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ARTICLE

Acoustic levitation as a tool for cell-driven self-organization of human cell spheroids during long-term 3D culture

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Abstract

Acoustic levitation, which allows contactless manipulation of micro-objects with ultrasounds, is a promising technique for spheroids formation and culture. This acoustofluidic technique favors cell-cell interactions, away from the walls of the chip, which leads to the spontaneous self-organization of cells. Using this approach, we generated spheroids of mesenchymal stromal cells, hepatic and endothelial cells, and showed that long-term culture of cells in acoustic levitation is feasible. We also demonstrated that this self-organization and its dynamics depended weakly on the acoustic parameters but was strongly dependent on the levitated cell type. Moreover, spheroid organization was modified by actin cytoskeleton inhibitors or calcium-mediated interactions inhibitors. Our results confirmed that acoustic levitation is a rising technique for fundamental research and biotechnological industrial application in the rapidly growing field of microphysiological systems. It allowed easily obtaining spheroids of specific and predictable shape and size, which could be cultivated over several days, without requiring hydrogels or extracellular matrix.

KEYWORDS

acoustofluidics, acoustic levitation, self-organization, spheroids, microphysiological system

1 | INTRODUCTION

Spheroids are *in vitro* multicellular spherical structures formed by self-assembly. Due to their three-dimensional (3D) arrangement, they exhibit a higher structural complexity than traditional two-dimensional (2D) cultures (Ryu, Lee, & Park, 2019). Although they display evident limitations, because they fail to mimic *in vivo* cell composition and size, spheroids provide a useful overview of cell behaviors and functions in 3D configurations. What they lack in complexity, they compensate in simplicity, reproducibility and ease of use.

Spheroids can be cultured in microfluidic chips, which provide several advantages: a controlled environment, the possibility of continuous

monitoring by the integration of live microscopy and sensors, an efficient control of fluid flow and shear stress and precise dosage during drug screening (Skardal, Shupe, & Atala, 2016; Sakai et al., 2014). These innovative microphysiological systems are an excellent tool for fundamental research, such as self-organization, organogenesis and developmental biology studies. Absorption, distribution, metabolism, excretion and toxicity (ADMET) tests for drug development are also keen to integrate spheroids into their pipeline. Furthermore, spheroids are the first step in the path towards engineering complex tissues for regenerative medicine (Clevvers, Lancaster, & Takebe, 2017; Takebe & Wells, 2019; Hafiz et al., 2021; Huch, Knoblich, Lutolf, & Martinez-Arias, 2017).

Several techniques have been developed to create spheroids, such as suspension cultures, hanging drops, low-attachment surfaces, scaffolds and magnetic levitation (Ramos et al., 2023; Li et al., 2019; Kelm, Timmins, Brown, Fussenegger, & Nielsen, 2003). Recently, acoustic levitation has emerged as an additional method to form and culture spheroids. This externally driven assembly technique (Soto et al., 2021) prevents direct contact or interaction of the cells with the solid walls. It consists in a contactless manipulation mediated by ultrasonic standing waves. The standing waves are obtained by matching the acoustic

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; ADMET, absorption, distribution, metabolism, excretion and toxicity; ALARA, as low as reasonably achievable; AR, aspect ratio; ARF, acoustic radiation force; EDTA, ethylenediaminetetraacetic acid; EGM2-mv, endothelial cell growth medium 2; EGTA, egtazic acid; FBS, fetal bovine serum; Hz, hertz; LSEC, liver sinusoidal endothelial cell; MEM, minimum essential medium; MSC, mesenchymal stromal cell; NCAM, neural cell adhesion molecule; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; PDMS, polydimethylsiloxane; ROCK, rho-associated protein kinase.

wavelength with the height of a resonant cavity. The use of ultrasounds does not require any marker or hydrogel.

Ultrasonic standing waves of a few megahertz can produce an acoustic radiation force (ARF) able to manipulate small particles as well as mammalian cells.

Up to now, acoustic levitation of cells had been mainly used to pattern cell structures into hydrogels, or to aggregate cells for a few minutes (Olofsson, Carannante, Takai, Önfelt, & Wiklund, 2021; Chen et al., 2019; Liu et al., 2007; Edwards, Bazou, Kuznetsova, & Coakley, 2007; Bazou, Coakley, Hayes, & Jackson, 2008; Bazou, 2010; Kuznetsova, Bazou, Edwards, & Coakley, 2009; Khedr et al., 2019; Comeau, Hocking, & Dalecki, 2016). We and others recently showed that this technique could be used to culture mesenchymal stromal cells (MSCs) in acoustic levitation up to 24 hours (Jeger-Madiot et al., 2021; Im, Kim, Lee, & Bhang, 2022).

In our devices, we used a 2 MHz ultrasound broadband transducer, in order to create a multi-mode acoustic standing wave in a cavity of 10 mm of height. Cells in suspension can be levitated and trapped into the multiple acoustic pressure nodes. The acoustic frequency is tuned to match the resonance condition leading in general to 30 levitation planes for an acoustic frequency of 2.2 MHz, resulting in the formation of 25 to 30 spheroids in acoustic traps.

In this case, cells are aggregated by the external acoustic radiation force, and then undergo a cell-driven self-assembly while being levitated. It has been shown that MSCs can then self-organize freely, from cell sheets to spheroids (Jeger-Madiot et al., 2021), similarly to what had been previously reported in magnetic levitation (Lee & Hur, 2014). MSCs were maintained in levitation during hours without affecting their viability or functionality (Jeger-Madiot et al., 2021).

We investigated for the first time longer cell culture times in acoustic levitation, up to 6 days. We performed a comparative analysis of three cell types. We continued the study of MSCs, which are broadly used for tissue engineering, cell therapy and tumor modeling (Ryu et al., 2019). We also investigated human umbilical vein endothelial cells (HUVEC), which are a lever of action to vascularize spheroids and upscale tissue engineering. Thirdly, considering the high potential of liver-on-chip for cell therapy, drug testing and fundamental research (Brooks et al., 2021; Beckwitt et al., 2018; Sae-be et al., 2023; Prior, Inacio, & Huch, 2019; Zuchowska, Kwapiszewska, Chudy, Dybko, & Brzozka, 2017), we levitated HepaRG cells (Andersson, Kanebratt, & Kenna, 2012).

