Liquefied Microcapsules as Dual-Microcarriers for 3D+3D Bottom-Up Tissue Engineering

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1. Introduction

Liquefied cell culture systems have emerged as an alternative to solve some specific drawbacks presented by the classical 3D tissue engineering strategies, namely the encapsulation of cells within hydrogels.[1] The main advantages of hydrogels as cell encapsulation matrices are their hydrophilic nature, and their ability to provide a 3D environment. However, key parameters when designing hydrogels include architectural factors, such as size and porous structure, which are limited to a specific range due to mass transportation concerns.[2] If cells encapsulated at the core are deprived from a proper diffusion of essential molecules, they will inevitably undergo apoptosis.[3] In the last years, we have been successfully exploring an alternative approach that combines the advantages of hydrogels, liquefied cell encapsulation systems must ensure the diffusion of molecules to avoid the formation of necrotic cores. The architectural design of hydrogels, the gold standard tissue engineering strategy, is thus limited to a microsize range. To overcome such a limitation, liquefied microcapsules encapsulating cells and microparticles are proposed. Microcapsules with controlled sizes with average diameters of 608.5 ± 122.3 µm are produced at high rates by electrohydrodynamic atomization, and arginyl–glycyl–aspartic acid (RGD) domains are introduced in the multilayered membrane. While cells and microparticles interact toward the production of confined microaggregates, on the outside cell-mediated macroaggregates are formed due to the aggregation of microcapsules. The concept of simultaneous aggregation is herein termed as 3D+3D bottom-up tissue engineering. Microcapsules are cultured alone (microcapsule 1) or on top of 2D cell beds composed of human umbilical vein endothelial cells (HUVECs) alone (microcapsule 2) or cocultured with fibroblasts (microcapsule 3). Microcapsules are able to support cell encapsulation shown by LiveDead, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS), and dsDNA assays. Only microcapsule 3 are able to form macroaggregates, as shown by F-actin immunofluorescence. The bioactive 3D system also presented alkaline phosphatase activity, thus allowing osteogenic differentiation. Upon implantation using the chick chorioallantoic membrane (CAM) model, microcapsules recruit a similar number of vessels with alike geometric parameters in comparison with CAMs supplemented with basic fibroblast growth factor (bFGF).
systems, and microcarriers. Besides solving the limited diffusion of hydrogels, liquefied capsules also allow the encapsulated cells to self-organize within the liquefied core, rather than pre-establishing their 3D organization. Additionally, liquefied capsules can be implanted by injection, thus allowing minimal invasive procedures, even when presenting macrosize diameters. We established the concept of liquefied and multilayered capsules, with promising results in vitro and in vivo for general or more applied tissue engineering strategies for cartilage and bone regeneration. The concept relies on the use of alginate hydrogels as temporary hydrophilic 3D structures to encapsulate cells and microparticles. After the build-up of a multilayered membrane, the core can be subsequently liquefied by a simple mild process. The nanostructure of this membrane assures the rapid exchange of biomolecules essential for long-term cell survival and signaling. The encapsulated microparticles act as cell microcarriers by providing the physical support required for cells to survive. Within the liquefied core, cells can move freely, interact directly with other coencapsulated cells, and, most importantly, create cell-mediated aggregates by recruiting the encapsulated microparticles. Of note, cells encapsulated in such bioencapsulation system showed enhanced metabolic activity, and proliferation compared to cells encapsulated in nonliquefied alginate hydrogels or in liquefied capsules without microparticles.

Here, we intend to boost the liquefied and multilayered capsules concept towards their clinical application by proposing a high rate production method. Additionally, we aim to design capsules with a bioactive outer membrane, which would present cell adhesive properties and allow to improve the agglomeration and integration of microcapsules within the surrounding tissue. Figure S1 in the Supporting Information shows a schematic representation of the production of the multilayered and liquefied microcapsules, and the experimental design of the present study. Electrohydrodynamic atomization (EHDA) principles were applied to produce microcapsules. Then, a multilayered membrane was produced by the assembly of three polyelectrolytes, namely poly(lysine), alginate, and chitosan, by the layer-by-layer (LbL) technique. The last alginate layer was then functionalized with the arginyl–glycyl–aspartic acid (RGD) domain, the most common peptide motif responsible for cell adhesion to the extracellular matrix (ECM). Then, microcapsules were placed on the top of previously formed 2D cell beds composed by human umbilical vein endothelial cells (HUVECs) alone or cocultured with fibroblasts. Inspired in a concept here termed as 3D+3D, we hypothesized that while within the inside of the liquefied core cells and microparticles would interact and create 3D microstructures, at the same time, microcapsules could interact to create macro 3D structures from the outside. Additionally, upon implantation, the bioactive outer membrane could also improve the microcapsules interaction with the surrounding tissue, contributing to a proper integration and regeneration. This hierarchical 3D macro- and micro-organization should be exclusively cell-mediated. Upon implantation, we envisage that the bioinspired vascularization substrate provided by the RGD-LbL of microcapsules would promote the recruitment of vessels and other cells involved in the cascade of the regenerative process, promoting tissue healing and integration. Simultaneously, new microaggregates of cells and microparticles could be form inside the microcapsules, which would also contribute to boost the healing process. Figure 1 summarizes the proposed 3D+3D concept allowing macro- and micro-simultaneous regeneration. The ability of microcapsules to allow cell adhesion on the outer LbL membrane was tested in vitro. For that, a cell bed composed by the sequential seeding of human fibroblasts and HUVECs was developed. The combination of the cell bed and the microcapsules system is here termed as microcapsule. Microcapsules cultured only with HUVECs (microcapsule) or alone (microcapsule) were used as controls. As a proof-of-principle, MC3T3-E1 cells were encapsulated with

