We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS BOOK CITATION INDEX INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter 11

3D Bioprinting: Surviving under Pressure

Dianne Eyvonn Godar

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73137

Abstract

Because 3D bioprinting using microextrusion was reported to yield cells with low viability (~40%) after pneumatic pressure (40 psi) printing through stainless steel nozzles, or blunt-end needles, with about 150 μm diameters (28 and 30G), we set out to improve the viability by coating the interior of the nozzles with silicone. For these studies, H9 human lymphoma cells were used to simulate human stem cells in suspension, and cell viability was measured using propidium iodide dye exclusion and flow cytometry. We tried to improve the viability by coating the inside of the 28 and 30G nozzles (1″ length) with silicone to protect the cell membranes from being damaged by the imperfections in the stainless steel nozzle. However, we discovered silicone coating had little effect on viability because imperfections in the nozzle were not the problem. Instead, the cells being placed in hypotonic 3% (w/v) alginate prepared in water prior to printing caused significant cell death (~25%) and considerably more (≥50%) after simulated printing under pressure. By preparing the alginate in isotonic solutions of either phosphate buffered saline or complete culture media, we could use pressures over five times (>220 psi) what most printing procedures use and obtain ~80% viability.

Keywords: force, hydrogel, hypotonic, isotonic, microextrusion, viability

1. Introduction

3D bioprinting is the wave of the future for constructing viable, functional, and biocompatible human organs that will be created from the patient's own stem cells so that antirejection drugs will not be needed after transplantation. Currently, there are three main 3D bioprinting methods: inkjet or "drop on demand," laser-assisted, and microextrusion [1].

Inkjet printing uses thermal or acoustic (piezoelectric) forces to create and eject droplets. Thermal inkjet bioprinting yields good cell viability (>85%), and although localized heating of 200–300°C occurs and the temperature at the head only rises 4–10°C for short durations

(~2 μs), cells can be heat shocked. The heat-shock protein chaperones can protect cells from dying [2] so that they have the potential of passing along DNA mutations that could ultimately result in a cancerous cell. Only a couple of degrees rise in temperature ≥2°C (~39°C) is needed to induce some heat-shock proteins [3]. Acoustic inkjet bioprinting applies voltage across polycrystalline piezoelectric ceramics to induce a rapid change in their shape that creates the pressure to eject droplets. However, the 15–25 kHz frequencies shock the cells: causing membrane damage and cellular lysis [4] and allowing molecules up to 40,000 Daltons (90 Å) in size to enter or exit the cell [5].

Laser-assisted bioprinting yields high cell viability (>95%) [6] and is nozzle-free so that the problem of clogging with materials or cells that other printing methods have is circumvented. In addition, it has microscale resolution of a single cell per drop. The drawbacks to laser-assisted bioprinting are low flow rates due to the high resolution requiring rapid gelation kinetics [7], time-consuming preparation of the ribbons used for printing, metal residues from vaporization of the metallic laser-absorbing layer during printing (nanoparticles), the complexity of making ribbons to print multiple cell types, and the high cost; more germane, it is not clear if this technology can be scaled up for larger tissue sizes other than skin [8], let alone organs.

Finally, microextrusion is the most common and affordable 3D bioprinting method that uses either pneumatic (air) or mechanical (screw or piston) forces to create pressurized dispensing systems [9]. However, this 3D bioprinting method is reported to yield the lowest cell viability of all three methods (40–80%) [10]. In previous studies, this low cell viability was completely attributed to the biofabrication mechanical forces or high pressures applied to the cells [11] rather than to structural imperfections inside the stainless steel nozzles creating membrane damage.

We reasoned if some of this membrane damage was actually due to the imperfections inside the stainless steel nozzle, then we might be able to improve the viability by coating their interiors with silicone. By minimizing these interior structural flaws, we would increase the viability during printing under pressure. However, in the course of our investigation, we found that neither the biofabrication of mechanical forces nor the structural flaws inside the nozzle were causing the reduction in viability but rather it was the hypotonic solution the cells were placed in when the alginate was prepared in water; the cells were placed directly into that hypotonic solution without first adjusting it with salt to be isotonic. This lysed many cells (~25%) until evidently the solution became isotonic from the released intracellular salts leaving the remaining cell population "bloated" or swollen and very sensitive to mechanical forces.

