

Alginate-PLL cell encapsulation system Co-entrapping PLGA-microspheres for the continuous release of anti-inflammatory drugs

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Abstract In spite of advances in cell microencapsulation technology in the past three decades, this approach still suffers from obstacles associated with its biocompatibility. We hypothesized that encapsulation system, which incorporates polymeric particles releasing anti-inflammatory drug in addition to the encapsulated cells, will result in improved biocompatibility, thus improving therapeutic efficacy. We have developed, optimized and studied a combined microencapsulation system in which Ibuprofen loaded PLGA microspheres (MS) are co-entrapped with cells. The combined system was developed and optimized in terms of Ibuprofen release profile, and the survival and proliferation of the co-encapsulated cells. The biocompatibility of the system was evaluated *in vitro* and *in vivo*. The developed system was shown to release Ibuprofen within two weeks, and support long-term cell viability. The combined system had improved the biocompatibility within the release period of Ibuprofen. All together, the co-encapsulation of anti-inflammatory loaded MS along with cells offers a clear advantage in the development of effective, long lasting cell based drug delivery systems.

The choice of the anti-inflammatory agent, or combination of several anti-inflammatory agents needs to be carefully optimized, as well as their release profile to achieve long-term biocompatibility.

Keywords Cell delivery device · PLGA · Drug delivery · Inflammatory response · Ibuprofen

1 Introduction

Microencapsulation of living cells is a promising approach for the continuous delivery of therapeutics. This technology is based upon the entrapment of cells within a polymeric matrix surrounded by a non-degradable semi-permeable membrane. The cells produce and secrete the therapeutic agent continuously, while the semi permeable membrane isolates the cells from the host immune system allowing the exchange of nutrients and waste (Chang et al. 1993; Read et al. 1999; Uludag et al. 2000; Machluf 2005a,b; Zimmermann et al. 2007). Alginate-Poly-L-Lysine (Alginate-PLL) system was successfully used for the entrapment of langerhans islets in 1980 by Lim and Sun (Lim and Sun 1980). Since then, extensive research has been performed using microencapsulation technology as a treatment for a wide variety of diseases, reaching significant achievements including clinical trials in diabetic patients and primates (Soon-Shiong et al. 1994; Dufrane and Gianello 2008), animal studies in cancer therapy (Joki et al. 2001; Visted et al. 2003; Visted and Lund-Johansen 2003) and more. Nevertheless, there are still obstacles preventing the clinical approval of the encapsulation technology. One of the major obstacles facing the polymeric encapsulation technology is the inflammatory response that eventually is evoked towards the transplanted microcapsules leading to the death

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of the encapsulated cells (Babensee et al. 1998; Orive et al. 2006). Different approaches have been taken to overcome these biocompatibility obstacles, including purification and modification of the polymers, and replacement of the cationic polymer (Bartkowiak and Hunkeler 1999; Omer et al. 2003; Bunger et al. 2005; Baruch and Machluf 2006; Mazumder et al. 2008). Other approaches included the reduction of the host initial response by temporal administration of immunosuppressant or anti-inflammatory drugs along with the administration of the encapsulated cells (Schneider et al. 2003; Bunger et al. 2005; Ricci et al. 2005; Blasi et al. 2006).

In the present study, we describe the design and characterization of a unique combined cell microencapsulation system, which incorporates a secondary drug release system that secret an anti-inflammatory drug. We hypothesize that such system can provide the release of therapeutic proteins from the encapsulated engineered cells concurrently with the continuous release of anti-inflammatory agents, thus avoiding the laborious need for constant administration of anti-inflammatory drugs that are needed to reduce host response against transplanted microcapsules. The studied system comprises alginate-PLL microcapsules which co-encapsulate living cells and Poly Lactic co-Glycolic Acid-microspheres (PLGA) loaded with Ibuprofen as a model for anti-inflammatory drugs. PLGA microspheres are non-immunogenic, biodegradable particles, which are extensively used for the delivery of many proteins and drugs (Alonso et al. 1994; Lisovsky et al. 1996; Mullerad et al. 2003; Seagal et al. 2003; Benny et al. 2005; Benny et al. 2008; Bae et al. 2009; Benny et al. 2009). Ibuprofen (2-(4-Isobutyl-phenyl)-propionic acid) is one of the most widely used non-steroidal anti-inflammatory drugs (NSAIDs). It is considered a safe NSAID, being associated with a relatively low risk of adverse gastrointestinal effects and renal effects at anti-inflammatory doses (Rainsford 2003). In this study we have produced and characterized Ibuprofen loaded microspheres and co-encapsulated them with cells in alginate-PLL microcapsules. We have studied the feasibility and the biocompatibility of the system *in vitro* and *in vivo* and compared its characteristics to those of alginate-PLL control system, which do not include PLGA particles.

2 Materials and methods

2.1 Cell culture

Baby hamster kidney cells (BHK-21, ATCC Rockville, MD, US) and mouse monocyte macrophage cells (RAW 264.7, ATCC Rockville, MD, US) were maintained in Dulbecco's modified Eagle medium (DMEM) supple-

mented with 10% FBS (Biological Industries, Israel). Cells were incubated at 37°C with 5% CO₂.

2.2 Preparation of Ibuprofen-loaded PLGA microspheres (MS)

Ibuprofen loaded microspheres were prepared using the double emulsion solvent extraction method with slight modifications (Benny et al. 2005). Briefly, 200 mg of PLGA 50:50 (Boehringer Ing. Germany) was dissolved in 0.5 ml of dichloromethane (Frutarom, Israel). A predetermined solution of Ibuprofen (Sigma, 0.5 gr/ml) was added to the dissolved polymer and the solution was homogenized using ultra-turax (type DI-18 IKA, Germany) for 1 min leading to the formation of the first emulsion (W/O). Polyvinyl alcohol of 85–89 kDa (Aldrich Chemical, Inc, USA) saturated with dichloromethane was rapidly added to the first W/O emulsion, and the solution was homogenized again for the second time for 20 sec. The resulting multiple W/O/W were mixed for 5 mins. and a volume of 50 ml of 0.1% w/v aqueous polyvinyl alcohol containing 5% (v/v) 2-propanol solution (J.T.Baker, Holland) was added. After 30 mins. of extensive stirring the microspheres were centrifuged, washed three times and lyophilized (Modulo Edwards). BSA-FITC loaded microspheres were prepared in the same conditions only with BSA-FITC instead of Ibuprofen. Empty microspheres were prepared in the same conditions only without Ibuprofen.

2.3 Size and morphological studies of PLGA microspheres

Scanning Electron Microscopy (SEM, Joel, JSM 5400) was used to evaluate the shape and surface morphology of the Ibuprofen loaded PLGA microspheres. Particles size distribution was analyzed using a Coulter LS 230 particle size analyzer. Samples were prepared by re-suspending 10 mg microspheres in distilled water. The results were reported as microsphere diameter determined by % volume distribution, and were analyzed by a model for ideal spheres.

