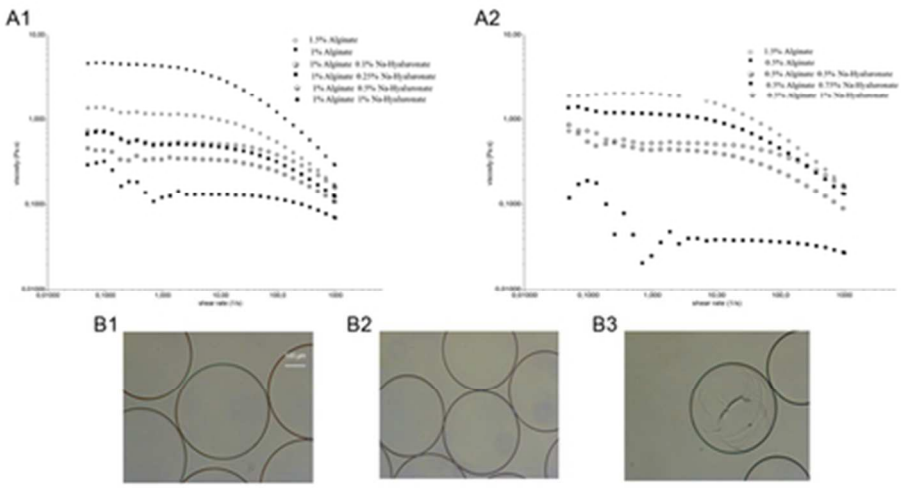

Figure 1.-(A) Rheological behavior comparison to 1.5% alginate of: (1)1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA and (2) 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, 0.5% alginate 0.5% HA and 0.5% alginate 1% HA. (B) Micrographs of microcapsules at the following compositions: (1) 1% alginate 0.1% HA, (2) 1% alginate 0.25% HA and (3) 0.5% alginate 0.5% HA. Note: Scale bar represents 100 μm.

38x20mm (300 x 300 DPI)

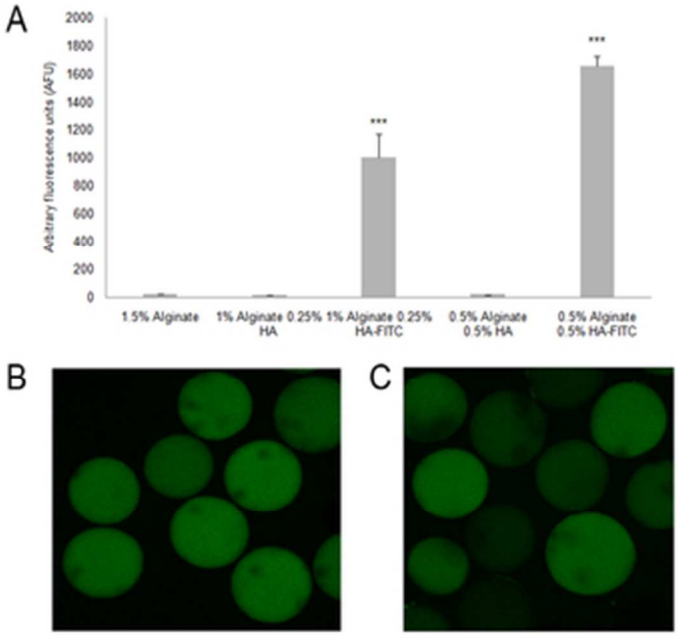

Figure 2.-Determination of HA content within microcapsules. (A) HA-FITC content quantification of 1.5 % alginate, 1% alginate 0.25% HA, 1% alginate 0.25% HA-FITC, 0.5% alginate 0.5% HA and 0.5% alginate 0.5% HA-FITC. Micrographs by means of confocal microscopy of (B) 1% alginate 0.25% HA-FITC microcapsules and (C) 0.5% alginate 0.5% HA-FITC microcapsules. Note: Values represent mean ± SD and *** represents $p < 0.001$.

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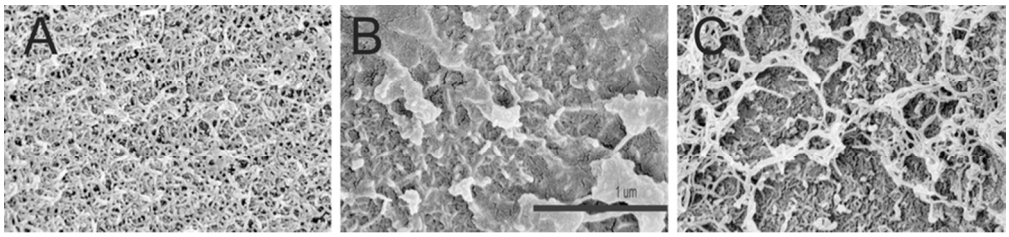

Figure 3.-Microcapsules surface micrographs by Scanning Electron Microscopy.(A) 1.5% alginate, (B) 1% alginate 0.25% HA and (C) 0.5% alginate 0.5% HA. Note: Scale bar represents 1 µm.

75x17mm (300 x 300 DPI)

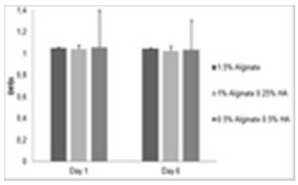

Figure 4.-Results from swelling assay of 1.5% alginate, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA microcapsules expressed as Df/Di: final diameter/initial diameter were Di corresponds to day 0 and Df is indicated in the abscises axe.

