The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells. *In vitro* and *in vivo* direct comparative study.

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Abstract

The inclusion of the tripeptide RGD (Arg-Gly-Asp) in otherwise inert biomaterials employed for cell encapsulation has been observed to be an effective strategy to provide the immobilized cells with a more suitable microenvironment. However, some controversial results collected during the last years, especially *in vivo*, have questioned its effectiveness. Here, we have studied the behavior of C_2C_{12} myoblasts immobilized in alginate-poly-L-lysine-alginate (APA) microcapsules with different densities of RGD. The use of these microcapsules offer the advantage of avoiding native proteins influence permitting to establish direct comparisons between *in vitro* and *in vivo* assays. Results suggest that RGD-modified matrices provide higher dynamism, achieving therapeutically more active biosystems not only *in vitro*, but also *in vivo*. The highest functionality of the immobilized cells *in vitro* was obtained with the lowest RGD density. However, higher RGD densities were required *in vivo* to obtain the same effects observed *in vitro*. Altogether, these results suggest the lack of *in vitro-in vivo* correlation when cell behavior is evaluated within different RGD-tailored cell-loaded scaffolds.

Keywords: Microcapsule, RGD, scaffold, ligand density, microenvironment.

1. INTRODUCTION

During the last decade, the behavior of entrapped cells and the functionality of 3D biosystems have become a major focus of interest in the field of therapeutic cell encapsulation, leading to increasingly sophisticated scaffold designs that provide the encapsulated cells with a more suitable and natural microenvironment [1-3]. One of the most employed molecules for such aim is the tripeptide arginine-glicine-aspartate (RGD), the principal integrin-binding domain present in natural adhesion proteins of the extracellular matrix (ECM) such as fibronectin, vibronectin or fibrinogen. The inclusion of RGD in otherwise inert biomaterials promotes the adhesion and survival of encapsulated cells, leading to mechanically optimized cell-based scaffolds, which enhance the long-term functionality of the cell-based biosystems [4-7]. Furthermore, the use of this short amino acid sequence offers several advantages over the previous mentioned native ECM molecules including the low risk of immune reactivity, the tight control over ligand presentation or the straightforward synthesis [8-10].

However, despite the demonstrated potency of this peptide sequence as bioactive molecule, recent investigations have shed controversial results concerning the effect of this adhesion moiety, opening an extended debate about its use [11-14]. While *in vitro* studies have confirmed the effectiveness of RGD peptides in enhancing cell function through the regulation of integrin-mediated signaling pathway, *in vivo* studies have been shown to be more variable [15-17]. This fact makes the so far used *in vitro* methods unreliable reporters of *in vivo* activity and, thereby, highlights the need for more *in vivo* studies in order to bring this therapy towards clinical reality. In this sense, researchers in the field are currently discussing the diverse factors that may influence in this lack of consistency between *in vitro* and *in vivo* results, including the background produced by the serum proteins adsorbed in the matrix [18,19] or the synergistic effect mediated by

the different physicochemical cues coming from the surrounded microenvironment [20,21]. Besides, the use of different RGD ligand types, densities or presentation patterns, may be additional parameters that introduce variability and confound the interpretation of the obtained results [22-24]. In addition, the effect of all these variables is much probably cell type dependent [25,26]. In fact, although numerous studies have been carried out to gain insight into the repercussion of the tripeptide RGD, the drawn conclusions are diverse and unalike [27,28]. The discrepancies regarding the therapeutic benefits of RGD as optimal strategy to modify biomaterials still continue, increasing the need for collecting these parameters in a unique comparative study.

The inability of alginate to support cell interaction and attachment of mammalian cells, together with the low protein adsorptive capacity of its hydrogels, makes this polymer an ideal platform for this type of study [29,30]. Moreover, one of the most studied 3D alginate scaffolds, namely alginate-poly-l-lysine-alginate (APA) microcapsules, represents an especially attractive model, as the semipermeable PLL membrane avoids/prevents the possible diffusion of serum proteins from the surrounding microenvironment [31]. Thus, it is possible to remove the "background noise" and isolate the variables under study. In addition, this biosystem, due to its biocompatibility and biosafety, offers reliable translation from *in vitro* to *in vivo* studies, allowing facile and direct comparison between both [32-34].

