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RESEARCH ARTICLE

EDTA enhances high-throughput two-dimensional bioprinting by inhibiting salt scaling and cell aggregation at the nozzle surface

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Abstract

Tissue-engineering strategies may be employed in the development of *in vitro* breast tissue models for use in testing regimens of drug therapies and vaccines. The physical and chemical interactions that occur among cells and extracellular matrix components can also be elucidated with these models to gain an understanding of the progression of transformed epithelial cells into tumours and the ultimate metastases of tumour cells. The modified inkjet printer may be a useful tool for creating three-dimensional (3D) *in vitro* models, because it offers an inexpensive and high-throughput solution to microfabrication, and because the printer can be easily manipulated to produce varying tissue attributes. We hypothesized, however, that when ink is replaced with a biologically based fluid (i.e. a 'bio-ink'), specifically a serum-free cell culture medium, printer nozzle failure can result from salt scale build-up as fluid evaporates on the printhead surface. In this study, ethylene diamine tetra-acetic acid (EDTA) was used as a culture medium additive to prevent salt scaling and cell aggregation during the bioprinting process. The results showed that EDTA, at a concentration typically found in commercially available trypsin solutions (0.53 mM), prevented nozzle failure when a serum-free culture medium was printed from a nozzle at 1000 drops/s. Furthermore, increasing concentrations of EDTA appeared to mildly decrease aggregation of 4T07 cells. Cell viability studies were performed to demonstrate that addition of EDTA did not result in significant cell death. In conclusion, it is recommended that EDTA be incorporated into bio-ink solutions containing salts that could lead to nozzle failure. Copyright © 2009 John Wiley & Sons, Ltd.

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1. Introduction

Drop-on-demand inkjet printing systems are capable of patterning a variety of cell types (e.g. primary rat hippocampal and cortical neurons, Chinese hamster ovary (CHO) cells and bovine endothelial cells) (Nakamura *et al*., 2005; Xu *et al*., 2005, 2006) and biomaterials (e.g. collage and alginate) (Boland *et al*., 2006). Based on these capabilities, inkjet systems have been proposed as microfabrication tools for organ replacement, printing multiple cell types and biomaterials in three-dimensional (3D) constructs with the geometric precision to produce biological structures, including vessels (Boland *et al*., 2003; Mironov *et al*., 2003; Wilson *et al*., 2003).

We have proposed the use of inkjet printing for a different application – the fabrication of *in vitro* tissue test systems (Burg and Boland, 2003; Chaubey *et al*., 2007; Parzel *et al*., 2008a,b). For example, the current 'gold standard' for tumour modelling involves the suspension

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of cells within gel-like matrices, mainly matrigel (Kasper *et al*., 2007; Sasser *et al*., 2007); although these systems appear to be superior to traditional 2-dimensional models, they lack the rigidity necessary to allow for normal functioning of anchorage-dependent breast cells, specifically adipocytes (Discher *et al*., 2005; Engler *et al*., 2004). Tissue-engineering strategies may be employed in the development of *in vitro* tissue models that are more structurally similar and therefore have enhanced potential for use in testing regimens of drug therapies and vaccines. Researchers can also employ these engineered tissue models to study the physical and chemical interactions that occur among cells and extracellular matrix (ECM) components and to better understand the progression of disease processes.

Previous *in vitro* studies have demonstrated the importance of spatial alignment of cells in culture when attempting to accurately mimic the *in vivo* microenvironment, which can include cell–cell and cell–ECM contacts in addition to physical forces and soluble factors (Wang *et al*., 2007). The ability to define cell placement and therefore spatial location within a 3D tissue can lead to better predictions of cell function and an overall increase in the stability of cell phenotype (Sodunke *et al*., 2007). Conventional cell-seeding methods are inadequate in the development of *in vitro* tissue test systems, because they involve the random placement of cells and therefore lack the precision necessary for spatial control. Conversely, microfabrication tools allow the creation of select biomaterial surface variations as well as the precise placement of cellular components (Khetani and Bhatia, 2006). Inkjet-based microfabrication, known as bioprinting, can provide a preliminary foundation for developing such *in vitro* breast tissue models. The reality is that, no matter what the intended application, ink-jet printing has many logistical barriers that must be addressed in order to realize the high-throughput precision fabrication of complex, 3D tissues. Khademhosseini and Langer previously discussed cell printing as a powerful 'bottom-up' tissue-engineering approach. They note that technical limitations associated with this method include nozzle clogging and sustainability of printed tissue (Khademhosseini and Langer, 2007). This manuscript addresses the former limitation, and provides a solution for the restricted number of drops, and correspondingly cells, that can be printed prior to printhead failure due to clogging.

