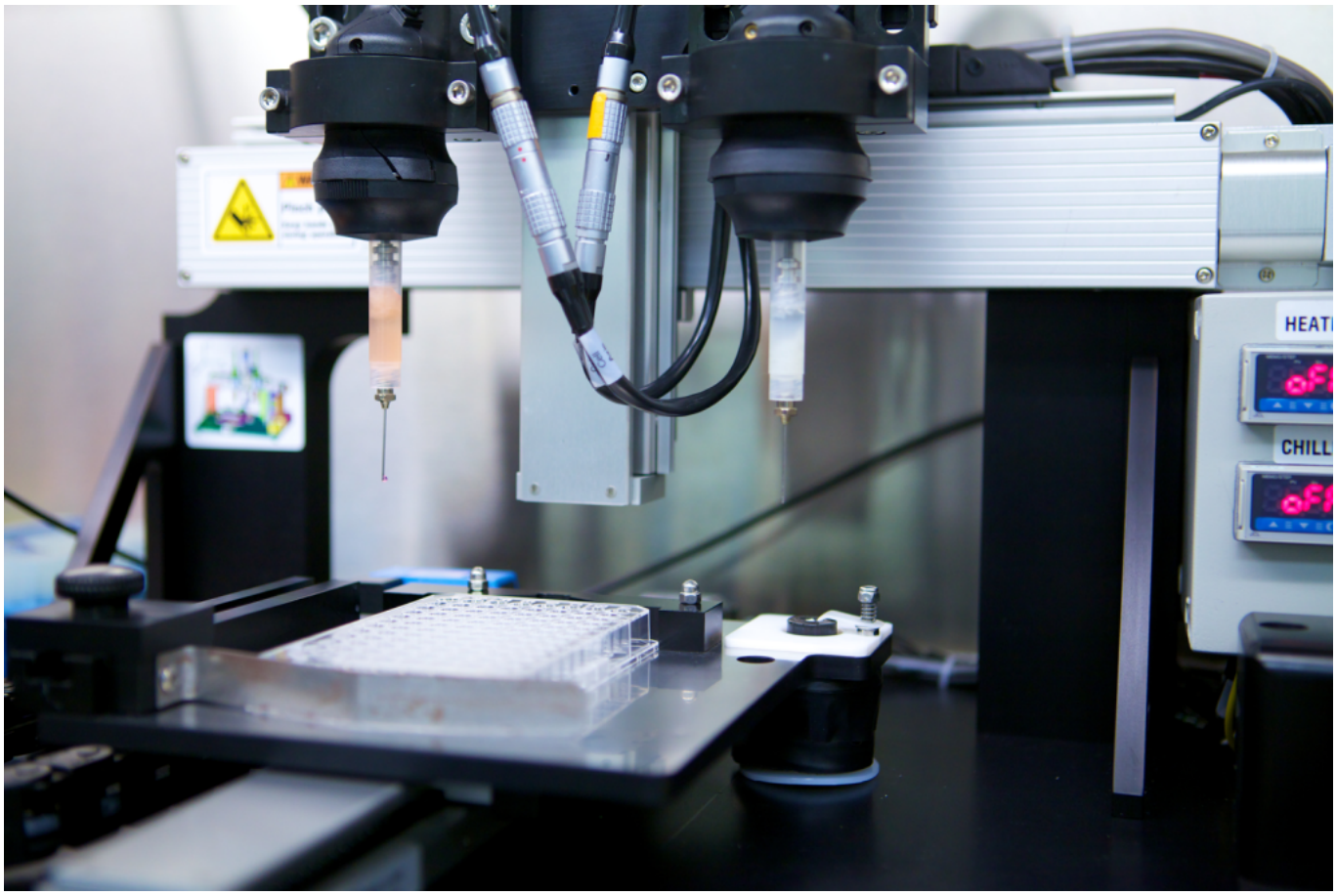
Minimizing Cell Death During the Extrusion Bioprinting of Gelatin-Alginate Bioinks



Organovo's NovoGen MMX syringe extrusion bioprinter

Source: 3D Printing Industry on Google Images

Abstract

This proposal seeks to minimize cell death while extrusion bioprinting with a gelatin-alginate bioink. Extrusion bioprinting was chosen over other types of bioprinting due to its accessibility and cost to researchers. Two different nozzles, cylindrical and conical, are examined to determine a mechanical aspect of extrusion bioprinting that can be modified to greatly minimize the cell death of bioprinted scaffolds. Gelatin-algiate bioinks can vary in concentration, and this concentration was also varied as a candidate solution to obtain the optimal concentration while maintaining a high cell survivability. The conical nozzle was chosen as the optimal printing nozzle with low shear stress, low cell damage, and highest cell viability. The 4% gelatin 5% alginate bioink was chosen as the optimal bioink concentration with optimal viscosity and high cell viability. Together, the use of this nozzle and this concentration bioink will greatly minimize the cell damage that occurs during extrusion bioprinting, boosting the quality of extrusion printing, and making it all-around more viable. Extrusion bioprinting, due to its improved cell death percentage, will be utilized more often by researchers – this will potentially accelerate the innovation of bioprinting as an overall technology towards the final goal of bioprinting a fully functioning organ.

Keywords: bioprint, bioprinting, bioink, bioprinted scaffold, cell death, cell death bioprinting, extrusion bioprinting, gelatin-alginate bioink, hydrogel bioprinting

Document Scenario: This engineering proposal examines extrusion bioprinting and how to optimize the mechanical aspect of the nozzle and the concentration of gelatin-alginate bioinks to minimize cell death in the printing of biomaterial scaffolds. This proposal would be written to a biotech company's executives that are looking to make advances in bioprinting technologies. Therefore, it would encourage researchers to utilize this technology as a step towards the penultimate goal of bioprinting: the development of fully functioning 3D printed organs. This model of optimization with gelatin-alginate based bioinks can be reused using various other bioinks. Thus, optimizing extrusion bioprinting is a crucial step to provide high quality bioprinting technologies that are widely accessible and as convenient as possible for researchers to use.

Executive Summary

Bioprinting is a new and innovative method of tissue engineering that seeks to create various functioning human biomaterials from bioinks - cells suspended in a malleable and nutrient rich liquid. Bioinks are extruded from a 3D printer into a scaffold, from where the cells chosen for the print grow into the desired biomaterial. There is a large discrepancy with the number of patients who need organ replacements versus those who can donate, as there are long waiting lists for certain organs. Ultimately, the goal of bioprinting is to eliminate this large desire for organs by printing fully functioning organs, and therefore save many lives. Bioprinting must still be innovated and improved upon to reach this goal of creating fully functioning organs, and therefore minimizing cell death is a crucial aspect of bioprinting.

Due to its accessibility and overall printing advantages over other types of bioprinting, extrusion bioprinting was focused on within this proposal - improving the printing style which is the easiest for researchers to implement in order to foster general innovation of the bioprinting field. Gelatin-alginate bioink was chosen to be examined in this proposal due to its common use in previous extrusion bioprinting research. Candidate solutions considered for the nozzle types in this proposal will be judged off design, shear stress, cell damage, cell viability, and the accessibility and cost of the nozzle type. For the comparison of different gelatin-alginate bioink concentrations, the different concentrations will be judged on solvent type, viscosity increase, degradation rate, and cell viability. The cost and accessibility comparison will examine the average cost and accessibility of the nozzles for use, which will then be considered with the lower cell death, which is a result of the minimization of shear stress, cell damage, and cell viability of the nozzle

The conical nozzle is the suggested nozzle type that will minimize cell death the during extrusion bioprinting because of its shape and low shear time. A 4% gelatin and 5% alginate bioink in a culture-medium solvent is the suggested concentration of gelatin-alginate bioink that will minimize cell death the most during extrusion bioprinting because of its high cell viability after 48 hours. Together, using a conical nozzle and placing the desired cell type within a 4% gelatin 5% alginate bioink will provide the researcher with a low cell-death percentage that will improve the quality and survivability of bioprinted scaffolds. Based on the data analyzed within this proposal, this would significantly improve the cell death percentage of extrusion bioprinting.

If successfully implemented, this proposal will improve the current existing extrusion bioprinting technologies and offer a formula to researchers wishing to improve their cell death percentage and create higher quality scaffolds. A broader impact of this proposal, if it is successfully implemented, is that this proposal will foster the accelerated innovation of the bioprinting field. The only major disadvantage of extrusion bioprinting is its tendency to kill more cells post-print in comparison to laser-assisted bioprinting, while also being the cheapest and easiest form of bioprinting to implement by researchers. On top of the extrusion style being the most accessible to researchers, this will give extrusion the best of both worlds and accelerate the innovation of bioprinting as an entire field by giving researchers cheap and high-quality equipment to try to attain a fully functioning organ.

