Monolithic 3D labs- and organs-on-chips obtained by lithography-based ceramic manufacture

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Received: 22 November 2016 /Accepted: 20 April 2017 /Published online: 17 July 2017
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Abstract In this study, we present a novel approach for the design and development of three-dimensional monolithic ceramic microsystems with complex geometries and with potential applications in the biomedical field, mainly linked to labs-on-chips and organs-on-chips. The microsystem object of study stands out for its having a complex three-dimensional geometry, for being obtained as a single integrated element, hence reducing components, preventing leakage and avoiding post-processes, and for having a cantilever porous ceramic membrane aimed at separating cell culture chambers at different levels, which imitates the typical configuration of transwell assays. The design has been performed taking account of the special features of the manufacturing technology and includes ad hoc incorporated supporting elements, which do not affect overall performance, for avoiding collapse of the cantilever ceramic membrane during debinding and sintering. The manufacture of the complex three-dimensional microsystem has been accomplished by means of lithography-based ceramic manufacture, the additive manufacturing technology which currently provides the most appealing compromises between overall part size and precision when working with ceramic materials. The microsystem obtained provides one of the most remarkable examples of monolithic bio-microsystems and, to our knowledge, a step forward in the field of ceramic microsystems with complex geometries for lab-on-chip and organ-on-chip applications. Cell culture results help to highlight the potential of the proposed approach and the adequacy of using ceramic materials for biological applications and for interacting at a cellular level.

Keywords Lithography-based ceramic manufacture · Labs-on-chips · Organs-on-chips · Ceramic materials processing · Biomedical microsystems · MEMS · Bio-MEMS

1 Introduction

Global health concerns require new ways of modeling and studying diseases and of assessing related therapies in a rapid and non-invasive way, all of which has promoted a growing demand of biomedical microdevices aimed at culturing different cell types, at learning from their mutual interactions and at applying such knowledge to the development of more efficient and realistic biomimetic platforms for modeling medical conditions and for drug testing [1]. The continued development and improvement of micro- and nano-manufacturing technologies is enabling the manufacture of biomimetic microsystems, using adequate materials and geometries for high throughput, for answering the aforementioned challenges. State-of-the-art cell co-culture devices and platforms include modified Petri dishes with some areas prepared for locating a cell-loaded gel amidst fluid; “Flocell®” devices with micro-perforated tubes placed in a bottle and loaded with saline solution; and, more frequently, “Transwell®” devices, consisting of a glass with a porous-membrane bottom placed inside a larger glass, again filled with buffer. In these transwell and flocell devices, different cell types can interact in a physiological way through the tube or membrane pores. Flocell

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devices allow for dynamic culture, as a fluid can be moved through the microtubes, but are much more complex and expensive to manufacture than transwell devices. On the other hand, transwell devices are simpler but the promotion of biomechanical dynamic cell culture conditions is still an unsolved issue. Regarding modified Petri dishes, obtaining 3D cell culture conditions is still a challenge difficult to overcome using planar geometries [1, 2]. In addition, the already mentioned devices are in many cases excessively complex, made of several parts manufactured separately, too large for the phenomena being studied and inadequate from the perspective of eco-efficient product development, making them more “chips on a lab” than “labs on a chip” [3]. Their sizes promote the use of high quantities of cells and reagents, many of which are extremely difficult and expensive to obtain, hence limiting the implementation of systematic studies.

In present study, we propose an alternative approach towards eco-efficient, straight-forward and biomimetic 3D cell co-culture, based on the development of a novel and versatile design of a micro-transwell device, which is obtained as a monolithic multi-scale device in a ceramic material adequate for cell culture trials. This monolithic approach is of interest, as recently highlighted [4–6], especially for handling fluids, as leakage is prevented thanks to the promotion of device integration, to the reduction of components and to the consequent elimination of joints between parts. As perceived from the aforementioned references, there is a growing concern towards more integrated design and manufacturing procedures linked to these engineering systems.

Additive manufacturing, working on a layer-by-layer approach and promoting “freedom of design”, thanks to the complex geometries typically attainable using these additive strategies, seems to be an interesting option towards 3D complex microfluidic systems with a reduced number of components and manufacturing steps, as well as for the development of new concepts based on more sophisticated geometries, which may turn out to promote efficiency, compactness and integration of functionalities. Some interesting and recent applications of these additive manufacturing technologies have been linked to the development of new concepts of cell culture systems [7] and of complex geometrical microfluidic systems [8], normally resorting to selective laser sintering/melting for a final metallic device, although polymeric, ceramic and composite powders can be also three-dimensionally sintered. New trends also describe the use of additive processes for obtaining design-controlled surface roughness and, hence, optimizing cell-material interactions [9].

In general, additive photo-polymerization processes, starting from a liquid resin or slurry, provide probably the best features, in terms of precision related to part size, from all additive manufacturing resources. Laser stereolithography, digital light processing, direct laser writing, two-photon polymerization and lithography-based ceramic manufacture are some examples of additive technologies working on the basis of additive photo-polymerization, which converts a liquid prepolymer or charged resin into a polymerized part or into a polymerized network with particles trapped inside. The liquid state procedure is also interesting for the development of 3D microfluidic systems and micro-vascular actuators [10, 11], as the un-polymerized liquid resin or slurry can be more easily removed from the micrometric channels, than in the case of working with a powder-based processes. In consequence, its application to the development of microfluidic systems may constitute a promising approach towards new microsystems, obtained as single part fully integrated devices in just one manufacturing step. However, working additively upon a liquid entails also relevant challenges, including the need of a supporting structure to avoid device collapse during manufacturing, the need to perform post-processing support removal steps and the need of well-trained designers to understand the limits of the technology and to design according to them.

