

Christian Polley*, Sophie Kussauer, Phillip Barkow, Robert Mau, Robert David and Hermann Seitz

Printing of vessels for small functional tissues – a preliminary study

Abstract: Vascularization of bioprinted constructs to ensure sufficient nutrient supply still remains to be a significant task in the tissue engineering community. In order to mimic functional tissue, it is necessary to be able to print vessels in various size scales, which places particularly high demands on the 3D printing technology and materials. In this preliminary study, we focused on the production of small hollow structures for the application in small functional units of living tissue. To fabricate hollow structures, the freeform reversible embedding of suspended hydrogels (FRESH) – method was utilized (Hinton et al.). A sodium alginate solution (5 % w/v) was used as a bioink. The scaffolds were fabricated with the Allevi 1 (Allevi Inc., PA, USA), a pneumatic extrusion-based bioprinter and plotted into a gelatine slurry serving as fugitive support. For first cell experiments, the bioink was loaded with immortalized mouse HL1-cells. A proof of concept could be shown since we were able to reliably create vessel-like structures with an inside diameter of 1.2 to 1.6 mm, a length of up to 8 mm and a wall thickness of 0.4 to 0.6 mm. In this study, the geometric requirements to print hollow structures for small functional tissues could be achieved. To expand the field of applications the resolution of the printing process has to be further improved. Moreover, the cell density should be increased to reach physiological cell numbers and extended with endothelial cells.

Keywords: 3D printing, hydrogels, bioprinting, vascularization,

<https://doi.org/10.1515/cdbme-2020-3121>

1 Introduction

The application of microfabrication techniques and 3D printing recently gained great attention in the medical, scientific community [1]. The idea of using 3D printing devices to engineer tissue or organs as mimicking substitutes for the native ones is promising and ambitious at the same time. Besides many challenges, such as cell density or the mechanical integrity of the scaffolds, one of the biggest challenges remains the supply of the cells with necessary nutrients. While simple structures can be supplied passively by diffusion processes under in vitro conditions, this is not sufficient for complex structures. The free diffusion length for the supply of incorporated cells in a hydrogel matrix is about 200-300 micrometers and thus very limited [2]. The construction of a vascular system consisting of microchannels is becoming indispensable and is in the focus of several studies applying different techniques ranging from the maturation of vessels in vitro to directly 3D printing of microchannels [3]. Since the human vascular system extends over several size scales, ranging from small capillaries up to larger vessels like the aorta or portal vein, unique demands are placed on the manufacturing technologies. In particular, smaller vessels for the supply of small structural subunits of organs, such as nephrons or liver lobules, or in vitro tumor models are difficult to fabricate due to the special demands on the material and the accuracy of the manufacturing process [4–6]. In this preliminary study we present first results for the production of small hollow structures on the basis of extrusion-based bioprinting into a gelatine support bath using freeform reversible embedding of suspended hydrogels (FRESH) [7].

***Corresponding author: Christian Polley:** Microfluidics, University of Rostock, Justus-von-Liebig Weg 6, 18059 Rostock, Germany, christian.polley@uni-rostock.de

Sophie Kussauer, Robert David: Department Cardiac Surgery, Rostock University Medical Center, Rostock, Germany

Phillip Barkow, Robert Mau, Hermann Seitz: Microfluidics, University of Rostock, Rostock, Germany

Sophie Kussauer, Robert David, Hermann Seitz: Department of Life, Light and Matter, University of Rostock, Rostock, Germany

2 Material and Methods

2.1 Preparation of the (bio)inks and the gelatine support bath

Firstly, a hydrogel ink without incorporated cells was prepared to investigate 3D printing performance and resolution in initial 3D printing experiments. Secondly, a cell-incorporated bioink was prepared to investigate cell viability after finishing the 3D printing process. Both inks are based on an aqueous sodium alginate solution (5% w/v). Sodium alginate (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was dissolved in distilled water at 20 °C utilizing a magnetic stirrer for 24 h. Blue dye (Pelikan, Schindellegi, Switzerland) was added to the ink (without cell-incorporation) to gain an adequate visualization of 3D printed structures. To prepare a bioink, immortalized murine HL-1 cells were incorporated in the alginate solution. This cell type was described first by Claycomb et al. in 1998 [8]. After harvesting the cells according to the manufacturer's instructions (Sigma Aldrich, SAFC, St.Louis, USA), the centrifuged cells (pellet) were resuspended in 5% alginate to a final cell concentration of 24×10^6 /ml alginate hydrogel. To prepare the gelatine support bath, 6.75 g of gelatine type A (Sigma-Aldrich, St. Louis, USA) was dissolved in 150 ml of calcium chloride (11 mM) at 27°C and then gelled at 4°C for 24 h. After gelation, a volume of 350 ml of calcium chloride (11 mM) was added to the gelatine block. This composition was blended for a time of 120 s utilizing a costumer-grade blender (Oster Manufacturing, Boca Raton, USA). Afterward, the blend was filled into 70 ml tubes and centrifuged at 4200 rpm for 2 min. The supernatant was removed and replaced with 11 mM calcium chloride at 4°C. The slurry was vortexed back into suspension and centrifuged again. This process was repeated until no foam was observed at the top of the supernatant. The foam is an indicator of soluble gelatine, which is left in the suspension. To evaluate particle size, 0.5 ml of the suspension were taken out of the blender at different blending times (30, 60, 90, 105, 120 s) and diluted with 10 ml of calcium chloride. The particle size was then measured manually with the use of a light microscope.

