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Using Bioprinting Technology to Develop a 3D In Vitro Liver Model

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14. ABSTRACT
The field of bioprinting has great potential for developing in-house, customizable organ models that would contribute greatly to the predictive toxicology effort at U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD). This technology incorporates traditional additive manufacturing techniques with relevant living cells to create physiologically relevant structures. This project used an Allevi 1 bioprinter (Allevi; Philadelphia, PA) in the DEVCOM CBC Makerspace Laboratory that had not been used for any prior projects. As a proof of concept to initiate bioprinting efforts at DEVCOM CBC, a liver model was developed using the Allevi 1 system. The bioprinter parameters were assessed and optimized for liver cells and biosupport materials. HepG2 cells and Pluronic F-127 were combined to serve as the bio-ink for the 3D in vitro model. To ensure liver cell viability and functionality after printing, a live/dead assay and human albumin enzyme-linked immunosorbent assays were performed. Fluorescence microscopy was used to confirm the bioprinted liver cells were metabolically and structurally viable after printing. This project was the first bioprinting effort at DEVCOM CBC. This effort yielded successful results and a vast potential for future opportunity harnessing this technology.

15. SUBJECT TERMS

Bioprinting	3D in vitro model	HepG2 cells
Computer-aided design (CAD)	Artificial liver model	Pluronic F-127 (PF127)

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PREFACE

The work described in this report was authorized under Section 2363 of the National Defense Authorization Act. The work was started in May 2022 and completed in December 2022.

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U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC) was previously known as U.S. Army Edgewood Chemical Biological Center (ECBC).

This report has been approved for public release.

Acknowledgments

Funding for this work was provided by the Director, DEVCOM CBC under the authorities and provisions of Section 2363 of the National Defense Authorization Act (FY 2018) to develop new technologies, engineer innovations, and introduce game-changing capabilities. Additional acknowledgements go to the DEVCOM CBC Makerspace Laboratory and Advanced Design Manufacturing team for their assistance and guidance throughout this project.

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EXECUTIVE SUMMARY

Bioprinting encompasses 3D-printing techniques with controls for biological aspects such as pressure and temperature to develop printed viable cell models. The Makerspace Laboratory at U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD) held an Allevi 1 bioprinter (Allevi; Philadelphia, PA) that had not been harnessed for any prior projects. In a collaboration with the DEVCOM CBC Threat Agent Science Division, a seedling project was funded to start bioprinting efforts. This proof-of-concept effort included development of a bioprinted model using liver cells (HepG2) and Pluronic F-127 (PF127). Because the bioprinter had not been used before, printing parameters had to be determined. This included designing several computer-aided design models and various volumes of printing materials. PF127 was used as a control biomaterial to optimize the bioprinter settings, which included build plate, pressure, temperature, print speed, and infill. HepG2 cells were added to the PF127 at various ratios, and it was discovered that a 1:6 ratio of cells to biomaterial was optimal for printing. The bioprinted models were tested for cell viability and function using a live/dead stain and human ELISAs. These assays showed that the cells exhibited 70% viability after the printing process and maintained liver function after 24 h. This was the first bioprinting project at DEVCOM CBC, and it has provided a baseline for future bioprinting projects. Future steps could include bioprinting a multicellular liver model that encompasses human primary cells to observe cell proliferation. This technology yields a vast potential for developing customizable, physiological organ models for predictive toxicology and regenerative medicine efforts.

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USING BIOPRINTING TECHNOLOGY TO DEVELOP A 3D IN VITRO LIVER MODEL

1. INTRODUCTION

1.1 Bioprinting Technology

Bioprinting technology combines traditional 3D techniques with living cells to create physiologically relevant models.[1] Rather than using inorganic materials, bioprinters construct structures by printing one layer of cells at a time, while maintaining cell viability and functionality.[2] By combining additive manufacturing methods with the biological aspect, bioprinting holds great potential for developing customizable in vitro organ models.

At the U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD), the Makerspace Laboratory houses an Allevi 1 bioprinter (Allevi; Philadelphia, PA) that had not been used prior to this project. This bioprinter features one core extruder that controls the printing of the cells with a platform that can hold various bases, such as a petri dish or six-well plate. The Allevi 1 bioprinter is controlled by Allevi's Bioprint Essential software, which allows a wireless connection to the bioprinter's network. This allows for control of the bioprinter from outside the biosafety cabinet.