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Problem Analysis

Organ donations are a common medical procedure which requires a donor and a recipient. There is a large imbalance of patients who require an organ transplant in contrast to the list of donors. In the United States, as of June 2017, there were only 5, 200 organ donors available to 120,000 patients who were on a waiting list for an organ. The bioprinting of biomaterials is a new and promising field that focuses on lab-assisted cell growth to create human tissue that has the potential to improve this donor-patient imbalance. Bioprinting (also 3D bioprinting) utilizes different types of cells and other biomaterials and is an effective and malleable method to create scaffolds. These scaffolds are where cell interaction and growth occur that are the building blocks for these engineered tissues (Derakhshanfar et al., 2018). An effective scaffold is one in which cells can adhere to and grow on to eventually form the desired organ or tissue.

Overview of problem and its significance

Bioprinting most commonly features a three-axis platform that moves according to a pre-determined shape and is the platform on which bioinks extrude onto. This mechanical platform is controlled by a co-ordinate plane and designed in a computer program. Bioprinting provides a great amount of control and efficiency to the creation of these scaffolds, which can ease some of the complexity that scaffolds feature. This makes it a more viable method than older methods which are less precise (Derakhshanfar et al., 2018).

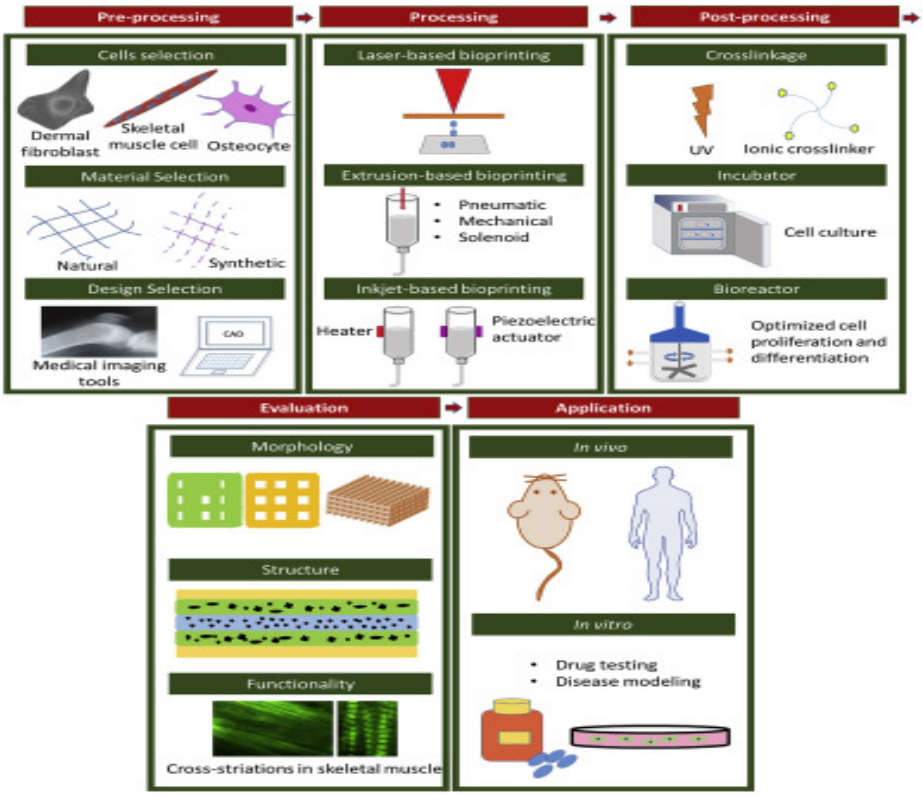

Figure 1. The main stages of bioprinting

Source: Boularaoui et al., 2020

Bioink

A 'bioink' is the name of biomaterial that is used in bioprinting. Different types of cells can be put into different bioinks – bioinks are the medium that carries various cell types while printing the scaffolds (Bociaga et al., 2019). The cells that are introduced to different bioinks varies based on all the wide applications of bioprinting, and thus the desired outcome of the print. As a result, the specific cell-type that is introduced to the bioink will be neglected in this proposal, but the properties of bioinks themselves will be analyzed.

Gelatin-alginate bioink

This proposal focuses on gelatin-alginate based bioinks. Gelatin-based bioinks are considered a type of hydrogel – made from three-dimensional polymers that have high water content and are therefore adept at containing biological material and being flexible. Hydrogels are a commonly used type of bioink due to their durability, and their strong simulation of natural tissues (much more than other synthetic biomaterials). To improve the structure durability of gelatin hydrogels, they are commonly combined with different hydrogels, especially alginate. This polysaccharide is a popular choice for this combination with gelatin and is being used in this proposal due to the wide use of this compound in previous extrusion bioprinting research. The concentration of this gelatin-alginate combination has been varied in previous research of bioprinting and has shown to affect the shear stress and cell survival. Therefore, varying the concentration of gelatin-alginate bioinks offers an inexpensive and convenient method to possibly maximize cell viability while extrusion printing with hydrogel bioinks (Bociaga et al., 2019).

Major bioprinting methods

There are four commonly utilized bioprinting techniques; inkjet bioprinting, stereolithography, laser-assisted bioprinting, and extrusion bioprinting. It is crucial to understand the advantages and disadvantages of each printing method to understand why extrusion printing is being focused on in this proposal.

Inkjet

Inkjet bioprinting is a method which utilizes droplets of bioink and that are dispensed using piezoelectric activators, thermoelectric bubbles, or pressure pulses. Inkjet printing consists of a heating element next to the chamber that dispenses the ink that causes the bioink to bubble and then expand and push out of the nozzle (Biazar et al., 2018). This method of printing is made possible through high temperatures in short periods of time and can print much more viscous fluids than extrusion-based printing. However, these high temperatures lead to an activity loss in the printed cells. As a result, inkjet's cell viability rate is 80-95 %, which is the highest average viability rate out of all the printing methods. There are three major different types of inkjet-based printing: thermal, piezoelectric, and pressure pulse printing. Because the nozzle cannot provide a continuous flow of bio ink, inkjet printing is not as accessible and has not been researched as much as extrusions-based printing (Derakhshanfar et al., 2018).

Stereolithography

Stereolithography is done utilizing polymers that are controlled by micromirrors which shape the polymers based on different rays of light. The ability of this printing method to be precisely controlled by light has large advantages of high printing quality, cell viability, and speed. The use of UV light is not as common as extrusion or inkjet-based printing because UV light is harmful for the DNA of human cells - UV light has been researched to cause skin cancer. As a result, there are some stereolithography printers that use visible light, however it is not a wide-reaching technology and is very much novel. Some advantages of stereolithography include its ability to print bioinks of any viscosity, its high accuracy, and its cell viability rate – greater than 90% (Derakhshanfar et al., 2018).

Laser-assisted

Laser-assisted bioprinting utilizes a pulsed-laser which evaporates the material and is therefore dispensed. Due to the use of the laser, and thermal damage on the cells, the cell survival rate is low (under 85%). However, research has been done on the best combination of bioink viscosity and laser energy which can improve this survival rate. Some of laser-assisted bioprinting's drawbacks include a high cost, and slower speed of printing as compared to stereolithography and inkjet printing (Derakhshanfar et al., 2018).

Extrusion

Extrusion bioprinting dispenses continuous beads of material in the x-y plane, which is then built up in the z direction, like the process of a regular 3D printer. Due to this extrusion process, this form of bioprinting is reliable and can be performed with relative ease to the other methods (Derakhshanfar et al., 2018). Furthermore, the ability to use a wide variety of materials, and the general affordability of extrusion printers, make it the most common form of bioprinting (Biazar et al., 2018).

Three common extrusion methods include pneumatic dispensing, piston-driven dispensing, and screw-driven dispensing. Pneumatic dispensing uses air pressure to force the bioink out of the nozzle, while piston-driven uses a vertical force to dispense the bioink, and screw-driven dispensing uses rotational force to dispense the ink. For extrusion bioprinting to be successful at printing, it must factor in: the modification of the viscosity, the bioink phase prior to extrusion, and the biofabriaction window that is specific to the bioink being used (Derakhshanfar et al., 2018). Due to the mechanical stresses that is placed upon cells while printing, extrusion printing has a cell survival rate of 40-86%, which is a major disadvantage, because it is smaller than that of inkjet printers (Biazar et al., 2018).

