Effects of living cells on the bioink printability during laser printing

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Laser-induced forward transfer has been a promising orifice-free bioprinting technique for the direct writing of three-dimensional cellular constructs from cell-laden bioinks. In order to optimize the printing performance, the effects of living cells on the bioink printability must be carefully investigated in terms of the ability to generate well-defined jets during the jet/droplet formation process as well as well-defined printed droplets on a receiving substrate during the jet/droplet deposition process. In this study, a time-resolved imaging approach has been implemented to study the jet/droplet formation and deposition processes when printing cell-free and cell-laden bioinks under different laser fluences. It is found that the jetting behavior changes from no material transferring to well-defined jetting with or without an initial bulgy shape to jetting with a bulgy shape/pluming/splashing as the laser fluence increases. Under desirable well-defined jetting, two impingement-based deposition and printing types are identified: droplet-impingement printing and jet-impingement printing with multiple breakups. Compared with cell-free bioink printing, the transfer threshold of the cell-laden bioink is higher while the jet velocity, jet breakup length, and printed droplet size are lower, shorter, and smaller, respectively. The addition of living cells transforms the printing type from jet-impingement printing with multiple breakups to droplet-impingement printing. During the printing of cell-laden bioinks, two non-ideal jetting behaviors, a non-straight jet with a non-straight trajectory and a straight jet with a non-straight trajectory, are identified mainly due to the local nonuniformity and nonhomogeneity of cell-laden bioinks. *Published by AIP Publishing.*

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I. INTRODUCTION

Laser printing, a versatile laser-induced forward transfer (LIFT)-based technique,¹ has been emerging as an orifice-free droplet-based direct-write technique for a variety of applications, such as microelectronics² and tissue engineering,^{3–7} to name a few. Compared with filament-based bioprinting techniques, such as microextrusion,^{8–11} droplet-based laser printing can be easily implemented for the fabrication of complex and heterogeneous constructs with a printing resolution defined by the morphology of printed droplets. Because of its orifice-free nature, laser printing can handle a wide range of viscous bioinks without suffering from possible clogging associated with other orifice-based drop-on-demand (DOD) bioprinting techniques such as

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inkjet printing.^{12–14} In particular, it has been applied to print various bioinks made from different biomaterials and biological materials including proteins,¹⁵ DNA,¹⁶ cell-encapsulating hydrogel beads,¹⁷ and living cells.^{3,18,19} Furthermore, laser printing has been successfully implemented to fabricate two-dimensional (2D) and three-dimensional (3D) cellular constructs.^{4,19,20}

In order to optimize the laser printing performance, the printability of different bioinks should be carefully studied. While the printing mechanism of Newtonian glycerol-based^{21,22} and viscoelastic alginate-water^{23–25} solutions has been investigated, unfortunately, thus far there is no work conducted to understand the printing dynamics during the laser printing of cell-laden bioinks. Cell-laden bioink contains living cells and is different from the aforementioned homogeneous Newtonian glycerol-based and viscoelastic alginate-water solutions in terms of the nature of fluids being printed: suspension versus solution. Macroscopically, suspended living cells change the rheological properties of bioinks and consequently the printing dynamics; microscopically, the interaction of suspended living cells may lead to cell aggregation, resulting in non-ideal jetting behaviors. The effects of living cells on the bioink printability during laser printing are to be elucidated for wide adoption of the laser bioprinting technique.

The objective of this study is to investigate the effects of living cells on the viscoelastic bioink printability during laser printing using a time-resolved imaging approach. Herein, the printability of viscoelastic bioink is defined as the ability to generate well-defined jets during the jet/droplet formation process as well as well-defined printed droplets on a receiving substrate during the jet/droplet deposition process. The effects of living cells on the bioink printability are mainly investigated by monitoring the jet/droplet formation and deposition dynamics under different laser fluences using a time-resolved imaging approach. This paper is organized as follows. First, the bioink preparation and related rheological property characterization as well as the laser printing experimental setup are introduced. Second, the jet/droplet formation and deposition processes of cell-laden bioinks are investigated and are further compared with those of cell-free bioinks. Third, the formation of non-ideal jetting behaviors during laser printing of cell-laden bioink has been discussed. Fourth, dimensionless number-based phase diagrams are proposed to classify different laser printing regimes. Furthermore, the observation during the laser printing of cell-laden bioinks is compared with that during inkjet printing. Finally, some conclusions are drawn regarding the effects of living cells on the bioink printability.

