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INKJET PRINTING OF THREE-DIMENSIONAL VASCULAR-LIKE CONSTRUCTS FROM CELL SUSPENSIONS

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INKJET PRINTING OF THREE-DIMENSIONAL VASCULAR-LIKE CONSTRUCTS FROM CELL SUSPENSIONS

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By
Changxue Xu
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Accepted by:
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ABSTRACT

Inkjet printing has found an increasing number of biofabrication applications, specifically organ printing, which has been emerging as a promising solution to the organ donor shortage. While some studies have been conducted to investigate various engineering problems associated with DOD inkjet printing of biological material-based fluids, the pinch-off, the cell-laden droplet formation, the effects of electric field on droplet formation, and challenges in 3D vascular-like construct fabrication haven’t been systematically investigated. The objective of this study is to investigate the pinch-off, the cell-laden droplet formation, the effects of electric field on droplet formation, and manufacturing challenges during fabrication using DOD inkjet printing.

The pinch-off process during DOD inkjet printing of viscoelastic alginate solutions is systematically investigated by studying the effects of sodium alginate (NaAlg) concentration and operating conditions on the pinch-off. It is found that there are four types of pinch-off during DOD inkjet printing of viscoelastic NaAlg solutions: front-pinching, exit-pinching, hybrid-pinching and middle-pinching. In particular, front-pinching is governed by a balance of inertial and capillary stresses, while exit-pinching is governed by a balance of elastic and capillary stresses. An operating diagram is constructed with respect to the Weber number (\(We\)) and a proposed \(J\) number (\(J = \sqrt{Oh \cdot El}\), where \(Oh\) is the Ohnesorge number and \(El\) is the elasticity number) to classify
regimes for different types of pinch-off.

The cell-laden droplet formation is studied and compared with the droplet formation of polystyrene bead-based suspensions. It is found that the breakup time increases but the droplet size, droplet velocity, and number of satellites decrease as the cell concentration increases. Compared to the polystyrene bead-based suspension, the ejected fluid volume is less, the droplet velocity is smaller, and the breakup time is longer using the cell-laden bioink.

The electric field-assisted droplet formation under piezoactuation-based DOD inkjet printing is investigated. It is found that droplet velocity increases and the droplet size decreases with the increase of the applied voltage. Pinch-off locations may vary depending on the applied voltage. The combination effect of the electric field and meniscus oscillation can be utilized to significantly reduce the droplet diameter. The electric field extends the capability of DOD inkjet printing to bioinks with high cell concentrations.

The gained knowledge of DOD inkjet printing has been further applied to vertical and horizontal printing of 3D vascular-like constructs using cell-laden bioink. It is found that the maximum achievable height of overhang structure depends on the inclination angle during vertical printing. To overcome the deformation-induced construct defect during horizontal printing, a predictive compensation approach has been
proposed to fabricate 3D tubular constructs horizontally. Alginate cellular tubes have also been successfully printed with a satisfactory post-printing cell viability of 87% immediately after printing and after 24 hours of incubation.

Overall, this dissertation provides a better understanding of the pinch-off of viscoelastic alginate solutions, cell-laden droplet formation, effect of electric field on droplet formation under piezoactuation-based DOD inkjet printing, and fabrication process of 3D vascular-like constructs from bioink. This work would help better fabricate tissue-engineered blood vessels with a complex geometry using DOD inkjet printing.
DEDICATION

This thesis is dedicated to my parents for their love and support.
ACKNOWLEDGEMENTS

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CHAPTER ONE

INTRODUCTION

1.1 Motivation and Background

Technological developments in the last twenty years have opened up the possibility of transplanting an increasing number of human organs to those in need [Farrell2011]. The number of organs available for transplantation, however, has not kept pace with such developments and the gap is widening more and more. As of March, 2010, the official U.S. kidney waiting list, maintained by the Organ procurement and Transplantation Network (OPTN), had reached about 84,100 patients, while an additional 2,200 were awaiting kidney-pancreas transplants [Beard2012]. Tissue engineering comprising tissue regeneration and organ substitution emerges as one of the most promising technologies in advanced medicine to address the organ donor shortage problem. The clinical goals of tissue engineering are to restore, repair, or replace damaged or lost tissues in the body, and the ultimate goal of tissue engineering is to design and fabricate functional human tissues and organs suitable for regeneration, repair and replacement of damaged, injured or lost human organs [Nichol2009] [Imani2012]. Organ printing, among different tissue engineering innovations, has emerged as a promising fabrication approach for making 3D tissue and organ constructs using cellular spheroids or bioink as building blocks, which utilizes layer-by-layer fabrication technologies to making 3D tissue-engineered living human organs. This fabrication technique is based on rapid prototyping (RP), which stands for techniques of reading in data from CAD drawings and building the 3D constructs layer by layer according to the
virtual design [Wust2011]. Significant progress has been made in recent years, which includes combinations of cells and polymers for engineering 3D tissue constructs and achievements in engineered simple tissues, such as the vascularized and heterogeneous cell-laden tissue constructs [Kolesky2014].

Organ printing is defined as a computer-aided, layer-by-layer additive biofabrication of 3D functional human tissue and organ constructs using self-assembling tissue spheroids as building blocks [Mehesz2011]. Organ printing has certain advantages: it is an automated approach that offers a pathway for scalable, reproducible, mass production of tissue engineered products; it allows for precise simultaneous 3D positioning of several cell types; it enables creation of tissue with a high level of cell density; it can solve the problem of vascularization in thick tissue constructs; and finally organ printing can be done in situ [Mironov2008].

Vascular networks deliver nutrients and oxygen to the organ systems in the body and are critical to the rise of large-scale multicellular organisms. The vascularization of tissue-engineered organs includes all essential elements of the intraorgan circulatory system. In general, the size of blood vessels varies from a diameter of about 25 mm in the aorta to only 8 µm in the capillaries [Blakemore2002]. Although tremendous progress has been made on 3D cellular printing, vascularization is still a big challenge, especially for large-scale tissue-engineered organs. Since vascularization is often correctly identified as a main technological barrier for building 3D organs [Mironov2003], the capacity to print a 3D tube with cells insides is not only a logical initial step but also a very important indicator of the overall feasibility of proposed organ printing technology.
In Fig 1.1 the schematic of envisioned fabrication of vascular trees is shown. Microspheres with cells inside are generated and are arranged spatially to form a layer of two-tensional (2D) annular pattern. The next layer is printed on the previous layer. All printed layers stack together to form a 3D tubular construct, which is then cultured in an incubator for tissue fusion. The final product is achieved after further tissue maturation. The research in this study focuses on the droplet formation and fabrication process of 3D tubular constructs.

3D tubular constructs can be achieved generally by two means: orifice-based printing and orifice-free printing. With respect to material shape during printing process, orifice-based printing includes droplet-based approaches, such as inkjet printing and hot-melt printing, and filament-based printing, such as robocasting (or robotic deposition), micropen writing, and fused deposition. Orifice-free printing mainly is laser printing which is jet-based [Lin2011]. Among the orifice-based and orifice-free printing methods, inkjet printing is favored for its scale-up potential, simple setup, and good process controllability [Herran2010] [Herran2012a] [Herran2012b] although it has a limited
capacity in delivering highly viscous fluids, which is not of concern herein.

There are two forms of inkjet printing: continuous inkjet (CIJ) printing and DOD printing shown in Fig. 1.2. In CIJ printing, a liquid is forced under a pressure through a small diameter orifice to form a liquid jet, and disturbances are supplied by a signal driver to control breakup of the liquid jet. The disturbances with a particular wavelength along the liquid jet synchronize the breakup of the liquid jet so that the size of each droplet and the spacing between them are the same, which eventually results in a very uniform stream of droplets [Martin2008]. The selected droplets are charged electrically on generation and the charged droplets are deflected by an amount depending on their charge when they pass through a fixed electric field. Uncharged droplets are steered into a gutter and recirculated, while charged droplets are directed onto the substrate. In DOD printing, droplets are generated only when required by propagating a pressure pulse in a fluid-filled chamber. Two methods are used to generate the pressure pulse responsible for fluid ejection and following droplet formation. 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. In a piezoelectric DOD printer, the pulse is formed by mechanical actuation of the piezoelectric element. For commercial applications CIJ printing is confined to applications in product marking and low resolution printing; most applications of interest to materials scientists use DOD printing, partially because of the risk of contamination that occurs during ink recirculation with CIJ [Derby2008].
Although inkjet printing has been widely used for fabrication of 3D constructs, there are some challenges needing to be carefully addressed. Firstly, understanding of pinch-off during DOD printing of viscoelastic alginate solutions is required. Secondly, the droplet formation performance during DOD inkjet printing of cell-laden bioink is lacking and a better understanding of the cell-laden droplet formation process can help control deposition resolution and optimize the operating conditions of DOD printing. In addition, a better understanding of the electric field-assisted droplet formation under
piezoactuation-based DOD inkjet printing will help to significantly reduce the droplet diameter and extend the capability of DOD inkjet printing to cell-laden bioinks with high cell concentrations. Finally, understanding of manufacturing challenges encountered during fabrication of 3D vascular-like constructs will help to effectively and efficiently fabricate 3D tubular constructs using proposed vertical printing and horizontal printing.

Recognizing the challenges described above, the objective of this study is to investigate the pinch-off during DOD inkjet printing of viscoelastic biomaterials, the droplet formation performance during DOD inkjet printing of cell-laden bioink, the effects of electric field on droplet formation without forming a Taylor cone during piezoactuation-based DOD inkjet printing, and manufacturing challenges encountered during fabrication of 3D vascular-like constructs using DOD inkjet printing. The main content of this work includes four parts:

1) The pinch-off process during DOD inkjet printing of viscoelastic alginate solutions is systematically investigated by studying the effects of sodium alginate (NaAlg) concentration and operating conditions on the pinch-off behavior and location in this study. For the first time, it is found that there are four types of pinch-off which may exist during DOD inkjet printing of viscoelastic NaAlg solutions: front-pinching, exit-pinching, hybrid-pinching and middle-pinching, as classified based on the pinch-off location. In particular, front-pinching is governed by a balance of inertial and capillary stresses, while exit-pinching is governed by a balance of elastic and capillary stresses experienced by a forming jet. The effective relaxation time, which is much smaller than the longest
relaxation time, characterizes the ligament thinning process. Furthermore, an operating diagram is constructed with respect to the Weber number (\( We \)) and a proposed \( J \) number (\( J = \sqrt{Oh \cdot El} \), where \( Oh \) is the Ohnesorge number and \( El \) is the elasticity number) to classify regimes for different types of pinch-off;

2) The droplet formation performance of cell-laden bioink is studied by investigating the effects of cell concentration on the breakup time, droplet size and velocity, and number of satellites. The droplet formation performance of comparable cell-laden and polystyrene bead-based suspensions is evaluated and further compared;

3) The electric field-assisted droplet formation under piezoactuation-based DOD inkjet printing is investigated by studying the effects of electric field on droplet volume charge density, droplet velocity and size, and pinch-off locations. In addition, the combination effect of the electric field and meniscus oscillation is utilized to significantly reduce the droplet diameter. The electric field is used to extend the capability of DOD inkjet printing to cell-laden bioinks with high cell concentrations; and

4) Vertical printing and horizontal printing are employed for fabrication of 3D vascular-like constructs. In vertical printing, a model is proposed to predict the maximum achievable height depending on the inclination angle of the overhang structure. In horizontal printing, a model is proposed to predict the cross-sectional deformation and predictive compensation is proposed to mitigate the cross-sectional deformation during horizontal printing. Alginate cellular tubes have been printed and the post-printing cell viability immediately after printing and
after 24 hours of incubation is tested.

1.2 Current State of Research

3D complex structures/constructs can be fabricated using a variety of techniques, such as inkjet printing [Boland2007] [Nishiyama2009] [Xu2012], laser printing [Schiele2010] [Riggs2011] [Yan2013], fused deposition modeling (FDM) [Zein2002], to name a few. Inkjet printing has been extensively used for layer-by-layer fabrication of 3D constructs due to its scale-up potential, simple setup, and good process controllability [Herran2010] [Herran2012a] [Herran2012b]. In this dissertation, DOD inkjet printing is employed to fabricate 3D vascular-like constructs. During the fabrication, two processes are classified as shown in Fig. 1.1: generation of droplets using DOD inkjet printing and layer-by-layer fabrication by precise control of the droplet movement. In the droplet formation process, alginate solutions widely used in 3D biofabrication are employed to study the pinch-off of viscoelastic alginate solutions during DOD inkjet printing. Then, cells are suspended in the alginate solutions to make cell-laden bioink, and the droplet formation performance of the cell-laden bioink is investigated. In addition, an electric field is introduced to piezoactuation-based DOD inkjet printing, and the effects of the electric field are studied. Finally, the generated cell-laden droplets are precisely positioned to form 2D layers which stack to each other to build 3D constructs. Therefore, in this section, current research is reviewed in the following aspects:

1) Ligament thinning and pinch-off location: focus on review of ligament thinning mechanism and pinch-off locations of Newtonian and non-Newtonian fluids;
2) Cell-laden droplet formation: focus on review of particle-laden droplet formation in dripping and DOD printing;

3) Electric field-assisted droplet formation under piezoactuation-based DOD inkjet printing: focus on review of electrohydrodynamic (EHD) jetting, especially EHD jet printing in a DOD fashion; and

4) Fabrication of 3D constructs: focus on review of different techniques for 3D construct fabrication and specific work on fabrication of vascular-like constructs.

1.2.1 Ligament Thinning and Pinch-Off Location

Pinch-off during fluid dispensing refers to the process during which a fluid jet or ligament disintegrates into droplet(s). The ligament pinch-off process, especially of viscoelastic fluids [Wagner2005], has received considerable attention recently because it is a key step for a variety of technological applications in biotechnology and drop-wise manufacturing. There has been a great practical and scientific interest to understand the physics underlying ligament/filament pinch-off which is a complex process controlled by capillary, viscous, elastic, and inertial effects.

The pinch-off process of Newtonian fluids has been widely studied during dripping [Henderson1997], inkjet printing [van Hoeve2010] [Castrejon-Pita2011], and liquid bridge breakup [Anna2000]. The ligament thinning of Newtonian fluids might be better evaluated based on the Ohnesorge number ($Oh$), which represents the ratio of viscous force to inertial and surface tension forces and is frequently used to define printability [Jang2009] [Yan2012]. Under a small $Oh$ number, meaning a negligible
viscous effect, Tirtaatmadja et al. [Tirtaatmadja2006] coupled the radial and axial force balances and simplified them to a balance of capillary and inertial terms by neglecting the viscous effect, resulting in a ligament decay following a power function with an exponent of 2/3. This power function has also been studied or verified experimentally [Chen2002b][Tirtaatmadja2006][van Hoeve2010] and computationally [Chen2002b][Castrejon-Pita2011]. The pinching at both the ends was reported during liquid bridge breakup [Vadillo2010], and such a pinch-off location can also be seen from other reported measurements during dripping [Tirtaatmadja2006] and continuous jetting [Christanti2002]. With a moderate Oh number, the viscous effect cannot be ignored. Eggers [Eggers1993] derived a similarity solution with a scaling form from the Navier-Stokes equation incorporating the inertial, viscous and capillary effects, resulting in a linear ligament decay following $D = 0.06 \frac{\gamma}{\mu} \Delta t$, where $D$ is the ligament diameter at the breakup location, $\gamma$ is the surface tension, $\mu$ is the viscosity, and $t$ is the time. This linear decay relationship has been verified experimentally [Kowalewski1996] and computationally [Castrejon-Pita2011]. With a high Oh number, the inertial effect may be ignored, and the ligament thinning is mainly controlled by the balance of viscous and capillary effects. A similarity solution was obtained from the Stokes equation by using the rational asymptotic expansions, resulting in a linear ligament decay $D = 0.14 \frac{\gamma}{\mu} \Delta t$ [Papageorgiou1995], which was further verified experimentally [McKinley2000] and computationally [Pozrikidis1999].

The pinch-off process of non-Newtonian fluids, especially viscoelastic polymer
solutions, has also been extensively studied during dripping [Chen2002b] [Tirtaatmadjia2006], continuous jetting [Oliveira2005] [van Hoeve2010] [Ardekani2010], and liquid bridge breakup [Anna2001] [Vadillo2010]. The thinning process of viscoelastic polymer ligaments during the aforementioned processes usually includes three consecutive stages: inertiocapillary thinning, elastocapillary thinning, and finite extensibility; their duration and dominance may vary due to material properties and operating conditions. During inertiocapillary thinning, the polymer chains start being stretched, and the elastic stress is still small. The capillary pressure is mainly balanced by inertial acceleration in the thinning ligament, resulting in a ligament diameter as a power function of jetting time [Tirtaatmadjia2006]. During elastocapillary thinning, the polymer stretching-induced elastic stress overshadows the inertial effect and competes with the capillary stress as the main stress component, resulting in an exponential function of jetting time [Wagner2005] [Tirtaatmadjia2006] [Clasen2009]. During finite extensibility, the polymer chains are fully stretched. The fluid behaves like a very viscous anisotropic Newtonian fluid characterized by the steady extensional viscosity of the fluid, leading to a linear ligament decay [Renardy1995] [Entov1997] [Clasen2009]. Vadillo et al. [Vadillo2010] studied the ligament pinch-off behavior of polystyrene solutions as a liquid bridge and reported three different pinch-off behaviors depending on the polymer concentration: at low polymer concentrations, pinch-off happened around both of the two bridge ends; at intermediate polymer concentrations, the ligament thinned until the Rayleigh instability appeared, and the pinch-off locations were along the thin ligament; and at high polymer concentrations, a long lasting uniform ligament was observed.
without identifiable pinch-off locations. Tirtaatmadja et al. [Tirtaatmadja2006] investigated the ligament thinning of polyethylene oxide solutions during dripping and reported that the ligament diameter decreased below the resolution of the imaging system before pinch-off was observed. Similar conclusion can also be made based on other ligament behavior observations during liquid bridge [Anna2001] and continuous jetting [Christanti2002] [Oliveira2005].

Different pinch-off locations have been reported such as end pinching at the both ends of a polystyrene-diethyl phthalate liquid bridge [Vadillo2010]; pinching at the mid-filament position of a polystyrene-diethyl phthalate liquid bridge if the polystyrene concentration is high enough [Tuladhar2008]; and front pinching (near the ligament head/forming droplet) and exit pinching (near the orifice) during inkjet printing sodium alginate solutions [Xu2013b]. Although these studies have mentioned various pinch-off locations, the possible pinch-off locations have been largely ignored, which should be systematically examined for the fabrication of monodisperse droplets.

1.2.2 Cell-Laden Droplet Formation

Recently, living cell-based biofabrication has emerged as a promising manufacturing research area with applications ranging from cell encapsulation [Orive2003] [Kauer2012] to cell/organ printing [Mironov2008] [Schiele2010] [Riggs2011]. Inkjetting has been pioneered to print different living cells including Chinese hamster ovary (CHO) cells [Xu2005], bovine vascular endothelial cells [Nakamura2005], rat heart endothelial cells (RHECs) [Khalil2009], mouse fibroblasts
[Xu2012], human fibroblasts [Khalil2005], human NT2 neuronal precursor cells [Xu2006], and human adipose-derived stem cells [Kim2010]. Therefore, it is of great importance to study the droplet formation process of cell suspensions during DOD inkjet printing.

Cell suspensions can be considered as a type of particle-laden ink. Particle-laden colloidal ink, mostly ceramics-based [Song1999] [Smay2002], has been used to print 3D patterns and structures. For better understanding of printing performance using particle-laden ink, studies have been devoted to investigating its droplet formation process during dripping and DOD printing of the ink with different particles laden.

In the dripping mode, Furbank et al. [Furbank2004] [Furbank2007] used the suspension of spherical poly (methyl methacrylate) (PMMA) particles with different particle volume fractions (up to 40%) to study the droplet formation process. It was found that at low particle volume fraction pinch-off structures were similar to that of the pure liquid, while at higher particle volume fraction the presence of particles in the thinning thread during necking resulted in thick cone-like structures. Particles suppressed the number of satellite droplets, but the few satellite droplets were much larger than that observed in pure liquid droplet formation. Bertrand et al. [Bertrand2012] used polystyrene suspensions with a volume fraction range from 15% to 50% to study the effect of particle volume fraction on droplet formation in dripping. It was reported that the final pinch-off location is closer to the nozzle at the higher volume fraction; with the increase of the volume fraction, the final detachment is faster due to larger difference in viscosity between the interstitial fluids and the shear viscosity of the suspensions.
In DOD inkjet printing, Reis et al. [Reis2005] used alumina particle suspensions with a volume fraction range from 20% to 40% to investigate droplet formation process during DOD inkjet printing, and reported that both the droplet velocity and volume decreased with the increase of particle volume fraction, and the characteristic time for the propagation of an acoustic signal was affected by the particle volume fraction. Tsai et al. [Tsai2008] used deionized water and 30 wt% silver suspensions to study effects of nanoparticles on droplet formation process during DOD inkjet printing. Observations showed that a higher driving pulse voltage was required for the silver suspension to form droplets. Compared to the droplet formation process of deionized (di) water, the droplet size of the silver suspension was smaller, the liquid column formed was thinner and longer, and the pinch-off time of the liquid column to form droplets was longer. Wang et al. [Wang2012] also studied DOD drop formation of pigment-laden suspensions by comparing Newtonian fluids and colloidal suspensions with the same low-shear-rate viscosity. It was concluded that the droplet formation dynamics of the colloidal suspension is similar to that of Newtonian fluid, with only slight systematic differences observed. The non-straight trajectories and non-axisymmetric ligaments were commonly observed due to the accumulated particles and non-ideal wetting condition on the nozzle plate.

