One-Step All-Aqueous Interfacial Assembly of Robust Membranes for Long-Term Encapsulation and Culture of Adherent Stem/Stromal Cells

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The therapeutic effectiveness and biological relevance of technologies based on adherent cells depend on platforms that enable long-term culture in controlled environments. Liquid-core capsules have been suggested as semipermeable moieties with spatial homogeneity due to the high mobility of all components in their core. The lack of cell-adhesive sites in liquid-core structures often hampers their use as platforms for stem cell-based technologies for long-term survival and cell-directed self-organization. Here, the one-step fast formation of robust polymeric capsules formed by interfacial complexation of oppositely charged polyelectrolytes in an all-aqueous environment, compatible with the simultaneous encapsulation of mesenchymal stem/stromal cells (MSCs) and microcarriers, is described. The adhesion of umbilical cord MSCs to polymeric microcarriers enables their aggregation and culture for more than 21 days in capsules prepared either manually by dropwise addition, or by scalable electrohydrodynamic atomization, generating robust and stable capsules. Cell aggregation and secretion overtime can be tailored by providing cells with static or dynamic (bioreactor) environments.

1. Introduction

The use of extracellular matrix (ECM)-mimetic bulk hydrogels has led to major advances on the exploration of the use of stem cells for tissue regeneration and in vitro organ fabrication.[1] These 3D polymer-based structures are usually flexible, transparent, rich in water, and easily tailored, both chemically and mechanically.[2] However, some major drawbacks associated with these structures are related with the lack of spatial freedom for cells to proliferate and migrate in order to create an organized tissue, and with the low diffusion capacity of essential molecules to these structures' core, compromising the proper biological function of encapsulated living cells over the whole depth of the biomaterial construct.[3] Semipermeable capsule-shaped membranes comprising a liquid core simultaneously allow the confinement of biological cargo, while enabling the diffusion of nutrients and essential molecules, such as oxygen (O₂) and carbon dioxide (CO₂). The first remarkable application of liquid-core capsules for cellular therapies was reported by Lim and Sun, targeting the immune isolation of pancreatic islets for transplantation of diabetic individuals.[4] Most methods described to produce liquid-core capsules rely either on the primary production of beads, often obtained by gelation, or in the use of oil-based emulsion/templating techniques. Gelled beads are used as sacrificial templates that support the formation of a limitative membrane, often by the deposition of antagonistic molecules.[5] Reactions driving the formation of these membranes usually occur either by complexation or precipitation of oppositely charged polyelectrolytes[6] and/or nanoparticles,[7] or by their layer-by-layer (LbL) deposition.[8] The removal of (semi)solid sacrificial templates is achieved in a sequential postprocessing step, and often requires the use of organic solvents,[9–11] ionic chelating agents,[12] or temperature cycles,[13] giving rise to multistep, complex, and time-consuming processes. Oil-based methodologies also rely on the formation of oil droplets to sustain the formation of membranes, showing detrimental effects on the biological cargo and difficult clinical translation.[14,15]

In the mid-1950s, aqueous two-phase systems (ATPSs) were presented as a biocompatible alternative for liquid–liquid extraction techniques capable of competing with traditional methodologies that often involve volatile organic solvents.[16] ATPS are characterized by a mixture of two incompatible hydrophilic materials—which may be two polymers, or a polymer and a salt—on aqueous solution. Depending on the concentration of the components, the systems may separate into two immiscible aqueous phases.[17] With the increasing need for safer and easier strategies to facilitate the encapsulation of molecules and biological cargo, ATPS emerged as a simple, versatile, and viable solution to produce a large variety of structures.[18]
ATPS’ phases have been enriched with reacting molecules to promote interfacial reactions. In this context, the complexation of polyelectrolytes/nanoparticles [19] and polyelectrolyte-polyelectrolyte [20] pairs were used to fabricate liquid-core capsules with permeable tailored membranes. The concept was materialized through the exploration of different techniques, including microfluidics [21,22] and electrospraying [23,24], culminating in the formation of multiscale structures. So far, biocompatible encapsulation devices fabricated using ATPS as all-aqueous emulsion supports has been restricted to scenarios where cells were grown in suspension conditions. While the studies by Weitz and co-workers [25] and Lee and co-workers [26] refer to the encapsulation of bacteria, a recent study by Qin and co-workers [27] showed that multicellular islet organoids (multicellular aggregates) could be assembled in complexed alginate/chitosan membranes.

Despite the value of liquified capsules as tools to promote the formation of multicellular aggregates, the long-term survival of adherent cells, namely clinically relevant mesenchymal stem cells (MSCs), is dependent on the provision of adhesion sites. The lack of ECM-mimetic cell-adhesive cues either directs cells into the formation of multicellular aggregates that tend to show poor diffusion properties, generating necrotic cores upon prolonged times of culture [28] or, upon insufficient establishment of cell contact to guide aggregates’ formation, individual cells tend to die from anoikis [29]. Correia et al. previously suggested the incorporation of polymeric microparticles inside liquified membranes made by the layer-by-layer technique, followed by the retrieval of sacrificial template, as an effective method to enable the adhesion, proliferation, and modulation of adipose-derived mesenchymal stem cells for cell differentiation [30,31].

Here, we propose a rapid one-step oil-free encapsulation method to generate a robust and permeable membrane with the shape of closed capsules. Phases of the well-described poly(ethylene glycol) (PEG)/dextran (Dex) ATPS were mixed with oppositely charged polyelectrolytes ε-poly-l-lysine (PLL) and alginate (ALG), respectively. Polyelectrolyte complexation promoted at the interface of the ATPS enabled the generation of insoluble and stable membranes using a dropwise pouring method to create millimetric capsules, and electrohydrodynamic atomization (EHDA) to form micrometric structures. The simultaneous encapsulation of animal cells—human umbilical cord (UC) Wharton’s jelly mesenchymal stem/stromal cells (hWJ-MSCs)—with or without poly(ε-caprolactone) (PCL) microcarriers enabled modulating and attaining high cellular viability up to 21 days after encapsulation, both in static or dynamic culture regimens. The versatile system may be adapted to additional polymeric combinations and used to compartmentalize sensitive cargo in various fields, including drug delivery, environmental applications, or food development.

