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Cite as: AIP Advances 9, 095055 (2019); https://doi.org/10.1063/1.5116371
Submitted: 24 June 2019 . Accepted: 16 September 2019 . Published Online: 26 September 2019

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Submitted: 24 June 2019 • Accepted: 16 September 2019 • Published Online: 26 September 2019

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ABSTRACT
Inkjet-based bioprinting have been widely employed in a variety of applications in tissue engineering and drug screening and delivery. The typical bioink used in inkjet bioprinting consists of biological materials and living cells. During inkjet bioprinting, the cell-laden bioink is ejected out from the inkjet dispenser to form microspheres with cells encapsulated. The cell distribution within microspheres is defined as the distribution of cell number within the microspheres. The paper focuses on the effects of polymer concentration, excitation voltage, and cell concentration on the cell distribution within microspheres during inkjet printing of cell-laden bioink. The normal distribution has been utilized to fit the experimental results to obtain the mean and standard deviation of the distribution. It is found that the cell distribution within the microspheres increases with the increase of the cell concentration, sodium alginate concentration, and the excitation voltage.

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I. INTRODUCTION AND BACKGROUND
Additive manufacturing, known as three-dimensional (3D) printing, was first presented in 1986 when photocrosslinkable polymers were utilized to successfully fabricate 3D structures based on a layer-by-layer manner. After two years, 3D printing was demonstrated to fabricate 3D synthetic tissues using biological materials collagen and fibronectin. In the past decade, 3D bioprinting has been gaining more and more attention in a variety of tissue engineering and regenerative medicine applications. 3D bioprinting utilizes different advanced manufacturing technologies to fabricate functional tissues and organs layer by layer using bioink containing biological materials, additives, and living cells. The fabricated tissues and organs are envisioned to be used for the replacement of damaged or injured human organs, which makes 3D bioprinting a promising solution to the challenge of organ donor shortage. The main advantages of 3D bioprinting include precise positioning of different cell types at desired locations, high cell densities, and no immune rejection problem.

Three main bioprinting techniques are widely used including inkjet-based, microextrusion-based, and laser-assisted bioprinting. Among these three bioprinting techniques, inkjet-based bioprinting is favored in this study because of the high cell viability and resolution, precise controllability, easy scale-up potential, high printing speed, to name a few. Inkjet printing can be mainly classified into two categories: continuous inkjet printing (CIJ) and drop-on-demand (DOD) inkjet printing. During CIJ printing, a liquid jet is ejected out from an inkjet dispenser under a pneumatic pressure and breaks into a stream of droplets due to the Rayleigh instability. The selected droplets are charged electrically and deflected using an electric field onto the substrate. The other droplets are steered into a gutter and recirculated. During DOD printing, droplets are generated only when required. There are two types of mechanism for droplet formation during DOD printing. In a thermal DOD printer, a microheater element vaporizes a small pocket of the fluid; the formation and collapse of the vapor bubble generates an acoustic pressure pulse which ejects the fluid out from the inkjet dispenser. In a piezoelectric DOD printer, the
piezoelectric element deforms the chamber of the inkjet dispenser to eject the fluid out. For bioprinting applications, the CIIJ printing is limited by the risk of contamination that occurs during ink recirculation, and the thermal DOD printing may cause damage or death of living cells. Hence, piezoelectric DOD printing is favored in this study due to good controllability and high cell viability.

Inkjet bioprinting have been widely used in tissue engineering and drug screening and delivery. The typical bioink used in inkjet bioprinting consists of biological materials and living cells. The biological materials are used to mimic natural extracellular matrix which provides support for cell attachment and proliferation. The biological materials used in inkjet bioprinting include collagen, fibroin, to name a few. Various living cells have been utilized in inkjet bioprinting, including embryonic motoneuron cells, human fibroblasts, and human amniotic fluid-derived stem cells (hAFSCs). Inkjet bioprinting has also been utilized to fabricate a variety of 3D structures, such as vascular-like constructs, cartilages, skin, bones, to name a few. Xu et al. utilized inkjet bioprinting to fabricate zigzag vascular-like constructs with fibroblasts encapsulated, and the cell viability after 72-hour incubation was above 80%. Cui et al. utilized a commercial inkjet printer to create an artificial cartilage from a bioink composed of human chondrocytes and poly(ethylene glycol) dimethacrylate. The bioprinted cartilage had similar mechanical properties and biochemical composition as the natural cartilages and could attach firmly with the surrounding tissues. Faulkner-Jones et al. reported negligible effects on post-printing cell viability and pluripotency when inkjet bioprinting was utilized to print human pluripotent stem cells (hPSCs). The bioprinted constructs with hPSCs encapsulated in alginate hydrogels were successfully directed into hepatic lineage under the differentiation protocol.