12x7mm (300 x 300 DPI)

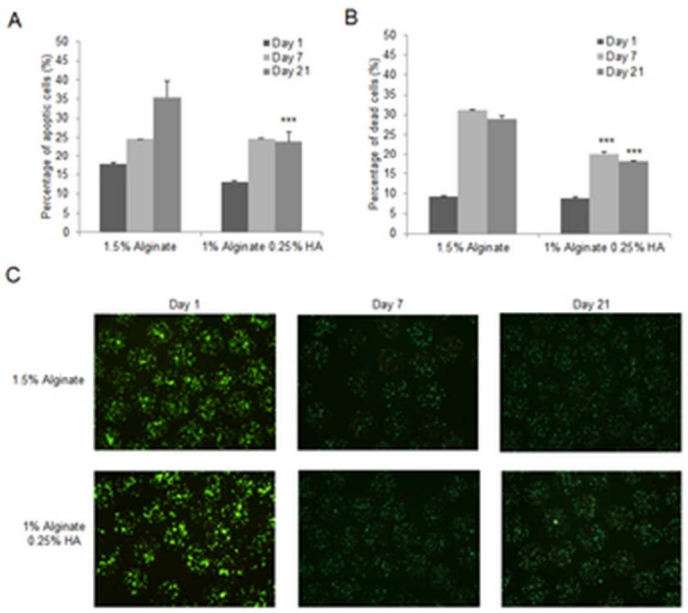

Figure 5.-Viability of D1-MSC-EPO encapsulated in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A)Early apoptotic cell quantification by means of flow cytometry after annexin/PI staining. (B) Dead cell quantification by means of flow cytometry aftercalcein/ethidium staining. (C) Micrographs of encapsulated cells after calcein/ethidium staining. Note: *: $p<0.05$ and ***: $p<0.001$. Scale bar represents 200 µm.

29x26mm (300 x 300 DPI)

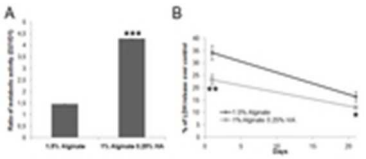

Figure 6.-Metabolic activity and membrane integrity of encapsulated D1-MSC EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A) Ratio of metabolic activity between day 21 (D21) and day 1 (D1) after encapsulation. (B) Membrane damage at day 1 and 21 after encapsulation. Note: Values represent mean ± SD. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

15x6mm (300 x 300 DPI)

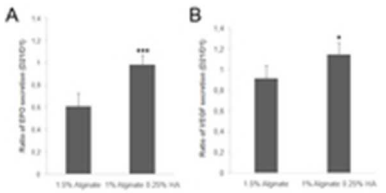

Figure 7.-Release of EPO and VEGF from encapsulated D1-MSC EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A) Ratio of EPO release between day 21 (D21) and day 1 (D1) after encapsulation. (B) Ratio of VEGF release between day 21 (D21) and day 1 (D1) after encapsulation. Note: Values represent mean ± SD. *: $p < 0.05$ and ***: $p < 0.001$.

15x7mm (300 x 300 DPI)

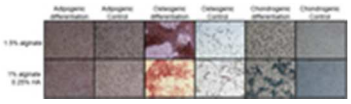

Figure 8. Differentiation potential of encapsulated D1-MSCs EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. Microscopic images at 4× amplification 3 weeks after differentiation.

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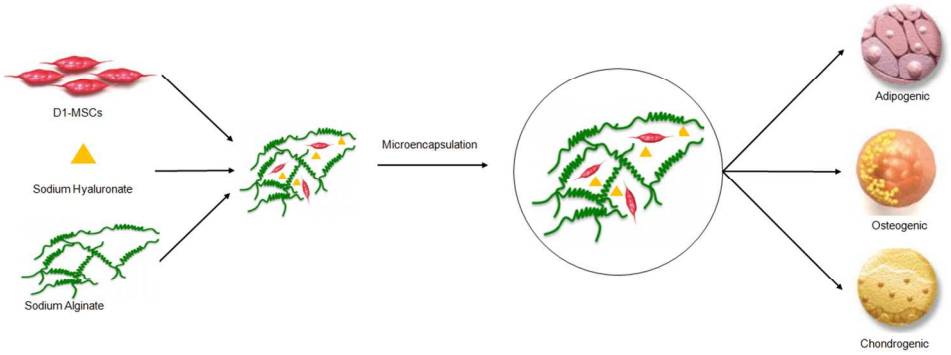

Abstract Graphic

112x44mm (300 x 300 DPI)

Alginate microcapsules incorporating hyaluronic acid recreate closer *in vivo* environment for mesenchymal stem cells

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Abstract

The potential clinical application of alginate cell microencapsulation has advanced enormously during the last decade. However, the 3D environment created by alginate beads does not mimic the natural extracellular matrix surrounding cells *in vivo,* responsible of cell survival and functionality. As one of the most frequent macromolecules present in the extracellular matrix is hyaluronic acid, we have formed hybrid beads with alginate and hyaluronic acid recreating a closer *in vivo* cell environment. Our results show that 1% alginate 0.25% hyaluronic acid microcapsules retain 1.5% alginate physicochemical properties. Moreover, mesenchymal stem cells encapsulated in these hybrid beads show enhanced viability therapeutic protein release and mesenchymal stem cells potential to differentiate into chondrogenic lineage. Although future studies with additional proteins need to be done in order to approach even more the extracellular matrix features, we have shown that hyaluronic acid protects alginate encapsulated mesenchymal stem cells by providing a niche-like environment and remaining them competent as a sustainable drug delivery system.

Keywords: alginate, microencapsulation, hyaluronic acid, mesenchymal stem cells

Abbreviations:

D1-MSCs-EPO: D1 mesenchymal stem cells

ECM: extracellular matrix

EPO: erythropoietin

HA: hyaluronic acid.

VEGF: vascular endothelial growth factor.

