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Preparation and *in vitro* and *in vivo* characterization of composite microcapsules for cell encapsulation

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Abstract

Cell encapsulation technology raises great hopes in medicine and biotechnology. Transplantation of encapsulated pancreatic islets represents a promising approach to the final cure of type 1 diabetes mellitus. Unfortunately, long-term graft survival and functional competence remain only partially fulfilled. Failure was often ascribed to the lack of biocompatibility generating inflammatory response, limited immunobarrier competence, hypoxia, and low β-cell replication. In the present work, ketoprofen loaded biodegradable microspheres, embedded into alginate/poly-L-ornithine/alginate microcapsules, were prepared in order to release ketoprofen at early stages after implantation. Morphology, size, *in vitro* release behaviour, and *in vivo* biocompatibility were assessed. The effect of some preparation parameters was also evaluated. Polymeric microspheres were spherical and smooth, two populations of about 5 and 20 μm of mean diameter characterized the particle size distribution. A high burst effect was observed for all preparations during *in vitro* release studies. Ketoprofen, plasticizing the polymeric matrix, could be responsible of this release behaviour. Alginate/poly-L-ornithine/alginate microcapsules were not modified upon ketoprofen loaded microspheres encapsulation and an optimal dispersion was obtained. Composite system showed good biocompatibility when a high molecular weight polymer was employed. Therefore a potentially suitable composite system for cell encapsulation was obtained. This system may be successfully used to release NSAIDs and other active molecules capable to improve cell system functional performance and life-span.

Keywords: Cell encapsulation; Alginate; Inflammation; NSAIDs; Ketoprofen; PLA; PLGA

1. Introduction

Transplantation of pancreatic islets embedded into semipermeable membranes, able to protect them from immune system injuries (referred as immunoprotection), has been proposed as final cure of type 1 diabetes mellitus (Orive et al., 2003; Calafiore, 2003). Different polymers have been used for islet encapsulation and immunoprotection with not always interesting or reproducible results (Calafiore and Basta, 1999). Photopolymerized poly(ethylene glycol) (PEG), water insoluble polyacrylates, silica sol–gel, sodium cellulose sulphate, agarose, chitosan, alginates are examples of polymers employed for islet encapsulation and immunoprotection (Calafiore and Basta, 1999). Among others, alginate/poly-cation microcapsules are surely the most investigated and probably one of the most promising device for allogeneic and xenogeneic islet immunoisolation. The value of alginate-based microcapsules is demonstrated by the fact that encapsulated rat islets restored glycaemia for about 1 year in diabetic mice after transplantation (Calafiore and Basta, 1999). To date, despite the success recorded in animal studies and the preliminary results in human pilot clinical trials (Soon-Shiong et al., 1994; Calafiore et al., 2006), the survival time of immunoprotected grafts is still too short to transfer this technology into clinical practice (de Vos et al., 2002a; de Groot et al., 2004).

The three main issues to which graft failure is generally ascribed are biocompatibility, immunoprotection, and hypoxia due to the great distance between the encapsulated islets and the blood supply (de Vos et al., 2002a; de Groot et al., 2004). Low

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β-cell replication has to be taken into account as well (Luca et al., 2003a).

As far as biocompatibility is concerned, implantation of empty microcapsules (without islets) can elicit a foreign body reaction. An acute inflammatory response, characterized by neutrophils as the primary cellular infiltrate, is followed by a chronic inflammation characterized by monocyte and lymphocyte. Monocytes, differentiated into macrophages, lead into the granulation tissue development (Babensee et al., 1998; Anderson and Langone, 1999).

In the specific case of intraperitoneally implanted empty alginate/poly-cation microcapsules, macrophages seem to be the main cells involved in the early inflammation and pericapsular overgrowth (de Vos et al., 2002b). They are recruited during the first day after transplantation and no changes in macrophage quantity are observed until day 5, while at day 7 their number starts to decrease (de Vos et al., 2002b). Different strategies have been developed in order to avoid acute inflammation and its harmful effects on transplanted islets (Omer et al., 2003; Bünger et al., 2005; Ricci et al., 2005).

Non-steroidal anti inflammatory drugs (NSAIDs) seem to be good candidates for this application since the more potent glucocorticoid drugs showed diabetogenic effects (Wajngot et al., 1992). Among others, Ketoprofen (KET) possesses interesting features to be accounted. In fact, it was successfully used to treat acute inflammatory response due to both surgical manoeuvres and the presence of biomaterials (Ricci et al., 2005).

Cyclo-oxygenase (COX) is the first enzyme in the formation of prostaglandins from arachidonic acid. Prostaglandins have a wide variety of physiological and pathophysiological effects, in particular the prostaglandin E2 in pancreatic islets is known to inhibit the insulin glucose-dependent release. It has been recently demonstrated that pancreatic islets, contrarily to most of other tissues, dominantly express the inducible form of COX, namely COX-2 instead of COX-1 (Sorli et al., 1998; Robertson, 1998). Therefore, the use of KET, a COX-1 selective compound (about 11 time more selective for COX-1), may circumvent potential undesired effects on islet glucose homeostasis (Mitchell and Warner, 1999).