2. Results

2.1. Liquid-Core Capsules Are Formed within the Interface of All-Aqueous Immiscible Phases

The formation of ALG/PLL hybrid capsules at the interface of the all-aqueous immiscible system was initially explored through the observation of the system’s visual features after a simple Phase I-in-Phase II dropwise addition strategy (Figure 1A,B) and, later on, by a scalable EHDA technique (Figure 1C). The transparent character of both initially separated solutions enabled the detection of the formation of an opaque interfacial membrane after the addition of the disperse ALG-loaded Phase I into the continuous PLL-loaded Phase II.

The formation of a stable membrane at the droplet’s interface that separates both aqueous immiscible phases was expected to be mainly ascribed to electrostatic interactions, at pH 7.4, of positively charged PLL (reported pk_\(_{\text{a}}\) ≈ \(9\)–\(10\)) and negatively charged ALG (reported pk_\(_{\text{a}}\) ≈ \(3\)–\(4\)). Previous studies based on the layer-by-layer technology show that the two polymers are capable of interacting, forming insoluble films [32,33]. The charge of each molecule was assessed in two different solutions: with the polyelectrolytes dissolved in DPBS, or in the corresponding ATPS’ phase, both after a pH adjustment to physiological value (7.4). While in DPBS PLL showed a net charge of +4.99 ± 0.3 mV and ALG of −57.80 ± 5.53 mV, the addition of the ATPS’ components (Dex, for ALG; PEG, for PLL) led to a significant decrease in the modular values of the net charge previously detected for each phase. PEG+PLL exhibited a net charge close to neutral of +0.98 ± 0.3 mV and the Dex+ALG solution followed the same trend, with a value of −4.99 ± 0.56 mV, which may be explained by the shielding effect of uncharged ATPS-composing polymers on charged polymers [34,35].

Figure 2II (PEG+PLL) showed much lower viscosity than Phase I (Dex+ALG) and both phases showed shear-thinning behavior (Figure S1, Supporting Information). These properties probably favored the easy dropwise addition of Phase I into Phase II, while enabling the formation of a continuous phase with extremely low viscosity under agitation, important to ensure physical distancing between formed capsules and to avoid their clumping during complexation. Millimetric capsules with an average diameter of 2.78 ± 0.10 mm and a coefficient of variation (CV) of 3.75% were obtained using alginate (1.5% (w/w)) and PLL (0.5% (w/w)) complexed for 15 min. Membrane’s analysis right after production allowed estimating a thickness of 132 ± 12 μm. Those membranes were resistant to a dynamic environment of DPBS under agitation, as well as to their contact with air (Figure 1D). Electrosprayed capsules processed using alginate (1% (w/w)) and PLL (0.5% (w/w)) showed a smaller diameter of 767.2 ± 85.3 μm (CV = 11.1%). Both macro- and microcapsules showed extensive hollow cores and continuous membranes with smooth outer surface and cross-sectional microporosity (Figure 1E).

2.2. Capsules Show Mechanical Robustness, Permeability, and Amenability to Integrate Hierarchical Structures

To explore the versatility of the developed system, we tested an array of different concentrations of the polyelectrolyte’s pair as well as different complexation times. Figure 2A summarizes the robustness’ features of macrocapsules complexed for 20 min using three concentrations of alginate [ALG] wt% = 0.5%, 1%, or 1.5% and three concentrations of polylysine [PLL] wt% = 0.5%, 1%, or 1.5%. Three out of the nine formulations yielded robust capsules, amenable to be easily handled: 1.5 wt% ALG + 0.5 wt% PLL, 1.5 wt% ALG + 1 wt% PLL, and 1.5 wt% ALG + 1.5 wt% PLL. Interestingly, capsules prepared with the lowest PLL...
Figure 1. Production and characterization of macro- and microcapsules assembled at the ATPS interface. A) Schematic representation of the complexation of alginate (ALG) and ε-poly-L-lysine (PLL) at the interface of the aqueous immiscible interface, leading to the formation of capsules. B) Schematic representation of the production of macrocapsules by dropwise addition of Phase I into Phase II, under agitation. C) Schematic representation of the production of microcapsules by electrohydrodynamic atomization of Phase I into Phase II, under agitation. D) Photographs of macrocapsules (produced by dropwise addition) and the ir opaque robust interfacial membrane immersed in DPBS, a phosphate-buffered saline solution (top), or placed on a dry surface exposed to air (bottom). The photographs show the stability of the capsules, either when wet or exposed to dry environments, showcasing the robustness of the system. E) SEM images of macro- and microcapsules with respective cross-sections, displaying the morphology and structure of the membrane.

concentration showed higher resistance to centrifugation cycles up to 769 × g (Figure 2B). Moreover, with the adaptation of the system for cell encapsulation in mind, that condition was selected for further characterization, since exposure to high concentrations of PLL has been reported as toxic[37,38] The possibility of reducing the complexation time was then assessed. Times of complexation below 10 min led to the formation of fragile capsules, unable to withstand contact with air or washing steps with DPBS. Upon centrifugation cycles, lower complexation times correlated with increasing rates of disrupted membranes (Figure 2C): with 20 min of complexation, ≈20% of the structures ruptured, increasing to 80% for a complexation time of 10 min. An intermediate point—15 min of complexation—ensured the rapid production of capsules with robust behavior and, consequently, this condition was selected for further experiments.