2.4 Ibuprofen loading efficiency in PLGA microspheres

The amounts of loaded Ibuprofen per unit weight of microspheres were determined as follow; fractions of 10 mg microspheres loaded with Ibuprofen were digested overnight with 0.1N NaOH, to increase PLGA hydrolysis rate. UV emission (OD320 nm) was used to determine total Ibuprofen loading in the microspheres and the amount of Ibuprofen released from the microspheres at different time points. The loading efficiency was obtained by calculating the percent of total Ibuprofen loaded in the microspheres, divided by the initial Ibuprofen amount added during the preparation of the microspheres.

2.5 Ibuprofen release from PLGA microspheres and combined microcapsules

For the *in vitro* release studies, 20 mg microspheres loaded with 50 mg Ibuprofen were incubated in PBS at pH 7.3 and maintained in a shaking incubator at 37°C. Every 2–3 days over a 30 day period, microspheres were recovered from the release media by centrifugation and the media was collected and replaced with fresh PBS. Ibuprofen concentration in the media was quantitatively determined by spectrophotometer at OD320 nm. The percent of the released protein of the total loaded Ibuprofen was calculated for every sample at each time point.

Additionally, the release of Ibuprofen from microspheres, which were co-encapsulated in Alginate-PLL microcapsules, was determined. Capsules were placed in a filter tube and were incubated in HEPES at pH7.4 (5 ml) in a shaking incubator at 37°C, every few days the medium was replaced by fresh one, and Ibuprofen concentration was measured. Ibuprofen concentration in the media was quantitatively determined by high-performance liquid chromatography (HPLC) using a C18 column (Agilent eclipse XDB-18C). The percent of the released protein of the total loaded Ibuprofen was calculated for every sample at each time point.

2.6 Preparation of combined microcapsules entrapping BHK cells and Ibuprofen loaded MS

BHK cells and PLGA microspheres were suspended in 1.2% (w/v) sodium alginate solution (Ultra Pure LVG, FMC Biopolymer, Norway) to a final ratio of 1×10^6 cells and 10 mg of microspheres per 1 ml of alginate. The suspension was sprayed through a 20G needle-air jet-head droplet-forming apparatus into a HEPES buffered calcium chloride (13 mM HEPES, 1.5% (w/v) CaCl_2 , pH7.4) solution, and was allowed to gel for 20 mins. Microcapsules were then reacted with 0.06% (w/v) PLL of 28.2 kDa (Sigma Aldrich) in saline for 10 mins. with gentle agitation. The microcapsules were washed three times in HEPES and cultured using appropriate medium in 37°C, 5% CO_2 incubator. Microcapsules with no PLGA MS were prepared using the same procedure.

2.7 Viability of encapsulated cells

Encapsulated cells viability was measured using Alamar Blue™ (AbD Serotec, UK). A volume of microcapsules equivalent to 100,000 encapsulated cells, at the day of encapsulation, was placed in 24 wells plate. Microcapsules were incubated in 1 ml of 10% (v/v) Alamar Blue™ for 4 hr, and then two samples of 100 μl conditioned media of each well was sampled to a 96 wells plate and read using a micro plate fluoremeter (Genios, Tecan).

2.8 *In vitro* biocompatibility study

The biocompatibility of microcapsules was assessed *in vitro* using macrophage ($\text{M}\Phi$) activation assay. In this assay the transcription of $\text{TNF}\alpha$ by $\text{M}\Phi$ was used as indication for the activation of the $\text{M}\Phi$. RT-PCR analysis was performed in order to quantify $\text{TNF}\alpha$ mRNA transcription. Raw macrophage cells were seeded in 6 well plates and 24 hr later microcapsules were added. Following additional incubation of 24 hr, the $\text{M}\Phi$ were harvested and RNA was extracted. Total RNA was extracted using Tri-Reagent (Sigma Aldrich), following standard protocol, and 1mg from the RNA solution was treated with DNase (RNase free, Promega) for 30 min at 35°C. For the synthesis of cDNA, 250ng of the DNA free RNA was taken and the synthesis was performed using random primers (AB-Gene, Epsom, UK). For the amplification of $\text{TNF}\alpha$, the following primers were used: 5'-GATTTGCTATCTCATACCAGGAGAA (sense) and 5'-GACAATAAAGGGGTCAGAGTAAAGG (antisense). Amplification of Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used as a control and was performed using the primers: 5'-ACCCAGAAGACTGTGGATGG (sense) and 5'-CTTGCTCAGTGTCTTGTCTG (antisense). PCR products were analyzed by 1% agarose gel electrophoresis. The results are shown as band intensity, as measured by Image J software, normalized to GAPDH band intensity and to the control.

2.9 *In vivo* biocompatibility study

C57 Black male mice, 6 weeks old, were transplanted with microcapsules subcutaneously. The treatment groups were: (I) combined microcapsules entrapping cells and Ibuprofen loaded PLGA MS, (II) combined microcapsules entrapping cells and empty PLGA MS, (III) microcapsules entrapping only cells, and (IV) control group in which serum free media was injected. Each treatment group consisted of 24 mice in which 6 mice were sacrificed at each time point. Mice were sacrificed at one, two, four and eight weeks post transplantation. At each scarification time point, images of the microcapsules within the mouse were taken prior to microcapsules retrieval. Half of the retrieved microcapsules were re-cultured and their morphology and tissue overgrowth were evaluated under microscope. In addition, the viability of encapsulated cells was assessed using FDA (Fluorescein diacetate). The second half was taken for histological analyses

2.10 Histology and Immuno histochemistry (IHC)

Retrieved microcapsules were dehydrated using a series of graded alcohols (30%–100%), and embedded in paraffin. Sections (10 μm) were stained using Hematoxylin & Eosin.

IHC was carried out using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Primary antibody mouse F4/80 antigen (1:100 AbD Serotec, UK) was used for the detection of M Φ . Detections were carried out using the DAB chromogen (Vector Laboratories) and sections were counterstained with hematoxylin. Negative control slides were obtained by omitting the primary antibody.

2.11 Evaluation of the immune reaction

The overall evaluation of the immune reaction towards transplanted microcapsules was performed blindly by a pathologist. H&E stained slides of each sacrifice time point and from each treatment group were evaluated. A Masson's trichrome staining was performed for further evaluation (data not shown). Each slide is scored according to the following parameters: width and components of outer capsule, collagen fibers in outer capsule, width and components of inner barriers, and collagen fibers in inner barriers. Each parameter is scored between 0 (no reaction) and 3 (severe reaction) and the total score for each slide is the sum of the four parameters scored. The presented score for each time point of each treatment is the average of the scores given to the slides of this time point.

2.12 Statistics

Results are presented as mean \pm SD or as percentage of mean of control \pm SD. Comparisons between groups were performed by using a two-tailed T-test. Statistical difference were reported when p value was at least p<0.05.