In addition, KET was found very efficacious in the selective inhibition of interleukin-8 (IL-8) chemotaxis (Bizzarri et al., 2001). IL-8 plays an important role in transplant immunology and its inhibition may be advantageous in encapsulated islet grafts for different reasons (Mukaida et al., 1998). Human islets have been found to express several immune modulating and pro-inflammatory mediators during *in vitro* culture. In this regard, IL-8 was expressed at early stages after islet isolation (Johansson et al., 2003). Moreover, lipopolysaccharides, present sometimes as contaminants of collagenase preparations (used for islets isolation) (Vargas et al., 1998), turned out a potent agonist for IL-8 release from monocytes (Lund and Østerud, 2004).

The aim of the present study was to formulate and to characterize composite microcapsules for cell encapsulation able to release KET early upon transplantation. For this purpose, KET loaded poly(lactic acid) (PLA) and poly(lactic-*co*-glycolic acid) (PLGA) microspheres (MS) were prepared using a solvent evaporation technique and the effect of some preparation parameters on MS characteristics has been investigated. Polymeric MS together with alginate/poly-L-ornithine/alginate capsules were used as components to build up a composite system. Empty composite microcapsule (without pancreatic islets) biocompatibility was evaluated *in vivo* as well.

2. Materials and methods

2.1. Materials

PLGA polymers (RESOMER®): RG504 H (inherent viscosity 0.50 dl/g, weight-average molecular weight (Mw) ~40–50 kDa), RG504 (inherent viscosity 0.54 dl/g, Mw ~40–50 kDa), RG502 H (inherent viscosity 0.17 dl/g, Mw ~10 kDa), RG502 (inherent viscosity 0.22 dl/g, Mw ~10 kDa) were supplied by Boehringer Ingelheim (Ingelheim, Germany). PLA (Mw ~106 kDa), PLGA (lactide/glycolide ratio: 50:50, Mw ~40–70 kDa) (PLGA 50:50), PLGA (lactide/glycolide ratio: 85:15; Mw ~90.1 kDa) (PLGA 85:15), KET, hydroxypropylmethyl cellulose (HPMC) (viscosity 80–120 cp 2% water solution) and poly-L-ornithine (Mw ~15–30 kDa) were purchased from Sigma–Aldrich corporation (St. Louis, MO). Sodium alginate high mannuronic (mannuronic acid (M): 61%; guluronic acid (G): 39%; polymer was comprised of dimeric blocks MM–GG–MM) was supplied from Stern Italia (Milan, Italy). Alginate was purified and sterilized with a multi step filtration, using pyrogen-free glassware, so as to obtain pharmaceutical grade product with a low endotoxin content (<100 EU/g). 0.22 μm Minisart® non-pyrogenic filters (Goettingen, Germany) were used to sterilize HPMC solutions, while methylene chloride (CH_2Cl_2) polymer solutions were filtered using a 0.2 μm polytetrafluoroethylene (PTFE) filter (Lida Manufacturing, Kenosha, WI). When not specified, all other chemicals and reagents were of the highest purity grade commercially available.

2.2. Microsphere preparation

The solutions used to produce MS were sterile filtered and all the equipment autoclaved.

KET loaded and blank PLGA or PLA MS were prepared by a solvent evaporation method (Gabor et al., 1999). Briefly, 250 or 416 mg of polymer (depending on the batch size) and a proper amount of KET (23% of theoretical loading) were dissolved in different volumes of CH_2Cl_2. CH_2Cl_2 volumes were varied to achieve polymer to solvent ratios of 1, 11, or 22% (w/v). The organic phase previously described (dispersed phase) was slowly injected into an aqueous phase (2.4%, w/v, solution of HPMC) (continuous phase) under mechanical stirring at 800 rpm. The resulting emulsion was maintained under continuous stirring for about 3 h to obtain the complete evaporation of the organic solvent and MS hardening. Finally, the MS were recovered by centrifugation (refrigerated centrifuge ALC, RCF Meter, 4233R International Equipment Company, Chicago, IL) (3000 rpm, 5 min, 4 °C), and washed three times with deionised water. MS, suspended in a small amount of deionised water, were immediately frozen and thereafter freeze dried for 12 h.

Different polymers and polymer concentrations were used for MS preparation in order to investigate their influence on particle characteristics, such as particle size morphology and KET release kinetic. In fact, the type of polymer (PLA or PLGA, PLGA at different molecular weights or different lactic-glycolic ratios or with different chain end groups) may have a very important role in drug release rate because of the different hydrophilic nature. In fact, the hydration rate and degree will be strictly related to the main release mechanisms: drug diffusion and polymer degradation. Since KET plasticizing effect was previously observed, polymer thermal behaviours were also investigated.

2.3. Scanning electron microscopy

MS surface structure and porosity were qualitatively investigated by scanning electron microscopy (SEM) using a Philips XL30 microscope (Philips Electron Optics, Eindhoven, NL). Samples were prepared by placing a droplet of an aqueous MS suspension onto an aluminium specimen stub. The samples were dried overnight and were sputter coated with gold prior to imaging (EMITECH K-550X sputter coater Ashford, Kent, UK). Coating was performed at 20 mA for 4 min.

2.4. Particle size distribution

An Accusizer C770 (PSS Inc., Santa Barbara, CA) using the technique "Single Particle Optical Sensing" was used to determine the size distribution of the various MS preparations. The lyophilized particles were suspended into deionised water and a small amount of Tween 80® was used as surfactant to prevent MS aggregation. Analyses were performed in triplicate and size was expressed as mean volume diameter ± standard deviation.

2.5. Thermal analysis

In order to characterize the MS thermal behaviour, differential scanning calorimetry (DSC) was performed by using a DSC821e calorimeter (Mettler Toledo, Switzerland) equipped with a refrigerated cooling system (RCS). The system was calibrated using an indium standard.

