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EXECUTIVE SUMMARY OF THE THESIS

3D Bioprinting of vascular structures for organoids models

TESI MAGISTRALE IN BIOMEDICAL ENGINEERING – INGEGNERIA BIOMEDICA

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1. Introduction

1.1. Organoids

Given the increasingly urgent need of in vitro models of human tissues, organoids are gaining a larger and larger foothold in biomedical research allowing to overcome the limitations associated with 2D cell cultures and animal models in mimicking human pathophysiology [1]. Organoids are indeed 3D self-organizing multicellular structures that recapitulate organ-specific architecture, cellular heterogeneity and functionality, an example is provided by intestinal organoids (Figure 1.1) that indeed resemble the structures and functions of their in vivo counterpart with all the different intestinal cell types properly distributed into the villus-like and crypt-like domains [2].

Organoids therefore represent a promising human-specific model system that provides unique opportunities for personalised medicine, drug testing, regenerative medicine and modelling of human development and disease. However, one of the main limitations in achieving completely functional organoids is the absence of a perfusing system that is crucial for their growth, long-term survival and, thus, maturation beyond the embryonic and fetal phase.

At present, in vivo transplantation into host animals is the only method that has been proven to successfully achieve a perfusable vasculature within organoids, but many strategies for in vitro vascularization are being studied, starting from pre-vascularization up to the engineering of capillary beds. The first one can be achieved through the co-colture of organoids with endothelial cells, or co-differentiation with mesodermal progenitor cells or mechanical stimulation, while for the second case the approaches can be divided into self-organized and pre-patterned methods [3].

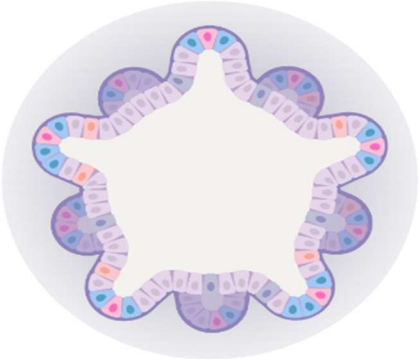

Figure 1.1 Scheme of an intestinal organoid

1.2. 3D Bioprinting

Although the promising results of these different approaches, the most ground-breaking technology is 3D bioprinting, which is the layer-by-layer deposition of a bioink, a biomaterial that can be mixed with living cells, to create 3D tissue-like structures starting from a CAD model. The latter is then sliced into layers generating the G-code commands that tell the printer how to move. Among the different bioprinting technologies, i.e., inkjet-based, laser-assisted and extrusion based bioprinting, the most used is pneumatic dispensing, that applies pneumatic pressure to extrude the bioink through a nozzle as a continuous filament. The optimization of the printing parameters, such as deposition speed, pressure and temperature, together with the nozzle size and bioink rheological properties, allow to increase the resolution of the printed construct, but at the same time can affect cell vitality and behavior and, thus, a compromise must be found.

The possibility to print filaments of different diameters make this technology very appealing for the perfusion of tissues models, as well as the use of bioinks, whose composition can improve vascularization, and the ability to control the spatial arrangement of the filaments to promote the formation of vascular networks [4].

For this reason, in the present master thesis 3D bioprinting is used with the aim to create a vascular network using a bioink that encapsulates endothelial cells, which are supposed to gradually migrate towards the periphery of the microfilaments generating a network of hollow endothelialized microfibers. Moreover, the grid is designed to host colon organoids and, thus, the network shows a central cavity that resembles the typical crypts and villi architecture of the intestinal lumen. Colon epithelial cells are then cultured in such a cavity for the purpose of giving rise to colon organoids.

In particular, this work has focused on the optimization of the printing process of the network, starting from the definition of the printing parameters and the lattice geometry, up to the G-codes modification. Then the bioprinting protocol has been optimized and biological experiments have been performed to evaluate endothelial cells survival inside the bioink filaments. In the end colon epithelial cells have been seeded inside the vascular network.