2.2 Rheological analysis

To assess rheological properties of the alginate bioink and the gelatine support bath, rheological measurements were performed using a HAAKE Mars II rheometer (ThermoFisher Scientific, Waltham, USA) with a plate-plate setup (plate-diameter of 35 mm) and a gap size of 0.5 mm. The viscoelastic

properties were measured with a dynamically oscillating temperature sweep ranging from 22°C to 37°C with a constant shear of 0.01 at a frequency of 1 Hz.

2.3 3D printing/bioprinting and cell culture

All 3D printing experiments were performed using the Allevi 1 (Allevi, Philadelphia, USA), a pneumatic extrusion printer, equipped with a cannula of a length of 1 inch and an inner diameter of $d = 250 \mu\text{m}$. First experiments were conducted without cells to investigate the performance of the 3D printing process utilizing the FRESH method [7]. During the printing process the cannula dips into the gelatine support bath which stabilises the deposited ink-material. Tubular, vessel-like test samples and cubic, scaffold-like test samples were 3D-printed (Figure 1). The structures were printed using following print parameters: printhead speed 2 mm/s, layer height 0,2 mm and an extrusion pressure of 25-30 psi. All geometries were previously modelled in Creo Parametric (PTC, Neeham, USA). Afterwards, the cell loaded ink was printed as a hollow structure into the gelatine slurry. Crosslinking was performed with 0.1M calcium chloride solution. The cell loaded printed structures were cultured in corresponding Claycomb culture medium under standard tissue culture conditions (37°C, 5% CO₂) before cell viability was analysed.

One day after printing the LIVE/DEAD™ Viability/Cytotoxicity Kit (Thermo Fisher Scientific, Waltham, USA) for mammalian cells was accomplished according to manufacturer's instructions and analyzed using the ELYRA PS. 1 LSM 780 confocal microscope (Carl Zeiss, Jena, Germany).

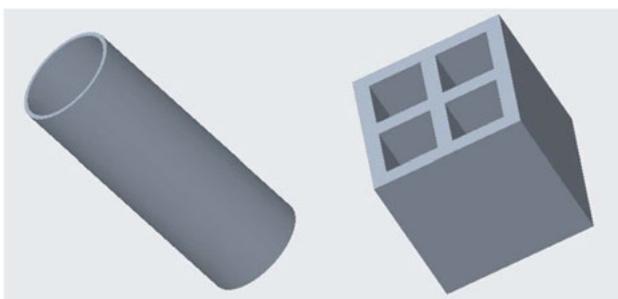


Figure 1:Left: CAD-model of tubular test sample (wall size of 0.25 mm; length of 8 mm, inner diameter of 2 mm); Right: CAD-model of cubic scaffold test sample (wall size of 0.5 mm, width of 5 mm)

3 Results

3.1 Rheological analysis

The composition of bioinks that determine their viscous and elastic properties is essential to control the shear stress in the nozzle. Figure 2 shows the elastic modulus G' and the loss modulus G'' for the 5 % w/v alginate solution over a temperature range from 22°C to 37°C. Both dynamic moduli show strongly temperature-dependent behavior and decrease significantly with increasing temperature. Overall, the viscous behavior in the ink (G'') predominates over the entire temperature curve. The gel state has not yet been reached, although the fluid is already very viscous. Very high viscosity can have adverse effects on the viability of cells during the extrusion process due to elevated shear stress in the nozzle. However, this can be reduced by adjusting the extrusion temperature or by varying the diameter of the nozzle. Where precisely the limits for printing with incorporated cells are is not yet fully understood and will be the subject of future studies in our group [9].

