Influence of 3D bioprinting on cell behaviour and capillary formation

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ABSTRACT: The lack of vascularization to support tissue substitutes remains a critical challenge for the clinical translation of tissue engineering. Cell nutrition can only be assured by diffusion at a maximum of 200 µm distance from vessels, therefore an effective revascularization of the fabricated tissues upon implantation is crucial for successful transplantation. 3D bioprinting is an emerging technology with remarkable potential to solve this challenge. However, the design of appropriate bioinks, which show simultaneously good printability and cytocompatibility, is still an issue. This study aimed at creating a bioink, which could be printed with high shape fidelity and promote the formation of capillary structures post-printing. For this, a blend of fibrinogen and hyaluronic acid was developed. Rheological characterization showed that this blend has improved viscosity properties for printing, when compared to its single constituents. Printing was conducted using a gelatine microparticle support bath and an extrusion bioprinter. With this system, the printing of complex structures with high resolution was possible and the incorporation of cells did not affect the shape fidelity of the constructs. Cell viability post-printing was maintained above 81% and a cell proliferation assay confirmed the recovery of proliferation 7 days post-printing. Following a 14-day incubation period, the formation of capillary networks was visible both in the printed hydrogels and in the non-printed moulded controls. Even though the formation of capillary structures proved to be more extensive in non-printed hydrogels, it was possible to show the high potential of this bioink for future vascularized tissue fabrication.

KEYWORDS: Extrusion 3D bioprinting, gelatine support bath, bioink, fibrinogen - hyaluronic acid, in vitro capillary formation

INTRODUCTION

Tissue engineering (TE) is an interdisciplinary field that intends to provide biological substitutes that can replace or restore anatomical function [1]. Clinical feasibility in TE has already been shown for tissues such as the skin [2] and cartilage [3], blood vessels [4] and bladders [5]. However, these tissues can be supported by the diffusion of nutrients from the host vasculature. Hence, one of the main challenges in TE is the creation of a functional vasculature within the TE construct, that is able to supply cells with nutrients and oxygen and remove metabolites [6]. Cells do not survive more than 200 µm away from capillaries, thus the long-term viability of TE tissues relies on its vascularization [7].

3D bioprinting is an emerging technology, which has allowed for impressive developments in the TE field. With the advent of 3D bioprinting and the possibility to obtain 3D computer-aided design models from computer tomography or magnetic resonance imaging scans, TE can be personalized to match the patients’ exact anatomy, therefore driving the advancement of personalized medicine [8]. Furthermore, the use of 3D instead of 2D cultures allows for a more realistic model of tissue-specific functions and the in-vivo microenvironment [9]. Extrusion-based bioprinting is currently the most used bioprinting technology due to its versatility for printing 3D structures and affordability [10]. Since in extrusion bioprinting the printing solution, or bioink, is dispensed through a micro-nozzle pneumatically or mechanically, the system can be adapted to extrude a wide range of bioinks with varying viscosities and cell concentrations [11]. Nevertheless, the resolution of extrusion bioprinting is considerably lower than for similar technologies, such as inkjet or laser bioprinting, which is one of its major limitations. To overcome this lack in resolution, embedded bioprinting recently emerged as a possible solution. Different viscous support baths have already been developed, based on Carbopol® polymers [12], on gelatine microparticles [13] or on nanoclay [14]. One of these techniques is the freeform reversible embedding of suspended hydrogels (FRESH), developed by Hinton et al, which relies on the development of a microparticle gelatine slurry. This slurry acts as a Bingham plastic during the printing process, supporting the printing of complex structures using soft biological hydrogels with high resolution, shape fidelity and cell viability [13].

Hydrogels are the most studied materials for bioprinting due to their cytocompatibility and non-immunogenicity [15]. A tuned printability for the chosen bioprinting technology, the ability to quickly crosslink, to have sufficient mechanical strength to hold its shape after printing, cytocompatibility and biodegradability are a few of the properties required for any hydrogel to be used as a bioink [11, 16, 17]. Finding hydrogels that are both cytocompatible and have the right properties for
biofabrication is a challenge [15]. In general, properties that enhance printability diminish cell compatibility and a good balance between these two requirements needs to be achieved for successful biofabrication [9]. Since the choice of the bioink should be made to closely match the functional and mechanical properties of the tissue it will replace, for vascularized tissue fabrication, the bioink and, later, the resulting hydrogel, need to be adapted to promote the formation of capillary-like structures. For this, bioinks must support the adherence, proliferation, migration and alignment of cells [18]. For bioprinting, though, bioinks need an intrinsic printability and must form a stable hydrogel post-printing. The combination of all these necessary properties is often unfeasible.