2. Materials and methods

2.1. Chemical formulations

The alginate solution was prepared using medium viscosity sodium alginate (Sigma, St. Louis, MO) dissolved in high-performance liquid chromatography grade water (Sigma Aldrich, St. Louis, MO) to make a 3% (w/v) solution as described previously [10, 11]. We also prepared 3% (w/v) alginate solutions in Dulbecco's phosphate buffered saline (PBS) without magnesium or calcium (GIBCO, Gaithersburg, MD) and in complete culture

http://dx.doi.org/10.5772/intechopen.73137

media (see below) with the addition of 1 mM ethylene diamine tetra acetic acid (EDTA; Sigma Aldrich, St. Louis, MO). The 1 mM EDTA was added to crosslink the 2 mM calcium present in the media so the alginate would not solidify. Note that this solution of alginate in complete culture media with EDTA did not solidify for over a year. We sterilized these solutions using either a 0.45 μm syringe filtration system (Nalge Nunc International Corporation, Rochester, NY) with the cell dispensing device described below (**Figure 1**) at the highest force (20 lbs) overnight or a 0.45 μm filtration unit with vacuum suction overnight. Additionally, we tested the viability after passing cells through the syringe and blunt-end needles using this force (20 lbs) in complete culture media or PBS (after washing the cells three times to remove any bound proteins that might afford membrane protection).

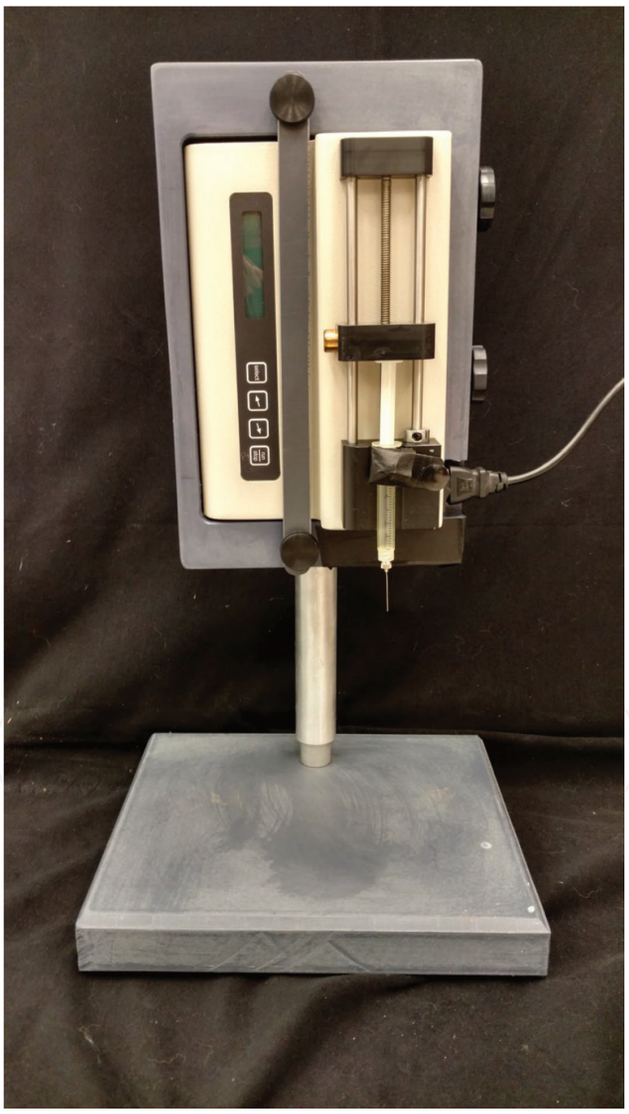

Figure 1. The KD scientific model 100 series screw-driven pressure pump with maximum force of 20 lbs is shown housed in a homemade holder attached to a vertical stand with a heavy base equipped with a 3-mL syringe and a 28G nozzle of 1″ in length that was used for the experiments shown in **Figures 2** and **4** (**Figure 3** has the 30G nozzle but same device).