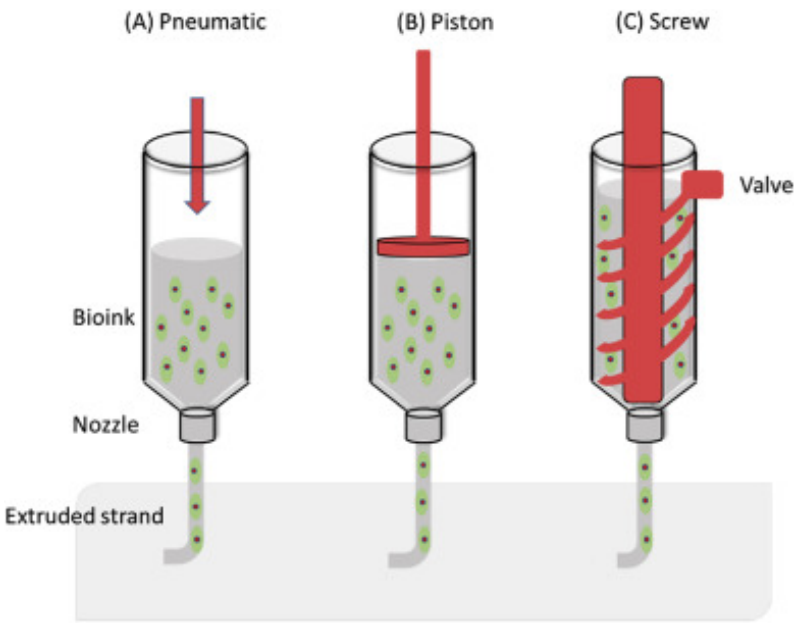

Figure 2. Types of extrusion bioprinting

Source: Boularaoui et al., 2020

Shear stress

Cell survivability and viability during the extrusion printing process is heavily dependent on shear stress, which is induced on the bioink during printing. Shear stress occurs when the bioink is released through a nozzle at a certain pressure and is a result of the bioink being pushed through the nozzle. A high shear stress is one of the most damaging factors that lowers cell viability and survivability. Cell damage is dependent on the magnitudes of shear stress and shear time, the amount of time that cells are exposed to the stress (Ning et al., 2018). Factors that affect cell survival and viability in extrusion printing include: the material concentration, the material flow rate, the pressure that is used to dispense the bioink, the nozzle size, and the nozzle moving speed (Derakhshanfar et al., 2018).

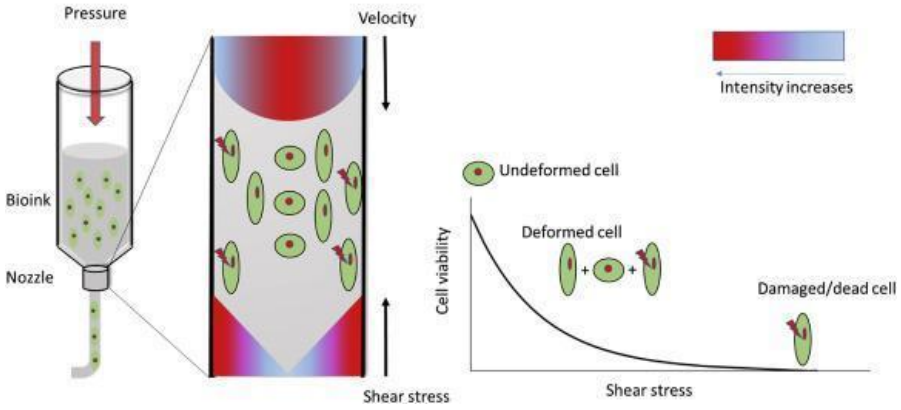

Figure 3. Shear stress

Source: Boularaoui et al., 2020

Extrusion nozzles

Extrusion printing utilizes the extrusion of bioinks through a specific nozzle. The nozzle of an extrusion printer is a crucial location where shear stress is placed on cells. Nozzles are one of the most important mechanical variables that can directly impact cell survivability (Li et al., 2011). Improving the nozzle, and the forces that it places on a bioink, in combination with the bioink concentration, could greatly minimize shear stress and cell death.

Why extrusion?

Extrusion printing, because of its convenience and affordability, could be the key to the acceleration of bioprinting technologies. This proposal will address the mechanical disadvantages of extrusion printing, and how to maximize the cell survival rate by manipulating both the nozzle used for printing and the concentration of the gelatin-alginate bioink. If extrusion printing was to be as reliable in cell survivability as inkjet printing, then it would be more favored by researchers. It could possibly accelerate the growth of the bioprinting field exponentially towards the point of creating fully functioning organs.

One of the largest obstacles hindering the advancement of extrusion bioprinting is cell survivability. This is a fundamental aspect of bioprinting, as dead and decaying cells are not functional and therefore minimizing the number of cells that die could greatly advance the technology.

As noted, minimizing shear stress limits cell damage during extrusion printing, leading to higher cell survivability and viability. The major variable in which shear stress can vary within is the extrusion nozzle. It is crucial to use the optimal nozzle type to minimize cell death and maximize cell viability while printing with gelatin-alginate bioinks. Secondly, extrusion printing places additional unnecessary shear stress on very viscous bioinks, due to the large amount of pressure required to extrude bioinks with a large viscosity (Derakhshanfar et al., 2018). As the dispensing pressure is increased, a greater shear stress is induced on the cells, which leads to a lower cell survivability (Biazar et al., 2018). The optimal concentration of gelatin-alginate in this hydrogel would prevent this bioink from becoming too viscous and minimize the cell death during a print.

The model of this proposal could also be applied to other types of bioinks in a similar analysis to determine the best nozzle and concentration of bioink to utilize while extrusion bioprinting.

STEM fundamentals of problem

It must be noted that the mathematical models explained below are theoretical. For this proposal, this model was chosen as a comprehensive model showing the importance of mechanical factors of the extrusion nozzle on shear stress. There are many theoretical models that have been proposed for the calculation of shear stress in extrusion bioprinting.

Pressure on the bioink is at its peak as it is extruding through the bioprinter nozzle. It is known that pressure is a value given from a certain force being placed over a certain area. Shear

stress can be characterized into a mathematical relationship that considers the shear stress and exposure time during bioprinting and return the percent of cell damage.

The general equation for cell damage due to shear stress is expressed in Equation 1, and the variables that are used to create Equation 1 will be explained following the parabolic model. In Equation 1, Esmaeil Biazar et al. described the equation for shear stress as "… a parabolic trend similar to that expressed by the exponential equation" (Ning et al., 2018).

$$D_s(\tau_s, t_s) = \left(1 - e^{-a_1 \tau_s^{b1} t_s^{c1}}\right) 100\%$$

Equation 1. Parabolic trend of cell damage due to shear stress and time

Source: Ning et al., 2018

A1, b1, and c1, are constants that can be experimentally defined. Here, the authors describe an equation for the percent of cell damage that "…increases from zero (no shear stress) to 100% along with increased stress and exposure time" (Ning et al., 2018).

Breaking down the components of this equation: the distribution of pressure in the nozzle tip of a bioprinter can be represented by:

$$\Delta P = P - P_{en}$$

Equation 2. Change of pressure in bioprinter nozzle

Source: Ning et al., 2018

Where the change in pressure, P, is defined by P and P_{en}. P being the air pressure applied in the nozzle during bioprinting and P_{en} being the "…pressure drop in the contractive region" (Ning et al., 2018).

In Equation 1, τ_s is the magnitude of shear stress, which can be given by:

$$\tau_s(r) = \frac{\Delta P r}{2L}$$

Equation 3. Shear stress distributed across a bioprinter nozzle

Source: Ning et al., 2018

Where L is the length of the nozzle tip of the bioprinter.