The mechanical resistance of the capsules with both size ranges was evidenced through the efficient encapsulation of micro- in macrocapsules (Figure 2D; Movie S1, Supporting Information), giving rise to an easy methodology to generate multiscale compartmentalized structures. This approach may enable the creation of complexly designed multicore devices to incorporate different cells and essential biomolecules, allowing a regulated coexistence in physically protected and semipermeable environments, not compromising the individual integrity and proper function of each individual system. This may be adapted either to mimic the intracellular environment of living cells within organelles, or for tissue engineering by taking advantage of important cocultures and essential regulatory signaling pathways.[39]

The permeability of 1.5% ALG + 0.5% PLL macrocapsules was assessed by the release of dextran–fluorescein isothiocyanate (FITC) with molecular weights ranging from 10 to 500 kDa. Release profiles obtained until 28 h of incubation suggest that capsules are permeable to linear molecules with a molecular weight of 150 kDa, although slower release profiles were observed with increasing the molecular weights of the dextran–FITC (Figure 2E; Table S1 in the Supporting Information can be consulted for calculations of permeability and coefficients of diffusion). Molecules with 500 kDa showed a total release of ≈20% of initially loaded mass, probably corresponding to entrapped molecules in superficial regions of the capsules, since this release was rapidly stabilized indicating that only a low portion of these molecules could diffuse from the capsules. Interestingly, the release profile for all molecules reached a plateau-like stage after short incubation times, around 200 min. The rapid diffusion of the smallest molecular weight molecule (10 kDa) suggests that capsules are easily permeable to low molecular weight
compounds essential for cell survival, including oxygen, nutrients, and cell’s metabolic waste.

2.3. Capsules Prepared in All-Aqueous Medium Enable the Loading of Microcarriers and the Long-Term Viability of Encapsulated Adherent Cells

We demonstrated the ability to encapsulate model molecules in mild conditions concerning pH, temperature, agitation, and time. The adequacy of the developed encapsulation method to withstand human cells’ encapsulation was further tested with adherent cells. The encapsulation of human primary stem/stromal cells with clinical relevance was assessed using hWJ-MSCs. Viable cells were detected after 1 day in culture, both under static and dynamic (rotational bioreactor) regimens (Figure 3A). Moreover, hWJ-MSCs adhered to plasma-treated and type I collagen-coated microcarriers at this early time point. The effect of microcarriers in macrocapsules was assessed under static conditions concerning cell viability, morphology, cell number, and metabolic activity (Figure S2, Supporting Information). hWJ-MSCs tended to aggregate in small-sized aggregates, overtime. In general, the size of these aggregates seemed smaller than the ones formed in the presence of microcapsules. Interestingly, though, the presence of microcarriers in macrocapsules did not dictate relevant differences in the metabolic activity profile or in the detected DNA overtime.

Culture under stirring led to the increase of hWJ-MSCs’ metabolic activity when normalized by the total amount of dsDNA (Figure 3B). While the maintenance of dsDNA content was observed in static culture, overtime (Figure S3, Supporting Information), a significant decrease in its detection was observed for cells cultured in bioreactors right after 3 days of culture (p < 0.05, Figure S3, Supporting Information). This effect may be related to a higher degree of compaction, as well as the increased tendency for cell agglomeration under a dynamic stimulus. Those structures may show a less effective permeability to oxygen and other vital molecules to their core. In fact, a relevant part of formed aggregates in dynamic culture conditions developed necrotic cores that occupied nearly half of their volume. Such dead cells on the inside of the aggregates may have their membrane ruptured due to necrosis, and loss of integrity of organelles, including cell nuclei. Indeed, the release of dsDNA to the outside of the nuclear membrane most likely drives its disintegration, which may justify the loss of quantified dsDNA in those samples.

Dynamic cell culture also promoted the effective and more rapid cell attachment to microcarriers, compared to static culture (Figure 3C; Movie S2, Supporting Information). Besides its role as adjuvants of cell aggregation, dynamic stirring conditions were assessed as modulators of the secretory profile of hWJ-MSCs, which have been reported as potent modulators of injured tissues, in part due to their ability to secrete proangiogenic and trophic factors capable of directing reperfusion and repair. The use of conditioned medium as a source of therapeutic molecules has been explored for the treatment of injuries and inflammatory diseases. We analyzed the composition of six molecules reported for their proregenerative and immunoregulatory potential, and compared how their concentration profile varied in static or dynamic culture conditions. The dissection of cell behavior under different culture regimens enabled tailoring MSCs’ secretory profile. Such data may be relevant for future applications as the in vitro production and collection of valuable
Figure 3. Viability and spatial organization of hWJ-MSCs encapsulated in macrocapsules containing PCL microcarriers. A) Live–dead assay for static and dynamic cell cultures. Living cells are stained in green, whereas dead cells are stained in red. B) Metabolic activity evaluation by MTS colorimetric assay for 1, 3, 7, 14, and 21 days in culture (results presented as means ± SD; n = 3 replicates for 4 capsules/replicate; statistical analysis performed by two-way ANOVA with Tukey’s post-test; statistically significant considered for * p < 0.05, ** p < 0.01, and *** p < 0.001), normalized by the quantification of dsDNA. C) DAPI/phalloidin staining was performed to assess cellular spatial organization inside the capsules. Cells adhered to PCL microparticles in both static and dynamic environments 1 day after encapsulation. DAPI stains the nuclei (in blue), and, in red, phalloidin marks the cytoskeleton’s F-actin filament. Bright-field images are also provided. D–I) Mass of regenerative and angiogenic growth factors detected in cell culture medium in static and dynamic culture regimens, overtime. Mass values are normalized to cell culture medium volume and number of capsules; n = 3 replicates, normalized to the content of 4 capsules/replicate. BDL: below detection limit.

secretome, or the direct use of these structures for revascularization and regeneration of damaged tissue, or the treatment of inflammatory diseases.\(^{[47,48]}\) Also, the secretion of prorregenerative molecules by hWJ-MSCs using this type of liquid-core system has never been characterized before, which may i) enable finding efficient ways of preconditioning cells to showcase higher regenerative profiles upon implantation,\(^{[49]}\) and ii) to predict the response of encapsulated cells in different locations and settings upon implantation. Indeed, exposing cells to distinct mechanical environments not only tailored their aggregation ability, but also had an impact on their secretory properties. As showcased in Figure 3D, the composition of cell-conditioned medium collected for 48 h was affected by the cell culture regimen. Interestingly, an analysis of medium composition, overtime, shows a general tendency for dynamic culture to decrease (mostly to values below detection limit) the concentration of such prorregenerative factors after 21 days of cell culture. In opposition, cells cultured in static regimen tend to maintain the secretion of these factors, overtime, with a tendency for their increase with longer culture periods. Therefore, the control over culture time, as well as mechanical regimen used for cell culture, may be used as simple tools to control cell culture medium composition with possible therapeutic applications.