Fibrin is a natural polymer with an intrinsic ability to support capillary formation [19]. Apart from the angiogenic potential, fibrinogen used as a bioink has an excellent cytocompatibility, comprising RGD motifs that promote cell-adhesion and growth factor binding sites [20–22]. Cell behaviour is known to be affected by the biochemical and structural properties of the fibrin gel [23]. It has been shown that there is an inverse correlation between matrix stiffness and capillary formation [23, 24]. Despite its cytocompatibility, its low viscosity and the requirement of two components (fibrinogen and thrombin) to achieve its polymerization both contribute to its poor extrudability. Therefore, new printing strategies, such as the FRESH technique are currently being used to improve resolution and shape fidelity. Furthermore, fibrinogen can also be blended with more viscous materials to improve its printability and shape fidelity [25]. Hyaluronic acid (HA) is a natural glycosaminoglycan which can not only improve the viscosity of fibrinogen for dispensing, but also interact with fibrin’s structure. Fibrin formed in the presence of high molecular weight (HMW) HA (=1500 kDa) is reported to have thicker fibres and larger pores, which has been shown to promote cellular migration, particularly of endothelial cells. In the presence of HMW HA, the fibrin gels form over a longer clotting time and are less rigid. However, HA was reported to inhibit fibrinolysis following plasminogen activation [26]. Besides improving dispensability, HA also has the potential to shield cells from the shear stresses on the walls of the printing nozzle.

Few studies have focused on the development of bioinks with angiogenic potential [18, 27]. Instead, the most common strategies involve the printing of vessels on the macroscale through the use of sacrificial bioprinting. Sacrificial bioinks, such as gelatine and Pluronic F127, are used to create channels within hydrogel matrices, which are later removed for seeding of the vascular cells [28]. Gao et al. have developed a coaxial extrusion nozzle which allows external flow of cell-laden alginate and internal flow of CaCl₂, thus creating endogenous perfusable microchannels [29]. Despite these advancements, the creation of the complex microvasculature network existent in organs is still a challenge, since the printing resolution of common bioprinters (150-200 µm) is an order of magnitude higher than the diameter of capillaries (10-20 µm) [1, 30]. Therefore, for capillary formation, pre-vascularization and the use of angioinductive bioinks are currently the most used strategies. Moulding is the most widely used in vitro pre-vascularization technique. It consists in the seeding and co-culturing of ECs and support cells, such as fibroblasts, in a scaffold, typically in a “gel” form. The anastomosis between the pre-formed capillaries and the host vasculature upon implantation has been shown to be accelerated using this technique [19]. It is likely that a combination of pre-vascularization and the fabrication of large vessels could be the ultimate solution to the vascularization challenge in TE and Lee et al. have already attempted this strategy with success [31]. In spite of the advancement that this study brought to the bioprinting vascularization field, the cells were not printed, therefore the effect of bioprinting on capillary formation has not been evaluated. In fact, few studies have been performed evaluating the effect of the printing process in cell function, despite its importance for future in vivo studies and applications. Therefore, the aim of this work was to develop a printable bioink with potential for capillary formation and to further evaluate the effect that the printing process has on cell behaviour and function.

**MATERIALS AND METHODS**

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated following established protocols [32]. Endothelial cell growth medium 2 (PromoCell) supplemented with 1% antibiotic-antimycotic solution (ABM, Gibco) was used for culturing and cells were used up to passage 4. Culture medium was exchanged every 2 to 3 days. Passaging was performed upon reaching 80%-90% confluence by using 0.04 mL/cm² of Trypsin/EDTA (PAN Biotech). Human dermal fibroblasts (HDF) were isolated from skin tissue, as described by Helmedag et al. [32]. Cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and 1% ABM, which was changed every 2 to 3 days. Passaging was performed as described for HUVECs. All cell cultures were maintained under incubation at 37°C in a vapour-saturated atmosphere with 5% CO₂.