3 Results

3.1 Characterization of Ibuprofen loaded microspheres and release kinetics of Ibuprofen from PLGA microspheres

Ibuprofen loaded PLGA-microspheres were produced using the 50:50 PLGA copolymer and a ratio of 1:4 Ibuprofen to polymer (Table 1). The loading efficiency of Ibuprofen was of 59%. As seen in Fig. 1(a), the produced MS exhibited a spherical, intact surface area. Moreover, a typical size-distribution graph obtained by Coulter counter for PLGA50: 50, indicates that the particles are of 30–50 μ m in diameter with a narrow size distribution (Fig. 1(b)). The release profile of Ibuprofen from PLGA-microspheres was

Fig. 1 Microspheres morphology, size and release kinetics. (a) SEM representative micrograph showing the morphology of PLGA50:50 microspheres. (b) Typical Ibuprofen-loaded microspheres size distribution. The graphs were obtained by Coulter counter light scattering measurement. (c) Release profile of Ibuprofen from microspheres composed of PLGA 50:50. Points, mean of three different experiments, expressed as a percentage of released Ibuprofen from the total amount of Ibuprofen loaded into the microspheres (after loading efficiency determination). Errors are standard error of the mean \pm S.D n=4

characterized by a continuous release for 14 days, with a mild burst during the first days of release (Fig. 1(c)).

3.2 Alginate-PLL co-encapsulation of cells with PLGA-microspheres

In order to evaluate the feasibility of the co-encapsulation system, PLGA 50:50 MS were co-encapsulated with BHK cells in alginate-PLL microcapsules. The combined PLGA-microcapsules were cultured and observed under a fluorescent microscope. Figure 2 depict BHK cells stained with Dil (red fluorescence), co-encapsulated with BSA-FITC loaded PLGA MS. As seen from the micrographs, there is a large fraction of BSA-FITC, which is still retained within the MS entrapped in the microcapsules for more than two weeks post encapsulation. The number of co-encapsulated cells increased with time, indicating that the cells proliferate within the microcapsules.

The next step in developing the co-encapsulation system was the co-encapsulation of Ibuprofen as a model for anti-inflammatory drug loaded PLGA MS and BHK cells in alginate-PLL microcapsules. The combined microcapsules were cultured and evaluated for Ibuprofen release and for encapsulated cells viability. The release profile of Ibuprofen from the combined microcapsules is shown in Fig. 3(a). More than 60% of the Ibuprofen was released within the first week of encapsulation, and 90% of the Ibuprofen was released by the end of the second week.

The viability of the co-encapsulated cells was assessed over time and compared to the viability of cells encapsulated in alginate-PLL without MS and to the viability of cells co-encapsulated with empty MS. As seen in Fig. 3(b), the viability of cells co-encapsulated with Ibuprofen-MS was similar to the viability of the control-encapsulated cells.

3.3 *In vitro* biocompatibility study

Macrophage (M Φ) activation assay was performed *in vitro* to obtain an indication for the biocompatibility of the

Table 1 Summary of microspheres properties

Polymer	Loading efficiency	Mean diameter	Ibuprofen
PGLA 50:50 Mw=20,000	59.8 \pm 6%	20–50 μ m	Mw=206 gr/mol

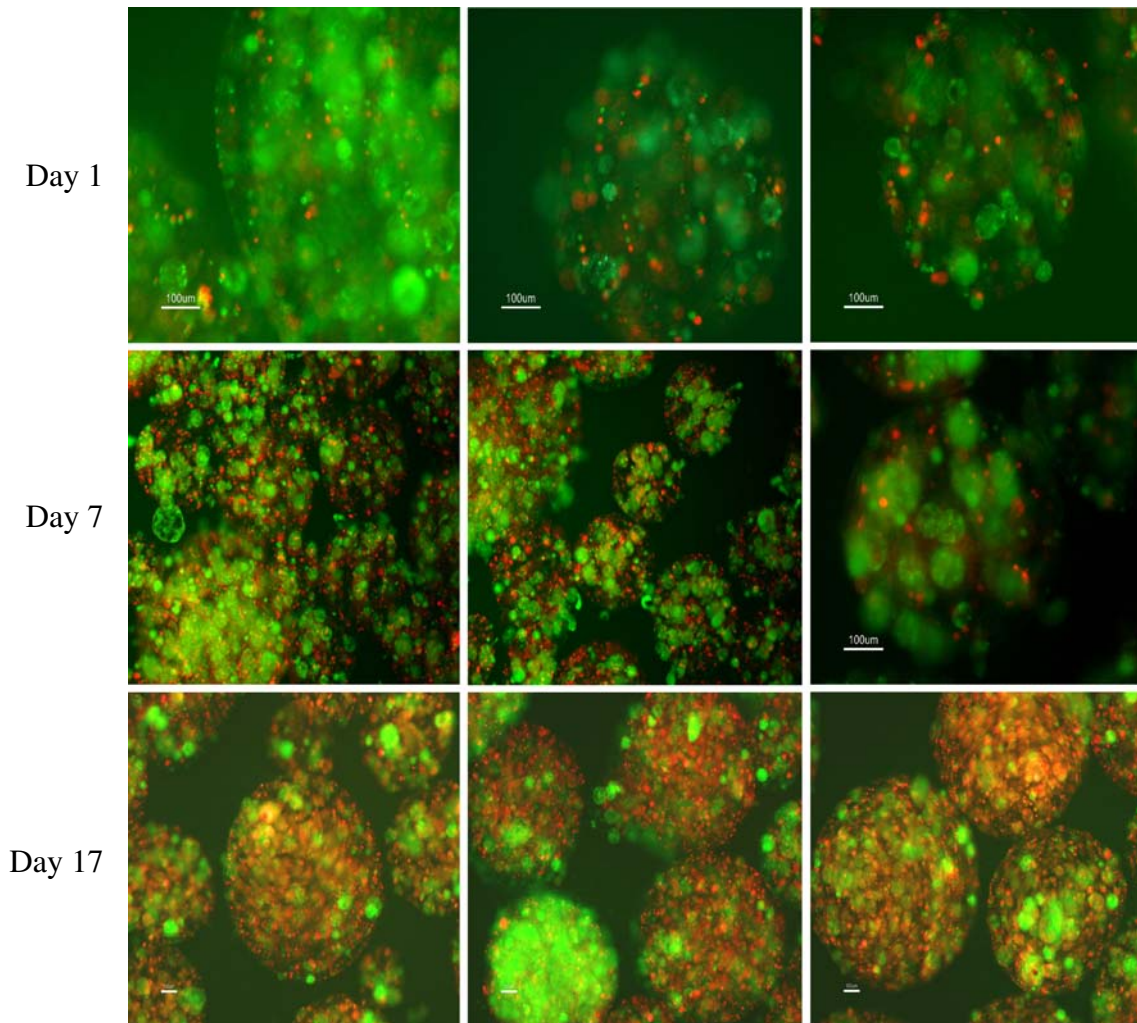
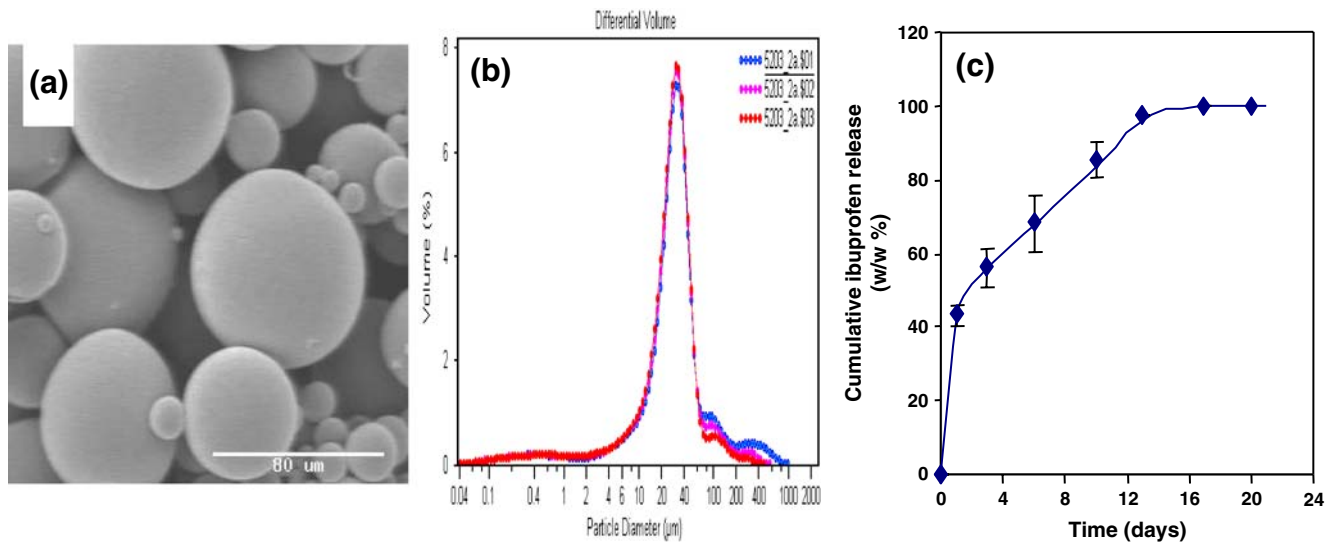


