ORIGINAL ARTICLE

Rational Design of a Triple-Layered Coaxial Extruder System: *in silico* and *in vitro* Evaluations Directed Toward Optimizing Cell Viability

Christian Silva[1]*, Carlos J. Cortés-Rodriguez[1], Jonas Hazur[2], Supachai Reakasame[2], Aldo R. Boccaccini[2]

[1]Department of Mechanical Engineering and Mechatronics, School of Engineering, Universidad Nacional de Colombia, Bogotá 111321, Colombia

[2]Department of Materials Science and Engineering, Institute of Biomaterials, University of Erlangen-Nuremberg, Erlangen 91058, Germany

Abstract: Biofabrication is a rapidly evolving field whose main goal is the manufacturing of three-dimensional (3D) cell-laden constructs that closely mimic tissues and organs. Despite recent advances on materials and techniques directed toward the achievement of this goal, several aspects such as tissue vascularization and prolonged cell functionality are limiting bench-to-bedside translation. Extrusion-based 3D bioprinting has been devised as a promising biofabrication technology to overcome these limitations, due to its versatility and wide availability. Here, we report the development of a triple-layered coaxial nozzle for use in the biomanufacturing of vascular networks and vessels. The design of the coaxial nozzle was first optimized toward guaranteeing high cell viability upon extrusion. This was done with the aid of *in silico* evaluations and their subsequent experimental validation by investigating the bioprinting of an alginate-based bioink. Results confirmed that the values for pressure distribution predicted by *in silico* experiments resulted in cell viabilities above 70% and further demonstrated the effect of layer thickness and extrusion pressure on cell viability. Our work paves the way for the rational design of multi-layered coaxial extrusion systems to be used in biofabrication approaches to replicate the very complex structures found in native organs and tissues.

Keywords: Three-dimensional bioprinting, Coaxial nozzle, Vascularized tissues, Tissue-engineered vessels, Biomaterials

***Corresponding Author:** Christian Silva, Department of Mechanical Engineering and Mechatronics, School of Engineering, Universidad Nacional de Colombia, Bogotá 111321, Colombia; casilvaca@unal.edu.co

Received: May 12, 2020**;** ***Accepted:*** June 22, 2020**;** ***Published Online*****:** July 24, 2020

Citation: Silva C, Cortés-Rodriguez C, Hazur J, *et al*., 2020, Rational Design of a Triple-Layered Coaxial Extruder System: *in silico* and *in vitro* Evaluations Directed Toward Optimizing Cell Viability, Int J Bioprint, 6(4): 282. DOI: 10.18063/ijb.v6i4.282.

1 Introduction

Three-dimensional (3D) bioprinting is an additive manufacturing technology that permits the spatiotemporal patterning of hydrogels embedded with cells, namely bioinks, into 3D structures[1,2]. Its goal is to fabricate cell-laden constructs that mimic tissues and organs, where cell viability is preserved and overall physiological functionality is replicated[1,3]. Among its several techniques, extrusion-based bioprinting (EBB) has emerged as the most promising additive manufacturing technique for achieving 3D structures of sufficient complexity, since it can work with a broad range of cell densities and printable materials[4,5]. Moreover, the versatility and affordability provided by EBB systems have contributed to its positioning as the most popular biofabrication technology among researchers worldwide for applications that range from cancer research and drug testing to tissue engineering[6].

Replicating complex internal tissue structures is, however, still a challenge for the available

biomanufacturing technologies[3,6]. In the case of EBB, the time lag between hydrogel extrusion and its subsequent crosslinking is one of the limiting factors in the formation of complex geometries[7,8]. This is mainly because the viscoelastic properties of extruded bioinks are often not sufficient to support these geometries before extensive crosslinking is applied[7,9,10]. Therefore, the shape fidelity of the printed constructs is greatly compromised, especially in the fabrication of hollow or highly detailed structures. Emerging techniques such as freeform reversible embedding of suspended hydrogels (FRESH)[11] and volumetric printing[12] have contributed to alleviating some of these limitations by allowing the formation of complex structures. In the FRESH technique, hydrogels are printed in a support bath of sacrificial microparticles, which provides structural support while the hydrogel is crosslinked and therefore guarantees a high degree of shape fidelity[13]. Despite the superior printing resolution achieved in constructs manufactured with this technique, results have not yet been reported when depositing cell-embedded hydrogels[3,14]. Similarly, volumetric bioprinting allows the fabrication of convoluted free-form geometries with the spatially selective exposition of cell-laden photocrosslinkable hydrogels to ultraviolet (UV) or blue light. In particular, this technique has demonstrated the rapid fabrication of anatomically relevant hollow structures with high cell viability[15]. However, the current technique cannot include multiple materials within the same bioprinting process and can lead to unwanted heterogeneous stiffness of the constructs, which greatly limits its exploitability.