II. BACKGROUND

During a typical ultraviolet (UV) laser-based bioprinting process, bioink to be printed is coated as the donor film on the bottom side of a UV light transparent quartz support. A focused UV laser pulse is guided through the quartz support and absorbed by an energy-absorbing material (water of bioinks in this study) at the interface of the quartz support and donor film, resulting in a high-temperature, high-pressure vapor bubble at the interface between the donor film and quartz support. As a result of the pressure of the laser-induced vapor bubble, part of the donor film may be propelled forward as a jet or droplet, which impinges and further deposits onto a receiving substrate. Generally, after the initial transient laser-matter interaction and bubble formation processes, there are two main sequential processes: jet/droplet formation and jet/droplet deposition. Thus far, most laser printing-related research efforts have focused on the analysis of the size and morphology of printed droplets.^{1,26,27} It is found that the resulting size and morphology depend on a number of operating parameters including the laser spot size,^{28,29} coating film thickness,³⁰ laser fluence,^{1,25,30,31} and direct-writing height (the stand-off distance between the donor film and receiving substrate).^{15,25,27,32} Accordingly, the jet/droplet formation^{21,22,24,33} and deposition²⁵ processes have also been studied to have a better understanding of the printing mechanism to fully realize the potential of laser printing.

The jet/droplet formation process, in particular, has been investigated during the laser printing of various bioinks, such as Newtonian glycerol-based^{21,22} and viscoelastic alginate^{23,24} solutions. For Newtonian glycerol-based solutions, three main jetting regimes have been identified during the jet/droplet formation process: (1) no material transferring, in which no material is transferred, (2) well-defined jetting, in which well-defined jetting/droplets may be obtained, and

(3) jetting with a bulgy shape/pluming/splashing, in which satellite droplets may appear.^{21,22} For viscoelastic alginate solutions, there is a unique jetting regime in addition to the three regimes as mentioned above, during which a jet with an initial bulgy shape may develop into a well-defined jet.²⁴ The jet/droplet deposition process has also been investigated for both Newtonian glycerol-based³⁴ and viscoelastic alginate²⁵ solutions. For glycerol-based solutions, the formation of single droplets on a receiving substrate is mainly due to the contact of ejected liquid jets with the receiving substrate.³⁴ For viscoelastic alginate solutions, the deposition process has been classified into three types based on the jet/droplet impingement type: (1) droplet-impingement printing, (2) jet-impingement printing with a single breakup, and (3) jet-impingement printing with multiple breakups.²⁵

The addition of living cells alters the nature of bioinks, further affecting the jet/droplet formation and deposition processes. Living cells can be considered as soft particles in bioink suspension. While the formation of droplets from particle-laden suspensions has been investigated during dripping,^{35–37} continuous inkjet (CIJ) printing,³⁸ and drop-on-demand (DOD) inkjet printing,^{39–42} most particles studied were hard such as alumina powders,³⁹ poly (methyl methacrylate) (PMMA) particles,^{35,36} pigments,⁴¹ silver nanoparticles,⁴⁰ and polystyrene particles.³⁷ The role of the particles in the overall droplet formation process has been investigated in terms of the resulting droplet size,^{35,36,40} droplet velocity,⁴⁰ number of satellites,^{35,36} and the formation of skewed trajectories and non-axisymmetric ligaments.⁴¹ Living cell-laden bioinks have also been studied during inkjetting;⁴² however, the material ejection (jet formation)⁴³ and jet breakup mechanisms during laser printing²⁵ are different from those during inkjet printing,⁴⁴ and the knowledge of printing dynamics during the laser printing of cell-laden bioinks is still elusive, which is the subject of this study.

III. MATERIALS AND METHODS

A. Bioink preparation

Typical bioinks are made from living cells, hydrogel, and/or other additives. As the most common cells of connective tissues in animals, fibroblasts have been widely used in biofabrication research.^{8,9,45} Alginate has received more and more attention in tissue engineering applications due to its biocompatibility and ease of gelation.^{46,47} Moreover, alginate can be chemically and physically modified to have an improved performance in cell adhesion^{48,49} and gel degradation^{49,50} and has been widely used in 3D bioprinting applications.^{11,13,14,19,45,51,52} As such, bioinks in this study were composed of fibroblast as the model cell and sodium alginate as the model hydrogel.

NIH 3T3 mouse fibroblasts (ATCC, Rockville, MD) were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Sigma Aldrich, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS) (HyClone, Logan, UT) in a humidified 5% CO₂ incubator at 37 °C, and the culture medium was replaced every 3 days as required. To prepare bioinks for printing, freshly 90% confluent flasks of 3T3 fibroblasts were washed twice with Dulbecco's phosphate-buffered saline (PBS; Cellgro, Manassas, VA) and incubated with 0.25% Trypsin/EDTA (Sigma Aldrich, St. Louis, MO) for 5 min at 37 °C to detach cells from the culture flasks. Then, the cell suspension was centrifuged at 1000rpm for 5 minutes at room temperature, and the resulting pellet was re-suspended in the DMEM complete cell culture medium with 1% penicillin and streptomycin (Sigma, St. Louis, MO). The re-suspended cells were adjusted to 1×10^7 cells/ml suspension for bioink preparation.