In the present work, C_2C_{12} myoblasts genetically engineered to secrete EPO were encapsulated in APA microcapsules with different RGD densities to further analyze the number of viable cells per capsule, the proliferation and the secretion of therapeutic factor either *in vitro* or *in vivo*. To the best of our knowledge, this is the first report involving in a unique and comprehensive study some of the prime factors that may have influence in the effect of RGD on the encapsulated cells, providing

comparative data between results obtained *in vitro* and *in vivo*. This is intended to shed some light on the existing debate about this issue in the field.

2. MATERIALS AND METHODS

2.1 Cell culture

C3H-mouse C_2C_{12} myoblasts, genetically modified to deliver EPO, were kindly provided by the Institute des Neurosciences (Ecole Polytechnique Federale of Lausanne, Switzerland). The cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% foetal bovine serum (FBS), L-glutamine to a final concentration of 2 mM, 4.5 g/L glucose and 1% antibiotic/antimycotic solution. Cells were plated in T-flasks, maintained at 37 ºC in a 5% $C0_2$/95% air atmosphere and passaged every 2-3 days. All reagents were purchased from Gibco BRL (Life technologies, Spain).

2.2 Incorporation of adhesion molecules into alginate

Alginate was chemically modified by the aqueous carbodiimide chemistry. A water soluble carbodiimide, (1-ethyl-dimethylaminopropyl) carbodiimide (EDC), was used to form amide linkages between amine containing molecules and the carboxylate moieties on the alginate polymer backbone, with a reaction efficiency of approximately 80% [8]. The total number of RGD peptides per alginate chain, defined as the degree of substitution (DS) [10], was altered by varying the concentration of RGD peptides in the coupling reaction, obtaining four different types of alginate: DS 0 (No modified alginate), DS 1 (0.112 mM), DS 5 (0.5 mM) and DS 10 (1.12 mM).

2.3 Cell microencapsulation

C_2C_{12} myoblasts genetically modified to release EPO were incorporated into 3D alginate-poly-l-lysine-alginate (APA) microcapsules using an electrostatic droplet generator with brief modifications of the procedure designed by Lim and Sun [35]. Briefly, cells were harvested from monolayer cultures using trypsin- EDTA (Life technologies), filtered through a 40 μm pore mesh and suspended in four different solutions of 1.5% (w/v) sodium alginate (DS 0, DS 1, DS 5, DS 10) at $5x10^6$ cells/ml density. The resulted suspensions were extruded in a sterile syringe through a 0.35 mm needle at a 5.9 mL/h flow rate using a peristaltic pump. The resulting alginate particles were collected in a 55mM $CaCl_2$ solution and maintained under agitation for 15 min after the end of the process to ensure complete gelation of all the beads. Then, the obtained particles were suspended in 0.05% PLL solution for 5 min, washed twice with 10 mL of manitol 1% and coated again with another layer of 0.1% alginate for 5 min. All the process was carried out under aseptic conditions at room temperature, and resulting microcapsules were cultured in complete medium at 37 ºC in a 5 % $C0_2$/95% air atmosphere standard incubator. Ultra pure low-viscosity high guluronic acid alginate (UPLVG) was purchased from FMC Biopolymer, Norway, and poly-l-lysine (PLL hidrobromide Mw 15 000–30 000 Da) was obtained from Sigma Aldrich (St. Louis,MO, USA).

2.4 Cell Viability

Cells entrapped into APA microcpasules were dyed with the LIVE/DEAD kit (Life technologies) following manufacturer's indications. After 30 min, fluorescence micrographs were taken using an epi-fluorescence microscope (Nikon TSM).

2.5 Quantification of the total number of living cells per capsule

In order to determine the exact number of living cells quantitatively, enclosed cells were firstly de-encapsulated with 500 µg/mL of alginate lyase (SigmaeAldrich). LIVE/DEAD kit (Life technologies) was used to differentiate living and dead cells. After incubation of samples for 20 min at room temperature and protected from light, cells were counted by means of flow cytometry (BD FACSCalibur) using Trucount Tubes (BD).). All samples were assayed in triplicate for all groups, and obtained values are shown as mean of 3 independent samples ± S.D per study group.

2.6 Measurement of EPO secretion

Encapsulated C_2C_{12} Myoblasts supernatants were assayed for EPO secretion using the Quantikine IVD Human Erythropoietin ELISA Kit purchased from R&D Systems (Minneapolis, MN). Standards and samples were run in duplicate according to the procedure specified in the kit. The EPO secretion of the equivalent of 1.5×10^4 cells/mL was measured for a 24 h release period in triplicate per study group, and results are expressed as mean ± S.D.