In addition to their viability, the dynamics of spheroids formation in acoustic levitation were studied for these various cell types. We evaluated the role played by the acoustic parameters, as well as biological mechanisms through inhibition of actin- or E-cadherin-mediated mechanisms, on the dynamics of spheroids formation. This study paves the way to investigate the potential of acoustic levitation for fundamental and applied research in cell biology.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Cells in 2D or levitation were cultivated in incubators at 37°C and 5% CO₂. All types of cells were amplified in 2D culture before levitation. Cells were passaged using 0,25% trypsin + ethylenediaminetetraacetic acid (EDTA) (Gibco, UK).

Adipose derived mesenchymal stromal cells (MSCs) were isolated from tissue of the thigh according to the protocol presented in (Jeger-Madiot et al., 2021). MSCs were isolated from the adipose tissue of a male donor, obtained at Necker-Enfants Malades hospital in Paris, France. The adipose tissue was the surgical leftover and was used for research purposes after a signed consent from the donor and his parents as the legal tutors, according to the French bioethical and medical research regulations. Cells were amplified in α -MEM supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaillé, France) and 1% antibiotic/antimycotic mix (Anti-Anti 100 \times , Gibco, Thermofisher, Waltham, United States). Cells were passaged at 80% confluency.

HUVEC, human umbilical vein endothelial cells (Lonza), were cultivated using Endothelial Cell Growth Medium 2 (EGM2-mv). Cells were passaged at 80% confluency.

HepaRG cells, provided by Biopredic International (Biopredic, St. Grégoire, France) were cultivated in basal hepatic cell medium (Biopredic) with 10% of HepaRG growth medium supplemented with antibiotics (Biopredic). Medium was renewed every 3 days. Cells were detached and passaged every 14 days.

Monolayers were cultured in 75 cm² culture flasks (Corning, Falcon, NY, USA). Levitated spheroids were formed and cultured in our acoustofluidic chip during several days.

2.2 | Microphysiological system: Acoustofluidic chip

The microphysiological system used consists in a spheroid-on-a-chip device placed in an incubator, with automated medium renewal and timelapse video recording (Fig. 1).

The chips were fabricated in Polydimethylsiloxane (PDMS, RTV 615, Neyco, Vanves), a transparent, gas permeable, biocompatible material that does not strongly reflect acoustic waves. PDMS (1:10 curing agent:base ratio) was poured in a plexiglass mold, heated at 70°C for 24h, then bonded with a plasma cleaner on a microscope glass slide acting as an acoustic reflector.

Waveform generators (Handyscope HS5, TiePie Engineering, Sneek, Netherlands) were used to power ultrasonic transducers (8 mm diameter, 2 MHz, SignalProcessing, Savigny, Switzerland) with a sinusoidal waveform of amplitude ranging from $U = 3 V_{pp}$ to $U = 8 V_{pp}$. Amplitudes higher than $8 V_{pp}$ induce a strong increase in temperature in the system that could hamper cell viability. Amplitudes below $3 V_{pp}$ are too low to levitate cells in our system. When using $3 V_{pp}$, the system is sensitive to

disturbances such as sudden moves. Therefore, the smallest amplitude ($3.5 V_{pp}$) was used for most cultures, in order to be as gentle as possible with the cells, according to the As Low As Reasonably Achievable principle (ALARA). The frequency used was $F_{ac} = 2.2$ MHz, a value contained in the levitation range from 1.5 to 2.5 MHz, as shown in (Jeger-Madiot et al., 2022; Dron & Aider, 2013) and maximizing the acoustic energy (matching of the resonance condition). After emission by the transducer, the acoustic wave travels through a thin oil layer, used as an acoustic matching layer, and a PDMS membrane, before reaching the bulk of the cavity filled with cell medium. The height of the cavities was $h = 10$ mm leading to 30 levitation planes, i.e. 25 to 30 spheroids per chip.

The chip was perfused with a constant flow of $10 \mu\text{L/h}$ with a syringe pump (Pump 11 Elite from Harvard Apparatus, Holliston, United States) in order to renew the medium every three days, to mimic the rate of medium change in 2D cultures.

Timelapses of the spheroids formation inside the chip were obtained using Dino-Lite Pro 630-1165 USB portable Microscopes (Dinolite company, Taipei, Taiwan). One image was recorded every 10 minutes during several days.

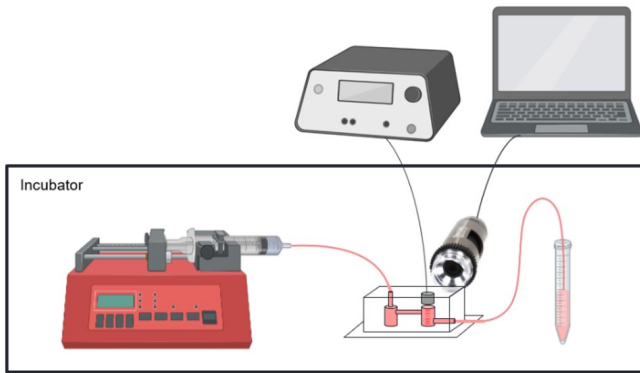


FIGURE 1 Acoustofluidic microphysiological system. The microphysiological system is placed inside an incubator (37°C , $5\% \text{CO}_2$). Culture medium is provided via tubing (flow : $10 \mu\text{L/h}$). The acoustic parameters are the same and kept constant during the culture (Amp = $3.5 V_{pp}$ / $F_{ac} = 2.2$ MHz). Created with BioRender.com.