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**Figure 1.** Schematic representation of the microcapsules system proposed. A) Overview of a liquefied and multilayered capsule composed by a perselective membrane that allows the entrance of essential molecules for cells survival and avoids the entrance of high molecules and other cells. B) Cells and microparticles encapsulated within the liquefied core can move freely. Inside the microcapsules, different cell phenotypes can be combined, and besides proliferation, cells can undergo differentiation. C) After implantation, microcapsules can easily adapt to a tissue defect, and agglomerate to form macrostructures able to fulfill a large defect.
surface modified poly(ε-caprolactone) (PCL) microparticles. Cell migration, proliferation, and differentiation were evaluated in vitro up to 21 d postencapsulation. The proangiogenic ability of the system was also tested in ovo using the chick chorioallantoic membrane (CAM) assay.

2. Results

2.1. Optimum Conditions for the Production of Spherical Microgels

To assess the optimum conditions for the production of microgels with the desired morphology and size, an alginate solution was electrosprayed under different flow rates (30, 40, 50, and 60 mL h\(^{-1}\)), polymer concentrations (1.5% and 2% w/v), distances between the tip to the collector (TTC 6, 7, and 8 cm), and applied voltages (10, 15, and 20 kV). A library of microgels with varying morphologies and sizes was obtained. Figure 2A shows representative images of the optimization process (constant parameters: 50 mL h\(^{-1}\), 2% w/v, 6 cm, and 10 kV) and a schematic representation of the EHDA set-up. The comprehensive understanding of the fundamentals of the EHDA process is well-described elsewhere.\(^8\)\(^{,}\)\(^9\)\(^{,}\)\(^10\) Our findings showed that the size of the microgels decreased as the polymer concentration increased. Additionally, below 2% w/v the microgels shifted from a circular morphology to a tear-like one. The stability of the jetting mode is highly affected by the voltage and the flow rate. Therefore, by controlling these two parameters monodisperse microgels can be obtained. For flow rates below 30 mL h\(^{-1}\) and applied voltages above 10 kV, polydisperse microgels were obtained (Figure 2A arrows). Higher flow rates also allowed to obtain microgels in a high-throughput fashion, which was a key factor in the optimization strategy implemented, since the obtained microgels were templates for the production of a cell encapsulation system composed by liquefied microgels. At a stable jetting mode, higher flow rates led to the formation of microgels with increased diameters. On the other hand, increasing the TTC distance decreased the strength of the electrical field, and thus higher and/or polydisperse microgels were obtained (Figure 2A arrows). Another key parameter influencing the EHDA process is the solution conductivity. Therefore, when microparticles were loaded into the alginate solution, the EHDA was affected, as shown in Figure 2B-(2). Therefore, in order to obtain spherical microgels the concentration of alginate was increased to 2.25% w/v. Then, by varying the TTC, microgels encapsulating spherical microgels (Figure 2B-(3)) with an average diameter of 50 ± 9 µm (Figure 2B-(4)) were obtained. As evidenced in Figure 2B-(5), monodisperse and circular microgels encapsulating microgels could be obtained after increasing the TTC distance from 6 cm, optimized for empty microgels, to 8 cm. At 7 cm, although spherical microgels could be obtained, the sample was heterogeneous (Figure 2B-(5) arrow). Below 7 cm and above 8 cm, microgels presented a tear-like morphology. After the production of the multilayered membrane, microcapsules were immersed in ethylenediaminetetraacetic acid (EDTA) to liquefy the alginate core. Consequently, the microparticles dispersed in the crosslinked alginate matrix of the spherical microgels (Figure S2A, Supporting Information), agglomerate at the bottom of the spherical microgels (Figure S2B, Supporting Information).