1.Introduction

Cell microencapsulation is a technology used for the sustainable controlled release of therapeutic proteins that has shown promising results in the future treatment of several diseases such as diabetes mellitus or Alzheimer´s disease. The outer layer of the microcapsules allows the flow of nutrients and oxygen into the core of the beads, while therapeutic proteins and waste are released from the cells outside. Moreover, the microcapsules represent a barrier to the recognition by the immune system, avoiding the entrance of immunoglobulins and, therefore, circumventing the immune rejection after encapsulated cell implantation [1, 2]. Although several biocompatible materials have been involved in cell microencapsulation such as agarose [3], chitosan [4] and hyaluronic acid [5], the most common is alginate because of its mechanical properties, and the isotonic solutions used instead of cytotoxic solvents [6]. Moreover, alginate microcapsules can be coated with polycations such as poly-L-Lysine (PLL), poly-D-lysine (PDL) and poly-L-ornithine (PLO) which provide higher resistance [7]. Among other applications, alginate microcapsules have been extensively studied in diabetes research by enclosing pancreatic islets with promising results. For example, diabetic patients lived without daily insulin injections for more than 9 months after intraperitoneal implantation of encapsulated pancreatic islets [8]. In addition, the incorporation of the chemokine CXCL12 into alginate encapsulated islets generated a long term (> 300 days) immune protective effect in allo- and xenogeneic transplantation, as well as a selective increase of intra-graft T_{reg} cells [9]. In Alzheimer´s disease, the implantation of microcapsules containing vascular endothelial growth factor (VEGF) secreting cells in double mutant amyloid precursor protein/presenilin mice, alleviated the symptoms for a period of three months by reducing the total brain amyloid-beta peptide load, and decreasing the apoptotic cell death in the cerebral cortex [10]. Moreover, nerve growth factor (NGF) secreting encapsulated cells showed no evidence of inflammation or device displacement after 12 months post-implantation [11]. Our research group has previously studied the microencapsulation of genetically engineered cells to secrete erythropoietin (EPO), showing that cell encapsulation allows the long term survival of cells and, therefore, an EPO

secretion up to 210 days, in allogeneic transplantation, and up to 98 days in xenogeneic transplantation [12-14].

Despite the promising results obtained with alginate in cell microencapsulation, this biomaterial does not provide the cell-matrix interactions required for cell adhesion and signaling, that could prolong even longer the cell survival and functionality [15, 16]. Cell matrix interactions are supported by extracellular matrix (ECM) components that supply mechanical and physiological support. Hence, different ECMs molecules have been tested in alginate encapsulated cells, such as laminin, collagen I or collagen IV, confirming an enhancement in encapsulated cell viability [17]. Other approaches trying to simulate the cell-matrix interactions provided by ECM are the short synthetic peptides derived from natural proteins that compose the ECM, for example the arginine-glycine-aspartic acid (RGD) peptide derived from fibronectin. This tripeptide offers advantages over the use of the whole protein like its simplicity, cost effectiveness, easy manipulation for functionalization and low immune response [18, 19]. Our group has shown its effectiveness enhancing the viability of several encapsulated cell types, such as myoblasts, fibroblasts or mesenchymal stem cells [20-22]. Moreover, it has been described that RGD in alginate microcapsules promotes the differentiation into bone cells when compared to unmodified alginate [23]. However, these short peptides do not accurately represent the ECM and do not provide the required signals for a complete reproduction of the cell environment *in vivo*. On this regard, another major component of the ECM distributed widely throughout connective, epithelial, and neural tissues is the hyaluronic acid (HA), which has been proposed for the preparation of biodegradable ECM-like constructs for tissue engineering applications [24].

Hyaluronic acid (HA) is a high molecular weight (MW) anionic non-sulphated glycosaminoglycan. It is integrated by the repetition of a disaccharide unit of an N-acetyl-glucosamine and a β-glucuronic acid, and interacts with cells via the surface receptor CD44. HA has been described for being involved in a wide variety of biological procedures like mediation of cell-signaling, regulation of cell adhesion and proliferation, and manipulation of cell

differentiation [25]. Hence, crosslinking of HA forms experimentally controllable hydrogels that provide a microstructure similar to native ECM [26]. Therefore, embedding cells within HA permits an appropriate structural support and protection, allowing cells to interact in 3D and enhance their viability [27]. Moreover, HA helps to reduce the immunogenicity of embedded cells because this biocompatible material reduce the adsorption of proteins [28], responsible of stimulating the recruitment of immune cells, such as macrophages. In terms of differentiation, HA promotes the differentiation towards murine chondrocytes when cultured either in 2D [29], or 3D, promoting the synthesis of cartilage tissue [30, 31]. Other authors have combined HA with other biomaterials. For example, the combination of HA with gelatin forms a biomimetic hybrid hydrogel after photocrosslinking gelation and mimics the ECM of native tissues, promoting the cell spreading of HUVEC cells, and improving their mechanical properties compared to their single component analogs [32]. Another example of hybrid HA microcapsules is the combination with heparin crosslinked by thiolated heparin and methacrylated hyaluronic acid via visible light mediated thiol-ene reaction. These hybrid microcapsules showed better spreading, proliferation, migration and differentiation of adipose derived mesenchymal stem cells than their respective single component analogs [33]. In summary, the combination of several biomaterials in microencapsulation technology seems to provide closer cell behavior to cells surrounded by ECM-like natural microenvironment than a single biomaterial. The optimal combination of the biomaterials that better mimic ECM still need to be determined.

In this work, we have identified the best combination of hyaluronic acid and alginate that forms hybrid microcapsules with similar physicochemical properties to alginate microcapsules, with the hypothesis that the presence of hyaluronic acid will mimic the natural ECM environment and, therefore, enhance the encapsulated cell viability and functionality. Hence, we have selected the formulation of the hybrid microcapsule based in the rheological behavior of an extensive number of combinations between hyaluronic acid and alginate, next studying more deeply the physicochemical characteristics of those combinations with similar rheological behavior to alginate. Finally, we have evaluated the beneficial effect of HA presence

in alginate encapsulated D1 mesenchymal stem cells genetically modified to secrete erythropoietin, in terms of viability and functionality.

2. Material and methods.

2.1. Materials

Ultra pure low-viscosity (20-200 mPa*s) and high guluronic (LVG) acid alginate (G/M ratio $\geq$ 1.5) with MW of 75-200 kDa was purchased from FMC Biopolymer (Norway). Poly-L-Lysine hydrobromide (PLL, 15-30 KDa) was purchased from Sigma-Aldrich (St Louis, MO). Hyaluronic acid and FITC labelled hyaluronic acid with a MW 1,1 MDa were purchased from Contipro (Czech Republic).

2. 2. Cell culture.

Murine D1 MSCs engineered to secrete erythropoietin (EPO) were grown with complete medium consisting of Dulbecco's modified Eagles's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin solution (Gibco) at 37 ºC in humidified 5% CO_2 atmosphere. Cells were passaged every 4–5 days.