Interestingly, encapsulated hWJ-MSCs in static culture conditions tended to form micrometric aggregates mediated by multiple cell–microparticle contacts after 40 days in culture, which
Figure 4. Viability and spatial organization of hWJ-MSCs encapsulated in microcapsules. A) Live–dead staining on microcapsules laden with hWJ-MSCs and PCL microcarriers for 1, 3, 7, 14, and 21 days in culture. B) Characterization of cell aggregates’ size and respective percentage of structures showcasing necrotic core (in red) in capsules containing microcarriers (n > 15 capsules). C) Characterization of cell aggregates’ size and respective percentage of structures showcasing necrotic core (in red) in microcarrier-free capsules (n > 15 capsules). D) Dispersion of the number of aggregates generated in microcapsules containing microcarriers over 21 days of cell culture. E) Dispersion of the number of aggregates generated in microcarrier-free microcapsules over 21 days of cell culture.

apparently adhered to the capsules’ inner wall, leading to their rupture after 57 days in culture (Figure S4, Supporting Information). With the increasing growth of those cell–microcarrier aggregates, the generated tension on the capsules’ membrane probably led to their localized rupture, allowing aggregates to escape from the capsules without the loss of their spherical structure. Microcapsules also enabled the encapsulation of hWJ-MSCs with efficacy and keeping high cellular viability. The live/dead assay was performed on encapsulated hWJ-MSC for 1, 3, 7, and 14 days in culture. Likewise, as observed in macrocapsules, encapsulated PCL microcarriers showcased attached cells, which then evolved into microaggregates (Figure 4A). Capsules’ diameter of 526 ± 74 µm (CV = 14.14%) were obtained, showing a significant reduction in size when compared to cargo-less microcapsules. Although adherent cells tend to go through anoikis in the absence of sites of adhesion, it has been previously shown that their culture in microcapsules may lead to the formation of multicellular aggregates that may be useful as organoids. The cell-interacting effect of microcarriers inside microcapsules was scrutinized by an analysis of the number (and its dispersion), size, and presence of necrotic core overtime (Figure 4). A morphological analysis of the organization of encapsulated cells at the first day of culture did not show any significant differences between samples containing or in the absence of microcarriers (Figure 4A). After 3 days of culture, microcapsules containing microcarriers led to the formation of significantly larger (p < 0.01) and more disperse in size (although not statically significant) cellular aggregates (Figure 4B). The tendency was kept up to 21 days of cell culture. Importantly, the presence of cell aggregates with necrotic cores could only be detected in the microcarrier-containing formulation at day 14, while in their absence they were detected much earlier, at day 3 (Figure 4B,C). This data suggests that cells probably rely in a less contractile state due to the presence of microcarriers, which delays the formation of necrotic structures. Despite being an often-overlooked aspect in the study of cellular aggregates for regeneration, avoiding the formation of necrotic cores may be adjuvant for the design and implementation of a long-term cell cultures, namely, for applications concerning secretome collection or localized delivery. A possible adjuvant effect of microcarriers on the dispersion of formed aggregates inside microcapsules was also assessed. As shown in Figure 4D,E, microcapsules with lower dispersion of aggregates’ numbers at early times of culture—with emphasis on day 1 and day 3—were the ones supplemented with microcarriers. Although, after 14 days of culture, the dispersion profile of microcapsules with and without microcarriers was similar, these results indicate that
microcarriers have an important role on inducing the rapid formation of less disperse microcapsule+cell aggregate hybrids.

3. Discussion

The design of new encapsulation strategies capable of surpassing current hurdles associated with conventional 3D single-phase hydrogels may facilitate and accelerate the clinical translation of cellular therapies. While cell encapsulation in liquid-core capsules seems to fulfill several requirements to outperform materials with bulk structures,[3,10] developing technologies compatible with their safe, scalable, one-step, and rapid production are still in great demand. Most technologies that aim at producing liquid capsules still rely either i) on the use of oil or other organic agents, difficult to remove during washing cycles with aqueous solvents and frequently associated with cytotoxicity, or ii) on the use of sacrificial templates to deposit conformal coatings, requiring multistep processing procedures, as well as the use of additional components that may impart the hybrid bio-logic systems with unpredictable behavior. Additionally, the incorporation of cell-adhesive microparticles in the core of liquidified capsules—necessary to generate a favorable environment for adherent cells—has been restricted to LbL capsule’s formation technologies, followed by chelating steps to promote cores’ liquefaction.[12,30,32]

In this study, we hypothesized that the encapsulation and long-term cell culture of clinically relevant human mesenchymal stromal cells[43,44] and solid microcarriers could be attained in a one-step all-aqueous procedure. The system would take advantage of the well-reported phase-separation’s properties of ATPS, as well as from their ability to promote an interfacial complexation of oppositely charged polyelectrolytes. Although the encapsulation of biological cargo, namely, bacteria and animal cells, has been reported using similar concepts,[25–27] it was not clear whether the encapsulation of polymeric microparticles would be feasible using these systems and if the majority of the cargo would be entrapped in the capsules’ liquid core. We showed that both cells and microparticles could be efficiently encapsulated and kept in the liquid core of capsules prepared with ALG and PLL, at physiologic pH and ionic strength, as well as at room temperature (RT).