Therefore, the length, L, of the nozzle, and the velocity of the flow of cells, V(r), can both be utilized to determine the time it takes cells to pass through the nozzle, t_s:

$$t_s = \frac{L}{V(r)}$$

Equation 4. Flow time of cells across a bioprinter nozzle

Source: Ning et al., 2018

The number of damaged cells that are exiting at the end of the nozzle, DCN, can be obtained. If the cell density is known, the volume of a cylinder is due to the change in the length of the nozzle Δl, multiplied by the cross-sectional area of the cylinder πR^2, multiplied by the cell density den.

$$DCN = den\Delta l \int_0^R 2\pi r D_s\left(\tau_s, t_s\right) dr$$

Equation 5. Exponential shear stress-induced cell damage law

Source: Ning et al., 2018

Lastly, using the relationship that "…the total cell number per unit volume can be expressed as $den\Delta l\pi R^2$, the total percent cell damage D_{st} can be calculated…"(Ning et al., 2018).

$$D_{st} = \frac{DCN}{den\Delta l\pi R^2} 100\%$$

Equation 6. Total percent cell damage due to shear stress

Source: Ning et al., 2018

The percentage of the cell damage that occurs from the bioprinting nozzle can be extrapolated from this equation, and thus it is important to be noted moving forward in the goal of minimization of shear stress. The proportional relationship between pressure in the bioprinting nozzle and shear stress should be kept in mind for the rest of this proposal.

Lessons from prior responses to the problem

Cell viability is a common topic that is researched in the field of bioprinting due to its utmost importance in the success of the printed scaffold. In this proposal, only the mechanical factors that are involved in the bioprinting process will be examined to maximize cell survival. A study examining cell damage before and after bioprinting by Liqun Ning et al. compared a developmental model of cell damage with experimental results. In the experiment, the model predicted the extent of the cell damage well. However, there was some variability in experimental results that suggested that "…interaction among cells (e.g., impact, abrasion, and friction)" also plays a role in cell damage, especially "as the cell density continuously increases"(Ning et al., 2018). Though it is important to remember that mechanical aspects of bioprinting are the major factor for cell death, minimizing just these factors will never result in a 100% cell survival rate.

In the same study, it was found that there was a higher percentage of dead cells than damaged cells because of shear stress. Instead of being in a damaged phase, cells are more likely

to immediately switch from normal to dead from the stresses of printing (Ning et al., 2018). For the focus of cell viability, there is a lesser number of damaged cells than already-dead cells.

The flow that comes from an extrusion printer is completely laminar. It maintains a constant pressure and flow rate that does not vary while the bioprinter is extruding bioink under the constant conditions. As a result, the shear stress will be constant under certain conditions and other factors, including the variation of flow, are not made more complex. For example, the flow of Drop-on-Demand printing varies with the pulse of the voltage (Shi et al., 2018).

Lastly, in a study conducted by Andreas Blaeser et al., it was discovered that "…printing pressure, hydrogel viscosity, and nozzle size" were three major factors that affected the extrusion of bioink (Blaeser et al., 2016). Also, it was discovered that "…high levels of shear stress affects cells immediately and furthermore can induce long-term alterations in the proliferation potential of the cell that have survived the dispensing process"(Blaeser et al., 2016). Minimizing shear stress is not only important for the immediate cell survival rate but should also be lowered to prevent long term cell replication issues.

Project objectives and constraints

The objective of this proposal is to determine the best way to minimize cell death and raise the cell survival rate during the bioprinting of gelatin-alginate bioinks. Extrusion printing is the most popular, accessible, and affordable form of bioprinting. Therefore, this proposal will focus on improving extrusion printing. The reason that this proposal has focused on gelatin-alginate bioinks is because of the wide use and accessibility of gelatin-alginate bioinks. Also, this bioink was chosen because of the amount of previous research available using gelatin-alginate bioinks over others.

Specifically, the goal is to raise the percent of cell survivability to be competitive with the range of inkjet printing (above 90% cell viability) while maintaining the other advantages of extrusion printers.

It will also be assumed that shear stress is the direct cause of cell death in the bioprinting process and that other lesser factors of cell death, including cell to cell interaction, are negligible.

This proposal will focus on maximizing the cell survival rate by minimizing the shear stress with different extrusion nozzle options, and varying concentrations of gelatin-alginate bioinks. It will also consider the accessibility and cost of using each proposed solution to researchers. That way, it is possible to maximize the number of researchers who could potentially utilize extrusion printing and the recommendations made in this proposal. The following factors are the biggest considerations that will be discussed with each proposed solution in this proposal:

-Shear stress (Pa)

-Cell damage percent

-Cell viability

-Accessibility and cost to researchers

Candidate Solutions

Scope of solutions considered

Though bioprinting is a novel method of creating biomaterial scaffolds, there are some solutions to minimizing cell death that have been studied that will not be covered in this proposal's solution section.

The first of these methods is bioink temperature variation of during bioprinting. In a study by J. Carlos Gómez-Blanco et al., a hydrogel bioink was printed at different extrusion temperatures. It was found that, when the gauge of the conical nozzle stayed constant, the shear stress lowered as the temperature increased. The comparison of different-sized nozzles was also included in this same study, and it was found that the influence of temperature on the shear stress of the bioink was reduced as the gauge of the nozzle increased. These findings suggested that the mechanical aspect of nozzle size had a much larger effect on the shear stress of the bioink compared to the variation of bioink temperature (Gómez-Blanco et al., 2020).

In a second study, the dispensing pressure was varied for multiple samples of bioink while trying to see its effect on the cell viability of the bioink. There was a clear increase in the shear stress and increase in flow rate as the pressure was increased. This trend can clearly be deduced from Figure 4, below. Therefore, a solution for minimizing cell death was suggested as to use the lowest dispensing pressure possible for the viscosity of the liquid to retain the highest cell viability (Paxton et al., 2017).

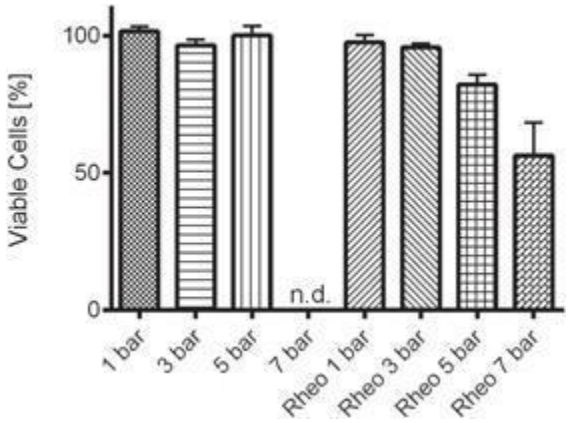

Figure 4. Cell viability percentage and dispensing pressure

Source: (Paxton et al., 2017).

In this same study, the relationship between shear stress and the residence time within the nozzle was studied for an 8% gelatin 1% alginate bioink. The shear stress rose as the time in the nozzle rose. Simultaneously, the bioink moved farther towards the tip from the center of the nozzle. Thus, this study also suggested that nozzle length was an important factor to minimize the bioink time in the nozzle – an important factor to minimize shear stress (Paxton et al., 2017).

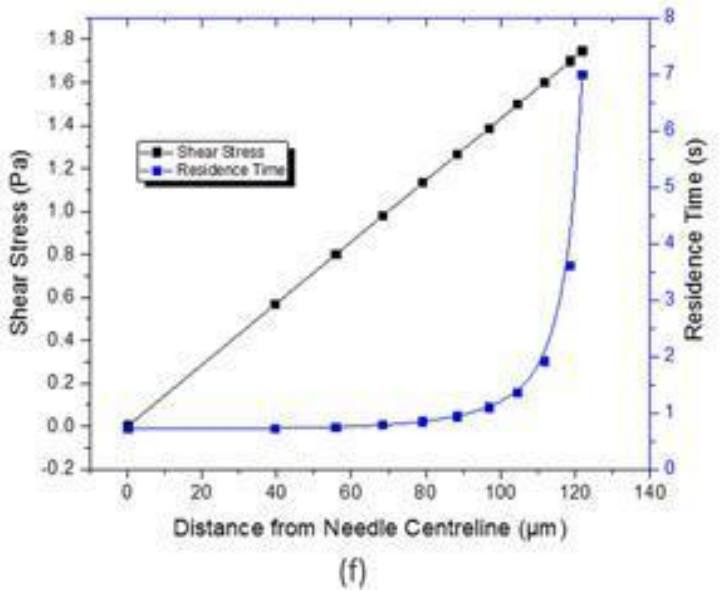

Figure 5. Shear stress and residence time

Source: (Paxton et al., 2017).