In order to analyze further on such limits and to expand the applications of the complex and detailed geometries attainable by means of additive photo-polymerization of ceramic slurries, this study is focused on the complete engineering design process of a monolithic multi-level and multi-chamber ceramic lab-on-chip obtained as a single part and as a fully integrated device in just one manufacturing step. The possibility of working with ceramics promotes biomedical applications and the attainable precision enables design features adequate for working with cells and studying their behavior in biomimetic conditions.

The procedure followed in our study also provides an example of systematic development of a biomedical microsystem using the CDIO (conceive, design, implement, and operate) methodology, in which design strategies oriented to additive manufacturing by photo-polymerization of ceramic slurries play a relevant role. The design, once optimized for its being manufactured using LCM (lithography-based ceramic manufacture), is versatile for lab-on-chip and organ-on-chip applications, as it includes a network of channels and a set of chambers adequately connected by a cantilever ceramic membrane. The complex design features, level of integration, overall part size and level of detail, characteristic of the developed microfluidic system, make this appliance one of the most remarkable examples of additive manufacturing applied to monolithic (bio-)microsystems currently available and probably a very unique one in the field of ceramic microsystems for cell culture applications, especially as regards the cantilever membrane, which is quite a very singular feature.

Once manufactured, a human mesenchymal stem cell (h-MSC) conditioned medium (CM), in fact a solution of growth factors generated by the own progenitor stem cells, is also used for improving the microsystem’s response and promoting cell adhesion, proliferation and viability in the cell culture
test. Preliminary validation, thanks to adequate cell culture of human mesenchymal stem cells (hMSCs) within the presented biomedical microsystem, after adequate bio-functionalization using the mentioned human mesenchymal stem cell conditioned medium, helps to put forward the potential of this and of similar monolithic ceramic microsystems. The attainable integration, thanks to working additively with adequate materials for biological purposes (ceramics and bioceramics), may help towards the industrialization of monolithic microsystems for performing cell studies in a more straight-forward “plug-and-play” form.

2 Materials and methods

2.1 Design process

Several computer-aided design and engineering (CAD) programs help with the development process of novel products. In our case, the geometry of the monolithic (organ-/lab-)on-chip under study is designed with the help of NX-8.5 (software commercialized by Siemens PLM Solutions). The design is aimed at single step manufacture and at final microfluidic systems manufactured as single parts. We take into account the particular features of the process (described in the following subsection) for obtaining computer-aided designs oriented to manufacture.

Figure 1 shows the schematic CAD design of a versatile biomedical microsystem, lab-on-chip device or organ-on-chip platform, aimed at enhanced cell co-culture strategies, object of present study. The inlets may allow for the incorporation of different cell types and growth or trophic factors (for instance parenchymal cells, endothelial cells, pericytes and vascular endothelial growth factor “VEGF”), which would be physically separated from other cells cultured upon the cantilever membrane (for instance adipose tissue cells, neurons, among other possibilities). Such a design may enable the in vitro development of several types of tissue models or physiological interactions using just a single and versatile multi-well, multi-channel and multi-layer microfluidic platform. The channels and lower chamber may help researchers to develop the vasculature, which may in turn potentially interact with specific cells from different tissues placed upon the cantilever membrane. The use of Boolean and pattern CAD operations may be used to develop, as a single macro-device, several of the proposed micro-platforms or micro-transwell devices, so as to help with experimental design and with the evaluation of potential factors of influence on cellular behavior and fate. The inlets and outlets may help researchers to culture under dynamic conditions and their design can be adapted to the required applications, showing in our example just circular openings for loading cells into them.

Main critical dimensions, designed according to the manufacturing precision of lithography-based ceramic manufacture (see next sub-Section 2.2), include 2 mm-wide channels and a $8 \times 8 \text{ mm}^2$ cantilever membrane with $350 \times 350 \mu\text{m}^2$ pores. In order to promote the stability of the cantilever, a thickness of 200 $\mu\text{m}$ (corresponding to eight manufacturing layers) is applied to the central cantilever membrane. The complete microsystem can be circumscribed by a box of $30 \times 30 \times 3 \text{ mm}^3$ (X, Y, Z, directions respectively). According to the detailed dimensions and to previous experiences [2, 9, 15, 16], lithography-based manufacture constitutes a very adequate micro-manufacturing technology for the development of the proposed biomedical microsystem in an additive way and using ceramic materials. The materials used in the process have also proved excellent for in vitro trials with cells [9].