Preparation of the gelatine microparticle support bath

The protocol followed was adapted from Hinton et al. [13]. Briefly, 250 mL of deionized water were pre-heated to 40-45°C on a hot plate in a glass mason jar (Ball Inc.) and 10g of gelatine (Type A from porcine skin, 300 Bloom, Allevi) and 0.4g of CaCl₂ (Allevi) were added and stirred until fully dissolved. The gelatine was stored overnight at 4°C. The next day, the remaining space of the jar (approximately 250 mL) was filled halfway with cold 11mM CaCl₂ solution and a spatula was used to separate the gelatine from the walls of the jar and introduce solution between the sides of the gelatine and the container. A rubber O-ring was placed on top of the mason jar and solution was filled to the top. The jar was placed at -20°C for one hour. The gelatine was blended at the
highest speed (III) of the blender (Osterizer MFG) for 60 s. After blending, approximately 40 mL of the resulting slurry were pipetted into 50 mL centrifuge tubes and 10 mL of cold 11 mM CaCl₂ solution were added and resuspended. Five centrifugation steps at 3800g and 4°C for 4 min were then performed. Between each centrifugation, the soluble gelatine on top and the supernatant were removed using a Pasteur pipette and a vacuum pump system. Cold CaCl₂ solution was used to refill the tubes and resuspend the slurry. After the centrifugations, the tubes with the slurry in suspension were stored at 4°C until printing. For printing, the suspension was centrifuged at 230 g for 5 min, the supernatant discarded, and the slurry poured into 24 well plates (VWR). Two Kim-wipes (Kimberly-Clark) were placed on top and allowed to saturate to remove excess fluid. For crosslinking the fibrinogen, 375 μL of thrombin (20 U/mL, Sigma) were pipetted into each well and mixed with a spatula to hold a final concentration of 5 U/mL of thrombin in each well.

**Preparation of the bioinks**

A fibrinogen-hyaluronic acid (FG-HA) ink was developed, adapted from the ink used by Hinton et al. [13]. It consisted of 10 mg/mL of fibrinogen from human plasma (Merck Millipore) and 5 mg/mL of high molecular weight hyaluronic acid from *Streptococcus Equi* (bacterial glycosaminoglycan polysaccharide, mol wt ~1.5-1.8 x 10⁶ Da, Sigma). To prepare it, the stock solution of fibrinogen was diluted with Tris-Buffered Saline (TBS) to the desired concentration and the HA powder was added directly to this fibrinogen solution. After vortexing several times, the ink was left in the incubator at 37°C for at least two days to fully dissolve the HA. For the preparation of cell-laden bioinks, cells (HUVECs and HDFs) were harvested and resuspended in a small amount of EGM2 medium. The correct volume of cells was added the previously prepared FG-HA ink to achieve a final concentration of 3x10⁶ cells/mL. The ink was then transferred to sterile 10 mL luer-lock plastic syringes (BD).

**Rheological characterization of the bioinks**

Rheological evaluation of the bioinks was performed using a Kinexus Ultra+ rheometer (Malvern), fitted with a cone and plate geometry. For measuring low-viscosity fluids (water and fibrinogen-only solutions) a 40 mm/1° cone geometry was used and for the higher viscosity inks (FG-HA and HA) a 20 mm/1° cone geometry was used. Both were used with a solvent trap to prevent drying of the samples. The shear rate was increased from 1 to 1000 s⁻¹ over a ramp time of 20 min. Measurements were performed at 25°C and the samples were allowed to stabilize for 5 min before starting with the measurements.

**3D bioprinting system**

Printing was performed using an Allevi 2 extrusion 3D bioprinter (Allevi, formerly Biobots, CA, USA).

The computer-aided design models of hollow and non-hollow cylinders were designed using SolidWorks 2016 software (Dassault Systèmes). The bifurcated vessel CAD model was retrieved from GrabCAD. All CAD files were exported from SolidWorks as stereolithography (STL) files to be further processed in the software Repetier-Host (Hot-World GmbH & Co). They were converted into gcode files for use with the 3D printer. For slicing and generating the gcodes, the software Slic3r (available within Repetier Host) was used. For printing, the previously bioink-loaded syringes were fitted with a stainless steel 12.7 mm length, 30 Gauge needle (EFD Nordson), unless otherwise stated. Printing pressure for this nozzle diameter ranged between 5.5 and 6.5 psi (0.38-0.45 bar). After calibration of the printer as described by the manufacturer, the printing process was started as soon as possible to avoid initiation of crosslinking and possible clogging of the nozzle inside of the support bath. Printing was carried out at room temperature (RT) and printing time was kept to a maximum of one hour in the case of cell-laden constructs to preserve cell viability. Sterility was maintained by printing inside a class II safety cabinet. Constructs polymerized for 20 min inside the slurry at RT and were then incubated at 37°C for 20 min to melt the support bath and further crosslink the gels. Printed structures were rinsed with PBS. Then, 1 mL of the appropriate cell culture medium type, supplemented with 0.16% w/v tranexamic acid (Carinopharm), was added to the wells of the plates that contained cell-laden constructs, and incubated for the duration of the experiments.

All the images used to show the 3D printed constructs were taken using a phone camera. Images were further processed with ImageJ software.