Fig. 2 Fluorescent microscope micrographs of microcapsules co-encapsulating BHK cells stained with Dil (*red*) along with BSA-FITC (*green*) loaded PLGA MS. Pictures were taken at day one, seven and seventeen days post encapsulation

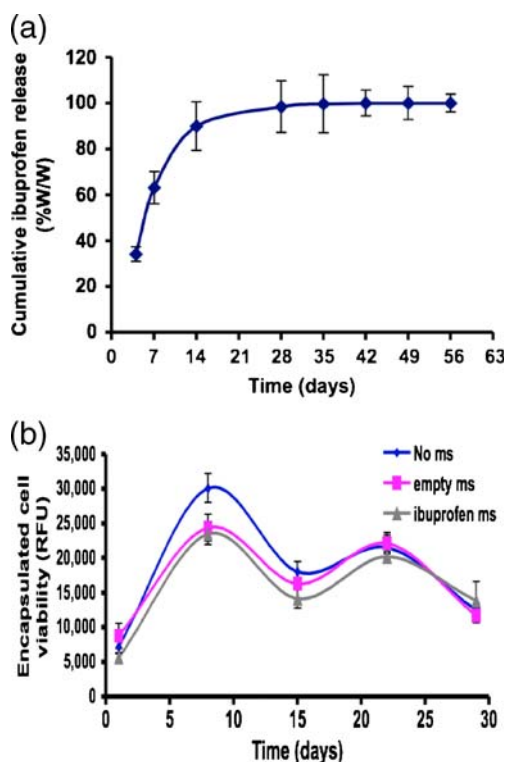


Fig. 3 Co-encapsulation of BHK cells and Ibuprofen PLGA-microspheres. **(a)** Ibuprofen release profile from a combined system. The release of Ibuprofen is measured using HPLC and expressed as a percentage of the total amount of Ibuprofen loaded into the microspheres. **(b)** Long-term viability of cells co-encapsulated with Ibuprofen-MS in comparison to the viability of cells co-encapsulated with empty MS and to the viability of cells encapsulated in microcapsules without MS. Cell viability was measured using Alamar Blue™ viability assay

combined system. The activation of $M\Phi$ treated with the combined microcapsules was compared to the activation of $M\Phi$ which were treated as follow: encapsulated cells without MS, only soluble Ibuprofen, only $IFN\gamma$ and LPS, encapsulated cells without MS together with $IFN\gamma$ and LPS, soluble Ibuprofen together with $IFN\gamma$ and LPS, combined microcapsules together with $IFN\gamma$ and LPS, and finally no treatment as negative control. Figure 4 demonstrates that the activation of $M\Phi$ incubated with the combined microcapsules is comparable to their activation following the negative control treatments. Moreover the $M\Phi$ activation is reduced when compared to the activation of $M\Phi$, which were treated with encapsulated cells with no MS as well as in comparison to the activation of $M\Phi$ following the positive control treatments.

3.4 *In vivo* biocompatibility studies

The biocompatibility of the combined microencapsulation system was evaluated *in vivo* in C57 mice. The microcapsules

were injected subcutaneously and mice were sacrificed at four time points and at each sacrifice time point the transplanted microcapsules were retrieved and analyzed.

As seen from Fig. 5(a), the transplanted microcapsules remained as a cluster in the transplantation site. One week post implantation, microcapsules with Ibuprofen MS appeared clear with no tissue surrounding them. Retrieved microcapsules with empty MS or no MS were characterized by slightly white tissue surrounding the cluster and vascularization one-week post implantation. At eight weeks time point, the microcapsules harvested from all the groups appeared as clusters and were surrounded by a white vascularized tissue. No bleeding was seen at the transplantation area in any of the mice.

All the retrieved microcapsules were imaged under light and fluorescent microscope following FDA viability staining (Fig. 5(b)). Microcapsules from all treatment groups and sacrifice time points remained intact. The retrieved microcapsules were characterized by varying levels of cells attachment, regardless of the treatment group or time point. The viability of the encapsulated cells as seen by the FDA staining did not vary between the different groups or time points.

Localization and quantification of macrophages in the formed tissue were performed using F4/80 antibody, a specific marker of macrophages (Fig. 6). Analysis of the stained sections of microcapsules revealed that large quantity of $M\Phi$ accumulated within the surrounding tissue. This had occurred in the first week, for the microcapsules, which did not contain MS and by the second week for the microcapsules, which contained empty and Ibuprofen loaded MS.