2.2. 2D Cell Bed and Microcapsules Set-Up

Microcapsules encapsulating surface modified microparticles and cells were successfully produced, and visualized by laser scanning confocal microscopy (LSCM) after overnight incubation at 37 °C in a humidified atmosphere of 5% CO\(_2\). The distribution of the encapsulated cells within the liquefied core of microcapsules was tracked by 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) nuclei staining (Figure 3A blue spots). Importantly, after the EHDA process, the encapsulated cells remained alive, and therefore the parameters of the process, did not jeopardize cell viability (Figure 3B). Prior to the transfer of microcapsules, the last step of the sequential seeding, the engineered cellular environment on the outside environment of microcapsules, termed as cell bed, was evaluated. For that, HUVECs were stained with Dil and their morphology and expression of CD31 was assessed when cultured alone or in a coculture with fibroblasts (Figure 3C,D). As shown, HUVECs-Dil in the coculture aligned within the extracellular matrix of fibroblasts and presented a more elongated morphology compared to the monoculture. Additionally, a higher expression of CD31 could be found in the coculture. To assess the colocalization of the different cells within the triple coculture system, each cell phenotype was previously stained with a lipophilic dye, namely with DiO, Dil, and DiD for fibroblasts, HUVECs, and MC3T3-E1, respectively. As shown, MC3T3-E1 encapsulated within the liquefied core of microcapsules is localized at the top of the cell bed composed by fibroblasts and HUVECs (Figure 3E). Interestingly, HUVECs and fibroblasts coexist within the same plane, as shown in Figure 3E zx and zy, showing that HUVECs occupied the space left by fibroblasts. Additionally, the alignment of HUVECs within the fibroblasts extracellular matrix is observed in Figure 3E xyz. Interestingly, in Figure 3E zx and zy fibroblasts and HUVECs could already be detected adhered to the microcapsules, although the majority still remained at the bottom of microcapsules, showing that cells migrated from the 2D environment towards the 3D structure of microcapsules.

Microcapsules with an alginate last layer lacking the RGD motif were also produced to assess if the previously formed 2D cell beds were able to adhere to the surface of such microcapsules. For that, microcapsules composed by a multilayered membrane ending in the poly(l-lysine) polyelectrolyte were produced. Poly(l-lysine) is widely used as a coating material known to favor cell adhesion. However, HUVECs alone or cocultured with fibroblasts remained adhered to the 2D surface and thus did not migrate towards the multilayered membrane of microcapsules (Figure S3, Supporting Information). Through the transparent core of the liquefied microcapsules, it is possible to observe that in the 2D cell bed composed by HUVECs alone, cells presented a rounded morphology (Figure S3A, Supporting Information), contrary to the stretched morphology observed for the 2D cell bed composed by fibroblasts and HUVEC (Figure S3B, Supporting Information).
2.3. In Vitro Bioperformance

The ability of microcapsules to perform as a dual microcarrier system for tissue engineering was assessed. The ability of microcapsules to support living cell encapsulation was assessed up to 21 d postencapsulation. Cell viability and proliferation were evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) (Figure 4A) and DNA (Figure 4B) quantification assays, respectively. Different microcapsule formulations cultured in basal (bas) or osteogenic differentiation (oste) media were tested, namely (i) microcapsule1 – microcapsules encapsulating MC3T3-E1 and containing a coculture of fibroblasts and HUVECs adhered to the external membrane, (ii) microcapsule2 – microcapsules encapsulating MC3T3-E1 and containing a coculture of fibroblasts and HUVECs adhered to the external membrane.
– microcapsules encapsulating MC3T3-E1 and containing HUVECs adhered to the external membrane, and (iii) microcapsule1 – microcapsules encapsulating MC3T3-E1 without cells on the external environment. Of note, MTS and DNA assays were quantified after transferring the samples in order to exclude the contribution resultant from the activity of cells that did not adhere to the membrane of microcapsules. For all the timepoints tested, microcapsule3 presented the highest metabolic activity levels, and microcapsule 1 the lowest. With time, MTS values increase evidencing the ability of microcapsules to support living cell encapsulation. Microcapsules cultured in basal or osteo media presented similar metabolic activity. Regarding the ability of cells to proliferate (Figure 4B), at day 1 no significant differences were found between all the formulations tested. At day 3, such differences began to appear, with microcapsules3 and microcapsules 2 presenting the highest values compared to microcapsule1. The higher DNA content of microcapsule3 at days 14 and 21 postencapsulation, can be explained by the adhesion of fibroblasts and HUVECs to the membrane. Likewise, microcapsules2 presented a higher DNA content compared to microcapsule1, which could also have occurred due to the adhesion of HUVECs. However only a slight increase could be detected. When cultured in the external environment of capsules alone, HUVECs presented a rounded morphology and a poor migrating ability compared to the coculture with fibroblasts. In fact, the adhesion of HUVECs to the membrane could hardly be detected, presenting a high similarity to microcapsule1 (Figure S4, Supporting Information). Contrarily, cells in the external coculture environment were able to adhere to the membrane of microcapsules (Figure 4D), and after overnight incubation cell were able to migrate from the 2D cell bed to the 3D environment provided by microcapsules (Figure 4D squares). With time, macroaggregates composed by the agglomeration of microcapsules could be detected (Figure 4E,H). These macroaggregates were cell-mediated by the action of fibroblasts and HUVECs, which started to migrate from the cell network towards the membrane of the microcapsules that after 8 h of incubation (Figure 4F,I). This behavior occurred independently from the cell culture media used, whether basal or osteo media. While macroaggregates were formed due to the agglomeration of microcapsules, encapsulated cells simultaneously formed microaggregates by recruiting the coencapsulated microparticles (Figure 4G,J; Figure S4, Supporting Information). Therefore, the proposed system allowed the simultaneous creation of cell-mediated macro (by agglomerating microcapsules) and micro (by agglomerating microparticles) aggregates.