**Stability and degradation of the printed hydrogels**

Triplicates of cell-laden and acellular FG-HA cylinders with 6 mm diameter and 2 mm height were fabricated. Constructs were cultured in EGM2 medium supplemented with 0.16% TXA in a vapour-saturated atmosphere at 37°C and 5% CO₂ and medium was changed three times per week. To study degradation rate, samples were weighted on days 0, 1, 4, 7 and 14 and the percent difference in the weight of the constructs in day x and day 0 was calculated as represented in equation (1).

\[
\% \text{ Mass Change} = \frac{m_{\text{day x}} - m_{\text{day 0}}}{m_{\text{day 0}}} \times 100
\]  

The samples’ diameter was measured with an Axio Zoom.V16 Stereo Zoom microscope (Zeiss), equipped with a 1.0x PlanNeoFluarZ objective. Images were processed using Zen 2 Pro software (Zeiss) and ImageJ.

**Cell viability and proliferation**

For evaluating cell viability, 6 mm diameter, 1mm height cylinders were printed using the described FG-HA bioink, inside the previously prepared support bath. A 200 μL cell-laden fibrin-HA gel was moulded into one of the empty wells as a control. Final concentrations in the moulded control were 10 mg/mL of fibrinogen, 2.5 mg/mL of HA, 5 U/mL of thrombin and 11 mM of CaCl₂. The same
cell concentration as used in the bioink was used in the final moulded gel (3×10^6 cells/mL). 200 µL of the cell-laden FG-HA bioink were directly pipetted into the gelatine slurry as a control to assess the influence of the support bath on cell viability. Following the printing process, the appropriate cell medium for each cell type was pipetted into each well and the plate was incubated for two hours at 37°C in a vapour-saturated atmosphere with 5% CO₂. LİVE/DEAD staining was performed by replacing cell culture medium with 300 µL of fresh medium supplemented with calcein-AM (AAT Bioquest) at a concentration of 2 µg/mL. Constructs were further incubated for 30 minutes, following which 3 µL of propidium iodide (Sigma) were added directly before imaging to each well, for a final concentration of 2 µg/mL. The stained hydrogels were imaged using an Axio Observer.Z1 inverted fluorescence microscope (Zeiss) equipped with a 10x/0.45 M27 Plan-Apochromat lens. Z-stacks were acquired containing 10 slices at 20 µm distance from each other. For each sample, 6 images at random locations were taken. Analysis of the images was performed with ImageJ. Z-stacks were reconstructed with the z-projection function of ImageJ, which projects multiple images onto a single plane. Cells were counted using the Cell Counter plugin of ImageJ and cell viability was calculated as the number of live cells divided by the total number of cells.

For evaluating the effect that the FRESH printing process has on the proliferation, a proliferation assay using the alamarBlue reagent (Invitrogen) was performed post-printing. As previously, cylinders were printed using the already described cell-laden FG-HA bioink. 200 µL of the cell-laden FG-HA bioink were directly pipetted into wells containing gelatine slurry and were used as non-printed controls. Measurements with alamarBlue assay were performed 2 hours, 1 day, 3 days, 7 days and 14 days post-printing, according to manufacturers’ protocol. Incubation with the alamarBlue reagent was performed for 3 hours, protected from light. The absorbance of the samples was analysed with a microplate reader (Infinite M200, Tecan) by transferring 100 µL of the medium of each condition to 96 well plates. Absorbance was measured at 570 nm and 600 nm. Samples were washed twice with PBS during 15 min, fresh culture medium was added and the plate was further incubated until the next measuring day. Medium was changed on the days before the measurements. The percentage reduction of alamarBlue was calculated as indicated by the manufacturer. Data was normalized to the values obtained 2 hours post-printing.

**Capillary formation assays and evaluation**

To study the effect that the developed bioink had on capillary-like network formation and to have non-printed controls for the FRESH angiogenesis assay, a 3D angiogenesis assay was performed by moulding cell-laden hydrogels in 48 well plates (VWR) and culturing them for 14 days. A moulded fibrin 5 mg/mL hydrogel that has been proven to be angioinductive was used as control [18]. Final concentration in the moulded control gel were of 5 mg/mL fibrinogen, 3 U/mL thrombin and 3.75 mM CaCl₂. Three different hydrogel concentrations were evaluated. For the fibrin 10 mg/mL, 5 U/mL and 11 mM CaCl₂ were used. For the fibrin-HA 10 mg/mL:1 mg/mL, HA was added to the previous mixture at the concentration of 1 mg/mL and for the fibrin-HA 10 mg/mL:2.5 mg/mL, HA was added at 2.5 mg/mL. The final concentration of HUVECs and HDFs on the gel was of 3×10⁶ cells/mL for each cell type. Gels were left to polymerize for 20 min at RT, followed by 20 min at 37°C. After this time, EGM2 medium, supplemented with 0.16% TXA was added to each well and plates were incubated for 14 days at 37°C in a vapour-saturated atmosphere with 5% CO₂.

