# 3D Printed Venous Valves, Biocompatibility and Functionality

Benjamin Holmes*,**, Huda Asfour**, Priyanka Koti**, Nara Muselimyan**, Narine Sarvazyan**

*Nanochon LLC, Burke VA, ben.holmes@nanochon.com
**The George Washington University Department of Physiology and Pharmacology, Washington DC, phynas@gwu.edu

## ABSTRACT

3D printing has been gaining popularity as a method of creating *heart* valves. However, to the best of our knowledge, no one was yet able to 3D print *venous* valves, particularly using cells or biocompatible materials. Toward this long-term goal, we pursued the following specific tasks. TASK 1 included exploration of several different valve designs, including a single flap valve, a two-flap valve, a floating ball valve and a tri-leaflet valve. Main dimensions included 5 mm diameter valve and a 1-mm wall thickness. Valves were printed from both silicone and polyurethane material, and demonstrated movable flaps in the presence of fluid. TASK 2 was to compare the viability of primary cardiac fibroblasts and their survival in multiple passages after seeding on to the surface of the optimized silicone cardiac valves. Analysis of cell viability was conducted with microscopy, immunocytochemistry, and bioluminescence imaging with Luciferin and CytoscanTM LDH assays. Finally, for TASK 3 we designed 3D circular structures to support formation of a ring of tissue-engineered cardiac muscle suitable for implantation around the outer circumference of a the valves. When placed around valve-containing vessel segments such self-beating rings can be potentially used to aid venous return.

Our findings bring creation of implantable venous valves one step closer to reality. Ability to replace and repair these vascular structures will be a major development for a broad spectrum of ailments associated with chronic venous disease.

***Keywords***: 3D bioprinting, venious valves, cardiovascular engineering, tissue engineering, luciferin assay

## 1 INTRODUCTION

Venous valves are essential to help return blood to the heart. They are components of what is called “skeletal muscle pump’. The latter combats the effect of gravity in upright individuals and works via compression of veins by surrounding skeletal muscle. For it to work, skeletal muscle pump requires functionally intact unidirectional valves within the veins. When the skeletal muscle pump mechanism fails (either due to lack of skeletal muscle activity, distention of veins, failure of venous valves, or a combination of the above), it leads to chronic venous insufficiency, which is also called chronic venous disease (CVD). CVD is one of the most widespread diseases in the Western world. The number of people who suffer from CVD is very large, with an estimated 25% of adult population having varicose veins, and 6% more advanced chronic disease. CVD can lead to chronic skin changes, phlebitis, venous stasis, ulceration and, ultimately, loss of a limb and death. Lower extremity ulcers are particularly common in diabetic patients, with venous disease accounting for majority of them. In United States alone, the annual cost associated with of CVD treatment is approaching $3 billion, constituting ~ 2% of the total health-care budget cost5. Today there are several treatments of CVD tailored to specific causes and symptoms. These procedures can be helpful, but they require continuous care and are associated with lower quality of life. Surgical options include vein stripping, sealing veins using radiofrequency or laser energy, or ultrasound-guided foam sclerotherapy. These procedures can be effective, particularly when treating individual vein segments. Downsides of these treatments include high recurrence rate, often at a different site, and surgical complications. Venous leg ulcers are primarily treated using compression, with only 40% to 70% healing after 6 months of treatment. Venous ulcers may get infected leading to cellulitis or gangrene and can eventually lead to amputation.

Thus far, there have been a number of efforts in the last two years to expand 3D printing to cardiac tissue engineering [1-3]. So far, the bulk of the project in the field have either focused on the 3D printing of hydrogels, cell laden hydrogels, and microporous meshes for cardiac patches [4-8]. Some others have also engineered bioinks out of decellularized cardiac tissue [9, 10], but in these cases as well, 3D printing was employed to make simple cross hatched structures using either primary cardiomyocytes, stem cells, or a combination of cardiac cells/progenitors and vascular cells or vascular progenitors. There has also been the use of sacrificial 3D structures to create vascular or channel networks within cast hydrogels,.