2.2. Cell culture

Human lymphoma H9 cells (ATTC, Manassas, VA) were cultured and maintained in the incubator at 37°C using complete culture medium: CO_2-independent media (GIBCO, Gaithersburg, MD) supplemented with 10% (v/v) heat inactivated, mycoplasma-tested, and endotoxin-free, fetal bovine serum (GIBCO, Gaithersburg, MD), 4 mM glutamine (GIBCO, Gaithersburg, MD), antibiotic (10,000 IU penicillin and 10,000 μg/mL streptomycin), and antimycotic (25 μg/mL amphotericin B) solution (Sigma, St. Louis, MO). We use CO_2-independent media so the cells do not undergo pH shock while being manipulated during or after experiments. This enabled us to leave the cells in culture tubes at 37°C using a constant temperature controlled heating block, Hema-Bath Block Module Heater Type 12,200 Dribath (Baxter Scientific Products, Deerfield, IL), under sterile conditions in the biosafety cabinet until monitored on the first day (0 and 4 or 6 h postexposure). For the 24-h time point, we diluted the samples 1:1 with CO_2-independent complete culture media and maintained the cells in 5 mL sterile culture tubes at 37°C in the heating block or in the incubator (results not shown). These suspension cells were grown and maintained below 1×10^6 cells/mL in culture and were usually used between 4 and 8×10^5 cells/mL for experiments with viability ≥90% as determined by dye exclusion of propidium iodide (PI) using flow cytometry.

Cells were centrifuged in 50 mL centrifuge tubes (Corning, Tewksbury, MA) at 300 × *g* for 7 min, and the media were aspirated to leave cell pellets that were loosened by quickly (2–3 s) vortexing at low speed. These cell pellets were very gently and briefly mixed in the viscous 3 mL of 3% (w/v) alginate solutions with a Pasteur pipet by swirling and slowly pipetting up and down three times to homogeneously disperse the cells as previously described [10]. The cell density was ~3×10^6 cells, as determined by hemocytometer readings, before mixing into 3 mL of alginate to give ~1×10^6 cells/mL.

2.3. Procedure for simulated microextrusion pressure printing

Cells were grown to 4–8×10^5 cells/mL in complete culture media whereby 100 mL was centrifuged at 300 × *g* for 7 min, media aspirated, and then the cell pellet was vortexed and suspended in one of the three, 3% (w/v) alginate solutions (H_2O, PBS, or complete culture media) or suspended in solutions of either PBS or complete culture media to a final density of ~1×10^6 cells/mL or 4–8×10^5 cells/mL, respectively. Different concentrations were used to know if high cell density afforded protection for the cells. For the PBS and complete culture media comparison studies, we washed the cells three times with either PBS or complete culture media, respectively, prior to microextrusion. For the T = 0 time point (actual time < 10 min), we simply collected the cells in a test tube containing 0.25–0.5 mL of complete culture media at room temperature. For the longer daily time points of 4 or 6 h, we put the test tubes at 37°C in a dry-block incubator under the biosafety cabinet. For overnight studies at 24 h, we further diluted the cells 1:1 with complete culture media and put them in tightly capped sterile 5 mL polypropylene culture tubes or T-25 flasks (Corning, Tewksbury, MA).

2.4. Microextrusion cell dispensing system

We employed a mechanical device that uses a screw to create a force with subsequent pressure dependent on the radius of the syringe. The KD Scientific Model 100 series (Harvard Apparatus, Holliston, MA) screw-driven pressure pump with maximum force of 20 lbs was housed in a homemade holder attached to a vertical stand with a heavy base (see **Figure 1**). We used syringes of various sizes (3–60 mL) with Hamilton blunt-end 28G or 30G needles (Harvard Apparatus, Holliston, MA) of different lengths: ½″, 1″, and 2″. These blunt-end needles are referred to as nozzles.