The 1×10^7 cells/ml cell suspension was mixed with 4% sodium alginate (Sigma-Aldrich, St. Louis, MO) in the DMEM complete cell culture medium with 1% penicillin and streptomycin at a volume ratio of 1:1, resulting in a final cell-laden bioink (2% alginate/DMEM + 5×10^6 cells/ml) with a cell concentration of 5×10^6 cells/ml and an alginate concentration of 2% for laser printing. Such a cellular bioink guarantees good structural quality of printed constructs while maintaining satisfactory cell viabilities.¹⁹ For comparison, cell-free bioink (2% alginate/DMEM solution) was also prepared as a control by dissolving 4% sodium alginate

(Sigma-Aldrich, St. Louis, MO) into the DMEM complete cell culture medium with 1% penicillin and streptomycin at a volume ratio of 1:1.

B. Bioink rheological property characterization

Bioink material properties, in particular, rheological properties, significantly affect the jet/droplet formation and deposition processes during laser printing. Herein, cell-laden (2% alginate/DMEM and 5×10^6 cells/ml) and cell-free (2% alginate/DMEM) bioinks were characterized in terms of the density, shear viscosity, surface tension, storage and loss moduli, and longest relaxation time. Using a cup-and-bob rheometer (ARES, TA Instruments, New Castle, DE), the shear viscosities of the cell-laden and cell-free bioinks were measured under steady shear tests, and the storage (G') and loss (G'') moduli were measured using oscillatory shear tests, characterizing the storage energy (the elastic effect) and the energy dissipated as heat (the viscous effect), respectively. The surface tension of the bioinks was measured using a pendant drop method (Attension ThetaLite 101, Biolin Scientific, AB, Sweden), and the density of the bioinks was measured by averaging the weight of 1 ml bioinks. In order to estimate the longest relaxation time, a high-speed camera (Photron Fastcam SA5, San Diego, CA) was used to capture filament thinning evolution images at 4000 frames per second (fps) during bioink dripping. The measurement results are shown in Table I and Fig. 1, and some rheological properties of 2% water-based alginate solutions²⁴ are also included in Table I for comparison. All the measurements were repeated three times, and error bars represent plus/minus one standard deviation in this study.

Figure 1(a) shows the average steady shear viscosities of the bioinks as a function of the shear rate. The measured zero-shear viscosity (η_0) increases from 205.9 cP for the cell-free bioink to 218.9 cP for the cell-laden bioink, and both the cell-free and cell-laden bioinks show the shear-thinning behavior mainly due to the dissolved sodium alginate.^{42,53} The storage (G') and loss (G'') moduli as a function of angular frequency are shown in Fig. 1(b), showing that both G' and G'' increase slightly with the addition of living cells. The increase of G' and G'' indicates that the addition of living cells introduces more elastic and viscous properties to bioinks. Specifically, the storage modulus increase is mainly due to the inter-particle contact and interaction,⁵⁴ while the loss modulus increase can be explained by the increase of hydrodynamic energy dissipation due to the distortion of the velocity field in the vicinity of particles,⁵⁵ which are living cells in this study. The surface tension of the cell-free and cell-laden bioinks is 44.4 ± 0.22 and 42.6 ± 0.15 mN/m, respectively. The decrease of the surface tension is attributed to the addition of living cells, which change the intermolecular interaction⁵⁶ and may be attached to the interface as titania particles in deionized water,⁵⁷ decreasing the internal energy and lowering the surface tension.

The relaxation time of viscoelastic fluids is one of the most important factors in understanding viscoelastic jet/droplet formation and deposition dynamics, and it is characterized based on the elasto-capillary thinning mechanism during dripping. During elasto-capillary thinning of viscoelastic ligaments, the ligament diameter ($D(t)$) follows an exponential decay as $D(t)/D_0 \approx (G_1 D_0 / 4\sigma)^{1/3} \exp(-t/3\lambda)$,^{58,59} where $D(t)$ is the minimum ligament diameter, D_0 is the initial ligament diameter, G_1 is related to the elastic modulus, σ is the surface tension, and

TABLE I. Material properties and associated dimensionless numbers of alginate solution and cell-free and cell-laden bioinks.

Solution or bioink	Density ρ (g/cm ³)	Viscosity η_0 (cP)	Surface tension σ (mN/m)	Longest relaxation time λ (μ s)	Oh	Ec
2% alginate	1.02	139.50	44.60	1649.70	2.39	7.07
Cell-free (2% alginate/DMEM)	1.02	205.90	44.36	2411.96	3.53	6.93
Cell-laden (2% alginate/ DMEM + 5×10^6 cells/ml)	1.02	218.96	42.62	2620.54	3.83	6.80

FIG. 1. (a) Shear viscosity and (b) storage (G') and loss moduli (G'') of cell-free bioink (2% alginate/DMEM) and cell-laden bioink (2% alginate/DMEM and 5×10^6 cells/mL).

λ is the longest relaxation time. Then, the longest relaxation time is found based on the slope ($-1/(3\lambda)$) of the log-log plot of the scaled minimum filament diameter [$D(t)/D_0$] and time (t) during bioink ligament thinning as shown in Fig. 2. The bioink was dripped through a nozzle with an inner diameter of 0.84 mm to form a droplet each time, during which the flow rate was precisely controlled at 0.1 ml/min using a syringe pump. The fitted longest relaxation time is 2411.96 μs and 2620.54 μs for the cell-free and cell-laden bioinks, respectively, showing that the cell-laden bioink has a longer relaxation time.