In our opinion, the proposed geometry is quite a versatile one for several lab-on-chip and organ-on-chip biomedical applications. For instance, the separation of a lower and an upper
chamber by a cantilever porous membrane, which is inspired on the typical structure of transwell assays, may promote cell co-culture applications aimed at reproducing the physiological interactions of different cell types within human organs in a biomimetic way. The circular inlets and outlets may also be used for obtaining dynamic cell culture conditions by connecting to adequate tubing and pumping networks, which can be of interest for promoting cell differentiation into different tissues and for enhancing the generation of vasculatures within in vitro models [12].

With lithography-based ceramic manufacture being a liquid phase additive manufacturing technology, the geometries must be in many cases obtained upon a network of supports and some space must be left for evacuation of the trapped resin during manufacture. Normally the slicing software, which works upon the .stl files for preparing the additive process, automatically provides a supporting network. In our case, we avoid the generation of supports, (which would fill most of the lower chamber when working with conventional slicing software) and incorporate, to the original part design, some vertical columns to support the central cantilever membrane and to provide structural integrity to the final devices. In fact, after the ad hoc incorporation of supports to the original CAD, we process the geometry with Lithoz’s proprietary software, for preparing the additive manufacture and for performing the slicing, setting off the automated support generation option. In consequence, no supports are generated below the membrane, but for those applied in a personalized way and highlighted in Figs. 2 and 3. Based on our experience with the lithography-based ceramic manufacturing system, cantilevers and over-hanging elements up to 7–8 mm can be constructed. Therefore, we design the supports with a maximum separation between them of 5–6 mm for feasible geometries, as shown in Fig. 2 by means of example and schematically comparing different alternatives in Fig. 3. The use of computational methods helps to optimize their positions and the selection of the best alternative, together with the results from prototyping stage.

This ad hoc design of support structures is a recent trend in the field of additive manufacturing, which can result in reduced consumption of materials and promote geometrical freedom of design even more. In fact, the support structures automatically generated with common slicing software usually overestimate the required supports and fill empty spaces with crowded networks of supports, which is inefficient for most fluidic devices and for several applications in energy, chemical and biomedical engineering. Apart from the special or personalized definition of support structures, the designs must count with exits for the un-polymerized trapped resin or slurry. In these devices, we take advantage of the inlets and outlets and of the porous central membrane for extracting the mentioned trapped resin or slurry after manufacture. Once the designs are ready, manufacturing is prepared with the help of in house developed slicing software that generates dynamic masks for three-dimensional layer-by-layer part generation using the materials and technology described in the following sub-section.

### 2.2 Manufacturing process

Towards the desired quality, lithography-based additive manufacturing of ceramics is probably the most suitable
option currently. This technology is based on the layer-by-layer selective curing of a photosensitive resin with homogeneously dispersed ceramic particles (slurry). The photopolymer acts as binder between the ceramic particles and makes the shaping of the part possible. The shaped form is produced as a “green” body that has to be further processed to obtain a part with higher relative density (99.45–99.95%). These post-processing steps include debinding (thermal decomposition of binder) and sintering into a compact ceramic part. This technology was developed at TU Wien [13–15] and is industrially developed by Lithoz GmbH.

2.3 Cell culture experiments

2.3.1 Additive manufacture of master prototypes or green parts

Once the design is optimized for 3D printing, manufacture is done by lithography-based ceramic manufacturing (LCM) [16]. The master models or green parts are manufactured, with previously prepared aluminum oxide (alumina, Al₂O₃) slurries, using the CeraFab 7500 machine from Lithoz GmbH (Fig. 4). The slurries are prepared with commercial Al₂O₃ powders. These powders are homogeneously dispersed, with the help of a dispersing agent, in a formulation containing reactive monomers and a solvent. In addition, the formulation contains a photo-initiator (typically less than 1 wt%). The photoinitiator reacts under an external energy source, in this case a LED emitting at 460 nm, which excites the initiator, creating radicals that chemically react with the monomers included in the mixture. The chain reaction forms the desired matrix of (meth)acrylate photopolymers that bind together the ceramic particles. The reaction occurs in a brief lapse of time, while a determinate section of the part is being projected with specific intensity and exposure time parameters. The slurry used for this research is commercially available under reference LithaLox HP 500 (Al₂O₃), which is developed and commercialized by Lithoz GmbH. The manufacturing process uses dynamic masks, which represent an individual cross section of the part being manufactured. The light engine uses high performance LEDs as light source and a DMD chip (digital mirror device) as dynamic mask with a resolution of 1920 × 1080 pixels. The corresponding pixel size at the building envelope is 40 × 40 μm. The fabrication of the part is done in sequential layer-by-layer manner. For each individual layer fresh slurry is applied on the building envelope via a dosage system and subsequent rotation of the vat. Afterwards, the building platform is lowered into the dispersion a distance of 25 μm to the bottom of the vat, which equals the thickness of an individual layer in the green body. Then, the space-resolved exposure of the slurry is done by the projection of an image corresponding to the cross section of the current layer. After printing, the parts are removed from the building platform and these green bodies are cleaned with solvent for several minutes until the non-polymerized slurry is removed from the cavities of the part.