Despite recent advances on the development of techniques that allow an increased structural complexity of constructs and novel hydrogel formulations that support bioprinting and maturation of tissues, functionality is a challenge that has not been fully addressed yet[3,16,17]. To engineer functionally relevant tissues *in vitro*, the current inability to recreate the 3D microenvironments seen *in vivo* is a key restriction that must be overcome. Among the attributes that bioprinted constructs must have to permit appropriate tissue maturation, vascular networks appear to be one of the most important[1-3,18]. The scalability of bioprinted constructs toward clinically relevant sizes is often limited by the accessibility of nutrients throughout the construct, as nutrient access and waste removal depend solely on diffusion-mediated transport. As a result, perfusable networks within bioprinted constructs are imperative to create tissues of clinically relevant size, as they will allow adequate nutrient availability and prevent waste accumulation in the innermost regions of the construct[19,20]. This, in turn, will facilitate the maturation of multilayered constructs and shorten the gap between native and *in vitro* functionality. In addition, the biomanufacturing of hollow tubular structures might also be beneficial for generating multilayered large and medium-diameter vascular grafts for use in either transplantation or disease modeling[21-23].

Accordingly, here we report the development of a triple-layered coaxial extruder system for the fabrication of layered tubular structures that allow the simultaneous dispensing of three different materials using EBB systems. Our aim was to study how different design parameters and bioprinting conditions of this system affect the viability of embedded cells upon extrusion. Computational analyses were initially implemented to optimize design parameters of the coaxial extruder system based on predicted pressure distributions, and these findings were then validated experimentally on bioprinting experiments using human cells in alginate-based hydrogels. In addition, the triple-layered design allowed immediate bioink crosslinking upon extrusion by including a crosslinking solution as the outermost layer of the printed tubular structures, and the formation of hollow structures by posterior removal of sacrificial material contained in the innermost layer.

2 Materials and methods

2.1 Triple-layered coaxial nozzle design and *in silico* evaluation

A first prototype of a triple-layered coaxial nozzle was fabricated by assembling commercially available nozzle parts (Nordson EFD, Dunstable, Bedfordshire, UK). A mixture consisting of a

cyanoacrylate solution and sodium bicarbonate was employed to adhere the different components into a single structure (**Figure 1A**). From a transverse view, the coaxial nozzle comprised three walls, two rings, and one cylinder, with Gauges (G) ranging from 13 to 25 G. This configuration led to three different flow channels, namely, channel *a*, *b,* and *c*, as shown in **Figure 1B**.

Based on this prototype, a computationally aided design model of the three flow channels was developed and studied using computational fluid dynamics simulations in COMSOL Multiphysics® software. A two-dimensional axisymmetric domain of flow channel *b* and a glass printing surface, with a 100 μm air interface in between, was modeled and simulated based on the overall design of the coaxial nozzle. This channel was of special interest as the nozzle is intended for the extrusion of single-layered tubular structures and the cell-laden hydrogel will be extruded through this channel. The hydrogel was conceived as a non-Newtonian fluid and its physical parameters, such as density and dynamic viscosity, were used as input for calibrating the models. Air was, however, conceived as a Newtonian fluid and its density and dynamic viscosity were also provided as input for the simulations. A parametric analysis was performed by varying the inlet pressure (P) of channel *b* between 10 and 70 kPa and the Gauge of this flow channel was fixed at 18 G. Outlet velocity and pressure were studied at the outlet of the flow channel and compared to previously reported literature to validate the design in terms of cell viability.

Three different coaxial nozzles were designed varying the area of the middle channel (namely, *b* in **Figure 1B**) and subsequently 3D printed using biocompatible photopolymer resins, namely, dental SG FLSGOR01 and dental LT clear (**Figure 1C**). The dimensions of each channel are reported in **Table 1**, along with the area of flow channel *b* (used

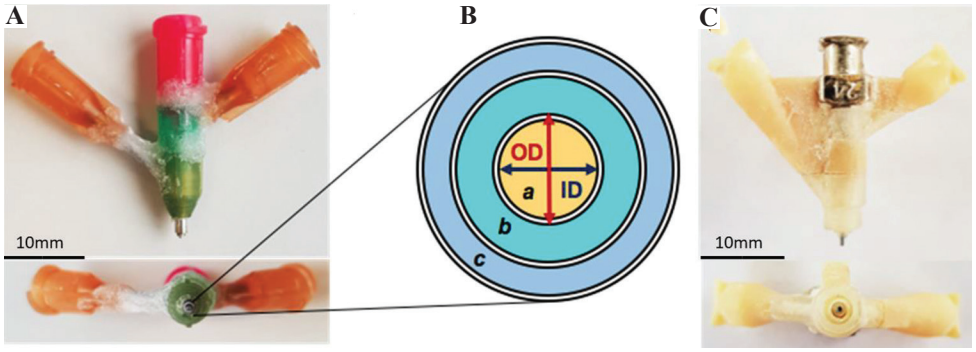

Figure 1. (A) Lateral and frontal view of the initial prototype of the triple-layered coaxial nozzle assembled with commercially available parts. (B) Schematic representation of the transverse view at the tip of the coaxial nozzle. The outer diameter and inner diameter of each channel were defined according to the results of the computational fluid dynamics simulations. (C) Three-dimensional printed coaxial nozzle.