The compatibility of the developed system with mild processing steps makes it promising not only for the encapsulation of cellular cargo, but also to broader applications involving sensitive molecules, including proteins and RNAs.[53,54]

To prove our concept, the immiscible ATPS dextran/PEG phases were used to mix oppositely charged polyelectrolytes. Despite the characteristic low interfacial tension of ATPS,[55] the dropwise addition of the dispersed Dex+ALG phase into the continuous PEG+PLL phase, under agitation, led to the formation of single capsules with firm spherical morphology, showing that the interfacial tension and probable immediate initiation of interfacial complexation was enough to ensure that the system was kept stable, avoiding droplet’s breakage. These characteristics allowed the adaptation of the system to the formation of miniaturized capsules with high production yield. This was achieved by using an electrospaying apparatus, which promoted the atomization of the Dex+ALG phase during its extrusion. The relatively low size polydispersion obtained after this process’ optimization suggests that, for both macro and micro setups, the generation of the primary droplets immediately mediated the formation of capsules. Electrospaying internal flow used in our study—10 mL h\(^{-1}\)—is 400-fold higher when compared to extrusion rates previously applied in microfluidics technologies.[27] The extreme difference in the viscosity of both solutions, with Phase I (≈15 Pa s, at 0.01 s\(^{-1}\)) about 10-fold more viscous that Phase II (≈2 Pa s, at 0.01 s\(^{-1}\); Figure S1, Supporting Information), with a consistent difference kept along a range of applied shear rates, may also contribute to the stabilization of the dispersed droplets. In an effort to explore the versatility of the system, the switching of the dispersed/continuous character of each phase was attempted. However, probably due to the much lower density of the PEG+PLL phase, as well as due to the high viscosity of the Dex+ALG phase, the entrance of PEG+PLL droplets in the Dex+ALG solution was not successful, as the PEG+PLL droplets tended to float at the air/Dex+ALG interface, hampering the formation of stable structures.

In the ALG and PLL-loaded phases, both polyelectrolytes can move freely in each solution and are able to partition to the contiguous aqueous phase at different extents, depending on their affinity. In fact, the control over polyelectrolytes’ affinity toward different phases has been suggested as a way to control capsules’ thickness and permeability properties.[20] Moreover, tailoring of the thickness of the capsule’s membrane has been achieved by varying the polyelectrolytes’ concentration. In a poly(allylamine hydrochloride)/poly(styrene sulfonate) (PAH/PSS) system, capsules thickness increased almost linearly with the polyelectrolyte’s concentration.[20] Here, the stability and robustness of the capsules was evaluated according to the systematic variation of both polyelectrolytes, as well as with the complexation times. Relevant aspects concerning the application of millimetric capsules in the context of cell encapsulation even for high cell densities (up to 25 million cells mL\(^{-1}\))—including their ability to withstand handling with a spatula (Figures S5 and S6, Supporting Information) and their resistance to contact with air—were visually inspected and systematically presented in a heatmap (Figure 2A). The effective formation of capsule-like structures and the achievement of robustness directly correlated with higher concentrations of the polyelectrolytes and seemed to be especially controlled by the alginate’s concentration. In fact, the use of 1.5% ALG enabled obtaining robust structures for 20 min of complexation, even in combination with the lowest concentration of PLL (0.5%). An interesting observation relied on the faster formation of robust micrometric structures by electrophoretic deposition, showcasing similar opacity to the optimized macrocapsules, but in periods of complexation as short as 5 min. Although the in/outflux of polyelectrolytes should be, in a primary analysis, mainly mediated by their molecular weight and molar concentration gradients, other phenomena should be considered. Chen and Dutches[56] stated that smaller sized droplets lead to a smaller diffusion boundary layer thickness, which reduces the diffusion time for polyelectrolytes to form the polymeric membrane in micrometric capsules. Furthermore, the curvature was also reported to have an impact on molecules’ transport to the interface, as in millimetric droplets (diameter higher than 1 mm) the spherical interface is of such order that the radius→∞ and approaches to a planar surface, creating a similar diffusion length scale for both phases, in contrast with the curvature in micro-
The ability to achieve robust structures with low concentrations of PLL was used as a criterion to select the 1.5% ALG + 0.5% PLL condition for cell encapsulation. High concentrations of PLL have been directly correlated to cytotoxic effects\cite{37,38} due to its high charge density. The polycationic interaction with the negatively charged cell membrane induces the formation of nanoscale holes and may increase its permeability by phospholipases’ activation.\cite{57,58} This formulation of polyelectrolytes was also tested for different complexation time. We sought the decrease of processing time, so a rapid method could be developed, while protecting cells from interacting with possible unreacted PLL that could enter the Dex + ALG droplet during the complexation reaction. While for complexation times lower than 10 min only fragile structures could be obtained, 15 min of reaction led to the formation of robust structures. Compared to LbL assembly of PLL/ALG/chitosan in liquified capsules, which takes 12 assembly steps (10 min per step),\cite{10} the structures described here outperform their robustness, with infrequent membrane rupture after exposure to rotational stimulus from 8 x g up to 769 x g, for 60 min. Compared to liquid-core capsules prepared by all-aqueous interfacial complexation, including structures prepared of synthetic strong polyelectrolyte pairs (e.g., PDMA and PSS, shell thickness \textapprox 150 nm)\cite{25} and weak polyelectrolytes (e.g., alginate and chitosan, shell thickness <1 µm), the macrocapsules characterized here show much higher thickness, measured in the order of hundreds of micrometers in the wet state. Importantly, to the best of our knowledge, this is also the first time that the robustness of liquid-core capsules fabricated by all-aqueous interfacial complexation is characterized and demonstrated.