C. Experimental setup and design of experiments

The laser printing apparatus herein consists of an argon fluoride (ArF) excimer laser (Coherent ExciStar, Santa Clara, CA; 193 nm, 12 ns full-width half-maximum, and 2 Hz repetition rate), an optical beam deliver system, a ribbon composed of a UV transparent quartz support (Edmund Optics, Barrington, NJ) and a 50 μm bioink donor film coated on its bottom side, and a poly-L-lysine coated glass slide (Polysciences, Warrington, PA) as a receiving substrate as shown in Fig. 3. Computer-controlled XY translational stages (Aerotech, Pittsburg, PA) were used to control the movement of the ribbon and the receiving substrate, respectively. The direct-writing height was fixed as 2.0 mm, the laser spot diameter was 150 μm , and more setup details can be found in a previous study.²⁵ For illustration, the right side of Fig. 3 shows some

FIG. 2. Scaled minimum filament diameter ($D(t)/D_0$) as a fitted exponentially decaying function of time.

FIG. 3. Laser printing setup schematic and two straight and Y-shaped cellular tubes¹⁹ [Adapted with permission from Xiong *et al.*, *Biofabrication* **7**, 045011 (2015). Copyright 2015 IOP Publishing] (right side) fabricated using laser printing.

representative straight and Y-shaped cellular tubes fabricated using laser printing in a previous study.¹⁹

As shown in Table II, two setups (Setups A and B) were implemented to investigate the effect of living cells on the bioink printability, and the laser fluence was adjusted in the range of 500~1500 mJ/cm² with an interval of 200 mJ/cm² after considering the 15% energy loss of the quartz support. In particular, Setup A was conducted to investigate the effects of laser fluence on the printing dynamics of the cell-laden bioink, and Setup B was implemented to study the effects of the living cells on the printing dynamics by comparing the jet/droplet formation and deposition processes during laser printing of cell-free and cell-laden bioinks under different laser fluences.

The jet/droplet formation and deposition processes were captured using a JetXpert imaging system (ImageXpert Inc., Nashua, NH) based on a time-resolved imaging approach.²⁴ The ImageJ software (National Institute of Health, Bethesda, Maryland) was utilized to process the captured images as well as optical micrographs of printed microarrays. The jet velocity U was calculated by a linear fitting of the jet head position dependence on the measurement time before the jet or droplet impinges upon a receiving substrate.^{24,60} Diameters of printed droplets were measured based on the average of five representative printed droplets. For those printed droplets with irregular shapes, their equivalent feature diameter was estimated based on their projected area on the receiving substrate. For those droplets accompanied by secondary droplets, their equivalent feature diameter was estimated based on their equivalent total volume found by assuming a constant contact angle and a spherical cap shape of all droplets.

IV. RESULTS

A. Representative observations during laser printing of cell-laden bioink

Suspended living cells affect the material properties of bioinks as well as the printing dynamics during laser printing. Figure 4 shows some representative time-resolved images of the jet/droplet formation and deposition processes as well as corresponding micrographs of printed droplets during the laser printing of the cell-laden bioink under different laser fluences. The inset of Fig. 4(c) shows a representative droplet embedded with living cells. During the jet/droplet formation process, most of the incident laser energy is converted into the kinetic energy

TABLE II. Design of experiments.

Setup	Bioink	Laser fluence (mJ/cm ²)
A	Cell-laden bioink (2% alginate/DMEM+ 5×10^6 cells/ml)	500, 700, 900, 1100,
B	Cell-laden bioink (2% alginate/DMEM+ 5×10^6 cells/ml), Cell-free bioink (2% alginate/DMEM)	1300, and 1500

FIG. 4. Time-resolved representative images during laser printing of cell-laden (2% alginate/DMEM and 5×10^6 cells/ml) bioink under laser fluences of: (a) 700 mJ/cm², (b) 900 mJ/cm², (c) 1100 mJ/cm², (d) 1300 mJ/cm², and (e) 1500 mJ/cm². (c) Inset shows a representative deposited droplet embedded with living cells. The scale bars are 200 μ m.

of the initial laser-induced bubble,⁴³ which is eventually converted into viscous dissipation, elastic, surface, and kinetic energies of the forming jet/droplet. As the incident laser fluence increases, the kinetic energy of the forming jet increases, resulting in a higher jet velocity. Depending on the jet velocity, a jet may experience the Rayleigh breakup, the first wind-induced breakup, the second wind-induced breakup, or even atomization, which significantly affects the jet morphology and breakup mechanism.²⁴

For Rayleigh breakup, the pinch-off of the filament is mainly due to the inertio-capillary force while the aerodynamic force caused by the relative motion between the liquid jet and ambient gas is negligible. As the jet velocity increases, the ligament may be subject to the first wind-induced breakup, during which aerodynamic force is large enough (as high as 10% of the surface tension force) to influence the jet breakup, and the perturbation on the filament has a higher growth rate, resulting in a faster breakup process and shorter breakup length. When the jet velocity further increases, the growth of perturbation is dominated by the aerodynamic force. Then, a jet may experience the second wind-induced breakup and atomization mechanisms, during which the most unstable perturbations turn to be those with shorter wavelengths than the ones during Rayleigh breakup and first wind-induced breakup. Under the second wind-induced and atomization mechanisms, the resulting drop size is much smaller than the jet diameter.