Table 1. Geometric data of the designed coaxial nozzles. ID and OD stand for inner diameter and outer diameter, respectively. The three flow channels of each nozzle are labeled as *a*, *b*, and *c*, as shown in **Figure 1B**.

Parameter	Nozzle 1			Nozzle 2			Nozzle 3		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Gauge (G)	23	18	14	25	18	14	20	15	13
ID (mm)	0.33	0.84	1.54	0.25	0.84	1.54	0.61	1.36	1.8
OD (mm)	0.64	1.27	1.83	0.52	1.27	1.83	0.91	1.65	2.41
OD – ID (mm)	0.32	0.43	0.29	0.27	0.43	0.29	0.30	0.29	0.61
Layer thickness (mm)		0.20			0.32			0.45	
Layer area (mm^2)		0.232			0.341			0.802	

for cell-laden hydrogel), which is continuously increasing from Nozzle 1 to Nozzle 3.

2.2 Preparation of hydrogels

For each of the channels (*a*, *b*, and *c*) described in section 2.1, different materials were used during the printing process. While channel *c* was perfused with $CaCl_2$ solution, the other channels were perfused with two different hydrogel compositions.

Channel *a* was used to print a support structure in the core, based on a methylcellulose-gelatin sacrificial ink, as described by Dranseikiene *et al*.[24] Briefly, the sacrificial biomaterial ink is composed of 9 % (w/v) Methylcellulose (Sigma, USA) and 5 % (w/v) gelatin (Sigma, USA) and was shown to exhibit good support characteristics after printing, while dissolving in culture conditions after 1 week.

The hydrogel used for printing with cells was an alginate-based bioink, prepared with a pre-crosslinking technique utilizing $CaCO_3$ and D-Glucono-δ-lactone (GDL). Concisely, a 2 % (w/v) alginate (VIVAPHARM® alginate PH176, JRS PHARMA GmbH & Co. KG, Rosenberg, Germany) solution was pre-crosslinked with 20 mmol/l $CaCO_3$ (Calcium carbonate precipitated for analysis EMSURE®, CAS 471-34-1, Merck KGaA, Darmstadt, Germany) and 40 mmol/l GDL (CAS 90-80-2, Merck KGaA, Darmstadt, Germany) at 4°C. After stirring continuously for 48 h, the hydrogel was allowed to warm up to room temperature and was subsequently mixed with cells (see section 2.4).

2.3 Setup for 3D bioprinting with coaxial nozzles

A commercially available fused deposition modeling 3D printer (Anycubic Prusa I3, ANYCUBIC 3D Printing, Shenzhen, China) was customized to allow the controlled deposition of hydrogels (**Figure 2**). For this purpose, three independent piston-driven extrusion systems were coupled to the machine and the feed rate (mm/s) was translated into pressure units (kPa) with the aid of an external system. Next, printheads suited for 12 mL Luer-lock syringes were adapted to the extrusion systems and their outlet tips were connected to the inlets of the 3D printed coaxial nozzles.