During inkjet bioprinting, the cell-laden bioink is ejected out from the inkjet dispenser to form microspheres with cell encapsulated, which are precisely deposited on the substrate to form 2D patterns and 3D constructs. Cell-laden droplet/microsphere formed, which are precisely deposited on the substrate to form 2D patterns. However, the comprehensive cell distribution within microspheres during inkjet bioprinting of cell-laden bioink is still missing.

During inkjet bioprinting, the cell-laden microspheres utilized as building blocks are precisely controlled to deposit on the substrate to fabricate 3D constructs. The cell distribution within microspheres is defined as the distribution of cell number within the microspheres. Uniform cell distribution is beneficial to post-printing cell viability as well as functionality of the fabricated tissues and organs. Despite of the previous droplet formation studies during inkjet bioprinting of cell-laden bioink, the investigation of cell distribution within microspheres is still missing. The paper focuses on the effects of polymer concentration, excitation voltage, and cell concentration on the cell distribution within microspheres during inkjet printing of cell-laden bioink. The rest of the paper is organized as follows. Section II presents the preparation of bioink and the experimental setup. Section III quantifies the cell distribution based on the normal distribution and presents the cell distribution results under different conditions including excitation voltage, polymer concentration, and cell concentration. Section IV summarizes main conclusions and proposes future work.

II. MATERIALS AND METHODS
A. Bioink preparation

Extracellular matrix (ECM) is a complex meshwork within all tissues and organs, composed of collagen, fibrillin, elastin, fibronectin, various laminins and integrins, and proteoglycans. ECM provides essential structural support, enable cellular attachment, and regulate cellular functions and behaviors. Biomaterials, in particular hydrogels, usually serve as an artificial ECM for cells to mimic the natural ECM in our body. Additionally, hydrogels possess excellent biocompatible properties and high-water content to protect cells. In this study, sodium alginate was selected to make up the bulk of the bioink. Fibroblasts were selected as the model cells in this study, because fibroblasts are the most common mammalian connective tissue cells. Hence, the bioink used in the study was composed of sodium alginate and 3T3 fibroblasts.

Sodium alginate powder (Sigma-Aldrich, St. Louis, MO) was dissolved into Dulbecco’s Modified Eagles Medium (DMEM; Sigma-Aldrich, St. Louis, MO) to make the bioink with different sodium alginate concentrations in the range of 0.5 – 2% (w/v). 3T3 fibroblasts were cultured with DMEM supplemented with 10% Bovine Calf Serum (BCS; Hyclone, Manassas, VA) and 1% antibiotic/antimycotic solution. The cells were collected using centrifugation at 1,000 rpm for 5 minutes and the cell pallet was suspended in the sodium alginate solutions to make the bioink with four cell concentrations of 5 × 10^5, 2 × 10^6, 5 × 10^6, and 8 × 10^6 cells/mL. More details regarding cell culture and bioink preparation can be found in our previous studies. The bioink was ejected out from an inkjet dispenser to form cell-laden droplets, which were deposited into a petri dish containing a crosslinking agent. The crosslinking agent used in this study was 2% (w/v) calcium chloride solution, which was prepared by dissolving the calcium chloride powder (Amresco, LLC, Solon, OH) into deionized water. The crosslinking mechanism is due to replacement of the sodium ions by the calcium ions, and the cell-laden droplets were crosslinked to form cell-laden microspheres. An inverted microscope was utilized to take images
of the cell-laden microspheres shown in Fig. 1. It is seen that the encapsulated cells can be observed clearly.