The experiments were performed using various pressures "P" that were calculated in pounds per square inch (psi) using Eq. (1):

$$P = \frac{F}{A} \tag{1}$$

where "F" is the applied force (maximum of 20 lbs was used) and "A" is the area (in inches squared) of the applied force. We performed various experiments using syringes of different sizes, 3, 10, 30, and 60 mL, having radii of 0.17 (8.59 mm), 0.285 (14.48 mm), 0.425 (21.59 mm), and 0.524 inches (26.6 mm), with areas of 0.09, 0.255, 0.568, 0.86 inches^2, yielding pressures of 220, 78, 35, and 23 psi, respectively. Pressure experiments were conducted at room temperature, and the cells were placed at 37°C after treatment until analyzed.

2.5. Chemical coating nozzles

In order to help minimize membrane damage incurred during pressurized simulated microextrusion printing, presumably from imperfections in the stainless steel, we coated the interior of the 28 and 30G blunt-end needles for 5–15 min at room temperature using ~10% (w/v) high molecular weight (500,000 g/mole) polydimethylsiloxane, trimethylsiloxy terminated (Gelest, Inc., Morrisville, PA) in high-performance liquid chromatography grade hexane (Sigma Aldrich, St. Louis, MO). To sterilize the blunt-end needles, we luer-locked them on the syringe, submerged them in 70% ethanol, and then used three 0.5 mL interior washes of 70% ethanol followed by three 0.5 mL interior washes of sterile 0.9% saline (isotonic).

2.6. Cell viability

Cell viability was assessed by the dye exclusion method using PI (Molecular Probes, Eugene, OR) at a final concentration of 1 μg/mL using a slightly modified procedure [12]. Briefly, the PI was added directly to the samples so as not to lose any of the representative cell populations or to create false positives by centrifuging and disrupting the membranes of partially damaged cells. Live cells completely exclude PI, while dead cells allow it to almost instantly pass through their membranes. Cell viability was quantified as percentages using flow cytometry.

2.7. Flow cytometry

The viability of 10,000 cells from each sample was determined by a FACSCanto II (Becton Dickinson, CA, USA) triple beam flow cytometer at medium flow rate in the PerCP-Cy5-5-A (equivalent to FL-2) channel using PI dye exclusion [12]. We gated on the single-cell population. To set the marker for the live cell population, we used cultured cells from the incubator (≥90%) and created the marker for the dead cell population (with viability <10%) using 10 mL of 4–8 × 10^5 cells/mL exposed overnight to a final concentration of 300 μM silver nitrate (unpublished results). The sham-exposed cells underwent the same treatment as the exposed cells except they were not put under pressure through the syringes and nozzles; the cells were carefully mixed in alginate or other solutions and then gently placed into the syringe and allowed to slowly drip out as small droplets.

For each experiment, the live and dead cell controls were used to set the gates for the live and dead cell populations in the PerCP-Cy5-5-A fluorescent channel. We used forward scatter characteristics (FSC-W and FSC-H) to distinguish between single and multiple, or clumped, cell populations. The gate was set on the single-cell population to collect 10,000 cells for further analysis in the PerCP-Cy5-5-A fluorescent channel.

2.8. Statistical analysis

The data in the text and figures are presented as the mean (n = 3) ± standard deviation (SD) computed using the Student's t-test for two samples assuming unequal variance and consider $p < 0.05$ to be significant.

3. Results

For these experiments, we used the pressure pump aligned vertically in a homemade holder attached to a stand with a heavy base at a maximum force of 20 lbs (see **Figure 1**).

After preliminary testing, we realized the hypotonic alginate solution was causing the decrease in viability, so we compared alginate prepared in three different solutions: H_2O, PBS, and complete culture media (as described in Section 2). **Figure 2** shows a very significant drop in viability compared to the control (95 ± 1%) when the cells were placed in alginate prepared in H_2O (Sham, 75 ± 11.6%, $p = 0.048$; 28G no coat, 21.8 ± 13.5%, $p = 0.0056$; 28G coat, 14 ± 1.3%, $p = 6.3 \times 10^{-8}$), while the cells placed in alginate prepared in either PBS (Sham, 94.3 ± 0.26, $p = 0.16$; 28G no coat, 92.9 ± 0.6%, $p = 0.025$; 28G coat, 87.8 ± 2.1%, $p = 0.0062$) or the complete culture media (Sham, 96.5 ± 0.4, $p = 0.063$; 28G no coat, 86.3 ± 2.8%, $p = 0.0075$; 28G coat, 85.5 ± 1.6, $p = 0.00045$) although sometimes significantly lower did not cause more than a 10% drop in cell viability using the same conditions: 3 mL syringe, 20 lbs of force giving 220 psi, and 28G nozzles of 1″ length. We show the data for 28G nozzles of 1″ length in **Figure 2**, but note that the ½ and 2″ lengths also did not cause a decrease of more than 10% in cell viability if the cells were placed in 3% (w/v) alginate prepared in PBS or complete culture media. Note that coating the nozzles did not improve the cell viability compared to those that were uncoated. The results for T = 0 are shown because the later time points did not yield more than a 10%