2. Materials and methods

The design of the 3D model of the constructs to be printed in the present study was performed using SolidWorks, a CAD software that allows to save the CAD model in the STL format. This STL file was then converted into a series of thin layers by a slicing software, PrusaSlic3r, and post-processed through Spyder, producing a G-code file that contains the instructions to tell the 3D printer exactly where to move and what speed to use to dispense the bioink in the desired pattern. The 3D bioprinter used to perform the experiments was the CELLINK INKREDIBLE +, it is a pneumatic-based extrusion bioprinter with dual heated (room temperature to 130°C) printheads and UV LED curing system (with 365 nm- and 405 nm-wavelengths) for constructs photo-crosslinking. Moreover, it provides a sterile printing environment without the need for a biological hood.

EAhy926 cells, a hybridoma line derived from human endothelium and A549/8 cells, were the first cells used to bioprint the vascular network, but then were substituted by other GFP cells to allow cell imaging through fluorescence microscopy. The lattice was thus printed using at first HUVEC GFP cells, primary human umbilical vein endothelial cells, then HEK293 GFP cells, immortalized human embryonic kidney cells, and lastly H5V cells, a murine endothelial cell line isolated from embryonal heart. Caco2 cells, an immortalized cell line of human colorectal adenocarcinoma cells, were instead resuspended in Matrigel droplets to fill the central cavity of the vascular network with the ultimate purpose of generating colon organoids.

CELLINK FIBRIN GEL and GelMA C are the bioinks that were used to bioprint the vascular network after being mixed with endothelial cells. The first one was chosen for its composition that supports vascularization through the creation of a fibrin network, the second for its transparency to allow cell imaging. Matrigel, a gelatinous protein mixture derived from mouse tumour cells, was instead used to be mixed with Caco2 cells before gelling to fill the vascular network cavity.

The mixing of the bioink with cells (10:1) was performed according to CELLINK bioprinting

protocol, which requires to connect through a luer lock two syringes that contain respectively the bioink and cell suspension, that can be mixed back and forward taking care not to introduce air bubbles and not to lose too much material [5]. When homogeneity is reached, the mixture is transferred to the cartridge, which is then capped with the nozzle and mounted into the printhead, that must be preheated to 26°C for GelMA C, while for CELLINK FIBRIN can be left at room temperature, where the latter is 20-25°C. Moreover, GelMA C UV protected cartridge requires to be heated for 15 min at 37°C in the incubator prior to cell mixing and left at room temperature for 20 min right after. Then the culture support must be cooled in the fridge for a whole night before printing and for 5 min after that to thermally gel the structures prior to photocuring, which is performed for 1 min using preferentially the 405 nm module to avoid cell damage. The nozzle size used in the present study was 0.41 mm, that ensures a good resolution and cell viability.
The image visualization and acquisition in brightfield and fluorescence was performed using the Eclipse Ti2 Inverted Microscope and NIS Element Viewer software. Fluorescence images were then processed using ImageJ to quantify fluorescence intensity, that is an indicator of cells viability, since only alive cells express the GFP protein. After RGB image splitting into separate channels, the green one was selected, then a mask was created and processed to select cells only without losing the original image. After being measured, this selection was applied to the original image to get the 'IntDen' value that measures fluorescence intensity.

3. Results and discussion

3.1. Definition of the structures and printing process optimization

Preliminary printings were performed using Nivea cream, a commonly used demonstration ink for extrusion bioprinting, to determine the suitable infill pattern of the grid, infill percentage, structure height and printing velocity. Different combinations of these parameters were tested and best results in terms of shape fidelity, split fibres and resolution were achieved with rectilinear pattern, 15% infill and a printing rate of 6 mm/s for 2 mm-high structures (5 layers).

Then the geometry of the internal hole of the grid, intended to host colon organoids, was studied and a flower-like hole with a 5 mm-external diameter was designed to resemble the colon crypts and villi geometry. Different hole geometries were tested into two kinds of 5 layered grids: grids with a 9.7x9.7 mm^2 -base and a single central hole and grids with a 15x15 mm^2 -base and four holes. Best results were obtained with a hole of 6 petals of 40° for both cases (Figure 3.1).