2.3 | Viability study

After turning the ultrasound off and removing the spheroids from the chip by aspiration, spheroids were centrifuged at $284 g$ during 5 minutes at 20°C . In order to assess the cell viability after 3 days of levitation, spheroids were stained with calcein and ethidium bromide (LIVE/DEAD mammalian viability kit, Montlucon, France) and observed in an Incucyte S3 imaging system (Sartorius). Culture medium was removed from 2D

cultures and spheroids, followed by dissociation with 0.25% trypsin during 15 minutes. After suspension in PBS (Eurobio, Courtaboeuf, France), viability of individual cells was evaluated via flow cytometry (Attune® NxT Acoustic Focusing Cytometer). Calcein+/Ethidium- cells are considered as "viable", Calcein-/Ethidium+ as "non-viable", double stained cells as "in initiation of cell death", and non-stained as "undetermined".

2.4 | Levitation with EDTA and Y-27632

The calcium and magnesium chelator ethylenediaminetetraacetic acid (EDTA, 0.1 mM) was used to modify the access of cells to calcium and disrupt calcium-mediated junctions such as E-cadherins.

To disrupt actin cytoskeleton functionality and remodeling, various concentrations ($40 \mu\text{M}$ and $60 \mu\text{M}$) of rho-associated protein kinase (ROCK) inhibitor Y-27632 were added to the culture medium.

2.5 | Shape and size description

To characterize the time evolution of the shape of the spheroids, we retrieved circularity (σ), aspect ratio (AR), width and height from ImageJ analysis.

The circularity σ of the object seen from the side is defined as:

$$\sigma = 4\pi \frac{A_r}{P_e^2} \quad (1)$$

with A_r the area of the spheroid and P_e its perimeter. By definition, $\sigma = 1$ corresponds to a perfectly circular object, while $\sigma = 0$ for a planar cell sheet.

To simplify the analysis, the aggregates will be considered as ellipsoids of major axis a , minor axis c (also the height of the ellipsoid) and transverse radius b (Fig.2). For a relatively flat ellipsoid, with $a = 10c$, the circularity is $\sigma = 0.2$

The Aspect ratio is then simply defined as :

$$AR = \frac{a}{c} \quad (2)$$

The surface area of the ellipsoid is calculated using the Knud Thomsen's Formula (relative error $< 1.42\%$) and using the hypothesis of a discoid ($b = a$) :

$$SA = 4\pi \left(\frac{a^{2p} + 2a^p c^p}{3} \right)^{1/p} \quad (3)$$

with $p = 1.6075$, a the half width of the spheroid and c the half-height from a side-view.

The corresponding volume was calculated using the formula:

$$V = \frac{4}{3} \pi a^2 c \quad (4)$$

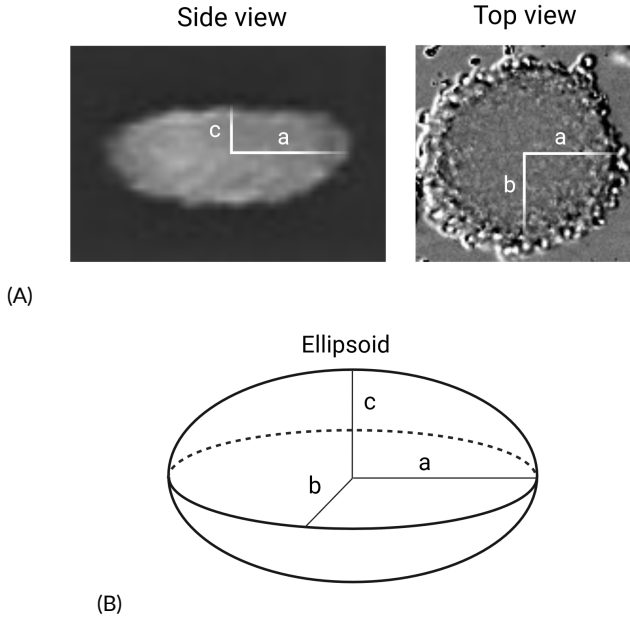


FIGURE 2 A) Side view and top view of a cell aggregate formed in acoustic levitation. B) Sketch of an ellipsoid with the three main radii.

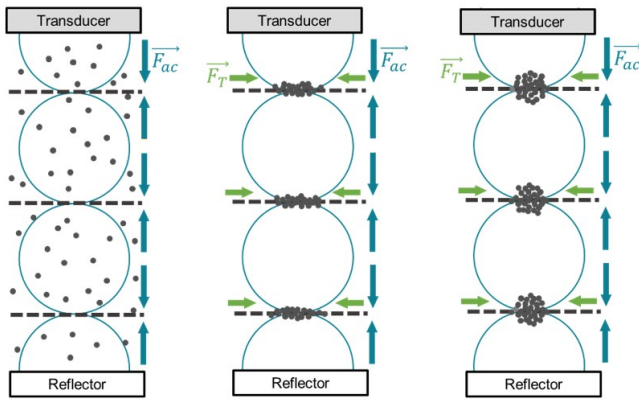


FIGURE 3 Sketches showing the three steps of acoustic manipulation (from left to right) by an ultrasonic standing wave of a few megahertz. First, the axial component (F_{ac}) of the acoustic radiation force (ARF) forces the particles or cells (with positive acoustic contrast factor) to move toward the acoustic pressure nodes. Once the particles or cells have reached the pressure nodes (levitation planes), they form monolayers thanks to the transverse component of the ARF (F_T). While particles form stable planar aggregates, cells spontaneously self-organize into spheroids that can be kept in acoustic levitation.

