Dynamic microfactories co-encapsulating osteoblastic and adipose-derived stromal cells for the biofabrication of bone units

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Keywords: adipose-derived stromal cells, liquefied microcapsules, dynamic environment, co-culture, microparticles, bone regeneration

Abstract
Cells with differentiation potential into mesodermal types are the focus of emerging bone tissue engineering (TE) strategies as an alternative autologous source. When the source of cells is extremely limited or not readily accessible, such as in severe injuries, a tissue biopsy may not yield the required number of viable cells. In line, adipose-derived stromal cells (ASCs) quickly became attractive for bone TE, since they can be easily and repeatedly harvested using minimally invasive techniques with low morbidity. Inspired by the multiphenotypic cellular environment of bone, we propose the co-encapsulation of ASCs and osteoblasts (OBs) in self-regulated liquefied and multilayered microcapsules. We explore the unique architecture of such hybrid units to provide a dynamic environment using a simple culture in spinner flasks. Results show that microtissues were successfully obtained inside the proposed microcapsules with an appropriate diffusion of essential molecules for cell survival and signaling. Remarkably, microcapsules cultured in the absence of supplemental osteogenic differentiation factors presented osteopontin immunofluorescence, evidencing that the combined effect of the dynamic environment, and the paracrine signaling between ASCs and OBs may prompt the development of bone-like microtissues. Furthermore, microcapsules cultured under dynamic environment presented an enhanced mineralized matrix and a more organized extracellular matrix ultrastructure compared to static cultures used as control. Altogether, data in this study unveil an effective engineered bioencapsulation strategy for the in vitro production of bone-like microtissues in a more realistic and cost-effective manner. Accordingly, we intend to use the proposed system as hybrid devices implantable by minimally invasive procedures for bone TE applications.

1. Introduction

Since Tissue Engineering (TE) principles were defined [1], significant progress has been performed in orthopedic research. The combination of cells and biomaterial-based scaffolds, in order to construct living tissues, has been viewed as a potential alternative to the current gold standard treatment, namely the autogenous bone grafting. Whereas classical approaches have not been entirely effective in the restoration of functional bone tissue, a novel generation of devices should instruct nearby cells to promote endogenous repair mechanisms while allowing an orchestrated spatiotemporal delivery of single or multiple factors [2, 3]. Therefore, prior to the design of novel regenerative approaches, a fundamental understanding of the inter-cellular network of native bone tissue is of utmost importance [4]. Moreover, the combination of cells with multilineage differentiation potential with biomimetic devices significantly broads their application for TE strategies. Among the different tissue sources, adipose-derived stromal cells (ASCs) quickly became attractive for bone TE, owing to a number of appealing features, such as being available in large quantities with diminutive donor site morbidity or patient discomfort [5, 6]. However, controlling cell multipotency and engineering bone in vivo remains a significant challenge, as it often leads to heterotypic and mechanically inferior osseous tissues. Inspired by the multiphenotypic cellular environment.
of bone, we hypothesized that self-regulated liquefied and multilayered microcapsules [7-10], already tested in vivo [11], loaded with ASCs and osteoblasts (OBs) could be a promising attempt. For that, microcapsules generated by electrohydrodynamic atomization (EHDA) were composed by (i) a multilayered membrane obtained through the layer-by-layer assembly of three polyelectrolytes, namely poly(L-lysine), alginate, and chitosan, (ii) a liquefied alginate core, (iii) surface functionalized poly(ε-caprolactone) micro particles (µPCL), and (iv) cells. While the multilayered membrane wraps all the cargo contents and ensures permeability to essential molecules for cell survival, the liquefied core maximizes their diffusion through the entire 3D construct, and thus the oxygen limitation size of 150–200 μm of tissue engineered constructs is eliminated. Within this unique liquefied environment, cells can move freely and construct 3D cell-mediated systems by recruiting surface functionalized microparticles according to their needs. Moreover, taking advantage of the unique confined liquefied core microenvironment, the proposed microcapsules were tested using a spinner flask. The production method of liquefied and multilayered microcapsules is represented in scheme 1. In vivo, the paracrine signaling between osteoblastic cells are imperative to the development, maintenance and adaptation of the skeleton. The biophysical stimulation provided by in vitro dynamic cultures were already shown to upregulate osteogenic markers, and stimulate bone mineralization [12, 13]. Therefore, the dynamic environment is expected to maximize the interaction between the different multiphenotypic cells and microparticles, while also mimicking the dynamic environment of native tissues. In this study, microcapsules encapsulating only ASCs (MONO microcapsules) or a co-culture with OBs (CO microcapsules) were cultured up to 21 d in culture medium with (OSTEO) or without (BASAL) osteogenic differentiation factors. Our hypothesis is that by recreating the specific microenvironment of the bone regenerative process inside microcapsules, new microtissues with superior quality and without requiring any osteogenic medium supplementation could be engineered.

2. Methods

2.1. Cell culture
ASCs (ATCC® PCS-500-011™) were cultured in α-MEM (minimum essential medium, ThermoFisher Scientific), supplemented with 10% of heat-inactivated FBS (Fetal bovine serum, ThermoFisher Scientific), 100 U ml⁻¹ of penicillin and 0.1 mg ml⁻¹ of streptomycin, and immersed in an acetic acid solution [12, 13]. Therefore, the dynamic environment is expected to maximize the interaction between the different multiphenotypic cells and microparticles, while also mimicking the dynamic environment of native tissues. In this study, microcapsules encapsulating only ASCs (MONO microcapsules) or a co-culture with OBs (CO microcapsules) were cultured up to 21 d in culture medium with (OSTEO) or without (BASAL) osteogenic differentiation factors. Our hypothesis is that by recreating the specific microenvironment of the bone regenerative process inside microcapsules, new microtissues with superior quality and without requiring any osteogenic medium supplementation could be engineered.