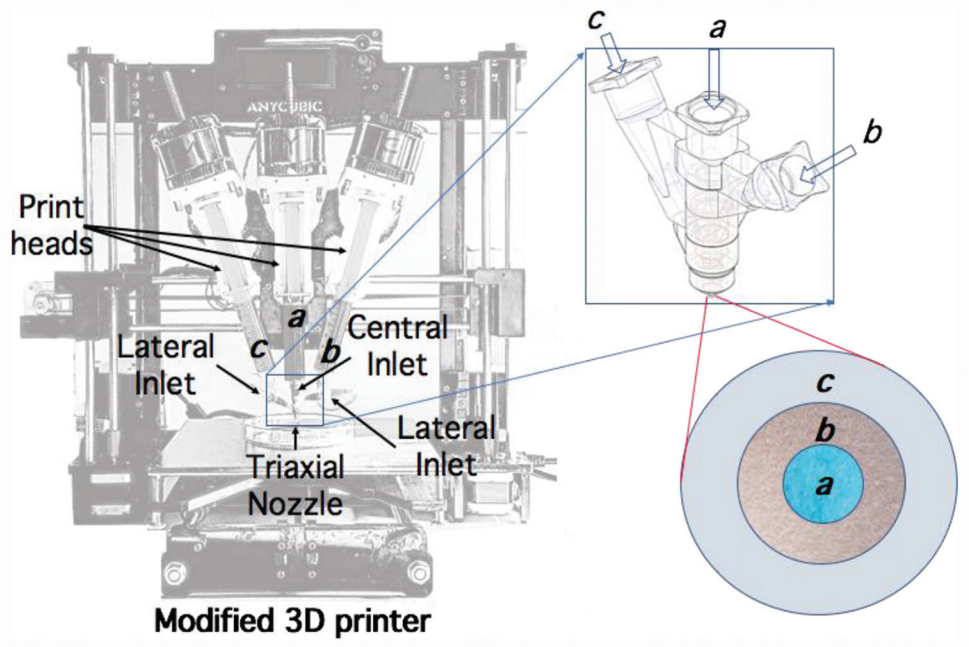

Figure 2. Three-dimensional (3D) bioprinter setup for bioprinting experiments. Three printheads were adjusted to a fused deposition modeling 3D printer with piston-driven extrusion systems and connected to the triple-channel coaxial nozzle. Each flow channel is labeled at the inlet and outlet of the coaxial nozzle for a better understanding of the reader.

2.4 Cell culture and embedding

Before bioprinting experiments, human bone osteosarcoma cells MG-63 (ATCC® CRL-1427™) were cultured in complete growth medium consisting of Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) Penicillin-Streptomycin (10,000 U/ml) and maintained in a CO_2 incubator at 37°C. Upon the culture reached a confluence of approximately 80–90%, cells were harvested with the aid of a 0.25 % (w/v) trypsin solution (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA). The cell concentration of the obtained suspension was then estimated by staining with trypan blue and hemocytometer counting. Subsequently, cells were carefully embedded in the alginate-based hydrogel. To perform this, the hydrogel (without cells) was filled into a 12 ml Luer-lock syringe and connected to another syringe containing an 11×10^6 cells/ml cell suspension. To guarantee homogeneous mixing, the two components were extruded back and forth at least 10 times. The volume ratio was 10:1 (hydrogel:cell suspension), resulting in a final cell density of 1×10^6 cells/ml in the bioink.

2.5 Bioprinting of tubular structures

For bioprinting, the coaxial nozzles were submerged in 70% (v/v) ethanol for 1 h before experiments and subsequently washed with sterile 1 × PBS in a biosafety cabinet. The modified 3D printer was thoroughly wiped with 70% (v/v) ethanol and exposed to UV germicidal light for 1 h inside a biosafety cabinet.

Each nozzle comprised three flow channels at the tip, namely *a*, *b,* and *c* in **Figure 1B**. Two different hydrogels and a crosslinking solution were employed for bioprinting hollow, tube-like structures. A methyl cellulose-based hydrogel was used as a sacrificial material for the lumen (flow channel *a* in **Figure 3**) and an alginate-based bioink embedded with human bone osteosarcoma MG63 cells was used for the middle tubular channel (flow channel *b* in **Figure 3**). Calcium chloride ($CaCl_2$) 0.1 M solution was expelled through the outer channel of the coaxial nozzle (flow channel *c* in **Figure 3**) since it served as a crosslinking agent for the alginate bioink. All materials were dispensed coaxially by mechanical extrusion of the three separate printheads simultaneously. The resulting