3 | THEORETICAL ASPECTS : ACOUSTIC RADIATION FORCE

To create an acoustic force field inside a cavity, one has to create an acoustic standing wave. The basic principle consists in emitting an acoustic wave inside the cavity, using an ultrasonic transducer. If a reflector is properly placed, facing the transducer at a distance h matching the resonance condition ($h = n\lambda_{ac}/2$, with λ_{ac} the acoustic wavelength), then an acoustic standing wave is created (Fig. 3). The particles or cells inside the cavity are then first submitted to the axial component of the ARF (F_{ac}) which forces them to move toward the acoustic pressure nodes (levitation planes) which are spaced by half a wavelength. Once the cells or particles have reached the acoustic levitation planes the transverse component of the ARF (F_T) forces them to move radially inside the levitation plane toward the local maximum of acoustic energy (located along the center of the cylindrical cavity) where they form an aggregate. The axial component is roughly 100 times stronger than the transverse component. The cells rapidly form a disk-like aggregate in which they can interact with neighboring cells. The confluency, as defined in a culture flask, is obtained within seconds. Once the discoidal aggregate is formed, it is consolidated by the Bjerknes force (short distance acoustic force between objects), as well as the cellular adhesion forces.

The most important component of the ARF, responsible of the acoustic levitation, is the axial component. It can be written as (Yosioka & Kawasima, 1955):

$$F_{ac} = \frac{\pi}{4} E_0 k d_p^3 F_Y \sin(2kz) e_z \quad (5)$$

where E_0 is the acoustic density energy, k is the acoustic wave number, d_p is the diameter of the particles / cells, F_Y is the acoustic contrast factor and z is the axial coordinate.

This expression is valid for circular, compressible particles, which is a reasonable approximation for cells floating in a medium. The particles are also considered as small compared to the acoustic wavelength (Rayleigh approximation). One can see from Eq. 1, that large particles will undergo a larger ARF than smaller particles. The acoustic contrast factor, which depends on the density and compressibility of the particles or cells, plays also an important role. It is defined as:

$$F_Y = F_K + F_Y = \frac{1 + \frac{2}{3}(1 - \frac{\rho_f}{\rho_p})}{2 + \frac{\rho_f}{\rho_p}} - \frac{\rho_f c_f^2}{3 \rho_p c_p^2} \quad (6)$$

where ρ_p and ρ_f are respectively the density of the particles and of the fluid, and c_p and c_f are the celerity of acoustic waves in the particles and the fluid. The larger the differences between the properties of the particles and the fluid are, the larger the ARF is. The acoustic contrast factor of cells is unknown, but it can be measured with a specific protocol based on the measurement of the acoustic focusing velocity of a given cell toward the acoustic pressure node (Bellebon et al., 2022). The averaged value of acoustic contrast factor of adipose tissue-derived MSCs was 0.15 after at least three passages of culture. Unfortunately, this protocol could not be carried out for the cells used in this study. If

the particles are passive (no propulsion, no interactions), with a positive contrast factor, they aggregate into a 2D disk which can be maintained in a steady position in acoustic levitation indefinitely. If the objects are living cells, it has been shown that they spontaneously self-organize into a spheroids, as shown by (Jeger-Madiot et al., 2021) with MSCs.

4 | RESULTS

4.1 | Self-organization: from cell sheets to spheroids

In the acoustofluidics chips, the ARF creates a confluent layer of cells in a few seconds, fostering immediate interactions between cells. Such cell clusters adopted a spontaneous collective behavior leading to self-organization. When maintained in acoustic levitation during several hours, aggregates of cells spontaneously self-organize, turning the originally 2D discoids into 3D spheroids.

Self-organization dynamics were shown to strongly depend on cell types (Fig. 4). While cell sheets formed during the first minutes of levitation always turned into spheroids after 60 hours, HepaRG spheroids were flatter than those obtained with MSCs and HUVEC (Fig. 4.A). We then evaluated the evolution of the circularity as a function of time (Fig. 4.B). The dynamics depends strongly on the cell type: MSCs and HUVEC spheroids are formed relatively quickly. The circularity of MSCs reaches its asymptotic value after 40 hours, while the circularity of HUVECs reaches its asymptotic value after only 6 hours in acoustic levitation. Nevertheless, spheroids of MSCs are less "circular" ($\sigma = 0.7$) than spheroids of MSC ($\sigma = 0.9$). Contrarily, HepaRG showed a very slow increase of the circularity, reaching $\sigma = 0.6$ after 60 hours in acoustic levitation.

Interestingly, the coculture of the HepaRG with HUVEC exhibited a different evolution, with a sharp increase of the circularity after 6 hours. Aggregates of cocultures with a 1:1 ratio of HepaRG and HUVEC displayed a behavior similar to HepaRG during the first 6 hours of culture, followed by a sharp increase up to 40 hours of levitation. These results demonstrated the synergy between the two cell types and the importance of the endothelial cells to accelerate the self-organization of hepatocytes.

After the initial increase, the circularities of the spheroids reached a plateau for all cell types. All asymptotic values of circularity were higher than 0.7 at the end of the culture in levitation. Nevertheless, the asymptotic value was again dependent on the cell type, since it was reached after 6 hours for HUVEC, 40 hours for MSCs, 40 hours for cocultures and 120 hours for HepaRG.

We then evaluated the aspect ratio (AR) of the spheroids (Fig. 4.C). For all studied cell types, AR decreased over time. Similarly, a decrease of surface area and volume was observed (Fig. 4.D and Fig. 4.E), indicating a compaction of the aggregates. In all cases the volume decreased but the reduction was particularly important for MSCs and HUVEC.

4.2 | Disruption of the self-organization mechanisms

In order to study more thoroughly the mechanisms responsible for the self-organization, either physical or biological, variations of voltage, and treatment with calcium chelator EDTA or ROCK-inhibitor Y-27632 have been performed.

4.2.1 | Influence of the amplitude of the ARF

It has been shown that ultrasounds, including those of frequency approaching 2 MHz, impacted various mechanosensitive ion channels involved in self-organization of tissues (Chu, Lim, Chien, Chen, & Wang, 2022; S. Kim et al., 2022; Wu, Lewis, & Grandl, 2017). In addition to the acoustic effects, cells also respond to various environmental mechanical stimuli, such as compression, substrate stiffness (Biswas, 2020) or shear-flow (S. Kim, Uroz, Bays, & Chen, 2021). Thus, it is of interest to investigate if variations in the applied ARF may influence the self-organization dynamics is of interest. To this purpose, experiments were performed with increasing voltage amplitude, for a given acoustic frequency. As shown in Eq. 1, the ARF depends linearly on the acoustic energy density E_0 . It has also been shown that the acoustic energy depends on the voltage as $E_0 \propto V_{pp}^2$ ((Dron & Aider, 2012; Barnkob, Iranmanesh, Wiklund, & Bruus, 2012)). It means that the amplitude of the ARF for 8 V_{pp} is more than 7 times higher than the ARF for 3 V_{pp} .