Figure 3. A) Overview of a liquefied microcapsule encapsulating MC3T3-E1 cells and polycaprolactone (PCL) microparticles visualized by laser scanning confocal microscopy (LSCM). Cell nuclei was stained with DAPI (blue). Scale bar is 200 µm. B) Microcapsules encapsulating living MC3T3-E1 cells stained with calcein (green) and PCL microparticles fluorescently marked with nile red. Scale bar is 200 µm. C) Immunofluorescence of CD31 (green) in a 2D coculture of fibroblast and HUVECs fluorescently marked with Dil (orange). Cell nuclei was stained with DAPI (blue). Scale bar is 50 µm. D) Immunofluorescence of CD31 (green) in a 2D culture of HUVECs fluorescently marked with Dil (orange). Cell nuclei was stained with DAPI (blue). Scale bar is 50 µm. E) LSCM of the triple sequential coculture system composed by microcapsules encapsulating MC3T3-E1 cells and PCL microparticles localized at the top of a 2D coculture of fibroblasts and HUVECs. Fibroblasts, HUVECs, and MC3T3-E1, were previously stained with DiO (green), Dil (orange), and DiD (blue). Scale bar is 100 µm.
Figure 4. A) Metabolic activity measured at 490 nm by MTS colorimetric assay read, B) cell proliferation evaluation by DNA quantification (µg), and C) alkaline phosphatase (ALP) activity quantification (mmol) of microcapsules encapsulating MC3T3-E1 cells and polycaprolactone (PCL) microparticles and in contact with (i) a coculture of fibroblasts and HUVECs (microcapsule³), (ii) a monoculture of HUVECs (microcapsule⁴), or (iii) alone (microcapsule¹). Results are presented as mean±standard deviation. Only statistically nonsignificant differences are marked, otherwise results are significantly different. D) Overview of a liquefied microcapsule encapsulating MC3T3-E1 cells and PCL microparticles. After 8 h of incubation, microcapsules were visualized by laser scanning confocal microscopy (LSCM). Cell nuclei was stained with DAPI (blue). The migration ability of cells from the 2D environment to the 3D structure provided by the RGD-membrane of microcapsules is highlighted by the yellow squares. Scale bar is 200 µm.

E, H) Macroaggregates of microcapsules assembled by fibroblasts and HUVECs after 7 d of culture in E) basal (bas) or H) osteogenic differentiation (osteo) media. Samples were visualized by fluorescence microscopy. F-actin filaments were stained by phalloidin (pink). Cell nuclei was stained with DAPI (blue). Scale bars are 200 µm. F, I) Interaction of the network created by fibroblasts and HUVECs and its interaction with the microcapsules after 3 d of culture in insets (F) bas or (I) osteo media. Samples were visualized by light microscopy. Scale bars are 100 µm. G, J) SEM images of the microaggregates composed by encapsulated MC3T3-E1 cells and PCL microparticles formed inside the liquefied core of microcapsules after 7 d of culture in G) bas or J) osteo media. Scale bars are 30 µm.
Additionally, the encapsulated cells were able to undergo cell differentiation towards the osteogenic lineage, as shown by the quantification of the activity of alkaline phosphatase (ALP) (Figure 4C). Of note, ALP activity peaks occurred at later time-points, namely at days 14 and 21 postencapsulation, but only when microcapsules were cultured in osteogenic differentiation medium.

2.4. Angiogenic Potential

The angiogenic potential of microcapsules was assessed after in ovo implantation using the CAM assay (Figure 5). Remarkably, results show that microcapsules were able to recruit a similar number of new microvessels compared to CAMs without microcapsules and supplemented with basic fibroblast growth factor-2 (bFG-2, control +). Since microcapsules were transferred to the CAM model in serum free α-MEM, a negative control of CAMs supplemented with culture medium was also used (control −). The number of microvessels recruited by microcapsules was significantly higher compared to control −, evidencing that such differences were prompt by the microcapsule system itself, due to their functionalized RGD LbL membrane. Of note, only microvessels with a diameter below 20 µm were considered for the quantification. Microvessels were quantified in a well-defined region of interest (ROI) for all conditions. The new vascular network formed within the ROI was similar to the vascular network formed by the control+ environment, in terms of the number of junctions (Figure 5D), although no significative differences were found for both conditions compared to control−. Additionally, microcapsules prompted the formation of a vascular network composed by vessels with increased lengths compared to the control−.

