Feasible Regions of Bioink Composition, Extrusion Pressure, and Needle Size for Continuous Extrusion-Based Bioprinting

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Bioprinting has many potential applications in drug screening, tissue engineering, and regenerative medicine. In extrusion-based bioprinting, the extruded strand is the fundamental building block for printed constructs and needs to be of good quality and continuous in structure. In recent years, many studies have been conducted on extrusion-based bioprinting. However, values of process parameters leading to continuous extrusion of strands have rarely been reported. In this paper, feasible regions of bioink composition, extrusion pressure, and needle size for continuous strand extrusion have been evaluated. The information on feasible regions for extruding continuous strands, provided in this paper, can be useful in deciding appropriate extrusion pressure and needle size for the bioink of different compositions (ratios of alginate:methylcellulose) in extrusion-based bioprinting.
[DOI: 10.1115/1.4048000]

Keywords: additive manufacturing, rapid prototyping and solid freeform fabrication

1 Introduction

Bioprinting can fabricate structures via layer-by-layer deposition of biomaterials. Bioprinting shows great promise for tissue regeneration and tissue research. Three main bioprinting techniques (extrusion-based, inkjet, and laser-based) have been developed [1]. The most widely used is extrusion-based bioprinting due to its affordability and compatibility with a wide range of biomaterials [2] compared to other techniques. Based on the way bioink dispensing system is actuated, extrusion-based bioprinter is classified as pneumatic-, mechanical- (piston or screw-driven), or solenoid-based [3]. Figure 1 illustrates the pneumatic extrusion-based bioprinting, where compressed air is used to extrude bioink through the syringe and out of the needle. Extrusion pressure can be changed during printing by varying the pressure of the compressed air. The strands are extruded during bioprinting, and constructs are fabricated layer-by-layer. The deposition of strands can be further controlled by varying print speed, which is the speed at which the printer head moves.

In extrusion-based bioprinting, the extruded strand is the smallest material unit that can be printed, and its dimension determines the resolution of printed constructs. Input parameters, including bioink composition [4], and process parameters (such as extrusion pressure, needle size, and print speed) affect the quality of the strand. Each input parameter has its feasible region. If the value of any input parameter is outside the feasible region, a continuous strand of acceptable quality cannot be printed.

Table 1 summarizes input parameters and their ranges in reported studies on extrusion-based bioprinting. In these publications, limited information was provided on why choosing a particular value of extrusion pressure for a particular needle size while printing with bioink of a given composition. Moreover, information on feasible regions of input parameters (such as extrusion pressure, needle size, and bioink composition) is largely not available.

This paper reports a comprehensive study on feasible regions of bioink composition, extrusion pressure, and needle size for extruding continuous strands. The rest of this paper is organized as follows. Section 2 describes the experimental procedure and conditions. Section 3 presents the definition and determination of feasible regions. Section 4 discusses the experiment results. Lastly, the conclusions are presented in Sec. 5.

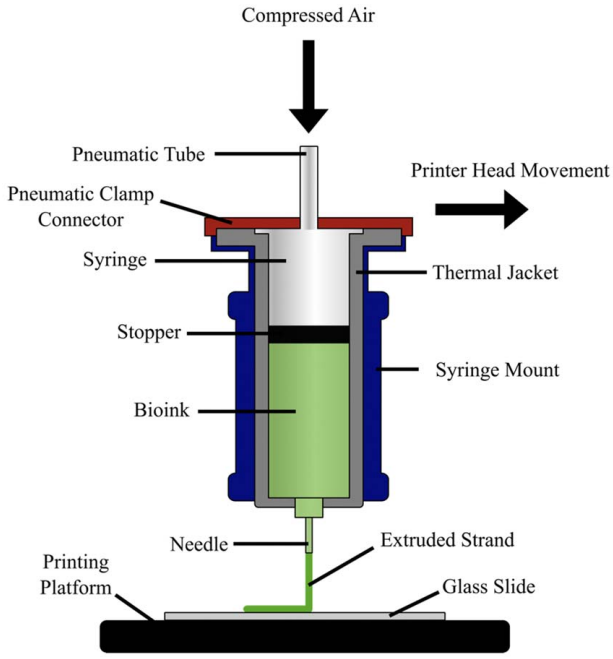

Fig. 1 Schematic of pneumatic extrusion-based bioprinting

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Manuscript received March 16, 2020; final manuscript received July 3, 2020; published online September 17, 2020. Assoc. Editor: Kevin Chou.