http://dx.doi.org/10.5772/intechopen.73137

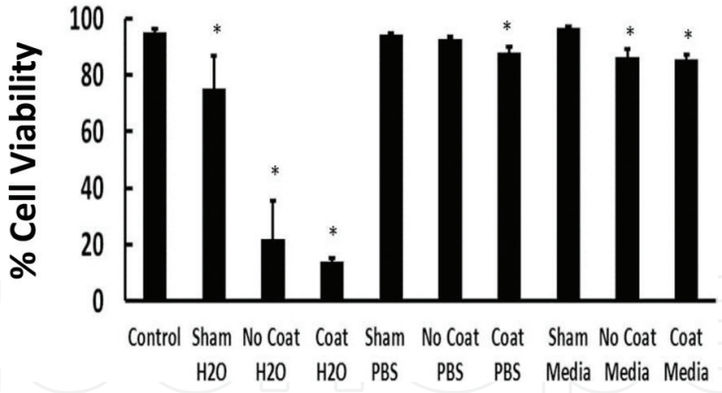

Figure 2. The viability of H9 cells immediately (T = 0 h) following direct cell writing biofabrication using the homemade microextrusion device shown in **Figure 1** equipped with a 3-mL syringe and 28G nozzle of 1″ length using maximum force of 20 lbs to produce 220 psi. Concentrated H9 cells were mixed with 3 mL of 3% (w/v) alginate prepared in different solutions: H_2O, PBS, and complete culture media with 1 mM EDTA. The "no coat" refers to the untreated nozzles and the "coat" refers to the nozzles coated with silicone as described in Section 2.The asterisks indicate significant differences from the controls.

decrease in viability, in agreement with what others found [10], and after 24 h, some of the surviving cells divided increasing the apparent viability.

As shown in **Figure 2**, the nozzle surface chemistry did not affect cell viability, but the hypotonic solution did, so we did not compare coated with uncoated nozzles in **Figure 3**. We wanted to know how a lower gauge nozzle with a smaller diameter would affect cell viability, so we used a 30G nozzle of 1″ length and placed the cells in 3% (w/v) alginate solutions as described previously: H_2O, PBS, and complete culture media with 1 mM EDTA. Using the same conditions employed for the 28G nozzle of 1″ length (i.e., 3 mL syringe and maximum force of 20 lbs yielding 220 psi), we did not observe more than ~8% decrease in cell viability in the alginate prepared in H_2O (14.1 ± 2.4%; $p = 6.9 \times 10^{-6}$), PBS (86 ± 10%; $p = 0.21$), or media