The effect of KET and preparation procedures on PLA and PLGA glass transition temperature (T_g) was evaluated in a set of samples prepared as described below. Samples of about 5 mg of raw polymer, blank MS, or KET loaded MS, exactly weighed, were sealed into holed lid aluminium pans. An empty holed lid aluminium pan was used as a reference.

Samples were subjected to two heating cycles, from 0 to 110 °C, at a 5 °C/min rate in static atmosphere. T_g was determined as the mid point between T_g onset and T_g offset in the second heating ramp in order to avoid any sample thermal history. Data were treated with STAR^e software (Mettler Toledo, Switzerland), and the results were expressed as the mean of two independent measures ± standard deviation.

2.6. Determination of encapsulation efficiency and microparticle recovery

Few milligrams of KET loaded MS were dissolved into CH_2Cl_2 in order to obtain a concentration of KET in the range of 3–15 μg/ml. KET concentration was determined by measuring the absorbance at 254 nm (Agilent 8453 UV–visible Spectroscopy System, Agilent Technologies, Germany) in a quartz cuvette and by subtracting blank MS absorbance values. PLA and PLGA solutions showed no significant absorbance at 254 nm. Encapsulation efficiency was calculated as the ratio between the experimental and the theoretical loading multiplied by a factor of 100. Microparticle recovery was calculated as weight percentage of the obtained MS with respect to the total amount of the solid materials used for the preparation.

2.7. Preparation and characterization of composite microcapsules

Solutions for microcapsule preparations were sterile filtered and all the equipments were autoclaved. Blank or KET loaded MS (0.8 mg/ml) were suspended in 1.6% sodium alginate solution and thoroughly, but gently, mixed in order to obtain an uniform suspension. The suspension was continuously aspirated, at a fixed flow rate, by a peristaltic pump and extruded through a single jet-head connected with air (4.5 L/min). The resulting microdroplets were collected in a 1.2% $CaCl_2$ water solution and immediately turned into water insoluble calcium alginate gel beads. The gel microbeads were washed twice in saline and then incubated with 0.12% PLO (coat I) (10 min). After washing in saline, the beads underwent a second incubation with 0.06% PLO (coat II) (10 min). After a final wash in saline, the beads were incubated with 0.04% sodium alginate (6 min). All steps were performed at room temperature (Calafiore and Basta, 1999).

Characterization of representative samples of composite microcapsules was performed by optical microscopy analysis. The integrity and the average size of the systems were thus evaluated.

2.8. In vitro KET release studies

In vitro KET release studies were performed in conical tubes without agitation in duplicate. A certain amount of polymeric MS (10 mg) or composite microcapsules (16 mg of encapsulated polymeric MS) were placed into 0.1 M phosphate buffer saline (PBS) (pH 7.4) (10 mL for free MS and 20 mL for composite microcapsules) under infinite sink condition and then incubated at 37 °C. At predetermined intervals, the entire volume of the buffer solution was removed, by using a syringe coupled with a 0.22 μm filter (Cameo 3AS STEPBIO SRL Bologna, Italy), collected, and replaced with fresh buffer. Before removal, PLA or PLGA MS were centrifuged at 1000 rpm for 5 min while the composite systems, because of their bigger size, higher weight, and fragility, were allowed to sediment for 5 min. The solutions were immediately assayed for KET concentration by

spectrophotometric analysis at 260 nm using a KET phosphate buffer calibration curve as reference.

2.9. In vivo biocompatibility study

Empty composite microcapsule (without pancreatic islets) biocompatibility was investigated. Thirty male CD-1 mice (weighing approximately 25 g) supplied from Charles River (Charles River Laboratories, Wilmington, MA), were divided in three groups composed of ten animals each (groups 1–3). The mice were acclimatized to the housing facility for 1 week prior to microcapsule implantation. The animals were housed in groups of two per cage in an air-conditioned room at $20 \pm 2\,^{\circ}C$ and maintained on a cycle of 12 h of light and 12 h of dark with food and water provided *ad libitum*. All the procedures were approved by the Bioethical Committee of the University of Perugia, Perugia, Italy.

Approximately 1 cm^3 of capsules per animal were introduced into the peritoneal cavity through a 16-gauge catheter via a small incision (3 mm) upon anaesthesia with pentobarbital. The abdomen was sutured by a two-layer suture. Group 1 was transplanted intraperitoneally with 1 cm^3 of conventional alginate/poly-L-ornithine/alginate capsules suspended in 0.5 mL of sterile saline and it served as a control. Groups 2 and 3 received both 1 cm^3 of composite capsules containing blank PLGA (preparation B) and PLA (preparation A) MS, respectively. At each time point two mice for each group were sacrificed and the capsules recovered by repeated peritoneal lavages. Microcapsule overgrown with macrophages and/or fibroblasts were detected under light microscopy examination. Capsule recovery was determined and the inflammatory response against capsules was quantified (de Vos et al., 2002b, 2003).

2.10. Statistical analysis

Data from MS characterization and *in vivo* studies were investigated by using a two-way and one-way ANOVA methods. In particular, encapsulation efficiency, release burst effect, and capsular cell overgrowth data were considered for statistical comparison. Two factors were picked up for each dataset: type of polymer and polymer concentration for the MS characterization study, and groups and post-transplant period for the *in vivo* study. Additionally, a post hoc all pairwise multiple comparison method was applied on each dataset in order to uncover single differences among the groups. In detail, a Fisher LSD method was performed on the cell overgrowth data and a Tukey test on the encapsulation efficiency and release data.