2.7 Cell proliferation assay

The equivalent of 2×10^4 cells/100 µL (≈100 microcapsule/ well) was placed into each well of 96-well plate. All groups were incubated with complete medium supplemented with 10% FBS except the negative control group, which was incubated with starving medium supplemented with 0.1% FBS. After 24 h, the encapsulated cells were incubated in the presence of 10 µM BrdU for an additional day, except non-specific binding control group. The third day cells were de-encapsulated using 500 µg/mL of alginate lyase (Sigma-Aldrich) and assayed for BrdU uptake using Cell

Proliferation Biotrak ELISA System (Amersham, NJ, USA) following manufacturer's indications. Absorbance measurements of the non-specific binding control group (without BrdU) were subtracted from the rest of the groups, and results were normalized with the corresponding negative control (microcapsules incubated with 0.1 % FBS) for each experiment. Data are shown as mean of 5 independent samples ± S.D per study group.

2.8 Cell morphology. Determination of F actin

Microcapsules (100 µl of capsules) were fixed in 4 % paraformaldehyde, washed in pre-warmed DPBS and permeabilized by 0.1 % Triton X-100 for 10 minutes. The cytoskeleton of encapsulated cells was stained with Alexa Fluor 488 phalloidin, a volume of 15 µL methanolic stock solution in 200 µl DPBS, for 30 min in the dark (Life technologies) containing 1 % bovine serum albumin to reduce nonspecific background. The nucleus of the cells were dyed with Hoechst (1 µg/ml) and the samples were analyzed by inverted confocal microscopy (Olympus Fluoview 500 Confocal Microscopy).

2.9 Microcapsule implantation and retrieval to evaluate explanted microencapsulated cells

Animal studies were carried out according to the ethical guidelines established by our Institutions, under an approved animal protocol (241/2012). Adult female Balb/c mice (n=6 per group) were anesthetized by isoflurane inhalation, and implanted subcutaneously with a total volume of 300 µL of cell-loaded microcapsules ($5x10^6$ cells/mL) suspended in PBS using a 20-gauge catheter (Nipro; Nissho Corp, Belgium). Animals were housed in specific pathogen free facility under controlled temperature and humidity with a standardized 12 h light/dark cycle and had access to food and water ad

libitum. At day 15 and 30 after implantation, 3 animals from each group were sacrificed and capsules were explanted. Briefly, a mix of collagenase H (2mg/ml) (Roche Diagnostics, Germany) and hyaluronidase (1mg/ml) (Sigma, St. Louis, USA) was prepared using DMEM. This enzyme solution was filtered-sterilized prior to use. Using 50 mL tubes, 5-6 mL of disgregation solution was added to around 3-4 mL of a microcapsule aggregate. Once tubes were carefully sealed, they were incubated in a shaker bath at 37 ºC at 100 rpm for 4 h. Once the surrounding tissue was disaggregated, the solution in the tubes was filtered using 40 µm pore size filters to recover tissue-free capsules.

2.10 Statistical analysis

Data are presented as mean ± S.D. One-way ANOVA and post-hoc test were used in multiple comparisons. The Bonferroni or Tamhane post-hoc test was applied according to the result of the Levene test of homogeneity of variances. In the case of non-normally distributed data, Mann-Whitney non-parametric analysis was used. All statistical computations were performed using SPSS 20 (SPSS, Inc., Chicago, IL).

3. RESULTS

3.1 Cell viability in vitro

In order to carry out a thorough characterization of encapsulated myoblasts *in vitro*, we first evaluated the number of living cells/cap by using flow cytometry. In this viability assay, slight intergroup differences were observed with a low statistical significance after 30 days of encapsulation without a clear trend during all the study (Fig. 1A). Moreover, the cells entrapped in all type of microcapsules showed a lower viability on the last day than by day 15. Fluorescence micrographs, taken in parallel to

flow cytometry assays, provided further evidence on our observations showing a similar green fluorescence in all type of elaborated microcapsules (Fig. 1B).

3.2 EPO secretion and cell proliferation in vitro

EPO secretion and BrdU uptake were analyzed in order to evaluate the functionality, proliferative capacity and behavior of immobilized cells. Overall, the RGD-coupled alginate microcapsules maintained higher values of therapeutic factor secretion than microcapsules without RGD, independently of their substitution degree. However, DS 1 was the group with the highest level of therapeutic factor secretion after 15 and 30 days of encapsulation, showing normalized EPO secretion values of 50.9 % ± 4.9 and 19.2 % ± 4.2 respectively. The differences were more evident when this group was compared to DS 0 and DS 10 groups ($p<0.001$) than to DS 5 group ($p<0.05$) (Fig. 2A). As expected, the results of proliferation activity indicated the highest DNA synthesis for the DS 1 group at day 30, as revealed by the 1.7 ± 0.1-fold higher BrdU uptake. The differences were even more evident when this group was compared to DS 0 and DS 10 groups ($p<0.001$) than to DS 5 group ($p<0.05$) (Fig. 2B). These results come along with those obtained in the therapeutic factor secretion assay.