To address this concern, the Hewlett-Packard HP26 cartridge and thermal printhead, driven by custom-designed interface electronics that mimic the behavior of the HP500 series consumer inkjet printers, was employed. We selected this thermal inkjet printhead because: (a) it has already been proved capable of printing viable cells and biomaterials (Roth *et al*., 2004); (b) it permits high-throughput microfabrication by providing a large number (50) of appropriately-sized (diameter 50 μm) nozzles in a small area; and (c) it is inexpensive and widely available. Before initial application of a new HP26 to printing, the cartridge is opened, the ink is drained and the cartridge and printhead are cleaned and sterilized. The ink is replaced with a biologically relevant fluid, such as cell culture medium or dilute hydrogel. These fluids are referred to as 'bio-inks'.

Inside each nozzle of a thermal inkjet printhead, such as the HP26, is a small thin-film resistor. To eject a drop from the nozzle, the resistor is heated with a short, precisely timed pulse of electrical current. The heat causes the adjacent ink to evaporate and form a small bubble and, as this bubble expands, a drop of liquid ink is ejected from the nozzle. As the drop is ejected, the bubble rapidly cools and shrinks, and ink from a reservoir refills the nozzle via capillary action. This process, which occurs in less than 3 μs, is illustrated in Figure 1. The process of applying current to a nozzle's thin film resistor in order to eject a drop is termed 'firing' the nozzle. In previous work using the HP26 printhead to print a serum-free cell culture medium, nozzles failed, i.e. the nozzles did not eject a drop when fired, after only a relatively short amount of printing time. This failure was generally attributed to clogging by adsorbed proteins (for which reason only serum-free medium is used) and cellular components, in addition to aggregated cells. After failure, the printhead may be sonicated in a bath of warm water, which moderately restores performance of the nozzles.

In order to maintain the clinical relevance for determining cell therapy protocols, *in vitro* cultures generally have a range of at least 10^7–10^9 cells (Enderle *et al*., 2005). Assuming that one cell is ejected per drop and that there are 50 nozzles per printhead, then a nozzle should be able to eject at least 1 million drops before failure, in order to approach clinical relevance. Experience with printing cell cultures with the HP26 printhead, using either the custom electronics or an HP500 series printer, showed that nozzle failure occurs significantly before that

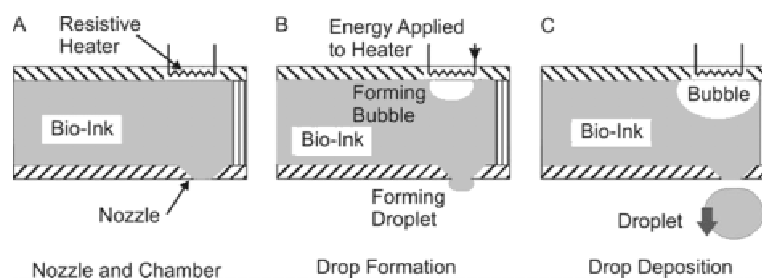

Figure 1. Thermal inkjet printer operation. A thin-film resistor heats the liquid print medium, causing a bubble to form. Pressure resulting from bubble formation forces the ejection of an ink drop through the nozzle

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lower limit. Moreover, nozzle failure was observed while printing simple salt solutions, where failure could not be attributed to adsorbed proteins and cellular components. These observations led to an alternative explanation for nozzle failure; as fluid evaporates during firing, salt is deposited on the print-head surface, affecting both fluid flow and drop formation, potentially resulting in degraded patterning, erratic cell ejection or complete nozzle failure.

2. Materials and methods

The experimental goals for this work were defined: (a) to enable a nozzle to print at least 1 million cells before failure by incorporating an additive in the bio-ink, to prevent the purported salt deposits inside the printhead; and (b) to provide an improved cleaning method for the cartridge and printhead. In this study, ethylene diamine tetra-acetic acid (EDTA) was used as a culture medium additive to prevent both salt scaling and cell aggregation during the bioprinting process. EDTA is a synthetic amino acid that acts as a chelating agent to trap heavy metal and mineral ions. It was hypothesized that this property of EDTA would inhibit ion crystallization, specifically crystallization of those ions which are present in commercially available cell culture mediums, thus preventing the degradation of printer performance. EDTA was selected for use in this study because of its already widespread use in cell culture as a calcium chelator, which can either enhance the function of trypsin or hinder the joining of cadherins among cells. All studies were performed by printing cells in two dimensions. The potential of these findings includes the construction of high-throughput, clinically relevant, two-dimensional (2D) cultures and the possible translation of these methods to facilitate creation of three-dimensional (3D) constructs.