1.2 Pluronic F-127 (PF127)

PF127 (chemical structure shown in Figure 1) is a nonionic copolymer that exhibits unique biomaterial properties for bioprinting applications.[3] The material has low toxicity and reverse thermal gelation properties, and it is biologically inert for a variety of cells.[4] It is advantageous for imaging as it is optically transparent. PF127 is in a liquid aqueous phase at colder temperatures and a solid phase at warmer temperatures, which makes this biomaterial ideal for bioprinting technologies. Past studies have found that PF127 forms an adequate gel around 20–30 °C during printing and can maintain its printed structure at 37 °C.[6] The proportions (weight per volume) of PF127 have also been well studied in the literature for tissue engineering applications.[5] Typically, PF127 is used in concentrations from 10 to 40% wt/vol for bioprinting purposes.[4] There are some drawbacks to using this material, such as its autofluorescence properties and rapid dissolution when mixed with excessive liquids. However, because of its availability, past uses in bioprinting, and ease of use, PF127 was selected for development of the bioprinted liver model.

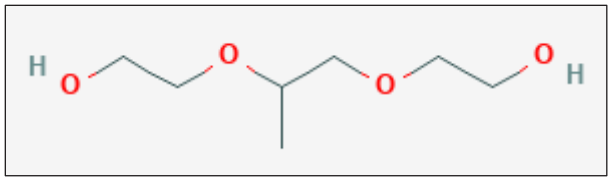

Figure 1. Chemical structure of PF127.
(From National Center for Biotechnology Information.[3])

2. METHODOLOGY

2.1 Computer-Aided Design (CAD) of the Models

SolidWorks CAD software (Dassault Systèmes; Waltham, MA) was used for the design of the various bioprinted models. A cube form was selected for initial proof-of-concept testing because it is easy to design and troubleshoot, should errors occur with printing. As a base model, a 7 × 7 × 2 mm^3 cube was developed to initiate bioprinting efforts. The total volume of this cube design is ~100 mm^3 for a standard measurement, which equates to 100 µL. This model was scaled up by a factor of 10, yielding an ~1000 mm^3 or 1 mL volume cube, as shown in Figure 2. When the design is bioprinted, the infill is another parameter that can be altered, depending on the size and design of the print.

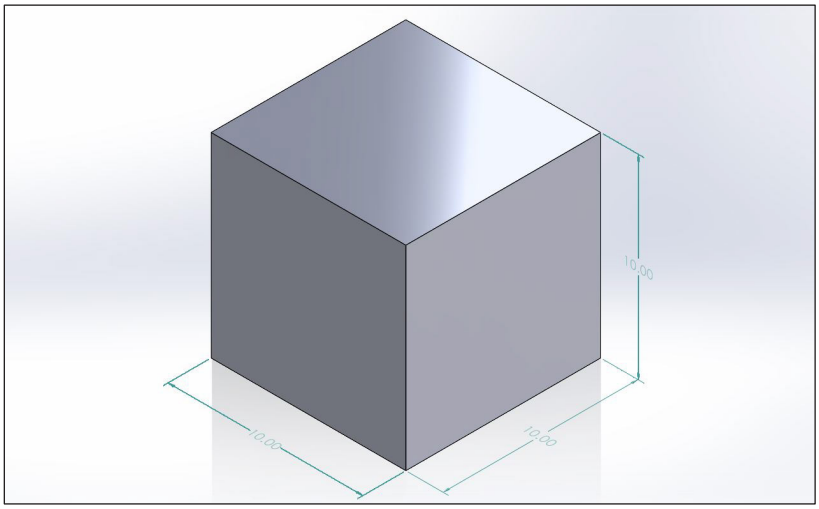

Figure 2. Cube model designed using SolidWorks CAD software.
Dimensions were 10 × 10 × 10 mm, and total volume was 1000 mm^3, or 1 mL.

2.2 Bioprinting PF127

Prior to this project, the settings and functions of the Allevi 1 bioprinter (Figure 3) had not yet been explored. The printer extruder controls the temperature and pressure of the material being dispersed. Temperature can be controlled from 4 to 160 °C, which allows for a wide array of biomaterials to be bioprinted. The machine is hooked up to an external air compressor that allows the user to control the extrusion pressure between 5 and 120 psi.