To determine the effect that the FRESH printing process has on capillary network formation, the cell-laden FG-HA bioink was printed in cylinders with 8 mm diameter and 2 mm height and structures were cultivated for 14 days. For all the capillary formation assays, medium was changed every 2 days.

**Immunostaining and Two-Photon Laser Scanning Microscopy**

The presence of vascular structures was assessed by immunohistochemical labelling of the adhesion molecule PECAM-1 (CD31). HUVECs were stained with mouse anti-CD31 (PECAM-1, 1:100, Sigma-Aldrich) and Alexa Fluor 594 goat anti-mouse IgG (1:400, Life Technologies). DAPI staining was performed at the concentration of 1.5 µg/mL. For visualization of the formed capillary like networks, a two-photon laser scanning microscope (TPLSM, Olympus Fluoview 1000MPE) was used with a 25x water immersion lens (NA 1.05, Olympus Optical) and a MaiTai Deep-See Titan-Saphir Laser (Spectra Physics). To quantitively evaluate lengths, surface areas, volumes and number of branches of the capillary like networks present, the images were analysed with Imaris software (Bitplane Inc.).

**Statistical Analysis**

Results obtained are presented as mean ± standard error of the mean (SEM) displayed as error bars. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software). To determine the p-values, a one-way ANOVA test followed by either Tukey’s or Dunnnett’s post-hoc test for multiple comparison were performed, appropriately. A student’s t-test was used when analysing only two datasets at a time. The significance level was denoted by asterisk symbols, and differences were considered significant when p-value was *<0.05, **<0.01, ***<0.001 and ****<0.0001.

**RESULTS AND DISCUSSION**

**Rheological properties of the FG-HA bioink**

Since viscosity is a property that affects the printability of the bioinks in a large scale, a study was performed to evaluate the viscosity of the FG-HA bioink used in this
work (Figure 1). All of the inks measured exhibited a shear-thinning behaviour typical of biopolymer solutions, particularly the inks containing hyaluronic acid (HA-5 and FG-HA). One interesting finding for the FG-HA bioink is that its viscosity is considerably higher than the viscosity measured for either of its components, HA 5mg/mL (HA-5) and fibrinogen 10 mg/mL (FG-10). Studies have shown that fibrinogen and HA can interact with each other in the presence of an external salt (which is present in the TBS used to dilute the fibrinogen), possibly due to electrostatic interactions between the negatively charged HA at neutral pH and the cationic sites in fibrinogen, resulting in the formation of a loose network. This effect has been attributed as responsible for the increase in the viscosity of HA in the presence of fibrinogen [33]. The dynamic viscosities of the HA-derived solution are in the range of values found in the literature (0.1-1 Pa s) [34]. Even though the FG-HA viscosity is on the lower range of viscosities used in extrusion bioprinting (0.03-6×10^-3 Pa.s), its value is higher than that of fibrinogen, which show the potential of this bioink for extrusion bioprinting.

![Graph showing dynamic viscosity of various inks.](image)

**Figure 1: Dynamic viscosity of the FG-HA bioink and its individual constituents.** Dynamic viscosity of distilled water was measured as a reference. Data presented as Average±SEM for each shear rate point measured (n=3 independent samples per ink).

**Printing results**

In this section, the goal was to show that the FRESH printing process allowed the printing of structures with high resolution using the developed FG-HA bioink, both with and without cells. Three main structures were chosen: hollow cylinders, bifurcated vessels and non-hollow cylinders, which were further used for the cellular experiments due to its higher stability. To facilitate the comparison of the printed results with the g code models from which they were generated, the latter are also provided (Figure 2, A-C). Printing results with the acellular FG-HA bioink were successful, even though constructs were quite fragile to be moved out of the support bath. Through imaging the non-hollow cylinders, it was possible to distinguish each individual printed strand of FG-HA ink as designed in the g code models of non-hollow cylinders. It was also possible to distinguish numerous air bubbles that are left trapped within the construct after melting the gelatine microparticles, resulting in a porous structure (Figure 2, D). The diameter of the cylinder-shaped constructs was approximately 15% larger than designed. The spreading of the printed strands in between the gelatine microparticles that may occur due to the low viscosity during the time it takes to crosslink might explain why the diameter of the cylinder-shaped FG-HA constructs was higher than expected. A thickness of approximately 1.54 mm was obtained and considered as the minimum printing resolution of FG-HA ink (Figure 2, E). Regarding the bifurcated vessel, it was possible to distinguish the hollow channel of the vessel in its larger portion as indicated by the arrow in Figure 2, F. The FRESH method was also successfully validated for the cell-laden FG-HA ink (Figure 2, G). Therefore, the success of FRESH printing complex structures with FG-HA is not affected by the use of cells. The printing of three constructs per model was performed to show the reproducibility of the results. These results are a step forward in the establishment of the FRESH technique since they were comparable to the ones obtained by Hinton et al. in the original FRESH article, further extended to the validation of the method for the printing of cell-laden complex structures with a FG-HA bioink [13].