3. Results and discussion

3.1. Preparation and characterization of polymeric MS

The effect of some preparation parameters on the MS properties was evaluated and the results are shown in Table 1. Different polymer chemical structures, intended as different lactide/glycolide ratios (50:50 or 85:15), different chain end groups (free or esterified "end-capped" carboxylic moiety), and different polymer molecular weights (10, 50, 40–70, 96.1, and 106 kDa) were investigated (Table 1). The influence of the polymer to organic solvent ratio on MS characteristics was examined as well.

Encapsulation efficiency and burst release data were submitted to ANOVA and post hoc pairwise multi comparison analysis to address possible differences among the groups. Two different factors were identified, such as polymer and polymer concentration. The data were divided into two different sets in which

Table 1
KET loaded, PLA and PLGA MS process parameters and characteristics

Preparation	Polymer	Polymer (mg)	Polymer/CH_2Cl_2 (%)	Encapsulation efficiency (%)	Experimental loading (%)	Mean volume diameter (μm)	"Burst effect" KET released (1 day)	KET released (15 days)
A	PLA, Mw ∼106 kDa	250	1	74	17.0	14.46 ± 1.12	69.1 ± 0.2	84.9 ± 3.0
B	PLGA (50:50), Mw ∼40–70 kDa	250	1	59.7	13.7	11.82 ± 0.96	92.8 ± 0.5	–[b]
C	PLGA (85:15), Mw ∼90.1 kDa	250	1	60.4	13.9	13.57 ± 1.07	93.5 ± 0.5	–[b]
D	PLA, Mw ∼106 kDa	416	11	91.6	21	24.42 ± 1.26	52.1 ± 3.3	51.3 ± 3.0
E	PLA, Mw ∼106 kDa	416	22	75.4	17.3	25.31 ± 1.15	49.5 ± 2.7	63.3 ± 2.5
F	PLGA (RG504 H), Mw ∼40–50 kDa	416	11	53.7	12.3	26.71 ± 5.68	61.6 ± 1.9	67.1 ± 1.1
G	PLGA (RG504 H), Mw ∼40–50 kDa	416	22	63	14.5	24.34 ± 2.18	45.6 ± 0.9	62.6 ± 2.4
H	PLGA (RG504), Mw ∼40–50 kDa, end-capped	416	11	62.3	14.3	18.91 ± 1.29	46.2 ± 1.5	60.5 ± 1.1
I	PLGA (RG504), Mw ∼40–50 kDa, end-capped	416	22	63	14.5	–	45.6 ± 7.9	70.4 ± 7.5
L	PLGA (RG502 H), Mw ∼10 kDa[a]	416	22	–	–	–	–	–
M	PLGA (RG502), Mw ∼10 kDa, end-capped[a]	416	22	–	–	–	–	–

[a] It was not possible to recover and to characterize the preparation.
[b] Release was complete after 2 days.

the first consisted of three batches for which polymer concentration was kept constant and the second of six batches for which polymer concentration was varied over two levels.

Significant differences ($p < 0.05$) were detected for both the encapsulation efficiency datasets. In particular, a significant interaction ($p < 0.001$) was observed between the polymer and the polymer concentration employed for MS preparation. As a consequence, RG504 H was seen to provide a significant lower encapsulation efficiency ($p = 0.018$) compared to RG504, when a low polymer concentration was used (11%), whereas at higher polymer concentrations (22%) this difference was null. On the contrary, the PLA polymer always showed a statistically significant higher efficiency with respect to PLGA, whether at low or high polymer concentrations. In addition, among the different polymers, only RG504 resulted not susceptible to a polymer concentration increase ($p = 0.779$), while an efficiency improvement was observed for RG504 H ($p = 0.005$) and a strong efficiency drop for PLA ($p < 0.001$). In the other hand, both PLGAs, 85:15 and 50:50, were found to provide significantly lower efficiencies compared to the PLA polymer, even when polymer concentration was as low as 1% ($p > 0.05$).

Preparations L and M (Table 1) were repeated several times, in both cases MS recovery resulted impossible as the MS irreversibly clamped together during centrifugation or filtration. The KET and water plasticizing effects were considered responsible for the aforementioned behaviour (Passerini and Craig, 2001; Blasi et al., 2005; Ricci et al., 2005).

In this regard, Table 2 shows the T_g values of PLA and different PLGAs as raw polymers, blank MS, and KET loaded MS. T_gs of blank MS were comparable to that of the raw polymers confirming the complete evaporation of CH_2Cl_2 and that the employed method had no effect on polymer thermal properties. On the contrary, a T_g reduction was observed when MS were loaded with KET (Table 2). KET plasticized all the investigated polymers.

In order to study the effect of KET on RG502 H, because of the impossibility to recover KET loaded MS, polymeric films obtained by solvent casting (loaded with 23%, w/w, of KET) were submitted to DSC analysis (Table 2). The T_g of the KET loaded RG502 H film was about 18 °C (Table 2) but previous data showed that the addition of water may further depress T_g of about 10 °C (Passerini and Craig, 2001; Blasi et al., 2005; Ricci et al., 2005). The low T_g of the system PLGA/KET/water was recognized as the main reason for MS aggregation, thus making the recovery impossible. In fact, being working temperatures around the polymer T_g, PLGA was in the rubbery state and, because of the higher polymer chain mobility, MS stuck together during the recovery process (Schade et al., 1995).