2.3.2 Sintering towards final ceramic parts

The printed parts, once they are free of non-cured slurry, are subjected to a thermal treatment with the aim of obtaining a final ceramic solid part, free of any organic material. In addition, as the ceramic particles are separated, green parts have lower density and mechanical properties than compact alumina. The elimination of the organic components for achieving final composition and properties is carried out as described in previous research [15]. In short, the thermal variation is controlled inside a high-temperature chamber furnace from 30 °C up to 400 °C. Firstly, the solvent included in the slurry is evaporated and/or decomposed. Subsequently, a slow
temperature variation is provided, so as to get an adequate decomposition of the binder, without causing internal stresses due to the formation of large amounts of gas at high temperature. In a second step, the Al₂O₃ particles are sintered. The part, already free of any organic component surrounding the ceramic particles, is heated to 1600 °C, hence achieving sintering and resulting in a final compact part. The whole process is schematized in Figs. 4 and 5.

2.3.3 Human mesenchymal stem cells (hMSCs) culture

The experiments with hMSCs are carried out following the indications approved by Research Ethic Committee at Autonoma University of Madrid (UAM). The hMSCs used were isolated from 1 to 2 ml from bone marrow-derived samples of healthy donors and were provided by Hospital La Princesa, the Jimenez–Diaz Foundation and Malaga University Biobank. Cells were isolated and culture expanded following processes described in previous research [17–19] with slight modifications. In short, culture media DMEM-low glucose (LG), 0.25% trypsin-EDTA and phosphate buffered saline (PBS) were prepared by the Research Service of the Molecular Biology Centre “Severo Ochoa.” Cells were seeded on Falcon plates and expanded in DMEM-LG adjusted to 10% FBS of selected lots (SIGMA, Spain) to 80% confluence. Medium was replaced twice a week and cells were cultured until 70–80% confluence in a 5% CO2-incubator (MRC, Iberlabo Spain) at 37 °C and 95% humidity. The medium was replaced twice per week.

2.3.4 hMSCs conditioned medium

For the preparation of each batch of CM-hMSCs, 8–10 p100 culture plates of hMSCs grown at 80% confluence in DMEM-LG and 10% FBS were used. Then, cells were washed thoroughly with PBS, incubated in DMEM-LG, starved of FBS, complemented with 2 mM pyruvate and incubated during the following 24 h. Afterwards, the culture medium was collected, cleaned of any floating cells in a bench centrifuge at 1500 rpm for 5 min. As described in an early work [20, 21], the clean supernatant was cooled down on ice for 30 min, centrifuged at 9000 rpm in a Sorvall centrifuge during 30 min to remove salt precipitations and then, the clean supernatant was kept in 2 mL aliquots at −30 °C until use. We avoided repeatedly freeze-thawing the samples. The activity of each hMSC-CM batch was measured by a triplicate wound-healing assay, using DMEM-LG-Pyr medium and DMEM-LG-2% FBS as controls.

2.3.5 Functionalization of 3D monolithic microsystems

The monolithic ceramic microsystems were exposed to the direct UV-light of a cabin (Teltair) and successively treated with 2 ml of (i) 95% ethanol, during 30 min and two times; (ii) PBS wash; (iii) 2 M HCl attack during 24 h for acid activation of surfaces; (iv) PBS wash until neutralization; (v) surface coating with CM-hMSCs during 24 h in cell incubator; (vi) removal of hMSC-CM and (vii) seeded with 10³ hMSCs drop by drop using the four chambers inlets of the ceramic lab-on-chip. After 30 min in a cell incubator, the monolithic ceramic microsystems were exposed to a growth media of DMEM-LG plus 10%FBS during 3 days following some successful experiences. In short, the process follows previous research with some slight modifications [22, 23].

2.3.6 Immunofluorescence

After cell culture, samples were processed as previously described [24, 25]. Briefly, the monolithic ceramic microsystems were rinsed twice with ice-cold PBS and fixed in 3.7% formaldehyde in PBS for 30 min at room temperature (RT), then washed and in PBS and either used or kept at 4 °C. The cells present upon the 3D printed biodevice were incubated with 0.5% Triton X-100 in CSK (cytoskeleton) buffer containing 10 mM Pipes pH 6.8, 3 mM MgCl₂, 100 mM NaCl, 1 mM EGTA, 0.3 M sucrose, and 1 mM PMSF for 30 min on ice to permeate and remove all cell soluble proteins, fixed with 3.7% formaldehyde and washed with PBS and kept at 4 °C until use for immune detection. For the former, cell preparations were blocked of unspecific proteins by incubation in PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton X-100 for 2 h at RT. Then, the blocking solution was removed and samples were equilibrated to PSB-1% BSA and used to incubate with mouse anti-α-tubulin (Sigma, Spain) at 1:2000 dilution during 45 min. Secondary antibody was anti-mouse labeled with Alexa 488 (Invitrogen) (Microscopy Service of MBC-CSIC-UAM). Nuclei were stained with DAPI (CALBIOCHEM) for 5 min. Immune staining was observed with an inverted IX81 Olympus associated with a DP72 digital camera and controlled by cellD software.
3 Results and discussion