**Stability and degradation of the hydrogels**

To study whether the printed constructs were stable post-printing in terms of its degradation rate and contraction, a degradation study was performed. Results showed a significant contraction of the cell-laden FG-HA hydrogels between day 0 and day 1, both in terms of weight and diameter (Figure 3, A-B). The reduction of hydrogel mass relative to the value measured two hours post-printing was statistically significant (p<0.01) for the cell-laden FG-HA hydrogel at all time points (Figure 3, A).

![Image showing printing results.](image)

**Figure 2: Results obtained from bioprinting the FG-HA bioink.** A-C: gcode models used for printing; D: Microscopic image of the structure of the printed cylinders; E: Top view of hollow and non-hollow cylinders with corresponding diameters and thickness; F: Printed bifurcated hollow vessel and G: Cell-laden structures printed. Scale bars: A-C, E-F: 1 cm; D: 2 mm.
This reduction happened mostly during the first 24 hours post-printing, stabilizing after this initial reduction. This can be partially attributed to the release of gelatine microparticles that are left captive inside the construct post-printing and that melt upon incubation. The higher extent of contraction exhibited by the cell-laden FG-HA hydrogels compared to the acellular hydrogels may seem to indicate that there was an interaction between the cells and the scaffold. It has already been reported that HDFs are able to reorganize fibrin fibrils into closely packed fibres, which result in a high contraction of the fibrin hydrogel occurring within few days of culture [35]. This phenomenon was verified throughout the experiments, as only hydrogels that contained fibroblasts (either in monoculture or in co-culture with HUVECs) visibly exhibited severe contraction, but not hydrogels containing HUVECs only (data not shown). Furthermore, the presence of HA may further enhance the contraction of the cell-laden hydrogels, which might be due to the cellular binding to HA via CD44 receptors, as has already been proven to happen in the case of collagen [36]. This would explain why there was a higher level of contraction in the case of FG-HA hydrogels, but not in the case of hydrogels without HA. Regarding hydrogel diameter (Figure 3, B), the contraction was statistically significant (p<0.0001) for the FG-HA cell-laden hydrogels as soon as one day post-printing. Two hours post-printing, the diameters of the cell-laden FG-HA hydrogels were already smaller than the diameter of the remaining hydrogels analysed, maybe implying that a significant contraction had already occurred during this period of time. To a less extent, this contraction was also significant acellular FG-HA hydrogels, from day 4 onwards. Hydrogels remained intact for the 14 days of the experiment. Further cultivation of the hydrogels beyond the end of the experiment (up to a month) proved their high resistance to degradation using the adopted cell culture conditions (data not shown).

Cell viability and proliferation post-printing

Using a co-culture of HUVECs and HDFs, we obtained a value of 87.3±10.2% cell viability post-printing, against the value 93±6.0% obtained for the moulded control and the value 90.2±4.1% obtained for the pipetted control. Both the printed and pipetted viability values were not statistically different from the moulded control, which implies that both the use of the support bath and the printing process did not impair cell viability. This high cell viability can be explained by the cell-compatible nature of the support bath, which is an aqueous, buffered environment [13]. Another important factor for the success of bioprinting concerns the ability to control the amount of cell proliferation, as both too little or too much proliferation can prevent the achievement of good results. Results showed that from day 4 to day 7, cell proliferation was significantly affected by the printing process, when comparing to the respective non-printed pipetted controls (Figure 4).

Even though cells were initially affected by the printing process, they seemed to recover to values similar to the non-printed controls after 14 days.