In summary, the resulting droplet size decreases [Furbank2004] [Furbank2007] [Tsai2008], the droplet velocity decreases [Tsai2008], and the number of satellites decreases [Furbank2004] [Furbank2007] too when a particle-laden fluid is dispensed. Unfortunately, there is no study investigating the droplet formation performance of cell-
laden suspensions, which has soft living cells inside and differs from hard particle-laden droplet formation studied in previous studies [Tay2001] [Furbank2004] [Furbank2007] [Tsai2008] [Bertrand2012] [Wang2012]. There are certain differences between cell-laden and particle-laden droplet formation. The first is the material properties of suspensions, which determine the droplet formation during DOD inkjet printing. The second is the volume fraction. During inkjet printing of cell-laden bioink, the cell volume fraction is usually much smaller than that of particle-laden suspensions. The third difference is the physical property difference between cells and rigid particles, such as deformability. The droplet formation performance during DOD inkjet printing of cell-laden bioink is still lacking, and a better understanding of the cell-laden droplet formation process can help control deposition resolution and optimize the operating conditions of DOD printing.

1.2.3 EHD Jetting

DOD jetting has two main drawbacks. First, the orifice tends to clog during processing viscous materials. Second, it is difficult to fabricate monodisperse droplets smaller than the orifice diameter, if needed, for a given orifice diameter. Even though it is doable to reduce the droplet diameter slightly by modifying the excitation waveform [Chen2002b], the achievable droplet diameter is usually limited by the nozzle orifice.

EHD technology has long been recognized as an effective approach to form droplets by utilizing electrostatic forces as the driving mechanism through an electrically charged fluid jet [Melcher1969] [Saville1997]. The resulting droplet size can be easily much smaller than the orifice size [Park2007] [Choi2008] [Kim2013]. It has also been
reported that EHD can facilitate the printing of viscous materials such as ceramic suspension (285 mPa.s) [Lee2008] and SU-8 photoresist (380 mPa.s) [Park2013].

Under representative EHD conditions, a cone-jet is usually formed, and such EHD jetting may result in a filament in electrospinning and many droplets in electrospraying. During electrospinning or electrospraying, the fluid being dispensed is exposed to an electric field resulting in electrical charge accumulation inside the forming jet. If the Coulombic repulsion of the charges overcomes the surface tension, usually a Taylor cone is formed and a thin jet is ejected from the cone apex. Depending on the material properties and operating conditions, the jet either breaks into a few droplets as electrospraying or stays as a filament to fabricate fibers as electrospinning. The EHD voltage is usually supplied in two modes: direct current (DC) mode [Park2007] including the pulsed DC mode [Chen2006] [Li2006], and alternating current (AC) mode [Nguyen2009]. The electric field in the DC mode utilizes the tangential electrostatic stress to form a thin jet from the apex of the Taylor cone; the electrostatic field in the pulsed DC mode is used to achieve DOD printing; and the electric field in the AC mode is used to prevent droplet deflection due to the same charge of the droplets.

The materials used in EHD are confined by the properties, especially conductivity. With perfect conductors or dielectrics, the electrostatic stress is perpendicular to the interface and there is no tangential electrostatic stress, which is responsible for EHD tip streaming [Saville1997] [Collins2008]. The extreme limit for conductivity is reported to be 1 S/m, at which the ions begin to escape from the liquid into the gas [de la Mora2007].

In order to DOD fabricate droplets, some setup modifications have been
introduced to the classical EHD configuration: adding a pulsating voltage [Choi2008] [Mishra2010] or including a piezoactuator [Kim2009] [Byun2010] [Kim2012] in order to control the droplet formation process externally. Under the EHD configuration using a pulsating voltage, a baseline DC voltage is used to deform the meniscus to nearly a conical shape without liquid jetting, and the voltage pulse induces a fast EHD jetting mode from the nozzle for a short duration. The duration of the pulse determines the ejected fluid volume. Under the EHD configuration using a piezoactuator, the piezoactuator mechanically deforms the meniscus and an electric field is responsible for fluid delivery from the nozzle to the substrate. Dots on the substrate were reduced from 151 µm to 59 µm with a frequency of 1 kHz controlled by the piezoelectric actuation [Kim2009] [Kim2012]. While both of the approaches have their pros such as droplet size reduction [Choi2008] [Mishra2010] [Byun2010] [Kim2012], the droplet size controllability has been a challenge. When a pulsating voltage is applied, the resulting droplets/dots are usually non-uniform [Byun2010] [Mishra2010]; when a piezoactuator is used, undesirable satellite droplets usually form as by-products [Kim2009] [Kim2012]. Due to the formation of cone-jets during the aforementioned EHD modifications, the uniformity of droplets cannot be easily guaranteed. For example, when a thin jet ejected from the apex of a Taylor cone breaks, numerous satellite droplets always form without a primary droplet. Electric field-assisted droplet formation without forming a Taylor cone under piezoactuation-based DOD inkjet printing is lacking and further experimental study is required.
1.2.3 Fabrication of 3D Constructs

Most liquid-based additive manufacturing techniques used in 3D fabrication, which may be potentially applicable for biofabrication applications, can be classified into two types: droplet-based and filament-based as shown in Fig. 1.3. The former can be implemented by using inkjet printing [Boland2007] [Nishiyama2009] [Xu2012] or laser printing [Schiele2010] [Riggs2011] to generate droplets as building blocks for deposition. The latter is to use extruded filaments to make parts instead of using droplets; and this technology is represented by fused deposition modeling (FDM) [Zein2002], extrusion [Hamid2011], and micropen printing [Lewis2006]. Tissues and organs are composed of different kinds of cells, which interact and express their respective functions based on their three-dimensional structures. Thus the position and arrangement of the components including cells are critical for tissue engineering; and it is necessary to develop effective technologies for the arrangement of the cells and the structure materials in 3D space [Henmi2008]. Compared with filament-based techniques, droplet-based techniques can more easily fabricate complex and heterogeneous parts with a resolution defined by the size of each droplet. As such, droplet-based techniques have been favored in many biofabrication applications [Boland2007] [Nishiyama2009] [Schiele2010] [Riggs2011] [Xu2012]. Among the droplet-based techniques, the inkjet-based technique is favored here for its scale-up potential, simple setup, and good process controllability [Herran2010] [Herran2012a] [Herran2012b], although it has a limited capacity in delivering highly viscous fluids, which is not of concern in this study.
Vascularization is often correctly identified as a main technological barrier for building 3D organs. The capability to print 3D cellular tubes is not only a logical first step towards successful organ printing but also a critical indicator of the feasibility of the envisioned organ printing technology. 3D tubular constructs can be printed vertically or horizontally based on the relative configuration between the moving direction of dispensing nozzle and the axis of the tube being printed. If the nozzle moves along the circumferential direction of the tube, the fabrication process is called vertical printing; if the nozzle moving direction is parallel to the tube longitudinal axis, the fabrication process is called horizontal printing. Most previous works on tube inkjetting were based on the vertical printing setup [Boland2007] [Nishiyama2009] [Xu2012], which can be easily implemented. Boland et al. [Boland2007] took the approach of printing the less
viscous calcium chloride solution into a tank of sodium alginate solution. The material was printed on an elevator which slowly moved downwards to build a branched construct. Nishiyama et al. [Nishiyama2009] directly printed the sodium alginate solution into calcium chloride solution to fabricate alginate tubes. It should be noted that some vascular constructs were also fabricated via the assembly of preformed solid cellular rods [Norotte2009] [Skardal2010]. Various vascular cell types were mechanically extruded to form either multicellular spheroids or cylinders of controllable diameter which were then printed layer by layer concomitantly. After post-printing fusion of the discrete units the single- or double-layered vascular tubes were fabricated [Norotte2009] [Skardal2010]. This printing process took a long time for cell fusion and the final tube outer surface is not smooth. In addition, it may not be easily extended to make 3D heterogeneous tissues or organs.

High cell viability during inkjetting is proved by many researchers. For example, human fibroblasts printed with a piezoelectrically driven glass tube printhead showed 95 to 98% viability, which went even higher at lower drive voltages [Calvert2007]. Xu et al. [Xu2005] utilized a modified HP 550C computer printer to print Chinese hamster ovary (CHO) cells and it was shown that the CHO cell viability was above 90% after printing and the cells retained their ability to function. Khalil et al. [Khalil2005] used a multi-nozzle biopolymer deposition system to print fibroblast mixed with 1.5% sodium alginate into the substrate of 5% calcium chloride. The survivability of the cells after the deposition was on average 85% and the cells were cultured and proliferated in 7 days after the deposition. Nakamura et al. [Nakamura2005] investigated the feasibility of
inkjet printing with bovine vascular endothelial cells and the results showed that living cells were safely ejected by inkjet printing onto culture disks, where they could adhere and proliferate. Three-dimensional cellular tubular constructs have been layer-by-layer fabricated using inkjetting [Henmi2008] [Xu2012].

Most previous work on fabrication of 3D vascular-like constructs is based on vertical printing. However, vertical printing itself may have unexpected challenges in fabricating 3D complex constructs. Therefore, there is a need to investigate the feasibility of horizontal printing for 3D tubular construct fabrication. Furthermore, understanding of manufacturing challenges encountered during fabrication of 3D vascular-like constructs will help to effectively and efficiently fabricate 3D tubular constructs using proposed vertical printing and horizontal printing.

1.3 Scope of Dissertation

As mentioned previously, there are several key challenges in the fabrication of 3D vascular-like constructs needing to be carefully addressed as follows:

1) Understanding of pinch-off during DOD inkjet printing of viscoelastic alginate solutions;

2) Understanding of effects of cell concentration on breakup time, droplet size and velocity, number of satellite droplets, and difference between cell-laden droplet formation and hard particle-laden droplet formation;

3) Understanding of effects of electric field on droplet volume charge density, droplet size and velocity, and pinch-off locations, and droplet generation
from cell-laden bioinks with high cell concentrations; and

4) Addressing and solving the manufacturing challenges during fabrication of
3D vascular-like constructs using vertical printing and horizontal printing, and analyzing post-processing cell viability.

1.4 Organization of Dissertation

This dissertation is aimed at enhancing a general understanding and providing a comprehensive investigation on the pinch-off during drop-on-demand (DOD) inkjet printing of viscoelastic alginate solutions, the droplet formation performance during DOD inkjet printing of cell-laden bioink, the effects of electric field on droplet formation without forming a Taylor cone during piezoactuation-based DOD inkjet printing, and manufacturing challenges encountered during fabrication of 3D vascular-like constructs using DOD inkjet printing. The layout of this dissertation is shown in Fig. 1.3. A typical vascular-like construct fabrication process using DOD inkjet printing is investigated by experimental study of the four types of pinch-off during DOD inkjet printing of viscoelastic alginate solutions, effects of cell concentration on droplet formation during DOD inkjet printing of cell-laden bioink, and effects of electric field on droplet formation during piezoactuation-based DOD inkjet printing. The gained knowledge of DOD inkjet printing has been further applied to proposed vertical printing and horizontal printing of 3D vascular-like constructs using cell-laden bioink, and the associated manufacturing challenges during fabrication are addressed and solved.
The organization of this dissertation is as follows:

- In Chapter 1, the motivation and objectives of this work are first introduced. The current research state is then reviewed. Finally, the scope of this dissertation is provided.

- In Chapter 2, the pinch-off process during DOD inkjet printing of viscoelastic alginate solutions is systematically investigated by studying the effects of sodium alginate (NaAlg) concentration and operating conditions on the pinch-off behavior and location. Four types of pinch-off
are observed based on the pinch-off location during DOD inkjet printing of viscoelastic NaAlg solutions: front-pinch, exit-pinch, hybrid-pinch, and middle-pinch. The associated ligament thinning is elucidated. Finally, a phase diagram in terms of $W e$ and $J$ is constructed to identify different regimes for the four types of pinch-off;

- In Chapter 3, the droplet formation performance of cell-laden bioink is studied by investigating the effects of cell concentration on the breakup time, droplet size and velocity, and number of satellites. The droplet formation performance of comparable cell-laden and polystyrene bead-based suspensions is evaluated and further compared;

- In Chapter 4, the electric field-assisted droplet formation under piezoactuation-based DOD inkjet printing is investigated by studying the effects of electric field on droplet charge density, droplet size and velocity, and pinch-off locations. In addition, the combination effect of the electric field and meniscus oscillation is utilized for droplet size reduction, and cell-laden bioinks with high cell concentrations are used to study the capability of the electric field-assisted DOD inkjet printing;

- In Chapter 5, vertical printing and horizontal printing are proposed to fabricate tubular constructs, such as zigzag tubes and $Y$-shaped tubes. Simple models are proposed to help better understand the fabrication process: one is for the maximum achievable height depending on the inclination angle of the overhang structure in vertical printing; the other is
for cross-sectional deformation in horizontal printing. In addition, predictive compensation is proposed to mitigate the cross-sectional deformation in horizontal printing. Finally, alginate cellular tubes are printed and cell viability immediately after printing and after 24 hours of incubation is tested; and

- In Chapter 6, the conclusions and future work of the dissertation are summarized.
CHAPTER TWO

PINCH-OFF LOCATIONS DURING DROP-ON-DEMAND INKJETTING OF ALGINATE SOLUTION

Abstract

The ligament pinch-off process, especially of viscoelastic fluids, has received considerable attention recently because it is a key step for a variety of technological applications in biotechnology and drop-wise manufacturing. This study is aimed at investigating various pinch-off locations as a function of material properties and operating conditions during drop-on-demand (DOD) inkjet printing of viscoelastic sodium alginate solutions. Four breakup types are identified: front-pinching, hybrid-pinching, exit-pinching, and middle-pinching. A dimensionless number $J$, which is defined as the square root of the product of Ohnesorge ($Oh$) and elasticity ($El$) numbers is proposed to represent the ratio of viscous and elastic effects to inertial and capillary effects. Based on the $J$ and Weber ($We$) numbers, a phase diagram is constructed to classify the regimes for different pinch-off types during DOD inkjet printing of alginate solutions. Some main conclusions are drawn as follows: (1) for very low sodium alginate concentrations such as 0.10 – 0.20%, front-pinching prevails at the voltage of 30 – 70V. The ligament thinning process is governed by a balance of inertial and capillary effects, following a power function with an exponent of 2/3; (2) for low concentrations such as 0.25 – 0.35%, with the increase of $We$, the pinch-off type may change from front-pinching to hybrid-pinching to exit-pinching; (3) for intermediate concentrations such as 0.50 – 1.00%, exit-pinching occurs at the voltage range of 30 – 70V. The ligament
thinning at the exit-pinching location is governed by a balance of elastic and capillary effects, resulting in the exponential decay process; and (4) for high concentrations such as 1.50 – 2.00%, both the viscous and elastic effects are dominant. The ligament thinning process near the ligament head/forming droplet is governed by a balance of viscous, elastic, and capillary effects while the ligament thinning process near the orifice is governed by a balance of elastic and capillary effects due to the high-frequency pressure wave. At small $We$, middle-pinching occurs. With the increase of $We$, middle-pinching turns to be exit-pinching.

2.1 Introduction

Different pinch-off locations have been reported such as end pinching at the both ends of a polystyrene-diethyl phthalate liquid bridge [Vadillo2010]; pinching at the mid-filament position of a polystyrene-diethyl phthalate liquid bridge if the polystyrene concentration is high enough [Tuladhar2008]; and front pinching (near the ligament head/forming droplet) and exit pinching (near the orifice) during inkjet printing sodium alginate solutions [Xu2013b]. Although these studies have mentioned various pinch-off locations, the possible pinch-off locations have been largely ignored, which should be systematically examined for the fabrication of monodisperse droplets.

The objective of this work is to study various pinch-off locations as a function of material properties and operating conditions during drop-on-demand (DOD) inkjet printing of viscoelastic sodium alginate solutions. DOD inkjet printing [Herran2010] [Herran2012b] has been favored due to its ease in droplet size control and satellite droplet
elimination by optimizing operating conditions such as the excitation pressure pulse. For the increasing popularity of DOD-based 3D printing technology, this study has selected DOD inkjet printing to study the pinch-off location during printing viscoelastic alginate solutions, which are well accepted for tissue engineering and drug delivery. The material properties were controlled by adjusting the sodium alginate concentration, and the operating conditions were determined by choosing different excitation voltages. The rest of the paper is organized as follows. First, the experimental materials and method are illustrated in detail. Then, four pinch-off types are classified and their associated mechanisms are discussed. Finally, a phase diagram is constructed in terms of a proposed $J$ number, representing the material properties and the Weber number ($We$) in order to classify the regimes for different pinch-off types during DOD inkjet printing of alginate solutions.

### 2.2 Experimental Design

#### 2.2.1 Materials

As a versatile biomaterial [Augst2006] [Murphy2013], alginate hydrogel has been used as scaffolds for tissue engineering, as delivery vehicles for drugs, and as model extracellular matrices for basic biological studies. Alginate, in particular, sodium alginate (NaAlg), has been used as a constituent of bioink in bioprinting [Nishiyama2009] [Xu2012] [Murphy2013] [Ringeisen2013]. For its broad bioprinting applications, sodium alginate solution has been chosen as a model system to study the pinch-off process during DOD inkjet printing.
The sodium alginate solutions were prepared by dissolving sodium alginate (Sigma-Aldrich, St. Louis, MO) into deionized (di) water to make the solutions with different concentrations of 0.10%, 0.15%, 0.20%, 0.25%, 0.30%, 0.35%, 0.50%, 1.00%, 1.50% and 2.00% (w/v). The density was measured by averaging the weight of 1 ml sodium alginate solution five times. The viscosity was measured using a rotational rheometer (ARES, TA Instrument, New Castle, DE), and the surface tension was measured based on the pendant drop method using a drop shape analysis system (Attension TL101, Biolin Scientific, Stockholm, Sweden) at room temperature. The density, viscosity, and surface tension information is listed in Appendix. The Appendix also provides the values of two relevant nondimensional numbers: Ohnesorge number 

\[ Oh = \frac{\mu}{\sqrt{\rho \gamma R}} \]

and elasticity number \( El = \frac{\mu \lambda}{\rho R^2} \) to represent the ratio of the viscous to inertial and surface tension effects and the ratio of the elastic to inertial effects, where \( \rho \) is the density, \( R \) is the nozzle radius of the inkjetting system (60 µm), and \( \lambda \) is the longest relaxation time. The material properties for each concentration and the associated \( Oh \) and \( El \) are shown in Table 2.1.
Table 2.1 Material properties of NaAlg solutions

<table>
<thead>
<tr>
<th>NaAlg concentration (w/v)</th>
<th>Density ρ (g/cm³)</th>
<th>Viscosity µ (cP)</th>
<th>Surface tension γ (mN/m)</th>
<th>Oh</th>
<th>El</th>
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<td>0.04</td>
<td>0.25</td>
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<td>71.5</td>
<td>0.07</td>
<td>0.49</td>
</tr>
<tr>
<td>0.25%</td>
<td>1.003</td>
<td>5.5</td>
<td>70.8</td>
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<tr>
<td>0.30%</td>
<td>1.004</td>
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<tr>
<td>0.35%</td>
<td>1.005</td>
<td>6.7</td>
<td>67.4</td>
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<td>0.50%</td>
<td>1.005</td>
<td>10.2</td>
<td>52.6</td>
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<td>1%</td>
<td>1.010</td>
<td>31.1</td>
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<tr>
<td>1.5%</td>
<td>1.014</td>
<td>75.2</td>
<td>45.7</td>
<td>1.43</td>
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<tr>
<td>2%</td>
<td>1.021</td>
<td>139.5</td>
<td>44.6</td>
<td>2.67</td>
<td>62.62</td>
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</table>

2.2.2 Method and Experimental Conditions

The DOD inkjet printing system herein was composed of three key sub-systems: a MicroFab nozzle dispenser with an orifice diameter of 120 µm (MJ-ABL-01-120-6mx) and its associated Jet Driver (MicroFab, Plano, TX), a pneumatic controller (MicroFab, Plano, TX), and an imaging system (ImageXpert, Nashua, NH). The excitation waveform was controlled using the MicroFab Jet Driver, and the pneumatic controller was used to adjust the backpressure of the fluid reservoir to obtain an ideal meniscus for good droplet formation. Once a pulsed voltage is applied to the nozzle piezoelectric element, a
pressure wave is generated to squeeze the nozzle, finally ejecting some fluid out to form droplets in a DOD mode. The droplet formation process was captured by the imaging system for time-resolved image analysis, and the jet velocity and breakup time were determined from the images captured. The image resolution herein is 1032 × 778 pixels, and one pixel is 3.2 µm, the minimum achievable feature resolution. The ligament length was determined using the ImageJ software developed by the National Institutes of Health. The DOD inkjetting setup is shown in Fig. 2.1.

During DOD inkjet printing, an excitation waveform was applied to the nozzle dispenser to form droplet(s), and the typical excitation waveform is bipolar, which consists of a succession of positive/negative square-wave pluses [Herran2012]. For the bipolar waveform, the second pulse of the wave is used to cancel some of residual
acoustic oscillations that may remain in the dispense nozzle after droplet ejection. The bipolar excitation waveform used in the study is defined as follows: excitation voltages in the range from 30V to 70V with an interval of 5 V, voltage rise/fall time 3 μs, dwell time 30 μs, echo time 30 μs, and frequency 50 Hz.

2.2.3 Key Characteristics of a Droplet Formation Process

The fluid being dispensed may experience several stages during a typical droplet formation process, and each stage may reveal some key morphological features of a forming jet/droplet. For easy reference, some of these features are defined as shown in Fig. 2.2. Two important locations are the origin $x_0$ which is the exit position of nozzle orifice and the jet/droplet leading point $x_l$, and these two locations are used to determine the droplet velocity ($U = \frac{x_l - x_0}{\Delta t}$) herein. Before the jet disintegrates from the nozzle, the connecting fluid thread is called ligament. After jet pinch-off, the trailing fluid behind the primary droplet is called tail.