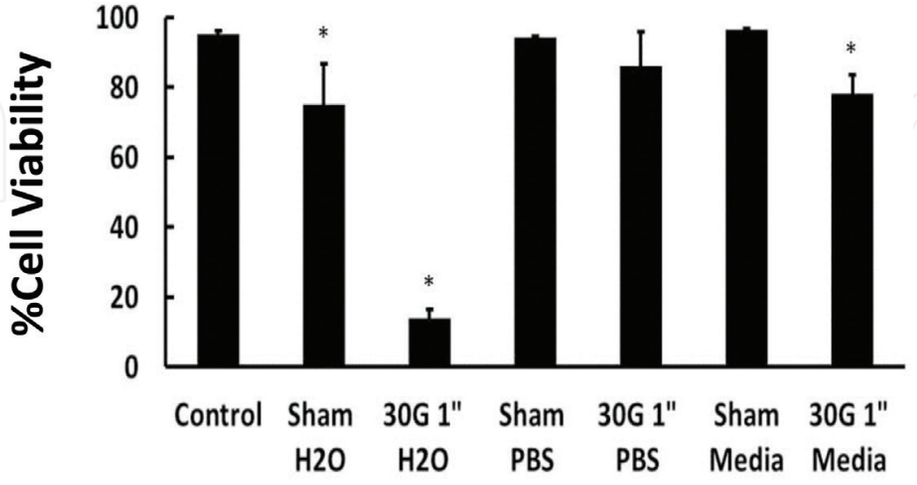

Figure 3. The viability of H9 cells immediately (T = 0 h) following direct cell writing biofabrication using the homemade microextrusion device equipped with a 3-mL syringe and 30G nozzle of 1″ length using maximum force of 20 lbs to produce 220 psi. Concentrated H9 cells were mixed with 3 mL of 3% (w/v) alginate prepared in different solutions: H_2O, PBS, and complete culture media with 1 mM EDTA. The asterisks indicate significant differences from the controls.

(78.3 ± 5.2%; p = 0.016) than after passing them through the 28G nozzle of the same length (**Figure 3**). Note that these differences in cell viability are not statistically significant from those obtained using the 28G nozzles (H_2O p = 0.22; PBS, p = 0.26; media, p = 0.051).

Finally, we wanted to see if we could decrease the cell viability by removing the alginate, as it might be affording protection by coating the cellular membranes. We show the results for 3-mL syringes with 28G nozzles of 1″ length (either coated or with no coat) but found no significant effect on cell viability even at the highest force of 20 lbs resulting in a pressure of 220 psi when in PBS or complete media (**Figure 4**). We also tried 30G nozzles of 1″ length and other syringe sizes, but they only slightly affected cell viability unless the cells were placed in a hypotonic alginate solution.

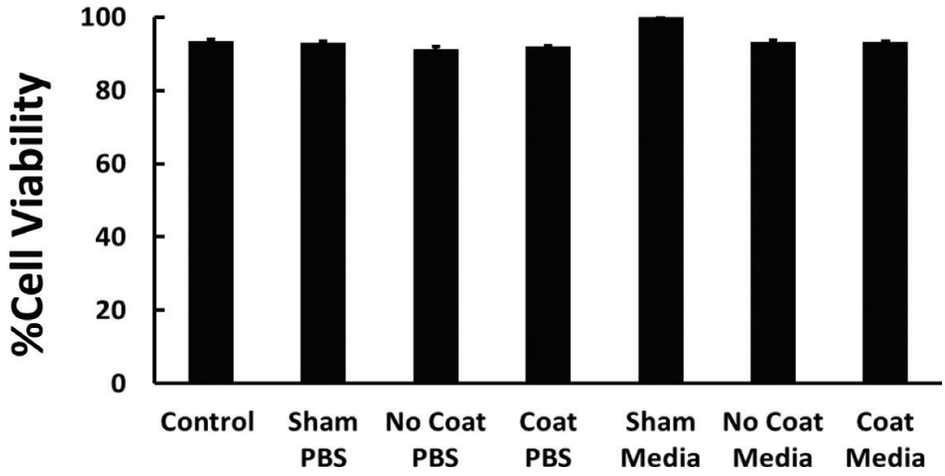

Figure 4. The viability of H9 cells was determined after control, sham, or passing the cells through the 3-mL syringe equipped with a 28G nozzle of 1″ length under 220 psi in either PBS or media in the absence of alginate. The cells were washed with either PBS or complete culture media three times prior to simulated microextrusion printing. The control and shams are as described in Section 2.