B. Effects of laser fluence on the printing dynamics of cell-laden bioink

The effects of the laser fluence on the jet/droplet formation and deposition dynamics during the laser printing of the cell-laden bioink are investigated. Three distinct jetting regimes are identified with the increase of laser fluence during the jet/droplet formation process: (1) undesirable no-material transferring {700 mJ/cm² [Fig. 4(a)]}, in which the kinetic energy supplied from the laser-induced bubble is lower than the threshold energy for jet formation, and the initial liquid jet recoils back into the coating film with no materials transferred; (2) desirable well-defined jetting with or without an initial bulgy shape (900, 1100, and 1300 mJ/cm² [Fig. 4(b)–4(d)]), in which the bubble kinetic energy is higher than the threshold energy, and the

ejected bioink is confined to a well-defined jet, which eventually breaks up into droplets due to the Rayleigh instability; and (3) undesirable jetting with a bulgy shape/pluming/splashing [Fig. 4(e)], in which the bubble kinetic energy overcomes the surface tension of the bioink film, resulting in bubble bursting and the formation of multiple disordered bioink jets, which lead to the formation of uncontrollable scattered satellite droplets on the substrate.

The jetting dynamics during the well-defined jetting regime are of fabrication interest and have been further analyzed. As shown in Fig. 5(a), the jet velocity increases monotonically with the laser fluence, mainly due to the increased incident laser energy. This observation is similar to those reported during laser printing of Newtonian glycerol^{21,22} and viscoelastic alginate²⁴ solutions. As reported, the jet velocity increases nearly linearly with the laser fluence during laser printing of Newtonian fluids.^{21,22} However, the change in jet velocity is sensitive to the applied laser fluence, and the slope of the jet velocity curve in Fig. 5(a) increases with the laser fluence. This is attributed to the shear-thinning property [Fig. 1(a)] of the cell-laden bioink. With the increase of laser fluence, the initial laser-induced bubble expansion generates a higher shear rate, leading to a reduced bioink viscosity and less energy loss due to viscous dissipation. As such, the resulting kinetic energy of the jet increases more than that during Newtonian fluid printing when the laser fluence increases, resulting in an increasing slope of the jet velocity curve.

Generally, there are two impingement-based printing types observed during the sequential jet/droplet deposition process: (1) droplet-impingement printing and (2) jet-impingement printing with multiple breakups. The jet breakup length determines the impingement types at a fixed direct-writing height during the deposition process. At a laser fluence of 900 mJ/cm² [Fig. 4(b)], the jet breakup length is smaller than the direct-writing height, which is considered as droplet-impingement printing. For droplet-impingement printing, the jet breaks up into several fragments before landing on the substrate, forming several secondary droplets scattering around as shown in Fig. 4(b). As the laser fluence increases (from 900 to 1100 and 1300 mJ/cm²), the jet does not break up before it lands on the substrate, which is considered as jet-impingement printing. For jet-impingement printing with multiple breakups, the jet keeps thinning and feeding into the primary droplet formed on the receiving substrate. This thinned jet may form a beads-on-a-string (BOAS) structure,²⁵ which means several liquid beads interconnected by thin liquid filaments. This BOAS structure breaks up into several fragments at a later time, which leads to the formation of secondary droplets around the main droplet as shown in Figs. 4(c) and 4(d). This observation is similar to that during the printing of 2% alginate solution.²⁵

During droplet-impingement printing, the primary droplet is surrounded by secondary scattered droplets with a similar size, while during jet-impingement printing with multiple breakups, a well-defined primary droplet is formed accompanied by several secondary droplets with much smaller sizes as shown in Fig. 5(b). Therefore, the jet-impingement printing with multiple breakups is preferred for better printing quality when compared with the droplet-impingement

FIG. 5. (a) Jet velocity and (b) printed droplet diameter as a function of laser fluence during laser printing of the cell-laden (2% alginate/DMEM and 5×10^6 cells/ml) and cell-free (2% alginate/DMEM) bioinks. The scale bars are 200 μm.

printing. The printed droplet size increases almost linearly when the laser fluence increases, which is consistent with the observations during the laser printing of glycerol,¹ polymer,⁶¹ and alginate²⁵ solutions.

Figure 6 further illustrates the jetting regimes/printing types as a function of laser fluence during the laser printing of cell-free and cell-laden bioinks. The solid and dashed lines are used to delineate different jetting regimes/printing types. As the laser fluence increases, the printing type changes from no material transferring to droplet-impingement printing to jet-impingement printing with multiple breakups to jetting with a bulgy shape/pluming/splashing during the laser printing of cell-free and cell-laden bioinks. It is noted that it requires a higher laser fluence to achieve the same printing type for cell-laden bioinks.