3D printing has been gaining popularity as a method of creating *heart* valves. However, to the best of our knowledge, no one was yet able to 3D print *venous* valves, particularly using cells or biocompatible materials. Venous valves are much smaller and more delicate structures as compared to heart valves, therefore they are particularly challenging to produce. Our studies represent the first steps toward creating biocompatible and implantable venous valves using 3D printing and tissue engineering tools. Toward this long-term goal, we pursued the following specific tasks. TASK 1 included exploration of several

different valve designs, including a single flap valve, a two-flap valve, a floating ball valve and a tri-leaflet valve. Main dimensions included 5 mm diameter valve and a 1-mm wall thickness. Valves were printed from both silicone and polyurethane material, and demonstrated movable flaps in the presence of fluid. TASK 2 was to compare the viability of primary cardiac fibroblasts and their survival in multiple passages after seeding on to the surface of the optimized silicone cardiac valves. Analysis of cell viability was conducted with microscopy, immunocytochemistry, and bioluminescence imaging with Luciferin and CytoscanTM LDH assays. A BioBot 3D bioprinter was used to print optimized valves from task 1, which were then incubated in 0.1% fibronectin solution for 24 hours at 37 deg C. Cells could be observed forming dense fibers on the outer walls of the valves, which beat spontaneously. Finally, for TASK 3 we designed 3D circular structures to support formation of a ring of tissue-engineered cardiac muscle suitable for implantation around the outer circumference of a the valves. When placed around valve-containing vessel segments such self-beating rings can be potentially used to aid venous return. Scaffolds were designed as 2-mm high rings, with a 6-mm outer diameter and a 5-mm inner diameter. The wall of the ring were printed around the cardiac valves, and then cast with cardiomyocyte laden fibronectin. Primary cardiac myocytes were cultured. Cardiac cells with and without fibrin were observed forming beating structures within the fibrin, forming coherent beating cell structures.

Our findings bring creation of implantable venous valves one step closer to reality. Ability to replace and repair these vascular structures will be a major development for a broad spectrum of ailments associated with chronic venous disease.

## 2 MATERIALS AND METHODS

A series of experiments were carried out, in order to both assess the viability of various bioprinting methods (direct cell printing and indirect culture on a 3D printed cconstruct), to evaluate the 3D printability of various venous valve designs and then to demonstrate viability of a more realized 3D valve construct. TASK 1 included exploration of several different valve designs, including a single flap valve, a two-flap valve, a floating ball valve and a tri-leaflet valve. Main dimensions included 5 mm diameter valve and a 1-mm wall thickness. Valves were printed from both silicone and polyurethane material, and demonstrated movable flaps in the presence of fluid. TASK 2 was to compare the viability of primary cardiac fibroblasts and their survival in multiple passages after seeding on to the surface of the optimized silicone cardiac valves. Analysis of cell viability was conducted with microscopy, immunocytochemistry, and bioluminescence imaging with Luciferin and CytoscanTM LDH assays. A BioBot 3D bioprinter was used to print optimized valves from task 1, which were then incubated in 0.1% fibronectin solution for 24 hours at 37 deg C. Cells could be observed forming dense fibers on the outer walls of the valves, which beat spontaneously. Finally, for TASK 3 we designed 3D circular structures to support formation of a ring of tissue-engineered cardiac muscle suitable for implantation around the outer circumference of a the valves. When placed around valve-containing vessel segments such self-beating rings can be potentially used to aid venous return. Scaffolds were designed as 2-mm high rings, with a 6-mm outer diameter and a 5-mm inner diameter. The wall of the ring were printed around the cardiac valves, and then cast with cardiomyocyte laden fibronectin. Primary cardiac myocytes were cultured.

Detailed description of the experimental steps can be found below.

### 2.1 Design of 3D venous valves

Several different valve designs were explored, including a single flap valve, a two-flap valve, a floating ball valve and a tri-leaflet valve. Main dimensions included 5 mm diameter valve and a 1-mm wall thickness. The tri-leaflet valve was eventually chosen based on anatomical similarity and the capabilities of the printer/materials. Valves were printed from both silicone and an experimen tal polyurethane variant, and demonstrated movable flaps in the presence of fluid. Valves where then tested for their ability to maintain viable cells on their surface.