HepaRG cells were levitated during 3 days with a constant voltage, with a steady voltage ranging from 3 V_{pp} to 8 V_{pp} . The evolution of the circularity as a function of time for 4 different voltages is shown in Fig. 4. Differences in circularity between the 4 chips were slight and statistically non-significant. As a consequence, one can conclude that the amplitude of the ARF does not play a significant role in the process leading to spheroids formation.

4.2.1.1 | Importance of the acto-myosin network and cell-cell adhesion in morphogenesis

The cross-talk between intracellular motor proteins myosins II and cytoskeletal actin filaments drives cell contractility, shape and migration. Furthermore, neighbouring cells are able to form strong adhesions by the mean of adherent junctions proteins such as E-cadherins (Bachir, Horwitz, Nelson, & Bianchini, 2017).

Previous studies on hepatic spheroids showed that it is possible to temper with the self-organization dynamics, by acting either on the cytoskeleton or on the E-cadherin cell-cell adhesion (Lin, Chou, Chien, & Chang, 2006; Luebke-Wheeler, Nedredal, Yee, Amiot, & Nyberg, 2009; Khedr et al., 2019). We investigated whether this disruption could be recreated in our acoustofluidics chip. Due to the rapid increase of spheroid circularity for HUVEC cells and MSCs, the plateau was achieved after only a few hours. Therefore, self-organization disruption was studied for shorter times of culture for MSCs and HUVEC (6 hours) than for HepaRG cells (60 hours).

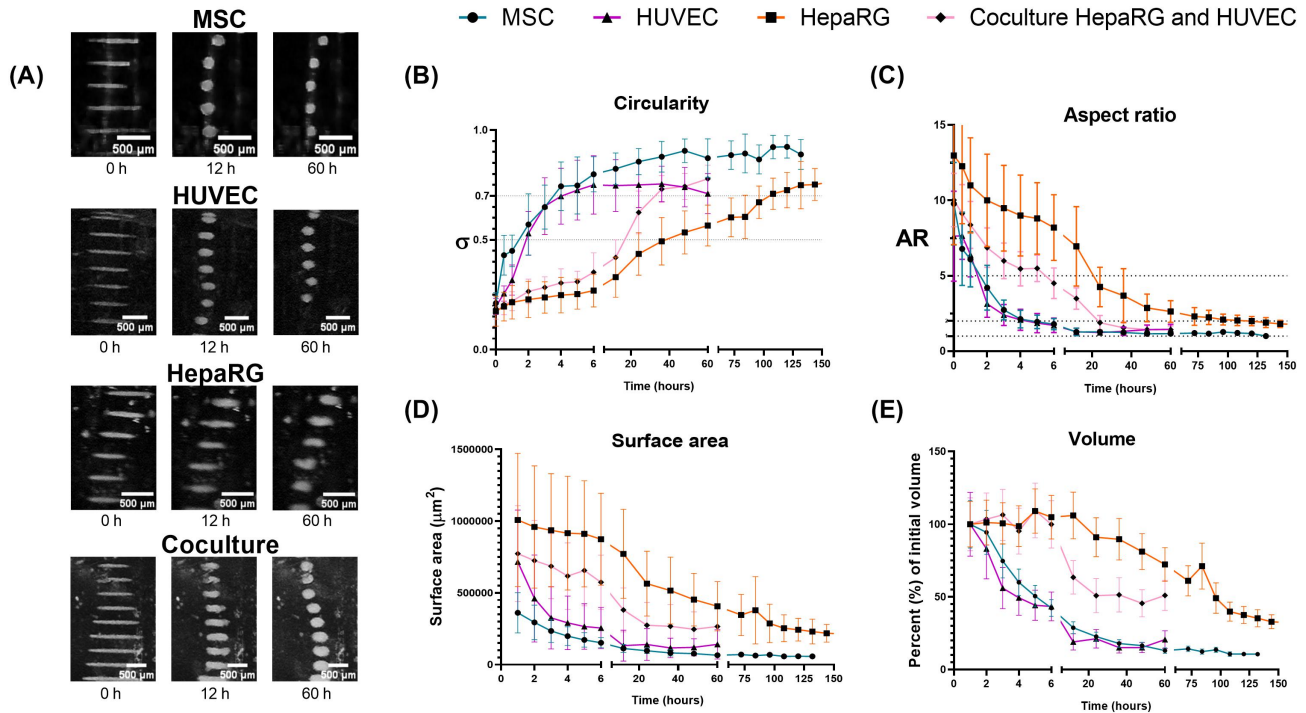


FIGURE 4 Shape and size analysis of spheroids ($n=20$) of different cellular composition: mesenchymal stromal cells (MSCs), human umbilical vein endothelial cells (HUVEC), HepaRG hepatic cell line, and coculture of 50% HepaRG and 50% HUVEC levitated during up to 6 days. The acoustic parameters are the same and kept constant during the culture ($U = 3.5\text{V} / F = 2.2\text{ MHz}$). (A) Side-views during 60 hours of levitation. The snapshots at 3 time steps show the evolution from cells sheets to spheroid over time (scale bars: $500\mu\text{m}$) for the three types of cells. (B) Time-evolution of the circularity (σ) of the aggregate (side views) showing the different dynamics of transformation of the cell sheets into spheroids. (C) Time evolution of the Aspect Ratio (AR) of the aggregates. (D) Time evolution of the surface area of the aggregates. (E) Time evolution of the volume of the aggregates compared to the initial volume.