Fig. 2.2. Illustration of a forming droplet
2.3 Pinch-Off during DOD Inkjet Printing

2.3.1 Pressure Wave

During DOD inkjet printing, the amplitude of excitation-induced pressure wave can be studied based on the force equilibrium inside the nozzle chamber. Fig. 2.3 shows a representative pressure wave at the nozzle orifice based on the meniscus shape during inkjetting. In order to form a droplet, the pressure at the nozzle tip has to overcome the steady and unsteady inertia, viscous resistance, elasticity, and pressure due to surface tension [Wijshoff2010]. For the pressure wave shown in Fig. 2.3, the first negative pressure pulse due to the rising edge of the excitation waveform retracts the meniscus, which is known as the fill-before-fire action [Wijshoff2010]. It is reflected at the reservoir inlet to become a positive pressure which is amplified by the falling edge of the excitation waveform to become a larger positive pressure to drive the fluid out from the nozzle. The second negative pressure pulse due to the echo of the excitation waveform is to cancel some of residual acoustic oscillations that may remain in the nozzle chamber. The detailed description of DOD inkjetting mechanism can be found in other relevant documents [Kwon2009] [Wijshoff2010].
During DOD inkjet printing, the generated pressure pulses propagate inside the nozzle reflecting at the reservoir and orifice, resulting in an oscillating pressure wave. Ligament thinning has been studied extensively theoretically [Bazilevsky1997] [Eggers1997] and experimentally such as dripping [Chen2002b] [Tirtaatmadjia2006], continuous jetting [van Hoeve2010] [Ardekani2010], and liquid bridge breakup [Anna2001] [Vadillo2010]. However, the high-frequency oscillating pressure wave makes the pinch-off process during DOD inkjet printing different from those aforementioned. Some careful examination of the pinch-off process during DOD inkjetting is greatly needed.

2.3.2 Possible Pinch-Off Locations

According to the Young-Laplace equation, the capillary pressure is proportional
to the curvature of the interface, and pinch-off usually occurs at the positions with the largest variation of the curvature where the pressure gradient is largest [Wijshoff2010]. During the DOD droplet formation process, the initial curvature variation may be generated at two locations: near the ligament head and near the nozzle orifice. At the location near the ligament head, the initial curvature variation results from a supercritical acceleration at the beginning of the droplet formation, which generates an additional maximum velocity in the ligament head to move it away from the rest of forming droplet [Wijshoff2010]. At the location near the nozzle orifice, the initial curvature variation originates from the jet constriction due to the acceleration [Badie1997]. The acceleration by the pressure wave affects the fluid near the nozzle orifice, causing an inward motion of the free surface near the orifice [Badie1997], which leads to a constriction. In addition to the aforementioned two pinch-off locations, a uniform thin ligament may be formed before pinch-off under some special conditions such as printing of highly elastic fluids, and eventually it breaks up due to the Rayleigh instability [Vadillo2010]. Then the pinch-off location can be some place along the thin ligament. These three pinch-off locations may combine and further determine possible pinch-off types during DOD inkjet printing.
2.3.3 Four Pinch-Off Types during DOD Inkjet Printing

After the pressure wave-induced initial curvature variation generates, the thinning process follows along the entire ligament under the interplay of the capillary, viscous, elastic and inertial stresses. During the DOD inkjetting of sodium alginate solutions, four different pinch-off types have been observed as shown in Fig. 2.5: front-pinching, hybrid-pinching, exit-pinching, and middle-pinching depending the sodium alginate and excitation voltage. It implies that different pinch-off mechanisms may prevail during the printing of viscoelastic fluids, which depends on the material properties and operating conditions, in particular, the sodium alginate concentration and excitation voltage.
Fig. 2.5. Four pinch-off types during DOD printing: (a) front-pinching, (b) hybrid-pinching, (c) exit-pinching, and (d) middle-pinching. Sodium alginate concentration and excitation voltage are listed as follows: (a) 0.30% and 35 V, (b) 0.30% and 42 V, (c) 1.00% and 50 V, and (d) 2.00% and 50 V. Each pinch-off location is marked using a dashed circle.
It is noted that only the first pinch-off event during a droplet formation process is of the interest herein. In particular, during front-pinching, the first pinch-off of ligament occurs near the ligament head/forming droplet (Fig. 2.5(a)). During exit-pinching, the first pinch-off of ligament occurs near the nozzle orifice (Fig. 2.5(c)). Under a critical condition, the first pinch-off of ligament occurs simultaneously near both the ligament head and the nozzle orifice, which is called hybrid-pinching (Fig. 2.5(b)). During middle-pinching, the ligament thins until its diameter is under the resolution of the imaging system. Eventually, the uniformly thin ligament breaks up, pinch-off occurs along the thin ligament but no exact pinch-off location(s) can be clearly identified. The nature of front-pinching is the Rayleigh instability, describing the growth of the primary disturbance, and the nature of exit-pinching is the hydrodynamic instability due to the external high-frequency pressure wave. It is noted that hybrid-pinching is a special case when the breakup times at the front-pinching and exit-pinching locations are the same; middle-pinching is a special case when the fluid in the ligament has enough time to drain into the forming droplet(s) and/or the nozzle orifice.

2.4. Analysis of Pinch-off Phenomena

2.4.1 Pinch-Off at Front-Pinching Location

Pinch-off at the front-pinching location during DOD inkjet printing is also usually observed during continuous inkjet (CIJ) printing [Bog1979] [Kow1996] [van H2010]. Front-pinching usually occurs using sodium alginate solutions with a
very low concentration such as 0.10 – 0.20% under the entire voltage range of 30 ~ 70V. The breakup time at the front-pinching location is usually shorter than that at the exit-pinching location. In this section, the mechanism of front-pinching is further explored by analyzing the ligament thinning process.

Effects of viscous and elastic forces

During the DOD inkjet printing of a viscoelastic fluid, the droplet formation process is always determined by the inertial, viscous, capillary, and elastic effects, and the influence of some effects may dominate over that of others. It is noted that the gravitational effect is negligible due to a very small Bond number \( \frac{DgR^2}{\gamma} \approx 10^{-3} << 1 \), where \( g \) is the gravitational acceleration) of forming droplets. During the experiments where front-pinching occurs, the sodium alginate concentration is usually less than 0.35%, resulting in \( Oh<1 \) and \( El<1 \) as seen from the Appendix. Therefore, due to the small \( Oh \) and \( El \) numbers, the viscous and elastic effects can be safely neglected during the pinch-off analysis, and the front-pinching process is mainly governed by a balance of inertial and capillary effects.

Front-pinching process

The jetting during DOD inkjet printing is caused by the nozzle deformation under the external excitation voltage. The positive/negative bipolar waveforms are applied herein to generate a train of pressure pulses. As observed, a cylindrical ligament
forms within the time range of 0 ~ 40 µs for very low-concentration solutions. Then the ligament thinning process follows, and the droplet starts forming around 40 µs (also approximately 40 µs for other very low-concentration solutions as measured). Fig. 2.6 shows the velocity information of a forming ligament/droplet at an excitation voltage of 50 V: significant velocity variation during 0 ~ 40 µs and relatively steady state after 40 µs.

![Graph showing velocity of the head of forming ligament/droplet](image)

Fig. 2.6. Velocity of the head of forming ligament/droplet using 0.10% sodium alginate solution at 50 V

In order to study the pinch-off mechanism, the ligament decay at the front-pinching location has been measured and shown in Fig. 2.7 when printing the 0.10% sodium alginate solution at different voltages. For the purpose of analysis, the ligament diameter at the front-pinching location at 40 µs is defined as the initial ligament diameter.
$D_f$ right before the ligament near the forming head starts thinning. For Fig. 2.7, the minimum diameter $D_{f_{\text{min}}}$ at the front-pinching location (necking position) is normalized by $D_f$, $t$ is the time, $t_c$ is the breakup time, and $\tau_c = \sqrt{\rho R^3 / \gamma}$ is the capillary time scale. In addition, the time is normalized using the capillary time scale $\tau_c$.

![Graph](image)

**Fig. 2.7.** Ligament diameter variation at front-pinching location under different conditions using 0.10% sodium alginate solution

During front-pinching, the viscous and elastic effects can be neglected due to the small $Oh$ and $El$ numbers; instead, it is sufficiently accurate to only consider the inertial and capillary effects for process modeling. Then the scaling law for $D_{f_{\text{min}}}$ can be described as follows [Tirtaatmadja2006] [Hoeve2010]:

...
\[ D_{f\text{min}}(t) \propto \left( \frac{t}{\tau_c} \right)^{2/3} \]  

(2.1)

As seen from Fig. 2.7, the linear regression fits of the experimental data results in a slope of 0.66 when printing the 0.10\% sodium alginate solution, which is close to the slope 2/3 as theoretically predicted. It confirms that the accurate breakup dynamics may be mathematically obtained based on the growth of the primary disturbance [Tirtaatmadja2006] for the 0.1\% sodium alginate solution. For solutions with the sodium alginate concentration from 0.10 – 0.20\%, the viscous and elastic effects start increase, but they are still relatively small compared to the inertial and capillary effects. As such, front-pinching is largely governed by a balance of inertial and capillary effects for 0.10 – 0.20\% sodium alginate solutions.

2.4.2 Pinch-Off at Exit-Pinching Location

Exit-pinching is frequently observed during DOD inkjet printing, in particular, during the printing of relatively high-concentrated sodium alginate solutions such as 0.50 – 1.00\% at the entire voltage range of 30 – 70V. During the pinch-off process at the front-pinching location, the forming droplet is far away from the nozzle orifice, which leads to less influence from the resultant pressure wave. However, during exit-pinching, the ligament thinning and droplet formation process is greatly influenced by the pressure wave.

Pressure wave during ligament formation and thinning process
As shown in Fig. 2.3, a typical pressure wave at the nozzle orifice, including the first negative, first positive, and second negative pressure peaks, and the subsequent residual vibration. Specifically, the first negative pressure is for meniscus retraction before firing, and the second negative pressure is to cancel some of the residual pressure wave inside the nozzle. The residual vibration must be as low as possible before the next droplet formation cycle starts [Wijshoff2010]. Before the second negative pressure ends (0 – 70 µs as observed), the magnitude of the first positive pressure (1.4×10^5 Pa) is estimated to be much larger than the capillary pressure \( P_c = \frac{2\gamma}{R} = 2.4\times10^3 \) Pa. The first positive pressure is estimated based on the pressure to overcome the inertia due to the Bernoulli pressure \( P_b = \rho \frac{U^2}{2} = 6.7\times10^4 \) Pa, the pressure for the fluid acceleration \( P_a = \rho R \frac{dU}{dt} = 7\times10^4 \) Pa, and the ink accelerates in 10 µs to the required speed as shown in Fig. 2.6), and the pressure due to surface tension \( P_a = \frac{2\gamma}{R} = 2.4\times10^3 \) Pa. The second negative pressure peak is half of the magnitude of the first positive pressure as experimentally designed in this study.

During the subsequent residual pressure wave (after 70 µs), the amplitude of the residual pressure wave is assumed small compared to the capillary pressure [Xu2007] [McIlroy2013]. The amplitude of pressure wave depends on the degree of viscous damping, and the viscous dissipation is reported to play an important parameter for damping the residual pressure wave when a pressure wave reflects at the nozzle orifice.
[Wijshoff2010]. During the exit-pinching process, the sodium alginate solutions are relatively high-concentrated and have a relatively high viscosity, which may effectively damp the residual pressure wave. Furthermore, the second negative pressure is to cancel some of residual acoustic oscillations that may remain in the nozzle chamber after droplet ejection. As such, the process after 70 µs is assumed to be dominated by the capillary pressure instead of the pressure wave.

**Exponential and linear decay during ligament thinning**

The ligament diameter at the pinch-off location during exit-pinching is shown in Fig. 2.8. There are two steps during the ligament thinning process: the first step is characterized by the exponential decay of ligament diameter, and the second step is represented by the nearly linear decay of ligament diameter due to the finite extensibility of polymer chain of the sodium alginate solution.
Fig. 2.8. Diameter changes at exit-pinching location using 0.50% and 1.00% sodium alginate solutions at 65 V

For sodium alginate solutions with a concentration of 0.50 – 1.00%, $Oh < 1$ and $El > 1$ representing a dominant elastic effect. The elastic stress is generated when the polymer chain of alginate solution is stretched. As such, exit-pinching is largely governed by a balance of elastic and capillary effects. The exponential filament decay at the exit-pinching location is due to the elastocapillary thinning mechanism, and such an exponential decay during DOD inkjet printing has also been observed elsewhere [Basaran2013]. Before the elastic stress due to polymer stretching is large enough to balance the capillary stress, the polymer deformation can be estimated as follows [Wagner2005]:

$$\frac{D_0}{D_{cr}} = \left( \frac{4\lambda\gamma}{\mu D_0} \right)^{\frac{4}{3}}$$  \hspace{1cm} (2.2)

where $D_0$ is the nozzle diameter, and $D_{cr}$ is the critical ligament diameter, which is estimated around 30 and 40 µm, respectively, for the 0.50% and 1.00% sodium alginate solutions. The measured diameters at 70 µs are 35 and 45 µm, respectively, which is in good agreement with the estimates.

As observed, with the increase of sodium alginate concentration, the exponential decay time increases; such as 60 µs for the 0.50% solution and 125 µs for the 1.00% solution. During exponential ligament thinning, the polymer chains are elongated in the
local region near the pinch-off location. With the decrease of ligament diameter, the capillary pressure increases, which requires the larger deformation of polymers to have a higher elastic stress to balance. The higher polymer concentration results in a higher elastic effect, which is able to balance the increasing capillary stress caused by the decreasing ligament diameter. Therefore, the time for the exponential decay turns longer with the increase of sodium alginate concentration.

As the filament continues to thin, it is observed that the ligament decay close to the breakup moment is nearly linear instead of exponential. During the exponential decay process, the deformation of polymer increases continuously until the polymer chains are fully stretched. Then the fluid behaves like a very viscous anisotropic Newtonian fluid whose viscosity is characterized by the steady extensional viscosity of the fluid. Therefore, the diameter decays almost linearly with time after an exponential decay period.

**Effective relaxation time**

The ligament thinning process is driven mainly by a balance between the capillary stress and elastic stress, which is due to polymer stretching. The ligament diameter at the exit-piningching location thins exponentially due to the elastocapillary thinning mechanism, which determines the scaling law for the ligament diameter decay process:

\[
D_{emin}(t) \propto e^{-\frac{t}{\lambda_e}}
\]

(2.3)

Where \(D_{emin}\) is the ligament diameter at the exit-pinching location, and \(\lambda_e\) is the effective
relaxation time used here to distinguish it from the longest relaxation time $\lambda$. As estimated from Fig. 2.8, the effective relaxation time for the 0.50% and 1.00% sodium alginate solutions is 36 $\mu$s and 37 $\mu$s, respectively, which are much smaller than the longest relaxation times (around 500 $\mu$s) observed during dripping. While the longest relaxation time characterizes the relaxation of the entire molecular chain, the shortest relaxation time characterizes the relaxation of one monomer. For the sodium alginate used in this study, its molecular weight is within the range of 12 – 40 kDa, and the amount of monomers in one molecule is estimated more than one hundred based on the average molecular weight (26 kDa). The estimated shortest relaxation time can be as small as several microseconds. As expected, the estimated effective relaxation time (such as 36 and 37 $\mu$s) based on the ligament exponential decay process is somehow between the shortest and longest relaxation times.

During dripping [Chen2002b] [Tirtaatmadjia2006], continuous jetting [Oliveira2005] [van Hoeve2010] [Ardekani2010], and liquid bridge pinching off [Anna2001] [Vadillo2010], the typical breakup time for polymer solutions is on the order of milliseconds. During DOD inkjet printing, the typical breakup time is on the order of microseconds, which is much shorter. This breakup time difference is mainly due to the influence of oscillating high-frequency pressure wave during DOD inkjet printing. One important characteristic of the pressure wave, which is usually ignored, is the high-frequency oscillation. The oscillation period as estimated from the meniscus oscillation is around 65 – 75 $\mu$s as observed, which is equivalent to an oscillation frequency of 13 – 15 kHz, and this oscillation frequency only depends on the nozzle geometry and acoustic
speed. On the other hand, the critical frequency $f_{cr}$ for the longest relaxation time is $1/\lambda$ [Mewis2012], and the 0.50% and 1.00% sodium alginate solutions in this study have the critical frequencies range of 1.5 – 2.3 kHz, which is much smaller than that of oscillating pressure wave (13~15 kHz). Actually, the period of the oscillating pressure wave is on the same order of the effective relaxation time instead of the longest relaxation time.

It is reported that there is a relaxation time capturing the polymer global chain unraveling mechanism depending on process conditions [Vadillo2012], and the dominating mode is the mode having the relaxation time nearest to time scale of the process [Entov1997]. Therefore, it concludes that during the experiments the mode with the effective relaxation time is dominant, and the contribution of the other available modes rapidly decays.

It should be noted that the sodium alginate used in this study is a natural product containing molecules with different molecular weights. When the external high-frequency pressure oscillation is applied, the response of small molecules is very quick due to their small relaxation time. For large molecules, the entire chain may not have enough time to respond, but a few monomers can show the effect of relaxation. Both lead to the exponential decay of ligament diameter with an effective relaxation time.

**Elastic stress**

During ligament thinning, the axial force balance on the ligament can be listed as follows by neglecting the viscous and inertial effects [Anna2001] [McKinley2005]:

\[
\frac{d}{dr} \left( r \frac{dF}{dr} \right) = \frac{F}{\lambda}
\]
\[
\frac{\gamma}{R_i(t)} + 6\mu_s \frac{R_i(t)}{R_i(t)} + fG(A_{zz} - A_{rr}) = 0 \tag{2.4}
\]

\[
f = \frac{L^2}{L^2 + 3 - tr(A)} \tag{2.5}
\]

where \( R_l \) is the ligament diameter at the exit-pinning location, dot means the differentiation operation, \( \mu_s \) is the solvent viscosity, \( A_{zz}, A_{rr}, \) and \( tr(A) \) are the axial component, radial component, and trace of the average second-moment configuration tensor \( A \), \( f \) is the finitely extensible nonlinear elastic (FENE) factor, \( G \) is the elastic modulus \( (G \sim \frac{\mu_p}{\lambda} \text{ [Anna2001]}), L \) is the finite extensibility of polymer chain, and \( \mu_p \) is the polymer contribution to the solution viscosity. For Equation (2.4), the first term represents the capillary pressure contribution, the second term represents the effect of the solvent viscous stress, and the third term is the elastic stress due to the polymer chain stretching. The polymer finite extensibility \( L \) can be estimated from the number of Kuhn-steps \( N_k \) in a polymer chain as \( L^2 = 3N_k \) [Clasen2009] where \( N_k \) is defined as follows [Tirtaatmadja2006]:

\[
N_k = \alpha \left( \frac{F^2}{C_{\text{mod}}} \right)^{0.5} \tag{2.6}
\]

where \( \alpha = 3M_w/M_0, M_w \) is the molecular weight, \( M_0 \) is the molecular weight of a monomer unit, \( \nu = 0.59 \) [McIlroy2013] is the solvent quality factor for a good solvent as in this study, \( F \) is the geometric factor \( F = \sin[\tan^{-1}(\sqrt{2})] \), and \( C_{\text{mod}} = C_{\infty} \left( \frac{F^2}{C_{\infty}} \right)^{2\nu-1} \) is the modified characteristic ratio with \( C_{\infty} = 53 \) being the characteristic ratio
During ligament thinning, the polymeric stress increases with the stretching of polymer chain until it reaches the finite extensibility limit. At the fully-stretched moment, \( f \) and \( A_{zz} \) satisfy the following relationship [Hoath2012]:

\[
f A_{zz} \approx \frac{U \lambda}{Z} L^2
\]  

(2.7)

where \( Z \) is the ligament length (\( Z \sim 1 \text{ mm} \) as observed in the experiments). It is assumed that the axial elastic strain of the polymer is larger than the radial strain as \( A_{zz} \gg A_{rr} \).

Then for the sodium alginate solution with a concentration of 0.50 – 1.00%, the solvent viscous term of Equation (2.4) is determined on the order of 10 Pa, and the capillary term is approximately 4.7 – 5.9 kPa. For the elastic term, it is estimated as 2.9 – 4.1 kPa using the effective relaxation time while 0.3 – 0.4 kPa using the longest relaxation time. Based on the values of these viscous, capillary, and elastic terms, it is concluded that the elastic stress determined based on the effective relaxation time balances the capillary stress, characterizing the exponential ligament decay process. That is, the elastic stress during exponential ligament decay should be estimated using the effective relaxation time instead of the longest relaxation time.

### 2.4.3 Middle-Pinching

Middle-pinching rarely happens during DOD inkjet printing of alginate solutions. It only occurs at high sodium alginate concentrations such as 1.50% and 2.00%, which have \( Oh>1 \) and \( El>1 \), meaning the dominant viscous and elastic effects. During middle-pinching, the ligament thins until its diameter is below the resolution of
the imaging system (3 µm). The ligament further thins and breaks up due to the Rayleigh instability. As summarized before, the breakup may occur any place along the thin ligament.

The ligament thinning process near the ligament head/forming droplet is governed by a balance of viscous, elastic and capillary effects. However, near the nozzle orifice, the viscoelastic behavior of the fluids is affected by the high-frequency pressure wave, and the ligament thinning process is exponential and governed by the elastocapillary thinning mechanism as shown in Fig. 2.9.