The solutions considered for this proposal are strictly addressing the mechanical aspects of the bioprinting nozzle and the concentration of the ink being used. Only three solutions could be chosen for the sake of this proposal, and all three solutions are within extrusion printing only. For the goal of improving existing extrusion printers without overly complicated and expensive additions to the bioprinters, this proposal does not address combining extrusion printing with other types of bioprinting. Also, this proposal is only addressing improving the cell death percentage of prints using gelatin-alginate bioinks to have a valid comparison of each solution.

Explanation of candidate solutions

The following explanations of the candidate solutions includes general description about the solution and its design, the shear stress, the cell damage, and the accessibility and cost to researchers of each possible solution: conical nozzle, cylindrical nozzle, and gelatin-alginate bioink concentration variation.

Cylindrical

The cylindrical nozzle is also called a straight nozzle.

Design

The cylindrical nozzle features a cylinder-shaped tip that is straight and does not vary in angle. Typically, as can be seen in Figure 6, the nozzle can typically be from metal, like a nozzle. Cylindrical nozzles are prone to become more clogged compared to conical nozzles (*Guide to Picking Your Bioprinting Needle: Support*). The cylindrical nozzle has completely uniform nozzle radius, which means it requires a constant amount of pressure throughout the entire

nozzle for a bioink to be extruded. This differs from conical nozzles, where there is a high pressure required to extrude the bioink, but only for a small portion of nozzle at the tip. This contributes to the shear stress distribution portrayed in Figure 8 (Li et al., 2011).

Figure 6. Cylindrical nozzle

Source: (Guide to Picking Your Bioprinting Needle: Support**).**

Mathematical model of cylindrical nozzle cell damage

Cell damage is represented by I_c and follows a similar model to that which is presented in Equation 6. The shear stress effect is expressed at the end of the nozzle, thus the total cell count for the length of the nozzle is $d \int_0^{D_{c/2}} 2\pi r I_s\left(\tau_c, t_c\right) dr$. The shear force is τ_c and the shear time is t_c (Li et al., 2011). The radial direction is *r*.

$$I_c = \frac{8 \int_0^{D_{c/2}} r I_s\left(\tau_c, t_c\right) dr}{D_c^{\,2}}$$

Equation 7. Cell damage in cylindrical nozzle

Source: (Li et al., 2011).

Shear stress

The shear stress in a cylindrical nozzle is generally positioned around the entire nozzle from the entrance to the exit. Higher shear stress occurs closer to the straight edges from the nozzle, moving from the center of the nozzle to the outer radius. The shear stress is constant throughout the entire nozzle. The closer cells are to the edge of the nozzle, the more cell damage will occur. Also, the time that cells are subjected to shear stress is longer than the more instant concentration of shear stress that occurs within a conical nozzle. Conical nozzles contain shear

stress forces that are higher for an instantaneous time – but only much closer to the tip of the nozzle. The stress in a cylindrical nozzle is lower than that of a conical nozzle, but the cells are exposed to this shear stress throughout the entire length of the nozzle (Billiet et al., 2014).

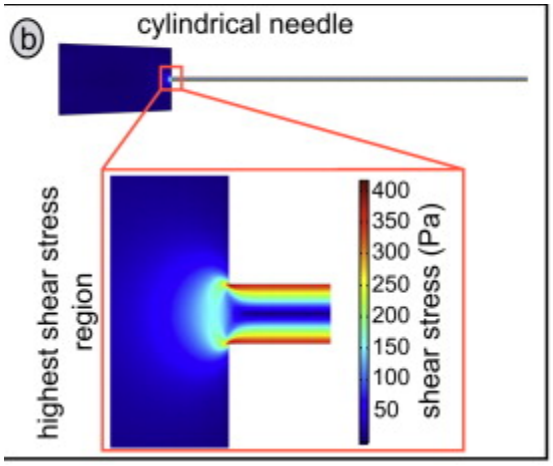

Figure 7. Cylindrical nozzle shear stress distribution

Source: (Billiet et al., 2014**).**

Cell damage

A study by Minggan Li et al. did a comparison of the cell damage that takes place in a cylindrical nozzle as the diameter is made larger and the flow rate is kept constant (for two different cell types in a gelatin-alginate bioink). This study showed that a cylindrical nozzle's cell damage decreases at a much larger rate than a conical nozzle's as the diameter was increased. A second comparison done in the same study kept the diameter at a constant 25 mm and varied the flow rate. The results of this comparison revealed the rate of the cell damage rose very quickly in comparison to a conical nozzle when the flow rate was increased for both cell types. This research suggested that there is more variability in cell damage when varying different aspects of a cylindrical nozzle (Li et al., 2011).

Accessibility and cost to researchers

Cylindrical nozzles are sold with a metal nozzle and a plastic body, with different gauge options for the nozzle. These nozzles are made with a stainless-steel nozzle sticking out of a polypropylene plastic body on the nozzle. The length of these nozzles also can vary depending on the interests of the purchaser. (*Guide to Picking Your Bioprinting Needle: Support*). These nozzles range from $40-$50 (depending on the gauge of nozzle) for a package of 50 nozzles. This nozzle is available online to be ordered from multiple websites. They attach to the extrusion mechanism of a bioprinter by a researcher using a female luer connection, which makes them easily accessible and usable by researchers (*Sterile high-precision blunt needles 27G, 50 pieces*). Existing extrusion printers can easily be adapted to use a cylindrical nozzle. Modifying an extrusion printer is much easier than modifying a more complicated mechanism such as an inkjet bioprinter, a laser-assisted bioprinter, or a combination of printing types - which would take many more resources to accomplish.

Table 1. Comparison of cylindrical nozzles

Cylindrical Nozzle Material	Number of Nozzles	Durability	Cost
Stainless steel nozzle, plastic body	50	Sterile & Disposable	$40.00-$50.00

Source: (Guide to Picking Your Bioprinting Needle: Support**)**

Conical

The conical nozzle is also called a tapered nozzle.

Design

The conical nozzle features a cone-shaped tip that can vary in angle. Typically, as can be seen in Figure 8, the conical nozzle is typically made of plastic or stainless steel. Plastic conical nozzles are used for bioprinting of hydrogel bioinks, while stainless steel tips are primarily used for high-temperature prints, such as those involving thermoplastics (*Guide to Picking Your Bioprinting Needle: Support*). The conical nozzle has a larger radius at the beginning, while it angles into a much smaller radius at the end. This design allows for a small amount of pressure (in comparison to a straight nozzle) being needed to dispense a bioink at the same flow rate (Li et al., 2011).

Figure 8. Conical nozzle

Source: (Guide to Picking Your Bioprinting Needle: Support**).**

Mathematical model of cell damage

The model for cell damage for a tapered nozzle must consider the conical nature of the nozzle but is otherwise like the model expressed for the cylindrical nozzle in Equation 7. The

cell damage is I_t and θ is the angle of the nozzle. In this model, τ_c and t_c are functions of *r*, and $I_t(\theta)$ is the double integral $I_t(\theta) = \int_R^{R-R_1} \int_R^{R-R_1} f(\tau_t, t_t)\tau'_t t'_t dr dr$. The shear stress effect is expressed at the end of the nozzle, thus the total dead cell count for the length of the nozzle is $\int_0^{\theta_0} 2\pi\theta R_1^{\,2} dI_t(\theta) d\theta$ (Li et al., 2011). The total cell damage percent for a tapered nozzle is:

$$I_t = \frac{2\int_0^{\theta_0} I_t(\theta) d\theta}{\theta_0^{\,2}}$$

Equation 8. Cell damage in tapered nozzle

Source: (Li et al., 2011).

Shear stress

The shear stress in a conical nozzle is generally centered around the conical portion of the nozzle towards the exit of the nozzle. As can be seen in Figure 9, high shear stress occurs closer and closer towards the tip. The shear stress is negligible at the entrance to the nozzle. The shear stress that conical nozzles place on an extruding bioink is high, but only for a short period of time (Billiet et al., 2014).