3. Discussion

Liquefied and multilayered capsules have emerged as an alternative bioencapsulation system in which cells and microparticles are encapsulated in a liquefied environment. The system is composed by four main components, each one with specific functions, namely (i) a permselective multilayered membrane, (ii) microparticles, (iii) cells, and (iv) a liquefied core. The membrane is composed by chitosan, poly(l-lysine), and alginate electrostatically interacting by the LbL technique. It acts as a physical barrier that mediates the interaction between the inside and outside environment of capsules, thus avoiding the entrance of large molecules and other cells, while ensuring the permeability of essential molecules for long-term cell survival. Microparticles provide adhesion sites required for different biological processes of anchorage-dependent cells, both dispersed in the liquefied core. We already demonstrated the superior biological performance of liquefied and multilayered microcapsules coencapsulating cells and microparticles when compared to classical alginate hydrogels or with liquefied and multilayered microcapsules without microparticles.[4] Results showed that the system presented an enhanced metabolic activity, cell viability, and proliferation up to 28 d of in vitro culture. Cells and microparticles, encapsulated within the liquefied environment of capsules can move freely, and macroaggregates containing cells and microparticles could be visualized by Scanning Electron Microscopy (SEM) or by fluorescence microscopy after F-actin staining. We believe that the established concept of liquefied and multilayered capsules as bioencapsulation system for regenerative medicine can find great applicability and value when used as building blocks to create complex 3D structures with clinical relevance. Therefore, we herein intend to take the concept of liquefied and multilayered capsules a step further.

Figure 5. Proangiogenic ability of microcapsules using the chick chorioallantoic membrane (CAM) assay. Optical microscopy of CAMs inoculated with A) microcapsule1, B) basic fibroblast growth factor-2 (bFG-2, control +), and C) α-MEM/1% penicillin/streptavidin (control −). Quantification normalized per area of a well-defined Region of Interest of the C) number of new recruited microvessels (<20 µm), number of junctions, and branch length (% relative frequency). Scale bars are 50 µm (**p < 0.01).
by proposing the combination of the high rate production of EHDA with the versatility and ease of the LbL technique to modify 3D structures. EHDA applied to produce cell encapsulation systems is often called bioelectrospraying (BES). Due to the low current of BES, the process enables to produce cell encapsulation systems with high cell viability. Xin et al.\textsuperscript{[11]} showed that up to 15 kV and a TTC of 15 cm, the viability and proliferation of six different cell types was not affected. Additionally, BES has also reported to do not affected the multilineage differentiation ability of mesenchymal stromal cells (MSCs).\textsuperscript{[12]} Herein, we used the BES technique to not only encapsulate cells but also microparticles. We optimized the parameters of the process to present the simplest set-up, rather than increasing its complexity by applying coaxial needles, high voltages or sheath gas.\textsuperscript{[13]} The proposed BES set up allowed to produce a library of spherical beads at high rates presenting different diameters (Figure 2). The rationale to select the optimized BES parameters to produce the hydrogel templates was a circularity close to 1 at the lowest voltage and highest flow rate, due to cell viability and scale-up concerns, respectively. Once the spherical hydrogels were obtained, a multilayered membrane was built by LbL technique. We have been employing this low cost and versatile technique to modify or construct 3D tissue engineering systems.\textsuperscript{[7,14–17]} Taking advantage of the versatility of the LbL technique, we conferred cell adhesion capability to the multilayered membrane simply by changing one of the polyelectrolyte solutions. Previously, we have reported the superior stability and an exponential regime growth of the multilayered membrane when combining three-polyelectrolytes, namely poly(L-lysine), chitosan, and alginate, compared to the classical two-system.\textsuperscript{[4]} Here, alginic combined with the well-known peptide sequence RGD was used to modify the last layer of the multilayered membrane of microcapsules. Our goal was to propose a dual-building block system by exploring the ability of microcapsules to produce microaggregates of cells and microparticles within the liquefied core, while at the same time, microparticles could be assembled by cells and create macro and more complex 3D structures. Our in vitro results show the successful production and bioperformance of such system. The superior organization of HUVECs when placed in a coculture with fibroblasts (Figure 3D) was attributed to the ability of the later to provide an immediate vascularization at the inside, the new microaggregates of cells and microparticles formed would contribute to accelerate the process. While aiding from the outside the healing of the tissue, acting with other cells involved in the cascade of the regenerative process. We envisage that upon implantation in vivo, the proposed microcapsules would stimulate the recruitment of blood vessels. Angiogenic capacity of biomaterials is a key asset to attain full tissue functionality, not only due to the transport of essential molecules for cell survival but also to allow a proper tissue integration.\textsuperscript{[26]} The promising results obtained, lead us to believe that the proposed system once implanted would easily adapt to lesions with complex shapes, and recruit new vessels while also interacting with other cells involved in the cascade of the regenerative process. While aiding from the outside the healing of the tissue, on the inside, the new microaggregates of cells and microparticles formed would contribute to accelerate the process.