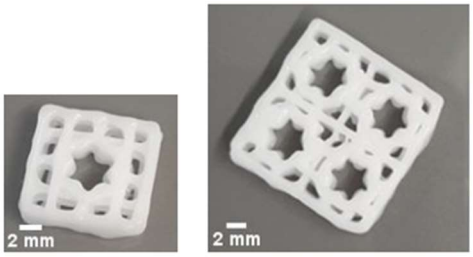

Figure 3.1 5-layers grids with single 40°-6 petals hole (A) and with four 40°-6 petals holes (B)

After that, aiming to print monolayer structures for cell imaging, the same kind of grids with one or four holes of different geometries were tested, but their height was 0.4 mm (1 layer), and since there was no overlapped layers collapse issue, printing speed could be set to 8 mm/s for perimeter and 6 mm/s for infill, allowing to obtain a thinner filament. Moreover, the infill was changed to 30% grid pattern to get the same type of final lattice as the 5-layers case. Best results were reached with 6 petals flower-like holes of 40° for the single hole case, while of 30° for the four holes case (Figure 3.2).

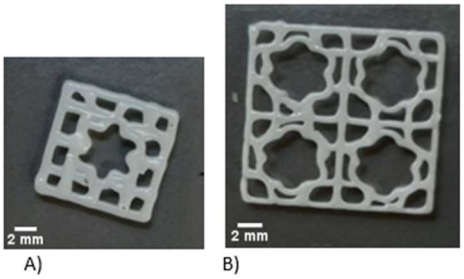

Figure 3.2 Monolayer grids with single 40°-6 petals hole (A) and with four 30°-6 petals holes (B)

These preliminary studies led therefore to the ultimate definition of the geometries of the grids to be printed:

- 1 layer (0.4 mm):
  - 9.7x9.7 mm^2 grid with single central 40°-6 petals flower-like hole

- 15x15 mm² grid with four 30°-6 petals flower-like holes
- High velocity (8mm/s for perimeter and 6mm/s for infill)
- 30% grid infill

▪ 5 layers (2 mm):
- 9.7x9.7 mm² grid with single central 40°-6 petals flower-like hole
- 15x15 mm² grid with four 40°-6 petals flower-like holes
- Slow velocity (6mm/s both for perimeter and infill)
- 15% rectilinear infill

Then different strategies to create and culture the vascular network with colon organoids right inside its cavity were established, from the simple lattice bioprinting with colon cells dispensed inside the hole, to 2-step bioprinting, where the network is printed first and then colon cells are printed inside the hollow, and dual printhead bioprinting with the simultaneous printing of endothelial cells with one extruder and of colon cells with the other.

The latter two cases also required the definition of the internal structures to be printed inside the holes of the four different grids. Flower-like and circular structures of different sizes were tested with 80% rectilinear infill, so that they could become a "full" environment enabling colon cells communication and migration. Circular structures gave better results in all the four cases, respectively with a diameter of 2.2, 2.5, 2 and 2 mm (Figure 3.3).

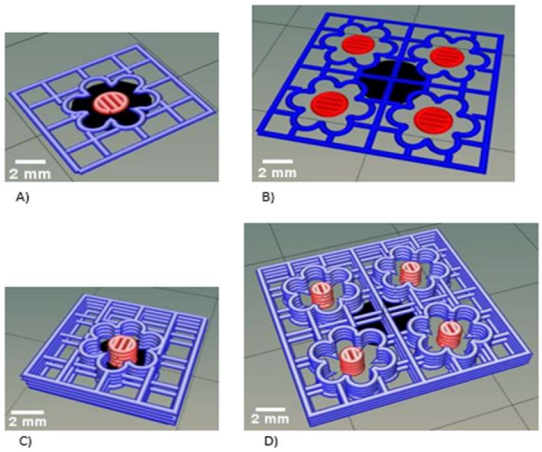

Figure 3.3 Final geometries of the monolayer (A and B) and 5-layers (C and D) structures

However, both the approaches reported some issues related to printhead movements during the printing process that led to grid damage and filament displacement. G-codes were thus modified to lower the printhead right in the hole centre and to lift, shift and lower it when moving from a hole to the other, but also at each extruder switch during the dual printhead printing. Such adjustments enabled to print both the inner and the outer structures without damaging each other.

Furthermore, the repetition of the G-code of the structure to be printed, together with the printhead displacement between the wells, allowed the simultaneous bioprinting of multiple constructs in a multi-well plate (Figure 3.4).