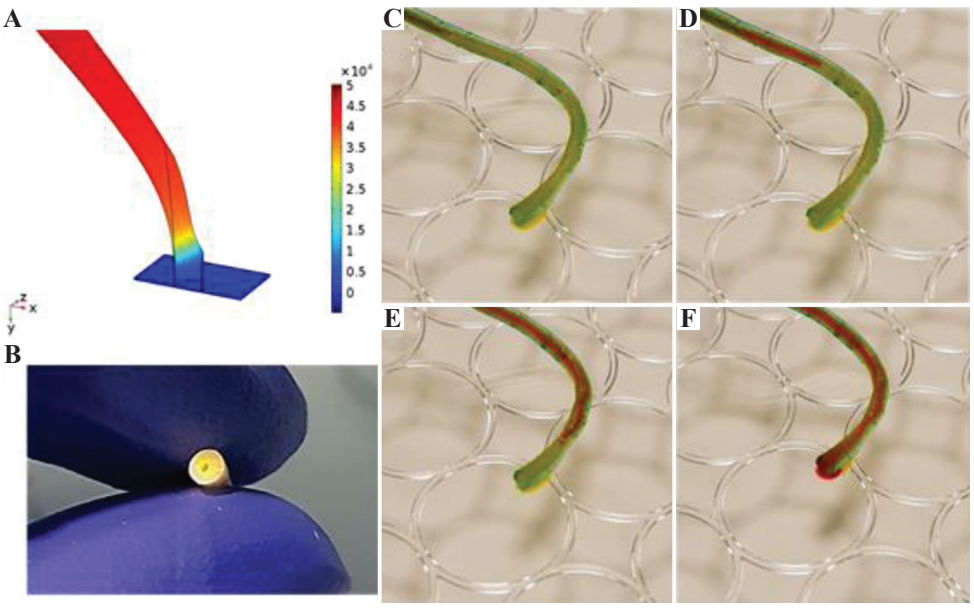

Figure 3. (A) Pressure distribution profiles along the geometry of one of the studied flow channels (flow channel *b*). Values on the color bar are displayed in Pa × 10^4. (B) Transverse view of one of the printed and perfused hollow cannular structures. (C), (D), (E) and (F) display one of the hollow cannular structures being perfused with 1 × PBS stained with red food coloring.

tubular structures were then perfused through the core channel with warm sterile 1 × PBS to wash away the sacrificial material.

This bioprinting procedure was performed with the three designed nozzles varying the extrusion pressure of the printhead connected to channel *b* since the cell-laden bioink was dispensed through this channel. The extrusion pressure of the other two channels was adjusted to achieve the same extrusion rate as that of channel *b*. Coaxial tubular structures were dispensed with three extrusion pressures: 26, 34, and 40 kPa, through each of the designed nozzles. Each combination of bioprinting parameters was performed in triplicates, resulting in a total of 27 extruded tubular structures.

2.6 Cell viability assessment

For studying the effect of the inlet extrusion pressure and the different coaxial nozzle geometries on cell survival, a Live/Dead (Sigma-Aldrich, St. Louis, MO, USA) assay was performed on the bioprinted tubular structures. Briefly, constructs were stained with calcein acetoxymethyl ester (calcein-AM) and propidium iodide (PI) immediately after bioprinting and fluid perfusion through the lumen, to visualize live and dead cells, respectively. The staining solution was prepared according to the manufacturer's instructions. Bioprinted constructs were subsequently submerged in it and incubated for 15 min at room temperature (~22ºC) protected from light. Samples were then washed with 1 × PBS and imaged using an epifluorescence microscope (ZEISS Axio Observer, Carl Zeiss AG, Oberkochen, Germany). One image from a random location on each sample was captured and later analyzed using the ImageJ software.

2.7 Statistical analysis

Data for cell viability was statistically analyzed with the aid of the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The statistical distribution of the data was first studied with the Shapiro-Wilk normality test and a two-way ANOVA with Tukey's multiple comparisons tests was subsequently performed.

3 Results and discussion

In silico simulations, varying the extrusion, pressure was performed to investigate the impact that the overall design of the coaxial nozzle might have on cell viability. Since this nozzle is intended for the fabrication of single-layered tubular structures, cell-laden hydrogels will only be extruded through channel b; however, the simulations were performed for all channels. Fluid velocity and pressure distribution through the entire channel geometry were collected from the simulations. The minimum and maximum fluid velocities at the outlet were 13 and 43 mm/s, respectively. Moreover, velocity appeared to remain constant through the entire geometry for all extrusion pressures studied and, in each layer/area, into the nozzle geometry. However, that was not the case for pressure distribution since it seemed to decrease as the fluid approached the air interface between the nozzle and the collecting glass slide. According to the simulations, pressure distribution values at the tip of the flow channels fell to between 2 and 10 kPa, while pressures between 20 and 64 kPa could be experienced at the upper most regions of the *in-silico* flow channels. These values were then compared to those validated experimentally by previous studies for ordinary[25,26] and coaxial[27] nozzles. Nair *et al.* reported that cell viability decreases exponentially as a function of increasing shear stress, with cell viability above 60 % for pressures below 100 kPa and nozzle diameters between (150 and 400 μm)[26]. Yu and colleagues investigated this same relationship on coaxial nozzles and obtained very similar results[27]. Although these estimations depend widely on the rheological properties of the studied hydrogel and on the specific response of the cells utilized, we might be able to predict the high viability of cells bioprinted with the present coaxial nozzle. The predicted values collected for pressures experienced by cells on the bioprinting process fall within a safe range for cells according to these previous studies.