2.2. PCL microparticles production and functionalization
Polycaprolactone micro particles (µPCL) were produced by emulsion solvent evaporation technique. Brie ﬂ y, a 5% w/v PCL (molecular weight (Mw) ~ 80,000, Merck) solution was prepared in methylene chloride (Honeywell). Then, the PCL solution was slowly added to a stirring 0.5% w/v polyvinyl alcohol (PVA, Merck) solution. After 2 d under agitation at room temperature (RT), the µPCL were collected, washed several times with distilled water, and sieved to obtain a diameter range of 40–50 μm. Afterwards, the surface of µPCL was modiﬁ ed by plasma treatment technique. µPCL were placed into a low pressure plasma reactor chamber (ATTO, Diener Electronic) in which air was used as gas atmosphere. A low-pressure glow discharge was generated at 30 V and 0.2–0.4 mbar for 15 min. Then, µPCL were immediately sterilized and immersed in an acetic acid solution (20 mM, Chem-Lab NV) containing collagen I (10 μg cm⁻², rat protein tail, ThermoFisher Scientiﬁc) for 4 h at RT.

2.3. Bioencapsulation setup
At 90% of conﬂ uence, ASCs (passage 6) and OBs (passage 6) were washed with phosphate buffered saline (PBS) solution, and detached using trypsin-EDTA solution (Merck) at 37 °C for 5 min. The two cell suspensions were centrifuged for 5 min at 300 g to discard the supernatant, and re-suspended (5 × 10⁶ cells ml⁻¹) in 2.5% w/v of low viscosity sodium alginate from brown algae (ALG, Merck) prepared in sodium chloride solution (0.15 M, NaCl, LabChem) with MES hydrate (25 mM, Alfa Aesar) containing surface-functionalized µPCL (30 mg ml⁻¹). Alginate microgels were then produced by EHDA technique (Spraybase, Avectas) using calcium chloride (0.10 M, CaCl₂, Merck) solution as the crosslinking bath for 10 min. The operating parameters for electrospray technique were 50 ml h⁻¹ of flow rate, 22 G needle, tip to collector distance of 8 cm, and 10 kV of voltage. Subsequently, layer-by-layer was performed using three different polyelectrolytes (0.3 mg ml⁻¹), namely poly(L-lysine) (PLL, Mw ~ 30 000–70 000, Merck), followed by ALG, and chitosan (CHT, NovaMatrix), to produce the multilayered membrane. The process was repeated until a 10-layered membrane was created. Ultimately, the liquefied core was obtained by chelation with EDTA solution (0.02 M, Merck) for 2 min at RT. The pH of all solutions was set to 6.7, excepting for CHT (pH 6.3). Two different formulations of liquefied and
multilayered microcapsules were obtained, namely a mono-culture of ASCs (MONO microcapsules), a n da a co-culture (1:1) of ASCs and OBs (CO microcapsules). Each obtained formulation was cultured in α-MEM medium, with (OSTEO) or without (BASAL) osteogenic supplementation. OSTEO medium was obtained by supplementing BASAL medium with ascorbic acid (50 μg ml⁻¹, Merck), β-glycerophosphate (10 mM, Merck) and dexamethasone (10 mM, ACROS Organics). Samples were cultured under static or a dynamic environment up to 21 d at 37 °C in a humidified 5% CO₂ air atmosphere. For static culture, plastic flasks were used. For dynamic culture, microcapsules were maintained in a spinner flask (Celstir, Wheaton) with double sidearms for good gas exchange, at 50 rpm. All solutions were sterilized by filtration using a 0.22 μm filter and the entire procedure was performed under sterile conditions.

2.4. Cell viability
The survival of the encapsulated cells was evaluated by live-dead fluorescence assay according to the manufacturer’s recommendation (ThermoFisher Scientific). Briefly, at 1, 7, 14, or 21 d post-encapsulation, samples were washed with PBS and then stained with the kit components at 37 °C for 20 min and protected from light. Afterwards, samples were visualized by fluorescence microscopy (Axio Imager 2, Zeiss).

2.5. Cytoskeleton F-actin staining
The F-actin network of the encapsulated cells in liquefied microcapsules was visualized after fluorescence phalloidin staining. At 1, 7, 14, or 21 d post-encapsulation, samples were washed with PBS and fixed in formalin (4% v/v) for 1 h at RT. Following 0.1% Triton X (Merck) permeabilization for 5 min at
RT, samples were incubated in Flash Phalloidin Red 594 (1:40 in PBS, Biolegend) for 30 min at 37 °C. DAPI counterstaining (1:1000, 1 mg ml⁻¹ in PBS, ThermoFisher Scientific) was performed for 5 min at 37 °C. Samples were visualized by fluorescence microscopy (Axio Imager 2, Zeiss).

2.6. Scanning electron microscopy and energy dispersive x-ray spectroscopy (SEM-EDS)

At 21 d post-encapsulation, the membrane of microcapsules was disrupted to expose the core content. Then, samples were fixed in 4% v/v formalin, and dehydrated in a graded series of ethanol. Afterwards, microcapsules were fixed with a carbon tape onto a graphite stub (Ted Pella) and sputtered by a thin film of carbon (K950X Turbo-Pumped Carbon Evaporator). Morphological and compositional analysis were carried out by scanning electron microscopy (accelerating voltage 15 kV, SEM Hitachi, SU-70 instrument) coupled with an energy dispersive x-ray detector (EDS Bruker, Quantax 400 detector). Calcium (Ca) and phosphorous (P) peaks were determined by EDS spectra using Esprit software. Ca/P ratio was calculated by deconvolution of Ca and P peaks after background subtraction. EDS spectra of lyophilized BASAL and OSTEO cell culture media were also performed as controls of the Ca/P values supplied to the microcapsules.