3.3 Cell morphology. Determination of F-actin

In order to obtain more detailed information regarding cell-ECM interaction, we next assessed the morphology of immobilized cells by confocal microscopy after staining the F-actin filaments with phalloidin Alexa Fluor 488. Photographs shown in Figure 3 demonstrate the presence of filopodia-like membrane extensions in the case of cells immobilized within RGD-coupled alginate matrices, being more prominent as RGD density increased. Contrariwise, microcapsules without RGD retained the typical round shape in enclosed cells with no detectable cytoplasm extensions.

3.4 Cell viability in vivo

In a second set of experiments, the effectiveness of RGD was assessed *in vivo* in order to study the influence of the physiological environment on the immobilized cells. Here, no statistical differences among the groups were obtained after 30 days of the study (Fig. 4A). Unlike *in vitro* study, the viability of immobilized cells *in vivo* increased over the course of 30 days. Fluorescence micrographs, taken in parallel to flow cytometry showed a similar green fluorescence in all type of microcapsules reflecting the quantitative data obtained in the previous assay (Fig. 4B).

3.5 EPO secretion and cell proliferation in vivo

In contrast to *in vitro* results, where the DS 1 group showed the highest secretion of the therapeutic factor, DS 5 group showed the highest EPO secretion *in vivo* by day 30, showing a normalized value of 35.9 % ± 5.8. This value resulted statistically significant when compared to DS 0 and DS 1 groups ($p<0.001$) (Fig. 5A). In accordance with the previous assay, the DS 5 group maintained the highest DNA synthesis, as revealed by 4.2 ± 0.5-fold higher BrdU uptake with respect to DS 0 group ($p<0.001$) (Fig. 5B).

3.6 Differences between in vitro and in vivo studies by day 30

For a better understanding/comprehension of the data obtained in this study, we elaborated a representative graphical analysis highlighting the differences between *in vitro* and *in vivo* assays (Fig. 6). For this purpose, the data obtained in each study with the DS 0 group was compared with the groups which contained different substitution degrees of RGD in order to observe the effect of different ligand densities on the behavior of entrapped cells compared to non-modified alginate. Although the viability assays pointed out no statistical significant influence of RGD matrices on the number of

living cells/cap either *in vitro* or *in vivo,* EPO secretion and proliferation profiles clearly showed that *in vitro,* DS 1 was the group which demonstrated the most prominent effect on encapsulated cell. *In vivo,* this effect resulted more noticeable in the DS 5 group.

4. DISCUSSION

In recent years increasingly sophisticated and tailored 3D bioscaffolds are being designed to compensate at least in part for the missing natural microenvironment of encapsulated cells. The RGD sequence, being the minimal adhesion ligand domain present in some ECM proteins, is one of the most commonly used molecule in this field due to its proved positive biological impact on the behavior of immobilized cells. However, some controversial results collected during the last years, especially *in vivo,* have questioned its effectiveness, opening an extended debate about its use. To address this, we immobilized erythropoietin (EPO)-releasing C_2C_{12} myoblasts within APA microcapsules in order to study the effect of different RGD densities both *in vitro* and *in vivo,* and with the aim of shedding some light on this topic of discussion.

As described in the literature, the adsorption of serum proteins − including integrin-binding native proteins such as fibronectin or vibronectin − into the biomaterials, may produce undesirable effects that lead to an increasing variability between *in vitro* and *in vivo* studies [36-39]. Importantly, the APA microcapsules employed in this study offer the advantage of avoiding such native proteins influence. This is given due to two main reasons: the low capacity of alginate gels to adsorb proteins; and the physical barrier provided by the semipermeable membrane of the microcapsules, which prevents the inward diffusion of serum proteins with molecular weights above the cut-off usually established in 70 KDa [29,31,40]. Thus, the employment of APA microcapsules in the present study permited the observation of RGD effects in an isolated way, removing the

background of native proteins that may mask, at least in part, the effectiveness of RGD to induce different cell responses.