To confirm these notions and to investigate the effect of nozzle geometry, specifically the flow channel Gauge, on cell viability, bioprinting

experiments were conducted with three different nozzles. As shown in **Table 1**, nozzles 1 and 2 allow the fabrication of single-layered hollow tubular structures of equal outer diameter (OD), but with different layer thicknesses. Likewise, nozzle 3 allows the fabrication of structures with a greater diameter and layer thickness than nozzles 1 and 2. These nozzles were subsequently 3D printed with biocompatible photopolymer resins (**Figure 1C**) and adapted to a commercially available and modified 3D printer for bioprinting experiments (**Figure 2**). These nozzles allow the fabrication of cannular structures of diameters in the range of 0.84–1.36 mm (OD) and 0.52–0.91 mm (ID), which fall within the average dimensions of human arteries[28]. As shown in **Figure 3**, single-layered cannular structures were successfully fabricated and perfused with a red-stained solution of 1 × PBS for demonstration purposes.

In addition to nozzle geometry, the effect of inlet pressure on cell viability was studied experimentally by varying the applied pressure of the mechanical extruder of flow channel *b* within 26–40 kPa. Three different values of extrusion pressures within this range were selected according to printing experiments with the alginate-based hydrogel. These values were 26, 34, and 40 kPa and the three of them fell within the material's printing window, as they allowed controlled and continuous deposition of a filament. An alginate-based bioink embedded with MG-63 cells was chosen for this evaluation since alginate is a widely used biocompatible material, easily extrudable and features rapid crosslinking upon exposition to divalent cations, which enables excellent shape fidelity in bioprinted constructs[29].

As shown in **Figure 4A and B**, most cells remain viable immediately upon deposition with all evaluated extrusion pressures and nozzles. The normal distribution of the data was first confirmed with the Shapiro-Wilk test ($P = 0.508$) before performing a two-way ANOVA on the data. Extrusion pressure ($P < 0.0001$) and nozzle geometry ($P < 0.0001$), as well as their interaction ($P < 0.001$), were found to have a significant effect on cell viability according to statistical analyses. Specifically, the viability of bioprinted structures through all nozzles seems to be significantly diminished with the rise of inlet extrusion pressure, as displayed in **Figure 4C**. In addition, all extrusion pressures evaluated through nozzle three yield significantly higher cell viability than nozzles 1 and 2, which suggests that a wider diameter in the tubular structures significantly reduces the stress to

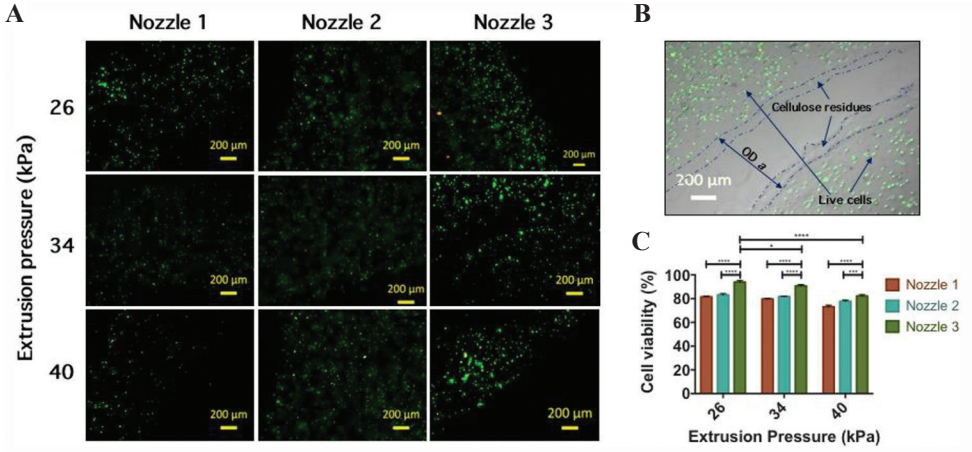

Figure 4. (A) Live/dead assay images of 3D printed cannular constructs with the three designed nozzles varying extrusion pressure of channel *b*. (B) Epifluorescent microscopy image of cannular structure after removal of the innermost sacrificial material. Cells embedded in the bioink extruded through channel *b* remain viable. (C) Cell viability of constructs immediately after bioprinting using the three designed nozzles and varying extrusion pressure between 26, 34, and 40 kPa. All configurations show high cell viability, but Nozzle 3 and low extrusion pressures yield the best results.

which cells are exposed during extrusion. Moreover, the viability of constructs extruded through nozzle 2 is only significantly different from those extruded through nozzle 1 when the inlet pressure is high (40 kPa), which indicates that layer thickness affects viability but to a lesser extent than the extrusion pressure. However, setting an 80 % cell viability threshold, these results suggest that all three coaxial nozzles could be used for the bioprinting of hollow tubular structures by applying an inlet extrusion pressure below 34 kPa.