2.7. Attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR)

At 21 d post-encapsulation, samples were fixed in 4% v/v formalin, and dehydrated in a graded series of ethanol. ATR-FTIR spectra were measured on a Bruker Alpha infrared spectrometer controlled by the OPUS software package (version 7.0). Background and sample measurements were performed in a range between 4000 and 5000 cm⁻¹ with a resolution of 4 cm⁻¹ and averaging 256 scans, at RT and with controlled humidity. Amide I bands were deconvoluted with the PeakFit software using the second derivative procedure as elsewhere described [14, 15]. After a linear baseline correction, the curve fitting was performed with Gaussian functions using a nonlinear least squares fitting routine (coefficient of determination r² > 0.999).

2.8. Lipophilic fluorescent labeling

ASCs and OBs were stained with 3,3′-diodioctadecylacarbocyanine perchlorate (DiO, ThermoFisher Scientific) and 1,1′-diiodoacetyl-3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD, ThermoFisher Scientific) lipophilic dyes, respectively. Prior to encapsulation, cells were detached by trypsin-EDTA treatment for 5 min at 37 °C, and subsequently centrifuged at 300 g for 5 min. Afterwards, cells were counted and re-suspended in each dye solution diluted in PBS (1 ml of PBS containing 5 μl of dye per 1 × 10⁶ cells) for 10 min at 37 °C. After encapsulation, microcapsules were fixed in 4% v/v formalin and counterstained with DAPI (1:1000 in PBS, 1 mg ml⁻¹, ThermoFisher Scientific) for 5 min at 37 °C. Ultimately, microcapsules were visualized by fluorescence microscopy (Axio Imager 2, Zeiss).

2.9. Osteopontin immunofluorescence detection

The secretion of osteogenic marker osteopontin (OPN) was analyzed by immunofluorescence detection. After 21 d of culture, microcapsules were subsequently washed with PBS, fixed in 4% v/v formalin for 1 h at RT, and permeabilized with 0.1% Triton X for 5 min. Then, non-specific binding was blocked by immersion of the samples in FBS (5% v/v in PBS) for 1 h at RT. Afterwards, microcapsules were incubated overnight at 4 °C with the primary antibody mouse anti-human osteopontin (1:200 in 5% FBS, Biolegend). Upon PBS washing, samples were incubated with the secondary antibody anti-mouse Alexa Fluor 647 (1:500 in 5% FBS, ThermoFisher Scientific) for 1 h at RT. Samples incubated only with the secondary antibody were used as controls. Ultimately, samples were counterstained with DAPI (1:1000 in PBS, 1 mg ml⁻¹, ThermoFisher Scientific) for 5 min at 37 °C, and visualized by fluorescence microscopy (Axio Imager 2, Zeiss).

2.10. OPN and VEGF cytokine quantification

The amount of OPN and human vascular endothelial growth factor (VEGF) released by the encapsulated cells was assessed by ELISA quantification assay. For that, the supernatants (1 ml) of cell culture media at 21 d of culture of the liquefied microcapsules were stored at −80 °C until analysis. Commercially available human OPN and VEGF ELISA development kits (Abcam) were performed according to the manufacturer’s specifications. The measurements were read at 450 nm in a microplate reader (Gen5, Synergy HT, Biotek).

2.11. Von Kossa and Masson’s Trichrome histological staining

At 21 d of culture, the presence of phosphate deposits and collagen was assessed by Von Kossa and Masson’s Trichrome staining, respectively. For that, microcapsules previously fixed in 4% of buffered formalin, were processed in an automatic tissue processor (STP120, Microm) and then embedded in paraffin. Histological section (5 μm) were obtained using a microtome (HM355E, Microm, ThermoFisher Scientific) and placed in adhesive slides (SuperFrost, ThermoFisher Scientific) for von Kossa and Masson’s Trichrome staining.

2.12. Hydroxyapatite fluorescence staining

At 21 d of culture, the presence of hydroxyapatite (HA) crystals was assessed using the OsteoImage™ Mineralisation Assay kit (Lonza) according to the manufacturer’s instructions. Samples were counterstained with DAPI (1:1000 in PBS, 1 mg ml⁻¹, ThermoFisher Scientific™) and visualized by fluorescence microscopy (Axio Imager 2, Zeiss).
2.13. Diameter measurements
The size of \( \mu \text{PCL} \), microcapsules, and \( \mu \text{PCL} \)-cell aggregates was measured by ImageJ image analysis software. Each value presented for \( \mu \text{PCL} \)-cell aggregates correspond to the mean of length and width, for a total of 10 samples for each condition in the different timepoints. For \( \mu \text{PCL} \) and microcapsules, exactly 100 and 50 diameter measurements were performed, respectively.

2.14. Statistical analysis
Statistical analysis was performed using two-way analysis of variance (ANOVA) with Tukey’s post-hoc test (GraphPad Prism 6.0). \( p \)-values < 0.05 were considered statistically significant (**\( p < 0.001 \); \( p < 0.01 \); \( p < 0.05 \)). All results are presented as mean ± standard deviation.