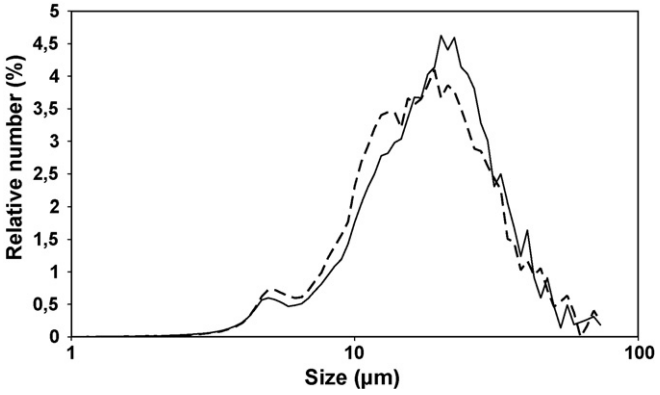

Fig. 1. Volume-weighted particle size distribution of the preparations D (dotted line) and E (solid line).

Fig. 1 shows the particle size distributions of preparations D and E. The MS seem to be mainly distributed in two populations around 5 and 20 μm and this trend was generally observed in all the analyzed preparations. Some differences on the relative abundance of MS in each population were observed when polymer to solvent ratio increased from 1 to 22%. As expected, the particle mean diameter increased with polymer concentration probably because of the enhanced polymer solution viscosity (Table 1). According to the Kurtosis coefficient values (data not shown), the preparations made with the lowest polymer to solvent ratio (1%) seemed constituted by only one population around 5 μm, whereas at higher concentrations (11 and 22%) the Kurtosis values were found to correlate with the existence of 2 or more populations.

Fig. 2 shows SEM pictures of the preparations C and D, while in Fig. 3 is reported the internal morphology of a particle belonging to formulation D. Particle morphology did not seem to be dramatically affected by the investigated parameters. MS were spherical and with a smooth surface (Fig. 2) and the internal structure was a compact matrix without visible pores (Fig. 3), as expected for MS obtained by a single emulsion process. KET crystals were not individuated into the matrix indicating that the active may be present either as an amorphous solid, or molecularly dispersed in the matrix. A similar morphology was observed for all the preparations (data not shown). Blank MS equivalent to preparations A–M were also analyzed. No differences were observed in morphology, mean particle size and particle size distribution when compared to loaded MS (data not shown).

Table 1 reports also the burst effects (KET released after 1 day) recorded during the *in vitro* release studies and the percentage of KET released after 15 days. Even if the data show a high

Table 2
Dry glass transition temperature of raw polymers, blank, and loaded MS

	PLA	RG504 H	RG504	RG502 H
Raw polymer, T_g	50.80 ± 0.30	43.94 ± 0.03	44.10 ± 0.19	40.44 ± 0.10
Blank MS, T_g	50.40 ± 0.20	44.05 ± 0.87	43.95 ± 0.02	40.25 ± 0.12
Loaded MS, T_g	32.10 ± 0.10	29.50 ± 0.63	27.44 ± 0.23	18.38 ± 0.20[a]

[a] Polymeric film obtained by solvent casting loaded with 23% (w/w) of KET.

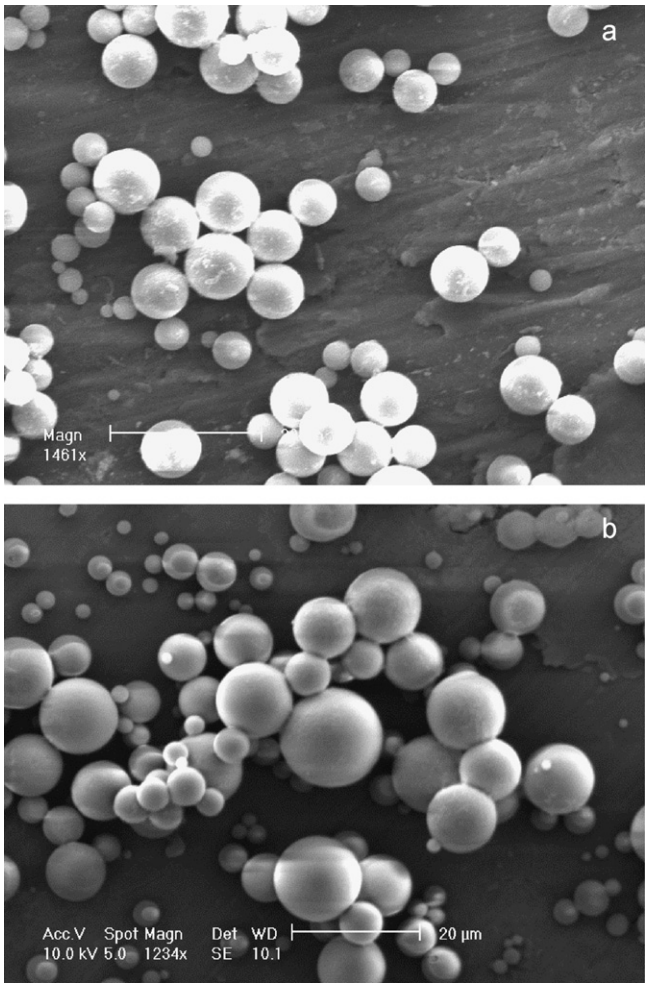

Fig. 2. SEM pictures of external morphology of KET loaded MS: (a) preparation C; (b) preparation D.

burst effect for all preparations, as it could be easy predicted, the largest amounts of KET released during the first day was found for the MS prepared with 1% (w/v) of polymer CH_2Cl_2 ratio (Table 1). In particular, B and C released almost all the encapsulated drug within the first day completing the release in 2 days (Table 1).