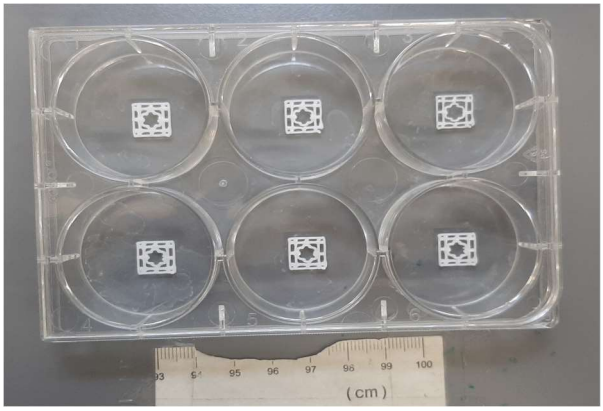

Figure 3.4 Printing of the same structure in a 6-wells plate

3.2. Bioprintings

Once defined all the geometries, CELLINK FIBRIN was the first bioink used to print the monolayer grid with a single central hole. The bioink allowed to achieve the same results of Nivea cream in terms of printability, shape fidelity and resolution, but it turned out to be too opaque for the cells to be clearly seen in bright-field microscopy. It was thus substituted with a translucent bioink, GelMA C, and, additionally, the grid height was reduced to 0.1 mm and GFP cells were used in order to be detected with fluorescence microscopy.

GelMA C was therefore mixed 10:1 with HUVEC GFP cells with a cellular density of 1.12×10^6 cells/ml to print the grids in a 6-wells plate. The structures were then crosslinked for 1 min using the 405 nm photocuring module, macroscopic and fluorescent images were acquired and culture medium was added to cover the grids. Images were captured for about 20 days, during which fluoresce microscopy showed an initial increase in cells number and fluorescence intensity, suggesting that cells were growing, then cells number and fluorescence intensity remained almost stable until they started

decreasing, symptom that cells were dying (Figure 3.5).

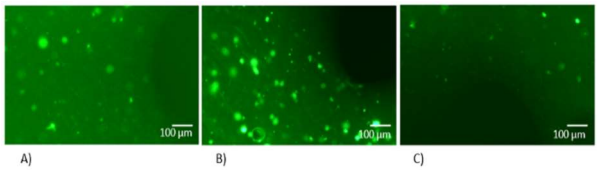

Figure 3.5 Fluorescence microscopy images of the grid filaments on day 0 (A), 6 (B) and 19 (C)

Visual assessment was further supported by the fluorescence quantification analysis (Figure 3.6).

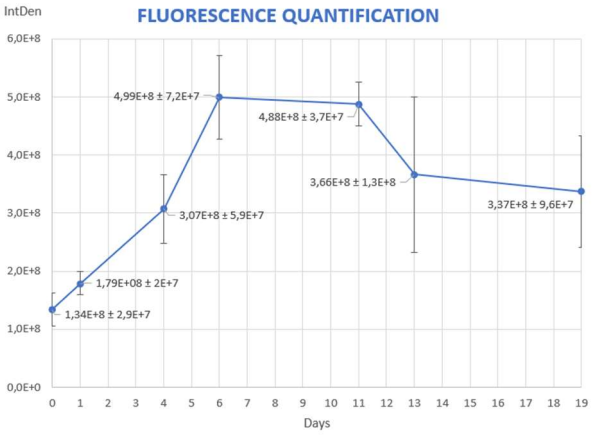

Figure 3.6 Graph of fluorescence intensity along the culture days (from day 0 to 19)

During these 20 days the bioink structures did not dissolve (Figure 3.7), shape and consistency were retained, demonstrating that 1-minute photocuring was a good compromise between hydrogel polymerization and cell survival. Moreover, no contamination occurred, proof that all the proceedings were performed in sterility conditions.

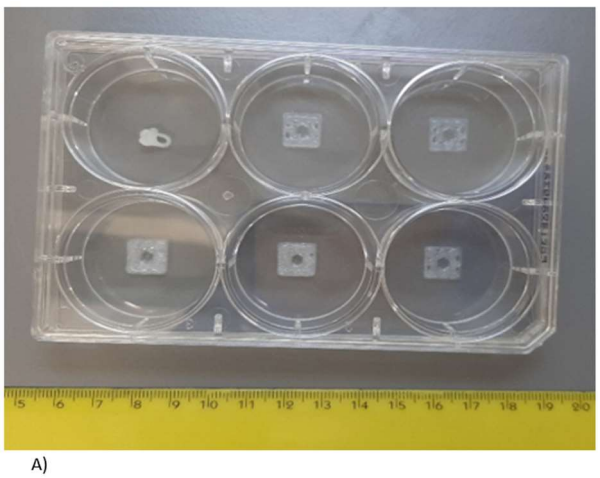

Figure 3.7 Macroscopic images of the printed grids on day 0 (A) 19 (B)