2.3. Cell microencapsulation.

The following solutions suspended in 1% mannitol of sodium hyaluronate,1–1,25 MDa (Contipro) and alginate (FMC Biopolymer) were performed: 1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA, 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, 0.5% alginate 0.5% HA,0.5% alginate 1% HA. Final solutions were filtered with a 0.20 µm syringe filter (Millipore, MA, USA).All the solutions were extruded in an electrostatic atomization generator (Nisco®) and the resulting alginate beads were completely gelled by agitation for 15 min in a 55 mM $CaCl_2$. Next, beads were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a second coating with 0.1% alginate for another 5 min. All the procedure was performed at room temperature, under aseptic conditions and in complete medium.

For cell microencapsulation, cells were suspended in the solutions of sodium hyaluronate and alginate obtaining $5x10^6$ cells/mL of solution following the aforementioned procedure for beads formation. The morphology and diameter of the microcapsules were assessed under an inverted optical microscopy (Nikon TSM).

2.4. Rheological properties.

The viscosity from all the solutions (1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA, 0.5% alginate 0.5% HA, 0.5% alginate 0.75% HA, 0.5% alginate 1% HA)were assessed on the rheometer AR1000 (TA instruments) with 40 mm flat plate geometry and compared to the viscosity of 1.5% alginate. Viscosity behavior was determined in 1 drop of 500 µL onto the rheometer platform by dynamic shear measurements in a frequency sweep range from 0,01 to 100 Hz at 20 °C. The gap between the upper plate and sample was set up to 1000 µm. Three independent measurements were conducted for each solution.

2.5. HA content determination within hybrid microcapsules.

HA FITC labelled (Contipro, 1.1 MDa) and alginate were suspended in 1% mannitol at the following solutions: 1% alginate 0.25% hyaluronic acid and 0.5%alginate 0.5% hyaluronic acid. 1.5% alginate solution was also prepared as control. Microcapsules from the three solutions were performed as previously described, but protected from light. Next, microcapsules were imaged under a fluorescent microscope (Olympus FV500) and their fluorescence was quantified at 488 nm excitation and 520 nm with an Infinite M200 TECAN plate reader. Three independent experiments were performed for each solution.

2.6. Scanning Electron Microscopy (SEM).

The surface of the different microcapsules was analyzed by SEM (Scanning Electron Microscopy). Samples were fixed using 2% glutaraldehyde in 0.1 M Sörenson buffer (pH 7.4), washed in iso-osmolar Sörenson/sucrose buffer and postfixed with 1% osmium tetroxide in Sörenson buffer. Microcapsules were washed three times, dehydrated through ethanol series and

washed three times in hexamethyldisilazane prior to air drying. Finally, samples were coated with gold using an Emitech K550X sputter coater. Microcapsules surface was imaged using a scanning electron microscope (Hitachi S-4800).

2.7.Swelling properties.

The osmotic resistance of alginate-HA hybrid microcapsules (1% alginate 0.25% HA; 0.5%alginate 0.5% HA) was determined by the increase of diameter of microcapsules in a sequential treatment with 1% (w/v) sodium citrate for 6 days and compared to 1.5% alginate microcapsules. Briefly, 100 µL of microcapsules were mixed with 900 µL of PBS and placed into a 24-well plate. Plates were incubated for 1 h at 37 ºC while shaking at 500 rpm. Next, supernatants were removed from each well, 800 µL of citrate solution added and incubated for 24 hours at 37 ºC. Finally, the diameter of 20 microcapsules/sample were quantified under an inverted optical microscopy (Nikon TSM). Results were expressed as Df/Di, where Df (final diameter) is the diameter of the microcapsules after citrate treatment and Di (initial diameter) is the diameter before citrate treatment. Three independent experiments were performed for each sample.

2.8.Early apoptosis quantification.

Early apoptosis of encapsulated D1 MSCs was quantified by means of Annexin-V-FITC apoptosis Detection Kit (Sigma-Aldrich). Encapsulated cells in 1.5% alginate, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA were analyzed at days 1, 7 and 21 post-encapsulation. Briefly, 200 µL of microcapsules were incubated with 1mg/ml alginate lyase (Sigma Aldrich) for 30 minutes at 37 ºC. The lysate was rinsed twice with DPBS and resuspended in binding buffer consisting of 10 mM HEPES/NaOH, pH 7.5 containing 0.14 M NaCl and 2.5 Mm CaCl2. Samples were stained with annexin V-FITC and propidium iodide for exactly 10 min at room temperature and protected from light. Unstained samples or stained only with annexin V-FITC or propidium iodide were established as controls. Apoptotic cells were quantified with a BD

FACS Calibur flow cytometer. At least three independent experiments were performed for each solution.

2.9. Quantification and imaging of cell viability.

Cell viability was quantified by means of LIVE/DEAD viability/cytotoxicity kit (InvitrogenTM) after 1, 7 and 21 days of encapsulation. Cells were released from microcapsules by alginate lyase treatment as described above and after rinsing with DPBS, they were resuspended in culture medium with 100 nM calcein AM and 8 nM ethidium homodimer-1. Solutions were incubated for 20 min at room temperature, protected from light and dead cells were quantified with a BD FACS Calibur flow cytometer. Unstained samples or stained only with 100 nM calcein AM or 8 nM ethidium homodimer-1 were used as controls. At least three independent experiments were performed for each solution.

For microscopy imaging a volume of 25 µL of microcapsules were rinsed twice in DPBS and resuspended in 500 µL of 0.5 µM calcein AM and 0.5 µM ethidium homodimer-1 in DPBS. Next, solutions were placed in a 96-well plate and incubated at room temperature protected from light for 45 minutes. Samples were observed under a Nikon TMS confocal microscope at the wavelength of excitation 495 nm/emission 515 nm (for calcein AM staining) and excitation 495 nm/emission 635 nm (for ethidium homodimer staining). Random images were analyzed with the Eclipse Net software, version 1.20.0.

2.10.Study of membrane integrity.