The analysis of the burst release detected significant, even though not remarkable, differences among the groups ($p = 0.014$), especially according to the polymer concentration employed ($p = 0.004$). Moreover, as a result of a significant interaction between the polymer used and concentration ($p = 0.009$), the behaviour of the polymeric MS was influenced also by the levels of the polymer concentration employed. In fact, while RG504 H and RG504 were not found to differ significantly at the higher polymer concentration level, in turn, RG504 H showed a much higher burst compared to RG504 ($p < 0.05$) at the lower concentration level. In addition, PLA did not differ significantly form PLGA polymers at higher concentrations ($p = 0.447$), while it showed a significantly lower burst release with respect to RG504 H ($p = 0.025$), but negligible differences with RG504 ($p = 0.183$), at a lower polymer concentration.

Among the polymers, only for RG504 H the burst release was found to decrease significantly when the polymer concentration was increased ($p < 0.001$). Moreover, both PLGA 85:15 and 50:50 seemed to differ consistently in burst release with PLA ($p < 0.05$) at 1% polymer concentration.

The high burst effect was partly due to the combined plasticizing effect on PLA and PLGA of both water and KET (Blasi et al., 2005; Ricci et al., 2005). Even in this case, plasticization may explain why polymers with so different molecular weights, hydrophobicity and lactic/glycolic ratio have release profiles almost overlapping (data not shown). Due to KET and water, the polymer during the release studies was mainly in its rubbery state (Ricci et al., 2005). It is known that the diffusion coefficient of small molecules in a polymer in the rubbery state is several orders of magnitude higher than that recorded in the glassy state (Karlsson et al., 2001). Therefore, in this condition, diffusion may represent the main mechanism controlling the release of the encapsulated molecule.

3.2. Preparation and characterization of composite microcapsules

Alginate microcapsules were prepared by an atomizing method (see details in Section 2) resulting in fabrication of "medium size" spherical beads that were extremely monodisperse with an average diameter of 600 ± 50 μm (Calafiore, 2002; Calafiore and Basta, 1999). Preparation A was chosen to be encapsulated into alginate based microcapsules in order to study the effect on KET release. Two blank MS preparations were embedded into alginate microcapsules to study the biocompatibility of the composite system *in vivo*. In particular, the blank MS of the preparations A and B were chosen because of the smaller particle size (Ricci et al., 2005). In fact, large MS due to their weight could lead to membrane deformations or ruptures responsible of a low biocompatibility. Indeed, membrane imperfections, such as tails and craters, were found to attract macrophages *in vitro* (Basta et al., 2004).

Composite microcapsules resulted elastic and transparent facilitating thus the internal microscopic observation useful for their characterization. Both PLA and PLGA MS were homoge-

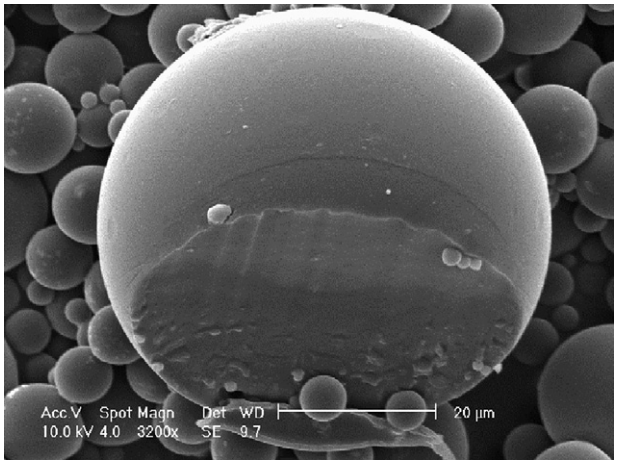

Fig. 3. SEM picture of internal morphology of KET loaded MS: preparation D.

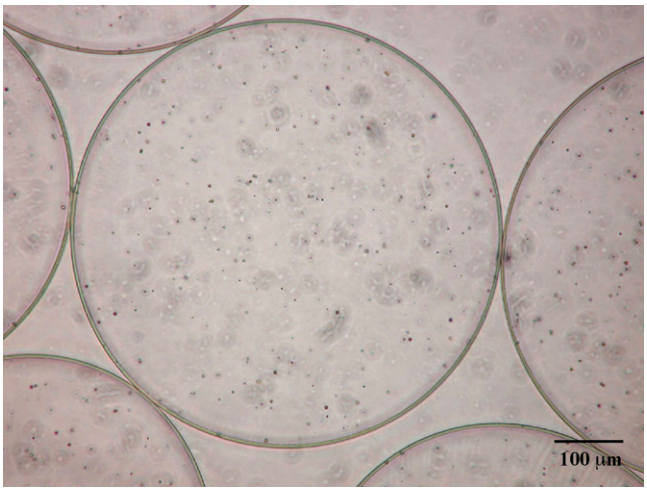

Fig. 4. Composite microcapsules containing KET loaded PLA MS.

nously dispersed into the alginate hydrogel and no technical problems were associated with membrane formation. Microscopic observation showed the complete absence of aggregates (Fig. 4) which could induce, if present, membrane deformation or rupture during the preparation process.