Table 1 Summary of input parameters and their ranges in reported studies on extrusion-based bioprinting

Input parameter	Reported range	Reference
Extrusion pressure	20–72 psi	[5–10]
Extrusion temperature	15–35 °C	[5–10]
Needle diameter	0.15–0.5 mm	[11,12]
Print speed	1.67–25 mm/s	[11,13]

2 Experimental Procedure and Conditions

2.1 Bioink Composition and Synthesis. The alginatemethylcellulose bioink was used in this study. Alginate is derived from the cell wall of brown algae, and methylcellulose is composed of linked glucose molecules. In this bioink, methylcellulose was used as a visco-modifier.

In this study, bioinks with different compositions of alginate: methylcellulose (Alg:MC) in 1:1.5, 1:2, 1:2.5, and 1:3 ratios by weight were synthesized, following the procedure reported in the literature [14]. The procedure is shown in Fig. 2.

(1) Step 1: 30 mg of alginic acid sodium salt (Sigma-Aldrich, USA) was added to 100 ml of deionized water in a glass beaker (VWR, USA).
(2) Step 2: The glass beaker was put on a magnetic stirrer (Model #1151049S; Fisher Scientific, USA) and stirred for 2 h.
(3) Step 3: The resulting alginate solution in the glass beaker was then heated to 80 °C on the magnetic stirrer (Model #1151049S; Fisher Scientific, USA). The desired amount of methylcellulose powder (Sigma-Aldrich, USA) was added to this heated solution. The solution was stirred for 30 min to allow methylcellulose powder to mix well into the alginate solution.
(4) Step 4: This alginate:methylcellulose solution was then sterilized by autoclaving at the temperature of 121 °C and the pressure of 20 psi for 20 min in an autoclave (LG 250 Sterilizer; Steris, USA). Autoclaving is the process of application of heat and pressure to ensure sterility.

In total, four beakers of bioink solutions were prepared. Each beaker contained the bioink with one composition of alginate:methylcellulose. These beakers were kept at room temperature for at least 2 h, based on the guidance provided in a reported study [15] to synthesize the bioink, before the bioinks were used for 3D printing.

Bioinks should exhibit shear-thinning behavior to be smoothly extruded from a needle and to retain the shape of the printed construct after deposition [13,14]. A rheological analysis was performed to study the shear-thinning behavior of the synthesized bioinks. TA Instrument DHR-2 rheometer (TA Instruments, USA) was used to perform rotational shear-viscosity measurements in flow mode with a shear rate ranging from 0.01 to 700 1/s using the cone-plate system. Measurements were performed at 24 °C. The parameters for the experiment were set via the TRIOS software (TA Instruments, USA). The software presented the results which were the average of three repetitive measurements. This number of repetitive measurements can be set in the software.

Figure 3 shows the relationships between viscosity and shear rate for the bioinks used in this study. It can be seen that the bioinks of different compositions had different viscosity values when the shear rate was zero. As the shear rate increased from zero to 700 1/s, their viscosity values decreased and became almost the same. Therefore, it was concluded that the bioinks exhibited shear thinning behavior and were suitable for bioprinting.

2.2 Design of Construct for Three-Dimensional Printing. The construct (the final product after bioprinting) to be bioprinted in this study was designed using the FUSION 360 software (Autodesk, USA). The design involved a straight line that was 5 cm in length. The STL file generated by FUSION 360 software was converted into a G-code file. This G-code file was then imported via a flash drive (a portable memory storage device) into the ALLEVI BIOPRINTER software (Allevi, Inc., Philadelphia, PA).