Our results illustrate the viability of manufacturing monolithic three-dimensional ceramic microsystems, whose complex geometries are oriented to studying cell-cell communication through a porous membrane and whose material chemistry is apt for culturing hMSCs, all of which is relevant for potential biomedical purposes, especially for lab-on-chip and organ-on-chip applications. The presented design is truly multi-scale and includes very relevant thickness transitions, which are always complex to manage and constitute unseen challenges in the field of additively obtained ceramic devices. The use of lithography-based ceramic manufacture proves to be an effective method for obtaining these geometries, with an important degree of resemblance between the final parts and the original designs and without the presence of cracks, even in the more reduced features, which is remarkable considering the debinding and sintering processes involved. The proposed microsystem, as appreciated from the cell trials, mimics the performance of transwell assays, with a membrane separating upper and lower chambers, through which the cells (from a single or several origins) can communicate and interact for mimicking some physiological conditions, but is obtained as a monolithic device in a straight-forward process. This promotes throughput, as the microsystem is in fact a reduced alternative to transwell devices, which normally consume larger quantities of cells and reagents. Our system, with a final size of $30 \times 30 \times 3 \text{mm}^3$ and with culture chambers, above and below the membrane, of just $8 \times 8 \times 0.75 \text{mm}^3$ is consequently more sustainable, according to trends in the use of microfluidics for modeling disease and studying cell-cell communication.

Some preliminary prototyping examples at scales 1:1 and 2:1 are included in Fig. 6a, obtained after the initial design from Fig. 1, which is has an unsupported membrane. These images show deflection (left) and collapse (right) of the cantilever membrane and highlight the importance of performing a re-design for the incorporation of ad hoc supports. The larger thickness of the membrane at scale 2:1 (Fig. 6a, left) prevents collapse, when compared to the thinner membrane at scale 1:1 (Fig. 6a, right). The manufacture at the different scales also helped us to analyze that the smallest features from the original design were attainable. Figure 6b shows the improved results, associated to the redesign presented schematically as the third solution of Fig. 3, towards the final 100% functional monolithic ceramic microsystem. Indeed, the use of ad hoc supports is a correct solution for achieving the desired geometry without compromising the microsystem's performance. Generating the supports with conventional 3D printing slicing software would suppose filling the lower chamber with a dense network of columns, which would prevent the cells from freely colonizing the chamber, entering to it through the lower channels.

The final model stands out for its similarity to the computer-aided design, for its symmetry, for the prevention of deflection in the re-designed cantilever micro-porous membrane and for the absence of inhomogeneous shrinking or structure collapse. After sintering, some contraction is appreciated, as further detailed below, but no cracks appear, hence helping us to validate the whole process for the development of three-dimensional biomedical microdevices, labs- and organs-on-chips using ceramic materials. The original size of the CAD file corresponds to a circumscribing box of $30 \times 30 \times 3 \text{mm}^3$, while the final debinded and sintered part is $28.6 \times 27.1 \times 2.9 \text{mm}^3$. This corresponds, from measured taken with the help of CAD software, to an original volume of 1.75 cm$^3$ for the whole microsystem and to a final volume of 1.45 cm$^3$, which corresponds to a global volume variation of a 18%, which is in accordance with the theoretical results for obtaining a relative density of around 99% after elimination of the polymeric phase and sintering. In general terms, this is in line with previous studies by our team [9], although in this case a slight difference in the contraction along the z axis (3.3%) can be appreciated, when compared with the contractions along x and y axes (with a mean contraction of 7.15%). This may be a consequence of liquid polymerization shrinkage during the additive process, which affects in a larger degree in the directions perpendicular to the construction, as the sintering process is expected to be more isotropic. Such shrinkage by polymerization can be minimized by...
maximizing the ceramic content within the slurry, although this makes the manufacture more complex as the particle content limits light penetration and consequent polymerization. These aspects have been further analyzed in previous studies, in which the objective was maximizing the content of ceramic particles [26].

In addition, the presence of a phase transformation at 1060 °C may also produce slight volume changes, which do not affect our end application, but which have to be considered, as they may produce undesired stresses and lead to crack appearance if the process is not performed with the adequate slow heating and cooling ramps advised by Lithoz and their material and process data sheets. In any case, these dimensional variations can be taken into account from the design stage, although in our case, the reduced geometrical mismatches do not affect overall performance, as the part in not aimed at interacting with other components.

Apart from the geometrical issues, it is important to mention that the obtained ceramic parts are even more compact than ceramic objects obtained with more traditional processes, which is really interesting for the development of microfluidic devices, especially for the labs- and organs-on-chips fields, with enhanced performance thanks to avoiding leakage. The process strikes for a manufacturing speed in the range of around 100 layers/h thanks to avoiding leakage. The process strikes for a manufacturing speed in the range of around 100 layers/h with a XY plane resolution of 40 μm and a layer thickness of 25 μm, hence being among the most precise and rapid procedures for the manufacture of high-quality extremely dense ceramic parts, even when compared with very recent developments [27]. As far as we know and based on the provided reference list, the presented microsystem, obtained by lithography-based ceramic manufacture, provides the best compromise between part size, degree of detail and compactness in the field of additive manufactured ceramic labs- and organs-on-chips currently available.

It is also important to highlight that the obtained geometry, although being quite versatile for cell culture applications, is just a conceptual example of the potential of the proposed design and manufacturing approach. More complex geometries, intricate networks of channels, additional layers and cantilever membranes, among other features and details, can be obtained following similar computer-aided design procedures, incorporating ad hoc support structures, leaving enough space for resin or slurry evacuation and applying lithography-based ceramic manufacture, either to ceramic and bioceramic slurries and to polymers and biopolymers, which constitutes and additional very unique feature of the technology used in this study. The presented process is aligned with current groundbreaking trends linked to light-weight design and to the minimization of support structures in additive manufacturing [28].