Fig. 5 shows the *in vitro* release profiles of the preparation A as free MS and as composite system (KET loaded PLA MS embedded into alginate/poly-L-ornithine/alginate microcapsules). Free MS showed a high burst effect, releasing about 70% of KET during the first day, and released almost 90% of the entrapped drug after 1 week of incubation (Fig. 5). When MS were encapsulated to form the composite microcapsules, the burst effect was lowered to 50%, with about 70% of drug being released after 1 week while the release profiles were similar (Fig. 5). Being membrane cut-off not an obstacle to KET diffusion, the lower burst was ascribed to the high alginate hydrogel viscosity (Calafiore and Basta, 1999).

3.3. *In vivo* biocompatibility studies

Table 3 and Fig. 6 show the results obtained for the *in vivo* biocompatibility study. Biocompatibility was evaluated as a

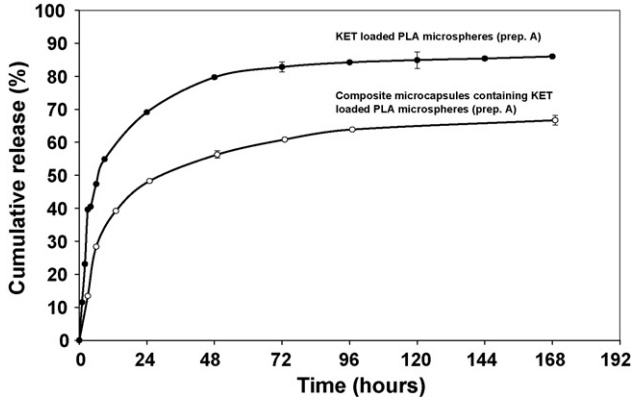

Fig. 5. *In vitro* KET release from PLA MS and PLA-composite microcapsules. Adapted from Ricci et al. (2005).

Table 3
Percentage of pericapsular overgrowth observed in the biocompatibility study

Day after implantation	Overgrowth		
	Group 1	Group 2[a]	Group 3[a]
9	3 ± 1	17 ± 2	5 ± 4
19	4 ± 2	25 ± 2	7 ± 2
34	0	20 ± 3	5 ± 3
41	3 ± 3	30 ± 2	8 ± 5
63[b]	0	0	0

[a] Significantly different from group 1 ($p < 0.05$).
[b] Significantly different from 19 to 41 days ($p < 0.05$).

function of the percentage of pericapsular overgrowth found on the explanted capsules (de Vos et al., 2002b, 2003).

Control microcapsules (Group 1) were practically free of inflammation and floated into the peritoneum with no significant adhesion to the abdominal organs (Table 3 and Fig. 6). Previous works showed the high biocompatibility of alginate when properly purified (Calafiore and Basta, 1999; de Vos et al., 2003). Groups 3 presented a very low inflammatory response comparable to that of the control group. The microcapsule recovery from groups 1 and 3 was comprised between 83 and 91%. Group 2 showed the highest pericapsular inflammation (Table 3 and Fig. 6) with a recovery between 74 and 85%. Statistical analysis evidenced a significant difference ($p < 0.05$) between the groups 2 and 3 and the group 1 and, for all the groups, a significant differences ($p < 0.05$) between day 63 and both days 19 and 41.

Surprisingly, at day 63 the percentage of pericapsular overgrowth of group 2 was equal to that of the control (Table 3). Differences in biocompatibility between PLGA and PLA composite microcapsules could be explained by the different polymer chemical structure and molecular weight, conferring to polymers a different degradation rate. Previous studies performed on PLA and PLGA 50:50, showed a mass loss after 60 days of ∼15 and ∼60%, respectively (Giovagnoli et al., 2004). PLGA 50:50 biodegradation by-products, such as lactic and glycolic acid, may be responsible for pH lowering. It is known that a local acidification, due to polyester degradation, leads to an inflammatory response around the device (Agrawal and Athanasiou, 1997; Sung et al., 2004). This suggested that higher molecular weight polyesters may be better tolerated than smaller polymers because of the slower degradation rate. In fact, the low degradation rate, generating small amounts of acidic by-products timewise, produces a limited acidification around the device that can be easily balanced (den Dunnen et al., 1997; Sung et al., 2004).

Biocompatibility of composite microcapsules seems to be correlated just with the degradation rate of polyesters; other factors, such as size, shape and surface characteristics seem to be less important. An interesting finding was the inflammatory response at day 63 (Table 3 and Fig. 6). In fact, it seems that the intensity of the inflammatory response slowed down even if the acidic by-products release was still in progress (Giovagnoli et al., 2004). This may be due to a complex evolution of this response, especially against "dynamic systems" such as biodegradable devices, changing their characteristics over time (den Dunnen et al., 1997; Sung et al., 2004). The aforementioned