The software allows the user to select features such as layer height, print speed, infill distance, and direction. These settings must be characterized for each type of biomaterial used for printing and cell type being used. Before cells were incorporated, the parameters for the biomaterial PF127 had to be optimized and validated. The PF127 available was 40% wt/vol in deionized water and was purchased from Allevi.

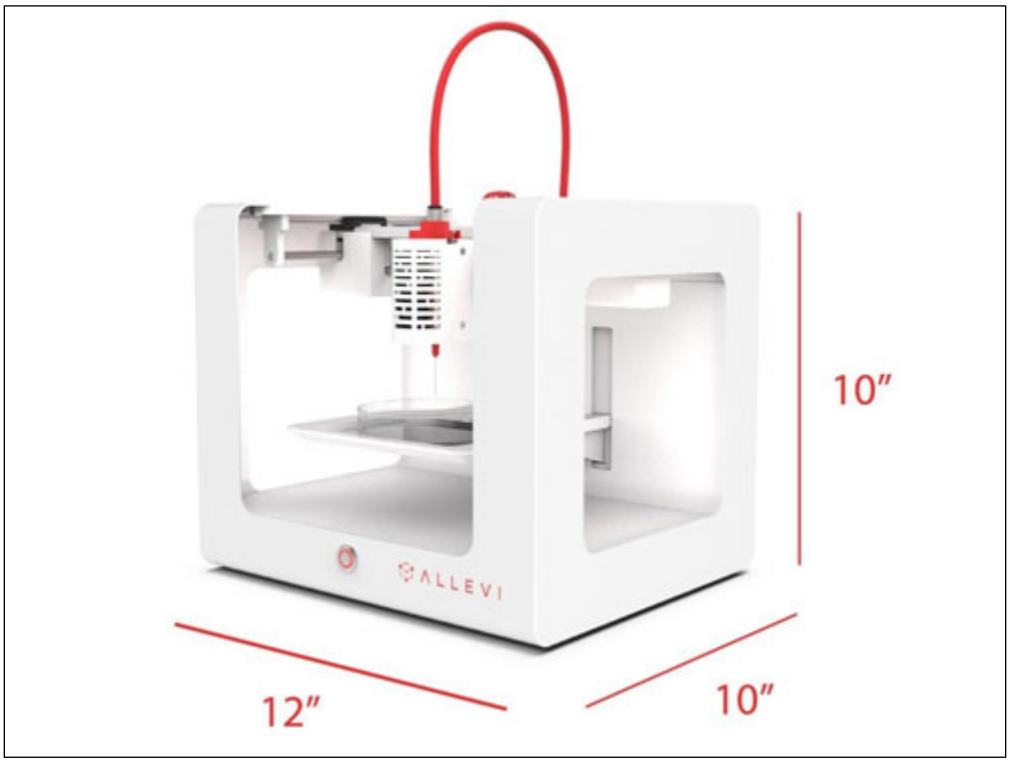

Figure 3. Allevi 1 bioprinter with dimensions.

2.3 Cell Culture

A commonly used human liver cell line, HepG2 cells (American Type Culture Collection [ATCC]; Manassas, VA) were thawed and expanded in Corning 75 cm² U-shaped canted neck, cell culture flasks (Corning Life Sciences; Tewksbury, MA). Eagle's minimum essential medium was supplemented with 10% fetal bovine serum for cell maintenance. Cells were passed with TrypLE Express enzyme (Gibco; Waltham, MA) on a weekly basis once confluence reached 70%, and fresh medium was exchanged every two to three days. Maintenance for each HepG2 cell line was discontinued after passage 10. For the bioprinting, HepG2 cells were passaged and reconstituted to the desired concentration in cell medium. All media and supplements were purchased from ATCC.