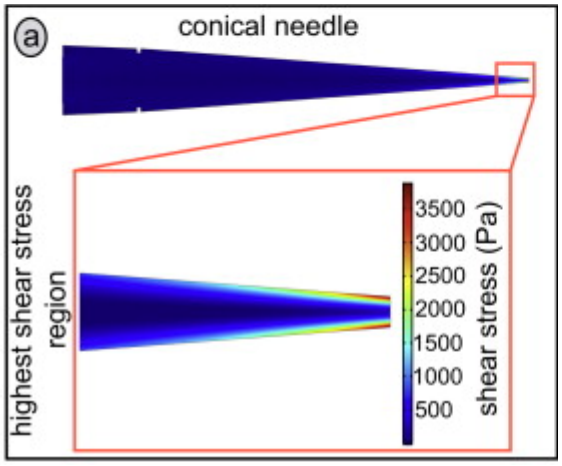

Figure 9. Conical nozzle shear stress distribution

Source: (Billiet et al., 2014**).**

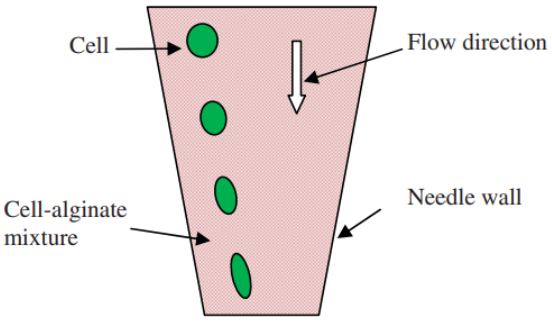

Figure 10. Cell damage in conical nozzle

Source: (Li et al., 2015**).**

Cell damage

A study showed that the cell damage in a conical nozzle decreases as its diameter gets larger and the flow rate is held constant. In this same study, when the nozzle diameter is held constant at 25 mm and the flow rate is varied, the cell damage rises as the flow rate increases for conical nozzles (Li et al., 2011).

Accessibility and cost to researchers

Conical nozzles are available in different gauges in both a plastic and metal version. The length of conical nozzles also varies and can be purchased based on the needs of the researcher. Full plastic conical nozzles range in gauge and length, and cost $30.00 - $40.00 for 30 plastic tips. Full plastic tips are the least durable form of tip, and if working with biomaterials, are most likely a one-time use. Metal conical nozzles with a metal tip but a plastic body range from $30.00 to $40.00 for one tip depending on whether the researcher would like the tip to be sterile or not. These have intermediate durability. For a full metal tip, which is the most durable and reusable (sterilizable) conical nozzle, it is $200.00 per nozzle (*Guide to Picking Your Bioprinting Needle: Support*). A full comparison of these conical nozzles can be seen in Table 2, below. All these conical nozzles are available online to be ordered from multiple websites. They are attached to the extrusion mechanism of a bioprinter by a researcher, which makes them easily accessible with existing extrusion printers. When compared to replacing an entire printer, or modifying a more complicated mechanism, changing the tip on an extrusion printer is very cheap and time efficient.

Table 2. Comparison of conical nozzles

Conical Nozzle Material	**Number of Nozzles**	**Durability**	**Cost**
Full Plastic	30	Lowest, disposable	$30-$40
Plastic Body & Metal Tip	1	Intermediate, reusable	$30-$40
Full Stainless Steel	1	Highest, reusable	$200

Source: (Guide to Picking Your Bioprinting Needle: Support)

Gelatin-alginate bioink solvent concentration variation

Material information

Gelatin is a protein that has a triple helix structure and has a large compressibility without damaging the material. Alginate is made from brown algae and bacteria. It is a natural copolymer that has a high extrusion capacity, and thus is particularly good for extrusion bioprinting. These two materials are combined to create the gelatin-alginate bioink, and these materials crosslink to create strong scaffolds. A combination of this crosslinking is depicted below for an example of a gelatin-alginate bioink in Figure 11. In previous studies this combination has led to a very high survival rate of cells suspended in this bioink during bioprinting (Bociaga et al., 2019).

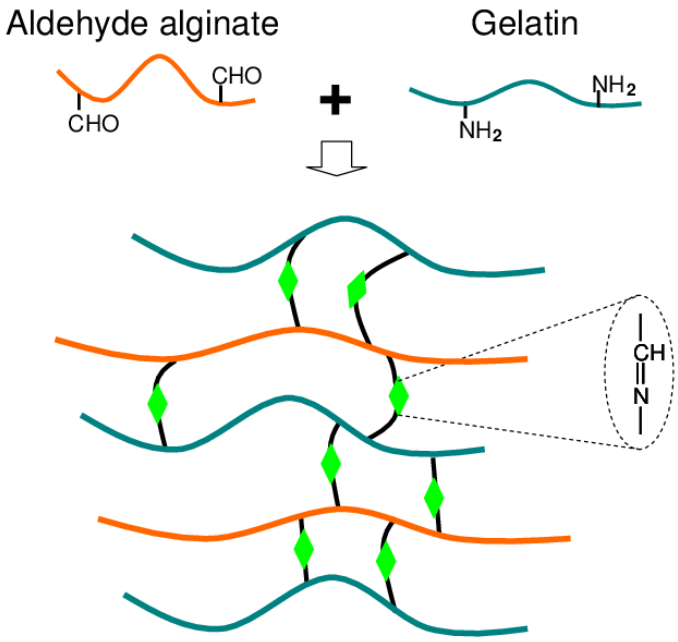

Figure 11. Gelatin-alginate crosslinking

Source: (Sun et al., 2013**).**

Concentration differences

In the creation of the gelatin-alginate bioink, the concentration of the bioink can be varied. It has been experimentally determined that this concentration plays a role on the cell viability and shear stress that is placed on the cells within the bioink. As the gelatin concentration is raised, the cell viability also is increased (Billiet et al., 2014). However, raising the concentration of the bioink also increases the viscosity of the bioink. As the viscosity is increased, so is the amount of pressure that is required to extrude the bioink - so the shear stress is also increased (Bociaga et al., 2019).

It was also determined that the solvent that is used with the gelatin-alginate solute influences cell viability. Though some would assume that water would be the most viable solvent, the culture medium solvent induces the highest cell viability (Billiet et al., 2014). In

Figure 12, it is demonstrated that the solvent will also affect the viscosity – (a) is a water solvent, and (b) is a culture medium solvent (Bociaga et al., 2019).

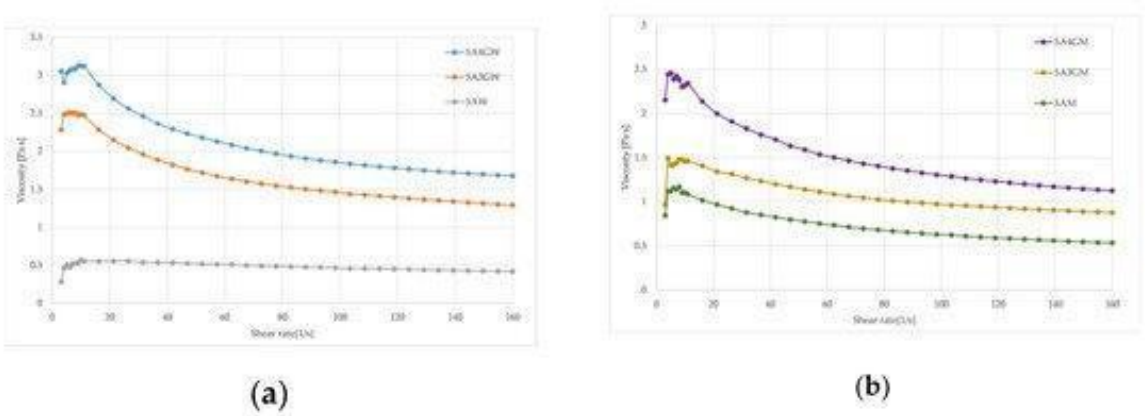

Figure 12. Effect of gelatin-alginate concentration on viscosity of bioink

Source: (Bociaga et al., 2019).

The degradation rate of the structure that is created from the gelatin-alginate bioink is impacted by the concentration of gelatin, alginate, and the solvent that the bioink is mixed with. The lower the degradation rate, the longer lasting a printed scaffold will be. As the concentration increases, the weight lost over time also increases, leading to a higher degradation rate. This is characterized below in Figure 13 (Bociaga et al., 2019).