C. Effects of living cells on printing dynamics

The effects of living cells on the jet/droplet formation and deposition dynamics have been investigated by printing the cell-laden and cell-free bioinks under different laser fluences. Compared to that of the cell-free bioink, the transfer threshold of the cell-laden bioink is higher as seen from Fig. 6, but the jet velocity and printed droplet size are smaller as shown in Figs. 7(a) and 7(b). As measured, the addition of living cells increases both the viscous and elastic properties of the cell-laden bioink, so more viscous energy is dissipated and more elastic energy is stored in the forming jet, which leads to a higher transfer threshold for the printing of the cell-laden bioink. Under the same laser fluence, more energy is consumed to overcome the higher viscous and elastic effects of the cell-laden bioink, leaving less kinetic and surface energies to the forming jet as well as fewer material transferred, resulting in a lower jet velocity and a smaller printed droplet diameter. At a fixed direct-writing height, for the printing of cell-laden bioinks, the lower jet velocity may allow a jet to have enough time to break up before it arrives at the receiving substrate. As shown in Figs. 7(c) and 7(d), under the same laser fluence of 900 mJ/cm², the cell-laden bioink [Fig. 7(d)] has a shorter jet breakup length than that of the cell-free bioink [Fig. 7(c)], changing the printing type from jet-impingement printing with multiple breakups to droplet-impingement printing.

D. Effects of living cells on the formation of non-ideal jetting behaviors

The jetting dynamics during laser printing is significantly affected by the bioink material properties, especially the rheological properties. The bioink used in this study is composed of biomaterials (sodium alginate) and living cells (fibroblasts) and is considered as a viscoelastic

FIG. 6. Jetting regimes/printing types as a function of laser fluence during laser printing of the cell-laden (2% alginate/DMEM and 5×10^6 cells/ml) and cell-free (2% alginate/DMEM) bioinks. The delineating solid and dashed lines are for illustration only.

FIG. 7. (a) Jet velocity and (b) printed droplet diameter of cell-laden and cell-free bioinks under different laser fluences, and time-resolved printing dynamics images under a 900 mJ/cm² laser fluence when using: (c) cell-free and (d) cell-laden bioinks. The scale bars are 200 μm.

cellular suspension instead of a homogeneous viscoelastic polymer solution. The cell-laden bioink (5×10^6 cells/ml) has a volume fraction ϕ of 0.88%, where $\phi = n\pi d_p^3/6$, n is the particle number density, and d_p is the particle diameter (15 μm for living cells). Then, it is a dilute ($\phi \leq 2\%$) particle-laden suspension, and the inter-cellular interaction is considered insignificant. However, the addition of living cells affects the jetting dynamics both macroscopically and microscopically. Macroscopically, suspended living cells change the material properties of bulk bioinks, such as the viscosity, storage and loss moduli, and relaxation time, which indirectly influence the jetting dynamics as observed in this study. Microscopically, suspended living cells may lead to the formation of cell aggregates, resulting in local nonuniformity and nonhomogeneity,^{62,63} which may result in non-ideal jetting behaviors during the laser printing of cell-laden bioinks.

During the experiments in this study, two types of non-ideal jetting behaviors are observed as shown in Fig. 8(a): a non-straight jet with a non-straight trajectory (case 1) and a straight jet with a non-straight trajectory (case 3). It is noted that when a straight jet is formed, it may have two fates: a straight jet with a straight trajectory (case 2) and a straight jet with a non-straight trajectory (case 3). Of these three jetting behaviors, the straight jet with a straight trajectory is considered desirable, while the other two are considered non-ideal. The occurrence of these three types of jets is further investigated based on the morphology of 100 forming jets under a 1100 mJ/cm² laser fluence. It is found that 81% of them are the straight jet with a straight trajectory (case 2), 11% are the non-straight jet (case 1), and 8% are the straight jet with a non-straight trajectory (case 3).

During laser printing, the initial laser-induced bubble expansion propels the underneath coating layer downwards, which forms a jet. The jet trajectory is dependent on the direction of the resultant ejection force, which is due to the inertial, capillary, viscous, and elastic forces acting on the jet. For ideal scenarios, the bioink film may have no cell aggregates, and the direction of the resultant ejection force is downward perpendicularly, resulting in the formation of straight jets as shown as Cases 2 and 3 in Fig. 8(a). However, cell aggregates, if formed, may induce local nonuniformity and nonhomogeneity in the bioink film, which can result in asymmetric flows during jet formation. As such, the resultant ejection force and the jetting direction deviate from the perpendicular centerline as shown as case 1 in Fig. 8(a).

FIG. 8. (a) Schematics illustrating the formation of different types of jets during the laser printing of the cell-laden bioink and (b) the lengths of straight segments of eight straight jets with a non-straight trajectory (case 3) under a laser fluence of 1100 mJ/cm^2 .