It is well known that life and death decisions at the cellular level are profoundly influenced by the proteins of ECM [41,42]. Indeed, extensive studies have proved the capability of RGD moiety to promote vital cellular functions such as adhesion, migration, survival, proliferation, differentiation, morphogenesis and gene expression by means of integrin-mediated signaling pathways [43-45].

In this work, the *in vitro* assays showed that although the number of living cells/cap did not change too much with the inclusion of different RGD densities in alginate matrices (Fig. 1), the cells enclosed within RGD-coupled alginates had more capacity to proliferate and secrete therapeutic factors (Fig. 2A-2B). In the current study, unlike many other works in the field, the viability was not evaluated by methods based on metabolic activity, achieving more accurate data. Thus, we also had the opportunity to know better the exact number of living cells per capsule and to show the necessity to improve the obtained worrysome results in the future. Moreover, although the viability was similar in both days (Fig. 1) the EPO secretion by day 30 was too much lower (Fig. 2A). This could be explained by the fact that although in the cytometry assay the cells might dye with green fluorescence as a living cell, its metabolic activity could be reduced. Anyway, the obtained results lead to the hypothesis that the living myoblasts entrapped in the presence of RGD were more active than those enclosed within non-modified alginate scaffolds which gives rise to more dynamic and functional biosystems. Such dynamism would cause higher rates of cell proliferation and cell death, resulting in a continuous replacement and renewal of the cell content. This phenomenon would have a notorious impact in therapeutic cell encapsulation, as newly

formed “fresh” cells would contribute to the increase of therapeutic activity (in terms of either duration or quality), while preventing the biosystem from aging.

Although further studies are required to analyze the biology and mechanism of the cell-matrix interactions, the analyses of actin filaments *in vitro* suggested that immobilized cells were able to establish interaction sites with alginate in microcapsules containing RGD, whereas cells enclosed within alginate matrices without RGD remained round (Fig. 3). Even if these filopodia-like extensions, indicators of cell spreading, were more prominent as RGD density increased, the highest proliferation and EPO secretion were obtained with the lowest RGD density (DS 1 microcapsules). Our findings come along with other previously reported results in the literature which revealed that intermediate levels of the triamino sequence are optimal to obtain the maximum proliferation rate of myoblasts *in vitro* [46]. In fact, as observed in other studies, while an optimal cell spreading was obtained with high densities of RGD, the maximum proliferation required lower adhesion ligand presentation [47]. This phenomenon was explained by assuming that the strong adhesions resulting from many bound receptors may impede cell division, producing an inhibitory effect when the employed densities of RGD are too high [48]. However, this theory is still no clear, and as mentioned previously, the RGD moiety may promote other vital cellular functions such as differentiation of enclosed cells hindering the proliferation of the cells.

In the current study, a total of 30 days of follow-up were required to achieve notorious differences between microcapsules elaborated with different densities of RGD. This may explain some of the discrepancies described in previous studies in which the time intervals evaluated were lower [25,26]. Indeed, depending on the specific application or study, the multiple effects of RGD may be expressed at different times, according also to the scaffold model and the cell type used. The results obtained

in the present work are specific to C2C12 myoblasts, one of the most studied cell line in the field. It is known that the RGD density of the matrices and the microenvironment may affect in a different way depending on the cell type [26]. Thus, future efforts should be focused on finding the optimal density of RGD for each cell type.

We next moved on to *in vivo* assays in order to test the influence of a physiological microenvironment on cells enclosed within RGD-enriched matrices. When the microcapsules were retrieved from the animals, there was no evidence of inflammation process neither differences on the volume or adherence in all types of microcapsules elaborated with different RGD densities (data not shown). With the aim of isolating this variable, we repeated the same experimental procedure carried out *in vitro*, and the differences between both types of studies by day 30 were collected in the Figure 6. Higher RGD densities seem to be required *in vivo* to obtain the same effects observed *in vitro*. Graph curves for all assayed parameters revealed clearly that enclosed cells reflected almost the same behavior shown *in vitro* but displaced to higher densities of RGD. This also includes the inhibitory effect produced at the highest RGD density (DS 10). In the particular case of proliferation, it must be taken into account that the inclusion of RGD led to a higher proliferation activity in all types of microcapsules *in vivo*, compared to the lower values obtained *in vitro*. These differences may be attributed to the complexity provided by the *in vivo* body fluids to the microenvironment where microcapsules reside, which may also influence the final outcome of RGD on encapsulated cells. In fact, it is well known that several growth factors and hormones may alter the integrin expression of cells, and that their receptors cooperate with integrins in the regulation of adhesion-mediated signaling networks [20,21,49]. Therefore, special attention must be paid to the synergistic effect of the

molecules coming from surrounding microenvironment, as this latter may vary according to the implantation site.