2.4 Bioprinting HepG2 Cells with PF127

No previous studies have focused on bioprinting models with HepG2 cells with PF127. The first step in this study was to find the optimal ratio of HepG2 cells and media to PF127 for functional bioprinting. The ratios of cells to PF127 were tested at 1:2, 1:4, 1:6, and 1:8 for printability, and 5×10^5 HepG2 cells/mL were prepared with each ratio of PF127 and mixed on ice. Once the mixture remained aqueous, it was transferred to a 10 mL syringe with a 30 gauge plastic needle tip. The bioprinter settings listed in Table 1 were used for test-printing the various ratios. It was determined that the 1:6 ratio was optimal for bioprinting HepG2 cells and PF127 (40% wt/vol). The lower ratios (1:2 and 1:4) were too thin to print, where the structure would not form. At higher ratios such as 1:8, the material was too solidified to extrude material from the needle tip.

Table 1. Bioprinting Settings for PF127

Feature	Setting
Layer height	0.2 mm
Print speed	6 mm/s
Infill type	Grid
Infill distance	1 mm
Direction	0°
Temperature	4 °C
Pressure	40 psi
Needle type	1/4 in. and 30 gauge
Build plate	100 mm^3 petri dish

2.5 Live/Dead Assay Procedure

To determine cell viability after printing, Live/Dead Viability/Cytotoxicity Kit assays (Invitrogen; Carlsbad, CA) were used. Using a modified protocol from Forsythe et al.,[5] prior to printing, the cells were spun down into a pellet. The cell pellet was washed with phosphate-buffered saline (PBS) and spun at 1200 *g* for 5 min. This was repeated three times. The pellet was then mixed with 2 mL of PBS, and two dyes were added: 2.0 µM calcein acetoxymethyl ester (AM; emission wavelength at 520 nm), and 4 µM ethidium homodimer (emission wavelength at 603 nm). The solution was allowed to incubate for 30 min at room temperature. After incubation, the cells were washed and spun down under the same conditions as the initial wash for a total of three times. Following staining, the cells were bioprinted into structures for imaging by fluorescence microscopy. The ethidium bromide (EB) fluoresces in the red spectrum, representing the dead cells, and the calcein AM green staining represents the metabolically live cells. EB indicates cell death as it is not able to diffuse unless the cell membrane is damaged or compromised. Calcein AM is a widely used live cell stain: nonfluorescent calcein AM converts to a green, fluorescent calcein through AM hydrolysis. Dead cells lack esterase activity, which makes calcein AM a useful live cell stain. The images were obtained with the Keyence BZ-X fluorescence confocal microscope (Keyence Corporation; Itasca IL), which provides both brightfield and fluorescence imaging.

2.6 Human Albumin SimpleStep ELISA Assay

To determine liver cell functionality after the bioprinting process, ELISAs were performed using the Human Albumin SimpleStep ELISA Assay Kit (Abcam; Waltham, MA) on three bioprinted samples. The bioprinted models were 1000 mm^3 cubes that were seeded at 1×10^6 cells/mL in accordance with the bioprinting settings and procedures in Section 2.4. Immediately after printing, 2 mL of cell media was added to the bioprinted models, which were placed under cell culture conditions for 24 h. A sample of cell media and PF127 (1:6 ratio) was used as a negative control, and a sample of HepG2 cells and media (1×10^6 cells/mL) was used as a positive control. Samples were collected and processed in accordance with the assay kit instructions.

2.7 Maintenance of the Bioprinted Structure

The bioprinted model was kept in a sterile petri dish and maintained in a cell culture incubator with 5% CO_2 at 37 °C and 90% relative humidity. These conditions served a dual purpose, to simulate the conditions the biostructures will be exposed to for storage and to prevent evaporation and contraction of the pluronic structures due to drying out. These conditions are required when printing with cells, to maintain cell life and metabolic activity. Media changes were attempted every two to three days, but there were issues with the bioprinted structure integrity after fresh media was added. This could be investigated in a future, follow-up study.

3. RESULTS

3.1 Bioprinted Models

Once the settings were optimized for the Allevi 1, PF127, and HepG2 cells, the models were printed and visualized. Before the models were analyzed on the microscope, images of the physical models were obtained, as shown in Figure 4. The two models had approximately a 10-fold difference in volume.