Despite their robust behavior, capsules processed under the optimized condition targeting cell encapsulation permitted the fast diffusion of linear molecules (here, model dextran–FITC) with molecular weights up to 150 kDa. Such feature not only allows envisioning these capsules as drug delivery reservoirs, but also as cellular containers, where cells are amenable to be continuously oxygenated, capable of exchanging nutrient and metabolic waste, and also receiving (low-molecular weight) protein-mediated signals, including cytokines and even antibodies\cite{59–62} from the capsules’ surrounding environment. The combination of i) mild processing technique, ii) robustness, iii) permeability to protein-sized molecules, and iv) compatibility with coencapsulation of cells and cell-adhesive microcarriers enabled envisioning this system as a promising platform for cellular encapsulation and long-term 3D culture, including in a bioreactor dynamic environment. In fact, the analysis of an early time point—1 day after culture—showed that the encapsulation process displayed low cytotoxicity. Longer-term analysis up to 21 days (and 60 days for static culture; Figure S4, Supporting Information) corroborated the adequacy of the system as a support for the expansion and culture of hWJ-MSCs. The effect of the addition of microcarriers as encapsulated cargo tailored cell response in microcapsules by accelerating the formation of cellular aggregates with lower number dispersion than their microcarrier-free counterparts. Additionally, the incorporation of microcarriers delayed the appearance of necrotic core structures in cellular aggregates. Stirred tank culture led to increased cell metabolic activity by the total number of cells, in agreement with previous literature’s reports that correlate shear stress and enhanced molecular exchange with faster cell expansion.\cite{63–66} Moreover, the secretion of key regenerative and angiogenic factors by hWJ-MSCs was tailored, overtime, by culturing cells in different regimes. The adequacy of the developed capsules as supports for long-term cell culture up to 60 days was proved. Cellular adhesion to the capsules’ walls was probably promoted by the presence of PLL in the biomaterial, previously reported as a promoter of cell adhesion.\cite{167} Encapsulated cells forming multicellular/microcarrier aggregates caused the rupture of the capsules’ walls after adhesion, in events apparently led by the mechanical stretching of the capsules by these micro- to millimetric-sized cell-rich newly formed structures. Interestingly, the escape of these cellular aggregates from the capsule environment did not lead to the catastrophic breakage of the biomaterial structure. One may take advantage of this unusual phenomenon to design innovative time-morphing cellular systems that initially acts on the basis of paracrine-only contact and, by the sole action of encapsulated cells (in this case, their ability to rupture a biomaterial involucrum), become cell-delivery vehicles.

The developed capsules with different size ranges and multi-compartment features may be used as versatile in vitro supports (with or in the absence of microcarriers) for cell expansion, retrieval of valuable conditioned medium, or cell differentiation. On the other hand, these structures may also act as permanent or temporary templates to generate cellular aggregates that may be implanted for regenerative and therapeutic purposes.

4. Conclusion

In summary, we report the one-step fabrication of electrostatically complexed liquid-core robust capsules in mild and cytocompatible conditions. Millimetric capsules were prepared by a simple dropwise addition, and miniaturized structures were obtained by an electrohydrodynamic atomization methodology compatible with continuous production and high yield. The rapidly fabricated capsules showcased permeability and enabled the successful encapsulation of hWJ-MSCs, with high clinical relevance, and their coentrapment with polymeric cell-adhesive microcarriers. The preparation of these hierarchically organized structures enabled long-term culture of viable hWJ-MSCs, including in a stirred tank configuration. We demonstrated the possibility of processing multiscale compartments with low quantity of biomaterials that could be used in bottom-up tissue engineering strategies.\cite{68} These results establish a proof-of-concept for the use of compartmentalized microcarrier-laden capsules for the culture of adherent cells under biotechnologically relevant setups, including 3D dynamic culture. This newly described technology may find application in stem cell expansion and differentiation, as well as in a myriad of therapeutic purposes and biotechnological applications based on cell encapsulation.

5. Experimental Section

Experimental Design: The production of liquid-core capsules was based on the electrostatic complexation of two oppositely charged polymers at the interface of an aqueous two-phase system. The composition of each ATPS’ phase followed the phase diagram reported elsewhere.\cite{69}
Two separate phases were prepared: i) a dispersed phase—Phase I—which consisted of Dextran Leuconostoc spp. (15% (w/w), Mw ≈ 500 kDa, Sigma-Aldrich) mixed with alginate (1.5% (w/v), alginic acid sodium salt from brown algae, 71238-0250, Sigma-Aldrich) dissolved in Dulbecco’s phosphate-buffered saline (DPBS, Corning, pH 7.4), and at RT. For cell encapsulation experiments, the solution was dissolved in cell culture medium; ii) a continuous phase—Phase II—comprising poly(ethylene glycol) (17% (w/w), Mw ≈ 8 kDa, Sigma-Aldrich) mixed with ζ-polyl-lysine (0.5% (w/w), Mw ≈ 4.7 kDa, Epolyly Pure, Handary S.A.) dissolved in DPBS, pH 7.4. The pH of both phases was adjusted after complete dissolution of the polyelectrolytes to pH 7.4 with 10 M NaOH.

Two methodologies were used to produce biocompatible capsules with distinct sizes: i) “macrocapsules” (millimetric-sized capsules) were fabricated by the dropwise addition of Phase I, using a 21G needle, to a stirring bath of Phase II, at 350 rpm. Complexation occurred for 15 min before washing cycles with DPBS, under agitation; ii) “microcapsules” (micrometric-sized structures) were prepared using an electrohydrodynamic atomization (Spraybase, Avectas) equipment. Phase II was used as a collector bath and complexation occurred for 5 min. The operating parameters were a flow rate of 10 mL h⁻¹, 22G needle, working distance (tip to collector distance) of 10 cm, and 10 kV of voltage.

ζ-Potential’s Measurements: The ζ-potential of the phases with and without polyelectrolytes, freshly made or stored for one week at 4 °C, was measured by Malvern Zetasizer Nano Zs equipment (Malvern Instruments Ltd., Malvern, United Kingdom) at 25 °C. Each value was obtained in quadruplicates as an average from three subsequent runs of the equipment with, at least, 10 measurements.

Rheological Characterization of Polyelectrolyte-Loaded ATPS: Phases: The response of both polyelectrolyte-loaded ATPS Phase I and Phase II to different shear rates was quantitatively assessed by a dynamic oscillatory rheology assay. The rheological measurements were collected using a Kineexus Pro+ Rheometer at room temperature, using a stainless-steel parallel plate geometry. Complex viscosity was recorded and analyzed.