4. Experimental Section

**Microparticles Production and Surface Functionalization:** PCL microparticles were produced by oil/water (o/w) emulsion technique, and surface modified by combining plasma treatment with collagen I as similarly described in our previous study\textsuperscript{[11]} PCL (5% w/v, M, 80 000, Merck) was dissolved in methylene chloride (CH\textsubscript{2}Cl\textsubscript{2}, Honeywell Riedel-de Haën). Under agitation, this solution was added to polyvinyl alcohol (0.5%, PVA, Merck). After 2 d at RT, the produced microparticles were subsequently collected, washed with distilled water, and dried in ethanol. Microparticles were then placed in a plasma reactor chamber (ATTO, Diener Electronic) fitted with a radio frequency generator. Air was used as...
the working atmosphere. A glow discharge plasma (0.4 mbar, 30 V) was created for 15 min. Subsequently, microparticles (500 mg) were sterilized in 70% v/v ethanol, and immersed in collagen (1200 µg, type I solution from rat tail, Merck) diluted in acetic acid (0.02 µL at 4 °C overnight. The diameter of microparticles was measured by ImageJ software.

**2D Cell Beds:** Human fibroblasts (primary dermal fibroblasts, PCS-201-012, ATCC) were cultured in DMEM-high glucose (ThermoFisher Scientific) supplemented with fetal bovine serum (FBS) (10% v/v, ThermoFisher Scientific) and antibiotic-antimycotic (1% v/v, ThermoFisher Scientific). At ~80% confluence, cells were detached using trypsin-EDTA (Merck) at 37 °C for 5 min. After 5 min of centrifugation at 300 g, the supernatant was discarded. Cells (10 000 cells per well) were suspended in culture medium and seeded in µ-slide eight wells (Ibidi). After 48 h of incubation 37 °C in a humidified atmosphere composed of 5% CO2, HUVECs (10 000 cells per well) were placed on top of fibroblasts. For that, HUVECs (PCS-100-010, ATCC) cultured in M199 supplemented with FBS (20% v/v), antibiotic-antimycotic (1% v/v), GlutaMAX (1% v/v, ThermoFisher Scientific), heparin sodium salt from porcine intestinal mucosa (180U, 10 mg mL\(^{-1}\), Merck) diluted in acetic acid (0.02 µL) in 70% v/v ethanol, and immersed in collagen (1200 µg/mL), type I solution from porcine intestinal mucosa (180U, 10 mg mL\(^{-1}\), Merck) buffered with MES hydrate (25 mM, pH 7). After 15 min at RT, microgels were dissolved in a stirring calcium chloride bath (0.1 M, type I solution from porcine intestinal mucosa (180U, 10 mg mL\(^{-1}\), Merck)) at pH 6.7. Alginate microgels encapsulating HUVECs were incubated in supplemented DMEM-high glucose and M199 (1:1). HUVECs were also cultured alone (10 000 cells per well) in supplemented M199 medium. After 48 h of HUVECs seeding, liquefied and multilayered microcapsules were placed on top of 2D cell beds.

**Liquefied and Multilayered Microcapsules:** MG3T3-E1 cells (Subclone 4, ATCC CRL-2593) were cultured in GIBCO MEM α (ThermoFisher Scientific) supplemented with FBS (10% v/v, ThermoFisher Scientific) and antibiotic-antimycotic (1% v/v, ThermoFisher Scientific). At ~80% confluence, cells were detached using trypsin-EDTA (Merck) at 37 °C for 5 min. After 5 min of centrifugation at 300 g, the supernatant was discarded. Cells were suspended in a solution containing sodium alginate (2.5% w/v, W201502, Merck), sodium chloride (0.15 M, NaCl, LabChem), and MES hydrate (25 mM, pH 6.7). Alginic microgels encapsulating cells (1 × 10\(^{-3}\) mL\(^{-1}\)) and microparticles (30 mg mL\(^{-1}\)) were obtained by EHDA at 10 kV, 8 cm, and 50 mL h\(^{-1}\). Total dsDNA quantification was performed using the assay kit Quant-IT PicoGreen (Life Technologies). Samples were aspirated and transferred to 1 mL centrifuge tubes. Following centrifugation at 300 g for 5 min, the supernatant was discarded and samples were suspended in phosphate buffered saline (PBS) containing MTS (1:6, 500 µL). After 4 h of incubation at 37 °C, 100 µL of each well (in quadruplicate) were transferred to a 96-well plate. The amount of formazan product was measured by absorbance at a wavelength of 490 nm using a multiwell spectrophotometer (Synergy HTX, Biotek).