Membrane integrity of encapsulated D1 MSCs was determined by the in vitro toxicology assay kit Lactic Dehydrogenase based (Sigma–Aldrich) at day 1 and 21 post-encapsulation following manufacturer recommendation. For the assay, 100 µL of microcapsules/sample were rinsed twice with culture medium, resuspended in 1 mL of complete medium and plated in two wells of a 24-well-plate. Wells were incubated for 90 minutes after adding 70 µL of lysis buffer to one well and 70 µL of culture medium to the other. Next, 50 µL of supernatant from each well was incubated with the kit cocktail mixture for 30 minutes, at room temperature and protected

from light. The absorbance was read out on an infinite M200 TECAN microplate reader at a wavelength of 490 nm, with absorbance reading at 690 nm as background. At least three independent experiments were analyzed for each condition.

2.11. Metabolic activity assay.

Metabolic activity was determined using Cell Counting Kit-8CCK-8 (Sigma–Aldrich) at day 1 and 21 after encapsulation following manufacturer recommendations. For CCK8 assay, 25 µL of microcapsules were rinsed, resuspended with 500 µL of culture medium and plated in 5 wells in a 96-well plate. After adding 10 µL of CCK-8 solution to each well, plates were incubated for 4 h at 37 °C inside a wet chamber. Absorbance was read out on an Infinite M200 TECAN plate reader at 450 nm with reference wavelength at 650 nm. Three independent test were analyzed for each condition.

2.12. EPO and VEGF quantification.

The secretion for 24 hours from culture supernatants at days 1 and 21 after encapsulation of EPO and VEGF was quantified. A volume of 100 µL of microcapsules were rinsed twice with culture medium, resupended in 1mL of medium and incubated for 24 hours at 37 ºC and 5% CO_2. Then, supernatants were collected. Next microcapsules were culture for 21 days, changing medium every 2 days, and rinsing twice 24 hours before collecting supernatant at the end of the culture. The EPO secretion from the supernatants was quantified by Quantikine IVD EPO ELISA kit (R&D Systems) while the secretion of VEGF was quantified by Human VEGF Standard ABTS ELISA Development Kit (Peprotech) following manufacturer recommendations. Three independent samples and controls for each condition were assayed. The results were expressed as D_{21}/D_1, where D_{21} (final value) is the amount of EPO or VEGF secretion by encapsulated cells at day 21 and D_1(initial value) at day 1.

2.13. Differentiation of mesenchymal stem cells.

Encapsulated D1-MSCs were differentiated into adipocytes, osteocytes and chondrocytes. Encapsulated cells were incubated for 5 days with MSCs culture medium,

changing medium every 3 days, at 37 ºC with 5 % CO2 atmosphere. After cells within microcapsules received alginate or alginate-HA stimulus for 5 days, de-encapsulated cells attached to the bottom of the plate were studied for differentiation. De-encapsulated D1-MSCs were incubated with complete mesenchymal stem cell medium as control in all the differentiations. For adipogenic differentiation, 200 µl of encapsulated D1-MSCs were incubated with adipogenic differentiation medium composed of DMEM-High glucose (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin solution (Gibco), 0.5 µM dexamenthasone (Sigma-Aldrich), 0.5 µM isobutylmethylxanthine (Sigma-Aldrich) and 50 µM indomethacin (Sigma-Aldrich). Encapsulated cells were incubated for 3 weeks, changing medium every 3 days, at 37 ºC with 5% CO_2 atmosphere. Cells were fixed with 10% formalin (Sigma-Aldrich) for 1 hour and stained with oil-red-C (Sigma-Aldrich) for 5 minutes at room temperature.

For osteogenic differentiation, 200 µl of encapsulated D1-MSCs were incubated with osteogenic differentiation medium composed of DMEM-High glucose (Gibco) and supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin solution (Gibco), 100 nM dexamenthasone (Sigma-Aldrich), 20 nm β-glycerophosphate (Sigma-Aldrich) and 0.5 µM L-ascorbic acid (Sigma-Aldrich). and 50 µM indomethacin (Sigma-Aldrich). Cells were incubated for 3 weeks, changing medium every 3 days. Attached cells were fixed with 10% formalin (Sigma-Aldrich) for 1 hour and stained with alizarin red-S (Sigma-Aldrich) for 5 minutes at room temperature.

Chondrogenic differentiation was tested incubating 200 µl of encapsulated D1-MSCs with chondrogenic differentiation medium composed of DMEM-High glucose (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin solution (Gibco), 10 ng/mL TGF-β1 (Sigma-Aldrich), 50 nM L-ascorbic acid (Sigma-Aldrich) and 6.25 µg/ml bovine insulin (Sigma-Aldrich). After changing medium every 3 days during 21 days, attached cells were fixed with 10% formalin (Sigma-Aldrich) for 1 hour and stained with alcian blue (Sigma-Aldrich) for 30 minutes at room temperature.

2.14. Statistics

Statistical analysis was performed with SPSS software, version 21.00.1. Data was expressed as means ± standard deviation and differences were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc Test when $p < 0.05$ after assessing their normal distribution.

3. Results.

3.1. Characterization of biomaterial.

First, we tried to search several hyaluronic acid/alginate composites that could show the same viscosity than 1.5% alginate, the standard concentration for cell encapsulation in our research group. On this regard, the rheological behavior of different hyaluronic acid and alginate mixtures at the following proportions were compared to 1.5% alginate: 1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA, 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, 0.5% alginate 0.5% HA and 0.5% alginate 1% HA. Thereby, 1% alginate 0.25% HA showed the most similar rheological behavior to 1.5% alginate and, therefore, similar viscosity (Fig 1.A). Moreover, close similarities to 1.5% alginate were detected with 1% alginate 0.1 %HA and 0.5% alginate 0.5% HA, while 1% alginate 0.5% HA, 1% alginate 1% HA, 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, and 0.5% alginate 1% HA displayed differences with 1.5% alginate (Fig1.A).