B. Experimental setup

The schematic of the inkjet-based bioprinting system is shown in Fig. 1. This inkjet-based bioprinting system consists of an inkjet dispenser, an excitation waveform generator, a pneumatic controller, a time-resolved imaging system, and a container with a crosslinking agent. The inkjet dispenser is piezoelectric. The mechanism of the droplet formation is the pressure pulses caused by the deformation of the piezoactuator. The excitation waveform generator applies a voltage signal to the piezoactuator resulting in the mechanical deformation. The excitation waveform used in this study is a bipolar waveform which is defined by several important parameters, such as rise time, dwell time, fall time, echo time, final rise time, frequency, and excitation voltage. The excitation voltage directly determines the jet behaviors and is chosen to investigate its effect on the cell distribution. The other parameters are fixed: rise time 3 μs, dwell time 25 μs, fall time 5 μs, echo time 30 μs, final rise time 3 μs, and frequency is 120 Hz. The pneumatic controller is utilized to optimize the back pressure to ensure flat meniscus at the nozzle tip. The typical back pressure in this study is -14 - -12 kPa. The imaging system captures the images of the droplet formation process in inkjet-based bioprinting. The captured images have a resolution of 640 x 480 pixels. The container has 2% (w/v) calcium chloride solution which is utilized to crosslink the cell-laden droplets to form microspheres. Under the microscope, the microspheres can be clearly observed, and the encapsulated cells are well recognized.

C. Experimental conditions

During inkjet printing, an excitation waveform was applied to the piezoactuator resulting in a complex pressure wave, which ejected the cell-laden bioink out from an inkjet dispenser to form cell-laden droplets. These cell-laden droplets were crosslinked by the calcium chloride solution to form cell-laden microspheres. Each microsphere may have different numbers of cells encapsulated. For example, in Fig. 1, some microspheres had no cells encapsulated, some had one cell encapsulated, and some microspheres had two encapsulated. The cell distribution is defined as the distribution of cell number within the microspheres. The objective of this paper is to investigate the effects of the printing variables on the cell distribution within the microspheres during inkjet printing of cell-laden bioink. There are two types of printing variables: bioink property-related variables and process-related variables. In this study, two bioink property-related variables selected were sodium alginate concentration and cell concentration, and one process-related variable selected was excitation voltage. All the three variables significantly affect the cell-laden droplet formation as well as the cell distribution within the microspheres.

Table I summarizes different printing conditions in this study. The sodium alginate concentration was 0.5 – 2% (w/v) with an interval of 0.5%. The sodium alginate concentration significantly affects the bioink rheological properties which further influence the jetting and pinch-off behaviors during the droplet formation process in inkjet printing. These four sodium alginate concentrations have been widely utilized in various inkjet-based bioprinting applications. The excitation voltage was 40 – 70 V with an interval of 10 V. The excitation voltage determines the jetting velocity which further influences the jet instability for satellite droplet formation. The cell concentrations used in this study were $5 \times 10^5$, $2 \times 10^6$, $8 \times 10^6$ cells/mL.

| TABLE I. Process conditions including sodium alginate concentration, excitation voltage and cell concentration. |
|---------------------------------------------------------------|
| Sodium alginate concentration (w/v) | 0.5%, 1%, 1.5%, 2% |
| Excitation voltage (V) | 40, 50, 60, 70 |
| Cell concentration (cells/mL) | $5 \times 10^5$, $2 \times 10^6$, $5 \times 10^6$, $8 \times 10^6$ |
5 × 10^6, and 8 × 10^6 cells/mL. Under each condition, 1,000 microspheres were randomly selected to quantify the cell distribution. The normal distribution equation was utilized to fit the experimental results to obtain the mean and standard deviation of the distribution.

III. RESULTS

A. Effect of cell concentration on cell distribution within microspheres

In this section, the effect of cell concentration on the post-printing cell distribution has been investigated. The cell concentrations selected are 0.5, 2, 5, and 8 × 10^6 cells/mL. These cell concentrations include low, medium, and high cell concentrations widely used in inkjet-based bioprinting. The other printing conditions are fixed: the excitation voltage 60 V, and the sodium alginate concentration 1% (w/v). The cell-laden bioink is ejected out from the inkjet dispenser into the 2% (w/v) calcium chloride to form cell-laden microspheres. The representative images of microspheres are shown in Fig. 2 using the bioink with different cell concentrations.

