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Cell Settling Effects on a Thermal Inkjet Bioprinter

Matthew E. Pepper, *Member*, IEEE, Vidya Seshadri, Timothy Burg, *Senior Member*, IEEE, Brian W. Booth, Karen J.L. Burg, *Senior Member,* IEEE, and Richard E. Groff, *Member*, IEEE

***Abstract*—This paper seeks to quantify cell settling in the print media reservoir of a bioprinter in order to determine its effect on consistent cell delivery per printed drop. The bioprinter studied here is based on the thermal inkjet HP26A cartridge, but any system that dispenses controlled volumes of fluid may be affected similarly. A simple model based on Stokes' law suggests that the cell concentration in the bottom of the reservoir should increase linearly up to some maximum and that the cell concentration in the printed drops should follow this trend. The results show that cell output initially followed the predicted increasing trend, but then peaked and decreased. The timing and rate of the decrease related to the number of use cycles for the cartridges. The results provide guidance for modifications to the printing process to ensure consistent printing of cells.**

I. Introduction

Over the last decade, bioprinting has emerged as a tool suitable for investigating cell–cell interactions. Current bioprinters are beginning to exhibit the high amount of spatial control, i.e. controlling cell placement location and cellular proximity [1] necessary to control the degree of homotypic and heterotypic cell–cell contact for *in vitro* studies [2]. These studies broaden understanding of many different types of cell-cell and cell– extracellular matrix (ECM) interactions, such as cancer proliferation, migration, metastasis, apoptosis or stem cell differentiation and function[1, 3]. Through automation, bioprinters have the potential to produce samples with precision and high throughput, enabling the creation of large datasets to support statistically significant conclusions [3].

Three of the main technologies in bioprinting, thermal inkjet (TIJ), piezoelectric inkjet (PEIJ), and pneumatic microvalve (PMV), require a cell suspension of some specific concentration to be loaded into a reservoir to supply the printing mechanism. While investigating printing performance over time, several papers have noted [4, 5] or documented [6] print failure or decreased cell output while printing over time periods greater than 10 to 20 minutes. This phenomenon has been attributed to the settling and aggregation of cells in suspension [4-7]. Implementing physical workarounds such as agitating the suspensions through vibration or stirring the cell suspension with a stir bar still resulted in unusually low or unpredictable cell output characteristics past the 20 minute mark [5, 6]. Even with intermittent stirring, cell aggregates over 200 um in size were still observed after 10 minutes [6]. Alternatively, the cell concentration in a suspension cannot be drastically reduced because some "cells per drop" criteria must be enforced to ensure the co-culture is produced with the requisite cell density to guarantee proper cell communication, growth, and spreading [5]. The dual constraints of acceptable printer and co-culture performance specify a narrow band for the cell concentration in suspension. This is seen directly in [4] where the correct concentrations of different cell types had to be finely based on the observed clogging tendencies of each cell type.

This paper examines the relationship between cell settling and the cell concentration in a printed drop. From this understanding it is expected that the consistency of cells per printed drop can be improved through i) management of the printing process, *i.e.* establishing a window of time for a particular bioprinting system in which the samples have consistent cell populations, ii) compensation in the printing process for predictable evolution in printed drop concentrations, and iii) additional insight into the requirements of reservoir stirring or agitation systems.

II. Cell Settling Model

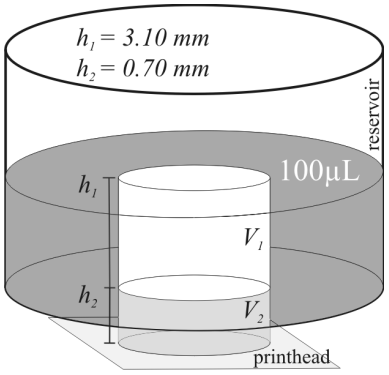

Figure 1 – a diagram of the inkjet cartridge reservoir and printhead shows the volumes of liquid in the reservoir (V_1) and above the print head (V_2). The total of these volumes is 100 µL. This ratio of these volumes estimates the final cell output concentration after settling.

An approximate model that assumes all cells of the same type settle at a constant rate can be used to frame our expectations for the effect of cell settling on printing. The diagram in Figure 1 shows the geometry of the reservoir area above the printhead for a TIJ bioprinter based on the HP26 cartridge (described below). Since the printer is drawing fixed volumes from the bottom of the reservoir it would be expected that the concentration of cells in a printed drop would reflect the concentration of cells near the printhead. The volume of liquid above the printhead is labeled V_2 and has associated height h_2 and the volume of liquid located directly above the printhead in the reservoir,

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Matthew E. Pepper, Richard E. Groff, and Timothy Burg are affiliated with the Department of Electrical and Computer Engineering and the Institute for Biological Interfaces of Engineering at Clemson University, Clemson, SC 29634-0915 USA (corresponding author Timothy Burg: e-mail: tburg@clemson.edu).