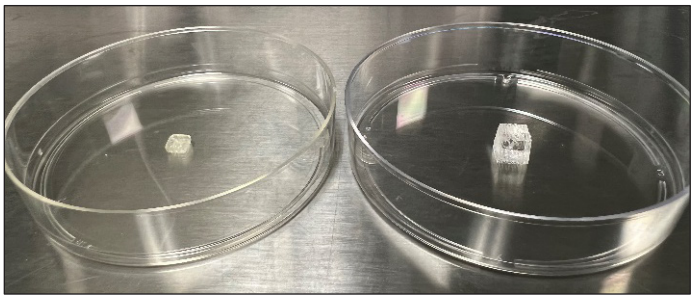

Figure 4. Bioprinted liver models: 100 mm^3 (left) and 1000 mm^3 (right).

3.2 Bioprinted Structure over Time

We investigated whether the customizable bioprinted models would hold structural integrity with the use of PF127 over time. Brightfield images were obtained of the bioprinted model for 35 days using the Keyence microscope at 4× magnification. As shown in Figure 5, the model retained its structural integrity while under cell culture conditions.

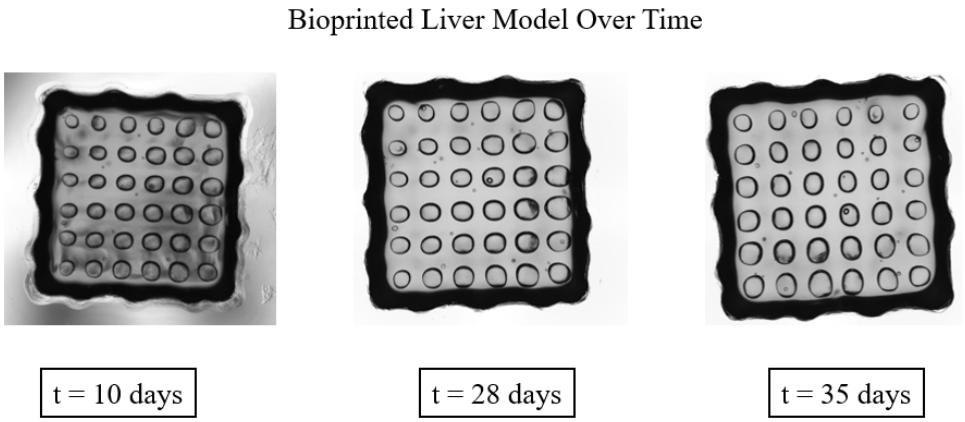

Figure 5. Images of the 100 mm^3 bioprinted liver model at three different time points spanning 35 days.

3.3 Live/Dead Assay and Evaluation

As described in Section 2.5, the cells were stained to ascertain cell viability before they were mixed with PF127 and printed. Prior to bioprinting, the cells were imaged for live/dead staining. Images were obtained (Figure 6) using the Keyence microscope at 4× magnification.

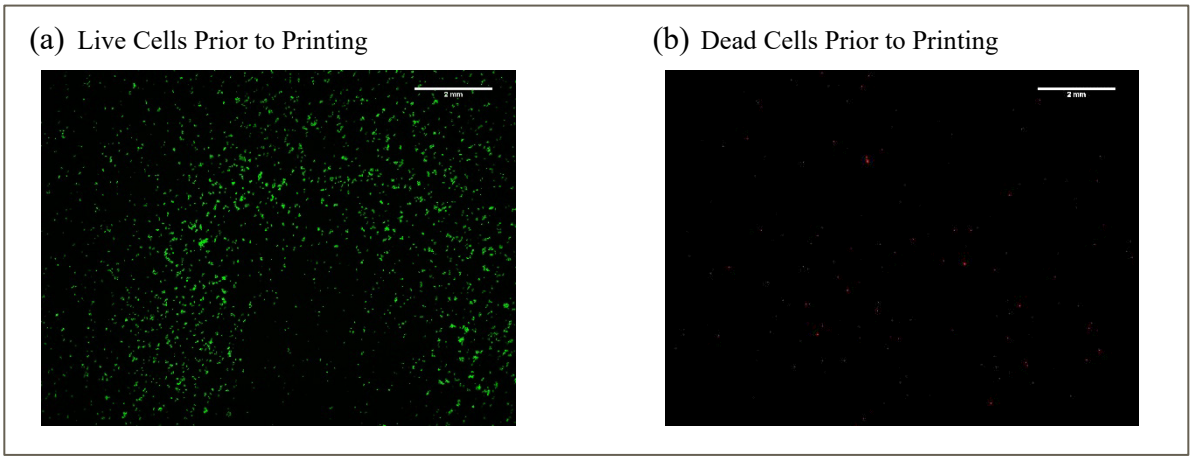

Figure 6. Stained HepG2 cells prior to bioprinting. (a) Calcein AM staining was used to indicate live cells, and (b) ethidium homodimer was used to indicate dead cells. Scale bar, 2 mm.