It should be pointed out that a straight jet does not guarantee a straight trajectory. In this study, the forming jet starts thinning when the jet diameter is around $60 \mu\text{m}$. During jet thinning, living cells may interact with each other and form cell aggregates. When the jet diameter is comparable to the living cell size (around $15 \mu\text{m}$), the effects of individual cells as well as cell aggregates on the jetting behavior have to be considered. The existence of cells and cell aggregates may perturb the symmetry of ligament flow, resulting in the formation of straight jets with a non-straight trajectory at a certain moment as shown as case 3 in Fig. 8(a). Specifically, Fig. 8(b) shows the length of the straight segment of the eight straight jets with a non-straight trajectory (case 3), and the average length of initially straight segments is $1076 \pm 99 \mu\text{m}$ and the average diameter at the tip of initially straight segments is $30 \pm 6 \mu\text{m}$. This indicates that the straight to non-ideal jet transition is consistent during the laser printing

of cell-laden bioinks, and the underlying reason of this transition is to be elucidated in a future study.

V. DISCUSSIONS

There are several potential sources to introduce cell injury during laser printing, such as UV radiation, heat exposure introduced by the laser-matter interaction-induced temperature rise, and/or mechanical stress. Of them, the possible cell injury is mainly attributed to the mechanical damage while the thermal and UV damages are considered negligible during laser printing.^{18,64,65} In particular, living cells may be subject to mechanical stress during the formation of bubbles⁶⁶ and jet/droplet, and/or the impact between cells and their receiving substrate during deposition.^{67,68} The mechanical stress-induced cell injury⁶⁹ may or may not be reversible depending on the magnitude and duration of the mechanical stress and the cell type. Such printing-induced cell injury is not within the scope herein and to be investigated in detail in a future study. This section focuses on the construction of phase diagrams to appreciate the printability of cell-laden bioinks as well as the comparison between inkjet and laser bioprinting.

A. Laser printing phase diagrams

To characterize the capillary thinning and breakup of free surface viscoelastic liquid filaments during the jet/droplet formation process, material property-based dimensionless Ohnesorge number (Oh) and elasto-capillary number (Ec) are derived based on the relative significance of three governing time scales: visco-capillary time scale $t_v = \eta_0 R / \sigma$, inertio-capillary or Rayleigh time scale $t_c = \sqrt{\rho R^3 / \sigma}$, and λ , where R is the characteristic length which is taken as the laser spot radius herein ($75 \mu\text{m}$). Of them, the Ohnesorge number ($\text{Oh} = \frac{t_v}{t_c} = \frac{\eta_0}{\sqrt{\rho \sigma R}}$) represents the ratio of viscous to inertial effect and the elasto-capillary number ($\text{Ec} = \frac{\lambda}{t_v} = \frac{\lambda \sigma}{\eta_0 R}$) represents the elastic to viscous effects. In addition, the Weber number ($\text{We} = \frac{\rho R U^2}{\sigma}$) is usually introduced as a process dynamics-related dimensionless number to study the jetting dynamics. For viscoelastic fluids, phase diagrams constructed based on these three dimensionless numbers have been frequently used to evaluate the printability of various fluids during laser printing.^{22,24,25}

Herein, the printability of the cell-free and cell-laden bioinks is mapped out in two (We, Oh) and (We, Ec) phase diagrams, respectively, which can be derived from the (We, Oh, Ec) space proposed for the laser printing of viscoelastic alginate solutions.²⁵ Different regimes have been identified to represent various printing types in the two (We, Oh) and (We, Ec) phase diagrams constructed for water-based alginate solutions.²⁵ In this study, the (We, Oh) and (We, Ec) phase diagrams are also constructed for the two bioinks: cell-free DMEM/alginate bioink in dashed symbols and cell-laden DMEM/alginate bioink in solid symbols. As seen from Fig. 9, the delineation of different printing types is similar based on the two phase diagrams of the cell-free alginate, cell-free DMEM/alginate, and cell-laden DMEM/alginate bioinks, indicating that the effects of the addition of DMEM and living cells on the bioink printability can be sufficiently captured by the material properties-related dimensionless numbers (Oh and Ec). The dashed lines in Fig. 9 separate the droplet-impingement printing, jet-impingement printing with multiple breakups, and jet-impingement printing with single breakup regimes from each other, and they are for illustration only based on the applicable experimental data. Generally, for given Oh or Ec numbers, as the We number increases, the printing type changes from droplet-impingement printing to jet-impingement printing with multiple breakups. At high Oh or low Ec numbers, representing high alginate concentrations, the formation of BOAS structures is suppressed. As a result, the printing type remains to be jet-impingement printing with single breakup. For a given We number, the printing type may change from droplet-impingement printing to jet-impingement printing with multiple breakups to jet-impingement printing with single breakup as the Oh number increases or the Ec number decreases, which reflects an increasing alginate concentration or the addition of living cells.

FIG. 9. Printing type: (a) as a function of We and Oh numbers and (b) as a function of We and Ec numbers.