4. Discussion

3D bioprinting using microextrusion is the most common and affordable way to print living cells. Microextrusion is a method of direct cell writing that can 3D bioprint using different substances like hydrogels, cell spheroids, and biocompatible polymers facilitating the deposition of multiple cell types with high resolution to accurately fabricate complex structures, like an ear, using computer-aided design software [13]. Among the multitudes of hydrogels, alginate is popular because it is inexpensive and is crosslinked using calcium to give it a solid structural form which can later be reversed using citrate or EDTA. One major advantage to microextrusion printing is the ability to print cells at high densities that are close to physiological conditions, which is needed to construct tissue-engineered organs, maintaining high cell viability using high pressures, and small nozzle sizes are required for fast printing speeds with high resolution. Because microextrusion 3D bioprinting was reported to result in a significant decrease in cell viability yielding between only 40 and 80% live cells in alginate

solution [10, 11], we decided to try and improve the cell viability by coating the nozzles with silicone in order to prevent membrane damage from nozzle imperfections and high pressures, as the latter was the proposed reason for the decrease in viability [14]. However, we discovered that neither the high pressures nor the membrane damage caused by nozzle imperfections was the reasons for the low cell viability; we found the low cell viability was really caused by preparing the hydrogel (alginate) solution in H_2O, which is a hypotonic solution that causes cell lysis and bloating (swelling). Some studies that reported low cell viability using microextrusion and alginate solutions did not state what solvent the hydrogel was dissolved in, but the fact that low cell viability was observed after printing using increasing pressures suggests a hypotonic solution was the culprit. This appears to be a recurring problem in this field because numerous scientists cite these findings and reproduce them using the same procedure.

During our investigation, we used a variety of syringes (3, 5, 10, and 60 mL) and nozzles (blunt-end needles; 28G with ½″, 1″, and 2″ lengths, and 30G with ½ and 1″ lengths) with only PBS or complete culture media containing H9 cells, but there was no effect on cell viability using the highest force (20 lbs) and 3 mL syringe to yield the smallest area (0.09 inches^2) for the highest pressure of ~220 psi, which is over five times the pressure (~40 psi) that is usually used and is over twice the pressure (~100 psi) most printers can accurately print. Only with the addition of alginate in H_2O did we see an adverse effect on cell viability; there was no effect when the alginate was prepared in either PBS or complete culture media (**Figures 2** and **3**) or if the cells were placed in PBS or complete culture media without alginate (**Figure 4**). The water created a hypotonic solution because the counter cationic ion, alginate cannot enter the cell like chlorine ions (and sodium anions) can because it is too big (MW 216.12 g/mole), so about 25% of the cells initially lysed to create an isotonic solution and the rest of the cells survived but became "bloated" (swollen) during the process. However, our results suggest the bloating made the remaining cells more sensitive to mechanical pressure and caused the observed pressure-dependent decrease in cell viability. By preparing the alginate solutions in PBS or other isotonic solutions like complete culture media, we demonstrate significantly higher cell viability. We also put the cells in PBS and complete culture media without the potential protection of the alginate to see if they would be killed by the pressure or shear force alone, but we did not see any cell lysis or death using the same system (3 mL syringe, 20 lbs, 220 psi) and 28G nozzles of 1″ length (**Figure 4**). In addition, we did not see any significant decrease in cell viability with ½″ or 2″ long nozzles (results not shown). Furthermore, the 1″ long 30G nozzle also did not cause any appreciable decrease in cell viability (~7% decrease compared to 28G, **Figure 3**).

The results presented here show the low cell viability found during some mircoextrusion 3D bioprinting studies using alginate was due to placing the cells in a hypotonic solution causing cell lysis and bloating that makes the cells more sensitive to mechanical pressure during printing, which has been modeled [14]. Here we show this problem can be easily resolved by using isotonic solutions like PBS or complete culture media (0.9% saline is also suitable). Furthermore, the so-called recovery or increase in cell viability after 24 h [10] can be attributed to the division of the living cells rather than the recovery of membrane or other cellular damage, as noted by the increase in total cell number. The reason there is a decline in viability with increasing pressure or decreasing nozzle diameter can be attributed to the increasing shear forces causing

increasing amounts of cellular damage resulting in increasing cell death via apoptosis [11] (and our unpublished observations) but only when the cells are placed in hypotonic solutions.

5. Conclusions

Microextrusion is an excellent 3D bioprinting method that can yield high cell viabilities (≥85%) similar to Inkjet printing using 28G nozzles of either 1/2″ or 1″ lengths for pressures up to 220 psi as long as the hydrogels or solutions are isotonic. Good viability of over 75% can also be achieved using 30G nozzles of 1″ or shorter lengths using 220 psi. Thus, cells can survive with good viability (~85%) under considerable pressure for short periods of time during the microextrusion 3D bioprinting process if they are in isotonic hydrogels.