Some important parameters for the design of biomimetic biomaterials such as optimal RGD density and the influence of the surrounded microenvironment are presented in this study. Although further investigation are needed to define the molecular and cellular basis of these observations, these types of screening studies provide meaningful information in order to explore the complexity entailed by cell-ECM interaction. Likewise, future studies should be focused on studying the efficacy of RGD taking into account other parameters such as cell type or implantation site in the animal.

CONCLUSION

This work adds further information to the existing debate about the therapeutic benefits resulting from the use of RGD. APA microcapsule design demonstrated to be a suitable model for the study of cell-RGD interaction due to its ability to exclude the influence derived from the adsorption of serum proteins. This also permitted to establish direct comparisons between *in vitro* and *in vivo* assays. RGD-modified matrices showed a higher dynamism to promote the renewal and replacement of the cell content and thereby achieve therapeutically more active biosystems. Finally, the present study showed clear differences between *in vitro* and *in vivo* assays, emphasizing the importance of the synergistic effect caused by the surrounding microenvironment and the difficulty to extrapolate *in vitro* results to *in vivo* reality.

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Figure captions

Figure 1 (A) *In vitro* percentage of living cells after 15 and 30 days of encapsulation. The number of living cells obtained for the day 0 was considered as 100% in each microcapsules group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). (B) *In vitro* Fluorescence micrographs taken by day 30. Scale bars = 100 µm.

Figure 2 (A) *In vitro* EPO secretion after 15 and 30 days of encapsulation. Therapeutic factor secretion levels obtained for the day 0 were considered as 100% in each microcapsule group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). (B) *In vitro* BrdU uptake after 0, 15 and 30 days of encapsulation. The results were normalized with those obtained with DS 0 group each day. Bar graphs symbolize the mean ± S.D (n=5). Statistical significance *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 3 Cytoskeleton organization of myoblasts and fibroblasts encapsulated in microcapsules elaborated with four different types of alginate *in vitro*. The cells inside APA microcapsules were stained with phalloidin Alexa Fluor 488 for F-actin (green) and Hoechst (blue) for nucleus. Scale bars = 20 µm.

Figure 4 (A) *In vivo* percentage of living cells after 15 and 30 days of encapsulation. The number of living cells obtained for the day 0 was considered as 100% in each microcapsules group, and all values were expressed in function of this percentage. (B)

In vivo Fluorescence micrographs taken by day 30. Bar graphs symbolize the mean ± S.D (n=3). Scale bars = 100 µm.

Figure 5 (A) *In vivo* EPO secretion after 15 and 30 days of encapsulation. Therapeutic factor secretion levels obtained for the day 0 were considered as 100% in each microcapsule group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). (B) BrdU uptake of myoblasts after 0, 15 and 30 days of encapsulation. The results were normalized with those obtained with DS 0 group each day. Bar graphs symbolize the mean ± S.D (n=5). Statistical significance $^{**}p < 0.01$ and $^{***}p < 0.001$.

Figure 6 Direct comparison between in vitro and in vivo studies showing the synergistic effect of the RGD density and the microenvironment on cell viability, EPO secretion and BrdU uptake. The data obtained in either in vitro or in vivo studies were normalized against their respective DS 0 control group in order to compare the behavior of encapsulated cells in these two microenvironments in function of RGD density (DS 1, DS 5 and DS 10). Bar graphs symbolize the mean ± S.D (Standard deviation is within the size of the symbols in the graph). Statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; DS 0 vs other groups (DS1, DS5 and DS 10).