![Fig. 2.9. Middle-pinching diameter information using 1.50% sodium alginate solution at 45V](image)

Middle-pinching actually is a special case when the fluid of the ligament has
enough time to flow into both the nozzle orifice and the main forming droplet. Two characteristics of middle-pinching are observed: fluid drainage back into the nozzle, and low, oscillating head velocity (Fig. 2.10) of the forming droplet. The head velocity oscillates and is less than 1 m/s when close to pinch-off. During middle-pinching, it is also observed (Fig. 2.11) that the ejected volume starts decreasing after 100 µs, indicating that the fluid of the ligament starts flowing back to the nozzle orifice due to the pressure difference caused by surface tension.

![Oscillating velocity graph](image)

Fig. 2.10. Head velocity of forming droplet using 1.50% sodium alginate solution at 45 V

 Apparently, long breakup time and low head velocity facilitate the drainage of the fluid of the ligament. The breakup time near the orifice should be long enough for the capillary pressure to drive some of the ligament fluid to flow back to the nozzle. The
head velocity of the forming droplet should be small enough so that the fluid of the ligament can stay connected with the ligament head. Only in this way can a uniformly thin ligament be formed before pinch-off, which eventually results in middle-pinching. It is observed in this study that the middle-pinching process only occurs using the 1.50 – 2.00% sodium alginate solutions under low excitation voltages (for example, 50 V for 1.50% solution and 55 V for 2.00% solution).

![Graph showing ejected fluid volume as a function of time](image)

**Fig. 2.11.** Ejected fluid volume as a function of time

### 2.4.4 Hybrid-Pinching

Hybrid-pinching happens when the front-pinching and exit-pinching occurs simultaneously. The breakup times at the front-pinching and exit-pinching locations compete with each other to determine the pinch-off type, which depends on the sodium
alginate concentration and initial ligament diameter. Under certain conditions, the two breakup times may be the same, resulting in hybrid-pinching. For low-concentration alginate solutions, the viscous and elastic effects are relatively small when compared to the inertial and capillary effects. As the sodium alginate concentration increases, the viscous and elastic effects start showing their influence by delaying the breakup time of front-pinching, which may be close to that of exit-pinching. While it is not common, hybrid-pinching is observed using the 0.30% sodium alginate solution at the excitation voltage within the range of 40 – 55 V and the 0.35% sodium alginate solution at the excitation voltage within the range of 34 – 36V.

2.4.5 Phase Diagram
To summarize the discussion on pinch-off, a decision tree is drawn as Fig. 2.12 based on the interplay of the capillary, viscous, elastic and inertial effects to illustrate the happening of the aforementioned four pinch-off types. The Weber number \( We = \frac{\rho RU^2}{\gamma} \), representing the ratio of fluid kinetic energy (operating condition-dependent property) to
surface energy (material property-dependent property), is selected to aid the determination of pinch-off type. While the excitation voltage doesn’t directly alter the mechanism of the ligament thinning process, it affects the ejected fluid volume and the resultant ligament diameter, which in turns affect the breakup time as well as pinch-off types. The effect of excitation voltage is included in the $We$ number in determining the pinch-off type. For Fig. 2.12, $We_1$, $We_2$, and $We_3$ depend on the sodium alginate concentration. For example, for the 0.25% alginate solution, $We_1 = 17.1$, $We_2 = 63$; for the 1.5% alginate solution, $We_3 = 3.3$.

For very low sodium alginate concentrations such as 0.10 – 0.20%, both the $Oh$ and $El$ numbers are very small, and the viscous and elastic effects are negligible, leading to front-pinching at the voltage of 30 – 70V. The ligament thinning process at the front-pinching location is governed by a balance of inertial and capillary effects, following a power function with an exponent of $2/3$.

For low sodium alginate concentrations such as 0.25 – 0.35%, both the $Oh$ and $El$ numbers are less than 1. Although the viscous and elastic effects start playing an important role during ligament thinning, they are still less significant when compared to the inertial and capillary effects. The pinch-off type is significantly affected by the fluid kinematics, which determines the initial ligament diameter near the ligament head. With the increase of $We$, the pinch-off type may change from front-pinching to hybrid-pinching to exit-pinching.

For intermediate sodium alginate concentrations such as 0.50 – 1.00%, $Oh < 1$ and $El > 1$, so the elastic effect starts to be dominant. As observed, exit-pinching occurs
at the voltage range of 30 – 70V. The ligament thinning at the exit-pinching location is governed by a balance of elastic and capillary effects, resulting in the exponential decay process.

For high sodium alginate concentrations such as 1.50 – 2.00%, $Oh > 1$ and $El > 1$, so both the viscous and elastic effects are dominant. The ligament thinning process near the ligament head/forming droplet is governed by a balance of viscous, elastic, and capillary effects while the ligament thinning process near the orifice is governed by a balance of elastic and capillary effects due to the high-frequency pressure wave. At small $We$, middle-pinching occurs since the fluid of the ligament flows back into the nozzle and joins the forming droplet. With the increase of $We$, middle-pinching turns to be exit-pinching due to the increase of the initial ligament diameter as well as the breakup time at the front-pinching location.

Furthermore, a dimensionless number $J$, which is defined as the square root of the product of $Oh$ and $El$ as shown in Equation (2.8), is proposed to represent the ratio of viscous and elastic effects to inertial and capillary effects. Based on the $J$ and $We$ numbers, a phase diagram as shown in Fig. 2.13 is constructed to classify the regimes for different pinch-off types during DOD inkjet printing of alginate solutions.

$$J = \sqrt{(Oh)(El)}$$ (2.8)
Fig. 2.13. Phase diagram in terms of $J$ and $We$

When $J$ is small ($< 0.2$), the elastic and viscous effects are almost negligible, and front-pinching usually occurs. When $J$ is in the range of $0.5 - 1.8$, the elastic effect becomes important, and exit-pinching dominates. The $J$ range of $0.2 - 0.3$ sees a mixed regime where front-pinching, exit-pinching, and hybrid-pinching may occur depending on $We$. As $We$ increases, the pinch-off type from front-pinching to hybrid-pinching and exit-pinching. When $We$ is small, the ejected fluid volume and the initial diameter at the front-pinching location are small, and front-pinching occurs. With the increase of $We$, the initial diameter at the front-pinching location increases, which leads to the increase of breakup time at the front-pinching location. When the breakup times at both the front-
pinching and exit-pinching locations are the same, hybrid-pinching occurs. When \( We \) is large enough, the breakup time at the front-pinching location turns larger than that at the exit-pinching location, exit-pinching occurs.

When \( J > 0.3 \), exit-pinching most likely happens. It should be noted that middle-pinching is favored only when \( J \geq 5 \) and \( We < 3 \). High \( J \) means that the viscoelastic effect is dominant, which significantly delays the ligament thinning process, so the capillary pressure has sufficient time to drive the flow to the forming droplet or the nozzle. Small \( We \) means that the fluid of the ligament is capable to connect and flow into the forming droplet. The combined effect results in a thin, uniform ligament before pinch-off, which eventually breaks due to the Rayleigh instability.

2.5 Conclusions

This work has studied various pinch-off locations as a function of material properties and operating conditions during DOD inkjet printing of viscoelastic sodium alginate solutions. Four breakup types have been identified: front-pinching, hybrid-pinching, exit-pinching, and middle-pinching. A dimensionless number \( J \), which is defined as the square root of the product of \( Oh \) and \( El \), has been proposed to represent the ratio of viscous and elastic effects to inertial and capillary effects. Based on the \( J \) and \( We \) numbers, a phase diagram is constructed to classify the regimes for different pinch-off types during DOD inkjet printing of alginate solutions. Some main conclusions are drawn as follows:
1) For very low sodium alginate concentrations such as 0.10 – 0.20%, both the $Oh$ and $El$ numbers are very small, and the viscous and elastic effects are negligible, leading to front-pinching at the voltage of 30 – 70V. The ligament thinning process at the front-pinching location is governed by a balance of inertial and capillary effects, following a power function with an exponent of 2/3;

2) For low concentrations such as 0.25 – 0.35%, although the viscous and elastic effects start playing an important role during ligament thinning, they are still less significant when compared to the inertial and capillary effects. With the increase of $We$, the pinch-off type may change from front-pinching to hybrid-pinching to exit-pinching;

3) For intermediate concentrations such as 0.50 – 1.00%, the elastic effect starts to be dominant. As observed, exit-pinching occurs at the voltage range of 30 – 70V. The ligament thinning at the exit-pinching location is governed by a balance of elastic and capillary effects, resulting in the exponential decay process. The effective relaxation time, which is much smaller than the longest relaxation time, characterizes the exponential decay; and

4) For high concentrations such as 1.50 – 2.00%, both the viscous and elastic effects are dominant. The ligament thinning process near the ligament head/forming droplet is governed by a balance of viscous, elastic, and capillary effects while the ligament thinning process near the orifice is governed by a balance of elastic and capillary effects due to the high-
frequency pressure wave. At small $We$, middle-pinching occurs. With the increase of $We$, middle-pinching turns to be exit-pinching due to the increase of the initial ligament diameter as well as the breakup time at the front-pinching location.
CHAPTER THREE

DROPLET FORMATION PROCESS DURING DROP-ON-DEMAND INKJETTING OF LIVING CELL-LADEN BIOINK

Abstract

Biofabrication offers a great potential for the fabrication of three-dimensional living tissues and organs by precisely layer-by-layer placing various tissue spheroids as anatomically designed. Inkjet printing of living cell-laden bioink is one of the most promising technologies enabling biofabrication, and the bioink printability must be carefully examined for it to be a viable biofabrication technology. In this study, the cell-laden bioink droplet formation process has been studied in terms of the breakup time, droplet size and velocity, and satellite formation using a time-resolved imaging approach. The bioink has been prepared using fibroblasts and sodium alginate with four different cell concentrations: without cells, 1×10^6, 5×10^6, and 1×10^7 cells/ml to appreciate the effect of cell concentration on the droplet formation process. Furthermore, the bioink droplet formation process is compared with that during the inkjetting of the polystyrene microbead-laden suspension under the identical operating condition to understand the effect of particle physical properties on the droplet formation process. It is found that: (1) as the cell concentration of bioink increases, the droplet size and velocity decrease, the formation of the satellite droplet is suppressed, and the breakup time increases; and (2) compared to the hard bead-laden suspension, the bioink tends to have a less ejected fluid volume, lower droplet velocity decrease, and longer breakup time.
3.1 Introduction

Cell suspension can be considered as a type of particle-laden ink. Particle-laden colloidal ink, most ceramics-based [Song1999] [Smay2002], has been used to print 3D patterns and structures. For better understanding of printing performance using particle-laden ink, numerous studies have been devoted to investigating its droplet formation process during dripping, CIJ printing or DOD printing of the ink with different particles laden. Such particles include poly(methyl methacrylate) (PMMA) particles [Furbank2004] [Furbank2007], silver nanopowders [Tsai2008], zirconia powders [Tay2001], polystyrene bead [Bertrand2012], and pigments [Wang2012], to name a few. It has been reported that the resulting jet had non-straight trajectories [Furbank2004] [Wang2012] and non-axisymmetric ligaments [Wang2012] and broke up closer to the orifice, meaning a short filament [Bertrand2012]. Generally speaking, the resulting droplet size decreases [Furbank2004] [Furbank2007] [Tsai2008], the droplet velocity decreases [Tsai2008], and the number of satellites decreases [Furbank2004] [Furbank2007] too when a particle-laden fluid is dispensed. Unfortunately, there is no study investigating the droplet formation performance of cell-laden suspension, which has soft living cells inside and different from hard particles studied in previous studies [Tay2001] [Furbank2004] [Furbank2007] [Tsai2008] [Bertrand2012] [Wang2012].

The objective of this part is to investigate the droplet formation process during DOD inkjetting of cell-laden fluids, namely bioink. For cell suspensions with low cell concentrations, the rheological properties of cell suspensions are measured. Then the effects of cell concentration on breakup time, droplet velocity and size are studied.
Finally, the inkjetting performance results using cell suspensions and polystyrene bead-based suspension are compared with the same particle concentration (5×10^6 particles/ml). For cell suspensions with high cell concentrations, the electric field has been introduced to facilitate the droplet formation process.

3.2 Experimental Design

3.2.1 Bioink Preparation

In additive biofabrication, typical bioink used is made from cell suspension, hydrogel, and/or other additives. Of various cells, fibroblast has been widely used in biofabrication and biomedical research [Norotte2009] [Skardal2010] [Pataky2012] [Xu2012] since fibroblasts are the most common cells of connective tissue in animals. As a versatile biomaterial [Augst2006] [Murphy2013], alginate hydrogel has been used as scaffolds for tissue engineering, as delivery vehicles for drugs, and as model extracellular matrices for basic biological studies. These applications require tight control of various material properties including mechanical stiffness, swelling, degradation, cell attachment, and binding or release of bioactive molecules. Control over these properties has been achieved by chemical or physical modifications of the polysaccharide itself or the gels formed from alginate. The utility of these modified alginate gels as biomaterials has been demonstrated in a number of in vitro and in vivo studies [Augst2006]. Alginate has been used widely as a hydrogel constituent of bioink in bioprinting [Khalil2005] [Nishiyama2009] [Xu2012], and a recent hydrogel evaluation study has proved its increasing applicability for bioprinting [Murphy2013]. As such, this study has used
fibroblast and sodium alginate (NaAlg) to prepare the bioink.

NIH 3T3 mouse fibroblasts (ATCC, Rockville, MD) were first cultured in Dulbecco’s Modified Eagles Medium (DMEM) (Sigma–Aldrich, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS) (HyClone, Logan, UT). The cultured cells were used to prepare cell suspensions with different concentrations of $2 \times 10^6$, $1 \times 10^7$ and $2 \times 10^7$ cells/ml as described by Xu et al. [Xu2012]. Different bioinks were prepared by mixing the cell suspensions and a solution of 2% (w/v) sodium alginate (Sigma-Aldrich, St. Louis, MO), which was prepared using DMEM, at a volume ratio of 1:1. The final bioinks had four different cell concentrations of 0, $1 \times 10^6$, $5 \times 10^6$ and $1 \times 10^7$ cells/ml with a sodium alginate concentration of 1% (w/v) since the typical cell concentration in inkjet printing is on the order of $10^6$ cells/ml [Xu2005] [Henmi2008] [Nishiyama2009].

In order to compare the influence of soft cells and hard particles on the droplet formation process during DOD inkjetting, polystyrene microbeads (Polysciences, Warrington, PA) were selected to make the hard particle-laden suspension for printing performance evaluation. The polystyrene microbeads were 15 µm in diameter, close to that of fibroblasts. However, they are considered as a hard material (3.7 GPa [Oral2011]) when compared to fibroblasts (4~5 kPa [Bushell1999]). Both polystyrene microbeads and 3T3 cells were suspended in 1% (w/v) sodium alginate solutions, respectively, to achieve a concentration of $5 \times 10^6$ particles or cells/ml.

The shear viscosity of the bioink was measured in a Couette geometry (bob diameter of 25mm and cup diameter of 27 mm), and the storage modulus $G'$ and loss modulus $G''$ were measured in a cone-plate geometry (plate diameter of 50 mm and cone
opening angle of 0.04 rad) using a rotational rheometer (ARES, TA Instrument, New Castle, DE). The surface tension was measured using a drop shape analysis system (DSA 10-MK2, Kruss HmbH, Hamburg, Germany) at room temperature based on the pendant drop method. The viscosity was determined based on the average of three measures of each bioink. The surface tension was determined based on the average of five measurements using 1 g/cm³ as the specific gravity for each bioink tested. After droplet formation, the droplets fabricated were further gelatinized using a 2% (w/v) solution of calcium chloride dihydrate (Sigma–Aldrich, St. Louis, MO) [Herran2012a] [Herran2012b].

3.2.2 Experimental Setup and Design

The inkjet printing process in this study was implemented using a MicroFab nozzle dispenser (MicroFab, Plano, TX), and the bioink was ejected using the MicroFab nozzle dispenser with an orifice diameter of 120 µm (MJ-ABL-01-120-6MX) in a DOD mode. The DOD pulse was controlled using the MicroFab Jet Driver, and a MicroFab pneumatic controller was used to adjust the backpressure of the fluid reservoir to obtain an ideal meniscus for good droplet formation. An imaging system (ImageXpert, Nashua, NH) was used to observe the droplet formation process from which the breakup time, droplet size, droplet velocity, and number of satellites were measured. The ligament length was determined using the ImageJ developed by the National Institutes of Health.
The dispensing head used has been commonly driven by a bipolar driving waveform consisting of a succession of two wave pluses: either positive/negative or negative/positive. The second wave pulse is to cancel some of residual acoustic oscillations that may remain in the DOD dispensing head after droplet ejection. The bipolar excitation waveform [Herran2012a] [Herran2012b] [Xu2012] was also employed in this study. The voltage of driving waveform was 45 V, and the voltage rise or fall times were 3 μs, and the dwell or echo time was in the range of 16 and 30 μs with an increment of 2 μs, which were all chosen to investigate the effects of operating conditions on droplet formation. The concentrations of the 3T3 cell suspension used in the experiments were $1 \times 10^6$, $5 \times 10^6$, and $1 \times 10^7$ cells/ml which are widely used in cell printing [Xu2005] [Moon2009]. The detailed experimental conditions are shown in Table 3.1.
Table 3.1. Experimental conditions for cell-laden droplet formation.

<table>
<thead>
<tr>
<th>Experimental parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation voltage (V)</td>
<td>45</td>
</tr>
<tr>
<td>Voltage rise and fall times (μs)</td>
<td>3</td>
</tr>
<tr>
<td>Dwell time (μs)</td>
<td>16, 18, 20, 22, 24, 26, 28, 30</td>
</tr>
<tr>
<td>Echo time (μs)</td>
<td>16, 18, 20, 22, 24, 26, 28, 30</td>
</tr>
<tr>
<td>Cell concentration (cells/ml)</td>
<td>0, 1×10⁶, 5×10⁶, 1×10⁷</td>
</tr>
<tr>
<td>Excitation frequency (Hz)</td>
<td>50</td>
</tr>
</tbody>
</table>

3.2.3 Key Characteristics of a Droplet Formation Process

During a typical droplet formation process, the fluid being dispensed may experience several stages, which reveal some key morphological features of a forming jet/droplet. As shown in Fig. 3.2, some of these features are defined for easy reference. Two important locations are the origin $x_0$ which is the exit position of nozzle orifice and the jet/droplet leading point $x_l$, and these two locations are used to determine the droplet velocity ($U = \frac{x_l - x_0}{\Delta t}$) herein. Before the jet disintegrates from the nozzle, the connecting fluid thread is called ligament. After jet pinch-off, the trailing fluid behind the primary droplet is called tail.
3.3 Material Properties of Bioinks

Material properties are very important for droplet formation process during DOD inkjet printing. It is known that for Newtonian fluids the important parameters during DOD inkjet printing are density, viscosity and surface tension [Jang2009] [Dong2006]. For viscoelastic fluids the additional important parameter is viscoelasticity [McIlroy2013] [Morrison2009] [Yan2011]. During our experiments the NaAlg is included to introduce viscoelasticity. Furthermore, cells suspended in the bioink increase the viscoelasticity [Adesanya2012] [Iordan2008]. The storage modulus and loss modulus are used to characterize the viscoelasticity. In this study, the measured material properties include density $\rho$, shear viscosity $\mu$, surface tension $\sigma$, and storage modulus $G'$ and loss modulus $G''$.

3.3.1 Shear Viscosity and Cell Deformation under Shear Flow
Generally, particle-laded suspension can be classified into three regimes based on the particle volume fraction $\phi$ [Rutgers1962]: dilute ($\phi \leq 2\%$), semi-dilute ($\phi \leq 25\%$) and concentrated ($\phi > 25\%$). Since the cell volume fractions of the four kinds of bioink in this study were 0, 0.18%, 0.88% and 1.77%, respectively, the bioink studied is considered as dilute suspension. The respective actual cell concentrations were 0, $1 \times 10^6$, $5 \times 10^6$ and $1 \times 10^7$ cells/ml. The results from measurements of the shear viscosity of the four kinds of bioink at different shear rates are shown in Fig. 3.3. The viscosity increases with the increase of the cell concentration at a given shear rate. When the suspended cells move with the bioink flow, the energy dissipation increases due to both the distortion of the flow field and the friction exerted by the bioink flow at the cell surface. Therefore, the viscosity increases with the cell concentration, and this observation is consistent with the viscosity measurement from prior literature studies [Pal2003] [Adesanya2012].

As seen from Fig. 3.3, the bioink demonstrates a shear-thinning property, i.e., the viscosity decreases with the shear rate. As reported, the concentrated suspension usually has the shear-thinning behavior due to the particle interaction [Mueller2010], and the cell suspension also shows the shear-thinning behavior due to the deformation of cells [Pal2003]. However, the bioink in this study is considered dilute, so the interaction between cells might not be pronounced. It is assumed that the measured shear-thinning behavior is mainly due to the sodium alginate solution which was a shear-thinning solution [Herran2012b].
It is known that the fibroblasts are highly elastic and easy to deform due to the small Young’s modulus of 4~5 kPa [Bushell1999]. For cell suspensions it is a good starting point to treat cells as deformable elastic particles in modeling the rheological behavior or cell suspensions. It is known that initially spherical elastic particles undergo the deformation in a shear flow and admit steady-state solutions characterized by the balance between the viscous force in the fluid and the elastic force in the solid. Gao et al. [Gao2012] modeled the particle as a homogeneous, incompressible, neo-Hookean elastic solid and analytically calculate the final shape of the particle subject to a shear flow:

$$\omega = (\sqrt{1 + G^2} - G)^2$$

Where $\omega$ is the aspect ratios of the elliptic particles and $G = \frac{\mu_0 \gamma}{S}$ is the shearelastic
number, $S$ is the shear modulus, $\mu_0$ is the viscosity, $\dot{\gamma}$ is the shear rate. Cell shear modulus $S$ is assumed to be 10 kPa [Fernandez2010]. When the bioink with the cell concentration of $1 \times 10^6$ cells/ml is used, the eventual $\omega$ is around 0.88 using $\mu_0=23$ cP and $\dot{\gamma} = \frac{\partial U}{\partial R} \approx \frac{3.5}{60 \times 10^{-6}} = 5.8 \times 10^4$ /s can be approximated as $U/R$.