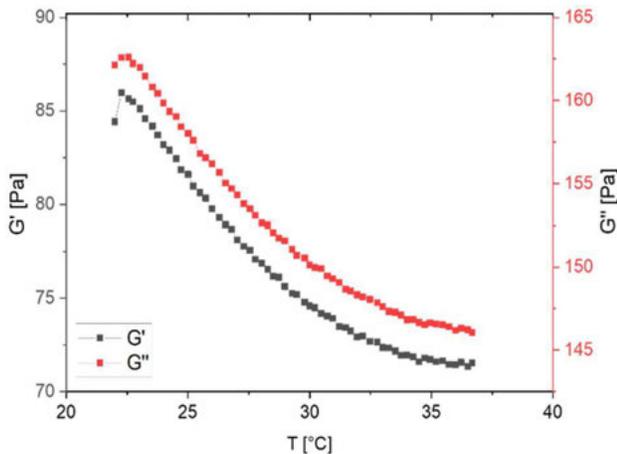


Figure 2: Oscillatory temperature sweep test of 5 % w/v alginate solution used as bioink.

3.1 3D printing/Bioprinting

By using the FRESH method, we were able to reliably 3D print tubes with a length of 8 mm, an inner diameter of 1,6 mm and a wall thickness of around 0.5 mm as well as small cubic hollow structures (Figure 3). The fabrication process showed only a slight deviation from the CAD File (Table 1). The 3D printed structures were all slightly smaller than the specification. This is mainly explained by the ionic cross-linking process which restructures the alginate molecule to

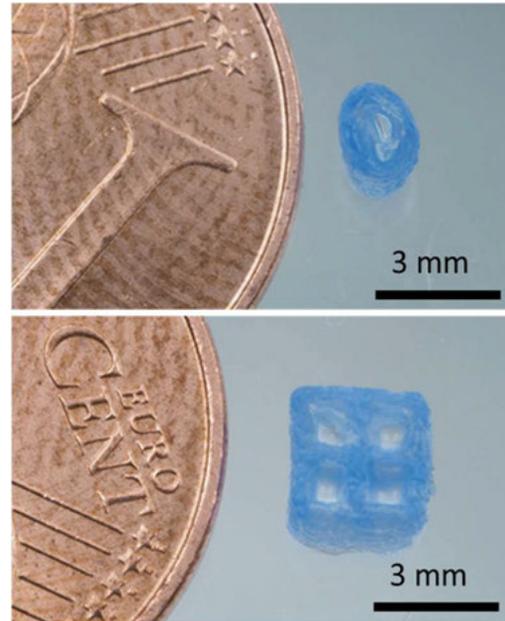


Figure 3: Top: 3D print of tubular test sample; Bottom: 3D print of cubic hollow structure.

incorporate the calcium ions. However, the crosslinking process is required to obtain mechanically stable structures. The shrinkage can be compensated by scaling. In addition, an adjustment of the printing parameters is still under investigation. By changing the layer height, for example, considerable improvements, especially in the direction of the z-coordinate, would be conceivable. Furthermore, a reduction of the particle size in the gelatine support bath could lead to a further increase in accuracy and also enable the implementation of even finer structures. The minimal particle diameter that we were able to achieve was 136 μm . Comparing the result with the work of Hinton et al., mean particle diameters of 55 μm are quite possible. An increase in resolution by reducing the cannula diameter is very questionable, as the viscosity of the ink used was already high leading to the necessary high pressure to achieve continuous extrusion. A further increase in pressure would probably have a significant negative effect on the viability of any incorporated cells in the bioink.

Table 1: Comparison of geometrical parameters of CAD model (cubic hollow structure) and the 3D printed scaffold test sample.

| Parameter | CAD model | 3D printed sample |
|-----------------------------------|-----------|-------------------|
| Width [mm] | 5 | 4.2 |
| Height [mm] | 5 | 4.1 |
| Bridge width xy [μm] | 500 | 430-550 |
| Bridge width xz [μm] | 500 | 720-780 |

The printing parameters presented here worked for the printing of HL-1 cells. After cultivation for one day under standard tissue culture conditions microscopy was performed. The microscopy images showed a wide distribution of cells within different layers of the printed structure. Moreover, LIVE/DEAD staining revealed high cell viability and related cellular survival during the printing process. Living cells appear green (Calcein dye) and dead cells appear red (Figure 4). Unfortunately, longer cultivation times could not be performed due to the instability of the structures and contamination issues. For future experiments, significantly longer cultivation times are planned. The switch to an endothelial cell line and the use of growth factors are also being considered. In addition, the simulation of a natural flow in a bioreactor could be useful.