During the droplet formation using the bioink with different concentrations, one primary droplet was formed, and one satellite droplet was formed. The size of the primary droplet and the satellite droplet was very close. At each cell concentration, 1000 microspheres were randomly selected to analyze the cell distribution. The detailed cell distribution using the bioink with different cell concentrations is shown in Fig. 3. At the very low cell concentration of 0.5 × 10^6 cells/mL, most microspheres are without cells, and the microspheres with one cell is only 7.4%. At the low cell concentration of 2 × 10^6 cells/mL, most microspheres are still without cells. But the percentage of the microspheres without cells is reduced. The percentage of the microspheres with cells significantly increases. The microspheres with one cell are 18.6%, and the microspheres with two cells are 3.1%. There are even 0.3% microspheres with three and four cells. At the medium cell concentration of 5 × 10^6 cells/mL, there are still 36.3% microspheres without cells. The microspheres with one cell, two cells and three cells are 30.5%, 20.5%, and 8.3%, respectively. The microspheres with more than three cells are 4.4%. At the high cell concentration, the microspheres with zero cell, one cell, two cells, and three cells are 31.1%, 32.6%, 15.1%, and 13.6%, respectively. There are more than 20% microspheres with more than three cells.

It is seen that with the increase of the cell concentration more and more cells are encapsulated within the microspheres, and the microspheres with few cells are reduced significantly. At the very low cell concentration of 0.5 × 10^6 cells/mL, there are no microspheres with more than two cells. As the cell concentration increases, the microspheres with more cells start to appear. At the medium cell concentration of 5 × 10^6 cells/mL, the microspheres with five cells are observed. At the high cell concentration of 8 × 10^6 cells/mL, the microspheres with eight cells can be observed. Moreover, the percentage of the microspheres with more than three cells increases monotonically. For example, as the cell concentration increases from 0.5 to 8 × 10^6 cells/mL, the microspheres with three cells increase significantly from 0% to 13.6%, and the microspheres with four cells also increase from 0% to 4.3%. At the same time, the microspheres with a low cell number are reduced significantly. For example, as the cell concentration increases from 0.5 to 8 × 10^6 cells/mL, the

![Representative images of microspheres using the bioink with different cell concentrations: (a) 0.5 × 10^6 cells/mL, (b) 2 × 10^6 cells/mL, (c) 5 × 10^6 cells/mL, and (d) 8 × 10^6 cells/mL. The other printing conditions are fixed: the excitation voltage 60 V, and the sodium alginate concentration 1% (w/v).](image-url)
FIG. 3. Cell distribution within the microspheres at different cell concentrations of (a) $0.5 \times 10^6$ cells/mL, (b) $2 \times 10^6$ cells/mL, (c) $5 \times 10^6$ cells/mL, and (d) $8 \times 10^6$ cells/mL.

microspheres without cells decrease significantly from 92.1% to 31.1%. It is noted that as the cell concentration increases from 0.5 to $8 \times 10^6$ cells/mL, the microspheres with two cells increase first from 0.5% to 20.5% and then decrease to 15.1%.

In order to better quantify the overall cell distribution, normal distribution has been implemented to analyze the mean and the standard deviation of the distribution. In the insert figure of Fig. 4, it shows the representative fitted normal distribution based on the experimental results at the cell concentration of $8 \times 10^6$ cells/mL. The fitted mean is calculated based on the formula $\mu = \sum_{x=0}^{n} xy$, where $x$ is the number of cells encapsulated within the microspheres, $y$ is the corresponding percentage, and $n$ is the maximum number of cells encapsulated within the microspheres at each cell concentration. The standard deviation is calculated based on the formula $\sigma = \sqrt{\sum_{x=0}^{n} (x - \mu)^2 \times y}$. The fitted standard deviation represents variation from the mean. A low standard deviation indicates that the cell number within the microspheres is very close to the mean, and a high standard deviation indicates that the cell number within the microspheres spreads out over a larger range. It is seen that as the cell concentration increases from 0.5 to 2 to 5 to $8 \times 10^6$ cells/mL, the fitted mean cell number within one microsphere increases from 0.08 to 0.26 to 1.15 to 1.39, respectively. The fitted mean cell number within one microsphere is nearly proportional to the cell concentration. Meanwhile, the standard deviation also increases from 0.30 to 0.53 to 1.16 to 1.39. When the cell concentration increases, more and more cells are encapsulated within one microsphere. But the cell distribution becomes more non-uniform due to the larger standard deviation. For example, at the very low cell concentration of $0.5 \times 10^6$ cells/mL, there are only the microspheres with zero, one, and two cells, and the microspheres with two cells is negligible. In contrast, at the high cell concentration of $8 \times 10^6$ cells/mL, the microspheres observed have a much larger range of cell number within one microsphere from zero to eight.