Production and Surface Characterization of Microcarriers: PCL microparticles were produced by an emulsion solvent evaporation technique, as described elsewhere. Briefly, a solution of PCL 80 kDa macromolecular weight (5% (w/v), Merck) in methylene chloride (Honeywell) was gradually added to a stirring solution of polyvinyl alcohol (0.5% (w/v), Merck), and left under agitation for 2 days at RT. A sieve was used to select microcarriers with diameters between 40 and 50 µm, which were thoroughly washed with distilled water, followed by 3 washing cycles with ethanol 100% (v/v) and dried overnight at RT. The surface of the microcarriers was first modified by ion coupled plasma treatment. Microparticles were placed inside a low-pressure plasma reactor chamber (Plasma System Corp.) and air was used as the working atmosphere to generate a glow discharge at 0.2–0.4 mbar, 30 V, for 15 min. The process was stopped every 5 min, so PCL microparticles were repositioned by shaking to improve the homogeneity of surface’s modification. Following this process, microcarriers were disinfect by an overnight immersion in 70% (v/v) ethanol at RT. After thoroughly washing with sterile DPBS, PCL microparticles were incubated in a solution of type I collagen (10 µg cm⁻², collagen type I from rat protein tail, Sigma-Aldrich, prepared in 20×10⁻³ M aqueous acetic acid solution (Chem-Lab NV)) for 4h, at 37 °C. The prepared microcarriers were then washed 3 times with sterile DPBS to remove excess collagen and stored at 4 °C until encapsulation assays.

Evaluation of Capsules’ Mechanical Resistance: The resistance of macromacroparticles to shear stress was evaluated using a rotational test. For each experimental condition, 10 capsules were placed in centrifuge tubes (in triplicates) containing DPBS (5 mL). The tubes were rotated at a speed of 8 x g for 60 min. Every 15 min, the number of damaged capsules was counted by naked eye observation. An additional rotation cycle at 769 x g for 15 min was performed and the number of damaged capsules was quantified. After selection of the best processing parameters for the production of macromacroparticles, the effect of cell density on its robustness was also assessed by encapsulating 15 million or 25 million of MC3T3-E1 (ATCC, LG Standards) cells, following the same methodology.

Characterization of Capsules’ Permeability’s Properties: Fluorescein isothiocyanate–dextran (Dextran–FITC, Sigma-Aldrich) with different average molecular weights—10, 40, 70, 150, and 500 kDa—were mixed with Phase I at a concentration of 1 mg mL⁻¹. Macromacroparticles were prepared using the previously described method using this solution. For release studies, DPBS (5 mL) was added to 10 capsules, in triplicates, and incubated at 50 rpm at 37 °C. For every time point, 100 µL of the release medium was collected to a white opaque reading plate (Corning) and 100 µL of fresh DPBS was added to the flask. Fluorescence was read at an excitation of 490 nm and an emission of 520 nm. Data were presented as the percentage of the released mass’ cumulative overtime, considering a total loading efficiency of dextran–FITC’s molecules (neglectable fluorescence values were detected on the Phase II solution retrieved after the preparation of capsules).

Macrocapsules’ Diameter’s Measurements: Macrocapsules’ diameter was measured for a total of 70 capsules using ImageJ image analysis software (NIH, USA).

Macrocapsules’ Thickness Measurement: Macrocapsules’ thickness was measured for a total of 25 capsules (5 from each of a total of 5 independent productions). Samples were observed under a stereomicroscope (Stemi 508, Zeiss) while a micrometer was used to measure their thickness while hydrated.

Microcapsules’ Diameter’s Measurements: The size of microcapsules was measured by analysis of microscopy images using the ImageJ image analysis software. The determined average diameter corresponded to the measurement of a total of 30 microcapsules, 10 microcapsules from each of a total of 3 independent productions.

Morphological Analysis of Capsules: Capsules were cut in their length to generate observable cross-sections using a surgical blade. Capsules were then dehydrated by immersion in an ethanol’s concentration’s gradient (30% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), 96% (v/v), and 100% (v/v)) for 15 min each and mounted on scanning electron microscopy (SEM) supports using a double-sided carbon tape. Further gold sputtering was performed for 3 min. SEM (Hitachi, SU-70 instrument) was used at an accelerating voltage of 8 kV.

Preparation of Hierarchical Structures: Microcapsules Encapsulated in Macrocapsules: Microcapsules were prepared by EHD using a collector bath of PEC (17% (w/w)) and PLL (0.5% (w/w)) in DPBS, under agitation, and a dispersed phase of dextran (15% (w/w)) and alginate (0.90% (w/w)) in DPBS. The operating parameters used were a flow rate of 10 mL h⁻¹, 24G needle, working distance of 10 cm, and 10 kV of voltage. The complexation occurred for 5 min. These microsized structures were thoroughly washed with DPBS and resuspended in Phase I (15% (w/v) dextran and 1.5% (w/v) alginate in DPBS) for the macromacroparticles production following the previously described procedure.

Cell Isolation and Characterization: One UC was obtained from healthy women undergoing Cesarean section for the worst infant delivery, either by C-section or vaginal birth, and after signing the informed consent. The collected tissues were obtained under a cooperation agreement between COMPASS Research Group from CICECO-University of Aveiro and Aveiro local hospital Centro Hospitalar do Baixo Vouga, after approval of the hospital Ethics Committee (EC). The UCs were transported to the laboratory facilities in phosphate-buffered saline (PBS, ThermoFisher Scientific) supplemented with penicillin–streptomycin (10% (v/v), ThermoFisher Scientific) and handled according to guidelines approved by the EC. Samples were processed under sterile conditions with 24 h after delivery. MSCs were isolated from the Wharton’s jelly (MSCs-WJ) using the explant method. For the use, the UC was washed with sterile PBS to remove blood and blood clots, and subsequently cut into small pieces of 3 cm. After removal of the vein and two arteries, small pieces of the WJ were transferred to cell adhesive petri-dishes. The tissues explants were incubated at 37 °C in a humidified atmosphere of 5% of carbon dioxide for 2 h. Minimum essential medium alpha (α-MEM) supplemented with antibiotic–antimycotic (1% (v/v), ThermoFisher Scientific) and heat-inactivated fetal bovine serum (10% (v/v), ThermoFisher Scientific) was added until immersion of the tissue pieces. After 24 h of culture and 24 h of culture, the tissue explants were removed from the petri-dish. At 90% confluence, WJ-MSCs were detached using trypsin–EDTA solution (ThermoFisher Scientific) and expanded at a density of 5 × 10⁶ cells cm⁻². Afterward, the successful isolation of WJ-MSCs was characterized by flow cyto.