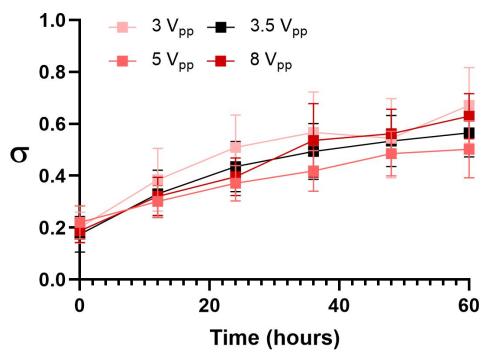


FIGURE 5 Circularity (σ) dynamic of HepaRG spheroids ($n \geq 6$) cultured in acoustic levitation with voltages ranging from 3 to 8 V_{pp} and constant acoustic frequency ($F_{ac} = 2.2\text{ MHz}$). Mean and standard deviation dynamic during 60 hours of levitation are shown.

4.2.1.2 | γ -27632 treatment

Cell contractility is mediated by the rho-associated protein kinase (ROCK), which enables the reorganization on the acto-myosin cytoskeleton. ROCK regulates notably cell contraction, motility, morphology and polarity (Sharanek et al., 2016). This mechanism plays a key role in the formation of spheroids ((Sodek, Ringuette, & Brown, 2009)). To evaluate the influence of contractility, the three cell types were treated with two different concentrations of ROCK-inhibitor γ -27632.

Circularity dynamic was decreased in the groups treated with $40\text{ }\mu\text{M}$ and $60\text{ }\mu\text{M}$ of γ -27632 (Fig. 6.A), for all cell types. These results confirm the important role played by the contractility of the cells in the self-organization process.

4.2.1.3 | EDTA treatment

Cell-cell adhesion is involved in spheroid self-organization and compaction. The adhesion is partly mediated by calcium-dependent E-cadherin homophilic binding in the cell types studied. E-cadherin is a calcium-dependent protein which plays a key role in cell-cell adhesion

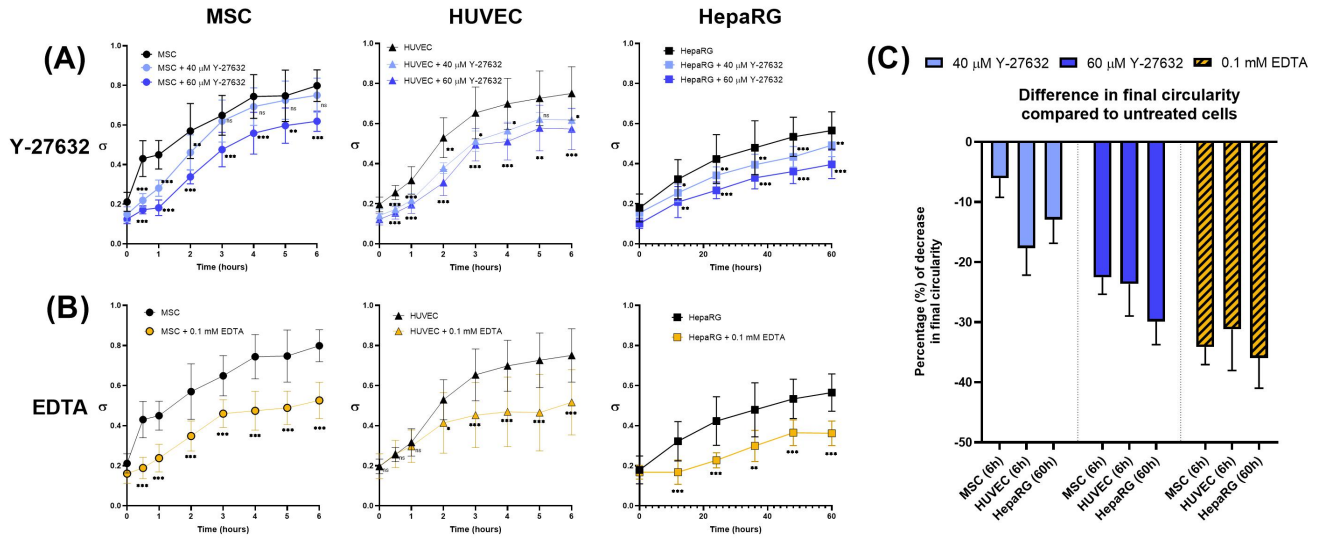


FIGURE 6 Evolution of circularity during treatment with EDTA and Y-27632 $A = 3.5 V_{pp} / F_{ac} = 2.2$ MHz. The time scale was adapted to the dynamics of the spheroid formation. (A) Circularity dynamic of MSCs, HUVECs and HepaRG spheroids ($n \geq 6$) treated with ROCK inhibitor Y-27632. (B) Circularity dynamic of spheroids treated with calcium chelator EDTA. (C) Percentage of difference in circularity compared to untreated cells at the end of the culture (6h for HUVECs and MSCs, 60h for HepaRG spheroids).

and tissue formation (Alberts et al., 2002). In order to affect calcium-dependent self-organization mechanisms, cells were treated with 0.1 mM ethylenediaminetetraacetic acid (EDTA).

We followed the evolution of circularity for the three cell types, with and without treatment with 0.1 mM EDTA (Fig. 6.B). For the three cell types, the treatment with 0.1 mM EDTA modified the circularity evolution during cell culture. At the end of the experiment, the circularity was decreased by more than 30% for the three cell types, confirming the important role played by E-cadherin in the self-organization process.

4.2.1.4 | Difference in final circularity

The Fig. 6.C shows the influence of the various treatments on the final circularities of the spheroids. It is computed as the difference in percentage compared to the untreated values. In all cases the asymptotic values are reduced and the percentage of reduction in final circularity is dependent on the dose and the treatment. Similar responses have been observed between the cell types. The maximum reduction is obtained with 0.1 mM EDTA for all cell types.