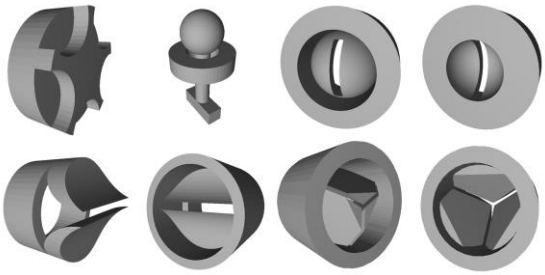

Figure 1: Various 3D designs for venous vavle replacements

Of the designs above (figure 1) the tri-leaf design was chosen. In this case, it was the most functional structure after pinting. Valves were evaluated by being docked to a 10 ml syringe filled to capacity with ultrapure water. The tri leaf design successfully allowed forward flow (45 drops per minute) while limiting backflow (15 drops per second). The average drop rate was recorded for 5 different printed valves. Tri-vavles were also printed from both Silicone and and expereimntal, nano-porous thermoplastic polyurethane material.

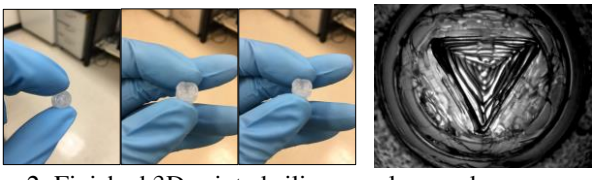

Figure 2: Finished 3D printed silicone valves and a magnified 10X light microscope of a valve, with cultured cardiomyocytes

## 2.2 Culture and viability of optomized 3D printed valve

The goal of of these experiments was to print 3D cardiovascular valves and assess cardiomyocyte viability via luciferin and LDH. First, valves were 3D printed from silicone on the Biobot, 6 samples in total. They were coated in 1a 5 ug/ml fibronectin solution for 1 hr. They were then washed twice in PBS and seeded with cardiomyocytes, 1 million per samples, in 1.5 ml of media per sample. Firefly luciferase oxidizes luciferin to produce light hat can be detected with bioluminescence imaging. Cardiomyoctyes were infected with luciferase on Day 1, seeded onto printed valves, and imaged on Days 3 and 5 to assess bioluminescence.

## 2.3 Design and evaluation of an advanced valve-cell structure

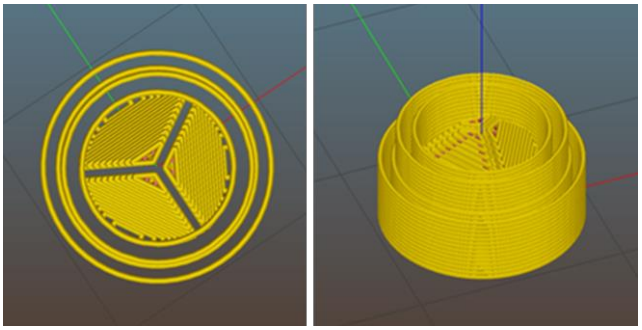

Figure 3: Designs for the valve, including a series of circular channels intended to hold cast cell laden fibrin

3D circular structures were designed to support formation of a ring of tissue-engineered cardiac muscle suitable for implantation around the outer circumference of a vein. When placed around valve-containing vessel segments such self-beating rings can be used to aid venous return. Scaffolds were designed as 1-mm high rings, with a 4-mm outer diameter and a 3.5-mm inner diameter. The wall of the ring also possessed a roughly 300 um channel designed to trap cells and foster cardiac tissue growth, as well as to encourage adequate fluid perfusion. Primary cardiac myocytes were cultured and seeded on samples printed on the BioBot from silicone, around the designed vavles, coated in fibronectin for 24 hours.

# 3 RESULTS AND DISCUSSION

## 3.1 Thermoplastic polyurethane / material selection

Thermoplastic polyurethane (TPU) is a versatile, flexible and biocompatable material that has been used in 3D printing based experiments for soft tissue constructs. Attempts were made to use TPU to print constructs. While it was very effective for printing structures, rings and valves, cardiac cells did not seem to respond to the material. Specifically, cells did not adhere well, forming benign spheroids, and never matriculating into coherent constructs. When samples were lifted and stained after five days, no adherent cells survived the staining process, and nothing could be observed. Cells (cardiomyocytes) did not form beating constructs. Thus Silicone was selected as the base material for ongoing experiments.