2.3 Bioprinter and Printing Conditions. ALLEVI 2 BIOPRINTER (Allevi, Inc., Philadelphia, PA), as shown in Fig. 4, was used to conduct the printing experiments. It is a pneumatic extrusion-based

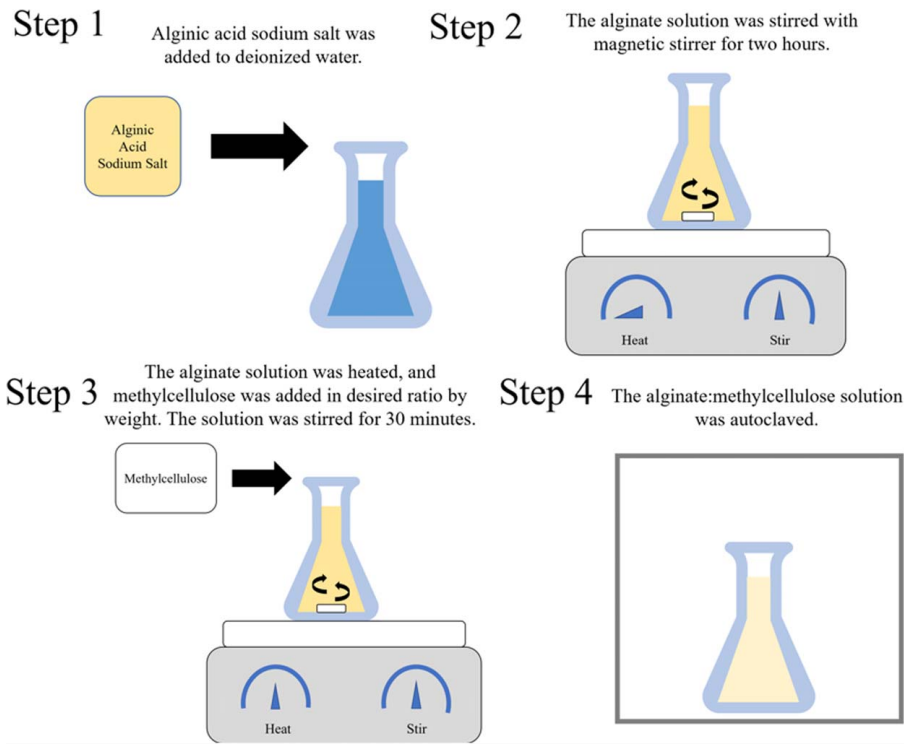

Fig. 2 Procedure for bioink synthesis

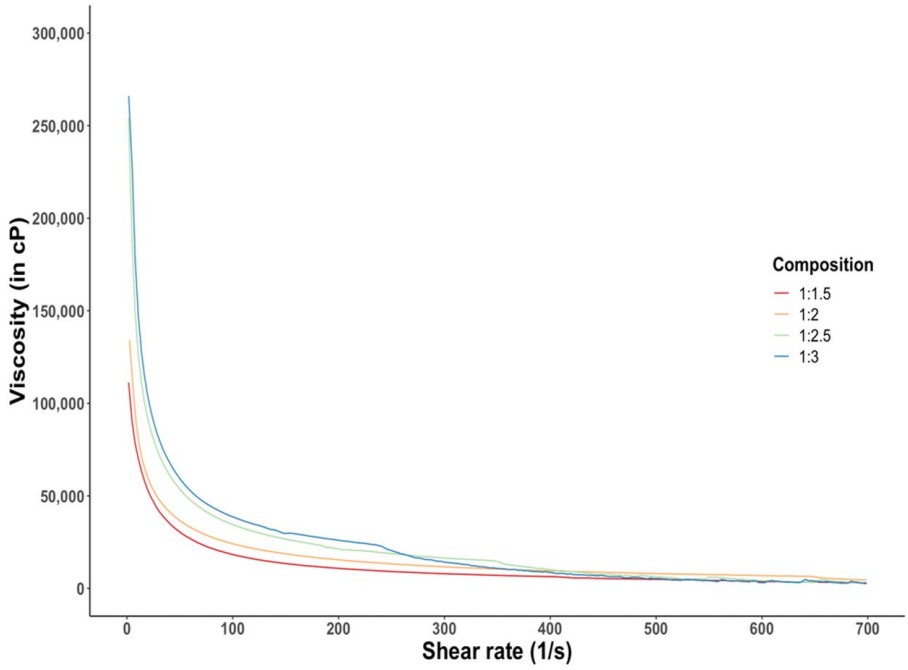

Fig. 3 Relationship between viscosity and shear rate

bioprinter. It was placed in a biosafety cabinet (Sterilgard III Advance, Baker Hughes, USA) to prevent contamination.

Based on past experiences with bioprinting, the following input parameters were selected for the study:

- Bioink composition
- Extrusion pressure
- Needle size

Table 2 shows the input parameters and their values. Bioink composition was varied by changing the ratio between alginate and methylcellulose. Four values of alginate to methylcellulose ratio were used: 1:1.5,1:2, 1:2.5, and 1:3 by weight. These values of alginate to methylcellulose ratios were selected based on the values used in a reported study using alginate:methylcellulose bioink [14].

Fig. 4 Allevi 2 bioprinter in a biosafety cabinet

Extrusion pressure was varied from 10 to 110 psi, with an increment of 10 psi. This extrusion pressure range was selected based on reported studies [7,9–14] with mammalian cells, and the assumption that algae cells are more robust than mammalian cells [15] and can withstand higher extrusion pressure. The extrusion pressure was varied via the ALLEVI software interface for the bioprinter.

Four values of needle size were chosen: 23, 25, 27, 30, and 32G. Table 3 depicts the relation between needle gauge and needle inner diameter. As the number of needle gauge increases, needle diameter decreases. 32G was chosen as the lower limit of the needle size range because smaller needle sizes would be detrimental to cell viability. When larger needle sizes are used, printed strands will have increased width, decreasing the dimensional accuracy of printed strands [10]. Needle sizes larger than 23G would print strands of insufficient dimensional accuracy.