Capillary formation assays

To assess the influence that both the designed bioink blend and the printing process itself have on capillary-like
network formation, in vitro angiogenesis co-culture assays with HUVECs and HDFs were performed for 14 days. Due to the solubility limitation of 5 mg/mL of the HA used in this work, the final concentration used for moulding (2.5 mg/mL) was not the same as had previously been used for printing (5 mg/mL). As thrombin and CaCl₂ are directly supplemented in the gelatine slurry support bath, their final concentrations in the printed constructs are unknown, which also prevents the exact replication of the FG-HA bioink in moulding. Morphological evaluation of the images obtained through TPLSM images confirmed the formation of capillary-like structures in all of the tested moulded hydrogels (Figure 5). With the increase in the concentrations of fibrinogen, thrombin and CaCl₂ (Figure 5, B), a decrease in the complexity of the vascular networks can be observed, compared to the control (Figure 5, A). The same observation is valid when evaluating the condition where there is the addition of 1 mg/mL of HA to the gel (Figure 5, C). However, results showed an overall increase in capillary complexity regarding capillary area, volume, length and number of branching points with the addition of 2.5 mg/mL of HA (Figure 5, D). This condition was the one of most interest, as it intended to replicate the same hydrogel conditions as would be further used in FRESH bioprinting. Quantitative evaluation showed a general increase in all measured parameters for the fibrin-HA (10 mg/mL:2.5 mg/mL) hydrogels in comparison with the fibrin (5 mg/mL) control (Figure 6). The mean value for the capillary area was of 28605.6±8068.2 µm² in the case of fibrin-HA (10 mg/mL:2.5 mg/mL) against the value of 9099.5±2665.5 µm² obtained for the fibrin control (Figure 29 A). Regarding capillary volume, the same trend was observed, with the value of 78455.6±14653.54 µm³ for fibrin-HA (10 mg/mL:2.5 mg/mL) statistically different (p=0.0170) from the value of 26555.56±9487.7 µm³ obtained for the fibrin control. Both capillary area and volume were the lowest for the fibrin-HA (10 mg/mL:1 mg/mL) hydrogel, which suggests that at this concentration HA has an inhibitory effect in capillary formation. It is possible that HA interacts with receptors present in endothelial cells, such as CD44 receptors, and that this mechanism requires an optimized concentration of HA to occur. These receptors may result in HA internalization, leading to its degradation by acid hydrolases inside the cells and the release of LMW fragments, which have been identified as able to induce angiogenesis [37,38]. The lower values relative to control obtained for fibrin (10 mg/mL) can be explained by the higher stiffness of the matrix for increasing fibrinogen and thrombin concentrations, as a higher stiffness is associated with a reduction in capillary formation [24]. Cross-sections of the images were obtained with Imaris and confirmed the presence of lumens, with diameters ranging between 4 and 21 µm. These values were higher in the case of the fibrin-HA (10 mg/mL:2.5 mg/mL) hydrogel, which proves the formation of thicker structures in this case.

Following the optimistic results obtained with the fibrin-HA (10 mg/mL:2.5 mg/mL) moulded gel, the next step was to FRESH print with the FG-HA bioink and evaluate whether the formation of capillary structures was still possible. Morphological evaluation of the images obtained with TPLSM showed a lower number of capillary structures present in the resulting hydrogels as well as a more inhomogeneous distribution of both the tubule-like formations (red) and the cells (blue), when compared with the moulded results (Figure 7). It was possible to distinguish two different types of structures in the printed gels. The typical capillary structures (Figure 7, A-B) and structures resembling the typical endothelial cell monolayer that forms in 2D cultures, with the CD31 signal present in the intercellular junctions (Figure 7, C).

Figure 5: TPLSM images of the moulded hydrogels and corresponding cross-sections obtained with Imaris to show the presence of lumen in the capillary structures formed within fibrin (5 mg/mL of fibrinogen, A), fibrin (10 mg/mL of fibrinogen, B), fibrin-HA (10 mg/mL of fibrinogen, 1 mg/mL of HA, C) and fibrin-HA (10 mg/mL of fibrinogen, 2.5 mg/mL of HA, D). Lumens are represented by yellow arrows. Scale bars: 100 µm.
It is possible that these monolayers formed due to the printing process, as no similar structures were found in the moulded gels. We hypothesize that since the printing process occurs through layer-by-layer deposition, the cells align within the hydrogel in that same manner. Quantitative evaluation revealed mean values for capillary area, of 33198.1±5186.2 µm², for capillary volume, of 83222.0±22962.4 µm³, for capillary length, of 550.5±623.7 µm and for number of branching points, of 5.26±5.69. Therefore, the structures present in the printed hydrogels were comparable to the ones presented in the moulded hydrogels in terms of capillary area and volume but with shorter length and lower number of branching points. Nevertheless, these decreases were not statistically significant (Figure 8). Lumens were present, even though in lower number and smaller diameter than in moulded gels (between 4 and 10 µm).