3. Results

3.1. Morphological analysis of microcapsules
The successful production by EHDA technique of spherical microcapsules encapsulating cells and \( \mu \text{PCL} \) was evaluated by optical microscopy (figure 1(A)). Microcapsules and \( \mu \text{PCL} \) presented diameters of 608.5 ± 122.3 \( \mu \text{m} \) and 45.7 ± 7.3 \( \mu \text{m} \), respectively. Regardless the distribution of microcapsules size (figure 1(A)), the microparticles:cells ratio is maintained since microparticles and cells are encapsulated at a ratio of 30 mg and 5 \( \times \) 10^6 per ml of alginate, respectively. The random distribution of the cells within the microcapsules core was confirmed by microscopic analysis. Prior to the encapsulation procedure, ASCs and OBs were fluorescently marked with lipophilic dyes. Both green and red fluorescence identifying respectively ASCs and OBs could be visualized after 1 and 7 d of culture (figure 1(B)). Live-dead assay shows that, up to 21 d post-encapsulation, the majority of encapsulated cells remained viable in all formulations (figure 1(C)), evidencing the mild conditions of the cell encapsulation technique proposed. Importantly, these results also evidence the ability of microcapsules for long-term cell survival, a major challenge for tissue engineering strategies aiming cell encapsulation.

3.2. In vitro evaluation of the microtissues
The fluorescence staining of F-actin filaments (figure 2(A)) evidences the interaction and structural organization of the encapsulated cells with the \( \mu \text{PCL} \) inside the compartmentalized and controlled environment of microcapsules, after one and seven days of culture. Interestingly, it is possible to observe that the dynamic environment led to the development of significantly larger aggregates of cells and \( \mu \text{PCL} \) comparing with static conditions (figure 2(B)), which evidences the maximized interaction between cells and \( \mu \text{PCL} \) inside of the microcapsules. These results were mainly visualized in the first days of culture, since, over time, the developed microtissues tend to achieve similar sizes.

3.2.1. Osteogenic potential evaluation
The late osteogenic marker OPN was visualized by immunofluorescence staining in MONO and CO microcapsules cultured in BASAL and OSTEO media under static and dynamic conditions at 21 d post-encapsulation. OPN is a calcium-binding glycoprophoprotein involved in bone remodeling and its expression is linked with extracellular osseous matrix mineralization [16]. Remarkably, the OPN marker was expressed in microcapsules cultured in BASAL medium under dynamic environment, evidencing that the combined effect of the shear stress and the cells coculture may prompt the development of bone-like microtissues (figure 3(A)). The profile of OPN and VEGF released by MONO and CO microcapsules cultured in the different conditions is shown in figure 3(B). The release of OPN was in accordance with the immunofluorescence staining. Despite no statistically relevance between static and dynamic conditions was observed, the release of OPN was higher in the microcapsules cultured under mechanical stimuli. Likewise, the release of OPN was higher for CO microcapsules, with statistical difference for microcapsules cultured in BASAL medium and under static environment. Additionally, VEGF is a vascular growth factor involved in the development of new blood vessels and, therefore, critical for bone regeneration [17]. In MONO microcapsules, the release of VEGF was significantly higher in microcapsules cultured in dynamic environment. Furthermore, for static conditions, the secretion of this growth factor was significantly enhanced in CO microcapsules over MONO microcapsules. The highest amount of released VEGF was observed for CO microcapsules, with similar values for all conditions.

3.2.2. Extracellular matrix (ECM) deposition evaluation
With increasing culture time, the protrusion of the core contents occurs, thus microaggregates composed by cells and PCL microparticles are released to the external environment. Consequently, such microaggregates from different microcapsules start to merge, creating macroaggregates, as schematically represented in figure 4(A). F-actin staining reveals the elongation of the cells while agglomerating various PCL microparticles. Masson’s Trichrome evidences the presence of collagen in the ECM of the macroaggregates. The structural analysis of the collagen-rich ECM was carried by FTIR-ATR through the deconvolution of the amide I band in the 1700–1600 cm\(^{-1}\) range aiming to assess qualitatively and quantitatively the different secondary structure contributions based...
on the band areas (Table S1, supporting information). Collagen typically presents five types of secondary structures, namely triple helix (∼1635 cm$^{-1}$), unordered structures (∼1645 cm$^{-1}$), α-helix (∼1655 cm$^{-1}$), β-turns (∼1670 cm$^{-1}$), and various absorption bands related to β-sheet conformers [18–22]. Bands around 1625 cm$^{-1}$ and 1680 cm$^{-1}$ are attributed to intramolecular β-sheet structures, while intermolecular β-sheets present a main band around 1615 cm$^{-1}$ with a minor contribution at 1690 cm$^{-1}$ [22]. At first glance, deconvoluted ATR-FTIR spectra of MONO microcapsules exhibit these typical features in the amide I region, which only seem to vary in intensity, and thus, confirming the presence of collagen. However, MONO microcapsules cultured in BASAL medium and static conditions (figure 4(B)) present an additional band at lower wavelength (ca. 19%) related to free amino acid residues [23]. This contribution is vanished under dynamic conditions and without any supplemental osteogenic differentiation factors (figure 4(D)), while α-helix (ca. 21%) and triple helix (ca. 24%) configurations increase compared to its static counterpart (15% and 14% for α-helix and triple helix, respectively). The influence of the dynamic stimulus on the structural organization of the collagen matrix was also clearly noticed in MONO microcapsules cultured in OSTEOMedium (figure 4(E)), with a decrease of disordered structure proteins (from 19% to 9%) and the prominence of triple and α-helices (overall 52%), which are typically the major secondary structures of collagen [22].

Interestingly, the amide I band of the different CO microcapsule formulations are significantly shifted to lower wavelengths (figures 4(F)–(I)). This shifting is indicative of stronger intermolecular hydrogen bonds attributable to protein aggregates and it is visualized by the additional contribution (blue shaded curve) that emerges at ∼1600 cm$^{-1}$ [24, 25]. The band intensity increases significantly under osteogenic differentiation factor supplementation (24%–27%) as compared to basal counterparts (6%–9%). Furthermore, these spectral features were confirmed by SEM analysis, showing elongated filaments in the nanometer scale for MONO microcapsules (figures 4(B)–(E)), characteristics
of collagen fibrils, while for CO microcapsules (figures 4(F)–(I)) the filaments are in a micrometer scale typical of protein aggregation into collagen fibers [26].