2100266 (10 of 11)

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tometry (BD Accuri C6 Plus). At 90% confluence, WJ-MSCs were detached (TrypLE® Express, ThermoFisher Scientific) and resuspended in DPBS containing bovine serum albumin (2% (v/v), Merk) and sodium azide (0.1% (v/v), TCI). Cells were then incubated with CD73 (PE-conjugated, Biolegend), CD90 (AlexaFluor 647-conjugated), CD34 (FITC-conjugated), and CD31 (APC-conjugated) antibodies according to manufacturer’s spec-
ifications (Biolegend). After 45 min at RT, samples were washed in DPBS, and resuspended in DPBS containing formaldehyde (1% (v/v), Sigma-Aldrich) and sodium azide (0.1% (v/v)) for analysis. Flow cytometry results confirmed the successful isolation of a population of MSCs highly expressing the stemness markers CD90 (92.3%) and CD73 (93.4%), while lacking the hematopoietic and endothelial markers CD34 (0.4%) and CD31 (2.1%), respectively.

Cell Encapsulation: The ATPS’ phases, without added polyelectrolytes, were filtrated with a 0.2 μm sterile filter (Whatman Puradisc Ø30 mm, Zmed). ALG and PLL powders were exposed to UV radiation for 40 min. The process was stopped after 20 min and both polyelectrolytes were repo-
ishoned by shaking to improve the homogeneity of sterilization. Afterward, they were respectively added to each ATPS’ phase, at the previously de-
scribed concentrations. Sterilized surface-functionalized PCL microcarri-
ers were then added at a concentration of 30 mg mL\(^{-1}\) to Phase I and resuspended using a micropipette. WJ-MSCs (passage 4) at 90% of con-
fluence were washed with DPBS and chemically detached using trypsin–
EDTA solution (Merck) for 5 min, at 37 °C. WJ-MSCs were suspended in Phase I with PCL microcarriers at a cell density of 5 × 10^6 cells mL\(^{-1}\). Macro capsules were then processed using the aforementioned method. After complexation, excess of Phase II was removed by 3 washing steps (5 min each) using sterile DPBS. Cell-laden macro capsules were cultured in supplemented α-MEM medium, in well-plates (static condition) or in a spinner flask at 50 rpm (Celstr, Wheaton). For static culture, 4 capsules were cultured in 1 mL of supplemented α-MEM medium, in triplicates for each time point. For dynamic assays, a total of 72 capsules were added to 75 mL of supplemented α-MEM medium. Both assays were conducted for 21 days, in a humidified 5% CO\(_2\) air atmosphere, at 37 °C. Cell culture medium was replenished every 2 days.

The preparation of solutions for EHDA processing followed the same procedure as the ones used for macrocapsules. The equipment’s oper-
ating parameters were set for a flow rate of 10 mL h\(^{-1}\), 22G needle with blunt end, working distance (tip of the needle-collector) of 10 cm, and 10 kV voltage. Cell-laden microcapsules were cultured in static complete α-MEM medium, in a well-plate for 14 days, in a humidified 5% CO\(_2\) air atmosphere, at 37 °C. Cell culture medium was replenished every 2 days.

Assessment of Cellular Organization and Morphology: For the spatial or-
ganization of encapsulated cells, phalloidin red (5:200 in PBS, Biolegend) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:1000, 1 mg mL\(^{-1}\) in DPBS, Thermo-Fisher Scientific) were used to display the F-actin distribution in cell’s cytoskeleton and their nuclei, respectively. Capsules in culture for 1, 3, 7, 14, and 21 days were washed with DPBS, fixed with formalin (4% (v/v)), for 2 h, at RT, and permeabilized for 5 min with Triton-X (0.1% (v/v), Merck) at RT. Samples were then incubated with phalloidin red for 45 min, at 37 °C, washed with DPBS, and finally stained with DAPI for 5 min, at RT. The structures were analyzed by fluorescence microscopy (Axio Imager 2, Zeiss).

DNA Quantification: Macrocapsules cultured for 21 days were ana-
yzed for their DNA amount in each studied timepoint. To promote cellu-
lar lysis and extract dsDNA, four capsules per time-point, in triplicates, were immersed in ultrapure water with Triton-X (0.2% (v/v)) and in-
cubated for 1 h at 37 °C. Afterward, samples were frozen at −20 °C and kept stored until quantification, following manufacturer’s specifications (Quant-iT PicoGreen dsDNA assay kit, Thermo Fisher Scientific). To plot a standard curve, a solution of a JDNA standard provided by the manu-
facturer was used. After 10 min of incubation at RT, fluorescence was read at an excitation wavelength of 485/20 nm and an emission of 528/20 nm, using a microplate reader.

Characterization of Growth Factors Secreted by Encapsulated hWJ-MSCs: The commercially available LEGENDplex Human Growth Factor Panel (Bi-
olegend), a bead-based multiplex assay based on the use of fluorescence-encoded beads suitable for use on flow cytometers, was used to deter-
mine the concentration an array of six human growth factors in cell culture medium after 1, 7, and 21 days of cell culture. Samples were prepared and analyzed accordingly to the manufacturer’s instructions.

Statistical Analysis: All data were expressed as mean ± standard devi-
tion. Two-way ANOVA test with multiple comparison test was applied to identify significant differences between static and dynamic conditions for the biological assays. A p-value < 0.05 was considered statistically signif-
icant using GraphPad Prism 6.0 software. MTS for cell metabolic activity analysis and DNA quantification results were grouped by timepoint, for static and dynamic environment, respectively, to analyze significant differences between conditions.