1% alginate 0.25% HA,1% alginate 0.1 %HA and 0.5% alginate 0.5% HA were selected as the compositions to be tested for encapsulation by means of an electrostatic atomization generator (Nisco ®). Spherical homogenous microcapsules were formed with all the compositions providing smooth surfaces similar to 1.5% alginate, with some wrinkled surfaces in microcapsules formed by 0.5% alginate 0.5% HA (Fig 1.B). The diameters shown at all the microcapsules independently of the composition was of 450 µm. We decided to discard 1% alginate 0.1% HA mixture for the following assays since it contains a low concentration of HA

and any effect detected with this mixture should be enhanced in the 1% alginate 0.25% HA composition.

With the selected compositions, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA, it resulted imperative to determine the content of HA within the microcapsules. So, we next proceeded to carefully analyze and quantify the content of HA inside them. On this regard, we elaborated microcapsules with FITC-labelled hyaluronic acid at the same molecular weight (1.1 MDa) than the aforementioned HA. FITC-labelled hyaluronic acid afforded us to quantify the HA content by measuring the fluorescence intensity and to observe the fluorescence under a confocal microscope. Thereby, we quantified double fluorescence intensity of 0.5% alginate 0.5% HA-FITC compared to 1% alginate 0.25% HA-FITC with no fluorescence intensity in their respective controls without FITC and in 1.5% alginate (Fig 2.A). The fluorescence of these composites was confirmed by means of confocal microscopy (Fig 2.B,C). No fluorescence was detected in their respective controls (data not shown). However, the higher intensity in 0.5% alginate 0.5% HA-FITC compared to 1% alginate 0.25% HA-FITC due to a higher presence of HA-FITC was not noticeable with this technology.

After determining the presence of HA within the microcapsules, we imaged the surface of the microcapsules by scanning electron microscopy (SEM) since smoother surfaces on the alginate microcapsules have shown better biocompatibility *in vivo*. Micrographs from SEM showed differences in the surface smoothness of the microcapsules indicating that the presence of HA induce to differences in the microcapsules surface (Fig 3), even when rheological behavior is similar. While, alginate microcapsules displayed a smooth and homogeneous surface, microcapsules containing HA displayed a wrinkled and heterogeneous surface, especially in 0.5% alginate 0.5% HA microcapsules. So, we decided to study if these differences could be reflected on the swelling behavior of the microcapsules. No significant differences were detected when these studies were performed 6 days along. After placing microcapsules into a monovalent ion solution like sodium citrate, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA showed similar expansion of the core diameter than 1.5% alginate

microcapsules (Fig 4). These results indicate that the presence of HA in the microcapsules does not affect the conversion of gel into liquid caused by a monovalent ion solution, independently of the HA concentration and, therefore, the rupture ratio *in vivo* of hybrid microcapsules will be similar to alginate microcapsules.

3.2. *In vitro* studies with encapsulated cells.

After observing that alginate HA hybrid microcapsules had similar rheological behavior and osmotic resistance than alginate microcapsules, we encouraged to assess the impact in the viability and bioactivity of encapsulated cells by the presence of HA within alginate microcapsules. We proceeded to encapsulate cells with the selected alginate-HA hybrid biomaterials. We chose to study D1-MSCs genetically modified to secrete EPO because, on the one hand, MSCs are being studied for clinical applications due to their immune tolerance properties and, on the other hand, their ability to secrete EPO allows their study as a sustainable drug delivery system. Thus, 1% alginate 0.25% HA microcapsules did not represent any issue in the encapsulation procedure of D1-MSCs-EPO while microcapsules composed by 0.5% alginate 0.5% HA agglomerated and did not display an appropriate core stability, releasing cells outside the capsule. Therefore, in the following assays we compared 1% alginate 0.25% HA to 1.5% alginate excluding the hybrid biomaterial composed by 0.5% alginate 0.5% HA from our futures studies.

First, we quantified the percentage of early apoptotic cells within the microcapsules for 21 days after cell encapsulation, by means of annexinV/propidium iodide staining and subsequent quantification by flow cytometry. Apoptotic cells percentage was lower in 1% alginate 0.25% HA than in 1.5% alginate the next day after encapsulation without statistical significance, showing the same percentage of apoptotic cells 7 days after encapsulation in both types of microcapsules (Fig 5A). However, a significant decrease of apoptotic cells ($p<0.05$) was detected in 1% alginate 0.25% HA encapsulated cells 21 days after encapsulation compared to 1.5% alginate (Fig5.A), indicating that the presence of HA within the alginate microcapsules influences apoptotic processes of encapsulated D1-MSC cells overtime. Next, we quantified if

the percentage of dead cells was also modified by the HA presence within the alginate microcapsules, by means of calcein/ethidium staining and subsequent flow cytometry. In correlation with apoptotic cell percentage, no significant differences were quantified in the percentage of dead cells the next day after encapsulation (Fig 5.B.). However, 7 days after encapsulation, the presence of HA in alginate microcapsules reduced significantly ($p<0.001$) the number of dead cells, even when apoptotic cell percentage did not show differences (Fig 5.B.). This result was also reflected at day 21 after encapsulation, when the presence of HA significantly ($p<0.001$) reduced the number of dead cells similarly to apoptotic cell percentage at this time point (Fig 5.B.). To verify these results, we stained encapsulated D1-MSC-EPO in 1% alginate 0.25% HA and 1.5% alginate with calcein/ethidium and observed them under fluorescent microscope. Micrographs obtained after staining confirmed the data displaying a higher number of alive cells in 1% alginate 0.25% HA capsules than in 1.5% alginate, especially at day 21 after encapsulation (Fig 5.C, green staining).

Because the main differences in viability were found at day 21 after encapsulation, we proceeded to quantify the metabolic activity at this time point. We quantified metabolic activity by the commercially available CCK8 assay studying the progression of cell metabolic activity from the first day after encapsulation until day 21. The increment in metabolic activity was 3-fold significantly higher ($p<0.001$) in D1-MSC EPO encapsulated in 1% alginate 0.25% HA capsules than in 1.5% alginate (Fig 6.A). We also analyzed and compared the membrane integrity of the encapsulated D1-MSC EPO in both types of microcapsules determining if there was a correlation with the viability and the metabolic activity detected. By means of the commercial assay Lactic Dehydrogenase based kit, we were able to detect that the percentage of membrane damage was always significantly lower ($p<0.01$ at day1 and $p<0.05$ at day 21) in D1-MSC EPO encapsulated in 1% alginate 0.25% HA capsules than in 1.5% alginate (Fig 6.B). These analyses confirmed that the presence of HA in alginate microcapsules not only improves the viability of the encapsulated D1-MSC EPO, but also improves their metabolic activity and the integrity of their membrane.