3.3.2 Surface Tension

The bioink surface tension is presented in Table 3.2, and it decreases with the cell concentration. The observed surface tension tendency of the bioink is consistent with other surface tension measurements of particle-laden suspension [Okubo1995] [Dong2003]. With the increase of cell concentration more cells are adsorbed to the interface to reduce the total free energy, resulting in a smaller surface tension value. The presence of particles such as living cells at the liquid-gas interface is expected to affect the overall surface tension. The concentration of particles in the suspension plays a vital role in the surface tension of suspension since the presence of any particle changes the intermolecular interaction [Waghmare2010]. The most energy favorable position for a particle is at the interface. Regardless that the particles are hydrophobic or hydrophilic, they tend to strongly attach to the interface [Dong2003]. After the particle adsorption to the interface, the internal energy decreases and the entropy increases; as a result, the interfacial tension decreases as the particle concentration increases.
Table 3.2. Fluid properties of different bioinks.

<table>
<thead>
<tr>
<th>cell concentration of Bioink</th>
<th>Viscosity at a shear rate of 50 s(^{-1}) (mPa(\cdot)s)</th>
<th>Surface tension (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>without cells</td>
<td>22.1</td>
<td>45.2±0.2</td>
</tr>
<tr>
<td>1×10(^6) cells/ml</td>
<td>23.0</td>
<td>44.4±0.2</td>
</tr>
<tr>
<td>5×10(^6) cells/ml</td>
<td>27.5</td>
<td>43.4±0.2</td>
</tr>
<tr>
<td>1×10(^7) cells/ml</td>
<td>30.6</td>
<td>42.3±0.1</td>
</tr>
</tbody>
</table>

3.3.3 Storage Modulus and Loss Modulus

The storage modulus \(G'\) and loss modulus \(G''\) quantify the stored energy (elastic effect) and the energy dissipated as heat (viscous effect), respectively, and their measurements are shown in Fig. 3.4. It can be observed that with the increase of cell concentration both the moduli increase, which is consistent with other reported results of particle-laden suspension [Pugh1994] [Mason1995] [Iordan2008] [Adesanya2012]. It is noted that there is no pronounced storage modulus difference among the bioink tested while there is a noticeable loss modulus difference as the cell concentration changes. The storage modulus difference is mainly from interparticle contact and interaction [Adesanya2012], and the bioink tested had a relatively low volume fraction. As a result, the cell interaction is not significant, resulting in a negligible storage modulus difference. For the bioink with 1×10\(^7\) cell/ml a few cell aggregations were observed, which represents the cell interaction. The loss modulus increases with the increase of cell concentration mainly due to the increase in hydrodynamic energy dissipation, which is introduced by the distortion of velocity field in the vicinity of each particle.
[Hosokawa2007]. The increase of storage and loss moduli also indicates the increase of both elastic and viscous effects, which affects the droplet formation process during printing.

![Graph showing storage modulus G' and loss modulus G'' under different angular frequencies](image)

Fig. 3.4. Storage modulus $G'$ and loss modulus $G''$ under different angular frequencies

### 3.4 Effect of Cell Concentration on Droplet Formation

#### 3.4.1 Representative Droplet Formation Process

The bioink was ejected into a 2% calcium chloride solution to form alginate microspheres embedded with cells. Fig. 3.5 shows some cell-encapsulated microspheres fabricated from the $1 \times 10^6$ cells/ml bioink using the driving waveform as follows: excitation voltage 45 V, voltage rise and fall times 3 μs, dwell time 20 μs, echo time 20
μs, and excitation frequency 50 Hz. The bioink droplet formation process was studied using a time-resolved imaging approach. Fig. 3.6 shows some representative droplet formation processes when the bioink with different cell concentrations (0, 1×10^6, 5×10^6, and 1×10^7 cells/ml) was printed, and Fig. 3.7 further illustrates the jet/droplet morphological evolution under different cell concentrations, including the head and tail positions and breakup time.

Fig. 3.5. Alginate microspheres embedded with living cells
As seen from Fig. 3.6, there are three main differences during the droplet formation process when the cell concentration changes: breakup time, ligament length at breakup, and number of satellite droplets, which are to be discussed in details in the following sections. Generally speaking, when the bioink without cells is printed, exit pinching [Xu2013b] during which the first breakup occurs near the nozzle orifice happens at 190 µs. As the cell concentration increases, the breakup time increases to 205 µs for the 10⁶ cells/ml bioink, 220 µs for the 5×10⁶ cells/ml bioink, and 240 µs for the 1×10⁷ cells/ml bioink. When the bioink without cells is used, it forms a long ligament with a large diameter. As the cell concentration increases, the ligament length is shorter, and the diameter is smaller at breakup. The formation of satellite droplets is also suppressed as the cell concentration increases. When the bioink without cells is used, a
long ligament is formed and eventually breaks into several satellite droplets. As the cell concentration increases, the ligament turns shorter and thinner at breakup, and fewer satellite droplets are formed. When the cell concentrations are $5\times10^6$ cells/ml and $1\times10^7$ cells/ml, only one droplet is formed.

Fig. 3.7. Jet/droplet morphological evolution under different cell concentrations: (a) without cells, (b) $1\times10^6$, (c) $5\times10^6$, and (d) $1\times10^7$ cells/ml

The ligament head and tail positions are shown in Fig. 3.7. In addition, three
critical times are illustrated: the breakup time at the nozzle orifice (exit pinching) to form the ligament tail ($t_1$), the breakup time of the ligament to form new satellites ($t_2$), and the merging time for the tail and primary droplet when no satellites are formed ($t_3$). Several conclusions can be drawn from Fig. 3.7. First, at a given time the position of forming filament/droplet decreases with the cell concentration, indicating the decrease of the ligament length as well as the droplet velocity. Second, the breakup time ($t_1$) increases with the cell concentration. Finally, the satellite droplets are suppressed with the cell concentration. Using the bioink with cell concentrations of 0 and $1 \times 10^6$ cells/ml, some satellites are formed at $t_2$ while using the bioink with cell concentrations of $5 \times 10^6$ cells/ml and $1 \times 10^7$ cells/ml, the tail eventually merges with the main droplet and no satellites are formed at $t_3$.

3.4.2 Effect of Cell Concentration on Droplet Formation

The bioink was ejected out from a nozzle dispenser due to a mechanical deformation introduced by the nozzle piezo-element under a bipolar waveform. Herein the effects of cell concentration on the bioink droplet formation process are carefully examined while the driving bipolar waveform was kept constant with the voltage of 45 V, voltage rise/fall time of 3 μs, dwell/echo time of 20 μs, and excitation frequency of 50 Hz. If not specified, the results presented in the following sections are all based on this waveform condition.

Effect of cell concentration on breakup time
The relationship between the breakup time ($t_1$) and the cell concentration is shown in Fig. 3.8. It is seen that the breakup time increases with the increase of cell concentration, which is consistent with the printing results using hard particle-laden suspension [Tsai2008]. As seen from Fig. 3.4, both the elastic and viscous effects increase with the increase of cell concentration. The increased elastic and viscous effects resist the capillary effect and delay the necking of ligament [Anna2001] [McKinley2005], resulting in a longer breakup time.

![Fig. 3.8. Effect of cell concentration on breakup time](image)

**Effect of cell concentration on droplet size and velocity**

The fluid properties influence the droplet formation process, subsequently the droplet size and velocity using a given nozzle dispenser under the same excitation condition. As seen from Fig. 3.9, both the droplet size and velocity decrease with the
increase of cell concentration, which is consistent with the observations during printing using hard particle suspensions [Derby2003] [Jang2009]. While the bioink is ejected out from the nozzle, the total energy applied is converted into the droplet kinetic energy, surface energy, elastic energy stored in the fluid, and energy due to viscous dissipation. As aforementioned, both the elastic and viscous effects increase with the increase of cell concentration. As a result, the viscous dissipation during the droplet formation process increases, and the elastic energy stored in the filament increases too; the remaining energy for the droplet surface energy and droplet kinetic energy decreases, resulting in a smaller fluid volume ejected [Dong2006] [Yu2007] and a lower droplet velocity [Yu2007] [Jang2009] as observed in this study.

Fig. 3.9. Effect of cell concentration on droplet size and velocity
**Effect of cell concentration on satellite formation**

After pinching off from the nozzle, the ligament either merges with the primary droplet or breaks up into several satellite droplets due to the Rayleigh instability. The viscous and elastic forces generally help resist the hydrodynamic instability mainly driven by the surface tension [McKinley2005]. As both the viscous and elastic effects increase, but the surface tension decreases with the cell concentration, all of which are in favor of reducing the growth rate of the most unstable disturbance. As a result, the satellite formation is suppressed and the jet is more stable during the droplet formation process as seen from Figs. 3.6 and 3.7, and this observation is consistent with the instability analysis results of the jetting of viscoelastic fluids [Brenn2000]. In short, it concludes that with the cell concentration the formation of satellite droplets is suppressed due to the increased viscous and elastic effects and the decreased capillary effect.

The number of satellite droplets, if formed, depends on the competing processes of ligament axial shortening and radial necking [Hoath2013], which is affected by a number of factors, notably the Ohnesorge number \((Oh)\) [Bhat2010] [Hoath2013] and for polymeric fluids, the polymer concentration and molecular weight [McIlroy2013]. With a large \(Oh\) number, a fluid tends to limit the satellite production by delaying the radial necking [Hoath2013]. As the cell concentration increases, the viscosity increases, and the surface tension decreases, which results in an increased \(Oh\) number and reduced number of satellite droplets. The elastic effect is also reported to reduce the number of satellite droplets computationally [Bhat2010] [McIlroy2013] and experimentally [Tirtaatmadja2006] by draining liquid from small droplet(s) into larger droplet(s).
Therefore, with an increased cell concentration, the number of satellite droplets is reduced due to the increase of the Oh number as well as the elastic effect.

Fig. 3.10. Effect of cell concentration on satellite number

**Effect of cell concentration on optimal dwell time**

Four different kinds of bioink (0, $1 \times 10^6$, $5 \times 10^6$ and $1 \times 10^7$ cells/ml) were dispensed to appreciate the effect of dwell time of excitation waveform [Herran2012a] [Herran2012b] on the droplet velocity at the moment of exit pinching when a ligament pinches off from the orifice. As seen from Fig. 3.11, the pinch-off droplet velocity changes with the dwell time, and there is a maximum which defines the optimal dwell time. The optimal dwell time herein is defined as the time when the reflected compression pulse inside the nozzle chamber passes through the position where the next
compression pulses generate, resulting in a maximum pinch-off droplet velocity.

During a typical piezoactuator-based inkjetting process, the piezo-element first enlarges and then compresses the nozzle as excited by the applied waveform. During nozzle enlarging, the piezo-element generates dilation pressure pulses propagating to both the ends of nozzle. The liquid reservoir, which connects and supplies the dispensing fluid to the nozzle, is assumed an open boundary condition. The reflected pressure pulse has a 180° phase shift, resulting in a compression pulse after reflecting back from the liquid reservoir. The nozzle orifice represents a large increase in acoustic impedance, and the pulse is reflected with the same sign. Then when the piezo-element compresses the nozzle, compression pressure pulses are generated and propagate in both the directions, canceling the dilation pulse reflected from the orifice and reinforcing the compression pressure wave reflected from the liquid reservoir. The resulting reinforced compression pulse drives the fluid inside the nozzle and eventually large enough to eject a droplet from the orifice.

The droplet velocity is a function of the dwell time of excitation waveform, and the pinch-off droplet velocity has a maximum as determined under the optimal dwell time, which is dependent on the acoustic wave speed in the fluid and nozzle dimension. The acoustic wave speed $a$ can be estimated using the following equation [Reis2005]:

$$a = \left( \frac{K_{\text{eff}}}{\rho} \right)^{1/2} \left( 1 + C_1 \frac{2rK_{\text{eff}}}{Eh} \right)^{1/2}$$  \(3.1\)

where $r$ and $h$ are the nozzle inner radius and wall thickness, $E$ is the wall Young’s modulus, $C_1$ is a geometrical factor, $K_{\text{eff}}$ is the bioink effective bulk modulus, and $\rho$ is the
bioink density. For a given nozzle, the optimal dwell time only depends on the acoustic wave speed which is a function of the bioink material properties. From Fig. 3.11, the optimal dwell time of the four kinds of bioink is within the range of 20 ~ 22 μs, meaning that the acoustic wave speed does not vary significantly for the bioink investigated. It is attributed to the low cell volume fraction of the bioink, which does not change significantly the effective bulk modulus and density of the fluid.

![Dwell time vs Droplet velocity](image)

**Fig. 3.11.** Relationship between the dwell time and droplet velocity

Four kinds of bioink with different cell concentrations (0, 1×10^6, 5×10^6 and 1×10^7 cells/ml) were used to study the effect of dwell time on droplet velocity to find the optimal dwell time for each bioink. Droplet velocity is defined as the velocity when the
ligaments pinch off from the orifice. In Fig. 3.11 it is shown that the droplet velocity is a function of the dwell time and there is a maximum which represents the optimal dwell time. From Fig. 3.11 the optimal dwell times are in the range of 20~22 μs and do not change for four bioinks with different cell concentrations. It is known that for a given nozzle during the experiments the optimal dwell time only depends on the acoustic speed. According to the above equation the acoustic speed is determined by the bulk modulus and the specific gravity of the bioink. Because the specific gravity of cells is close to water [Wang2006] the specific gravity of the bioinks is assumed to be same to be 1 g/cm³. Considering the small cell volume fraction the effective bulk modulus only depends on the fluid [Szczech2004] and does not change with the cell concentration. Therefore the acoustic speed does not change with the cell concentration, which is proved by the experimental data.

3.4.3 Discussion on Non-Ideal Jetting Behavior of Bioink and Effect of Particle Physical Properties

Non-ideal jetting behavior of bioink

Non-ideal jetting behaviors were frequently observed during inkjetting: non-straight flying trajectory as seen in Fig. 3.12. This non-ideal jetting behavior is attributed to accumulated cells near the orifice and the non-ideal wetting condition at the nozzle tip. During the experiments, wetting on the surface around the nozzle tip is usually observed. After a droplet is ejected, some residual liquid is left at the nozzle tip. For the cellular bioink, some cells may adhere at the nozzle tip, causing non-ideal wetting. When a
ligament thins, it may deviate from the nozzle center of the nozzle. The position of the ligament may be different from that being newly dispensed upon the effect of a subsequent pressure wave, affecting the droplet formation process in terms of the droplet trajectory and breakup time. It should be noted that wetting is a very common issue during inkjet printing.

![Image of 100 µm](image)

**Fig. 3.12. Typical non-ideal jetting behavior (5×10^6 cells/ml)**

**Effect of particle physical properties**

The bioink droplet formation process is further compared with that during the inkjetting of the polystyrene microbead-laden suspension under the identical operating conditions to better understand the effect of living cells on the droplet formation process. The average particle size for both the suspensions was around 15 µm, and the particle concentration was 5×10^6 particles/ml. As seen from Fig. 3.13, there is less liquid dispensed, and the droplet velocity is lower during bioink inkjetting. It is found that the bioink breakup time is also longer than that of the polystyrene microbead-laden suspension (195 µs vs. 220 µs).
To appreciate the observed droplet formation differences, the storage and loss moduli of the two suspensions were measured and are presented in Fig. 3.14. It can be seen that the storage modulus $G'$ for the two suspensions is very close while the bioink has a little higher $G'$, meaning that a similar elastic effect may occur during inkjetting. However, the bioink has a much higher loss modulus $G''$, meaning that the significant viscous effect of the bioink leads to a longer breakup time, smaller droplet, and lower droplet velocity as observed. The higher loss modulus of the bioink is probably due to the cell tank-treading motion [Fischer1980]. When the ratio of the cell inner to outer liquid viscosities is small and the shear rate is high, a tank-treading motion of the cell membrane is expected,
resulting in additional energy dissipation [Fischer1980] [Pal2003]. As such, it is concluded that using the bioink the ejected fluid volume and droplet velocity decrease, and the breakup time increases due to the storage and loss moduli differences of the two suspensions.

![Graph showing storage and loss moduli of cell and bead suspension](image)

Fig. 3.14. Storage and loss moduli of cell and bead suspension

### 3.5 Conclusions

The cell-laden bioink droplet formation process has been studied in terms of the breakup time, droplet size and velocity, and satellite formation using a time-resolved imaging approach. The bioink has been prepared using 3T3 cells and sodium alginate, and four different cell concentrations have been investigated: without cells, $1\times10^6$, $5\times10^6$, $1\times10^7$, and $5\times10^7$ cells/ml.
and $1 \times 10^7$ cells/ml to appreciate the effect of cell concentration on the droplet formation process. Furthermore, the bioink droplet formation process is compared with that during the inkjetting of the polystyrene microbead-laden suspension under the identical operating condition to understand the effect of particle physical properties on the droplet formation process. Some main conclusions are drawn as follows:

1) With the increase of the cell concentration of bioink, the shear viscosity increases, the surface tension decreases, and both storage modulus and loss modulus increases. There is no pronounced storage modulus difference among the bioink tested while there is a noticeable loss modulus difference as the cell concentration changes;

2) As the cell concentration of bioink increases, the droplet size and velocity decrease, the formation of the satellite droplet is suppressed, and the breakup time increases; and

3) Compared to the hard bead-laden suspension, the bioink tends to have a less ejected fluid volume, lower droplet velocity decrease, and longer breakup time due to its higher storage and loss moduli.
CHAPTER FOUR

ELECTRIC FIELD-ASSISTED DROPLET FORMATION USING PIEZOACTUATION-BASED DROP-ON-DEMAND INKJET PRINTING

Abstract

Droplets with a diameter from a few to hundreds of micrometers have found increasing applications in various fields. For inkjet printing, it is always a great need to control and reduce the droplet size for a given nozzle diameter and print viscous fluids by avoid clogging. This study has investigated the electric field-assisted droplet formation process under piezoactuation-based drop-on-demand (DOD) inkjet printing. For the better control of droplet monodispersity, the Taylor cone is intentionally suppressed from happening to avoid undesirable satellite droplets. The droplet formation process of deionized water has been investigated, and some main conclusions are drawn as follows: 1) with the increase of the applied voltage, the droplet velocity increases and the droplet size decreases, 2) the pinch-off location may be different depending on the applied voltage; and 3) the combination effect of the electric field and meniscus oscillation can be utilized to significantly reduce the droplet diameter to less than one-fifth of the orifice diameter. The electric field also demonstrates its capability in facilitating the DOD inkjet printing of high-concentrations cell-alginate suspensions.

4.1 Introduction

Nozzle-based jetting has two main drawbacks. First, the orifice tends to clog
during processing viscous materials. Second, it is difficult to fabricate monodisperse droplets smaller than the orifice diameter, if needed, for a given orifice diameter. Even it is doable to reduce the droplet diameter slightly by modifying the excitation waveform [Chen2002a], the achievable droplet diameter is usually limited by the nozzle orifice. Electrohydrodynamic (EHD) technology has long been recognized as an effective approach to form droplets by utilizing electrostatic forces as the driving mechanism through an electrically charged fluid jet [Melcher1969] [Saville1997]. The resulting droplet size can be easily much smaller than the orifice size [Park2007] [Choi2008] [Kim2012]. It has also been reported that EHD can facilitate the printing of viscous materials such as ceramic suspension (285 mPa.s) [Lee2008] and SU-8 photoresist (380 mPa.s) [Park2013]. However, none of these studies have investigated the effects of high voltage on the formation of a single droplet during piezoactuation-based DOD inkjetting, which is the interest of this study. In this study, a high voltage is introduced to assist the droplet formation process during piezoactuation-based DOD inkjet printing. The effects of high voltage on the charge density, droplet velocity and size, and pinch-off position are studied. Furthermore, high voltage has been applied to DOD fabricate cellular droplets from high-concentration cell suspension, which is a difficult-to-print fluid.

4.2 Materials and Method

4.2.1 Mechanism of EHD Jetting

During DC mode EHD jetting, two setups are generally employed for droplet charging: conduction and induction. Under the conduction condition (Fig. 4.1 (a)), a
voltage, typically on the order of kV [Hayati1986], is directly connected to the nozzle, and mobile ions in the fluid accumulate near the interface due to the external electric field. The charged interface is subject to the surface charge-induced electrostatic stresses: normal electrostatic stress which balances the surface tension and tangential electrostatic stress which drives the fluid downstream. During the droplet formation process, positive charges are trapped in the droplets, resulting in positively charged droplets. Under the induction condition (Fig. 4.1 (b)), a voltage is connected to a substrate electrode. Due to the high potential of substrate, the negative charges are induced to the fluid interface. When the fluid is dispersed into droplets, the induced negative charges are trapped on the droplets, resulting in negatively charged droplets.