Total dsDNA quantification and ALP assay were performed using the assay kit Quant-IT PicoGreen (Life Technologies). Samples were aspirated and transferred to 1 mL centrifuge tubes. Following centrifugation at 300 g for 5 min, the supernatant was discarded and samples were suspended in ultrapure sterile water (1 mL per sample) for cell lysis. After 1 h at a 37 °C shaking water bath, samples were frozen at −80 °C at least overnight. Samples were defrosted and used according to the specifications of the kit. A standard curve for DNA analysis was generated with the provided dsDNA solution. After 10 min of incubation at RT, fluorescence was read at an excitation wavelength of 485/20 nm and 528/20 nm of emission using a microplate reader (Synergy HTX, Biotek). Samples resultant from cell lysis were also used for the quantification of the activity of alkaline phosphatase. A substrate solution (pH 9.8) was prepared by dissolving 4-nitrophenylphosphate disodium salt hexahydrate (0.2% w/v, Merck) in diethanolamine (1 M, Sigma-Aldrich). Each sample (20 µL, in triplicate) was mixed with the prepared substrate solution (60 µL). After 45 min at 37 °C protected from light, the reaction was stopped (80 µL) with sodium hydroxide (2 M) and EDTA (0.2 × 10\(^{-3}\) M). A standard curve with a range of concentrations was prepared by diluting 4-nitrophenol solution (10 × 10\(^{-3}\) M, Merck) in the stop solution. Absorbance was read at 405 nm in a microplate reader (Synergy HTX, Biotek). Statistical analysis was performed using two-way analysis of variance (ANOVA) with Tukey’s post-hoc test (GraphPad Prism 6.0). A p-value < 0.05 was considered statistically significant. Only nonsignificant differences (ns) were marked to facilitate the interpretation of the data. All results are presented as mean ± SD.

**SEM:** Microcapsules were fixed at RT in formalin (10% v/v) for 30 min, and subsequently dehydrated in increasing gradient series of ethanol (10 min each). The membrane of microcapsules was destroyed to expose the core contents. After gold sputtering, samples were visualized (25 kV, S4100, Hitachi).

**CD31 Immunofluorescence Staining of 2D Cell Beds:** Following 48 h of HUVECs seeding, 2D cell beds were fixed in formalin (10% v/v) for 30 min at RT, and permeabilized for 5 min at RT with Triton X-100 (0.1% v/v, Merck). Prior to cell seeding, HUVECs were labeled with Dil (1 mL containing 2 × 10\(^{-3}\) M per 1 × 10\(^{6}\) cells) at 37 °C for 15 min. 2D cell beds composed by fibroblasts cocultured with HUVECs-DiL or HUVECs-DiL alone were then immersed in FBS/PBS (5% w/v) for 1 h at RT to block nonspecific binding, and then incubated overnight at 4 °C with the primary antibody mouse anti-human CD31 (Biologend, 1:100 in 5% FBS/PBS). After PBS washing, samples were incubated for 1 h at RT with the secondary antibody anti-mouse AlexaFluor 488 (Biologend, 1:500 in 5% FBS/PBS). After PBS washing, samples were counterstained with DAPI (1 mg mL\(^{-1}\) diluted 1:1000 in PBS, ThermoFisher Scientific) for 5 min at RT, and analyzed by fluorescence microscopy (Axio Imager 2, Zeiss).
F-Actin Filaments Fluorescence Staining: Microcapsules were fixed at RT in formalin (10% v/v) for 30 min, and permeabilized for 5 min at RT with Triton X-100 (0.1% v/v). Samples were stained with phalloidin (Flash Phalloidin Red 594, 1:40 in PBS, Biolegend) for 45 min at RT, and then counterstained with DAPI (1 mg mL\(^{-1}\) diluted 1:1000 in PBS) for 5 min at RT. Microcapsules were analyzed by fluorescence microscopy (Axio Imager 2, Zeiss).

Calcein-AM Fluorescence Staining: Microcapsules encapsulating MC3T3-E1 and PCL microparticles stained with nile red were immersed in a PBS (1 mL) solution containing calcein-AM (1 µM) for 15 min at 37 °C. After PBS washing, samples were immediately visualized by fluorescence microscopy ( Axio Imager 2, Zeiss).

CAM Assay: The CAM assay was used to evaluate the angiogenic response of microcapsules. After EHDA production, microcapsules (total infused: 10 mL alginate) were incubated at 37 °C in 5% CO\(_2\) in serum-free Gibco MEM (20 mL) supplemented with antibiotic-antimycotic (1% v/v). After an overnight incubation, microcapsules were implanted in fertilized chicken (Gallus gallus) eggs, obtained from commercial sources, which were previously incubated horizontally at 37.8 °C in a humidified atmosphere for 10 d. A square window was cut in the shell after removal of 2–2.5 mL of albumen, to allow detachment of the developing CAM. The window was sealed with adhesive tape and the eggs returned to the incubator. Microcapsules (20 µL) were transferred to a 3 mm silicone ring under sterile conditions. CAMs without microcapsules and with GibcoMEM α (20 mL) supplemented with antibiotic-antimycotic (1% v/v) were used as positive and negative controls, respectively. The eggs were resealed and returned to the incubator for 3 d. After removing the silicone ring, CAMs were excised from the embryos and visualized ex-ovo under light microscopy (SZX16 coupled with a DP71 camera, Olympus). The number of new vessels (<20 µm in diameter) growing radially towards the inoculation site were counted blinded. Statistical analysis was carried out using GraphPad Prism 6 software. Data are presented as mean ± standard deviation (SD) from ten eggs (n = 3). A using two-way analysis of variance (ANOVA) with Kruskal–Wallis test was used. A p-value < 0.05 was considered statistically significant.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
The authors acknowledge the funding from the European Research Council (ERC) for project ATLAS (ERC-2014-ADG-669858), and the Portuguese Foundation for Science and Technology (FCT) for project CIRCUS (PTDC/BTM-MAT/31064/2014 and SFRH/BD/129224/2017). 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. This work was also partly supported by Japan Society for the Promotion of Science (JSPS) Bilateral Open Partnership Joint Research Projects. The authors are also grateful to A. Sofia Silva (Figures 3A and 4D) and Vitor M. Gaspar (Figure 3E) for LSCM imaging. Image acquisition was performed in the LiM facility of iBiMED, a node of PPBI (Portuguese Platform of BioImaging) with grant agreement number POCI-01-0145-FEDER-022122.