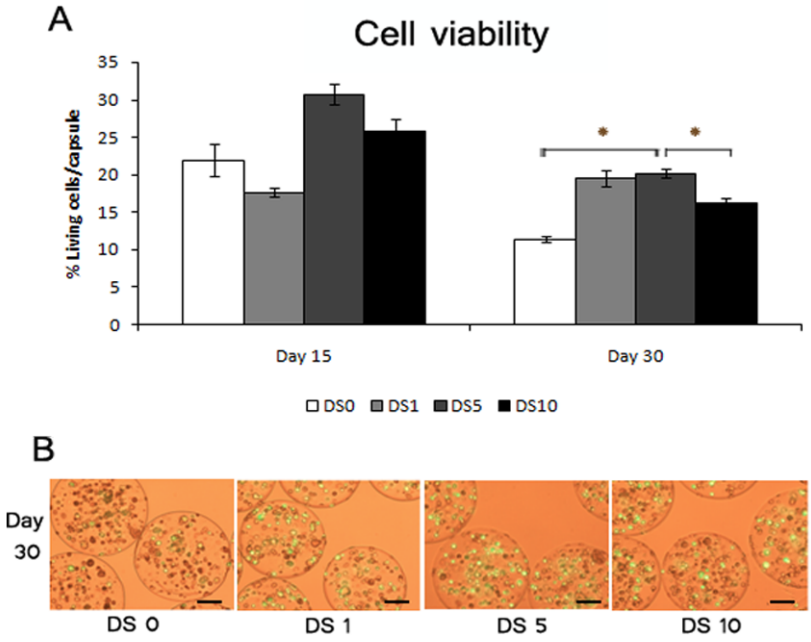

Figure 1.

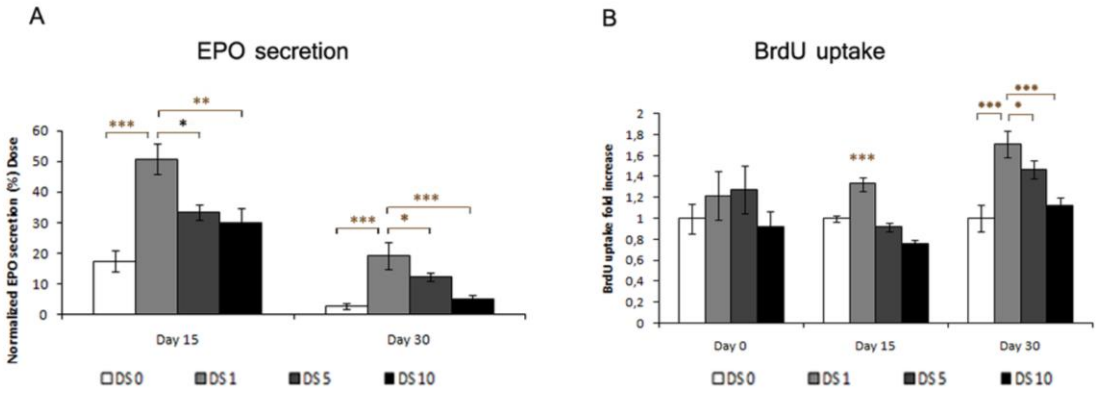

Figure 2.

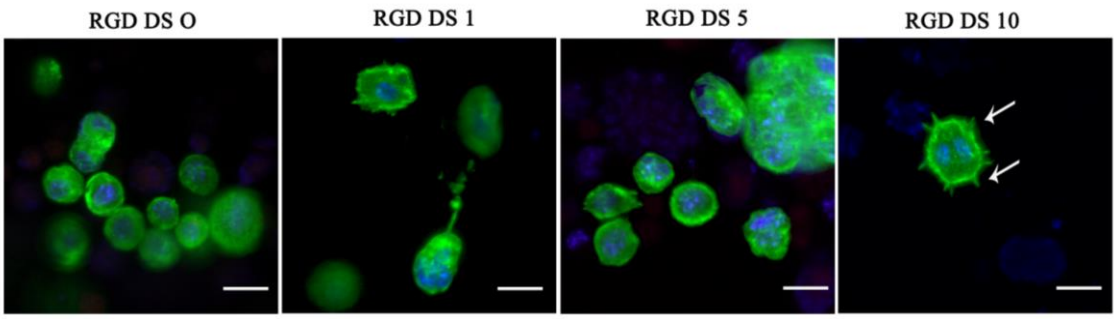

Figure 3.