Current challenges are linked to additionally improving the attainable degree of precision, while maintaining the capability of manufacturing large objects. The possibility of obtaining nano-porous cantilever and integrated membranes can be useful for expanding the applications of these monolithic ceramic labs-on-chips. Nanometric details can even help to interact with micro-organisms, not just at a cellular, but also at a molecular level. Potential applications include the development of enhanced organs-on-chips, the manufacture of biological traps and the development of selective filters capable of capturing bacteria, viruses and pathogens for subsequent studies [29–31]. The optimal biological properties of ceramic materials may result in improved performance, as our cell culture results, detailed further on, help to put forward. It is also important to mention that, thanks to working additively, design-controlled devices and repeatable manufacturing results can be obtained. The proposed process is straight-forward, single-step and leads to more integrate and simpler (in terms of components and connecting elements) microfluidic devices, with which it is easier to interact.

Regarding cell culture trials, results from hMSCs interacting within the monolithic ceramic microsystem are shown in the images of Fig. 7. To this end, the ceramic microsystems were exposed to conditioned medium produced by hMSC, as reported in early work, then seeded with cells and incubated with DMEM low glucose and 10%FBS during 48 h. Figure 7a shows an overall view of the microsystem and marks the zones magnified in images 6b, 6c, 6d and 6e. Figure 7b shows cells adhered to the inlet well. Figure 7c, d focuses more on the perpendicular channels of the microsystem, showing cells on the upper and lower levels of the microsystem. Figure 7e focuses on a pore of the cantilever membrane, showing cells reaching such pore and interacting through it. The nuclei are stained in blue (DAPI) and their rounded forms without blebs indicate that the cells are in healthy conditions and well adhered to the porous scaffold. It is important to note that more than 20 cells/mm² can be appreciated. The images also help to show the good energetic behavior of the cells, with clearly expanded cytoskeleton configurations (stained in green: α-Tubulin—Ph488) typical of healthy hMSCs, both in the upper and lower levels of the microsystem, as more than 80 expanded and active cells can be appreciated in images 6b, 6c and 6d.

We would like to mention that hMSCs are adherent cells. If they do not have metabolic energy to adhere, hMSCs become in less than an hour spheroid, apoptotic, die and appear floating in the cell culture assays disturbing microscope imaging. In our case, we did not found a relevant number of dead and floating cells within the microsystem and we could not appreciate any
spheroid-like cells, all of which helps to validate the good biological performance of the material used and of the conditioned medium functionalization. As an example of such behavior, recent publications have shown how the number of cells adhered is reduced if mitochondria have diminished electron transport, as consequence of Pt interfering with Cu entry to cells and mitochondria, and how this is directly linked to the progressive spheroidization of cells [32]. We believe that in our case cellular metabolic activity is adequately exposed by means of the described exploration of cytoskeleton morphologies and by the lack of spheroid-like cells. Additional studies will be carried out using improved confocal microscopy facilities and performing osteochondral differentiation, which may let us provide more details about the three-dimensional configuration of the cells within the whole microsystem and its potential for modeling complex physiological interactions, but we understand that the provided validation shows promising results. Exploring the co-culture of different cell types and the communication among them is also a proposed continuation path, in accordance with recent progresses in cell-culture microsystems [33–35].

In short, our results show that the cells and the monolithic ceramic microsystems are excellent companions for potential organ-on-chip developments. The hMCSs-seeded microsystem and its channel configuration and lower and upper chambers separated by the micro-porous cantilever membrane may result very adequate in order to allow the permeation of nutrients and debris, to promote oxygenation, to enable adaptation and to provide cellular communication systems, capable of locally inhibiting the immune system and of activating tissue repair, following the surrounding fluid dynamics, although additional assessments need to be performed.

Last but not least, the hMCSs-seeded monolithic ceramic microsystem may offer interesting possibilities to study cellular mechanisms present in different types of tissues, specially the interactions between bone and cartilage, although they may well be useful for studying other types of tissues, including adipose, endothelial and neuronal cell types and interactions thereof, in devices with additional levels or with a higher degree of geometrical complexity aimed at promoting biomimicry. The use of other ceramic materials for the monolithic microsystem, such as zirconia, tricalcium phosphate and hydroxyapatite, which can be also processed using lithography-based ceramic manufacture, may be another interesting line for continuation, but the first trials using alumina have provided remarkable insights.