Karen J.L. Burg, Vidya Seshadri, and Brian W. Booth are affiliated with the Department of Bioengineering and the Institute for Biological Interfaces of Engineering at Clemson University, Clemson, SC 29634 USA.

V_1, has associated height h_1. First, Stokes' law can be used to estimate the cell settling velocity of each cell by assuming the cells are small particles in a slow velocity fluid flow. Stokes' law is defined as

$$v = \frac{(\rho_p - \rho_f)}{18\mu} g D_p^2 \quad (1)$$

where ρ_p is the density of the particles, ρ_f is the density of the fluid, μ is the fluid's viscosity, g is gravitational acceleration, and D_p is the average diameter of the particles.

The settling velocity can be used with the initial concentration, C_0, to model the concentration in the printhead as

$$C_2(t) = \begin{cases} C_0\left(1 + \frac{vt}{h_2}\right) & \text{for } t < \frac{h_1}{v} \\ C_0\left(1 + \frac{h_1}{h_2}\right) & \text{for } t \geq \frac{h_1}{v} \end{cases} \quad (2)$$

The model in (2) predicts that the concentration in the print area will linearly increase until it reaches a constant steady state value after time t, when all cells from V_1 have completely settled into V_2.

III. Materials & Methods

The bioprinting system, described in detail in [8], uses modified Hewlett-Packard (Hewlett-Packard Company, Palo Alto, CA) (HP) 26A thermal inkjet cartridges. Previous work done in [9] found the anti-scalant EDTA could be added to the cell suspension to significantly decrease the probability of nozzle failure over a span of 25 minutes.

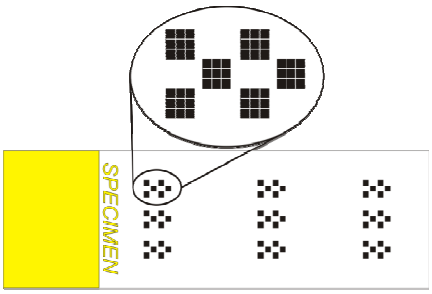

Figure 2 – a sample slide showing nine patterns, each composed of six squares, 3 pixels by 3 pixels. Sample slides in the fourth experiment contained only two rows of patterns, six sample patterns total.

A. Cell Settling Study

The cell output characteristics of the bioprinting system were examined by tracking the change in number of cells per pattern (which is used to calculate the number of cells per drop) over a time span of fourteen minutes. Slides of samples were produced, one immediately after loading cells into the cartridge and then one every two minutes thereafter. Each slide had nine printed samples, each sample was composed of a simple pattern of six squares, 3 pixels x 3 pixels each, 54 pixels total (Figure 2). The two minute interval between slides was chosen to correspond to the typical time required by the TIJ bioprinter to produce one slide of patterned co-cultures, and the fourteen minute experiment duration was based on previous observations of acceptable bioprinter performance using a 8.0×10^6 cells/mL D1 cell (described below) suspension.

Five HP26 cartridges labeled A, B, C, D, and E were chosen from a set of cartridges used in previous cell printing experiments along with two new cartridges labeled F and G. These cartridges were modified from their original form and prepared for printing by removing the top, inner bladder, and reservoir filter. Prior to use in previous experiments, cartridges A, B, C, D, and E were cleaned using a set Cleaning Method, which consisted of a 15 minute soak in Cool Soak Stain and Rust Remover (Burnishine Products, Gurnee, IL), a 15 minute soak in Instrument Lubricant (Burnishine Products), then followed by 15 minutes of sonication (Branson Ultrasonics Corp., Danbury, CT). Each cartridge was then prepared for an experiment using the Experiment Preparation procedure, in which each cartridge was filled with ink and a verification pattern was printed to ensure proper performance of all nozzles. After printing, each cartridge was sonicated for 10 minutes and vacuum dried. The sonication steps in these methods was omitted when preparing Cartridges F and G.

1) Cell Culture

D1 murine mesenchymal stem cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured according to the manufacturer's suggested protocol. Briefly, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose (ATCC), 10% fetal bovine serum (FBS; Mediatech, Herndon, VA), 1% antibiotic/antimycotic, and 1% fungizone (Invitrogen). The culture medium was replaced every 48-72 hours, and cells were maintained at 37ºC with 5% CO_2.