These cells were qualitatively evaluated and showed ~95% viability before they were prepared for bioprinting. Immediately after printing, the models were imaged on the Keyence microscope (Figure 7) and then placed in a cell culture incubator. The PF127 material is slightly autofluorescent; however, the signal from the calcein AM stain was clearly present, indicating the presence of live cells throughout the bioprinted structure (Figure 7a). The ethidium homodimer staining (Figure 7b) indicates dead cells were present. Comparing the live and dead cells using an overlaid image of the two fluorescence channels (Figure 7c) shows that after bioprinting, there were more live cells than dead. It was estimated by qualitative evaluation that the cells showed ~70% viability after the printing process. In a future study, a quantitative method, such as running a lactate dehydrogenase-based assay with cells prior to plating and then after dissolving the bioprinted structure, could be implemented for a cell viability comparison. Cell death was expected, as the cells underwent mechanical stress being extruded from the printer as well as dramatic temperature shifts when they were mixed with the PF127. As a result, this work produced a bioprinted liver model that was validated to contain viable liver cells immediately after printing.

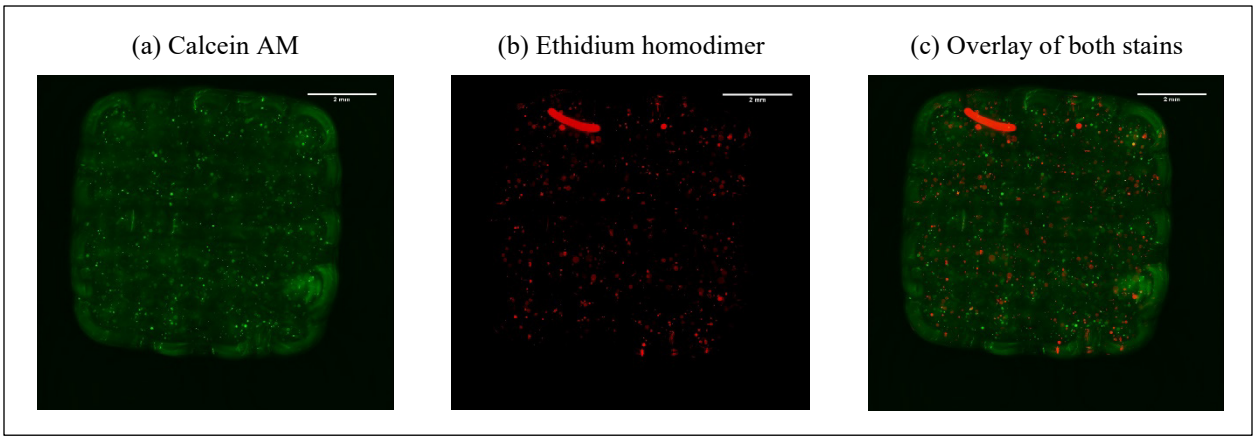

Figure 7. The 100 mm^3 liver model imaged with (a) calcein AM, (b) ethidium homodimer, and (c) both stains overlaid. Scale bar represents 2 mm.

3.4 Human Albumin ELISA

A one-way analysis of variance (ANOVA) test was performed using Prism 9 software (GraphPad; Boston, MA) to determine the statistical significance between the bioprinted samples with cells versus the control, a bioprinted model with just PF127. As shown in Figure 8, statistical significance was confirmed for the bioprinted liver samples versus the control ($p < 0.0001$). This showcases that the HepG2 cells in the bioprinted form maintain liver function after 24 h.