4.3 | Viability

A high cellular viability in the spheroid is essential to maintain a normal cell behavior. We first assessed cell viability of the whole spheroids. LIVE/DEAD confirmed that cell viability was preserved at the surface of the spheroids for the three cell types (MSCs, HUVEC and HepaRG), as shown in Fig. 7.

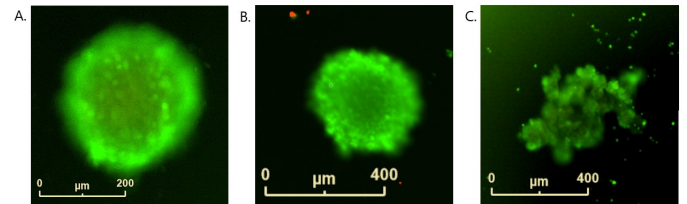


FIGURE 7 Spheroids of MSCs (A), HUVECs (B) and HepaRG hepatic cell line (C) after 3 days of acoustic levitation ($U = 3.5 V_{pp} / F_{ac} = 2.2$ MHz), stained with calcein and ethidium (Live/dead mammalian viability kit).

We then evaluated single cell viability by flow cytometry, after spheroid dissociation, and compared the results to the ones obtained with MSCs, HUVEC, and HepaRG cultured in 2D or low attachment. As shown in Fig. 8, cell viability was not impacted by a culture of 3 days in levitation, since more than 80% of cells were viable.

A similar approach was used to evaluate the impact of Y-27632 and EDTA on cell viability. While no significant decrease of cell viability was observed on MSC and HUVEC spheroids after 6 hours of levitation, the proportion of dead HepaRG cells was significantly increased after 3 days of exposition to Y-27632 and EDTA, showing a death-inducing effect of this long-term treatment (Fig. 9).

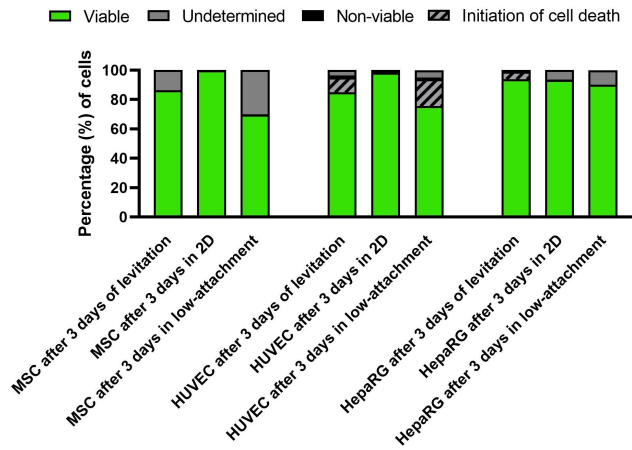


FIGURE 8 Viability of MSCs, HUVECs and HepaRG cells after 3 days in acoustic levitation ($3.5 V_{pp} / F_{ac} = 2.2$ MHz), 2D culture or in low-attachment, followed by spheroid dissociation. Viability was estimated with calcein and ethidium staining (Live/dead mammalian viability kit), and proportions of single stained and double stained cells were estimated by flow cytometry.

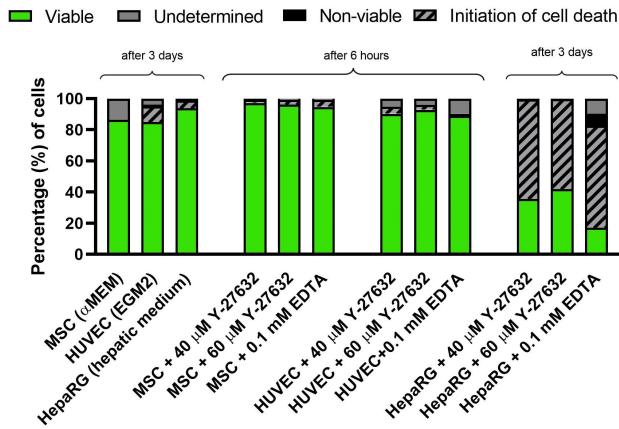


FIGURE 9 Cell viability after levitation, assessed by flow cytometry, in the presence or absence of calcium chelator EDTA and actin inhibitor Y-27632.

5 | DISCUSSION

Reported potentially harmful side effects of ultrasound treatment are numerous: temperature increase, formation of cavitation bubbles, increase in cell membrane permeability or shear stress created by acoustic streaming (Wiklund, 2012; Du et al., 2022). However, these side effects are only observed when high amplitude ultrasounds are used. In our

setup, all experiments are carried out with a very low amplitude ($U < 8V_{pp}$), so that all these potential side effects are negligible. According to the transducer manufacturer instructions, use of the device is safe because the ultrasonic power is of the order of few milliwatts. Exposure to ultrasounds in this frequency range at small amplitudes has been reported in both *in vivo* (Willemetz, 1990; Levenson, Pessana, Garipey, Armentano, & Simon, 2001; Grigioni et al., 2001) and *in vitro* applications (Devendran, Carthew, Frith, & Neild, 2019; Wiklund, 2012; Khedr et al., 2019; Hultström et al., 2007; Vanherberghen et al., 2010). Viability in our system is excellent. Further research, however, is still needed for functional validation.

Size is a limiting factor for spheroids. Hypoxic core has been reported on a number of spheroids with large diameters, the upper limit being generally considered to be $500 \mu\text{m}$ (Glicklis, Merchuk, & Cohen, 2004; Moshksayan et al., 2018). Due to the small size of our spheroids (diameters lower than $300 \mu\text{m}$ at all times) and based on our previous findings on MSC spheroids (Jeger-Madiot et al., 2021), core necrosis is not an issue in our system.

Self-organization is a key characteristic of spheroids and organoids. The recapitulation of self-assembly and polarity is essential for mimicking an organ (Huch et al., 2017).