Acknowledgements

The author thanks Dr. Girish Kumar for help with flow cytometry, Randy Bidinger for making the apparatus that securely housed the pressure pump device, Dr. Stephen J. Merrill for help with statistical analysis, and Dr. Shelby Skoog for critically reviewing this manuscript.

Conflicts of interest

None.

Source of funding

Food and Drug Administration

Disclosure statement

No competing financial interests exist.

Disclaimer statement

The findings and conclusions in this paper have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination or policy. The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products by the Department of Health and Human Services.

Abbreviations

EDTA	ethylenediaminetetraacetic acid
PBS	phosphate buffered saline
PI	propidium iodide
psi	pounds per square inch
SD	standard deviation

Author details

Dianne Eyvonn Godar

Address all correspondence to: dianne.godar@fda.hhs.gov

US Food and Drug Administration, Center for Devices and Radiological Health, Silver Spring, MD, USA

References

[1] Murphy SV, Atala A. 3D bioprinting of tissues and organs. Nature Biotechnology. 2014;**32**:773-785

[2] Beere HM. "The stress of dying": The role of heat shock proteins in the regulation of apoptosis. Journal of Cell Science. 2004;**117**:2641-2651. Review

[3] Oehler R, Pusch E, Zellner M, Dungel P, Hergovics N, Homoncik M, Eliasen MM, Brabec M, Roth E. Cell type-specific variations in the induction of hsp70 in human leukocytes by feverlike whole body hyperthermia. Cell Stress & Chaperones. 2001;**6**:306-315

[4] Cui X, Boland T, D'Lima DD, Lotz MK. Thermal inkjet printing in tissue engineering and regenerative medicine. Recent Patents on Drug Delivery & Formulation. 2012;**6**:149-155. Review

[5] Cui X, Dean D, Ruggeri ZM, Boland T. Cell damage evaluation of thermal inkjet printed Chinese hamster ovary cells. Biotechnology and Bioengineering. 2010;**106**:963-969

[6] Hopp B, Smausz T, Kresz N, Barna N, Bor Z, Kolozsvári L, Chrisey DB, Szabó A, Nógrádi A. Survival and proliferative ability of various living cell types after laser-induced forward transfer. Tissue Engineering. 2005;**11**:1817-1823

[7] Guillotin B, Guillemot F. Cell patterning technologies for organotypic tissue fabrication. Trends in Biotechnology. 2011;**29**:183-190. Review

[8] Michael S, Sorg H, Peck CT, Koch L, Deiwick A, Chichkov B, Vogt PM, Reimers K. Tissue engineered skin substitutes created by laser-assisted bioprinting form skin-like structures

in the dorsal skin fold chamber in mice. PLoS One. 2013;**8**:e57741. DOI: 10.1371/journal.pone.0057741. Epub 2013 Mar 4

[9] Chang CC, Boland ED, Williams SK, Hoying JB. Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies. Journal of Biomedical Materials Research. Part B, Applied Biomaterials. 2011;**98**:160-170. Review

[10] Chang R, Nam J, Sun W. Effects of dispensing pressure and nozzle diameter on cell survival from solid freeform fabrication-based direct cell writing. Tissue Engineering. Part A. 2008;**14**:41-48

[11] Nair K, Gandhi M, Khalil S, Yan KC, Marcolongo M, Barbee K, Sun W. Characterization of cell viability during bioprinting processes. Biotechnology Journal. 2009;**4**:1168-1177

[12] https://www.rndsystems.com/resources/protocols/flow-cytometry-protocol-analysis-cell-viability-using-propidium-iodide [Accessed on 04/05/2016]

[13] Peltola SM, Melchels FP, Grijpma DW, Kellomäki M. A review of rapid prototyping techniques for tissue engineering purposes. Annals of Medicine. 2008;**40**:268-280. Review

[14] Yan KC, Nair K, Sun W. Three dimensional multi-scale modelling and analysis of cell damage in cell-encapsulated alginate constructs. Journal of Biomechanics. 2010;**43**:1031-1038