The D1 cells were labeled with Hoescht 33342 trihydrochloride trihydrate (Invitrogen). The D1 cells were suspended in 2 mL of DMEM at 16.0×10^6 cells/mL. Next, 10µL of the stock Hoescht solution (concentration: 1mg/mL) was added to the cell suspension resulting in a dye concentration of 5µg/mL. The cell suspension was incubated for 30 minutes at 37 ºC and 5% CO_2. The cell suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the cell pellet resuspended in 1mL of serum free DMEM (SF-DMEM). After fluorescent labeling of the cells, the 16.0×10^6 cells/mL D1 cell suspension was filtered using a 40 µm sterile cell strainer to remove any large cell clumps (Becton Dickinson, Franklin Lakes, NJ) and stored on ice.

2) Printing Experiments

In the first experiment, twenty four glass microscope slides (VWR International, Westchester, PA) were wiped with a 70% EtOH solution, labeled, and placed in sterile petri dishes. Before loading the cartridge reservoir with cell suspension, the cell suspension was vortexed (Henry Troemner LLC, Thorofare, NJ) to insure homogeneity of the

suspension. Immediately, 50 µL of cell suspension was removed and combined with 50 µL HBSS (Invitrogen, Grand Island, NY) with 1.06 mM ethylene diamine tetraacetic acid (EDTA; Invitrogen) solution to form 100 µL of 50% SF-DMEM and 50% HBSS, containing D1 cells at 8.0×10^6 cells/mL with 0.53 mM EDTA. Experiments were conducted at 20℃.

Experiment	Cartridge	Slides	Interval (min.)	Use Cycles
E1	C	8	2	6
E1	D	8	2	6
E1	E	8	2	5
E2	A	11	1	11
E2	B	8	2	9
E3	D	8	2	7
E3	E	8	2	6
E3	B	8	2	10
E4	F	8	2	1
E4	G	8	2	1

Table I – experiment plan and the previous usage of each cartridge

In the first experiment (E1), the cell suspension was pipetted into the cartridge reservoir of cartridge C, D, or E, the firing chambers primed, and the cartridge inserted into the bioprinter. The first experiment slide was removed from its Petri dish and printed immediately after cartridge insertion. Seven slides were successively printed at two-minute intervals. After printing, each slide was returned to its Petri dish. When printing was complete, the cartridges were cleaned and dried according to the Cleaning Method. Each slide was inspected with a Zeiss Axiovert 40 CFL microscope (Carl Zeiss AG Oberkochen, Germany) equipped with a 50 W Xenon lamp and the number of cells in each of the 9 samples was hand counted and recorded.

In the second experiment (E2), A and B were chosen because these cartridges had almost twice as many previous uses as C, D, and E (Table I), allowing investigation into the possible effects of cartridges wear and cleaning. Cartridges A and B were cleaned and verified according to the Cleaning Method and Experiment Preparation method. Eleven glass slides were prepared as above for cartridge A and eight glass slides for cartridge B. The cell suspension was prepared and labeled as above. Cartridge A was used to create 11 slides, each printed at a 1 minute interval, to investigate if the cell output per sample would change with increased printing frequency. Cartridge B printed eight slides at 2 minute intervals. Instead of hand counting all samples on each slide, each sample was imaged using the Zeiss Axiovert 40 CFL microscope, captured using an AxioCam MRC 5, and processed with Zeiss AxioVision LE 4.6. The cell counts of all samples for all slides of A and B were calculated using image processing techniques implemented in Matlab R2009b (Mathworks Inc, Natick, MA).

A third experiment (E3) was performed to collect additional data from cartridges B, D, and E and compare it with their performance data from the first and second experiments. Cell suspension preparation and labeling was the same as above. Each cartridge generated eight slides of 9 samples each every two minutes. The samples were imaged and counted as in experiment 2.

In a fourth experiment (E4), two new cartridges F and G were used to produce 24 slides each, consisting of 3 trials of 8 slides, printed at 2 minute intervals. All cell suspensions and slides were prepared as in previous experiments. Each cartridge was cleaned between experiments using the Cleaning Method, but without the sonication steps. Six samples per slide were printed as opposed to nine (Figure 2) to reduce the number of images that must be counted while maintaining statistical significance. All samples were imaged and analyzed as in experiment 2.

IV. RESULTS & DISCUSSION

The mathematical settling model in (1) and (2) predicts that the concentration of particles in suspension in the print area will linearly increase until it reaches a constant steady state value. The measured geometry of the HP26 cartridge suggests $C_2(\infty) = 5.4C_0$, which indicates that the particle output should linearly increase due to settling to over five times its initial value then remain. The model parameters can be found in Table II. The D1 cell density was estimated from literature that measured Chinese Hamster Ovary (CHO) cells [10]; D1 and CHO cells are both mammalian cells of similar size thus similar densities are assumed. The viscosity of the SF-DMEM/HBSS/0.53 mM EDTA solution was measured without particles using a size 50 glass capillary viscometer. Wall effects and particle effects were not taken into consideration as the concentration of the suspension was below 10×10^6 cells/mL [11].