Assuming an ideal uniform cell distribution (each microsphere has the same number of cells encapsulated), the theoretical cell number within one microsphere can be estimated based on the formula $\mu_t = \frac{4}{3} \pi R^3 C$ where $\mu_t$ is the theoretical cell number within one microsphere, $C$ is the selected cell concentration, and $R$ is average radius of the formed microspheres. Fig. 4 shows both the theoretical and experimental results of cell number within one microsphere under different cell concentrations. It is seen that the experimental mean cell numbers within one microsphere are very close to the
FIG. 4. Effect of the cell concentration on the mean cell number within one microsphere. The insert figure shows the fitted normal distribution based on the experimental results at the cell concentration of $8 \times 10^6$ cells/mL.

The theoretical values. At the cell concentration of $8 \times 10^6$ cells/mL, the microspheres have size of $68.86 \pm 4.82 \mu m$ in diameter. The corresponding theoretical cell number within one microsphere is 1.37, and the experimental mean cell number within one microsphere is 1.39. The difference is only 1.5%. However, at the very low cell concentration of $0.5 \times 10^6$ cells/mL, the diameter of the microspheres is 61.87 $\pm$ 3.81 $\mu m$. The corresponding theoretical cell number within one microsphere is 0.06, and the experimental mean cell number within one microsphere is 0.08, which results in a difference of 33.3%. The difference is much larger mainly due to limited cell distribution data points.

B. Effect of polymer concentration on cell distribution

In this section, the effect of sodium alginate concentration on the post-printing cell distribution within the microspheres has been investigated. The sodium alginate concentrations selected are 0.5%, 1%, 1.5%, and 2% (w/v) which are widely used in a variety of inkjet-based bioprinting applications. The other printing conditions are fixed: the excitation voltage 60 V, and the cell concentration $3 \times 10^6$ cells/mL. The cell-laden bioink is ejected out from the inkjet dispenser into the 2% (w/v) calcium chloride to form cell-laden microspheres. 1000 microspheres are randomly selected to analyze the cell distribution at each sodium alginate concentration, and the results are shown in Fig. 5.

It is seen from Fig. 5 that with the increase of the sodium alginate concentration the microspheres with low cell numbers decreases and the microspheres with high cell numbers increases. As the sodium alginate concentration increases from 0.5 to 2% (w/v), the microspheres without cells decrease significantly from 65.1% to 48.1%. On the contrary, with the increase of the sodium alginate concentration, the microspheres with two cells increases significantly from 5.2% to 13.9%, and the microspheres with three cells also increases from 1.1% to 4.9%. The microspheres with one cell are considered as the medium case between a low cell number and a high cell number. The microspheres with one cell almost have the same percentage at different sodium alginate concentrations. It is also seen from Fig. 5 that when the sodium alginate concentration increases from 0.5 to 2% (w/v), the maximum cell number within the microspheres increases from 3 to 4 to 5 to 6.

The normal distribution has been utilized to fit the experimental results at different sodium alginate concentrations, and the mean and the standard deviation of the distribution are obtained to characterize the cell distribution within the microspheres. In the insert.
figure of Fig. 6, it shows the representative fitted normal distribution based on the experimental results at the sodium alginate concentration of 2% (w/v). In Fig. 6, it shows that with the increase of the sodium alginate concentration the mean cell number within one microsphere increases. The main reason is that with the increase of the sodium alginate concentration the diameter of the microspheres increases. For example, at the sodium alginate concentration of 0.5% (w/v) the diameter of the microspheres is 61.07 μm while at the sodium alginate concentration of 2% (w/v) the diameter of the microspheres is 91.08 μm. At the low sodium alginate concentration (e.g., 0.5% (w/v)), the viscoelasticity of the bioink is negligible. The droplet formation process is governed by the balance of the inertial stress and capillary stress. The ejected ligament is very long, and quickly breaks up into several droplets due to the Rayleigh instability. The size of these droplets is usually similar, which is mainly determined by the ligament diameter. On the contrary, at the high sodium alginate concentration (e.g., 2% (w/v)), the viscoelasticity of the bioink is dominant. The droplet formation process is governed by the balance of the viscoelastic stress and the capillary stress. The ejected ligament is short, and the breakup time is significantly delayed by the bioink viscoelasticity. The fluid within the ligament has enough time to flow into the primary droplet. Usually only one primary droplet is formed, and the size of this primary droplet is large.