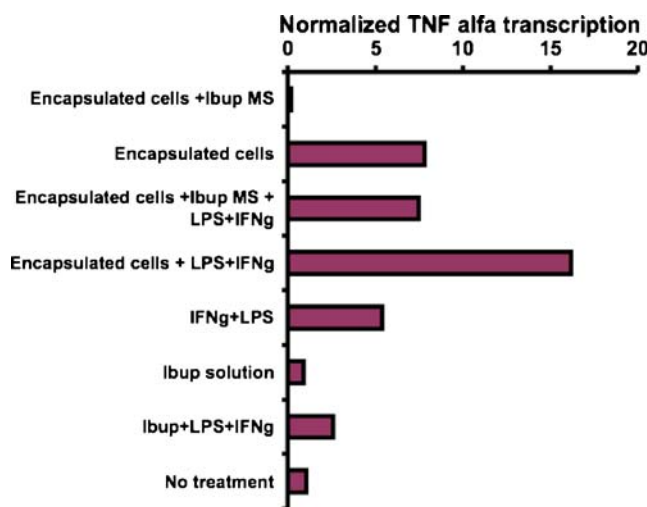


Fig. 4 *In vitro* biocompatibility assay. Macrophages were treated with combined microcapsules and compared to macrophages treated with different combinations of microcapsules, Ibuprofen solution and LPS + $IFN\gamma$. Transcription of TNF α was evaluated using RT-PCR analysis

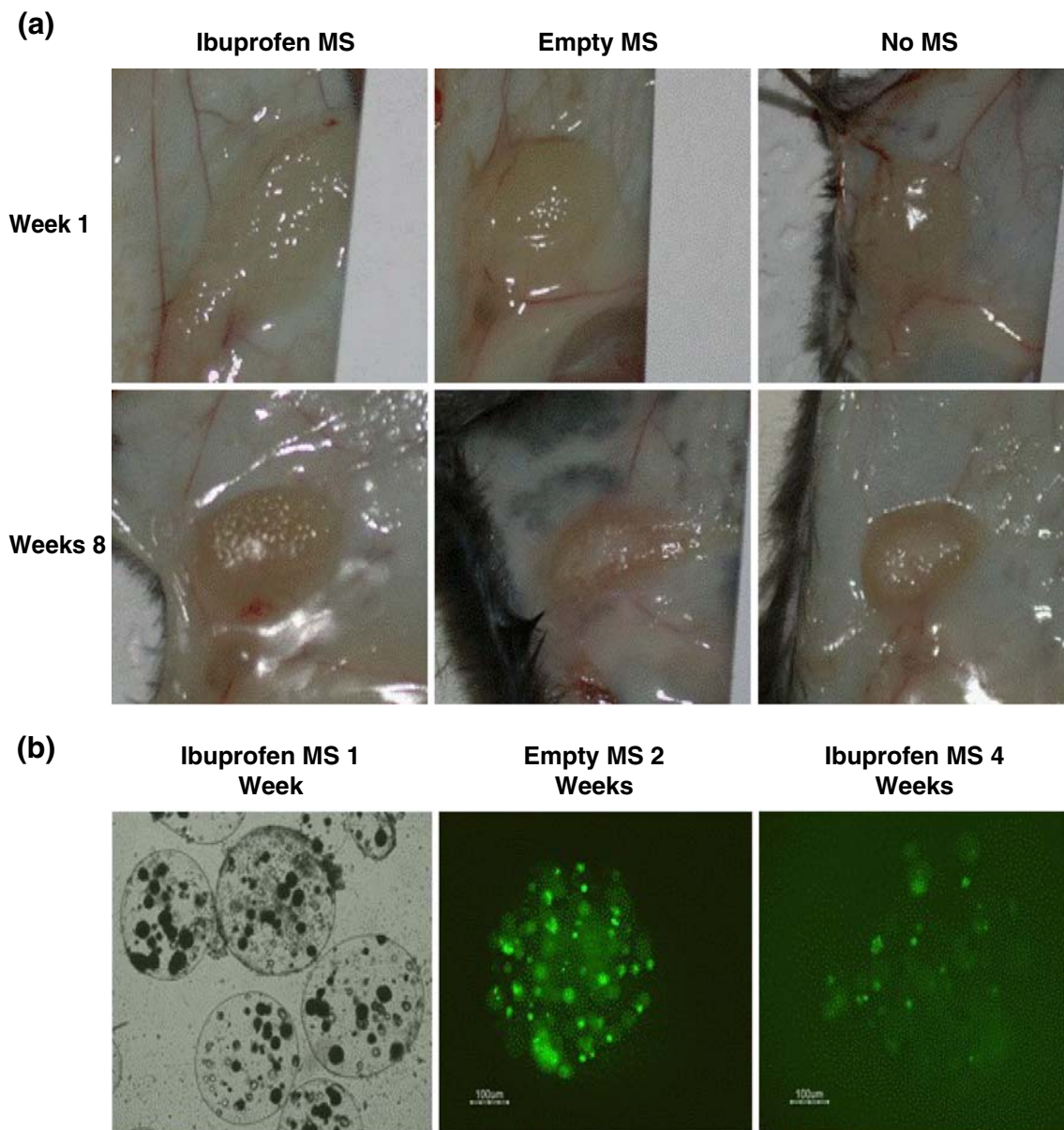


Fig. 5 *In vivo* biocompatibility studies. **(a)** Micrographs of microcapsules clusters in transplantation site in 1 and 8 weeks post transplantation. Treatment groups are: Alginate-PLL microcapsules entrapping cells + Ibuprofen MS, Alginate-PLL microcapsules entrapping cells + empty MS, and Alginate-PLL microcapsules entrapping cells with no MS. **(b)** Microcapsules retrieved from mice at the termination of the experiments. Light microscope micrographs (I) and fluorescent microscope micrographs of FDA stained microcapsules (II,III)

Hematoxylin and Eosin (H&E) staining was used to visualize and evaluate the following indices: The extent of tissue which surrounded the implant, the width of the cellular tissue between microcapsules, the different cell types composing the formed tissue and the amount of fibers in it, and the vasculature in the transplant area (Fig. 7).

High levels of cellular overgrowth and fibers formation were observed in all of the treatment groups starting from the first week, except the group of microcapsules with Ibuprofen loaded MS in which it could be observed starting from the second week. Thick cellular layers were seen

between the microcapsules and surrounding the implant. The cell types present at the formed tissue were macrophages, giant cells, fibroblast, lymphocytes and endothelial cells forming small capillaries (Fig. 7(a)).