3.2.3. Mineralization evaluation
After analysis of the extracellular matrix of the μPCL-cell aggregates, we were looking for the presence of mineralization. However, in all MONO microcapsules
no evidence could be found, even in MONO microcapsules cultured in OSTEO medium under dynamic environment (figure S1, supporting information is available online at stacks.iop.org/BF/12/015005/mmedia) despite its osteogenic differentiation ability and the presence of an organized extracellular matrix ultrastructure. On the other hand, mineralization evidences could be found in CO microcapsules. The chemical characterization of the microtissues formed inside CO microcapsules was analyzed by energy dispersive spectroscopy (EDS) after 21 d of culture. For microtissues developed in BASAL microcapsules, results show that phosphorous (P) and calcium (Ca) contents were higher in dynamic cultures (figure 5(A)). Likewise, the same results were shown for microtissues developed in OSTEO medium (figure 5(B)). Interestingly, nodules-like structures were observed by SEM (figures 5(A1) and (B1)), and further analyzed by EDS mapping, which evidenced a P and Ca enriched ECM (figures 5(A2) and (B2)). However, by the analysis of Ca/P ratio differences could be noticed (figure 5(C)). In BASAL microcapsules (figure 5(C) black line), the average of Ca/P content ratio was 4.92 and 2.65 for static and dynamic conditions, respectively. For CO microcapsules in OSTEO medium (figure 5(C) grey line), the average of Ca/P content ratio decreased to 3.97 (static) and 1.50 (dynamic). These results suggest that CO microcapsules in OSTEO medium and under dynamic environment allowed to develop hydroxyapatite-like minerals (HA), with a Ca/P ratio similar to the native HA (1.67) [27]. Furthermore, the elemental analysis by chemical mapping shows the distribution of P (red) and Ca (green) in microcapsules cultured under mechanical stimulation. The overlap of both elements, mainly observed in OSTEO medium, is represented in yellow (figures 5(A2) and (B2)). Additionally, in CO microcapsules it is possible to visualize HA nodules stained in green (figure 5(D)). Of note, higher amount of HA nodules could be found in formulations cultured in dynamic environment or in OSTEO medium. In accordance, von Kossa staining shows an enhanced mineralization in microcapsules cultured under dynamic environment by the staining of the phosphate deposits marked in black (figure 5(E)).

4. Discussion

We have previously developed a cell encapsulation system under static conditions that solved issues related with oxygen and nutrients diffusion as well as ensured physical support for anchorage-dependent cells [7–11]. Additionally, in vitro and in vivo studies
demonstrated the osteogenic potential of the developed capsules co-encapsulating ASCs and endothelial cells, even in the absence of dexamethasone and ascorbic acid, two major osteogenic differentiation factors. However, such capsules presented an average diameter of 1.8 mm and microbiomaterials are currently shown to regulate cell microenvironment in spatial and temporal aspects, crucial for regenerative medicine purposes [28]. So, in order to overcome the requirements concerning the macro size of the liquefied and multilayered capsules, electrohydrodynamic atomization (EHDA) technique was performed. As shown, microcapsules encapsulating ASCs, OBs and μPCL were successfully obtained, presenting a privileged microenvironment for the development of microtissues. In all conditions, cells adhered to the surface of the μPCL, proliferated, and deposited extracellular matrix in such a way that μPCL were assembled in 3D micro-constructs. The multilayered membrane allowed an appropriate diffusion of the osteogenic factors, facilitating the development of microtissues. Figure 4. (A) Schematic representation of the aggregation of microtissues derived from different microcapsules. Over time the microtissues from different microcapsules begin to aggregate, creating macroaggregates of cells and polycaprolactone microparticles (μPCL). Fluorescence images correspond to phalloidin staining (F-actin, red) counterstained with DAPI (cell nuclei, blue). Scale bars correspond to 100 μm. Masson’s trichrome staining of a representative histological section from MONO microcapsules. Collagen is stained in blue. Scale bar corresponds to 50 μm. (B–I) Bone matrix analysis of MONO and CO microcapsules after 21 d of culture in BASAL and OSTEO medium and under static and dynamic environments. Representative SEM images of the encapsulated cells and μPCL. Scale bars correspond to 25 μm. Gaussian deconvolution of the different contributions of the secondary structure of collagen for MONO and CO microcapsules obtained by curve fitting the corresponding IR spectra in the Amide I region. The fitted curve is shown as a solid black line, closely overlapping the experimental data trace, shown as a dotted black line. The shaded blue curve in CO and dynamic microcapsules is attributable to the development of protein aggregates.
essential molecules for the long survival of the encapsulated cells. In addition, to take advantage of the liquefied core microenvironment, the proposed microcapsules were tested under dynamic environments using spinner flasks. We hypothesized that the fluid flow could increase the interactions between cells and μPCL while improving mass transfer inside the compartmentalized microcapsules. Usually, the flow produced by spinner flasks remains restricted to the periphery of the 3D scaffolds and the distribution of cells and essential molecules is sparse [29, 30]. However, the liquefied core feature of the compartmentalized and multilayered microcapsules allowed the freely dispersion of cells and μPCL.

Therefore, the interactions of the cellular components are enhanced by the hydrodynamic shear provided by the uniform and continuous rotation of the culture medium. As demonstrated in F-actin filaments staining, the dynamic environment led to the development of significantly larger aggregates of cells and μPCL comparing to static conditions. After only 1 d, the dynamic flow allowed the recruitment of almost all cells and μPCL, demonstrating the maximum efficiency of spinner flasks in developing microtissues.