There were 220 unique combinations of parameter values. For example, one combination would comprise bioink composition of 1:2 (Alg:MC), extrusion pressure of 50 psi, and needle size of 27G. For each of the 220 combinations of input parameters, three replications were printed.

3 Definition and Determination of Feasible Regions

Feasible regions were determined based on the quality of the strand being extruded. The quality of the extruded strand was categorized as follows:

Good (with a score of 3): the strand was printed in a continuous manner without any surface breaks, as illustrated in Fig. 5(*a*).

Table 2 Input parameters and their values

Parameter	Value
Bioink composition (ratio of Alg:MC by weight)	1:1.5, 1:2, 1:2.5, 1:3
Extrusion pressure (psi)	10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110
Needle size (G)	23, 25, 27, 30, 32

Table 3 Conversion between needle gauge and needle inner diameter

Needle gauge	Inner diameter (mm)
23	0.337
25	0.260
27	0.127
30	0.159
32	0.108

Fair (with a score of 2): the printed strand had minor surface breaks and was not completely continuous, as illustrated in Fig. 5(*b*).

Poor (with a score of 1): no strand was extruded, as illustrated in Fig. 5(*c*).

There were, in total, 220 combinations of input parameters. For each combination, three replications of strands were printed and the average score of all the three replications was used as the final score for the strand quality for that particular combination. If the average was below 2.7, the combination was marked outside the feasible region. If the average was above 2.7, the combination was marked in the feasible region. The value of 2.7 was selected as cutoff since its average score for three replications where at least two replications have to be Good (with a score of 3) and one has to be Fair (with a score of 2).