The main goal of this research is to optimize the development of triple-layered coaxial nozzles for facilitating the fabrication of biomimetic tissues and organ-like constructs for tissue engineering and regenerative medicine applications. Furthermore, the enhanced development of triple-layered coaxial nozzles can help to solve the issues regarding vascularization, which remain as one of the key bottlenecks of the field[30]. The next step in our research is to apply these same concepts in the development of a four-layered coaxial nozzle, whose advantages compared to the coaxial nozzle presented here will be remarked. With one more layer, it will be easier to close mimic the complexity of the vascular network, taking into account that the current state of the art tissue conventional culture technique is limited to only triple co-culture (3 types of cells). Advancements in tissue culture techniques are necessary to address the bottleneck of maturing bioprinted multi-cellular 3D tissue constructs into functional tissues with a wide range of cells and biomaterials with differentiated layer co-culture within one single bioprinted construct.

The novelty of this work is adding value in the research field of bioprinting with a triple-layered coaxial nozzle development that has the potential to closely mimic the complexity of vascular networks found in the native human body in terms of histological and morphological of this vascular constructs, as well this development can replicate the wall thickness of a native blood vessel that generally comprised three layers with the innermost tunica intima layer made by continuous endothelium cells followed by the middle tunica layer made of elastic, smooth muscle cells and an outermost tunica adventitia layer made of surrounding fibroblast and collagen; an ideal tissue-engineered blood vessel should consist of those three layers, this coaxial nozzle can reduce the wall thickness of this kind of tissues obtained in other investigations[31] close mimicking the wall thickness of small arteries and veins in a real human body.

4 Conclusions

In silico simulations were performed for studying the pressure distribution exerted on cells during the bioprinting process, as well as the outlet velocity at the tip of three different flow channels. Our results confirmed those of previously reported studies and demonstrate the usefulness of *in silico* experiments in helping to optimize *in vitro* experiments. The results can be useful in guiding the future development of improved multi-layered coaxial nozzles.

Three triple-layered coaxial nozzles with different Gauges were first studied *in silico* regarding varying pressures and then successfully designed for the fabrication of single-layered hollow tubular structures of different dimensions. All nozzles displayed adequate bioprinting conditions to guarantee cell viability above 80 % in alginate-based hydrogels when extrusion pressure was kept below 34 kPa, meaning they are all suitable for bioprinting with bioinks with similar composition or rheological properties to the one studied in this project. Moreover, herein reported coaxial nozzles to allow the formation of perfusable cannular structures with dimensions that fall within the range of human arteries, which means they could be further exploited for the fabrication of multicellular vascular networks and vessel-like constructs with applications on tissue engineering. Our work paves the way for the rational development of coaxial nozzles useful for bioprinting multi-layered vascular channels or vessel-like constructs that truly resemble those found in native organs and organisms.

Acknowledgments

We would like to acknowledge the financial support received from the Colombian Administrative

Department of Science, Technology, and Innovation, COLCIENCIAS, and the National Doctorate Scholarship Program-647 and by the German Academic Exchange Service (Deutscher Akademischer Austauschdienst). DAAD, Research Grants-Short Term Grants, 2018. "No conflict of interest was reported by all authors."

Conflicts of interest

No conflicts of interest were declared by all authors.