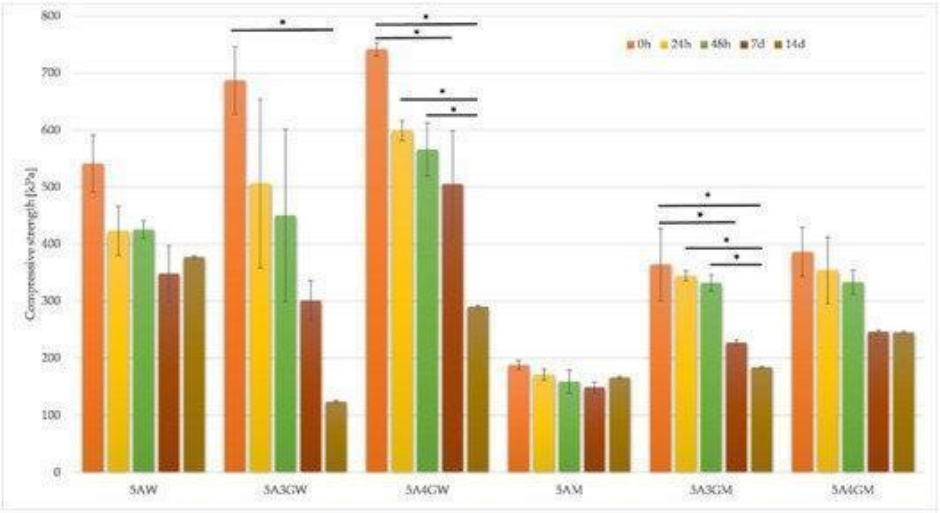

Figure 13. Degradation rate % of printed gelatin-alginate bioinks over time

Source: (Bociaga et al., 2019**).**

Thus, finding the correct balance of gelatin concentration, alginate concentration, and bioink solvent is crucial in maximizing the cell viability and minimizing the cell death.

Cell Viability

The cell viability over time gets lower the more time passes after a cell is extruded. For the gelatin-alginate bioinks, cell viability decreasing over time can be attributed to cells that enter the micropores of the gelatin-alginate bioink, where they are more likely to die. Therefore, the cell viability differs with the different porosity of gelatin-alginate bioinks, varied by their concentration (Bociaga et al., 2019).

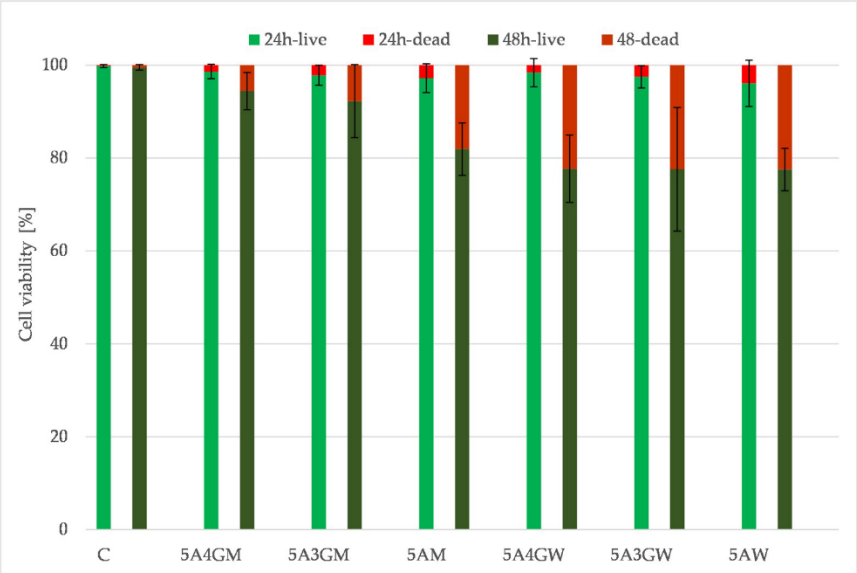

Figure 14. Cell viability of gelatin-alginate bioinks with varied concentrations

Source: (Bociaga et al., 2019**).**

Comparative assessment of candidate solutions

Comparison of Conical and Cylindrical Nozzles

Shear Stress

The shear stress originating from conical and cylindrical nozzles compared with 1 bar of pressure placed on a hydrogel bioink (10 w/v% gelatin methacrylamide):

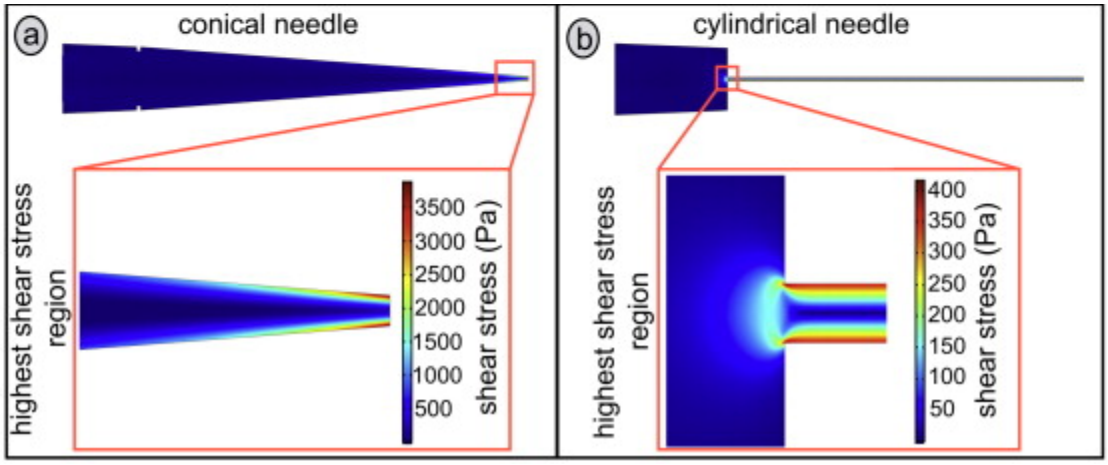

Figure 15. Shear stress of conical and cylindrical nozzle

Source: (Billiet et al., 2014**).**

Cell damage

The cell damage originating from conical and cylindrical nozzles extruding a 5% alginate gelatin-alginate bioink at different diameters with two different cell types: Schwann (a) and 3T3 (b):

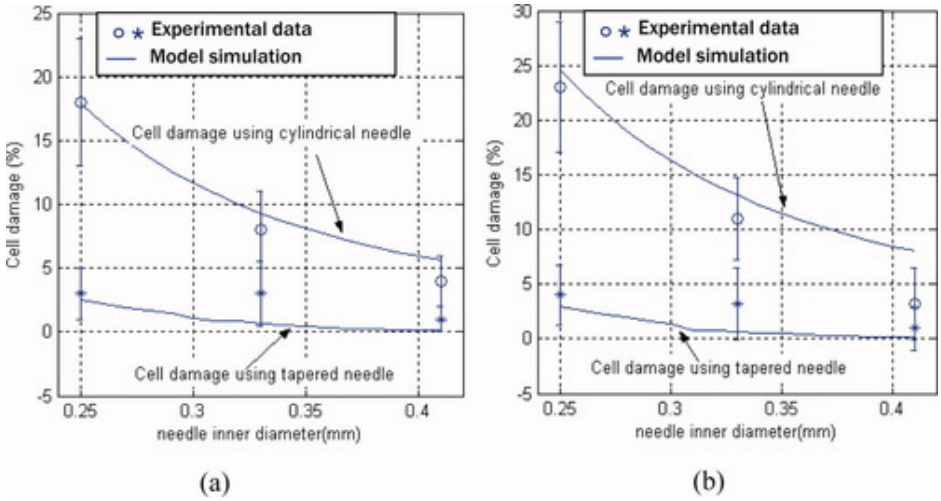

Figure 16. Cell damage of conical and cylindrical nozzle with varying diameter

Source: (Li et al., 2011**).**

The cell damage originating from conical and cylindrical nozzles extruding a 5% alginate gelatin-alginate bioink at different flow rates with two different cell types: Schwann (a) and 3T3 (b):

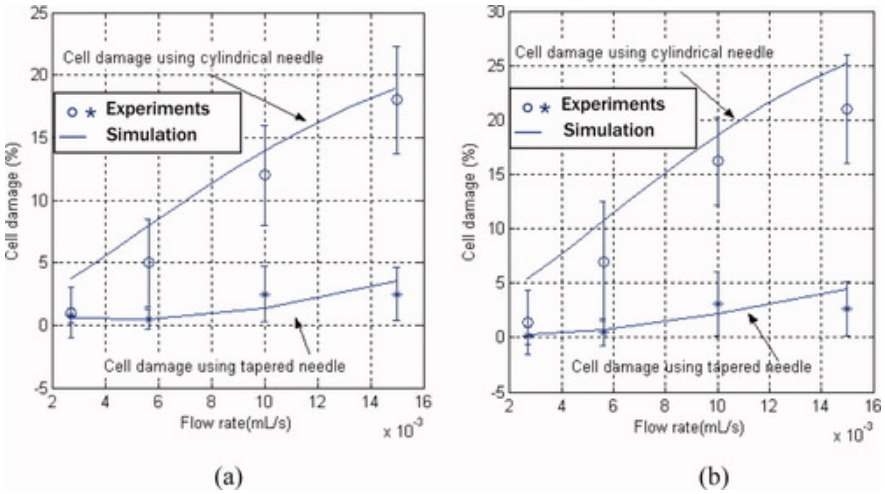

Figure 17. Cell damage of conical and cylindrical nozzle with varying flow rate

Source: (Li et al., 2011**).**

Cell viability

The cell viability originating from conical and cylindrical nozzles at different concentrations of the hydrogel bioink: gelatin-methacryloyl physical gel (GPG):