The same experiment was then performed first using HEK GFP cells and then again HUVECs GFP, but the bioink was too liquid to obtain grids with the desired shape and resolution, mainly because the too high room temperature affected the viscosity. The bioprinting protocol was therefore adjusted reducing the duration of the permanence in the incubator and cooling the cartridge in the fridge before printing, allowing to achieve a less liquid bioink and thus better results in terms of printability and shape fidelity, but also preventing dissolution in culture medium.
The same grids were then printed using GelMA C mixed with H5V cells as well as GelMA C alone. Further protocol adaptation, together with a low printing pressure, enabled to reach in both cases very well-shaped grids with a high resolution and shape fidelity, that did not dissolve in warm medium even in non-optimal environmental conditions (Figure 3.8).

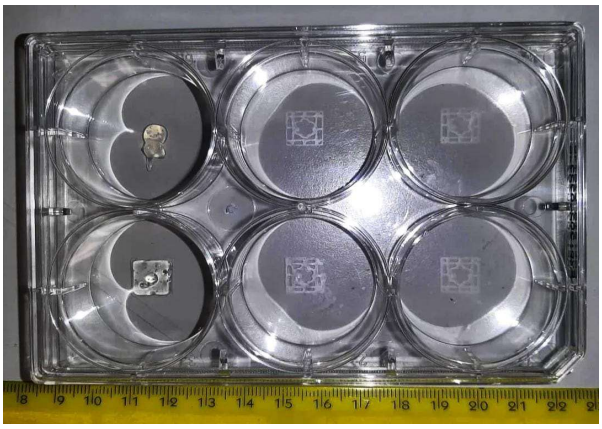

Figure 3.8 Grids printed using GelMA C mixed with H5V cells

However, such good results were achieved with the extra precaution of a longer photocuring (2 min), that unfortunately caused cell death and which might have been unnecessary. Regrettably, it could not be verified whether the protocol adjustments, together with the correct crosslinking duration (1 min), could effectively yield good results also in terms of cell viability.
Nevertheless, after grids printing, the cavities of these grids were filled with Caco2 cells-laden Matrigel droplets of 10 μl with a cell density of 10^5 cells/ml. The procedure optimization considerably reduced bubbles formation in Matrigel, allowing to see cells in brightfield microscopy.

4. Conclusions

This thesis work allowed to define the suitable printing parameters as well as the geometry of the network designed to host colon organoids, but also that of the internal structures printed right inside the lattice. G-codes modifications enabled to print both the inner and the outer structures without damaging each other, along with the bioprinting of multiple constructs in a muti-well plate. Moreover, the choice of the proper nozzle and bioink, together with the optimization of the bioprinting protocol, allowed to print high-resolution constructs, which did not dissolve in culture medium enabling endothelial cells proliferation and survival as well as the seeding of colon epithelial cells.
However, the 3D printer resolution did not allow to print filaments with an even smaller diameter that might better mimic that of in vivo capillaries. Moreover, this printed vascular network alone will not be enough to achieve a fully perfusable system, but an in vitro pre-vascularization, followed by the anastomosis between external vessels and internal vasculature, would be essential. Therefore, the future of this work could be the co-colture of Caco2 cells together with endothelial cells to create pre-vascularized organoids right inside the lattice hollows and then anastomose the internal vessels with the printed vascular network.
Prior to this, further printings and characterizations are mandatory, as well as supporting fluorescence quantifications. Then, next step would be the bioprinting of all the other remaining prepared constructs using the endothelial cells-laden bioink and the culture with colon cells-laden Matrigel droplets up to capillary and organoids formation. Moreover, the influence of such a network on organoids growth should be evaluated in all the different cases. Additionally, confocal microscopy could be used to detect cells in multi-layered constructs. After that, also 2-step and dual printheads bioprintings should be experimented with cells.
Last but not least, although this thesis has focused on colon organoids, geometry changes could be carried out to adapt this vascular network to the vascularization of a different kind of organoid.

References

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