4 Results and Discussion

Figure 6 is one way to show the feasible regions obtained from this study. Each data point shows the minimum extrusion pressure required to print a continuous strand for a given needle size and a give bioink composition. For example, using the 32G needle size and bioink with alginate:methylcellulose ratio of 1:1.5, a continuous strand was possible only if the extrusion pressure was 100 psi. Using the 23G needle size, continuous strands were printed for alginate:methylcellulose ratios of 1:1.5, 1:2, 1:2.5, and 1:3 with a minimum extrusion pressure of 20 psi, 60 psi, 70 psi, and 50 psi, respectively.

Figure 7 is another way to show the feasible regions. It shows the average scores of strand quality of the three replications for each of the 220 unique combinations of the three input parameters. Figure 7(*a*) shows the feasible regions of needle size and extrusion pressure for bioink with alginate:methylcellulose ratio of 1:1.5. There was no extrusion for needle size of 32G until the extrusion pressure was raised to 100 psi. For a needle size of 30G, continuous extrusion was feasible only if extrusion pressure was 70 psi or higher. An extrusion pressure of 50 psi or higher was required for continuous extrusion with needle sizes of 27G and 25G. An extrusion pressure of 20 psi or higher was required for continuous extrusion when printing with a needle size of 23G.

Figure 7(*b*) shows feasible regions of needle size and extrusion pressure for bioink with alginate:methylcellulose ratio of 1:2. When printing with a needle size of 32G, there was no continuous extrusion for the entire extrusion pressure range studied (from 10 to