Besides the improved mass transfer, the fluid flow also provided physiologically relevant physical signals to cells. For example, the late osteogenic marker OPN could be visualized in MONO microcapsules cultured in the absence of osteogenic differentiation factors, evidencing that cell differentiation was prompted by the presence of shear stress. Several studies have been reporting that cell behavior is influenced by mechanical stimulation [31, 32]. The shear stress trigger mechanotransduction pathways in cells that converts mechanical signals into biochemical signals. In particular, osteoprogenitor cells have been shown to be mechanosensitive. Mechanical stimulation has been shown to enhance the osteogenic differentiation of cells by displaying increased calcium deposition, ALP activity and bone specific genes expression [12, 32, 33]. Importantly, herein aggregates of cells and microparticles could be generated inside microcapsules and under dynamic stimulation due to the unique liquefied and confined environment of such cell encapsulation system.

The effect of mechanical stimulation added to the microcapsules culture was also observed in the complexity of the newly deposited ECM. Natural bone is a
hierarchical organized tissue composed by an organic phase, mainly constituted by collagen fibrils from type-I collagen molecules (~90%), and an inorganic phase, comprised predominantly by nanocrystals of carbonated hydroxyapatite (HA) that are distributed along the collagen fibrils [4, 34]. After SEM image analysis, it was possible to notice the presence of thin and elongated filaments in the ECM of cell-μPCL aggregates that could correspond to collagen fibrils formation in MONO microcapsules (excepting in basal/static condition (figure 4(B))). These collagen fibrils, with typical diameters in the nanometer range, are formed from the building-block aggregation of triple-helical tropocollagen molecules [26, 35]. From this point of view, ATR-FTIR spectra are corroborated by SEM analysis. The mechanical stimulation in MONO microcapsules prompted the enhancement of triple helix structures content, absent in static conditions, and hence, achieving the primary hierarchical level of collagen, shown by collagen fibrils formation. It is worth noting that the dynamic environment, as well as, the presence of osteogenic differentiation factors from the OSTEO medium, extinguished the presence of free amino acids in MONO microcapsules. Furthermore, for CO microcapsules cultured in OSTEO medium, the content of triple and α-helix contributions decreased (figures 4(G) and (I)). These findings suggest that triple and α-helices assembled into highly ordered structures with stronger intermolecular hydrogen bonds, resulting in collagen aggregates. As showed in figure S3, for PCL microparticles there is no absorption bands in the 1700–1500 cm⁻¹ range (figure S3 square), indicating that the contributions observed in the spectra of the different biosystems, after 21 of culture, are unambiguously related with proteins secreted by the extracellular matrix. Additionally, SEM images analysis shows that CO microcapsules cultured in OSTEO medium (figure S2(a), supporting information) are covered by a thicker extracellular matrix with randomly distributed fibers in a micrometer scale (protein aggregates), while MONO microcapsules exhibit a homogenous and smooth ECM morphology. It is also notable that cross-sections of CO microcapsules (figure S2(b), supporting information) showed the abundance of mineral crystals embedded in the collagen matrix. These results are more evidenced in CO microcapsules under mechanical stimulation, as shown by the presence of fluorescently marked HA crystals (figure 5(D)) and von Kossa staining (figure 5(E)). Likewise, EDS analysis suggests that the dynamic environment allowed to develop apatite-like minerals, with a Ca/P ratio similar to the native HA of the bone matrix [27]. In fact, bone tissue possesses a precise collagen-mineral balance content. In the early stages of mineralization, the nucleation of HA crystals occurs in the gap zones of collagen molecules, while in the latter stages, the crystal deposition cover the overlap zones [35, 36]. Besides the mechanical stimuli added to the culture of microcapsules, we hypothesized that by increasing the cellular complexity through the co-encapsulating of bone-forming OBs with ASCs, a well-orchestrated cell-to-cell interaction would occur. Consequently, inside the liquefied and multilayered microcapsules, microtissues could be formed in a more realistic fashion of the native bone regenerative process. Several studies have demonstrated that osteoblasts have a great ability to modulate cell differentiation without requiring any osteogenic supplementation [37–39]. Here, we demonstrate that within the hierarchical and compartmentalized environment of microcapsules, co-encapsulated OBs and ASCs can interact in such a way that leads to the development of microtissues presenting a ECM enriched with collagen fibrils and HA crystals. CO microcapsules also release the highest amount of VEGF, compared to MONO microcapsules, since both ASCs [40–42] and OBs [43–45] are known to be involved in blood vessels formation by secreting pro-angiogenic factors, such as VEGF. This growth factor is not only involved in cell differentiation towards the endothelial lineage, but it also seems to play an important role in the osteogenic healing capacity of ASCs [17, 46]. Of note, the VEGF measured was detected in the culture medium, and thus it indicates that the VEGF released by the encapsulated cells was able to cross the multilayered membrane of microcapsules. Therefore, we believe that such microcapsules can also act as angiogenic inducers, which upon implantation may stimulate the recruitment of the host vessels, and ultimately contribute to the vascularization and integration of the formed microtissues.

Altogether, the present study showed the potential of the proposed microcapsules for bone regeneration. Here, we demonstrated that the mechanical stimulation added to the microcapsules suppressed the need of osteogenic supplementation factors for osteoblastic differentiation occur. In fact, the liquefied environment allowed to the encapsulated cells to freely move inside of the microcapsules, to proliferate, to self-organize in a 3D structure and to develop a bone-like ultrastructure. Considering the reported findings, we believe that microcapsules can be promptly implanted into a bone injury, after 24 h of in vitro mechanical stimulation. Additionally, we intend to use the proposed microcapsules as hybrid devices implantable by minimally invasive procedures due to their injectability provided by the liquefied core. These compartmentalized units might facilitate the implantation of cells, avoiding their dispersion to other regions of the body, while encapsulated μPCL act as cells adhesion sites, allowing cells to adhere, proliferate and create bone-like tissues.

