenomena. This burst was followed by a period of minimal release due to the hydrophobicity of PCL (major component of the blend: 70%), which causes a barrier for water penetration and consequently preventing the diffusion of the drug through the polymer matrix. At this stage, the drug was released faster from the sample in presence of enzymes, indicating that the enzymatic degradation of the SPCL microparticles increases the release rate of the entrapped DEX. These results indicate that the release of DEX from the SPCL microparticles is firstly controlled by diffusion and then by the degradation of the polymeric matrix. The dependence

of the drug release with the degradation of the system along the time makes the SPCL microparticulate system more adequate for sustained rather than controlled drug release.

The main concern of biodegradable polymers when proposed for biomedical applications, is the potential toxicity of their leachables and degradation products. The slightly cytotoxicity of the developed system for osteoblast-like cells, may be due to the presence of residual solvent. Previous studies [12] have attributed similar results to components added and or formed during the processing.

## 5 Conclusion

Considering the overall results obtained in this study, it is expected that the developed SPCL microparticulate system is a suitable carrier for the sustained release of drugs, with the advantage of being susceptible to enzymatic degradation, thus being ultimately digested in the body, avoiding the surgical removal. These results are indicative of the potential of the developed system to be used in the controlled release of growth and differentiation factors with relevance in the field of bone regeneration.

**Acknowledgments** E. R. Balmayor is beneficiary of Marie Curie PhD grant under the Alea Jacta EST Project (MEST-CT-2004-008104). This work was partially supported by the European Network of excellence (NoE) EXPERTISSUES (NMP3-CT-2004-500283).

## References

1. G.A. Silva, P. Ducheyne, R.L. Reis, J. Tissue Eng. Regen. Med. **1**, 4 (2007)
2. A. Tuncel, K. Ecevit, K. Kesenci, E. Piskin, J. Polym. Sci. A: Polym. Chem. **34**, 45 (1996)
3. A. Kamyshny, S. Magdassi, Colloids Surf. B Biointerfaces **18**, 13 (2000)
4. M. Shinkai, J. Biosci. Bioeng. **94**, 606 (2002)
5. G.A. Silva, O.P. Coutinho, P. Ducheyne, R.L. Reis, J. Tissue Eng. Regen. Med. **1**, 97 (2007)
6. G.A. Silva, A. Pedro, F.J. Costa, N.M. Neves, O.P. Coutinho, R.L. Reis, Mater. Sci. Eng. C. **25**, 237 (2005)
7. P.B. Malafaya, G.A. Silva, R.L. Reis, Adv. Drug Deliv. Rev. **59**, 207 (2007)
8. M.C. Bissery, in *Microspheres and Drug Therapy: Pharmaceutical, Immunological and Medical Aspects*, ed. by S.S. Davis, L. Illum, J.G. McVie, E. Tomlinson (Elsevier, New York, 1984), p. 217
9. S.P. Baldwin, W.M. Saltzman, Adv. Drug Deliv. Rev. **33**, 71 (1998)
10. R.E. Eliaz, J. Kost, J. Biomed. Mater. Res. **50**, 388 (2000)
11. H.S. Azevedo, R.L. Reis, in *Biodegradable Systems in Tissue Engineering and Regenerative Medicine*, ed. by Reis, S. Roman (CRC Press, New York, 2005), p. 178
12. A.P. Marques, R.L. Reis, J.A. Hunt, Biomaterials **23**, 1471 (2002)
13. P.B. Malafaya, C. Elvira, A. Gallardo, J. San Roman, R.L. Reis, J. Biomater. Sci. Polym. Ed. **12**, 1227 (2001)
14. G.A. Silva, F.J. Costa, N.M. Neves, O.P. Coutinho, A.C.P. Dias, R.L. Reis, J. Biomed. Mater. Res. A **73**, 234 (2005)
15. L.F. Boesel, J.F. Mano, C. Elvira, J. San Roman, R.L. Reis, in *Biodegradable Polymers and Plastics*, ed. by E. Chiellini, R. Solaro (Kluwer Academic/Plenum Press, New York, 2003), p. 243
16. M.E. Gomes, V.I. Sikavitsas, E. Behravesh, R.L. Reis, A.G. Mikos, J. Biomed. Mater. Res. A **67**, 87 (2003)
17. R.L. Reis, A.M. Cunha, in *Encyclopedia of Materials: Science and Technology*, vol. 11, ed. by K.H.J. Buschow, R.W. Cahn, M.C. Flemings, B. Ilschner, E.J. Kramer, S. Mahajan, P. Veyssière (Elsevier, New York, 2001), p. 8810
18. H.S. Azevedo, F.M. Gama, R.L. Reis, Biomacromolecules **4**, 1703 (2003)
19. S.C. Mendes, R.L. Reis, Y.P. Bovell, A.M. Cunha, C.A. van Blitterswijk, J.D. de Bruijn, Biomaterials **22**, 2057 (2001)
20. N.M. Neves, A. Kouyumdzhiev, R.L. Reis, Mater. Sci. Eng. C **25**, 195 (2005)
21. E.R. Balmayor, K. Tuzlakoglu, H.S. Azevedo, R.L. Reis, in *Proceedings of the European Materials Research Society Fall Meeting*, Warsaw, Poland, September 2006, ed. by Conference Engine Pielaszek Research, Poland, 2006, p. 219
22. G.A. Silva, F.J. Costa, N.M. Neves, O.P. Coutinho, A.C.P. Dias, R.L. Reis, J. Biomed. Mater. Res. **73**, 234 (2005)
23. T.K. Ghose, Pure Appl. Chem. **59**, 257 (1987)
24. J. Zeltinger, J.K. Sherwood, D.A. Graham, R. Mueller, L.G. Griffith, Tissue Eng. **7**, 557 (2001)
25. A.J. Salgado, O.P. Coutinho, R.L. Reis, Tissue Eng. **10**, 465 (2004)
26. R.L. Reis, S.C. Mendes, A.M. Cunha, M. Bevis, Polym. Int. **43**, 347 (1997)
27. R. Langer, N.A. Peppas, AIChE J. **49**, 2990 (2003)