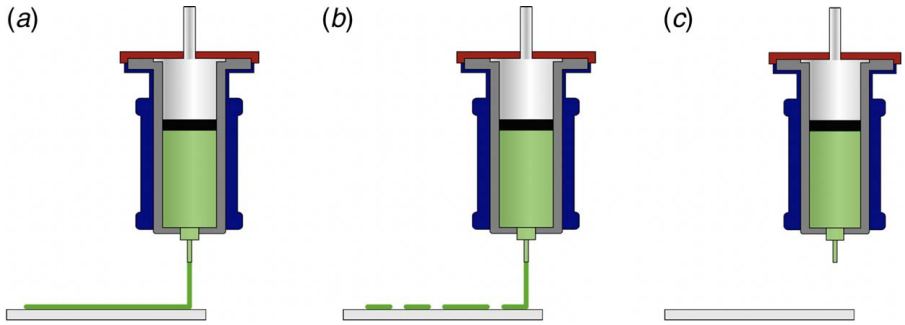

Fig. 5 Quality of extruded strand: (*a*) good, (*b*) fair, and (c) poor

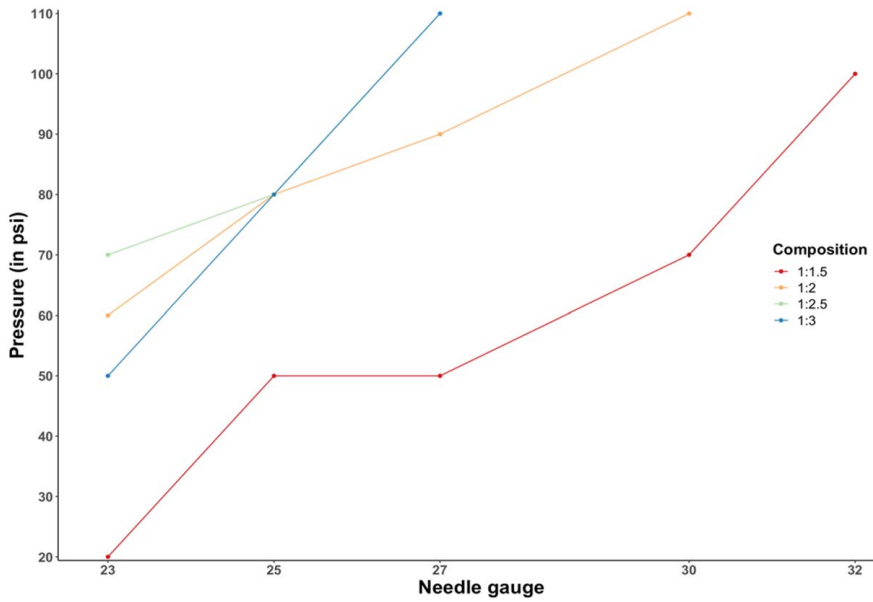

Fig. 6 Minimum extrusion pressure required to print a continuous strand

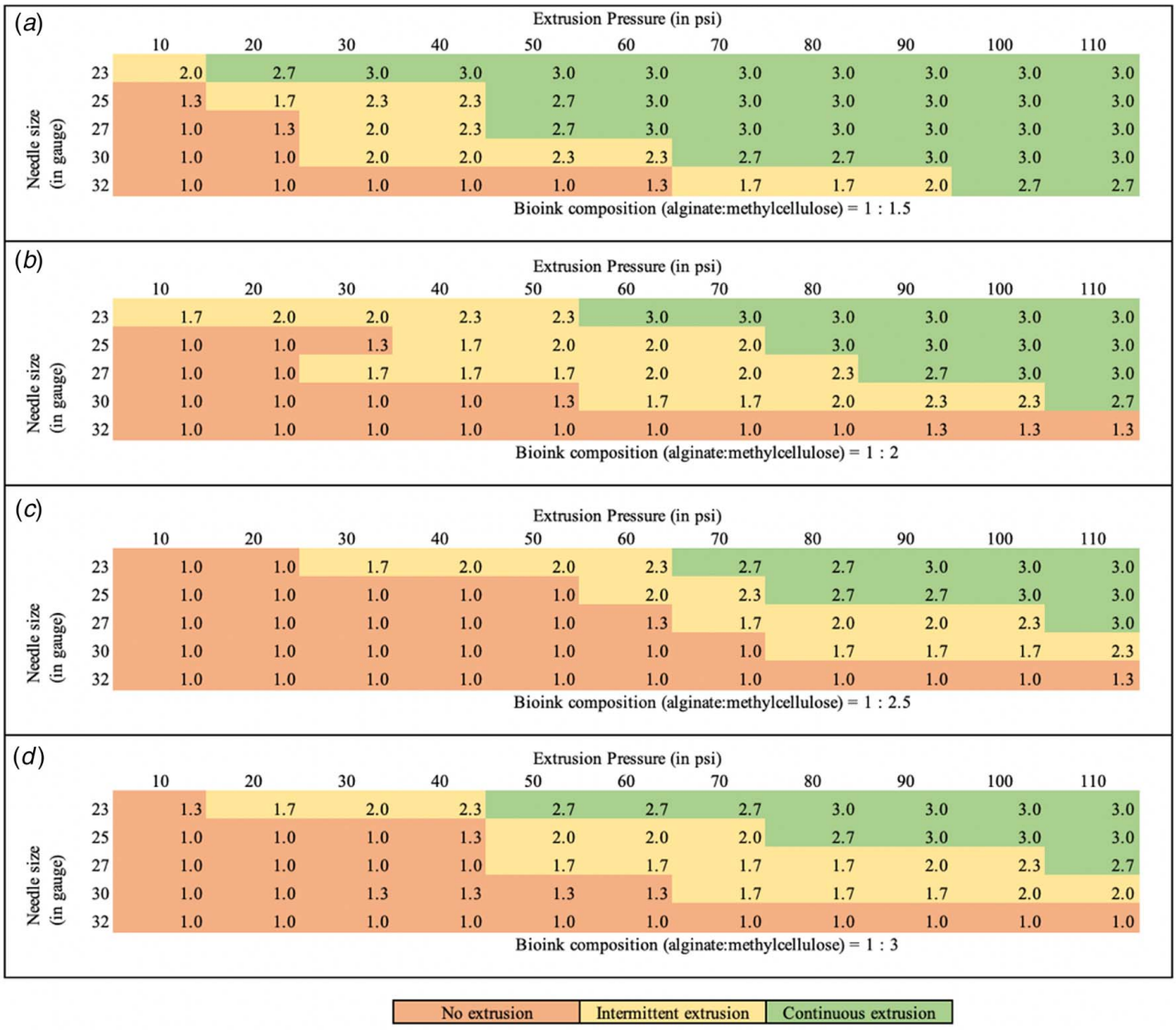

Fig. 7 Feasible regions of bioink composition, extrusion pressure, and needle size