References

1. Murphy SV, Atala A, 2014, 3D Bioprinting of Tissues and Organs. *Nat Biotechnol*, 32:773–85.
2. Pati F, Gantelius J, Svahn HA, 2016, 3D Bioprinting of Tissue/Organ Models. *Angew Chem*, 55:4650–65.
3. Heinrich MA, Liu W, Jimenez A, *et al*., 2019, 3D Bioprinting: From Benches to Translational Applications. *Small*, 15:1–47.
4. Jiang T, Munguia-Lopez JG, Flores-Torres S, *et al*., 2019, Extrusion Bioprinting of Soft Materials: An Emerging Technique for Biological Model Fabrication. *Appl Phys Rev*, 6:11310.
5. Paxton N, Smolan W, Böck T, *et al*., 2017, Proposal to Assess Printability of Bioinks for Extrusion-based Bioprinting and Evaluation of Rheological Properties Governing Bioprintability. *Biofabrication*, 9:4.
6. Ozbolat IT, Hospodiuk M, 2016, Current Advances and Future Perspectives in Extrusion-based Bioprinting. *Biomaterials*, 76:321–43.
7. Hölzl K, Lin S, Tytgat L, *et al*., 2016, Bioink Properties before, during and after 3D Bioprinting. *Biofabrication*, 8:32002.
8. Jungst T, Smolan W, Schacht K, *et al*., 2016, Strategies and Molecular Design Criteria for 3D Printable Hydrogels. *Chem Rev*, 116:1496–539.
9. Williams D, Thayer P, Martinez H, *et al*., 2018, A Perspective on the Physical, Mechanical and Biological Specifications of Bioinks and the Development of Functional Tissues in 3D Bioprinting. *Bioprinting*, 9:19–36.
10. Hospodiuk M, Dey M, Sosnoski D, *et al*., 2017, The Bioink: A Comprehensive Review on Bioprintable Materials. *Biotechnol Adv*, 35:217–39.
11. Hinton TJ, Jallerat Q, Palchesko RN, *et al*., 2015, Three-dimensional Printing of Complex Biological Structures by Freeform Reversible Embedding of Suspended Hydrogels. *Sci Adv*, 1:9.
12. Kelly BE, Bhattacharya I, Heidari H, *et al*., 2019, Volumetric Additive Manufacturing via Tomographic Reconstruction. *Science*, 363:1075–9.
13. Li J, Wu C, Chu PK, Gelinsky M, 2020, 3D Printing of Hydrogels: Rational Design Strategies and Emerging Biomedical Applications. *Mater Sci Eng R Rep*, 140:100543.
14. Lee A, Hudson AR, Shiwarski DJ, *et al*., 2019, 3D Bioprinting of Collagen to Rebuild Components of the Human Heart. *Science*, 365:482–7.
15. Bernal PN, Delrot P, Loterie D, *et al*., 2019, Volumetric Bioprinting of Complex Living-Tissue Constructs within Seconds. *Adv Mater*, 31:42.
16. Levato R, Jungst T, Scheuring RG, *et al*., 2020, From Shape to Function: The Next Step in Bioprinting. *Adv Mater*, 2020:1906423.
17. Moroni L, Burdick JA, Highley C, *et al*., 2018, Biofabrication Strategies for 3D *In Vitro* Models and Regenerative Medicine. *Nat Rev Mater*, 3:21–37.
18. Ravnic DJ, Leberfinger AN, Koduru SV, *et al*., 2017, Transplantation of Bioprinted Tissues and Organs: Technical and Clinical Challenges and Future Perspectives. *Ann Surg*, 266:48–58.
19. Ke D, Murphy SV, 2019, Current Challenges of Bioprinted Tissues Toward Clinical Translation. *Tissue Eng Part B Rev*, 25:1–13.
20. Kang HW, Lee SJ, Ko IK, *et al*., 2016, A 3D Bioprinting System to Produce Human-scale Tissue Constructs with Structural Integrity. *Nat Biotechnol*, 34:312–9.
21. Distler T, Ruther F, Boccaccini AR, *et al*., 2019, Development of 3D Biofabricated Cell Laden Hydrogel Vessels and a Low-Cost Desktop Printed Perfusion Chamber for *In Vitro* Vessel Maturation. *Macromol Biosci*, 19:9.
22. Jia W, Gungor-Ozkerim PS, Zhang YS, *et al*., 2016, Direct 3D Bioprinting of Perfusable Vascular Constructs Using a Blend Bioink. *Biomaterials*, 106:58–68.
23. Gao G, Park JY, Kim BS, *et al*., 2018, Coaxial Cell Printing of Freestanding, Perfusable, and Functional *In Vitro* Vascular Models for Recapitulation of Native Vascular Endothelium Pathophysiology. *Adv Healthc Mater*, 7:1–12.
24. Dranseikiene D, Schrüfer S, Schubert DW, *et al*., 2020, Cell-laden Alginate Dialdehyde Gelatin Hydrogels Formed in 3D Printed Sacrificial Gel. *J Mater Sci Mater Med*, 31:3–7.
25. Blaeser A, Campos DF, Puster U, *et al*., 2016, Controlling Shear Stress in 3D Bioprinting is a Key Factor to Balance Printing Resolution and Stem Cell Integrity. *Adv Healthc Mater*, 5:326–33.
26. Nair K, Gandhi M, Khalil S, *et al*., 2009, Characterization of Cell Viability during Bioprinting Processes. *Biotechnol J*, 4:1168–77.

27. Yu Y, Zhang Y, Martin JA, *et al*., 2013, Evaluation of Cell Viability and Functionality in Vessel-like Bioprintable Cell-laden Tubular Channels. *J Biomech Eng*, 135:1–9.
28. Widmaier EP, Raaff H, Strang KT, 2014, Vander's Human Physiology. 13th ed. McGraw Hill, New York.
29. Curley CJ, Dolan EB, Otten M, *et al*., 2019, An Injectable Alginate/Extra Cellular Matrix (ECM) Hydrogel Towards Acellular Treatment of Heart Failure. *Drug Deliv Transl Res*, 9:1–13.
30. Wei LN, Chee KC, Yu FS, 2019, Print Me An Organ! Why We Are Not There Yet. *Prog Polym Sci*, 97:1–45.
31. Schoneberg J, De Lorenzi F, Theek B, *et al*., 2018, Engineering Biofunctional *In Vitro* Vessel Models Using a Multilayer Bioprinting Technique. *Sci Rep*, 8:1–13.