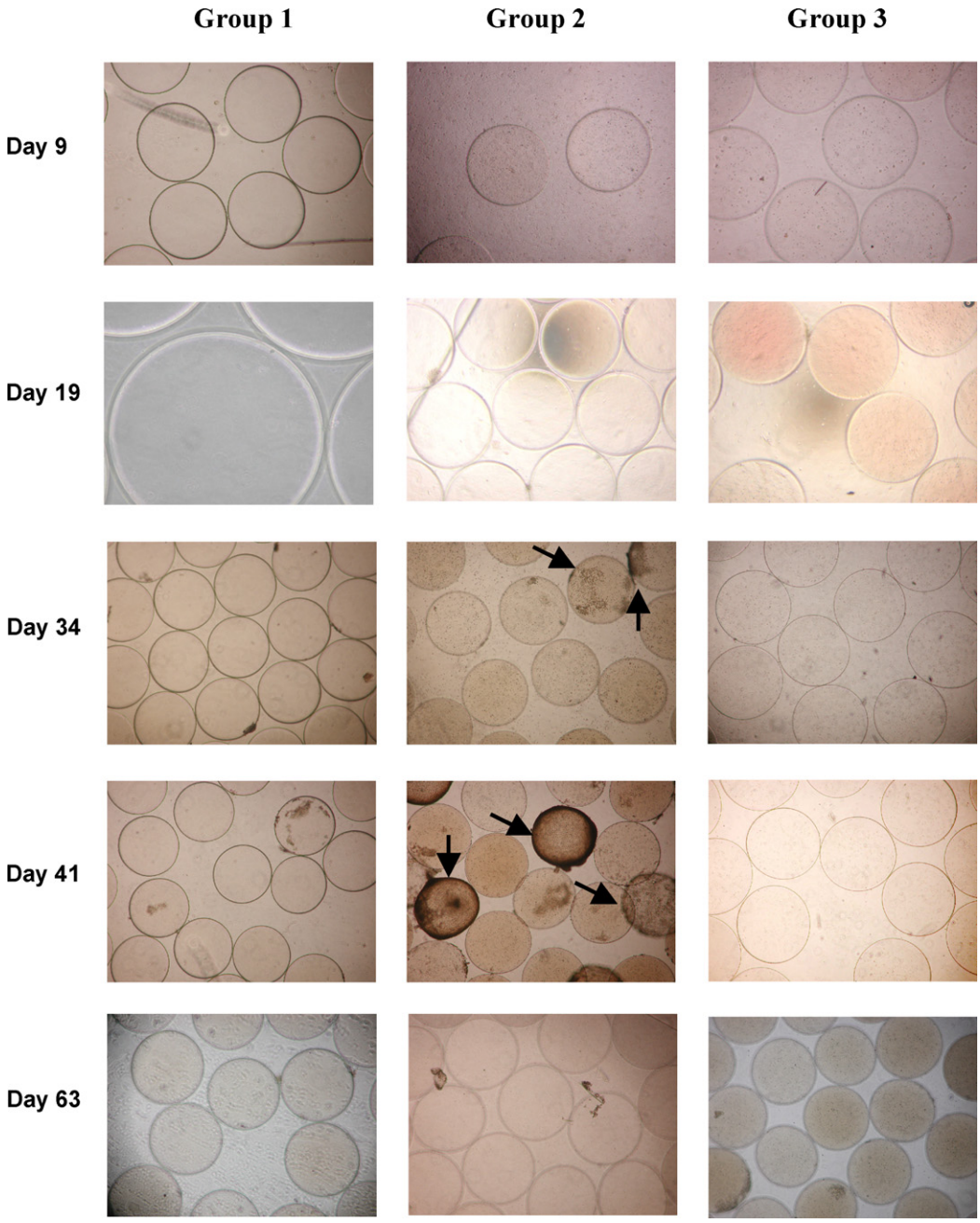

Fig. 6. Optical microscopy pictures of the explanted microcapsules (black arrows indicate pericapsular overgrowth).

device is formed by two main parts: the stable alginate/poly-L-ornithine/alginate capsule and the enveloped biodegradable MS. The early inflammation, due to both surgical trauma and the presence of the foreign material, disappears within 2 weeks (Bünger et al., 2005); while acidic by-products continue to stimulate inflammation. The inflammatory response changes over time in intensity and type of the recruited inflammatory cells (den Dunnen et al., 1997). On the contrary, inflammatory cell migrations and the correlated angiogenesis were found to be negatively affected by the acidic environment (Sung et al., 2004). The inflammatory cells respond to the acidic environment in different ways depending upon time or acidity with mechanisms that are only partially understood (den Dunnen et al., 1997; Sung et al., 2004).

Surprisingly, most of the PLGA composite microcapsule membranes at day 63 were still intact even if an overgrowth, and the related oxidative stress, were observed during most of the implantation period. This confirms the chemical stability of alginate/poly-L-ornithine/alginate microcapsules and the mildness of the inflammatory reaction induced. It can be concluded, at least preliminarily, that the pH decrease and the ongoing inflammation did not alter the membrane characteristics and did not expose the high immunogenic PLO moieties to the host, thus not giving rise to biocompatibility issues.

In conclusion, a composite system intended for cell encapsulation, composed by polymeric MS embedded into alginate/poly-L-ornithine/alginate microcapsules, was successfully prepared and characterized. Polymer concentration showed to have slight influence on the particle size and on the release behaviour of MS. The KET plasticizing effect on PLA and PLGA may explain the high burst effects observed for all the investigated preparations. KET was released from composite microcapsules in a controlled fashion over 2 weeks.

As far as biocompatibility is concerned, PLA composite systems showed better performances than those containing PLGA. The aforementioned approach, also referred as multifunctional (Luca et al., 2003b), was successfully used to release NSAIDs in order to prevent acute inflammation (Ricci et al., 2005), but it could also be applied to other active molecules, such as radical scavengers (Luca et al., 2003b; Giovagnoli et al., 2005), immunomodulators (Luca et al., 2001), and growth factors (Luca et al., 2001), capable to improve cell functional performance and life-span.

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