B. Comparison between laser and inkjet printing of cell-laden bioinks

Of various 3D bioprinting techniques, laser printing^{19,70} (for the printing of highly viscous inks) and inkjet printing^{12,13} (for the printing of less viscous inks) enable the fabrication of complex heterogeneous constructs using printed droplets as building blocks. To qualitatively compare the difference regarding the effects of cells on the jetting dynamics during laser and inkjet printing, their jetting behaviors are compared when inkjet printing 1% alginate/DMEM and 5×10^6 cells/ml with a viscosity of 27.50 cP as shown in Fig. 10(a) and laser printing 2% alginate/DMEM and 5×10^6 cells/ml with a viscosity of 218.96 cP. It should be noted that the viscosities of the two bioinks for inkjet and laser printing are different and the comparison is only to qualitatively evaluate some main differences. The inkjetting process utilized a bipolar excitation waveform^{13,42} with an excitation voltage of 45 V and an excitation frequency of 50 Hz. The incident laser pulse for laser printing had a laser fluence of 1100 mJ/cm² and a 2 Hz repetition rate. In addition to their different working mechanisms, there are two main jetting behavior differences as observed: the average jet velocity and the formation of non-ideal jetting behaviors.

First, the resulting average jet velocity is different between inkjet and laser printing. During inkjet printing, a bipolar excitation waveform is applied to a piezoelectric actuator, which deforms the nozzle dispenser, generating pressure pulses. The pressure pulses propagate

FIG. 10. Time-resolved images showing (a) an inkjetting printing process (1% alginate/DMEM and 5×10^6 cells/ml),⁴² (b) non-ideal jetting during bioink inkjetting (1% alginate/DMEM and 5×10^6 cells/ml),⁴² (Reprinted (adapted) with permission from (Langmuir 30, 9130 (2014)). Copyright (2014) American Chemical Society), and (c) schematic of typical non-ideal jetting behaviors during inkjetting. The scale bars are 200 μ m.

inside the nozzle chamber and superimpose together to result in a complex pressure wave,⁴⁴ which results in a jet velocity of 3.31 m/s as measured. During laser printing, the energy of the incident laser pulse is absorbed by the energy-absorbing material, forming a high-temperature, high-pressure vapor bubble. The expansion of the bubble results in a jet velocity of 16.33 m/s as measured. As such, laser printing usually results in a jet with a much higher jet velocity (on the order of 10 m/s) than that during inkjet printing (on the order of 1 m/s).

Second, the mechanism for the formation of non-ideal jetting behaviors is different. Inkjet printing is an orifice-based printing technique, which ejects materials to form a liquid jet through an orifice. In contrast, laser printing is an orifice-free printing process, which catapults materials away from a coating film to form liquid jets. During the inkjet printing of cell-laden bioinks, jets with a non-straight trajectory have been reported [Fig. 10(b)]⁴² and are illustrated in Fig. 10(c), which is mainly attributed to accumulated cells near the orifice and the non-ideal wetting condition at the nozzle tip.⁴² During the laser printing of cell-laden bioinks, the non-ideal jetting behaviors are mainly due to the local nonuniformity and nonhomogeneity of cell-laden bioinks as discussed in this study. It is noted that accumulated cells and the non-ideal wetting at the nozzle tip during inkjetting only affect the trajectory of ligament tail instead of the forming droplet. Because the ligament tail has a much lower velocity/kinetic energy than that of a forming droplet, the overall deviation of jetting trajectory, if have, during inkjet printing is minimized. During laser printing, however, the higher jet velocity promotes any non-ideal jetting behavior once initiated.

VI. CONCLUSIONS AND FUTURE WORK

The effects of living cells on the bioink printability have been studied using a time-resolved imaging approach when printing the cell-free (2% alginate/DMEM) and cell-laden (2% alginate/DMEM and 5×10^6 cells/ml) bioinks under different laser fluences. With the increase of laser fluence, three main jetting regimes are identified during the jet/droplet formation process: no material transferring, well-defined jetting with or without an initial bulgy shape, and jetting with a bulgy shape/pluming/splashing. During the well-defined jetting, two impingement-based deposition and printing types are identified: droplet-impingement printing and jet-impingement printing with multiple breakups.

Some main conclusions are drawn as follows: (1) for desirable well-defined jetting regimes, when the laser fluence increases, the jet velocity, jet breakup length, and printed droplet size increase, and the printing type improves from droplet-impingement printing to jet-impingement printing with multiple breakups; (2) compared with cell-free bioink printing, the transfer threshold of the cell-laden bioink is higher while the jet velocity, jet breakup length, and printed droplet size are lower, shorter, and smaller, respectively. The addition of living cells transforms the printing type from jet-impingement printing with multiple breakups to droplet-impingement printing; and (3) two non-ideal jetting behaviors, a non-straight jet with a non-straight trajectory and a straight jet with a non-straight trajectory, are identified mainly due to the local nonuniformity and nonhomogeneity of cell-laden bioinks. Future work may focus on the following aspects: (1) simulation of the laser printing of cell-laden bioinks; and (2) characterization of the happening of non-ideal jetting behaviors.

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