CONCLUSIONS AND FUTURE WORK

The development of bioinks suitable for 3D bioprinting in terms of printability, resolution and stability, which are also able to promote cell adhesion, migration and differentiation is, to date, still a challenge in the 3D bioprinting field [18]. This is particularly true when the goal is to find a bioink that, besides the abovementioned properties, has potential for de novo capillary formation. Only a few authors have reported a successful development of bioinks with both printing and angiogenic potential and, to our knowledge, none have studied the effect that the 3D bioprinting process itself has on the cell ability for 3D vessel formation [18, 27]. Therefore, our goal was to create a bioink that allowed the printing of complex structures, while maintaining the potential to induce 3D capillary formation.
With the FRESH technique, the FG-HA bioink developed was used to print complex, high resolution structures, which revealed high shape fidelity when compared to the respective CAD designs and were able to be handled post-printing, despite its soft nature. This process was also validated for the printing of a cell-laden FG-HA bioink, with similar outcomes, which was a step forward on the current state of the art [13].

A degradation study performed for 14 days revealed high contraction of the crosslinked cell-laden FG-HA hydrogels especially between day 0 and day 1 post-printing. This effect was confirmed by a more than 50% reduction in the hydrogel mass as well as a reduction of approximately 30% in hydrogel diameter. The initial reduction of mass may partly be due to the melting of trapped gelatine microparticles from the support bath and due to the interaction of cells, particularly fibroblasts, with the surrounding matrix. Cells may interact specifically with HA, possibly via CD44 receptors. The contraction observed is a feature which will require correction in further studies, as it could severely impair the use of this bioink for in vivo applications. This may imply the design of CAD files which account for this contraction. An improved support bath could also prevent the decrease in weight attributed the gelatine microparticles, and, possibly, improve the overall contraction process. It would be interesting to verify if it would still be possible to print this bioink using a Carbopol support bath, which, with particles as small as 7 μm, could lead to better results [12].

Regarding cell compatibility, non-printed FG-HA hydrogels showed high cell viability (93.9±6.0%), which confirmed the inherent cytocompatibility of this bioink. Furthermore, it was possible to maintain a cell viability above 87% post-printing, which suggests that this hydrogel can shield cells against the shear stresses associated with the printing process. Even though cell viability was not severely affected by the printing process, long-term effects were visible by performing a proliferation assay for 14 days. Printed cells showed lower proliferation rates compared to the respective non-printed controls in all measurement days. Nevertheless, cell proliferation recovered by day 7 and seemed to slowly continue to increase until day 14.

A fibrin-HA (10 mg/mL:2.5 mg/mL) hydrogel, with similar constitution to the one used for printing was used as a non-printed moulded control and showed intrinsic ability to promote the formation of complex and branched vascular networks. Quantitative evaluation showed up to 3-fold increase in area, volume, length and number of branching points of the formed capillary structures, when compared to the fibrin control, previously established as angiogenic [18]. FRESH printed hydrogels showed lower potential for capillary formation. This may have occurred not only due to the printing process itself impairing cellular function but also due to the higher concentration of HA present in the FG-HA bioink. Nevertheless, some tubule-like structures were present and quantitative analysis showed that these structures were comparable to the one present in non-printed controls in terms of area and volume. Further work should verify if printing with the same HA concentrations as used for moulding would be feasible. This would allow to verify whether the decrease in capillary formation which occurred in the case of printed constructs was due to the higher concentration of HA used for printing or due to the printing process itself. In the case of loss of printing resolution, factor XIII or transglutaminase could be used to reduce the crosslinking time of the hydrogel. Our results seem to indicate that HA stimulates angiogenesis in a dose-dependent manner and that there is an optimal concentration of HA for this mechanism to occur. We have hypothesized that, during the 14 day cultivation period, cells encapsulated in the hydrogel may bind to HA via CD44 receptors, which are also involved in HA internalization, leading to its degradation by acid hydrolases [37]. This has already been proven to occur in the case of liver endothelial cells, which release LMW HA compounds into the medium post-uptake of HA [39]. Further work should study the mechanism by which this process occurs and verify this hypothesis. Furthermore, stimulation with VEGF could be performed to see if the formation of capillary structures would be enhanced. Perhaps, as suggested by other authors, VEGF will be required after the first few days, to maintain the established vessels and potentiate their growth [40]. Different cell sources could also be used to improve results, namely lung fibroblasts were reported to exhibit improved results in capillary assays in comparison with dermal fibroblasts [31]. Another interesting study to perform would be a chick chorioallantoic membrane assay. The chorioallantoic membrane is an extraembryonic membrane present in the chick, which is commonly used in vivo to study vessel formation. Hydrogels can be placed on this membrane through a hole cut in the egg shell [41]. After incubation, this method could be used to evaluate whether the pre-vascularized network present in the hydrogels could be an advantage with regards to a quicker integration between the TE constructs and the host vasculature.

To conclude, we were able to demonstrate that the FG-HA bioink, when printed using the FRESH technique, has a high potential for future vascularized tissue fabrication.

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