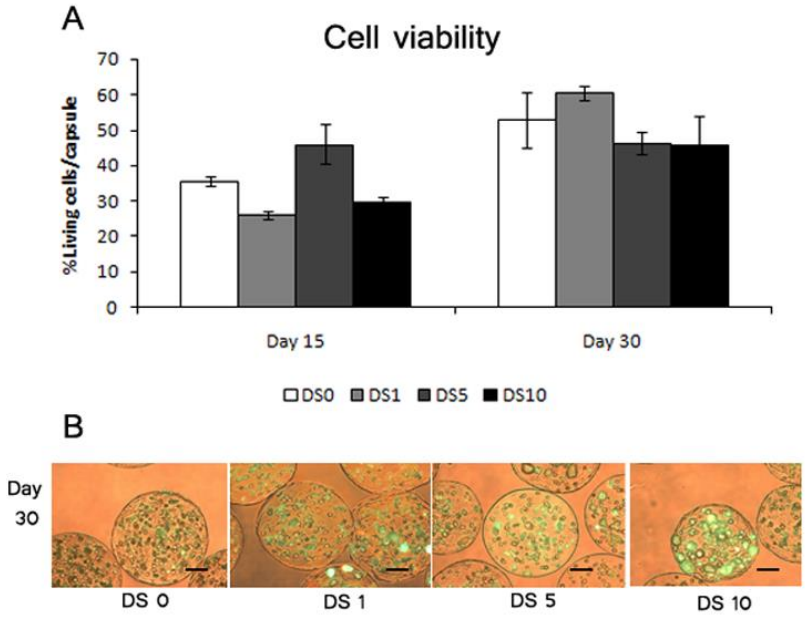

Figure 4.

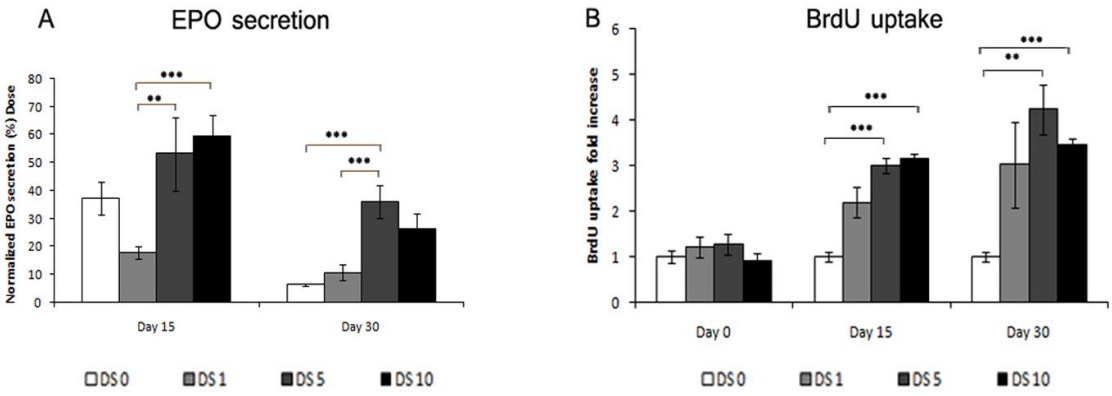

Figure 5.

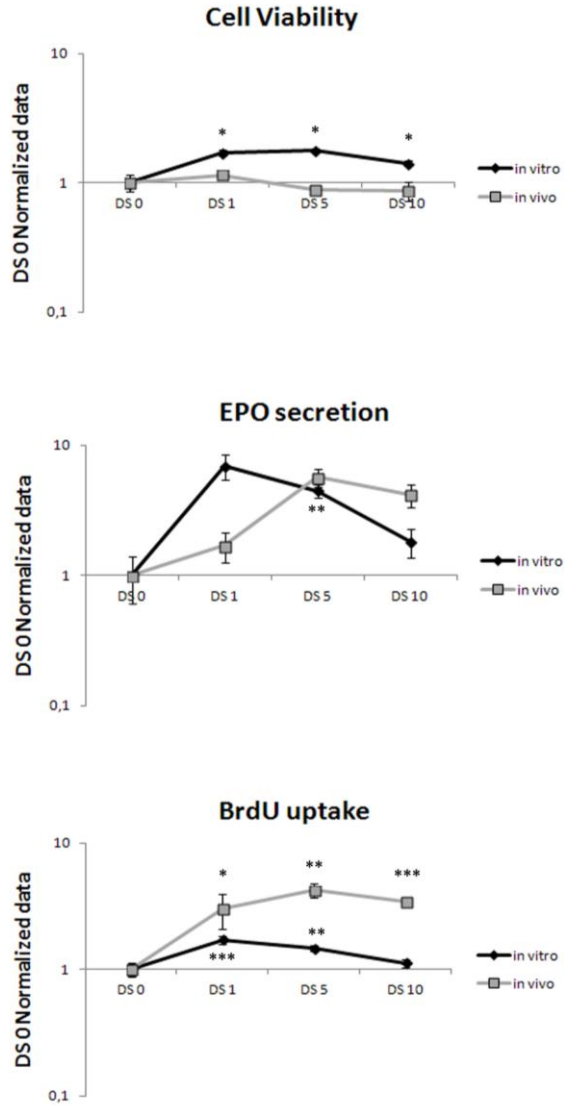

Figure 6.