110 psi). For the needle size of 30G, continuous extrusion was feasible only at an extrusion pressure of 110 psi. An extrusion pressure of 90 psi or higher was required to print in a continuous manner with the needle size of 27G. There was no continuous extrusion for needle size of 25G if extrusion pressure was below 80 psi. For needle size of 23G, the continuous extrusion was feasible only if the extrusion pressure was 60 psi or higher.

Figure 7(*c*) shows the feasible regions of needle size and extrusion pressure for bioink with alginate:methylcellulose ratio of 1:2.5. For needle sizes of 32G and 30G, there was no continuous extrusion for the entire pressure range from 10 to 110 psi. A minimum pressure of 110 psi was required for continuous extrusion when printing with a needle size of 27G. For needle size of 25G, continuous extrusion was feasible only if extrusion pressure was 80 psi or higher. Extrusion pressure of 70 psi or higher was needed to continuously extrude with a needle size of 23G.

Figure 7(*d*) shows feasible regions of needle size and extrusion pressure for bioink with alginate:methylcellulose ratio of 1:3. There was no continuous extrusion when printing with a needle size of 32G and 30G for the entire range of extrusion pressure from 10 to 110 psi. For needle size of 27G, continuous extrusion was not feasible until extrusion pressure reached 110 psi. A minimum pressure of 80 psi was required for continuous extrusion when printing with a needle size of 25G. When using needle size of 23G, continuous extrusion occurred only if the extrusion pressure was 50 psi or higher.

From Fig. 7, it can be observed that quality scores for strands can be different between repetitions even for the same combination of parameters. The difference in quality scores is because for a combination of parameters the scores can lie on the border of being good or fair. The repetitions help to differentiate in these borderline cases. The higher resolution of printed strands requires the use of needles with a smaller size. When a smaller needle size is used, a higher extrusion pressure is usually required in order to continuously extrude bioink [16]. On the other hand, the use of high extrusion pressure might negatively affect cell viability [4,7]. Thus, the trade-off between resolution and cell viability needs to be accounted for when selecting needle size.

5 Conclusions

An experimental study has been conducted to determine feasible regions of bioink composition, extrusion pressure, and needle size in extrusion-based bioprinting. Feasible regions are defined as the ranges of input parameters that result in continuous extrusion of the strand. The combinations of input parameters that were most likely to be in feasible regions were bioinks with a lower ratio of

alginate:methylcellulose (lower viscosity), higher extrusion pressure, and larger needle size.

Certain parameter ranges are more likely to result in continuous extrusion of strand, but they may cause other issues during bioprinting. For example, larger needle sizes tend to print strands of poor resolution, and higher extrusion pressures are more likely to damage cells in the bioink. Thus, the trade-off between resolution and cell viability needs to be accounted for while deciding on needle size and extrusion pressure. The information on feasible regions for extruding continuous strands, provided in this paper, can be useful in deciding appropriate extrusion pressure and needle size while printing with bioink of given composition (alginate:methylcellulose ratio) in extrusion-based bioprinting.

Acknowledgment

The authors thank Mr. Jackson Sanders (an undergraduate student in the Industrial and Systems Engineering Department at Texas A&M University) for his assistance in making the schematic illustrations (in Figs. 1 and 5).

Funding Data

- The authors acknowledge funding from the Air Force Office of Scientific Research (AFOSR) (Grant No. FA9550-18-1-0233).

Conflict of Interest

There are no conflicts of interest.

Data Availability Statement

The datasets generated and supporting the findings of this article are obtainable from the corresponding author upon reasonable request. Data provided by a third party are listed in Acknowledgments. No data, models, or code were generated or used for this paper.

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