Considering potential industrial impacts, apart from the positive features of the developed microsystem and of related design and manufacturing methods already covered, it is interesting to compare the detailed microsystem with existing devices and with current trends in the field of organs-on-chips [33–35], in order to find some additional directions for improving. We have highlighted that the monolithic microsystem provides benefits in terms of integration, leakage prevention and throughput promotion by working with small amounts of cells and reagents, when compared with common transwell devices (in terms of size and integration) and with typical organ-on-chip platforms (in terms of reduction of...
components). However, it is also true that, in some cases, such level of integration may limit the versatility of the final device, as in many organ-on-chip platforms the possibility of rearranging the disposition of elements helps to perform a wide set of studies with a reduced number of components. Usually, cell-culture platforms and related microsystems are made of transparent materials (i.e. glass, polystyrene or PDMS), which help to visualize cell-cell and cell-material interactions in a better way, and include membranes made of more flexible materials with smaller pore sizes, for interacting at a real cellular or even molecular level in a more biomimetic way. Working towards “plug-and-play” kits of ceramic microsystems, towards the manufacture by lithography-based processes of transparent biomaterials (including biopolymers and bioglasses) and towards increased levels of detail, may help to promote the industrial impact of the concepts and technological combinations presented here, probably as a complement to existing technologies.

4 Conclusions

In this study, we have presented a very promising approach for the design and development of three-dimensional monolithic ceramic biomedical microsystems with complex geometries and with potential applications in fields linked to labs-on-chip and organs-on-chips. The microsystem object of study stands out for its having a complex three dimensional geometry, for being obtained as a single integrated element, hence reducing components, preventing leakage and avoiding post-processes, and for having a cantilever porous ceramic membrane aimed at separating cell culture chambers at different levels, which imitates the typical configuration of transwell assays.

The design has been performed taking account of the special features of the manufacturing technology and includes ad hoc incorporated supporting elements, which do not affect overall performance, for avoiding collapse of the cantilever ceramic membrane during debinding and sintering. The manufacture of the complex three-dimensional microsystem has been accomplished by means of lithography-based ceramic manufacture, the additive manufacturing technology which currently provides the most appealing compromises between overall part size (up to several centimeters) and precision (down to details of around 50 μm) when working with ceramic materials. The microsystem obtained provides one of the most remarkable examples of monolithic bio-MEMS and, as far as we know, a step forward in the field of ceramic MEMS with complex geometries for lab-on-chip and organ-on-chip applications. Cell culture results help to highlight the potential of the proposed approach and the adequacy of using ceramic materials for biological applications and for interacting at a cellular level, if the manufacturing process provides enough precision, as is our case thanks to the use of lithography-based ceramic manufacture.

We aim to continue our research searching for new appliances based on these geometries, design procedures and additive ceramic manufacturing technologies. Our computer-aided designs are placed at the disposal of colleagues who would like to further explore with us the potentials of these monolithic biomedical microsystems. The described lithography-based ceramic manufacturing process can be used for many other fields, which may benefit from the use of microdevices with complex geometries, including energy engineering, transport applications and aerospace solutions, in which the use of ceramics is of interest due to the high environmental temperatures involved.

Acknowledgements Present research was supported by the “TOMAX: Tool-less manufacture of complex geometries” project, funded by the EU Commission under grant agreement number 633192 - H2020-FoF-2014-2015/H2020-FoF-2014 and led by Prof. Dr. Jürgen Stampfl from the Technical University of Vienna.

We are indeed grateful for the detailed review and for reviewers’ comments and improvement proposals, which have helped us to incorporate interesting references, to take into account relevant issues and to enhance overall paper quality.

Compliance with ethical standards The experiments with hMSCs have been performed with the approval and following the guidelines of the Research Ethic Committee of Autonoma University of Madrid (UAM).