During the first minutes of cell culture in acoustic levitation, self-assembly is mainly driven by the ARF (externally-driven assembly), leading to the initial cell sheet layer formation. Generator-controlled variations in voltage can increase or decrease the speed of cell sheets formation (modification of the acoustic energy), whereas modulations in frequency affect axial position of spheroids (Jeger-Madiot et al., 2022). It creates cell-cell interactions very quickly, which is one of the major interests of cell culture in acoustic levitation.

It also provides an environment where cells are free to self-organize without contact with cavity walls. Microenvironment stiffness has been proven to have an impact on cell morphology, proliferation, differentiation and functionality, for MSCs (Kozaniti, Deligianni, Georgiou, & Portan, 2021), endothelial (Vania et al., 2020) and hepatic cells (Serna-Márquez et al., 2020). Whereas in many self-organization studies the organoids are grown in contact with plastic surfaces, Matrigel® or hydrogels mimicking the extracellular matrix, in our case the cells self-organize only in contact with the medium or other cells, without mechanical contact with a stiff environment.

Once in the acoustic levitation plane, cells are contained in a so-called "acoustic trap", in an environment in which they can interact only with each other. This autonomy in organization has been advised in order to allow the cells organize as freely as possible (Sthijns, LaPointe, & van Blitterswijk, 2019). Even if the short-term self-assembly of cell sheets is primarily ARF-driven, our findings indicate that the long-term self-organization into spheroids is mainly cell-driven.

Previous studies have found that the initial rapid aggregation was followed by a compaction after a delay period of 2 to 4 hours (Lin et al., 2006). A compaction was also observed in our system, as volume decreases over time for all cell types.

The difference of self-organization between cell types can be explained by distinct biophysical properties. Cell adhesion abilities differ between cell types, leading for instance to different migration capacities (Parsons, Horwitz, & Schwartz, 2010). Contractile forces, generated by the acto-myosin interactions (Discher, Janmey, & Wang, 2005) differ according to the cell type. MSCs and LSECs (liver sinusoidal endothelial cells) are twice as contractile as HepaRG cells (Zhao et al., 2015). Notably, when cocultured, the more contractile cell type is able to drag the other cell type (Sakai et al., 2013; Takebe et al., 2012), which leads to stronger contractions. This explains the intermediary self-organization dynamic we observed when HepaRG and HUVEC were cocultured. Our results pointed out the importance of the cross-talk between the two cell types in our acoustic levitation system.

We showed that changes in the amplitude from 3 V_{pp} to 8 V_{pp} did not affect the long-term behavior of the HepaRG cells. Higher voltages could be needed to observe an impact of the ARF on the self-organization of HepaRG, but possible heating should be monitored and taken care of.

Self-organization from cell sheets to spheroids in other methods of culture has been reported to rely on cytoskeleton reorganization and cell-cell adhesion (Keller & Shook, 2011).

Even if their localization can be distinct between cell types, actin filaments generally form stress fibers in 2D cultures (Tzanakakis, Hansen, & Hu, 2001), and are located next to the cellular cortex in 3D cultures (Leite et al., 2012). The cortex localization has also been reported in acoustic levitation. For instance, HepG2 cells can form stable cell-cell connections in levitation within 10 minutes, showing membrane spreading and increase in junctional F-actin (Edwards et al., 2007). Distributions of filamentous actin, N-cadherin and neural cell adhesion molecule (NCAM) in neural cells are evolving in the first few minutes of acoustic levitation (Bazou, Foster, Ralphs, & Coakley, 2005 May-Jun), while chondrocytes form functional gap junctions in less than one hour (Bazou et al., 2006), showing the importance of cytoskeleton remodeling in levitated cells.

Self-assembly of fibroblast cell sheets into spheroids was reported to be delayed by a treatment with 10 μM of Y-27632, and completely blocked by a treatment with 100 μM of this compound (E. J. Y. Kim, Kotorkevich, & Hiragi, 2018). On the contrary, (Kojima, Takeuchi, & Sakai, 2011) showed that treatment with a higher concentration (200 μM) do not prevent migration of HepG2 and MS1 cells nor spheroid formation in low-attachment plates. Even if in the scientific literature distinct cell types display different sensitivities to the treatment, in our study, all cell types displayed a significant decrease of circularity for a concentration of 100 μM of Y-27632.

Treatment with 0.1 mM EDTA slows down the spheroid formation process in our system. Previous findings have shown that E-cadherin inhibition blocks spheroid formation in classical 3D culture (Luebke-Wheeler et al., 2009)) and in acoustic levitation (Tait et al., 2019; Khedr et al., 2019). Our treatment did not completely inhibit self-organization, probably because the EDTA dose was not sufficient to chelate all calcium ions. We did not use higher concentrations because they resulted

in massive cell death. EDTA chelates several compounds, including Ca^{2+} and Mg^{2+} ions. Other more specific molecules such as egtazic acid (EGTA) or anti-E-cadherin antibodies could be investigated in future studies.

We here showed that self-organization mechanisms are similar in our acoustofluidic chip, because they are impacted by treatment with EDTA and Y-27632. Cell-cell adhesion and cytoskeleton remodeling were shown to be critical for self-organization of the three cell types.

This first long-time study also opens up interesting prospects for cell culture in general. Indeed, cell types performing poorly in 2D, such as primary hepatocytes might thrive in acoustic levitation.

In conclusion, we demonstrated that our microphysiological system of cell culture in acoustic levitation is well-fitted for biotechnological applications and for further fundamental studies. It may then be used for building blocks for tissue engineering, or may serve as an *in vivo*-like model to study self-organization and organogenesis.

AUTHOR CONTRIBUTIONS

L.R., L.A., N.J., D.R.G., J.L. and J-L.A. conceived and planned the experiments. L.R., L.A., N.J., and D.R.G. performed the experiments. J-L.A. and J.L. supervised the project. All authors discussed the results and contributed to the final manuscript.

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FINANCIAL DISCLOSURE

None reported.

CONFLICT OF INTEREST

The authors declare no potential conflict of interests.

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