## 3.2 Silicone tri-vavle viability and functionality

3D printed silicone vavles showed excellent results when analyzed via LDH assay.

| ROI | Image Layer | Total Flux [p/s] | Avg Radiance [p/s/cm²/sr] | Stdev Radiance | Min Radiance | Max Radiance | Flux-Background |
|---|---|---|---|---|---|---|---|
| ROI 1 | Overlay | 3.45E+06 | 1.09E+05 | 7.44E+04 | 1.53E+03 | 3.28E+05 | 3.23E+06 |
| ROI 2 | Overlay | 3.83E+06 | 1.22E+05 | 7.55E+04 | 9.59E+03 | 3.89E+05 | 3.62E+06 |
| ROI 3 | Overlay | 4.13E+06 | 1.31E+05 | 9.76E+04 | 1.54E+03 | 4.87E+05 | 3.91E+06 |
| ROI 4 | Overlay | 3.79E+06 | 1.20E+05 | 9.06E+04 | 9.27E+03 | 4.88E+05 | 3.58E+06 |
| ROI 5 | Overlay | 4.09E+06 | 1.30E+05 | 1.05E+05 | -4.29E+03 | 4.81E+05 | 3.88E+06 |
| ROI 6 | Overlay | 4.56E+06 | 1.45E+05 | 8.77E+04 | 2.06E+04 | 4.22E+05 | 4.34E+06 |
| ROI 7 | Overlay | 2.15E+05 | 6.86E+03 | 1.48E+04 | -3.15E+04 | 7.82E+04 | Background |

Figure 4: LDH readings after 24 hours

After 48 hours there were slight drops in luminescence. There was also visable spreading of light intensity. This suggests that valves wee viable / compatable with cardyomyocytes but that a more stable 3D culture envirnment was needed.

| ROI | Image Layer | Total Flux [p/s] | Avg Radiance [p/s/cm²/sr] | Stdev Radiance | Min Radiance | Max Radiance | Flux-Background |
|---|---|---|---|---|---|---|---|
| ROI 1 | Overlay | 2.18E+06 | 1.02E+05 | 5.53E+04 | 5.31E+02 | 3.50E+05 | 2.12E+06 |
| ROI 2 | Overlay | 2.65E+06 | 1.24E+05 | 6.87E+04 | 5.39E+02 | 3.52E+05 | 2.58E+06 |
| ROI 3 | Overlay | 2.72E+06 | 1.27E+05 | 4.23E+04 | 2.69E+04 | 2.91E+05 | 2.66E+06 |
| ROI 4 | Overlay | 3.05E+06 | 1.43E+05 | 4.89E+04 | 1.92E+04 | 2.91E+05 | 2.98E+06 |
| ROI 5 | Overlay | 2.73E+06 | 1.29E+05 | 5.58E+04 | 6.45E+03 | 3.33E+05 | 2.67E+06 |
| ROI 6 | Overlay | 2.87E+06 | 1.35E+05 | 5.40E+04 | 2.02E+04 | 2.95E+05 | 2.81E+06 |
| ROI 7 | Overlay | 6.22E+04 | 2.92E+03 | 1.30E+04 | -3.69E+04 | 4.62E+04 | Background |

Figure 5: LDH readings after 48 hours

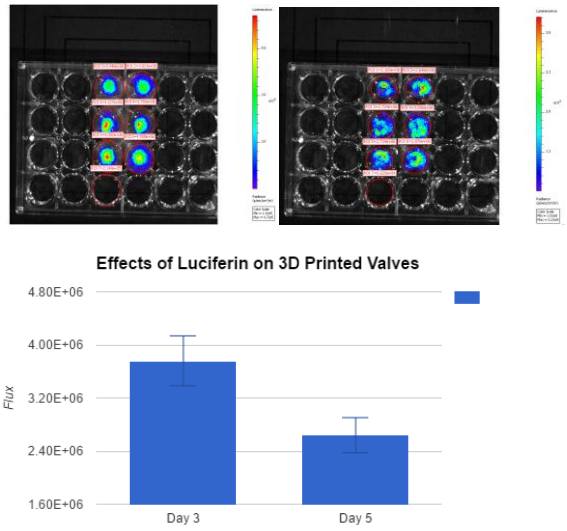


Figure 6: From top left, LDH light emitted after 24 hours, LDH light emitted after 48 hours and average light readings of day 3 and day 5.