5. Conclusion

Inspired by the multiphenotypic cellular environment of the native bone, we developed a bioencapsulation...
system that proved to be an effective strategy for the in vitro creation of microtissues expressing different bone biomarkers. Overall, the co-encapsulation of ASCs, OBs and μPCL led to development of differentiated bone-like microtissues, even in the absence of dexamethasone, ascorbic acid and β-glycerophosphate, the three classical supplements of osteogenic differentiation medium. Additionally, the free dispersion of co-encapsulated cells and microparticles prompted by the mechanical stimulation, recreates the dynamic environment of the native bone tissue. Here, we demonstrated the possibility of an engineered self-regulated osteogenic device that can be further envisioned as an injectable procedure for bone repair.

Acknowledgments

Sara Nadine acknowledges financial support by the Portuguese Foundation for Science and Technology (FCT) with doctoral grant SFRH/BD/130194/2017. This work was supported by the European Research Council grant ERC-2014-ADG-669858 for the project ‘ATLAS’ and FCT grant agreement PTDC/BTM-MAT/31064/2017 for the project ‘CIRCUS’. The costs resulting from the FCT hirings is funded by national funds (OE), through FCT – Fundação para a Ciência e a Tecnologia, L.p., in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, FCT Ref. UID/CTM/50011/2019, financed by national funds through the FCT/MCTES.