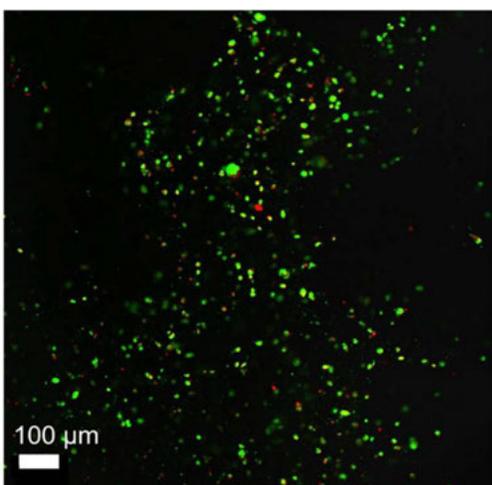


Figure 4: LIVE/DEAD of printed (32,5 psi) cells after one day of cultivation. A high number of living cells (green) and individually distributed dead cells (red) can be seen.

Conclusion

In this preliminary study, we were able to produce small vessel-like structures using the FRESH-method which could be used to provide the nutrients for small functional tissues. We still see more potential in regards to resolution and printing fidelity by optimizing the printing and slurry production to reach particle sizes down to 20 μm . To ensure the further functionality of the 3D-printed tissue, the cell density should be increased to physiological cell numbers using a higher amount of the corresponding target cells mixed with the addition of endothelial cells. Moreover, the rheological properties of bioinks and the printability assessment will be investigated further in order to provide an insight into the

effect of the rheological properties of bioinks on cells and to determine optimal printing parameters. All in all, the FRESH method is a very good and easy-to-use method for extrusion-based bioprinting experiments. It offers the capability to produce vessel-like structures of different sizes and has additional potential to increase the resolution [10].

Author Statement

Research funding: This study is funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – SFB 1270/1 - 299150580. **Conflict of interest:** Authors state no conflict of interest. **Informed consent:** Informed consent has been obtained from all individuals included in this study.

References

- [1] S. V Murphy, A. Atala, 3D bioprinting of tissues and organs., *Nat. Biotechnol.* 32 (2014)
- [2] B. Aleksander Skardal, J. Zhang, L. McCoard, S. Oottamasathien, G.D. Prestwich, G.D. Prestwich, A. Skardal, J. Zhang, L. McCoard, S. Oottamasathien, Dynamically Crosslinked Gold Nanoparticle – Hyaluronan Hydrogels, *Adv. Mater.* 22 (2010)
- [3] M.D. Sarker, S. Naghieh, N.K. Sharma, X. Chen, 3D biofabrication of vascular networks for tissue regeneration: A report on recent advances, *J. Pharm. Anal.* 8 (2018)
- [4] X. Mu, W. Zheng, L. Xiao, W. Zhang, X. Jiang, Engineering a 3D vascular network in hydrogel for mimicking a nephron, *Lab Chip.* 13 (2013)
- [5] D. Kang, G. Hong, S. An, I. Jang, W.S. Yun, J.H. Shim, S. Jin, Bioprinting of Multiscaled Hepatic Lobules within a Highly Vascularized Construct, *Small.* 1905505 (2020)
- [6] C. Polley, R. Mau, C. Lieberwirth, J. Stenzel, B. Vollmar, H. Seitz, Bioprinting of three dimensional tumor models: a preliminary study using a low cost 3D printer, *Curr. Dir. Biomed. Eng.* 3 (2017)
- [7] T.J. Hinton, Q. Jallerat, P.R. N., H.J. Park, M.S. Grodzicki, H.-J. Shue, H.M. Ramadan, A.R. Hudson, A.W. Feinberg, Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended ... Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels, (2015).
- [8] W.C. Claycomb, N.A. Lanson, B.S. Stallworth, D.B. Egeland, J.B. Delcarpio, A. Bahinski, N.J. Izzo, HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998)
- [9] S.J.L. Teng Gao, Gregory J. Gillispie, Joshua S. Copus, Anil Kumar Pallickaveedu Rajan Asari, Young-Joon Seol, Anthony Atala, James J. Yoo, Optimization of gelatin-alginate composite bioink printability using rheological parameters: a systematic approach To, *Biofabrication.* (2018) 1–46.
- [10] A. Lee, A.R. Hudson, D.J. Shiwardski, J.W. Tashman, T.J. Hinton, S. Yerneni, J.M. Bliley, P.G. Campbell, A.W. Feinberg, 3D bioprinting of collagen to rebuild components of the human heart, *Science* (2019).