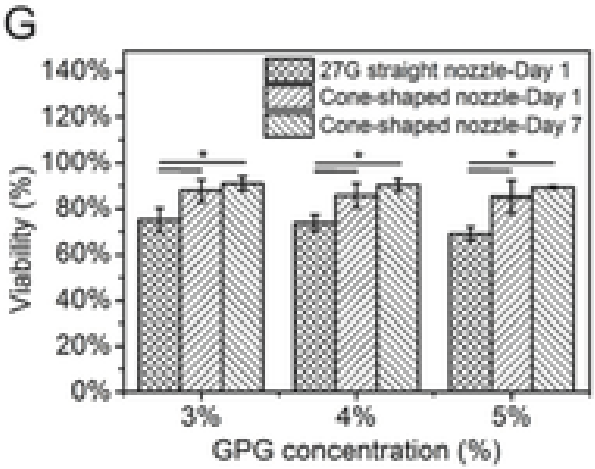

Figure 18. Cell damage of conical and cylindrical nozzle with varying flow rate

Source: (Liu et al., 2017).

Comparing only the conical and cylindrical nozzle cell viability after Day 1, the conical nozzle has a much higher cell viability at every different concentration, ranging from approximately 83%-95%, while the cylindrical nozzle is lower for every concentration, ranging from 70%-80% cell viability (Liu et al., 2017).

Comparative assessment of conical and cylindrical nozzles

Table 3. Comparative assessment of conical and cylindrical nozzles

	Conical Nozzle	Cylindrical Nozzle
Shear Stress (Pa)	~3250 Pa for (<1 mm)	~350 Pa for (>16 mm)
Cell Damage Range (%) w/ Varied Diameter	1%-4%	8%-18%
Cell Damage Range (%) w/ Varied Flow Rate	1%-5%	6%-19%
Cell Viability Range (%)	83%-95%	70%-80%
Accessibility	Online Order	Online Order
Cost per Lowest-Quality, Disposable Nozzle	$1.00-$1.33	$0.90 - $1.00
Cost per Highest-Quality, Reusable Nozzle	$200.00	N/A

Comparative assessment of gelatin-alginate concentrations in water solvent

Table 4. Comparative assessment of gelatin-alginate concentrations in water solvent

Gelatin Concentration %	Alginate Concentration %	Viscosity Increase	Viscosity (Pa*s)	Degradation Rate % after 14 days	Cell Viability After 48 Hours
4.0%	5.0%	4x	3.2 Pa*s	8.0%	78%
3.0%	5.0%	3x	2.5 Pa*s	6.0%	77%
0.0%	5.0%	Lower than culture-medium	0.5 Pa*s	2.9%	77%

Comparative assessment of gelatin-alginate concentrations in culture-medium solvent

Table 5. Comparative assessment of gelatin-alginate concentrations in culture-medium solvent

Gelatin Concentration %	Alginate Concentration %	Viscosity Increase	Viscosity (Pa*s)	Degradation Rate % After 14 days	Cell Viability After 48 Hours
4.0%	5.0%	1.5x	2.5 Pa*s	5.9%	95%
3.0%	5.0%	0.5x	1.5 Pa*s	5.2%	93%
0.00%	5.0%	Higher than water	1.2 Pa*s	2.2%	82%

Project Recommendations

The proposed solution will combine the optimal nozzle type and the optimal gelatin-alginate bioink concentration from the comparative assessment of solutions above.

Proposed solution

Based on the following comparison completed above in the Table 3, the nozzle that should be chosen to minimize cell death in extrusion bioprinting is the conical nozzle. This is due to its ability to minimize cell damage and maximize cell viability range much more effectively than the cylindrical nozzle. The conical nozzle has a much lower period of shear stress in comparison to the cylindrical nozzle. While the cylindrical nozzle's shear stress has less magnitude, it is for almost 16x the duration of the conical nozzle. Therefore, the overall cell death is lower when utilizing a conical nozzle. Also, the conical nozzle is like the cylindrical nozzle in terms of its ease of distribution and the cost of the nozzle to researchers.

Based on the following comparison completed above in the Table 4 and 5, the 4% gelatin and 5% alginate bioink with a culture-medium solvent should be utilized out of all the concentrations of the gelatin-alginate bioink. These concentrations have the lowest degradation rate after 14 days and cell viability after 48 hours in comparison to all the other concentrations. Also, the viscosity is increased by only 1.5x, which is lower than 5% gelatin 5% alginate in a water solvent. This minimizes the cell damage that could occur because of increased shears stress due to extruding the bioink at a higher pressure.

Together, extrusion printing of gelatin-alginate bioinks has the lowest cell death when using the mechanical parameter of a conical nozzle and using a 4% gelatin 5% alginate bioink with a culture-medium solvent.

Design and implementation challenges

To physically implement the proposed solution, mechanical knowledge of an extrusion bioprinter is needed to be able to successfully install the conical nozzle onto a bioprinter. To implement the use of a 4% gelatin 5% alginate bioink, researchers must be familiar with concentrations of different materials and how to modify them with percentage concentrations when creating the bioink. This is common knowledge - a basic lab requirement for researchers - so there are minimal challenges for the implementation of these solutions.

To write this this proposal, very specific parameters of extrusion bioprinting were used. Secondly, a very specific type of bionk was utilized in comparison to the wide range of bioinks that are a viable option for extrusion printing. As can be seen from the data provided in Table 3, the conical nozzle can be utilized with other types of bioinks and still provide a better cell death percentage than using a straight nozzle.

Anticipated project outcomes and impacts

Researchers who implement the solutions recommended in this proposal will have access to a high-quality method of extrusion printing. This improved method has a much lower cell

death percentage than previous extrusion printing, so it will lead to better results in the printing of biomaterials for various research. Secondly, the higher quality of cell viability and survivability will make the extrusion printer a more viable option in comparison to the inkjet bioprinter. Therefore, researchers can use cheaper and more commonly used equipment and get the same results as if they were using an inkjet bioprinter. Research involving bioprinting could become more widespread because of this accessibility, and there will be more research occurring that involves the bioprinting of biomaterials. As research increases, bioprinting will move closer to its goal: printing a fully functioning organ.

This proposal can be used as a model document for researchers who wish to minimize cell death during extrusion bioprinting using different types of bioinks and comparing different mechanical aspects of the bioprinter that directly affect shear stress. Therefore, this comparison is fluid - it can be applied to multiple other different types of bioinks or mechanical aspects of bioprinting by researchers who are looking to improve extrusion printing even farther.

Glossary

Bioink	Liquid combination of solvent and desired cell type that is ejected by bioprinter to bioprint scaffolds.[1]
Conical	In the shape of a cone.[4]
Cylindrical	In the shape of a cylinder.[4]
Degradation Rate	The rate at which the scaffold printed using bioprinting loses quality and viability.[1]
Extrusion	Bioink being pushed through a certain size of nozzle to eject the bioink in the desired pattern.[2]
Hydrogel	Polymer that is commonly used in bioprinting as a solvent within a bioink.[1]
Inkjet	Type of bioprinting that uses an activator in the nozzle to turn bioink into tiny droplets during the bioprinting process.[2]
Shear stress	Force upon bioink caused by pressure placed upon it or the bioink rubbing against the bioprinter nozzle – causes damage to bioink and cells within.[3]
Stereolithography	Type of bioprinting that uses precisely placed mirrors during the printing process to create the photopolymerization of a bioink to create layers of the bioink in the desired pattern.[2]
Viability	The ability to survive, reproduce, and complete desired function.[1]
Viscosity	The ability of a bioink to flow – higher if liquid has more resistance to flow, and lower if the liquid has less resistance to flower. More pressure required during extrusion of bioink if higher than if lower.[2]

[1] Derakhshanfar et al., 2018
[2] Ning et al., 2018
[23] Gómez-Blanco et al., 2020
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