The immune reaction was quantified, blindly, by a pathologist. A relative score was given to the sections of each treatment group and each time point. As seen in Fig. 7(b), severe immune reaction was developed towards both control groups (no MS and empty MS) starting from the first week and remaining through the eight weeks of the study. In the Ibuprofen MS group, the immune reaction

was significantly lower than in the control groups. The immune reaction was quantified, blindly, by a pathologist. A relative score was given to the sections of each treatment group and each time point. As seen in Fig. 7(b), severe immune reaction was developed towards both control groups (no MS and empty MS) starting from the first week and remaining through the eight weeks of the study. In the Ibuprofen MS group, the immune reaction

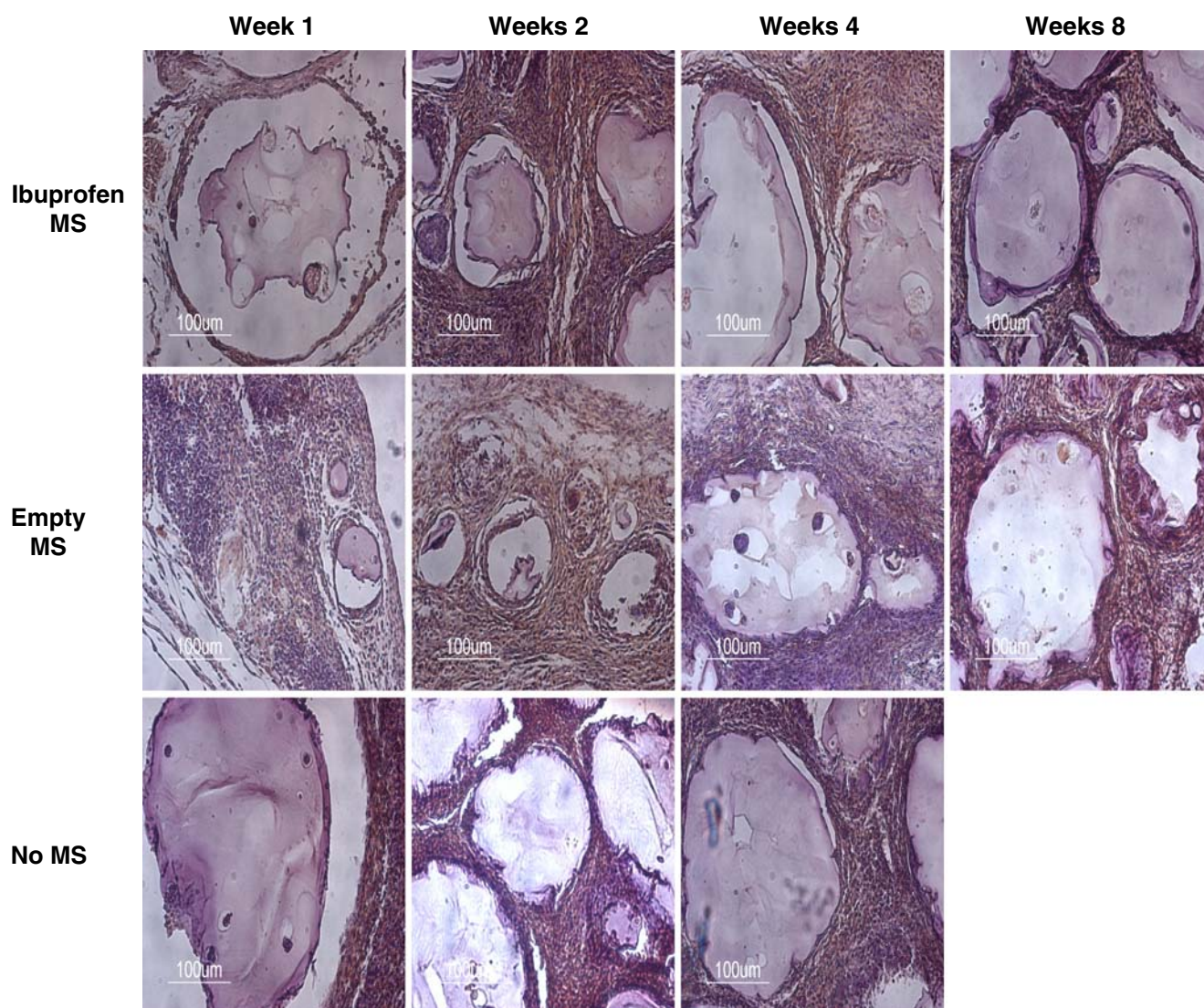


Fig. 6 Immunohistochemistry of macrophage. Mice were treated with alginate-PLL microcapsules entrapping cells + Ibuprofen MS, alginate-PLL microcapsules entrapping cells + empty MS, or alginate-PLL microcapsules entrapping cells with no MS. The mice

were sacrificed 1, 2, 4 and 8 weeks post transplantation. The implants were paraffin embedded and immunostained against F4/80, a macrophage specific marker

developed during the first week was lower than in the reference groups. However, by the second week the immune response had increased and reached the level of the reference groups.

4 Discussion

Extensive work has been performed in recent years aiming at reducing or eliminating the immune reaction towards encapsulated cells implants. Many studies focused on improving the biocompatibility of the polymeric component of the encapsulation system. These studies have achieved huge progress in reducing the immune reaction, but failed

to eliminate it. Additional possible approach toward eliminating the immune reaction is to actively suppress the inflammatory process against the transplanted microcapsules rather than improve the system biocompatibility. In the present study, we were motivated to address this important problem by designing a combined cell microencapsulation system, which incorporates a drug delivery system secreting an anti-inflammatory drug. The hypothesis was that the co-encapsulation of anti-inflammatory drug delivery system within the microcapsules would provide a local and continuous release of the anti-inflammatory agent to the transplantation site, thus decreasing the inflammatory reaction towards the microcapsules and improve the system's long-term efficacy. Ibuprofen was chosen as a