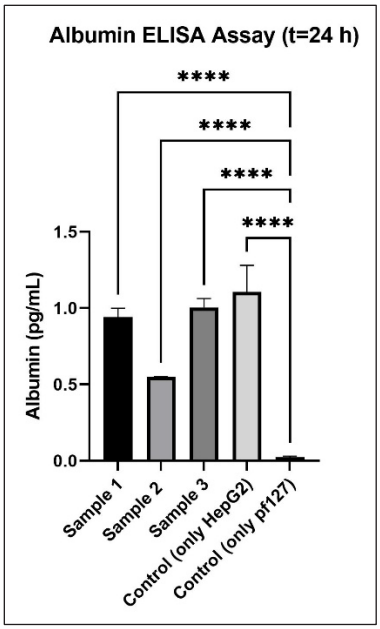

Figure 8. One-way ANOVA analysis for differences in albumin production from bioprinted samples (n = 3).

3.5 Customizing the Liver Model

A unique and extremely beneficial feature of bioprinting is the customizability of each model. Because each model has its own structural support, it is easy for the user to scale their models. To show this, we designed a cube that is 1000 mm^3, or 1 mL in volume, a 10-fold increase compared to the original 100 mm^3 model. To achieve this volume, the CAD file was designed to be 10 × 10 × 10 mm. This larger model was printed with the same conditions as in Table 1. Figure 9 shows a brightfield image in 4× magnification of the structure for the larger model and the corresponding calcein AM staining that shows live cells.

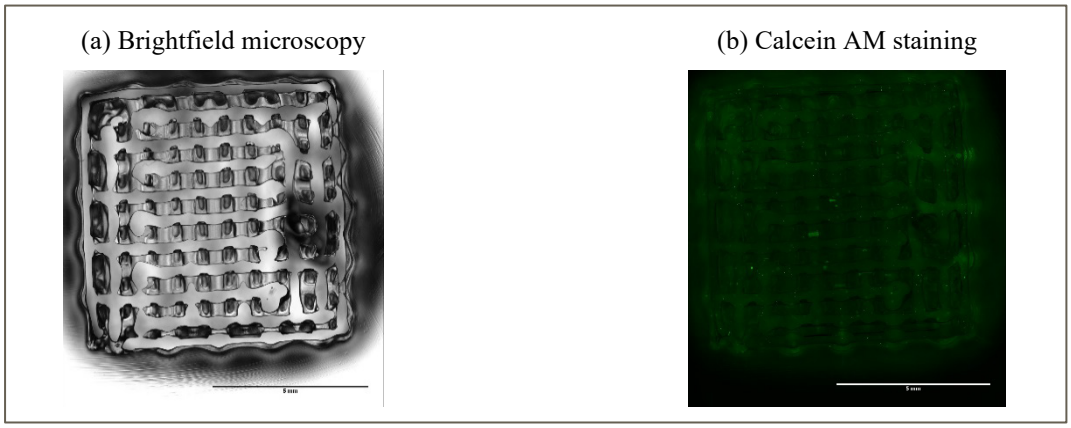

Figure 9. Upscaled 1000 mm^3 liver model, shown by (a) brightfield microscopy and (b) fluorescence microscopy with calcein AM staining in green with 4× magnification. Scale bar, 5 mm.

4. CONCLUSION

In this study, we initiated the first bioprinting efforts at DEVCOM CBC by developing a bioprinted liver cell line model. HepG2 cell survival and function after printing was proven with our optimized bioprinting protocol and provided a foundation for use of the Allevi 1 bioprinter. Some staining limitations were identified during this first attempt at bioprinting. Because PF127 showed fluorescence when stained for the live/dead assay, it was difficult to distinguish live cells from PF127. An additional study to address this limitation would be to transfect the cells prior to bioprinting with a fluorophore tagged to the cytosol and evaluate cell death through this mechanism.

Many future potential studies could stem from this project, including bioprinting a more complex multicellular liver model or expanding into the use of different cell types, such as primary cells, for a more physiologically accurate model. DEVCOM CBC currently houses several microphysiological systems for which bioprinting would be extremely beneficial. Bioprinting allows the user to customize the model to their own standards rather than relying on industry settings. Bioprinted organ models would be extremely useful tools for ongoing predictive toxicology studies for compounds of interest.

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ACRONYMS AND ABBREVIATIONS

AM	acetoxymethyl ester
ANOVA	analysis of variance
ATCC	American Type Culture Collection
CAD	computer-aided design
DEVCOM CBC	U.S. Army Combat Capabilities Development Command Chemical Biological Center
EB	ethidium bromide
PBS	phosphate-buffered saline
PF127	Pluronic F-127
wt/vol	weight-to-volume ratio

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