### 3.3 Complex construct with silicone valve and cast cardyomyocyte laden fibronectin

In order to solve the previously observed culture issues, vavles were printed with incorporated rings, and cast with cell laden fibrin. 3D circular structures were designed to support formation of a ring of tissue-engineered cardiac muscle suitable for implantation around the outer circumference of a vein. When placed around valve-containing vessel segments such self-beating rings can be used to aid venous return. Scaffolds were designed as 1-mm high rings, with a 4-mm outer diameter and a 3.5-mm inner diameter. The wall of the ring also possessed a roughly 300 um channel designed to trap cells and foster cardiac tissue growth, as well as to encourage adequate fluid perfusion. Primary cardiac myocytes were cultured and seeded on samples printed on the BioBot from silicone, around the designed vavles, coated in fibronectin for 24 hours. Cardiac cells with fibrin could be observed adhering to structures on the inner and outer walls of the rings and in the channels, forming spontaneously beating cell structures

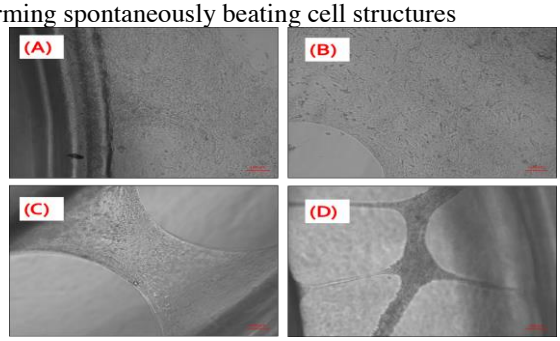


Figure 7: Cardiomyocytes cultured in the "cardiac rings" of the full construct. (A-B) cells forming beating monolayers after 5 days and (C-D) beating 3D filaments forming between the inner and outer walls of the rings after 7 days.

## REFERENCES


1. Jung, J.P., D.B. Bhuiyan, and B.M. Ogle, *Solid organ fabrication: comparison of decellularization to 3D bioprinting*. Biomater Res, 2016. 20(1): p. 27.
2. Duan, B., *State-of-the-Art Review of 3D Bioprinting for Cardiovascular Tissue Engineering*. Ann Biomed Eng, 2016.
3. Cheung, D.Y., B. Duan, and J.T. Butcher, *Current progress in tissue engineering of heart valves: multiscale problems, multiscale solutions*. Expert Opin Biol Ther, 2015. 15(8): p. 1155-72.
4. Yeo, M., et al., *An Innovative Collagen-Based Cell-Printing Method for Obtaining Human Adipose Stem Cell-Laden Structures Consisting of Core-Sheath Structures for Tissue Engineering*. Biomacromolecules, 2016. 17(4): p. 1365-75.
5. Kang, L.H., et al., *Optimizing Photo-Encapsulation Viability of Heart Valve Cell Types in 3D Printable Composite Hydrogels*. Ann Biomed Eng, 2016.
6. Lee, H.J., et al., *A New Approach for Fabricating Collagen/ECM-Based Bioinks Using Preosteoblasts and Human Adipose Stem Cells*. Adv Healthc Mater, 2015. 4(9): p. 1359-68.
7. Gaetani, R., et al., *Epicardial application of cardiac progenitor cells in a 3D-printed gelatin/hyaluronic acid patch preserves cardiac function after myocardial infarction*. Biomaterials, 2015. 61: p. 339-48.
8. Gaetani, R., et al., *Cardiac tissue engineering using tissue printing technology and human cardiac progenitor cells*. Biomaterials, 2012. 33(6): p. 1782-90.
9. Jang, J., et al., *3D printed complex tissue construct using stem cell-laden decellularized extracellular matrix bioinks for cardiac repair*. Biomaterials, 2016. 112: p. 264-274.
10. Jang, J., et al., *Tailoring mechanical properties of decellularized extracellular matrix bioink by vitamin B2-induced photo-crosslinking*. Acta Biomater, 2016. 33: p. 88-95.
11. Hinton, T.J., et al., *3D Printing PDMS Elastomer in a Hydrophilic Support Bath via Freeform Reversible Embedding*. ACS Biomater Sci Eng, 2016. 2(10): p. 1781-1786.
12. Hinton, T.J., et al., *Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels*. Sci Adv, 2015. 1(9): p. e1500758.