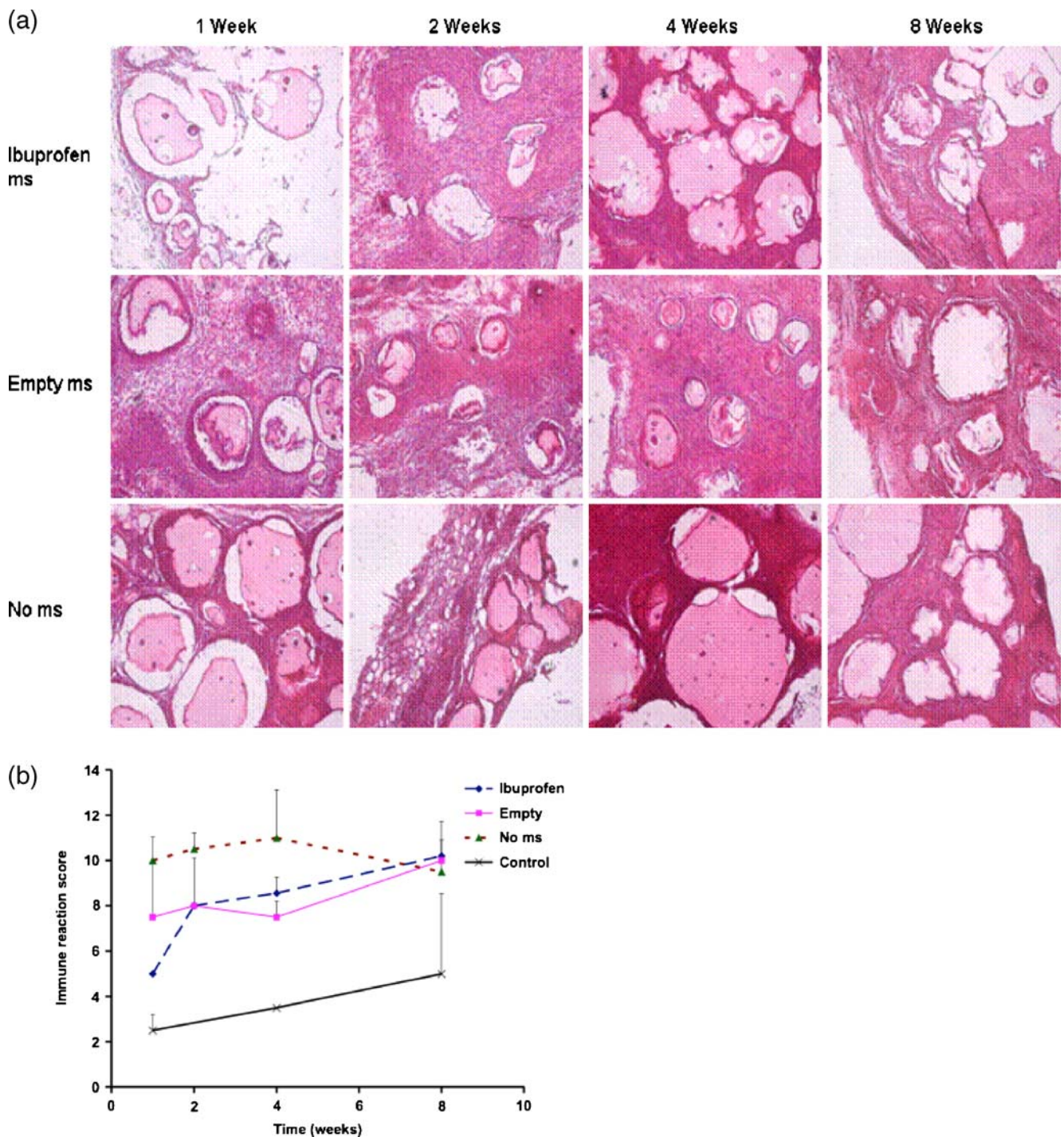


Fig. 7 Immune reaction scoring. **(a)** Histological analysis of the retrieved microcapsules from the different groups at 1, 2, 4 and 8 weeks post transplantation. The implants were paraffin embedded and H&E stained. **(b)** Immune reaction was scored for each treatment

group with time. Relative scores were blindly determined by a pathologist for each of the treatment groups in each time point, based on stained sections of retrieved microcapsules

model anti-inflammatory drug due to its safety and wide use. We have previously shown the efficacy of PLGA microspheres (MS) drug release system for the continuous delivery of therapeutics *in vitro* and *in vivo* (Benny et al. 2005; Benny et al. 2008; Benny et al. 2009). Therefore, we

chose to incorporate Ibuprofen loaded PLGA MS as a controlled release system within alginate-PLL microcapsules which entrap the cells. The incorporation of PLGA microspheres in transplanted devices was previously reported in the field of tissue engineering (Perets et al.

2003; Kempen et al. 2008; Tan et al. 2009) and protein delivery (Edelman et al. 2000). The concept of co-encapsulation of anti-inflammatory factors within cell entrapping microcapsules was first applied by Bunger et al. (Bunger et al. 2005), who have encapsulated the steroid dexamethasone directly in APA microcapsules. This resulted with the deletion of tissue response towards the microcapsules. However, when cells were co-encapsulated with the dexamethasone, the anti-proliferative effect of dexamethasone prevented cell proliferation within the microcapsules. Another combined cell microencapsulation system was recently reported by the Calafiore group (Ricci et al. 2005; Blasi et al. 2006), who have microencapsulated ketoprofen loaded PLGA MS in alginate-PLO system with the intention to co-encapsulate pancreatic islets. They have found that encapsulated PLGA MS resulted with more cellular overgrowth than plain alginate-PLO microcapsules. In their study, the encapsulation of cells was not demonstrated.

The first step in developing a combined microencapsulation system was, to produce Ibuprofen loaded PLGA MS and characterize the particles. The produced MS exhibited a spherical, intact surface area and a narrow size distribution of about 30 μm . The loading efficiency of Ibuprofen was of 59%, and its release profile from the MS was characterized by a continuous release for 14 days, with a mild burst during the first days of release. These characteristics are favorable for the co-encapsulation system in which the MS had to be small enough to be adequately entrapped within the microcapsules and on the other hand, large enough to retain the Ibuprofen for several days. Our results demonstrate that PLGA MS are still present in the microcapsules seventeen days post encapsulation. The encapsulation process did not affect significantly the Ibuprofen release profile, which was still continuous and lasted for 14 days. A parameter of great importance to the system, is its ability to maintain the viability of encapsulated cell. Cell viability can decrease either as a result of acidic conditions caused by the MS degradation products or as a result of the Ibuprofen activity. To date, no cell encapsulation system, which releases anti-inflammatory drug, reported long-term cell viability. Our results clearly show that PLGA MS or Ibuprofen do not affect the viability of the encapsulated cells. Thus, the next significant step was to address the system biocompatibility. Following preliminary *in vitro* studies, in which co-encapsulated Ibuprofen was shown to decrease M Φ activation; *in vivo* studies were performed to evaluate the biocompatibility of the combined encapsulation system. The biocompatibility of cells co-encapsulated with Ibuprofen PLGA MS was compared, at different time points, to two reference systems: encapsulated cells with no PLGA MS and cells co-encapsulated with empty PLGA MS, and to a control group. Our results which were blindly analyzed by a pathologist, demonstrated almost no response

towards the transplanted combined microcapsules with Ibuprofen loaded PLGA MS in the treatment group, one week post transplantation. The tissue, which surrounded the microcapsules of this group, was thin and poor in collagen fibers. In contrast, explants from all the other groups exhibited a higher immune response. This was seen in the morphology of the microcapsules clusters, which were vascularized in some cases, and were often surrounded by a white and rigid tissue. In addition, the histology and immunohistochemistry of these microcapsules sections revealed a high degree of cellular overgrowth surrounding the whole transplant and between individual microcapsules. The formed tissue was rich in macrophages, giant cells, fibroblasts, lymphocytes and collagen fibers. The only implants, which did not show severe fibrosis, were of the combined microcapsules with Ibuprofen loaded PLGA MS, one-week post transplantation. This group's immune reaction score was significantly lower than the scores given to the other groups. This is possibly due to the short-term inhibition of the immune response by the secreted Ibuprofen. However, once all the Ibuprofen has been released, the host immune system is promoted by the presence of the alginate-PLL microcapsules, which contain cells. Thus a higher immune reaction is seen two weeks post transplantation, reaching the levels of the reference groups. This explanation is consistent with the release profile of Ibuprofen from the combined system, showing that 63% of the Ibuprofen is released within one week and 90% is released within two weeks. However, the release of Ibuprofen started at the day of encapsulation, when the microspheres are transferred into an aqueous media, rather than at the day of transplantation. Thus the release profile within the host, starts at day two of the presented release profile (Fig. 3). This suggested explanation is contradictory to Bunger et al. (Bunger et al. 2005), who claimed that a two weeks release of anti-inflammatory drug is enough for the deletion of tissue response. However, they have used dexamethasone that is a much more potent anti-inflammatory drug than Ibuprofen. Moreover, they have implanted microcapsules that did not contain cells and thus did not include the cellular component of microcapsules immunogenicity. Between the two reference systems, our results show increased immune reaction towards the microcapsules, which did not contain PLGA MS. This finding is inconsistent with the Calafiore group (Ricci et al. 2005; Blasi et al. 2006) ones, who found that encapsulated PLGA MS resulted with more cellular overgrowth than plain microcapsules. Nevertheless, their microcapsules did not include cells and their MS were about ten fold smaller than the ones used in our study.