We also aimed to study the application of the hybrid biomaterial as a sustainable drug delivery system by taking advantage of the ability of D1-MSCs EPO to secrete EPO. Thus, we compared the progression of EPO release by encapsulated D1-MSCs EPO in 1.5% alginate microcapsules and 1% alginate 0.25% HA microcapsules from the first day after encapsulation until day 21. The hybrid 1% alginate 0.25% HA microcapsules provided 2-fold significant increment ($p < 0.001$) in EPO release than 1.5% alginate microcapsules (Fig 7.A.), indicating that the presence of HA, through its influence in the viability, metabolic activity and membrane integrity, helps to improve the release of a therapeutic protein secreted by encapsulated genetically modified cells, and therefore improves the capacity of alginate microcapsules as aa sustainable release system.

The presence of HA in alginate microcapsules could also be influencing the release of endogenous proteins secreted by MSCs. Hence, we compared the progression of VEGF release by encapsulated D1-MSCs EPO in 1.5% alginate microcapsules and 1% alginate 0.25% HA microcapsules from the first day after encapsulation until day 21. Hybrid 1% alginate 0.25% HA microcapsules provided higher VEGF release increment ($p < 0.05$) than 1.5% alginate microcapsules (Fig 7.B.). However, this increment was lower than the increment observed in EPO, indicating that the presence of HA in alginate microcapsules influences both the release of transgenic and endogenous proteins in encapsulated MSCs, but this influence is higher on transgenic than endogenous proteins.

Finally, we compared the potential of encapsulated D1-MSCs within 1.5% alginate and 1% alginate 0.25% HA into . three mesoderm lineages: adipogenic, osteogenic and chondrogenic. After culturing encapsulated D1-MSCs in complete mesenchymal stem cell medium for 7 days, attached cells were exposed to differentiation media for 21 days and next stained. The presence of vacuoles characteristic of adipogenic differentiation was detected with no qualitative differences between D1-MSCs from 1.5% alginate and 1% alginate 0.25% HA (Fig 8A). The calcified deposition identifying osteogenic differentiation was also detected in both type of microcapsules without qualitative differences (Fig 8B). However, higher amount of

sulfated proteoglycan deposits, indicative of functional chondrocytes, were displayed in 1% alginate 0.25% HA than in 1.5% alginate matrix (Fig 8C), suggesting that the incorporation of HA in alginate matrixes upgrades D1-MSCs potential for chondrogenic differentiation.

4. Discussion.

Cell encapsulation technology allows the continuous release of therapeutic factors avoiding the need of repeated drug administration. It has succeeded in the treatment of several pathologies showing high potential for its clinical application. However, before being translated from bench to bedside, several challenges still need to be overcome, such as the development of a matrix containing proteins from the ECM that mimics a closer natural cell environment and enhance cell survival and functionality. HA is one of the major components of the ECM [24], representing a good candidate in microencapsulation technology in order to enhance cell signaling, regulation of cell adhesion and proliferation, as well as manipulation of cell differentiation [25]. Therefore, we decided to study the incorporation of this macromolecule within alginate microcapsules since alginate has been the most commonly used biomaterial in cell encapsulation [6].

We selected HA with a MW of 1.1 MDa because at this MW, HA-coated cell cultures show the highest cell adhesion rate, decreasing the cell adhesion and proliferation when MW is increased [25]. Thus, we elaborated different solutions by mixing alginate and HA at different proportions to compared their rheological behavior with 1.5% alginate in order to find suitable mixtures that display similar viscosity properties of non-newtonian fluid like 1.5% alginate. We chose 1% alginate 0.25% HA and 0.5% alginate 0.5% HA due to, on the one hand, their similar viscosity behavior to 1.5% alginate along the frequency range studied and, on the other hand, their ability to form microcapsules by an electrostatic atomization generator. High HA concentrations increased viscosity over 1.5% alginate ratios when mixed with either 1% or 0.5% alginate, maybe due to a higher presence of hydrogen bonding between hydroxyl groups along the chains [34]. However, low HA concentrations were not able to increase solutions viscosity to 1.5% alginate values, precluding their use in cell encapsulation. In fact, when alginate

increased from 0.5% to 1%, it was enough to add HA at 0.25% instead of 0.5% to reach the same viscosity than 1.5%.alginate. We confirmed the presence of HA inside the selected microcapsules by imaging and by quantifying the emitted fluorescence from microcapsules after their performance with HA-FITC. The fluorescence of 0.5% alginate 0.5% HA was significantly higher than the one obtained from 1% alginate 0.25% HA but it did not exactly double, indicating that a release of HA could occur in 0.5% alginate 0.5% HA microcapsules. The breakage during the performance of 0.5% alginate 0.5% HA microcapsules could explain the release and, therefore, a lower HA-FITC intensity than expected.

We detected differences in the uniformity and the cross linking among the surface of both HA-alginate combinations and 1.5% alginate after forming the microcapsules. In spite of the observed differences, all samples showed a smooth surface, which have shown to provide better biocompatibility than rough surfaces [35]. We next confirmed that surface differences were not reflected in the swelling behavior, indicating that the osmotic resistance of all the microcapsules was enough to preserve them in an *in vivo* environment, avoiding a cell exposure to the host [36]. The similarities between the macromolecular structure of alginate and hyaluronate, allowing the formation of an alginate network where hyaluronic maybe accommodated, might explain the same swelling behavior among all microcapsules [37].