The theoretical cell number within one microsphere can be estimated based on the microsphere size and the cell concentration. Fig. 6 shows the comparison of the theoretical and experimental results of cell number within one microsphere at different sodium alginate concentrations. It is seen that at the sodium alginate concentrations of 0.5% and 1% (w/v) the experimental results are greater than the theoretical results while at the sodium alginate concentrations of 1.5% and 2% (w/v) the experimental results are smaller than the theoretical results. The main reason is due to different flow directions at the pinch-off location near the nozzle orifice reported in our previous studies. At the relatively low sodium alginate concentrations (e.g., 0.5% and 1% (w/v)), the viscoelasticity of the bioink is negligible and the jet velocity is large. The bioink at the pinch-off location near the nozzle orifice flows from the nozzle to the ligament. The ejected ligament is short, and the breakup time is significantly delayed by the bioink viscoelasticity. The fluid within the ligament has enough time to flow into the primary droplet. Usually only one primary droplet is formed, and the size of this primary droplet is large.

The theoretical value utilizes the microsphere size to calculate the mean cell number within one microsphere. Hence, the theoretical value is smaller than the experimental value. The higher the sodium alginate concentration is, the more the bioink flows back. It is seen from Fig. 6 that at the sodium alginate concentration of 1.5% (w/v) the difference between the theoretical value and the experimental value is 5.2% while at the at the sodium alginate concentration of 2% (w/v) the difference increases to 26.9%.

C. Effect of excitation voltage on cell distribution

In this section, the effect of excitation voltage on the post-printing cell distribution within the microspheres has been investigated. The excitation voltages selected are 40 V, 50 V, 60 V, and 70 V, which are widely used in a variety of inkjet-based bioprinting applications. The other printing conditions are fixed: the sodium alginate concentration 1% (w/v), and the cell concentration 3 × 10^6 cells/mL. The cell-laden bioink is ejected out from the inkjet dispenser into the 2% (w/v) calcium chloride to form cell-laden microspheres. 1000 microspheres are randomly selected to analyze the cell distribution under each excitation voltage. The normal distribution has been utilized to fit the experimental results at different excitation voltages, and the mean and the standard deviation of the distribution are obtained to characterize the cell distribution within the microspheres. The effect of excitation voltage on the post-printing cell distribution within the microspheres is shown in the figure of Fig. 6, it shows the representative fitted normal distribution based on the experimental results at the sodium alginate concentration of 2% (w/v).
Fig. 7. It is seen that at 40 V and 50 V the mean cell number within one microsphere is very similar, and after 50 V the mean cell number within one microsphere increases with the increase of the excitation voltage. At 40 V, only one primary droplet was formed. At 50 V, 60 V and 70 V, one primary droplet and one satellite droplet were formed during the droplet formation process. The size of the primary droplet and the satellite droplet was very similar. Typically, with the increase of the excitation voltage the microsphere diameter increases. For example, as the excitation voltage increases from 50 V to 60 V to 70 V, the microsphere diameter increases from 67.2 μm to 72.1 μm to 74.8 μm, which results in an increase of the mean cell number within one microsphere from 0.52 to 0.71 to 0.85, respectively. However, at 40 V and 50 V, the formed microsphere diameters are 68.6 μm and 67.2 μm, respectively. The similar microsphere diameters are due to different droplet formation: at 40 V only one primary droplet was formed while at 50 V one primary droplet was formed with one satellite droplet with a similar size. It is also seen from Fig. 7 that the experimental value of the mean cell number within one microsphere is greater than the theoretical value, which is mainly due to the shrinkage of the droplet during the crosslinking. The difference between the experimental value and the theoretical value increases from 1.1% to 8.3% to 20.3% to 28.7% as the excitation voltage increases from 40 V to 50 V to 60 V to 70 V.