The main difference between the conduction and induction setups is the way by which the charges flow. For the former, the charges flow from the voltage supply by conduction while for the latter the charges are induced by the electric field. Compared to the conduction setup, the induction setup has fewer safety concerns since the liquid is grounded. Furthermore, there should be no electrical current through the power supply as well as the electrical energy that needs to be supplied by the power supply under the induction setup [Zhao2005]. For piezoactuation-assisted EHD jetting, the induction setup also prevents the undesirable interference between the driving signal of piezoelectric element and external high voltage. Therefore the induction setup, widely used as reported in the literature [Zhao2005] [Nguyen2009], is implemented in the study to investigate the effect of electric field on the droplet formation process.
4.2.2 Experimental Setup and Materials

As shown in Fig. 4.2, the proposed high voltage-assisted DOD inkjet printing system included a piezoactuator-based 110 μm nozzle with a metallic cover (Engineering Arts, Phoenix, AZ) to dispense the fluid, a high voltage source (Glassman High Voltage PS/FC40P03.0-11, High Bridge, NJ) to supply an electric field in an induction configuration, and an imaging system (ImageXpert, Nashua, NH) to visualize the droplet formation process. A voltage up to 11 kV was connected to an aluminum cylindrical substrate with a diameter of 25 mm and a height of 5 mm, and the nozzle was connected to the ground, forming an electric field between the substrate and the nozzle. Deionized water, commonly used as solvent in biological applications, was used in the experiments as a proof-of-concept liquid to study the effect of high voltage on the droplet formation.
process. The deionized water (Millipore, Billerica, MA) used in this study has the following properties: relative dielectric \( \varepsilon_r \) as 78.39 [Shankar2007], density \( \rho \) as 1 g/cm\(^3\), viscosity \( \mu \) as 1 mPa∙s, and surface tension \( \gamma \) as 73 mN/m. Then cell suspension with a cell concentration of 3×10\(^7\) cells/ml in a 1% sodium alginate solution was used to test the performance of the proposed high voltage-assisted printing system during the printing of highly viscoelastic fluids such as high-concentration cell suspension. The protocol for cell-alginate suspension preparation can be found in a previous study [Xu2012].

The imaging system was used to capture the images of droplet formation process and measure the droplet size. The ligament length of forming jet/droplet was determined using ImageJ developed at the National Institutes of Health. The droplet velocity was estimated by dividing the ligament length at pinch-off over the breakup time, and the droplet acceleration was calculated by dividing the velocity difference during a 40 µs duration immediately after pinch-off over the recording duration (40 µs).

In addition, a pneumatic controller was used to adjust the back pressure of the fluid reservoir to obtain an ideal meniscus for good droplet formation. The back pressure was ranging from -2.0 to -1.2 kPa with an interval of 0.2 kPa to guarantee a good droplet formation condition. The excitation waveform used in the experiments is a tripolar excitation which was reported to reduce the droplet size [Chen2002a] and minimize the formation of satellite droplets [Gan2009]. The detailed excitation waveform is shown as the inset of Fig. 4.2.
4.3 Effects of Electric Field on Droplet Formation

When a high voltage is applied between the nozzle and the conductive substrate, the charges are induced and accumulated at the droplet-air interface, which in turn results in an electrostatic stress. During the droplet formation process, since both the Ohnesorge (Oh) and Bond (Bo) numbers are small in this study, the viscous and gravitational effects are neglected. In addition, it is assumed that the deionized water is incompressible and Newtonian, the air is a perfectly insulating fluid, and the liquid bulk (deionized water) is electrically neutral.

Fig. 4.3 shows the four different representative droplet formation processes under different external voltages, clearly showing different pinch-off locations and there is no Taylor cone formed during the entire process. When no voltage is applied, the pinch-off location is next the nozzle orifice as exit pinching. At 2 kV, the pinch-off location appears close to the forming droplet, and, the ligament retracts back into the
nozzle after pinch-off. At 7 kV, two pinch-off locations are observed: the first pinch-off occurs close to the forming droplet, which resembles to what happens at 2 kV; after the first pinch-off, the remaining ligament further deforms under the applied electric field, and the second pinch-off occurs near the nozzle orifice due to EHD instability to form a satellite. At 9 kV, the electrostatic stress is high enough to overcome the surface tension to make the forming droplet an ellipsoid. There is no identifiable ligament formed, and pinch-off occurs some place between the nozzle orifice and the forming droplet.

**Fig. 4.3.** Representative droplet formation images under different voltages

**4.3.1 Effect of Applied Voltage on Charge Density**
To model the motion of charged droplet during a droplet formation process, the gravity, the drag force, and the electrostatic force should be all taken into account. Since the droplet is much smaller compared to the writing height between the nozzle and the substrate, the electric field $E$ is assumed constant during the droplet formation process.

To estimate the droplet charge, the following equation of motion is applied:

$$ma = mg + F_e - F_d$$

(4.1)

where $m$ is the droplet mass, $a$ is the droplet acceleration, $F_e$ is the electrostatic force, and $F_d$ is the drag force. The drag force is defined as:

$$F_d = \frac{1}{2} \rho_a U^2 AC_d$$

(4.2)

where $\rho_a$ is the air density, $U$ is the droplet velocity at pinch-off, $A$ is the cross-sectional area of the droplet, $C_d$ is the drag coefficient as $\frac{24}{Re} \left(1 + 0.15Re^{0.687}\right)$ for the Reynolds number $Re = \frac{\rho DU}{\mu} \leq 800$ [Levy2001], and $D$ is the effective droplet diameter as measured. The electrostatic force $F_e$ is defined as follows by neglecting the dipole contribution [Andrukh2011]:

$$F_e \approx QE$$

(4.3)

where $Q$ is the droplet charge. Therefore, the droplet charge $Q$ can be derived from the equation of motion:

$$Q = \frac{ma - mg + F_d}{E}$$

(4.4)

where $mg = \frac{1}{6} \pi D^3 \rho g$, and $g$ is the gravitational acceleration.
The induced electric field is simulated using the procedure as described in [Andrukh2011] with the following geometrical information: the nozzle with a length of 10 mm and diameter of 1 mm, the conductive substrate with a diameter of 25 mm and the height of 5 mm, the writing height \( d \) of 10 mm between the nozzle and substrate. The system is simulated using COMSOL 3.5a within a 100 mm diameter cylinder which is 6 times larger than the writing height. At 0~6 kV, the droplets are assumed spherical; at 8~10 kV, the droplets are assumed ellipsoidal. The dimensions and positions of the spherical and ellipsoidal droplets are based on the measurements. The boundary conditions are as follows: the nozzle is connected to the ground, and the substrate is connected to the applied voltage; at the droplet surface, the continuity condition of electrical displacement field is applied [Andrukh2011]; the enclosing cylinder has zero charge. As simulated, the electric fields are 0, 4.4×10⁶, 6.0×10⁶, 1.1×10⁷, 1.9×10⁷, and 2.7×10⁷ V/m, respectively, under an applied high voltage \( \phi \) from 0 to 10 kV with an interval of 2 kV.

Once the droplet charges are determined based on Equation (4.4), the droplet volume charge density is estimated by dividing the droplet charges by the droplet volume. From Fig. 4.4, it can be seen that the droplet volume charge density increases with the increase of the applied voltage, and the estimated droplet volume charge density is on the order of C/m³.
4.3.2 Effects of Applied Voltage on Droplet Velocity and Size

The effects of applied voltage on the droplet velocity and diameter (primary droplet, if applicable) are shown Figs. 4.5 and 4.6. It is shown that the droplet velocity increases with the applied voltage. When the applied voltage is lower than 6 kV, the effect of the high voltage is not significant compared to that of the surface tension, and the droplet velocity change is not pronounced and the droplet shape is still almost spherical. The increase of droplet velocity is mainly due to the electrostatic force-induced acceleration, resulting in longer ligaments. When the applied voltage is higher than 6 kV, the droplet shape changes noticeably under the effect of electrostatic force. The electric Bond number $\Gamma = \frac{\varepsilon \epsilon_0 E^2 R}{2 \gamma}$ ($R$ is the nozzle radius), the square of the ratio of capillary to electric times represents the ratio of electrostatic effect to the surface tension effect. At 8 kV, $\Gamma = 1.2$ which means that the electrostatic stress is comparable to the capillary stress.

Fig. 4.4. Droplet volume charge density as a function of applied voltage
in influencing the droplet formation. As such, the droplet is deformed to be ellipsoidal such as the one shown in Fig. 4.3 (9 kV) due to the elongation effect of electrostatic force. As the droplet is elongated, the head of the forming droplet travels more distance, resulting in a noticeable velocity increase as shown in Fig. 4.5 when the applied voltage is higher than 6 kV.

![Droplet velocity as a function of applied voltage](image)

**Fig. 4.5.** Droplet velocity as a function of applied voltage

Droplet diameter is normalized by dividing it by the droplet diameter measured under the zero voltage inkjetting condition. The normalized droplet diameter decreases with the applied voltages, and it reaches less than 80% at 10 kV as seen from Fig. 4.5. When the applied voltage is lower than 6 kV, a forming droplet usually has a ligament connecting to the nozzle before pinch-off. After pinch-off, the remaining ligament may retract to the nozzle instead of merging with the primary droplet or forming a satellite. As a result, the droplet size is usually smaller due to the ligament retraction. When the
applied voltage is larger than 6 kV, the electrostatic stress is too large to pull the ligament away to form a droplet earlier than those under the lower voltage conditions. As such, less fluid is dispensed before forming a droplet, resulting in an even smaller droplet.

Fig. 4.6. Dimensionless droplet size as a function of applied voltage

4.3.3 Effect of Applied Voltage on Pinch-Off Locations

When the fluid is ejected from the nozzle, usually a ligament with a forming droplet is generated first, and then the ligament breaks up from the nozzle orifice or the forming droplet. According to the pinch-off locations under different external voltages (Fig. 4.3), four distinguishable regions are identified as shown in Fig. 4.7. For better discussion, the normalized pinch-off location is introduced as the ratio of the ligament length at the first breakup moment to the nozzle diameter.
Regime 1 (Voltage: 0 ~ 1 kV and $\Gamma = 0.02$): the pinch-off process is mainly driven by the surface tension, and pinch-off occurs close to the nozzle orifice. When the fluid is ejected out from the nozzle, the ligament head has a positive downward velocity while the meniscus near the nozzle orifice retracts due to the following negative pressure pulse, which results in a ligament with the minimum diameter near the nozzle orifice. The curvature becomes small at the transition from the meniscus to the ligament where the ligament has the minimum diameter near the nozzle orifice. The largest curvature change happens at this transition, resulting in the largest capillary pressure. Pinch-off usually occurs at the position with the largest variation of curvature where the pressure gradient is largest [Wijshoff2010]. Therefore, pinch-off occurs near the nozzle orifice as exit-pinching [Xu2013b].

Regime 2 (Voltage: 2 ~ 6 kV, $\Gamma = 0.06$~0.4): the electric field is strong enough
to affect the droplet formation process, and the resulting pinch-off location is close to the ligament head. Due to the following negative pressure pulse during inkjetting, a flow reversal occurs near the nozzle orifice [Xu2007]. As the applied voltage increases, the surface charge density and electrostatic stress increases at the interface between the ligament and the nozzle orifice. The increased electrostatic stress counteracts the reversal flow, preventing the ligament from pinching off next the nozzle orifice. On the other side, the ligament and forming droplet connection point is formed by the nearly spherical droplet and the nearly vertical liquid thread, which is approximately 90° where the electric field and charge density vanish around the vicinity of the connection point [Notz1999]. As such, the electrostatic effect is less significant at the interface between the ligament and the forming droplet and pinch-off occurs near the forming droplet instead of being close to the nozzle orifice. Due to the small electric Bond number, the remaining ligament retracts to the nozzle after pinch-off.

Regime 3 (Voltage: 7 kV and $\Gamma$ around 0.75): there are two pinch-off locations: near the nozzle orifice and near the forming droplet. Pinch-off occurs first near the forming droplet, similar to that under the applied voltage of 2~6 kV. After pinch-off, the ligament is subject to the electrohydrodynamic instability caused by the external strong electric field and the consequent faster growth rate of instability, which causes the ligament pinch-off before it may retract into the orifice. The second pinch-off eventually results in a satellite droplet, which is not desirable for droplet monodispersity.

Regime 4 (Voltage: 8 ~ 10 kV and $\Gamma = 1.2$~2.4): under a high voltage, the droplet changes from spherical to ellipsoidal, indicating that the electrostatic stress is
comparable to the capillary stress. The electric Bond number is larger than 1, indicating a large electrostatic force responsible for droplet shape change. There is no obvious ligament connecting the orifice and droplet, and pinch-off occurs within a relatively narrow region between the nozzle orifice and the forming droplet.

Regime 5 (Voltage: ≥11 kV): wetting may occur at the nozzle tip mainly due to electrowetting. During electrowetting, the contact angle is governed by the Young-Lippmann equation

$$\cos \theta - \cos \theta_0 = \frac{\varepsilon_d \varepsilon_0 \phi}{2 \gamma d}$$

[Millefiorini2006], where $\theta$ is the contact angle under an applied voltage, $\theta_0$ is the contact angle without an applied voltage, $\varepsilon_d$ is the dielectric constant of the dielectric layer (air), and $\varepsilon_0$ is the vacuum permittivity. Under this special voltage condition, $\cos \theta = \cos \theta_0 + 0.74$ when $\varepsilon_d = 1$, $\varepsilon_0 = 8.85 \times 10^{-12}$ F/m, $d = 10$ mm, and $\phi = 11$ kV. Clearly, the contact angle under a high electric field is much smaller, resulting in the wetting phenomenon at the nozzle tip as seen from Fig. 4.8.

![Orifice wetting at 11 kV](image)

Fig. 4.8. Orifice wetting at 11 kV
4.4 Discussion on Droplet Size Reduction and Printing of High-Concentration Cell Suspension

4.4.1 Effect of Voltage on Droplet Size Reduction

As discussed, the electric field may reduce the droplet diameter by more than 20%. Furthermore, the electric field and meniscus oscillation are combined to reduce the droplet size even more significantly. Using an excitation voltage 40 V instead of 60 V, only meniscus oscillation can be observed, but no droplet forms under no external electric field. When a 5 kV voltage is applied, the charged liquid is subject to a strong electric field, and the electrostatic stress exerted by the electric field facilitates the droplet formation process. As seen from Fig 4.9, the instability is still not strong enough to break up the ligament at 160 µs. Fortunately, this instability propagates to the ligament head where a cone forms due to the tangential electrostatic stress. Near the apex of the cone, the diameter is small enough for the perturbation to break up the ligament. Eventually a small droplet with a diameter of 20 µm is generated at the apex of the cone. In short, when the excitation waveform alone is not capable of forming droplets under some conditions, the electric field may provide additional aid to facilitate the droplet formation process.
Most industrial DOD inkjet printheads generate droplets with a diameter similar to the nozzle orifice. Generally, in order to generate small droplets, the orifice diameter should be reduced. However, small nozzles are difficult to fabricate and easy to clog [Castrejon-Pita2012]. Alternatively, the waveform can be adjusted to generate droplets with a diameter smaller than the nozzle orifice. Chen et al. [Chen2002a] reported that the droplet diameter was reduced from 42 µm to 16 µm using a tripolar excitation waveform. Castrejon-Pita et al. [Castrejon-Pita2012] used a single square negative pulse to reduce the diameter of the droplet $R_d$, with respect to the nozzle diameter $R_n$, up to a ratio of $R_n/R_d \approx 2.5$. In this study, the droplet diameter is reduced significantly with a $R_n/R_d$ ratio of 5.5, which provides a special approach for droplet size reduction for a given nozzle.

4.4.2 Printing of High-Concentration Cell Suspension

High-concentration 3T3 cell suspension ($3 \times 10^7$ cells/ml) is also tested for printing in this study. Under the nominal printing conditions without electric field, the suspension still cannot be printed even using the achievable maximum amplitude of the excitation waveform (148V: 100 V and -48 V). However, cell-laden droplets are successfully generated at 4 kV as shown in Fig. 4.10.

When the viscoelastic cell suspension is ejected from the nozzle orifice, the total energy of the pressure wave due to the deformation of the piezoelectric element is converted to the energy for viscous dissipation as well as the elastic energy stored in the
forming ligament/droplet and the droplet surface energy and kinetic energy. Due to the large viscoelastic effect of the high-concentration suspension, the remaining energy for the droplet kinetic energy is limited during DOD inkjet printing, resulting in a short ligament length and small velocity as shown in Fig. 4.10(a). When the negative pressure pulse retracts the meniscus, the ligament head is easily affected, and the velocity of the ligament head may even become negative [Xu2007], which eventually drives the ligament to flow back into the nozzle orifice and leads to no droplet formation. However, when the 4 kV voltage is applied, the electric field accelerates the charged interface, which results in the longer ligament and larger ligament velocity. Even during meniscus retraction, the velocity of ligament head can still keep positive. Eventually, pinch-off occurs near the nozzle orifice, and the cell-laden droplet successfully forms as shown in Fig. 4.10(b). Printing of suspensions with high cell concentrations has been a big challenge during DOD inkjet cell printing. The proposed approach provides a promising fabrication technique to this challenge and extends the capability of DOD inkjet printing to high-concentration cell suspensions.
Fig. 4.10. (a) Without droplets using the excitation waveform only, and (b) droplet formation at 4 kV

4.5 Conclusions

This study has investigated the electric field-assisted droplet formation process under piezoactuation-based DOD inkjet printing. For the better control of droplet monodispersity, the Taylor cone is intentionally suppressed from happening to avoid undesirable satellite droplets. The droplet formation process of deionized water has been investigated, and some main conclusions are drawn as follows:

1) With the increase of the applied voltage, the droplet velocity increases and the droplet size decreases. From 0 to 6 kV, the increase of droplet velocity is due to the acceleration of the charged liquid meniscus due to the electric field, and the decrease of droplet size is due to the ligament retraction into the nozzle.
From 8 to 10 kV, the increase of droplet velocity is mainly due to the droplet shape change, and the decrease of droplet size is due to smaller breakup time;

2) The pinch-off location may be different depending on the applied voltage. With the applied voltage there are five different regions according to different pinch-off locations. In the first region, the pinch-off occurs near the nozzle orifice. In the second region, the pinch-off occurs near the forming droplet. In the third region, after the pinch-off near the forming droplet, the ligament breaks from the nozzle orifice due to electrohydrodynamic instability before the ligament is retracted back into the nozzle. In the fourth region, the pinch-off occurs near the nozzle orifice since there is no obvious ligament between the nozzle orifice and the forming droplet due to droplet shape change. In the fifth region, the wetting problem occurs probably due to the reduced contact angle caused by electrowetting.

3) The combination effect of the electric field and meniscus oscillation can be utilized to significantly reduce the droplet diameter to less than one-fifth of the orifice diameter. The bioink with a high concentration (3×10^7 cells/ml) is not able to be printed using DOD inkjet printing only. However, the electric field-assisted DOD inkjet printing is proved to generate cell-laden droplets successfully, which extends capability of DOD inkjet printing to bioink with high cell concentrations.
CHAPTER FIVE

FABRICATION OF 3D VASCULAR-LIKE CONSTRUCTS

Abstract

The capability to print 3D cellular tubes is not only a logical first step towards successful organ printing but also a critical indicator of the feasibility of the envisioned organ printing technology. In this study, vertical printing and horizontal printing have been proposed for fabrication of the vascular-like alginate tubes, which mimic typical vascular constructs. In addition, associated manufacturing challenges are briefly discussed. It has been found that: 1) the maximum achievable height of overhang structure depends on the inclination angle of the overhang structure during vertical printing; 2) a model for cross-sectional deformation during horizontal printing is proposed and the experimental result and model prediction are compared to show a good agreement; 3) a predictive compensation approach has been proposed to mitigate the cross-sectional deformation during horizontal printing to show a good result; and 4) alginate cellular tubes have also been successfully printed with a satisfactory post-printing cell viability of 87% immediately after printing and after 24 hours of incubation.

5.1 Introduction

In Chapters 2, 3 and 4, droplets are generated. In this chapter, the generated droplets are used to form a two-dimensional (2D) pattern as one layer by precisely controlling the positions of the droplets, and then 3D constructs can be built by layer-by-
layer fabrication. During inkjet printing, 3D vascular-like constructs can be printed vertically or horizontally based on the relative configurations between the moving direction of dispense nozzle and the axis of tube being printed. If the nozzle moves along the circumferential direction of tube, the fabrication process is called vertical printing; if the nozzle moving direction is parallel to the tube longitudinal axes, the fabrication process is called horizontal printing. Vertical printing or horizontal printing alone is almost impossible to fabricate tubular constructs with complex geometry since the true vascular network is very complex. For example, shown in Fig. 5.1(a) the kidney intraorgan vascular tree is very complex. Even the typical tubular construct in the vascular tree can’t be fabricated only using vertical printing or horizontal printing. Therefore, there is a need to investigate the feasibility of both vertical printing and horizontal printing for 3D tubular construct fabrication. In this chapter, vertical printing and horizontal printing are employed to fabricate 3D vascular-like constructs. The manufacturing challenges during the fabrication processes are investigated, specifically the process-induced tube failure in vertical printing and the cross-sectional deformation in horizontal printing. A predictive compensation approach is proposed to mitigate the cross-sectional deformation. Post-printing cell viability is tested to show a satisfactory result.
Fig. 5.1. (a) Kidney intraorgan vascular tree [Mironov2009], (b) basic tubular construct of a vascular network, and (c) two representative parts of the tubular construct

5.2 Vertical Printing

5.2.1 Materials and Method

Materials

Since 3D cellular tubes are envisioned as living blood vessel replacements [Kelm2010], a starting point is to fabricate complex tubular constructs by mimicking the native blood vessel anatomy. A typical blood vessel includes three layers: intima, media and adventia, and fibroblasts are the key component of adventia. As such, fibroblast cells have been widely used in biomanufacturing and biomedical research [Norotte2009] [Skardal2010] [Pataky2012]. This study has also used fibroblast cells as the model cell in developing the inkjetting-based 3D tube fabrication technology.

NIH 3T3 mouse fibroblast cells (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 cellular bioink for printing, freshly confluent flasks of 3T3 fibroblasts were washed once 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 the cells from the culture flasks. Then the cell suspension was centrifuged at 1000 rpm for 5 minutes at room temperature, and the resulting pellet was resuspended in DMEM complete cell culture medium. The resuspended cells were adjusted the cell concentration to 6×10⁶ cells/ml.