Conflict of Interest
The authors declare no conflict of interest.

Keywords
3D systems, bottom-up tissue engineering, cell encapsulation, layer-by-layer, microcapsules

Received: September 1, 2019
Revised: September 28, 2019
Published online: October 11, 2019

[1] C. R. Correia, R. L. Reis, J. F. Mano, Adv. Healthcare Mater. 2018, 7, 1071444.
[2] J. Li, D. J. Mooney, Nat. Rev. Mater. 2016, 1, 1.
[3] O.-W. Merten, Philos. Trans. R. Soc., B 2015, 370, 20140040.
[4] C. R. Correia, R. L. Reis, J. F. Mano, Biomacromolecules 2013, 14, 743.
[5] C. R. Correia, P. Sher, R. L. Reis, J. F. Mano, Soft Matter 2013, 9, 2125.
[6] C. R. Correia, S. Gil, R. L. Reis, J. F. Mano, Adv. Healthcare Mater. 2016, 5, 1346.
[7] C. R. Correia, R. P. Pirraco, M. T. Cerqueira, A. P. Marques, R. L. Reis, J. F. Mano, Sci. Rep. 2016, 6, 21833.
[8] C. R. Correia, T. C. Santos, R. P. Pirraco, M. T. Cerqueira, A. P. Marques, R. L. Reis, J. F. Mano, Acta Biomater. 2017, 53, 483.
[9] S. K. Boda, X. Li, J. Xie, J. Aerosol Sci. 2018, 125, 164.
[10] N. Nguyen, C. Claesen, G. Van den Mooter, J. Pharm. Sci. 2016, 105, 2601.
[11] Y. Xin, G. Chai, T. Zhang, X. Wang, M. Qu, A. Tan, M. Bogari, M. Zhu, L. Lin, Q. Hu, Y. Liu, Y. Zhang, Biomed. Rep. 2016, 5, 723.
[12] Z. McCrea, Y. Amantinho, S.-A. Cryan, S. O’Dea, J. Med. Biol. Eng. 2018, 38, 497.
[13] J. Wang, J. A. Jansen, F. Yang, Front. Chem. 2019, 7, 258.
[14] J. Borges, L. C. Rodrigues, R. L. Reis, J. F. Mano, Adv. Funct. Mater. 2014, 24, 5624.
[15] J. M. Silva, N. Georgi, R. Costa, P. Sher, R. L. Reis, C. a. Van Blitterswijk, M. Karperien, J. F. Mano, PLoS One 2013, 8, 1.
[16] S. M. Oliveira, V. E. Santo, M. E. Gomes, R. L. Reis, J. F. Mano, Biomaterials 2015, 48, 56.
[17] T. D. Gomes, S. G. Caridade, M. P. Sousa, S. Azevedo, M. Y. Kandur, E. T. Oner, N. M. Alves, J. F. Mano, Acta Biomater. 2018, 69, 183.
[18] M. N. Nakatsu, R. C. A. Sainson, J. N. Aoto, K. L. Taylor, M. Atkenhead, S. Pérez-del-Pulgar, P. M. Carpenter, C. W. Hughes, Microvasc. Res. 2003, 66, 102.
[19] B. Andrée, H. Ichanti, S. Kalies, A. Heisterkamp, S. Strauß, P.-M. Vogt, A. Haverich, A. Hilfiker, Sci. Rep. 2019, 9, 5437.
[20] X. Fan, Y. Teng, Z. Ye, Y. Zhou, W.-S. Tan, J. Cell Sci. 2018, 131, jcs216135.
[21] M. C. Moorer, J. P. Stains, Curr. Osteopor. Rep. 2017, 15, 24.
[22] L. I. Plotkin, T. Bellido, Bone 2013, 52, 157.
[23] F. Villars, B. Guilhotin, T. Amédée, S. Dutoya, L. Bordenave, R. Bareille, J. Amédée, Am. J. Physiol.: Cell Physiol. 2002, 282, C775.
[24] Y. Chen, M. Chen, T. Xue, G. Li, D. Wang, P. Shang, J. X. Jiang, H. Xu, J. Cell. Physiol. 2019, 234, 19824.
[25] B. Guilhotin, R. Bareille, C. Bourlet, L. Bordenave, J. Amédée, Bone 2008, 42, 1080.
[26] N. Mitrousis, A. Fokina, M. S. Shoichet, Nat. Rev. Mater. 2018, 3, 441.