After our extensive physicochemical evaluation of the new hybrid microcapsules, we proceeded to encapsulate D1-MSCs EPO to determine the beneficial impact of the presence of HA within alginate microcapsules. The first bottleneck we met was the plugging up of the electrostatic atomization generator when trying to encapsulate D1-MSCs EPO in 0.5% alginate 0.5% HA, that when overcome, generated microcapsules that agglomerated and released cells outside the capsules, precluding future studies. However, 1% alginate 0.25% HA encapsulated MSCs displayed homogenous round microcapsules with smooth shape. The presence of HA in the aforementioned microcapsules reduced the percentage of apoptotic MSCs overtime compared to 1.5% alginate, which could be mediated by the endocytic internalization of HA through the surfaced receptor CD44 expressed in MSCs, and subsequent protection of DNA

from damage [38]. In fact, it has been proven that the pretreatment of chondrocytes with hyaluronic acid decreases mitochondrial DNA damage while enhancing DNA repair capacity, cell viability, preservation of ATP levels and amelioration of apoptosis [39]. This beneficial effect seems to be mediated by the CD44 receptor, since anti-CD44 antibody at saturating concentrations abolishes the protective effects of hyaluronan, suggesting that CD44 mediates this mechanism [40]. The reduction in apoptotic cells was also reflected in a reduction in cell death in the hybrid microcapsules, showing a higher number of alive cells. HA has shown to directly influence the activation of cell proliferation. High concentration of HA (> 2mg/ml) causes the release of endogenous growth factors, stimulating cell-cell interactions, that results in faster cell proliferation *in vitro* [41]. Thus, the growth rate of adipose derived MSCs shortly supplemented with HA is increased in culture at early passages, contributing to their lifespan extension, with a marked reduction of cellular senescence and a prolonged differentiation potential [42]. Moreover, the presence of HA in the hybrid microcapsules increased the metabolic activity of encapsulated MSCs, similarly to tendon derived cells exposed to HA [43], while reducing the membrane damage suffered by MSCs the next day after encapsulation [44]. All together afford us to conclude that HA protects encapsulated MSCs from the high stress derived from the encapsulation process.

We also aimed to evaluate the controlled released of a therapeutic protein from our hybrid microcapsules, thanks to the ability of the genetically modified MSCs to secrete EPO. Thus, we could quantify that MSCs increase EPO secretion for 21 days when allocated in microcapsules containing HA, similarly to hydrogels formed by 100% HA [45]. This increase of transgenic protein secretion was also correlated with an endogenous protein increment, reflecting that the secretion enhancement might be related with the boost of metabolic activity.

Finally we assessed the MSCs potential to differentiate into adipogenic, osteogenic and chondrogenic lineages shown in 2D [46]. It has been previously described the alginate encapsulated MSCs are able to differentiate into the above mentioned lineages [47] but we have demonstrated that the presence of HA into an alginate matrix promotes the differentiation of

MSCs into chondrocytes maybe due to an induction of aggrecan and proteoglycan accumulation, nodule formation, and inhibition of TNF-alpha induced inhibition of chondrogenic differentiation [29].

5. Conclusions.

Our work have shown that it is possible to produce hybrid microcapsules of 1% alginate 0.25% HA containing MSCs that retain 1.5% alginate physicochemical properties, while mimicking a natural ECM environment, which helps to enhance the viability and functionality of encapsulated cells. However, future studies need to be performed with the inclusion of other proteins derived from ECM in order to improve these beneficial effects, while maintaining the physicochemical properties of the microcapsules.

We can conclude that HA protects MSCs when encapsulated within alginate, providing a niche-like environment and improving the beneficial effects of alginate microcapsules after encapsulated MSCs implantation. Encapsulated MSCs into such bio-artificial niches are protected and remain competent in terms of cell delivery or sustained drug release systems.

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DECLARATION OF INTEREST STATEMENT

The authors report no conflicts of interest

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FIGURE LEGENDS

Figure 1.-(A) Rheological behavior comparison to 1.5% alginate of: **(1)**1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA and **(2)** 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, 0.5% alginate 0.5% HA and 0.5% alginate 1% HA. **(B)** Micrographs of microcapsules at the following compositions: **(1)** 1% alginate 0.1% HA, **(2)** 1% alginate 0.25% HA and **(3)** 0.5% alginate 0.5% HA. Note: Scale bar represents 100 µm.

Figure 2.-Determination of HA content within microcapsules. **(A)** HA-FITC content quantification of 1.5 % alginate, 1% alginate 0.25% HA, 1% alginate 0.25% HA-FITC, 0.5% alginate 0.5% HA and 0.5% alginate 0.5% HA-FITC. Micrographs by means of confocal microscopy of **(B)** 1% alginate 0.25% HA-FITC microcapsules and **(C)** 0.5% alginate 0.5% HA-FITC microcapsules. Note: Values represent mean ± SD and *** represents $p < 0.001$.

Figure 3.-Microcapsules surface micrographs by Scanning Electron Microscopy.**(A)** 1.5% alginate, **(B)** 1% alginate 0.25% HA and **(C)** 0.5% alginate 0.5% HA. Note: Scale bar represents 1 µm.

Figure 4.-Results from swelling assay of 1.5% alginate, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA microcapsules expressed as Df/Di: final diameter/initial diameter were Di corresponds to day 0 and Df is indicated in the abscises axe.

Figure 5.-Viability of D1-MSC-EPO encapsulated in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A)Early apoptotic cell quantification by means of flow cytometry after annexin/PI staining. (B) Dead cell quantification by means of flow cytometry aftercalcein/ethidium staining. (C) Micrographs of encapsulated cells after calcein/ethidium staining. Note: *: $p<0.05$ and ***: $p<0.001$. Scale bar represents 200 µm.

Figure 6.-Metabolic activity and membrane integrity of encapsulated D1-MSC EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A) Ratio of metabolic activity between day 21 (D21) and day 1 (D1) after encapsulation. (B) Membrane damage at day 1 and

21 after encapsulation. Note: Values represent mean ± SD. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

Figure 7.-Release of EPO and VEGF from encapsulated D1-MSC EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A) Ratio of EPO release between day 21 (D21) and day 1 (D1) after encapsulation. (B) Ratio of VEGF release between day 21 (D21) and day 1 (D1) after encapsulation. Note: Values represent mean ± SD. *: $p < 0.05$ and ***: $p < 0.001$.

Figure 8. Differentiation potential of encapsulated D1-MSCs EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. Microscopic images at 4× amplification 3 weeks after differentiation.