Model Parameters	Symbol	Values
Cell Density (g/cm^3)	ρ_p	1.051
Particle Diameter (µm)	D_p	13
Gravitational Acceleration (m/s^2)	g	9.8
Solution Viscosity (cP)	μ	1.036 (20℃)
Solution Density (g/cm^3)	ρ_f	0.998

Table II – model parameters used for the cell and bead settling output models

In Figure 3, the number of cells per sample was normalized by the initial cells per sample to remove effects due to variation in the initial suspension concentration between experiments and examine how cell settling affected the "cells per drop" output of the cartridges over time. The normalized average cell output of all cartridges follows the predicted cell settling output closely until they begin to diverge after 4 to 8 minutes. This phenomenon was not due to cell depletion, as no slide contained more than 3000 cells between all of its printed patterns and no experiment came close to depleting the approximately 100,000 cells located in the column of liquid above the printhead.

With the output profiles of A, B, C, D, and E consistent between experiments, grouping the cell output profiles by previous usage (Table I) rather than experiment showed

three distinct average cell output profiles for the more heavily used cartridges (A and B), moderately used cartridges (C, D, and E), and new cartridges (F and G). The heavily and moderately used cartridges had performed previous experiments; they were cleaned and prepped using the Cleaning Method and Experiment Preparation method. The sonication step, present in both methods, can pit hard materials [12]; sonication could be pitting the surfaces of the cartridge printhead and firing chamber walls, promoting cell attachment, leading to the decrease in cell output. Compared to the cell output model in Figure 3, it appears the longer a cartridge is exposed to sonication the more pronounced the performance decrease.

Future experiments will only use cartridges with less exposure to sonication than the moderately used cartridges (<2 hours) to maximize the number of samples with comparable cell populations. The output profiles of the moderately used and new cartridges suggest that 3 to 4 slides of samples containing comparable cell populations can be produced as long as cartridges that have seen similar amounts of moderate use and sonication are paired. These samples could be printed between 2 and 10 minutes after cartridge loading. Refining the cleaning and preparation methods to use less sonication will improve cartridge performance and increase a cartridge's useful life.

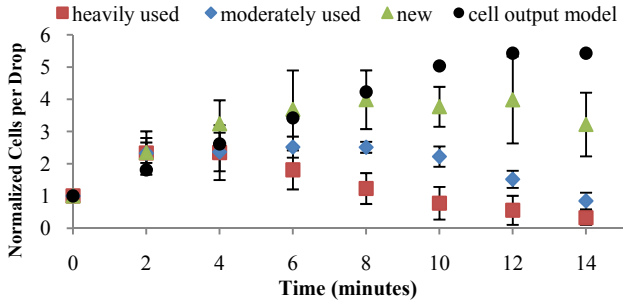

Figure 3 – chart comparing the cell output of heavily used (A and B), moderately used (C D and E), and new cartridges (F and G). The error bars represent standard deviation of average cells per sample from the different trials of each cartridge group (N > 9 for all data points).

In order to compensate for varying cell concentration due to settling and aggregation, the cell output profile (Figure 3) should be characterized for the desired cell type and initial suspension concentration. Given the output profile, several approaches can be taken to produce samples with consistent cell population sizes. For example, some methods are:

i) Print during a pre-specified time window over which variation in cell concentration is acceptable.

ii) Use the cell output profile to vary the number of drops deposited per location to ensure that a consistent number of cells are deposited.

iii) Develop a reservoir stirring or agitation policy to keep concentration within bounds. The effects of stirring on cell activity require further study.

Incorporating one or more of these methods into a bioprinting system should allow the generation of large numbers of samples with comparable cell populations.

V. Conclusion

Generating large datasets of patterned co-cultures is an important bioprinter milestone. This work indicates that cell settling is an important factor that must be addressed to achieve this milestone. A simple cell settling model was shown to predict the effect of cell settling over an initial printing period starting from a uniform concentration. It was also observed that other effects such as cell aggregation or attachment eventually dominated the settling effects on the "cells per drop" behavior. The model is general enough to be adapted to examine cell settling effects in other systems.

Comparing cells per drop over time between three sets of cartridges with heavy, moderate, and no exposure to sonication showed that the less a cartridge is exposed to sonication the longer its output follows the cell settling output model. New cleaning procedures minimizing sonication should increase the number of use cycles per cartridges as well as maintain output consistency.

By more fully characterizing the processes of bioprinting, we are now able to estimate the number of cells per drop and compensate for evolution in cell number. This knowledge is necessary to produce the largest number of comparable samples while operating within the time constraints imposed by cell settling and subsequent aggregation.

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