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References

[1] Langer R and Vacanti J P 1993 Tissue engineering. Science 260 5110
[2] Goldberg R E 2009 Spatiotemporal delivery strategies for promoting musculoskeletal tissue regeneration J. Bone Miner. Res. 249
[3] Dang M, Saunders I, Niu X, Fan Y and Ma P X 2018 Biomimetic delivery of signals for bone tissue engineering Bone Res. 6 1
[4] Lopes D, Martins-Cruz C, Oliveira M B and Mano J F 2018 Bone physiology as inspiration for tissue regenerative therapies Biomaterials 185 240 – 75
[5] Shen F H, Werner B C, Liang H, Shang H, Yang N, Li X, Shimer A L, Balian G and Katz A J 2013 Implications of adipose-derived stromal cells in a 3D culture system for osteogenic differentiation: an in vitro and in vivo investigation Spine J. 13 1
[6] Ciuffi S, Zonefrati R and Brandi M L 2017 Adipose stem cells for bone tissue repair Clin. Cases Miner. Bone Metab. 14 2
[7] Correia C R, Gil S, Reis R L and Mano J F 2016 A closed chondromimetic environment within magnetic–responsive liquefied capsules encapsulating stem cells and collagen II/ TGF-beta3 microparticles Adv. Healthc. Mater. 5 11
[8] Correia C R, Pirraco R P, Cerqueira M T, Marques A P, Reis R L and Mano J F 2016 Semipermeable capsules wrapping a multifunctional and self-regulated Co-culture microenvironment for osteogenic differentiation Sci. Rep. 6 21883
[9] Correia C R, Reis R L and Mano J F 2013 Multilayered hierarchical capsules providing cell adhesion sites Biomacromolecules 14 3
[10] Correia C R, Sher P, Reis R L and Mano J F 2013 Liquefied chitosan-alginate multilayer capsules incorporating poly( lactic acid) microparticles as cell carriers Soft Matter 9 7
[11] Correia C R, Santos T C, Pirraco R P, Cerqueira M T, Marques A P, Reis R L and Mano J F 2017 In vivo osteogenic differentiation of stem cells inside compartmentalized capsules loaded with co-cultured endothelial cells Acta Biomater. 53 15
[12] Hoey D A, Tormey S, Ramcharan S, O’Brien F J and Jacobs C R 2012 Primary cilia-mediated mechanotransduction in human mesenchymal stem cells Stem Cells 30 11
[13] Knippenberg M, Helder M N, Doubli B Z, Semeins C M, Wuisman P I and Klein-Nulend J 2005 Adipose tissue-derived mesenchymal stem cells acquire bone cell-like responsiveness to fluid shear stress on osteogenic stimulation Tissue Eng. 11 11 – 22
[14] SYSTAT PeakFit (Systat Software, San Jose, CA) (https://systatsoftware.com/products/PeakFit)
[15] Yang H, Yang S, Kong J, Dong A and Yu S 2015 Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy Nat. Protocols 10 3
[16] Denhardt D T and Noda M 1998 Osteopontin expression and function: role in bone remodeling J. Cell Biochem. Suppl. 30 1 92 – 102
[17] Hu K and Olsen B R 2016 The roles of vascular endothelial growth factor in bone repair and regeneration Bone 91 30 – 8
[18] Petibois C, Gouspillou G, Webbe K, Delage J P and Deleris G 2006 Analysis of type I and IV collagens by FT-IR spectroscopy and imaging for a molecular investigation of skeletal muscle connective tissue Anal. Bioanal. Chem. 386 7 – 8
[19] Belbachir K, Noroos R, Gouspillou G and Petibois C 2009 Collagen types analysis and differentiation by FTIR spectroscopy Anal. Bioanal. Chem. 395 3
[20] Terzi A et al 2018 Effects of processing on structural, mechanical and biological properties of collagen-based substrates for regenerative medicine Sci. Rep. 8 1
[21] Petibois C 2017 3D quantitative chemical imaging of tissues by spectromics Trends Biotechnol. 35 12
[22] Petibois C and Deleris G 2006 Chemical mapping of tumor progression by FT-IR imaging: towards molecular histopathology Trends Biotechnol. 24 10
[23] Mikály J, Deak R, Szigiártó I C, Bóta A, Beke-Somfai T and Varga Z 2017 Characterization of extracellular vesicles by IR spectroscopy: Fast and simple classification based on amide and CH stretching vibrations Biochim. Biophys. Acta 1859 3
[24] Seshadri S, Khurana R and Fink A L 1999 Fourier transform infrared spectroscopy in analysis of protein deposits Anal. Biochem. 269 349 – 60
[25] Shi v B, Seshadri S, Liu J, Oberg K A, Uversky V N and Fink A L 2013 Distinct beta-sheet structure in protein aggregates determined by ATR-FTIR spectroscopy Biochem. J. 52 31
[26] Gautieri A, Vesentini S, Reddaelli A and Buehler M J 2011 Hierarchical structure and nanomechanics of collagen microfibrils from the atomistic scale Up Nano Lett. 11 2
[27] Bett J A S, Christner L G and Hall W K 1967 Hydrogen held by solids. XVL Hydroxypapite catalysts J. Am. Chem. Soc. 89 22
[28] Bae H et al 2014 Development of functional biomaterials with micro- and nanoscale technologies for tissue engineering and drug delivery applications J. Tissue Eng. Regen. Med. 8 1
[29] Uebersax L, Hagenmuller H, Hofmann S, Gruenblatt E, Muller R, Vunjak-Novakovic G, Kaplan D L, Merkle H P and Meinel L 2006 Effect of scaffold design on bone morphology in vitro Tissue Eng. 12 12
[30] Sladkova M and De Peppo G M 2014 Bioreactor systems for human bone tissue engineering Processes 2 2
[31] Huang H, Kamm R D and Lee R T 2004 Cell mechanics and mechanotransduction: pathways, probes, and physiology Am. J. Physiol. Cell Physiol. 287 1
[32] Meinel L, Karageorgiou V, Fajardo R, Snyder B, Shinde-Patil V, Zichner L, Kaplan D, Langer R and Vunjak-Novakovic G 2004 Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium Am. J. Biomed. Eng. 32 1
[33] Kim H J, Kim U J, Leisk G G, Bayan C, Georgakoudi I and Kaplan D L 2007 Bone regeneration on macroporous aqueous-derived silk 3D scaffolds Macromol. Biosci. 7 5
[34] McKee M D, Addison W N and Kaartinen M T 2005 Hierarchies of extracellular matrix and mineral organization in bone of the craniofacial complex and skeleton Cells Tissues Organs 181 3–4
[35] Katz E P and Li S T 1973 Structure and function of bone collagen fibrils J. Mol. Biol. 80 1
[36] Landis W J, Hodgens K J, Arena J, Song M J and McEwen B F 1996 Structural relations between collagen and mineral in bone as determined by high voltage electron microscopic tomography Micron. Res. Tech. 33 2
[37] Birmingham E, Niebur G L, McHugh P F, Shaw G, Barry F P and McNamara I. M. 2012 Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche Eur Cell Mater. 23 13–27
[38] Ehnert S, van Griensven M, Unger M, Scheffler H, Falldorf K, Fentz A-K, Seeliger C, Schröter S, Nussler A K and Balmayor E R 2018 Co-culture with human osteoblasts and exposure to extremely low frequency pulsed electromagnetic fields improve osteogenic differentiation of human adipose-derived mesenchymal stem cells Int. J. Mol. Sci. 19 4
[39] Rozila I, Azari P, Munirah S B, Wan Safwani W K Z, Gan S N, Nur Azurah A G, Jahendran J, Pingguan-Murphy B and Chua K H 2016 Differential osteogenic potential of human adipose-derived stem cells co-cultured with human osteoblasts on polymeric microfiber scaffolds J. Biomed. Mater. Res. A 104 2
[40] Kang J H, Gimble J M and Kaplan D L 2009 In vitro 3D model for human vascularized adipose tissue Tissue Eng. A 15 8
[41] Verseijden F, Jahr H, Posthumus-van Sluijs S J, Hagen T I T, Hovius S E, Seynhaeve A L, van Neck J W, van Osch G J and Hofer S O 2009 Angiogenic capacity of human adipose-derived stromal cells during adipogenic differentiation: an in vitro study Tissue Eng. A 15 2
[42] Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Tenmm-Grove C I, Bovenkerk J E, Pell C L, Johnstone B H, Considine R V and March K L 2004 Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells Circulation 109 10
[43] Huang B et al 2016 Osteoblasts secrete Cxcl9 to regulate angiogenesis in bone Nat. commun. 7 13885
[44] Deckers M M L, van Bezoijen R L, van der Horst G, Verseijden F, Jahr H, Posthumus-van Sluijs S J, Hagen T I T, Hovius S E, Seynhaeve A L, van Neck J W, van Osch G J and Hofer S O 2009 Bone morphogenetic proteins stimulate angiogenesis in bone derived vascular endothelial growth factor a Endocrinology 143 4
[45] Oliveira-Navarrete R, Hzyz S L, Gittens R A, Schneider J M, Haithcock D A, Ulrich P F, Slosar P J, Schwartz Z and Boyan B D 2013 Rough titanium alloys regulate osteoblast production of angiogenic factors Spine J. 13 11
[46] Behr B, Tang C, Germann G, Longaker M T and Quarto N 2011 Locally applied vascular endothelial growth factor a increases the osteogenic healing capacity of human adipose-derived stem cells by promoting osteogenic and endothelial differentiation Stem Cells 29 2