Taking the results all together, we can conclude that the co-encapsulation of Ibuprofen loaded MS along with the encapsulation of the cells may improve short-term system

performance. Further improvement of the combined encapsulation system is required in order to provide long-term efficacy. This may be achieved either by a more prolonged release of the anti inflammatory drug or by the use of a combination of NSAIDs with different release profiles.

References

- M.J. Alonso, R.K. Gupta, C. Min, G.R. Siber, R. Langer, *Vaccine* **12** (4), 299 (1994)
- J.E. Babensea, J.M. Andersonb, L.V. McIntirea, A.G. Mikosa, *Adv. Drug Deliv. Rev.* **33**, 111 (1998)
- S.E. Bae, J.S. Son, K. Park, D.K. Han, *J Control Release* **133**(1), 37 (2009)
- A. Bartkowiak, D. Hunkeler, *Ann. N. Y. Acad. Sci.* **875**, 36 (1999)
- L. Baruch, M. Machluf, *Biopolymers* **82**(6), 570 (2006)
- O. Benny, M. Duvshani-Eshet, T. Cargioli, L. Bello, A. Bikfalvi, R.S. Carroll, M. Machluf, *Clin. Cancer Res.* **11**(2 Pt 1), 768 (2005)
- O. Benny, S.K. Kim, K. Gvili, I.S. Radzishovsky, A. Mor, L. Verduzco, L.G. Menon, P.M. Black, M. Machluf, R.S. Carroll, *FASEB J.* **22**(2), 488 (2008)
- Benny, O., L. G. Menon, G. Ariel, E. Goren, S. K. Kim, C. Stewman, P. M. Black, R. S. Carroll and M. Machluf, *Clin. Cancer Res.*, (2009)
- P. Blasi, S. Giovagnoli, A. Schoubben, M. Ricci, C. Rossi, G. Luca, G. Basta, R. Calafiore, *Int. J. Pharm.* **324**(1), 27 (2006)
- C.M. Bunger, B. Tiefenbach, A. Jahnke, C. Gerlach, T. Freier, K.P. Schmitz, U.T. Hopt, W. Schareck, E. Klar, P. de Vos, *Biomaterials* **26**(15), 2353 (2005)
- P.L. Chang, N. Shen, A.J. Westcott, *Hum. Gene Ther.* **4**, 433 (1993)
- D. Dufrane, P. Gianello, *Transplantation* **86**(6), 753 (2008)
- E.R. Edelman, A. Nathan, M. Katada, J. Gates, M.J. Karnovsky, *Biomaterials* **21**(22), 2279 (2000)
- T. Joki, M. Machluf, A. Atala, J. Zhu, N.T. Seyfried, I.F. Dunn, T. Abe, R.S. Carroll, P.M. Black, *Nat Biotechnol* **19**(1), 35 (2001)
- D.H. Kempen, L. Lu, T.E. Hefferan, L.B. Creemers, A. Maran, K.L. Classic, W.J. Dhert, M.J. Yaszemski, *Biomaterials* **29**(22), 3245 (2008)
- F. Lim, A.M. Sun, *Science* **210**(4472), 908 (1980)
- M. Lisovsky, Z. Estrov, X. Zhang, U. Consoli, G. Sanchez-Williams, V. Snell, R. Munker, A. Goodacre, V. Savchenko, M. Andreeff, *Blood* **88**(10), 3987 (1996)
- M. Machluf, *Cell based delivery system for anti antiangiogenic therapy* (Harding, Intercept publisher, Biotechnology and Genetic Engineering, S. E, 2005a)
- Machluf, M. (2005). Protein therapeutic delivery using encapsulated cell platform. *Focus on Biotechnology*. V. Nedovi and R. Willart, Springer. **2**.
- M.A. Mazumder, F. Shen, N.A. Burke, M.A. Potter, H.D. Stover, *Biomacromolecules* **9**(9), 2292 (2008)
- J. Mullerad, S. Cohen, D. Benharroch, R.N. Apte, *Cancer Investig.* **21** (5), 720 (2003)
- A. Omer, M. Keegan, E. Czismadia, P. De Vos, N. Van Rooijen, S. Bonner-Weir, G.C. Weir, *Xenotranspl.* **10**(3), 240 (2003)
- G. Orive, S.K. Tam, J.L. Pedraz, J.P. Halle, *Biomaterials* **27**(20), 3691 (2006)
- A. Perets, Y. Baruch, F. Weisbuch, G. Shoshany, G. Neufeld, S. Cohen, *J. Biomed. Mater. Res. A* **65**(4), 489 (2003)
- K.D. Rainsford, *Int. J. Clin. Pract. Suppl.* **135**, 3 (2003)
- T.A. Read, V. Stensvaag, H. Vindenes, E. Ulvestad, R. Bjerkvig, F. Thorsen, *Int. J. Dev. Neurosci.* **17**(5-6), 653 (1999)
- M. Ricci, P. Blasi, S. Giovagnoli, C. Rossi, G. Macchiarulo, G. Luca, G. Basta, R. Calafiore, *J. Control. Release* **107**(3), 395 (2005)
- B.L. Schneider, F. Schwenter, W.F. Pralong, P. Aebischer, *Molec Ther* **7**(4), 506 (2003)
- J. Seagal, E. Edry, Z. Keren, N. Leider, O. Benny, M. Machluf, D. Melamed, *J. Exp. Med.* **198**(10), 1609 (2003)
- P. Soon-Shiong, R.E. Heintz, N. Merideth, Q.X. Yao, Z. Yao, T. Zheng, M. Murphy, M.K. Moloney, M. Schmehl, M. Harris et al., *Lancet* **343**(8903), 950 (1994)
- H. Tan, J. Wu, L. Lao, C. Gao, *Acta Biomater.* **5**(1), 328 (2009)
- H. Uludag, P. De Vos, P.A. Tresco, *Adv. Drug Deliv. Rev.* **42**, 29 (2000)
- T. Visted, T. Furmanek, P. Sakariassen, W.B. Foegler, K. Sim, H. Westphal, R. Bjerkvig, M. Lund-Johansen, *Hum. Gene Ther.* **14** (15), 1429 (2003)
- T. Visted, M. Lund-Johansen, *Expert Opin. Biol. Ther.* **3**(4), 551 (2003)
- H. Zimmermann, S.G. Shirley, U. Zimmermann, *Curr. Diab. Rep.* **7** (4), 314 (2007)