Alginate, particularly, sodium alginate, has been used as a constituent of bioink in bioprinting [Khalil2005] [Nishiyama2009] to facilitate gelatinization. While alginate is not an ideal material for living tissue construction, it is a good hydrogel material for proof-of-concept studies. In this study, sodium alginate was also used to make bioink for cellular tube fabrication. The cell suspension was mixed with the solution of 2% sodium alginate (Sigma-Aldrich, St. Louis, MO) at a volume ratio of 1:1, resulting in a final bioink solution of 3×10⁶ cells/ml. The 2% sodium alginate solution was prepared using DMEM. The resulting bioink, including 3T3 cells, sodium alginate and cell medium, had a cell density of 3×10⁶ cells/ml and a sodium alginate concentration of 1% (w/v), which was identified for good printability [Herran2012]. The printed construct was gelatinized using a 2% (w/v) solution of calcium chloride dihydrate (Sigma–Aldrich, St. Louis, MO).

The cell viability of post-printing cells was assessed immediately after printing and 3 days after printing. The cell pellet was collected by centrifuging at 1000 rpm for 5 minutes after liquefying the printed cellular tubes with the solution of 0.055 M sodium
citrate (Sigma Aldrich, St. Louis, MO) and resuspended in a 20 μl DMEM complete cell culture medium. The controls were unprinted 3T3 cells, routinely cultured in 35 mm petri dishes in a humidified 5% CO₂ incubator at 37°C. All the cells were evaluated using 20 μl 0.4% trypan blue stain (Sigma Aldrich, St. Louis, MO) and viewed using an optical microscope. Transparent cells were considered live while blue cells were considered dead since viable cells with intact cellular membrane excluded the blue dye. All were counted twice to get an average cell viability value.

Setup of 3D inkjet printing system

A platform-assisted 3D inkjet bioprinting system, as shown in Fig. 5.2, has been proposed to fabricate 3D complex constructs such as zigzag tubes and hollow pyramids. The bioprinting system was composed of four key subsystems: motorized XY stages (Aerotech, Pittsburgh, PA) attached with a computer-controlled nozzle dispenser (MicroFab, Plano, TX), a motorized Z stage (Edmund optics, Barrington, NJ) attached with a Z-shape platform where constructs were printed, a container containing the calcium chloride solution, and an imaging system to visualize the droplet formation process during printing (ImageXpert, Nashua, NH). Both the droplet size and velocity were measured by the imaging system.

The bioink, supplied via a reservoir, was ejected using a 120 μm nozzle dispenser (MicroFab MJ-ABL-01-120-6MX dispense head, driven by a sleeve piezoelectric element) in a DOD mode. The DOD pulse was controlled using a MicroFab Jet Driver, and a pneumatic controller was used first to adjust the backpressure (2.0 to -
1.0 kPa) of the fluid reservoir by decreasing at an interval of 0.2 kPa to obtain an ideal meniscus for good droplet formation. The XY stages were precisely controlled to define the dispense head location for planar feature printing, and the Z stage was controlled moving down vertically to match the printing speed for each layer. The orifice of the dispense head was kept 5 mm above the construct surface being made, which was always at the fluid level. Ideally, once a layer is printed, the Z-stage attached platform should move down by a distance of the layer thickness for the gelatinization of each newly deposited layer. In this study, the Z-stage moved down vertically at a constant speed by matching the printing speed, resulting in a deposited layer of 70 µm. The submerged layer was sequentially gelatinized by the calcium chloride solution, forming a gelled cell-calcium alginate layer. In essence, it was a layer-by-layer printing process.
Driving waveform and droplet formation process

The dispense head used has been commonly driven by a bipolar excitation waveform consisting of a succession of two wave pluses: either positive/negative or negative/positive [Herran2012]. The second wave pulse is to cancel some of residual acoustic oscillations that may remain in the DOD dispense head after droplet ejection. Based on the droplet formation results, a good excitation wave was identified as the good operating conditions for the following fabrication experiments: voltage 45 V, voltage rise and fall times 3 µs, dwell time 30 µs, echo time 30 µs, excitation frequency 50 Hz. It was determined based on their good droplet formation performance and higher droplet speed which helped improve the droplet positioning accuracy and feature resolution. It should be pointed out that there is a possible variation of good waveforms for different nozzles even their orifice size is the same. Some representative images of the drop formation process under the chosen operating conditions are shown in Fig. 5.3. The ligament ruptured from the orifice at 235 µs, and the cellular droplet well developed at 410 µs with a velocity of 1.8 m/s. The droplet size was approximately 106 µm in diameter.
To better improve the applicability of 3D inkjet bioprinting, some lines were first printed in order to investigate the effect of nozzle speed on the line alginate structure. Then straight and zigzag cellular tubes were fabricated using the proposed 3D inkjet bioprinting system. Since the volumetric percentage of cells in each droplet was very small (0.34%), it is assumed that the droplet formation process will not be affected by cells. As such, only DMEM-based sodium alginate solution was used for the printing of lines.

**Line printing**

The purpose of line printing was to study the effect of the dispense head speed on the printing quality and identify a good dispense head speed for a given excitation frequency. The 1% (w/v) sodium alginate-medium solution was directly printed into a container with the 2% (w/v) calcium chloride-water solution. In order to print a continuous line, the dispense head speed must be lower than $D_d \times f$, where $D_d$ is the
droplet diameter and $f$ is the excitation frequency. Since the formed droplet was around 100 µm in diameter and the excitation frequency was 50 Hz herein, the dispense head speed should be lower than 300 mm/min.

As such, five dispense head speeds, namely 300, 280, 250, 200 and 150 mm/min, were tested in straight line printing, and the printing results are shown in Fig. 5.4. When the dispense head speed was 300 mm/min, droplets were separated from each other and floating inside the calcium chloride solution. There were no continuous gel lines as expected.

The dispense head speed was decreased to 280 and 250 mm/min at which a continuous gel line could be successfully formed; however the printed gel lines had a non-uniform width. When the dispense head speed was 200 mm/min, the printed gel line was considered as a good one which had no gap between neighboring droplets and had a relatively uniform line width. When the dispense head speed was further lowered to 150 mm/min, the gel line width turned to be non-uniform again. As such, the dispense head speed was selected as 200 mm/min for the following printing fabrication studies.
Cellular zigzag tube printing

Scaffold-free printing of 3D constructs has been an exciting manufacturing challenge, and this is also critical to help achieve the envisioned goal of organ printing. This study has proposed to fabricate 3D cellular zigzag tubes with a controllable overhang structure as shown in Fig. 5.5. A zigzag tube includes three parts: the bottom straight segment, the middle overhand segment, and the top straight segment.

Fig. 5.4. Gel lines printed using the 120 µm dispense head

Overhang structure
Such designed 3D cellular tubes were constructed by layer-by-layer printing using the proposed platform-assisted 3D inkjet bioprinting system. During bioprinting, the cell droplets were positioned precisely along the XY plane as the constituent elements to form a layer of annular pattern which was printed on the top of a previous gelatinized cell alginate annular layer at a given tube height. Simultaneously, the Z-shape platform moved downwards to build the height of the printed 3D tube. While the dispense head had a 3 mm diameter circular movement at the speed of 200 mm/min, the motorized Z stage lowered downwards at a constant speed of 0.02 mm/s to buildup the tube along the Z direction. All the layers stacked to each other to form a 3D structure. Specifically, the bottom segment was first made, followed by the overhang and top segments. During the printing of overhang structure, the dispense head moved towards left with a distance \( d \) of 20 \( \mu \)m after each layer deposition. Finally, the top straight segment was printed on the top of the overhang structure, forming the complete zigzag tube.

There were 210 layers deposited for the whole tubular structure shown in Fig. 5.5, and its height was 10 mm. It is found that some overhang structures can be fabricated by carefully selecting the operating conditions with the help of buoyant force from the calcium chloride solution. Fig. 5.5(b) shows such an overhang structure with an overhang angle \( \alpha \) of 63° and an overhang height \( H \) of 5 mm. By a close examination of the printed tube, it can be seen that the outer surface of tube had a wavy profile (Fig. 5.6)
since the each building block was a round droplet ejected from the dispense head. The longitudinal profile of tube wall resembled well with that of gel lines shown in Fig. 5.6. As a proof-of-concept study, this resulting knowledge helps print tissue-engineered blood vessels with complex geometry and structures.

![Wavy surface profile](image)

Fig. 5.6. Printed cellular tube and part of its enlarged outer surface

### 5.2.3 Discussion on Failure in Overhang Printing

Scaffold-free fabrication of overhang structures has been well recognized as one of key challenges in 3D construct printing [Pataky2012]. During the printing of the overhang segment in this study, one kind of process failures is observed due to the moment imbalance. The failure happens when the moments due to the gravity and buoyant forces of the printed structure are not in balance with respect to the pivot point $o$ shown in Fig. 5.7. Then the printed structure may flip down around the point $o$. As a starting point, this section analyzes the possible imbalance based on the failure
The free body diagram of the overhang structure and the bottom segment is shown in Fig. 5.7, where $F_d$ is the impact force due to the droplet, $m_d g$ is the droplet weight, $m_1 g$ and $m_2 g$ are the weight of the bottom and overhang segments, respectively, $g$ is the gravitational constant, $F_{b1}$ and $F_{b2}$ are the buoyant forces of the bottom and overhang segments, respectively, $F_s$ is the supporting force, $u$ is the impact velocity, $D$ is the tube diameter and $h$ is the height of the bottom segment. It is assumed that 1) the adhesion between the printed tube and the platform is negligible, 2) the drag due to the calcium chloride solution is also negligible, and 3) only the bottom and overhang segments are considered. Due to the symmetry of the printed structure, the printed 3D structure is simplified to a 2D problem in the YZ plane.
The effect of droplet impact should also be determined whether its contribution should be included in predicting the moment imbalance. After a droplet lands on the top layer of the overhang structure, its maximum impact force can be approximated as

\[ F_b = \frac{4}{3} R^{1/2} E^{*} \delta^{3/2} \] [Johnson2003] by assuming that the droplet and overhang structure are elastic and have the same Young’s modulus \((E = 22 \text{kPa} \ [\text{Khalil2005}])\) and Poisson’s ratio \((\nu = 0.5 \ [\text{Wang2005}])\), where \(R\) is the droplet radius, \(E^{*} = \frac{E}{2(1-\nu^2)}\) and

\[ \delta = \left( \frac{15m_{\mu}R^2}{16R^{1/2}E^{*}} \right)^{2/5}. \]

For a droplet with a diameter of around 100 µm and an impact velocity of 0.47 m/s as measured in this study, this maximum impact force is around \(8.85 \times 10^{-6} \text{N}\) (on the order of \(10^{-6}\)) and is negligible when comparing with that of a typical structure (10 mm) being printed (on the order of \(10^{-4} \text{N}\)). Here the droplet density is taken as \(0.99 \times 10^3 \text{ kg/m}^3\) [Bacabac2005]. As such, the impact force is not included in determining the process failure if only based on the first mechanism.

Then the critical condition of interest is when the printed structure is to lose its equilibrium and flip around. The moment of the bottom segment \((M_1)\) is \((-m_{bi}g + F_{bi})\frac{D}{2}\) and the moment of the overhang segment \((M_2)\) is \(\int_{h}^{H} (-\rho_{o} + \rho_{c}) g \pi D t l \, dz\), where \(\rho_{o}\) is the density of the overhang structure, \(\rho_{c}\) is the density of CaCl$_2$ solution, \(t\) is the wall thickness of the overhang structure and \(l\) is the perpendicular distance from the point \(o\) to the center of gravity of each layer of the overhang segment. Then the relationship
between the maximum achievable $H_{\text{max}}$ and $\alpha$ can be expressed as follows based on the moment balance:

$$H_{\text{max}} = \frac{D \tan \alpha + \sqrt{D^2 (\tan \alpha)^2 + 4Dh \tan \alpha}}{2}$$

(5.1)

The comparisons of $H$ between the theoretical predictions and experimental results are shown in Fig. 5.8 when $h$ was 3 mm, demonstrating a good match while the model always little underestimate $H$. This underestimation is attributed to the negligence of possible gel and structure adhesion between the printed tube and the platform.

![Comparison of results and predictions](image)

**Fig. 5.8.** Comparisons of the experimental results and predictions of achievable maximum height

### 5.3 Horizontal Printing

#### 5.3.1 Materials and Method
Alginate, particularly, sodium alginate, has been used as a constituent of bioink in bioprinting [Nishiyama2009] [Xu2012]. In this study, sodium alginate (Sigma-Aldrich, St. Louis, MO) was also used to make 1% (w/v) sodium alginate bioink for tubular construct fabrication, and the cross-linking solution was 2% (w/v) calcium chloride (Sigma-Aldrich, St. Louis, MO).

The platform-assisted inkjet 3D printing system used in this study was described in our previous work used for vertical printing configuration [Xu2012]. In this study, horizontal printing configuration was used. Under the horizontal printing configuration, shown in Fig. 5.9, a 120 µm MicroFab dispense nozzle moves parallel to the tube longitudinal axes as the primary motion during the fabrication of each layer. The alginate droplets were positioned precisely along the XY plane as the constituent elements to form a layer of polygonal pattern. The Z stage was controlled moving down vertically as the secondary motion. Ideally, once a layer is printed, the Z-stage attached platform should move down by a distance of the layer thickness for the gelation of each newly deposited layer. Next layer was deposited on the top of a previous gelled alginate layer at a given radial height of tube until the 3D tubular structure was fabricated. During horizontal printing, the NaAlg droplets reacted with CaCl₂ solution in the substrate to produce alginate-based tubular structure. The other product of this reaction is sodium chloride (NaCl). The solution inside the substrate including both CaCl₂ and NaCl was also used as the supporting material which supplied the buoyant force to balance the gravity of the printed structure. The big advantage of liquid supporting material was easily dumping. The experimental conditions are defined as follows [Herran2012] [Xu2012]: excitation
voltage 60V and -45V, voltage rise and fall times 3 μs, dwell time 30 μs, echo time 30 μs, excitation frequency 50 Hz, air gap between the nozzle tip and CaCl\(_2\) solution 20 mm.

Fig. 5.9. Horizontal printing setup

5.3.2 Conventional Horizontal Printing

During conventional horizontal printing [Xu2012b], the circular cross section of tubular constructs was horizontally sliced into different layers whose coordinates were precisely determined based on the mathematical description of circular shape. As shown in Fig. 5.10, such an approach has led to a deformed construct due to a combined effect of gravity and elastic deformation of printed overhang structures, buoyant force, and
droplet impact force. The cross section of the printed tube was irregular instead of circular. By taking the fourth quadrant structure as an example, Fig. 5.10 illustrates why such a deformed construct was formed. At the beginning of the process, the vertical deformation was more evident due to the more mass being deposited per the nozzle feed distance, resulting in a concave shape mainly due to the gravitational effect. As the printing process moved on, the nozzle had a higher feed speed towards the center line (Z axis) in order to precisely interpolate the top portion of the tube, and the nozzle feed between adjacent layers became larger. The mass being deposited per the nozzle feed distance was smaller, and the buoyant force might be comparable to the gravity and the impact force for this portion being printed. As a result, a convex shape and even material buildup were observed. It is noticed that the deformation of the bottom half was not so pronounced due to the supporting effect of the platform.

Fig. 5.10. Deformed tubular construct and schematics illustrating its forming process
5.3.3 Cross-Sectional Deformation during Horizontal Printing

During horizontal printing the free end of the printed tube undergoes deflection due to the droplet impaction, the gravity and buoyant force of the printed tube. The deflection accumulates layer by layer in a dynamic process, which eventually results in the cross-sectional deformation of the printed tube. This is a very complicated process. As a starting point, this section proposes a model to predict the cross-sectional deformation during horizontal printing based on the linear elastic behavior of calcium alginate [Wang2005].

Fig. 5.11. (a) Typical cross section of the printed tube in horizontal printing and (b) the definitions of diameter difference in the $Y$ direction $\Delta Y$

In Fig. 5.11 (a) it shows a typical cross section of the printed tube in horizontal
printing. It is seen from this figure that the severe cross-sectional deformation occurs in horizontal printing. In order to model the cross-sectional deformation, in Fig. 5.11(b) the comparison between the ideal cross section and the cross section of the printed tube is shown to define the diameter difference in the Y direction \( \Delta Y \).

![Free body diagram of the printed tube in horizontal printing at \( 0 \leq \alpha \leq \frac{\pi}{2} \)](image)

The free body diagrams of the printed tube for \( \alpha \in [0, \frac{\pi}{2}] \) is shown in Fig. 5.12, where \( o \) is the cross-sectional center of the tube, \( A \) is the printed base, \( B \) is the free end, \( R \) is the tube radius, \( f_d \) is the impact force due to the droplet impaction, \( f_b \) is the buoyancy force, \( mg \) is the weight of the printed tube, \( g \) is the gravitational constant. A segment of the tube is cut for the slab analysis. It is assumed that (1) the bottom of the printed tube is considered as a fixed end since it adheres to the platform; (2) the gelation process is instantaneous; (3) the shear deflection is negligible [Ugural2003]; and (4) the tube exhibits the linear elastic behavior [Wang2005] and is isotropic and homogeneous. Due
to the symmetry of the printed structure, the tube is simplified as a thin curved beam with a fixed end and a free end in the YZ plane.

When the droplet impacts with the free end the deflection is caused. In this case, the buoyancy force \( f_b \), gravity force \( G_t \) and droplet impaction \( f_d \) are taken into account for the deflection. Since the gravity and buoyancy forces are in the opposite directions we consider the difference of the gravity and buoyancy forces: \( G_t - f_b = (\rho_h - \rho_c) V_t g \) where \( \rho_h \) is the density of the printed tube (calcium alginate), \( \rho_c \) is the density of CaCl\(_2\) solution, and \( V_t \) is the volume of the printed tube. The difference of the gravity and buoyancy is around \( 7 \times 10^{-6} \) N for a half printed tube with a diameter of 5 mm and a length of 5 mm when \( \rho_h = 1.06 \) g/cm\(^3\) [Salsac2009]. For a droplet with a diameter of around 100 µm and an impact velocity of 4 m/s as measured in this study, this maximum impact force is around \( 1.2 \times 10^{-4} \) N [Xu2012]. Therefore, in this case the gravity and buoyancy forces of the printed tube are negligible compared with the droplet impact force and the impact force is the only factor considered for the deflection.

A theoretical model for the deflection of the free end under the droplet impaction is developed based on Castigliano’s second theorem which is a method widely used for determining the displacements of a linear elastic system. According to Castigliano’s second theorem \( \delta_v = \partial U/\partial V \) and \( \delta_H = \partial U/\partial H \) are used to estimate the resultant diameter difference where \( \delta_v \) is the tangential deflection of the beam, \( \delta_H \) is the axial deflection of the beam, \( U \) is the strain energy, and \( H \) and \( V \) are the tangential and normal components
of the impact force at the free end. The stain energy $U$ is defined as $U = \int \frac{M^2}{2EI} ds$ where $M$ is the moment due to the droplet impaction, $E$ is the Young’s modulus, $I$ is the area moment of inertia, and $s$ is the curve $AB$ shown in Fig. 5.12. At $0 \leq \alpha \leq \frac{\pi}{2}$, the moment $M$ is $M = VR \sin \alpha + HR(1 - \cos \alpha)$. The tangential deflection $\delta_h$ and the axial deflection $\delta_v$ are calculated as follows:

$$\delta_h = \frac{\partial U}{\partial H} = \frac{1}{EI} \int (VR \sin \alpha + HR(1 - \cos \alpha))R(1 - \cos \alpha) R d\alpha \quad (5.2)$$

$$\delta_v = \frac{\partial U}{\partial V} = \frac{1}{EI} \int (VR \sin \alpha + HR(1 - \cos \alpha))(R \sin \alpha) R d\alpha \quad (5.3)$$

The deflection in the $Y$ direction $\Delta Y$ is derived:

$$\Delta Y = -\delta_h \cos \alpha + \delta_v \sin \alpha \quad (5.4)$$

For a thin beam, $E$ is replaced by $\frac{E}{1 - \nu^2}$ [Bhushan2004] where Poisson’s ratio $\nu$ is 0.5 [Salsac2009]. The area moment of inertia is defined as $I = \frac{Lt^3}{12}$ where $L$ is the length of the tube, $t$ is the tube thickness.

The diameter difference in the $Y$ direction $\Delta Y$ is defined as the horizontal deflection of the curved beam at $\alpha = \pi/2$. For a tube with a length of 5 mm and a thickness of 0.6 mm, the comparison of $\Delta Y$ between the theoretical prediction and experimental result is shown in Fig. 5.13, demonstrating a good match. It is also seen that the experimental result is always larger than the model prediction, probably due to the weight and buoyant force of the printed tube.
Fig. 5.13. Comparison of $\Delta Y$ between the experimental result and theoretical prediction

5.3.4 Horizontal Printing with Predictive Compensation

As described, conventional horizontal printing may produce a deformed cross section due to the fact that the circular interpolation does not account for the deflection of the overhanging tube wall being printed. Fortunately, this deflection can be predicted and mitigated by printing with predictive compensation as illustrated in Fig. 5.14. The basic idea is that the nozzle follows a non-circular printing trajectory with some deformation allowance built in, resulting in a nearly circular shape after deformation.