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Lanza R, Langer R, Vacanti J (2014) Principles of tissue engineering, 4th edn. Elsevier
2. Díaz Lantada A (2016) Handbook on microsystems for enhanced control of cell behaviour: fundamentals, design and manufacturing strategies, applications and challenges. Springer
3. Jenkins G, Mansfield CD (2013) Microfluidic diagnostics: methods and protocols. Springer
4. Preechaburana P, Filippini D (2011) Fabrication of monolithic 3D micro-systems. Lab Chip 11:288–295
5. Gelber MK, Bhargava R (2015) Monolithic multilayer microfluidics via sacrificial molding of 3D-printed isomalt. Lab Chip 15(7):1736–1741
6. Hengsbach S, Diaz Lantada A (2014) Rapid prototyping of multiscale biomedical microdevices by combining additive manufacturing technologies. Biomed Microdevices 16(4):617–627
7. Schmieder F, Ströbel J, Rösl M, Grünzner S, Hohenstein B, Klotzbach U, Sonntag F (2016) 3D printing—a key technology for tailored biomedical cell culture lab ware. Curr Dir Biomed Eng 2(1):105–108
8. Waheed S, Cabot JM, Macdonald NP, Lewis T, Guijt RM, Paul B, Breadmore MC (2016) 3D printed microfluidic devices: enablers and barriers. Lab Chip 16:1993–2013
9. De Blas Romero A, Pfaffinger M, Mitteramskogler G, Schwentenwein M, Jellinek C, Homa J, Diaz Lantada A, Stampfl J (2016) Lithography-based additive manufacture of ceramic biodevices with design-controlled surface topographies. Int J Adv Manuf Technol, May (Online first) 1–9
10. Waldbaur A, Rapp H, Länge K, Rapp BE (2011) Let there be chip. Towards rapid prototyping of microfluidic devices: one-step manufacturing processes. Anal Methods 3(12):2681–2716
11. Diaz Lantada A, De Blas Romero A, Chacón Tanarro E (2016) Micro-vascular shape-memory polymer actuators with complex geometries obtained by laser stereolithography. Smart Mater Struct 26:065018
12. Hasan A, Paul A, Vrana NE, Zhao X, Memic A, Hwang YS, Dokmeci MR, Khadhosseini A (2014) Microfluidic techniques for development of 3D vascularized tissue. Biomaterials 35(26):7308–7325
13. Gruber H et al (2006) Rapid-prototyping method and radiation-hardenable composition of application thereto. PCT/AT2006/000271, WO 2007002965 B1
14. Patzer JF (2011) Generative Fertigung von keramischen Bauteilen für dentale Anwendungen. Dissertation, TU Wien
15. Felzmann R, Gruber S, Mitteramskogler G, Tesavibul P, Boccaccini AR, Liska R, Stampfl J (2012) Lithography-based additive manufacturing of cellular ceramic structures. Adv Eng Mater 14(12):1052–1058
16. Schwentenwein M, Homa J (2015) Additive manufacture of dense alumina ceramics. Appl Ceram Technol 12(1):1–7
17. Lennon DP, Haynesworth SE, Bruder SP, Jaiswal N, Caplan AI (2006) Human and animal mesenchymal progenitor cells from bone marrow: identification of serum for optimal selection and proliferation. In Vitro Cell Dev Biol 32:602
18. Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem 98(5):1076–1084
19. Oguea S, Muñoz J, Obregon E, Delgado-Baeza E, Garcia-Ruiz JP (2002) Prolactin is a component of the human sinovial liquid and modulates the growth and chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. Mol Cell Endocrinol 190(1–2):51
20. Caplan AI (2013) Adult mesenchymal stem cells and the NO pathways. Proc Natl Acad Sci 110(8):2695–2696
21. Alarcón H, Ynsa MD, Dang ZY, Torres-Costa V, Manso-Silván M, Wu JF, Breeze MBH, García-Ruiz JP (2015) Conditioned bio-interfaces of silicon/porous silicon micro-patterns lead to the chondrogenesis of hMSCs. RSC Adv 5:92263–92269
22. Diaz Lantada A, Alarcón Iniesta H, Pareja Sánchez B, García-Ruiz JP (2014) Free-form rapid-prototyped PDMS scaffolds incorporating growth factors promote chondrogenesis. Adv Mater Sci Eng 612976
23. Diaz Lantada A, Alarcón Iniesta H, García-Ruiz JP (2015) Composite scaffolds for osteochondral repair obtained by combination of additive manufacturing, leaching processes and hMSC-CM functionalization. Mater Sci Eng C: Mater Biol Appl 59:218–227
24. Romero-Prado M, Blázquez C, Rodriguez-Navas C, Muñoz J, Guerrero I, Delgado-Baeza E, García-Ruiz JP (2006) Functional characterization of human mesenchymal stem cells that maintain osteochondral fates. J Cell Biochem 98:1457
25. Javed A, Guo B, Hiebert S, Choi JY, Green J, Zhao SC, Osborne MA, Stifani S, Stein JL, Lian JB, van Wijnen AJ, Stein GS (2000) Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBF (alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. J Cell Sci 113(Pt 12):2221
26. De Blas Romero A (2015) Optimization of photocurable zirconia slurries. Master’s Degree Thesis (J. Stampfl & A. Diaz Lantada (advisors)), TU Wien & Technical University of Madrid
27. Eckel ZC, Zhou C, Martin JH, Jacobsen AJ, Carter WB, Schaedler TA (2016) Additive manufacturing of polymer-derived ceramics. Science 351(6268):58–62
28. Li Z, Zhang DZ, Dong P, Kucukkoc I (2016) A lightweight and support-free design method for selective laser melting. Int J Adv Manuf Technol doi: 10.1007/s00170-016-9509-0
29. Soman P, Lee JW, Phadke A, Varghese S, Chen S (2012) Spatial tuning of negative and positive Poisson’s ratio in a multilayer scaffold. Acta Biomater 8(7):2587–2594
30. Warkiani ME et al (2014) Slanted spiral microfluidics for the ultrafast, label-free isolation of circulating tumor cells. Lab Chip 14:128–137
31. Lee W, Kwon D, Choi W, Jung GY, Au AK, Folch A, Jeon S (2015) 3D-printed microfluidic device for the detection of pathogenic bacteria using size-based separation in helical channel with trapezoidal cross-section. Sci Rep 5:7717
32. Garcia-Ruiz JP, Mateusanz Garcia AI, Perez Souza A, Souza Castelo P (2015) Thiosemicarbazone-Pt(I) complex causes a growth inhibitory effect on human mesenchymal stem cells. Med Chem 11(7):670–675
33. Huh D, Hamilton GA, Inger DE (2011) From 3D cell culture to organs on chips. Trends Cell Biol 21(12):745–754
34. Wilhelm I, Fazakas C, Krizbai IA (2011) In vitro models of the blood-brain barrier. Acta Neurobiol Exp 71:113–128
35. Huh D, Kim HJ, Fraser JP, Shea DE, Khan M, Bahinski A, Hamilton GA, Inger DE (2013) Microfabrication of human organs-on-chips. Nat Protoc 8:2135–2157