During inkjet bioprinting, the cell-laden bioink is ejected out from an inkjet dispenser to form cell-laden droplets, which are crosslinked by the calcium chloride solution to form cell-laden microspheres. Each microsphere may have different numbers of cells encapsulated. This study investigates the effects of the printing variables (namely cell concentration, the polymer concentration, and the excitation voltage) on the post-printing cell distribution within the microspheres during inkjet printing of cell-laden bioink. Based on the results, it is seen that the mean cell number within one microsphere is determined by the cell concentration and microsphere size, and both are related to the droplet formation process. During inkjet-based bioprinting, the bioink used is composed of the biological material and living cells, which is considered as a suspension. The cell-laden droplet formation is a very complex process. The living cells play a critical role in ligament thinning and pinch-off during the droplet formation process. During inkjet-based bioprinting, the bioink is jetted from the nozzle with a specific orifice size, and the cell size of 15 μm is large enough to affect the bioink flow during the droplet formation process. Moreover, the cell-cell interaction and cell motion significantly affect the ligament thinning and pinch-off processes due to the large size of cells. Therefore, the living cells significantly affect the droplet formation process as well as the post-printing cell distribution in both macroscopic and microscopic ways. This study is the first study investigating the post-printing cell distribution during inkjet printing of cell-laden bioink. The next study will focus on the relation between the cell-laden droplet formation and the post-printing cell distribution.

During inkjet-based bioprinting, the microspheres utilized as building blocks are precisely deposited on the substrate to
fabricate 3D constructs based on a layer-by-layer manufacturing mechanism. The cell distribution within the microspheres significantly affects the cell distribution within the fabricated 3D constructs. Uniform cell distribution within the microspheres is beneficial to the post-printing cell viability as well as the following tissue fusion and maturation into functional tissues/organs. However, the results in this study show that the cell distribution within the microspheres is highly non-uniform. The standard deviation fitted from the experimental results is high, indicating that the cell distribution spreads out over a larger range. Yamaguchi et al. implemented the push-pull excitation waveform which was proved to be effective for stable ejection of one cell per droplet during inkjet bioprinting. The bioprinting process was reported to be reliable and stable to achieve uniform one cell per droplet. It is noted that the cell concentration was used only 1 × 10^4 cells/mL, which is much lower than the typical cell concentration on the order of 10^6 cells/mL in inkjet-based bioprinting. Optimization of the excitation waveform provides a promising approach to reduce and mitigate the large standard deviation of the cell distribution within the microspheres.

IV. CONCLUSIONS

This study is the first paper focusing on post-printing cell distribution within microspheres during inkjet printing of cell-laden bioink. The effects of the printing variables (namely cell concentration, the polymer concentration, and the excitation voltage) on the post-printing cell distribution within the microspheres have been investigated. The cell distribution within the microspheres has been quantified using the normal distribution in terms of the mean cell number within one microsphere and the associated standard deviation. It is found that 1) with the increase of the cell concentration both the mean cell number and the standard deviation increase. At the very low cell concentration of 0.5 × 10^3 cells/mL, the difference between the theoretical cell number and the experimental mean cell number 33.3% due to limited cell distribution data points; 2) with the increase of the sodium alginate concentration the mean cell number within one microsphere increases. At the low sodium alginate concentration (e.g., 0.5% and 1% (w/v)), the theoretical cell number is smaller than the experimental mean cell number due to shrinkage of the microspheres. At the high sodium alginate concentration (e.g., 1.5% and 2% (w/v)), the theoretical cell number is greater than the experimental mean cell number due to backflow of the bioink in the ligament; and 3) at 40 V and 50 V the mean cell number within one microsphere is very similar to similar microsphere sizes during different droplet formation processes. After 50 V the mean cell number within one microsphere increases with the increase of the excitation voltage due to the increase of the micropore diameter.

Future work may include: 1) multiphase simulation of inkjet bioprinting of cell-laden bioink, 2) effect of the excitation waveform on cell distribution, 3) effect of sedimentation on post-printing cell distribution, and 4) effect of cell distribution on post-printing cell viability, migration, and proliferation.

ACKNOWLEDGMENTS

This work was partially supported by the National Science Foundation (CMMI-1762282).
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