The entire process can be mainly divided into two steps as described below by taking the fourth quadrant structure as an example. First, the nozzle had no feed motion, and a vertical base structure was printed. Second, the overhanging layers were printed.
During this phase, the nozzle fed towards the center line (Z axis) with a feed ($\Delta x'$) larger than that ($\Delta x$) during conventional horizontal printing as depicted in Fig. 5.14(b) for a tube with the same diameter. For instance, a conventional circular interpolation yielded a $\Delta x$ between the layers 6 and 7 of 9.3 µm, while the interpolation with predictive compensation yielded a $\Delta x'$ of 154.2 µm. Each layer thickness, which was kept constant, was controlled around 50 µm. While the compensation allowance may be determined experimentally or analytically, it was determined experimentally during this preliminary study. The vertical structure added a height to the upper half of the tube and deformed slightly to provide a gradual transition in slope to be circular when the overhanging layers started being printed. Because $\Delta x'$ was sufficiently large between all layers during overhang layer printing under predictive compensation, the concavity observed in conventional horizontal printing was mostly eliminated. The deformation during printing was adequately mitigated by the deformation allowance of the printing trajectory, resulting in a nearly circular tube which is good enough for a vascular construct. As observed in conventional horizontal printing, the deformation of the bottom half was not so pronounced.
(a) Vertical base structure being printed
Nozzle feed direction
Droplet
Designed deposition location
Actual deposition location
Overhanging layers being printed
Deformed construct with predictive compensation

(b) Printing trajectory without predictive compensation (ideal circular shape)
Printing trajectory with predictive compensation
Deposited material at the same nth overhanging layer
Where overhanging layers start being printed under predictive compensation

(Overhanging layer number)
Fig. 5.14. (a) Illustration for horizontal printing with predictive compensation and (b) comparison of the nozzle feeds under conventional and predictive compensation configurations

5.4 Comparison with Other Bioprinting Techniques

Blood vessels in humans are in a wide range of the diameter from several micrometers for the capillaries to 25mm for the aorta [Blakemore2002]. Typical blood vessels include three layers which are composed of different types of cells. Both vertical printing and horizontal printing used in this paper are inkjet-based. Inkjet printing has many advantages including the flexibility and versatility for different shapes of 3D tubular constructs, simple experimental setup, scale-up potential, and good process controllability [Herran2012a] [Xu2012]. Other applicable techniques for the fabrication of 3D vascular-like constructs may include rapid casting [Miller2012], laser printing [Yan2013b], and extrusion [Khalil2005]. Table 5.1 lists some advantages and disadvantages of different 3D construct fabrication techniques.
Table 5.1. Comparison of different techniques used for 3D vascular-like construct fabrication

<table>
<thead>
<tr>
<th>Approaches</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid casting</td>
<td>• Capability for fabrication of complex perfusable channels</td>
<td>• Difficulty in fabrication of small channels and removal of sacrificial materials</td>
</tr>
<tr>
<td></td>
<td>• High productivity</td>
<td>• Process complexity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Inapplicability for blood vessel fabrication</td>
</tr>
<tr>
<td>Laser printing</td>
<td>• Capability for highly viscous materials</td>
<td>• Low Productivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Unstable droplet formation process and non-uniform ribbon coating</td>
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<tr>
<td></td>
<td></td>
<td>• Relatively low post-printing cell viability</td>
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<tr>
<td></td>
<td></td>
<td>• Relatively high cost</td>
</tr>
<tr>
<td>Extrusion</td>
<td>• Simple experimental setup</td>
<td>• Long time required for fusion</td>
</tr>
<tr>
<td></td>
<td>• High productivity</td>
<td>• Difficulty in extending to make 3D heterogeneous structures</td>
</tr>
<tr>
<td></td>
<td>• Capability for fabrication of multilayer tubular construct</td>
<td></td>
</tr>
<tr>
<td>Inkjet printing</td>
<td>• Flexibility and versatility</td>
<td>• Restriction of fluid viscosity</td>
</tr>
<tr>
<td></td>
<td>• Simple experimental setup</td>
<td>•Difficulty for large and complex structure fabrication</td>
</tr>
<tr>
<td></td>
<td>• Scale-up potential</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Good process controllability</td>
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</tbody>
</table>
During rapid casting, a biocompatible sacrificial material made from mixtures of inexpensive and readily available carbohydrates was used to print a 3D carbohydrate-glass lattice which was dissolved in the cell media to form many channels inside the hydrogel to serve as the vascular network [Miller2012]. This technique is capable for fabrication of complex perfusable channels with a high productivity. However, several challenges may exist for this method. One challenge is the channel resolution. Fabrication of very small channels might be difficult. Even though the small channels can be fabricated, the sacrificial material is difficult to remove and the fabricated small channels are easy to be blocked by the surrounding soft hydrogel. Another challenge is the process complexity of this technique. The printing process was at 110 °C which may not be applicable for cells. Furthermore, after printing, additional coating layers might be required for preventing disruption of surrounding hydrogel and the potential for osmotic damage to encapsulated cells. In addition, this technique may not be applicable for the blood vessel fabrication. The vascular network fabricated by this technique is only the perfusable channels. Although the endothelial cells were seeded in the vascular lumen, the fabricated channels are quite different from real blood vessels consisting of three layers.

For laser printing, the big advantage is it capability for printing of highly viscous fluids [Yan2013b]. However, the productivity of laser printing for fabrication of 3D constructs is still relatively slow. Another problem during laser printing is the unstable droplet formation process due to the non-uniform laser energy for each pulse and non-uniform ribbon coating, which results in non-uniform droplet size and difficulty to
control the resolution of the printed structure [Yan2013b]. Other disadvantages for laser printing include the relatively high cost of experimental setups and low cell viability [Lin2009].

For extrusion, it advantages include simple experimental setup, high productivity, and capability for fabrication of multilayer tubular constructs [Norotte2009]. Norotte et al. [Norotte2009] and Skardal et al. [Skardal2010] used the extruded cylindrical filaments to fabricate the vascular constructs. However, the fabrication process needed at least several days for the post-printing fusion of the discrete unities, and this approach may not be easily extended to make 3D heterogeneous structures [Xu2013a]. Therefore, compared to the above techniques, inkjet printing is more widely used for 3D construct fabrication due to its advantages including flexibility and versatility for different shapes of 3D tubular constructs, simple experimental setup, scale-up potential, and good process controllability [Herran2012a] [Xu2012]. Usually inkjet printing is performed with air contact. However, Campos et al. [Campos2013] proposed for the first time the concept of submerged 3D bioprinting in a hydrophobic, high-density fluid which mechanically supported the printed hydrogel constructs during the 3D printing process. It was reported that this method is applicable for the 3D printing of large constructs embedded with cells, which is a big advantage compared to other techniques. However, this method is restricted highly to the materials used in the experiments. The fluid should be cytocompatible, highly oxygenated, hydrophobic, and with high density. Furthermore, for the 3D multilayer constructs, the various types of hydrogels used for different layers should be sufficiently different, so that the hydrogels
remain non-miscible within these layers during printing. Despite the problems, this technique still serves as a complementary approach and supplies a good idea for 3D large construct printing.

Vertical printing and horizontal printing used in this paper are flexible and versatile approaches for 3D tubular construct fabrication. The resolution of the printed 3D tubular constructs depends on the droplets generated from a dispensing nozzle with the orifice diameter of 120 µm. The generated droplets are controlled by the moving stages precisely to deposit in the right position. The whole fabrication process is relatively fast compared to other methods mentioned above. However, there are still two limits for fabrication of 3D constructs using inkjet printing. One is the restriction of viscosity range for the dispensing nozzle. The other restriction is the difficulty to fabricate 3D large and complex structures. It is envisioned in a previous study [Xu2013a] that combination of vertical printing and horizontal printing supplies a possible way to fabricate a tubular construct with both vertical and horizontal branching features. However, the fabrication of 3D large and complex constructs using inkjet printing is still a great challenge.

5.5 Cell Viability

5.5.1 Cell Viability Test Procedure

The cell viability of post-printing cells was assessed immediately after printing and 3 days after printing. The cell pellet was collected by centrifuging at 1000 rpm for 5 minutes after liquefying the printed cellular tubes with the solution of 0.055 M sodium citrate (Sigma Aldrich, St. Louis, MO) and resuspended in a 20 µl DMEM complete cell
culture medium. The controls were unprinted 3T3 cells, routinely cultured in 35 mm petri dishes in a humidified 5% CO₂ incubator at 37°C. All the cells were evaluated using 20 μl 0.4% trypan blue stain (Sigma Aldrich, St. Louis, MO) and viewed using an optical microscope. Transparent cells were considered live while blue cells were considered dead since viable cells with intact cellular membrane excluded the blue dye. All were counted twice to get an average cell viability value.

The printed cellular tubes, cultured inside Petri dishes with the complete DMEM, were incubated in a humidified, 37°C, 5% CO₂ incubator. Cell viability was tested to evaluate any possible inkjet-induced cell death right after printing and 24 hours after printing. For simplicity, only the bottom 3 mm straight segments of a zigzag cellular tube were printed and evaluated after liquefying them using 0.055 M sodium citrate (VWR, West Chester, PA). The control was the unused bioink, which was then incubated at the same condition. Each test had three repeats.

### 5.5.2 Cell Viability Test Results

As seen in Fig. 5.15, the cell viability of both the printed cells (or post-printing cells) and the control didn’t change too much over the 24 hour period while the control had a higher cell viability than that of the printed cells (91% vs. 87%). The student’s t-test shows that the two-tailed P value equals 0.0191, meaning the cell viability difference between the printed cells and the control is statistically significant. Depending on the operating conditions, it was also reported that inkjetting does not bring any pronounced damage to cells [Xu2005] [Calvert2007] [Saunders2008] [Nair2009]. The cell death in
this study may be due to the operating conditions selected, which led to high shear stress to cells being printed. It was found that the cell viability of printed cells further decreased to 85% after 48 hours and 82% after 72 hours. The printed cells were encapsulated inside alginate microspheres, which made a cellular tube. The transport kinetics of nutrients through the alginate microsphere membrane and/or the accumulation of waste products in alginate microspheres may inevitably lead to the death of a certain number of the encapsulated cells [Lahooti2000]. Nevertheless, a post-printing cell viability of 80% or higher is still good enough for inkjetting to be a viable bioprinting technology. Future study will study how to optimize the operating conditions to mitigate the process-induced cell damage and study the possibility to replace sodium alginate with other biodegradable and biocompatible hydrogels.

![Cell viability graph](image)

Fig. 5.15. Cell viability of post-printing and control cells (Inset: stained cells after liquefying)
5.6 Conclusions

Both vertical printing and horizontal printing have been used for fabrication of 3D vascular-like constructs, which is a preliminary step towards the envisioned organ printing. The associated manufacturing challenges during fabrication are studied in this chapter. The conclusions are summarized as follows:

1) 3D Vascular-like constructs are fabricated successfully using both vertical printing and horizontal printing;

2) the maximum achievable height of overhang structure depends on the inclination angle of the overhang structure during vertical printing;

3) During horizontal printing, cross-sectional deformation is modeled and the comparison with the experimental results shows a good agreement;

4) To overcome the deformation-induced construct defect during horizontal printing, a predictive compensation approach has been proposed to fabricate 3D tubular constructs horizontally; and

5) Alginate cellular tubes have also been successfully printed with a satisfactory post-printing cell viability of 87% immediately after printing and after 24 hours of incubation.
CHAPTER SIX
CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

This dissertation investigated the pinch-off during drop-on-demand (DOD) inkjet printing of viscoelastic alginate solutions, the droplet formation performance during DOD inkjet printing of cell-laden bioink, the effects of electric field on droplet formation without forming a Taylor cone during piezoactuation-based DOD inkjet printing, and manufacturing challenges encountered during fabrication of 3D vascular-like constructs using DOD inkjet printing. The major conclusions of this dissertation are summarized in the following discussions.

6.1.1 Pinch-Off Locations during DOD Inkjetting of Alginate Solution

Various pinch-off locations have been studied as a function of material properties and operating conditions during drop-on-demand (DOD) inkjet printing of viscoelastic sodium alginate solutions. Four breakup types are identified: front-pinching, hybrid-pinching, exit-pinching, and middle-pinching. A dimensionless number $J$, which is defined as the square root of the product of Ohnesorge ($Oh$) and elasticity ($El$) numbers is proposed to represent the ratio of viscous and elastic effects to inertial and capillary effects. Based on the $J$ and Weber ($We$) numbers, a phase diagram is constructed to classify the regimes for different pinch-off types during DOD inkjet printing of alginate solutions. Some main conclusions are drawn as follows: 1) for very low sodium alginate concentrations such as 0.10 – 0.20%, front-pinching prevails at the voltage of 30 – 70V.
The ligament thinning process is governed by a balance of inertial and capillary effects, following a power function with an exponent of 2/3; 2) for low concentrations such as 0.25 – 0.35%, with the increase of We, the pinch-off type may change from front-pinch-off to hybrid-pinch-off to exit-pinch-off; 3) for intermediate concentrations such as 0.50 – 1.00%, exit-pinch-off occurs at the voltage range of 30 – 70V. The ligament thinning at the exit-pinch-off location is governed by a balance of elastic and capillary effects, resulting in the exponential decay process. The effective relaxation time characterizing the exponential decay is much smaller than the longest relaxation time; and 4) for high concentrations such as 1.50 – 2.00%, both the viscous and elastic effects are dominant. The ligament thinning process near the ligament head/forming droplet is governed by a balance of viscous, elastic, and capillary effects while the ligament thinning process near the orifice is governed by a balance of elastic and capillary effects due to the high-frequency pressure wave. At small We, middle-pinch-off occurs. With the increase of We, middle-pinch-off turns to be exit-pinch-off.

### 6.1.2 Droplet Formation Process during DOD Inkjetting of Living Cell-Laden Bioink

The cell-laden bioink droplet formation process has been studied in terms of the breakup time, droplet size and velocity, and satellite formation using a time-resolved imaging approach. The bioink has been prepared using 3T3 cells and sodium alginate, and four different cell concentrations have been investigated: without cells, $1 \times 10^6$, $5 \times 10^6$, and $1 \times 10^7$ cells/ml to appreciate the effect of cell concentration on the droplet formation
process. Furthermore, the bioink droplet formation process is compared with that during the inkjetting of the polystyrene microbead-laden suspension under the identical operating condition to understand the effect of particle physical properties on the droplet formation process. In this study, it has been observed that: 1) with the increase of the cell concentration of bioink, the shear viscosity increases, the surface tension decreases, and both storage modulus and loss modulus increases. There is no pronounced storage modulus difference among the bioink tested while there is a noticeable loss modulus difference as the cell concentration changes; 2) as the cell concentration of bioink increases, the droplet size and velocity decrease, the formation of the satellite droplet is suppressed, and the breakup time increases; and 3) compared to the hard bead-laden suspension, the bioink tends to have a less ejected fluid volume, lower droplet velocity decrease, and longer breakup time due to its higher storage and loss moduli.

6.1.3 Electric Field-Assisted Droplet Formation Using Piezoactuation-Based DOD Inkjet Printing

This study investigated the electric field-assisted droplet formation process under piezoactuation-based DOD inkjet printing. For the better control of droplet monodispersity, the Taylor cone is intentionally suppressed from happening to avoid undesirable satellite droplets. The droplet formation process of deionized water has been investigated, and some main conclusions are drawn as follows: 1) with the increase of the applied voltage, the droplet velocity increases and the droplet size decreases; 2) The pinch-off location may be different depending on the applied voltage. With the applied
voltage there are five different regions according to different pinch-off locations. In the first region, the pinch-off occurs near the nozzle orifice. In the second region, the pinch-off occurs near the forming droplet. In the third region, after the pinch-off near the forming droplet, the ligament breaks from the nozzle orifice due to electrohydrodynamic instability before the ligament is retracted back into the nozzle. In the fourth region, the pinch-off occurs near the nozzle orifice since there is no obvious ligament between the nozzle orifice and the forming droplet due to droplet shape change. In the fifth region, the wetting problem occurs probably due to the reduced contact angle caused by electrowetting; and 3) the combination effect of the electric field and meniscus oscillation can be utilized to significantly reduce the droplet diameter to less than one-fifth of the orifice diameter. The electric field also extends the capability of DOD inkjet printing to high-concentrations cell-alginate suspensions.

6.1.4 Fabrication of 3D Vascular-Like Constructs

In this study, vertical printing and horizontal printing have been proposed for fabrication of the vascular-like alginate tubes, which mimic typical vascular constructs. In addition, associated manufacturing challenges are briefly discussed. It has been found that: 1) the maximum achievable height of overhang structure depends on the inclination angle of the overhang structure during vertical printing; 2) a model for cross-sectional deformation during horizontal printing is proposed and the experimental result and model prediction are compared to show a good agreement; 3) a predictive compensation approach has been proposed to mitigate the cross-sectional deformation during horizontal
printing to show a good result; and 4) alginate cellular tubes have also been successfully printed with a satisfactory post-printing cell viability of 87% immediately after printing and after 24 hours of incubation.

6.2 Research Contributions

While some studies have been conducted to investigate various engineering problems associated with DOD inkjet printing of biological material-based fluids, the pinch-off during DOD inkjetting of viscoelastic alginate solutions, the droplet formation performance during DOD inkjetting of cell-laden bioink, and the effects of electric field on droplet formation without forming a Taylor cone during piezoactuation-based DOD inkjet printing haven’t been systematically investigated. In addition, manufacturing challenges during 3D vascular-like construct fabrication using DOD inkjet printing are still lacking. The research work in this dissertation fills in these gaps, which helps to better fabricate tissue-engineered blood vessels with a complex geometry using DOD inkjet printing.

The main contributions of this dissertation are summarized as follows:

1) The pinch-off process during DOD inkjet printing of viscoelastic alginate solutions has been systematically investigated. For the first time, it is found that there are four types of pinch-off which may exist during DOD inkjet printing of viscoelastic NaAlg solutions: front-pinching, exit-pinching, hybrid-pinching and middle-pinching, as classified based on the pinch-off location. The effective relaxation time is identified to characterize the
exponential decay governed by the elastocapillary thinning instead of the longest relaxation time. An operating diagram is constructed with respect to the We and a proposed $J$ number to classify regimes for different types of pinch-off.

2) Cell-laden droplet formation during DOD inkjet printing has been systematically studied. The effects of cell concentration on the breakup time, droplet size and velocity, and number of satellites have been investigated. Furthermore, the droplet formation performance of comparable cell-laden and polystyrene bead-based suspensions is evaluated and further compared to show the effect of particle physical properties.

3) The electric field-assisted droplet formation under piezoactuation-based DOD inkjet printing has been studied. The effects of the applied voltage on droplet volume charge density, droplet size and velocity, and pinch-off locations have been experimentally determined. In addition, the combination effect of the electric field and meniscus oscillation has been utilized to significantly reduce the droplet diameter to less than 1/5 of the orifice diameter. The electric field has been proved to extend the capability of DOD inkjet printing to cell-laden bioinks with high cell concentration.

4) Vertical printing and horizontal printing have been proposed for fabrication of the vascular-like alginate tubes, and the associated manufacturing challenges have been addressed. Process-induced failure during vertical printing and cross-sectional deformation during horizontal printing have been modeled.
Predictive compensation has been proposed to mitigate the cross-sectional deformation during horizontal printing. Cell viability immediately after printing and after 24 hours of incubation has been tested to show a satisfactory result.

6.3 Future Work

While this dissertation reveals some interesting phenomena during fabrication of 3D vascular-like constructs such as pinch-off, cell-laden droplet formation, electric field-assisted droplet formation, and manufacturing challenges during vertical printing and horizontal printing, there are still many open fields needed to further investigate. To enhance understanding of the phenomena in inkjet printing of 3D vascular-like constructs, the recommended future studies are summarized as follows:

6.3.1 Pinch-Off Locations during DOD Inkjetting of Alginate Solution

This study focuses on the first breakup during DOD inkjet printing of viscoelastic alginate solutions. The following problems are of great interest to further understand the pinch-off during DOD inkjet printing, such as: 1) the analytical and computational modeling of ligament thinning process during DOD inkjet printing of viscoelastic fluids to understand the underlying pinch-off physics; 2) the following breakup of the ligament for possible satellite formation after the first breakup; and 3) effect of meniscus oscillation caused by the oscillating pressure wave on the pinch-off near the nozzle orifice.
6.3.2 Droplet Formation Process during DOD Inkjetting of Living Cell-Laden Bioink

In this study, cell-laden droplet formation during DOD inkjet printing has been studied by investigating the effects of cell concentration on droplet formation process. Future studies may need to include as follows: 1) theoretical modeling of the bioink droplet formation process to compare with the experimental observations, 2) improving cell-laden droplet formation performance of bioink with high cell concentration by preventing cell sedimentation and aggregation, 3) printing of other bioink in addition to the fibroblast-sodium alginate suspension to evaluate any droplet formation process differences, and 3) study of the cell injury and functionality variations during inkjet printing.

6.3.3 Electric Field-Assisted Droplet Formation Using Piezoactuation-Based DOD Inkjet Printing

Future work may focus on the control of undesirable wetting phenomenon under extremely high voltages, the physical understanding of the proposed electric field-assisted DOD inkjetting setup, the understanding of mechanism for the combination effect of the electric field and meniscus oscillation, and post-processing cell viability and the cell injury and functionality variations during electric-field-assisted DOD inkjet printing.
6.3.4 Fabrication of 3D Vascular-Like Constructs

In this study, 3D vascular-like constructs have been successfully fabricated using proposed vertical printing and horizontal printing. Alginate cellular tubes have been successfully printed with a satisfactory post-printing cell viability. Future work may focus on: 1) detailed analysis and modeling of the failure mechanisms during the printing of overhang structures; 2) improvement of the model of cross-sectional deformation during horizontal printing; 3) printing using bioink with high cell concentrations; 4) post-printing test including cell functionality testing, tube fusion and mechanical property characterization of printed tubes; 5) fabrication of vascular-like constructs including three different layers using different types of cells, mimicking the blood vessel; 6) printing of large and complex vascular networks; and 7) Printing of adipose-derived stem cell and the following differentiation.
REFERENCES