2.1. EDTA effectiveness

A procedure that rapidly fires a single nozzle was used to determine the range of concentrations of EDTA in serum-free Dulbecco's modified Eagle's medium (SF-DMEM) that would prevent nozzle failure. A 0.5 M stock solution of EDTA was diluted in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, and was subsequently combined with SF-DMEM to yield final EDTA concentrations of 0.27, 0.53 and 1.06 mM. These concentrations are within the range typically present in commercially available trypsin solutions. Bio-inks were printed, i.e. fired, at a frequency of 1000 Hz, from a single nozzle onto glass microscope slides in successive fixed-length test periods. After each period, the slide was observed for evidence of printed media. Firing test periods for bio-inks containing EDTA were 5 min long, while the periods for SF-DMEM, which causes nozzles to fail much more quickly, were 1 min. Solutions were tested at 1000 drops/s for a maximum of 25 min (1.5×10^6 total drops) or until the nozzle became completely clogged, whichever condition occurred first. Samples were given qualitative scores and corresponding numerical values of yes (1, indicating a well-defined dot of medium), yes with spatter (0.5, indicating initial nozzle failure) or no (0, indicating no medium printed). Spatter within a pattern was defined as satellite drops and irregular printing that presumably result from various levels of clogging, which can cause erratic spraying of the bio-ink during ejection. Figure 2 depicts a three-line pattern printed from a single nozzle in ink, first from a clean nozzle (left) and subsequently from the same nozzle, which was partially clogged after using a 30 s printing procedure with SF-DMEM. Bio-inks were tested for a maximum of 25 min (1.5×10^6 total drops) or until complete nozzle failure occurred. A separate cartridge was used for each of the three trials within a control or experimental group (12 total cartridges); there was a maximum of five firing test periods in each individual group of a particular trial, unless the cartridge failed to print. The number of drops ejected during a period is estimated as the numerical score times the number of drops targeted per period. The estimated number of drops successfully ejected from a nozzle during the test is given by equation 1:

$$\text{No. drops ejected} = \text{no. seconds/test period} \times \text{firing frequency} \times \sum \text{numerical scores} \quad (1)$$

Data were plotted and evaluated using one-way ANOVA with a significance level of $p < 0.05$ and a sample size of $n = 3$.

2.2. Cell culture

D1 murine mesenchymal stem cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured according to the manufacturer's suggested protocol.

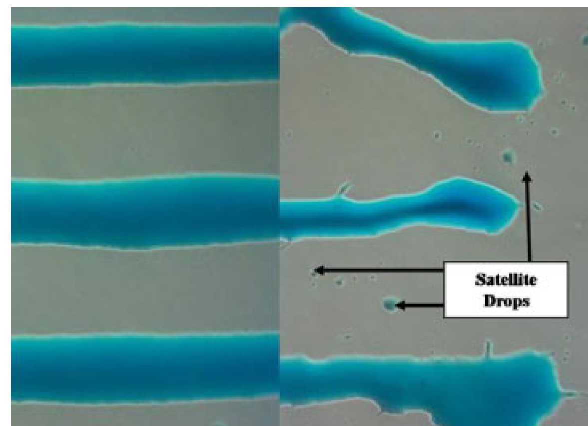

Figure 2. Comparison of a three-line ink pattern with spatter (right) and without (left). Spatter indicates that a nozzle is in the initial stages of failure. The pattern with spatter was printed after rapidly firing SF-DMEM from the nozzle for 30 s. This demonstrates how even short-term use of SF-DMEM can cause significant pattern disruptions. Total magnification, ×100

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Briefly, cells were maintained in DMEM containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate and 4.5 g/l glucose (ATCC), and 500 ml were supplemented with 50 ml fetal bovine serum (FBS), 5 ml antibiotic/antimycotic and 1 ml fungizone. The culture medium was replaced every 48–72 h as required, and the cells were stored in an incubator at 37 °C and 5% CO_2. Cells from a non-metastatic murine mammary cancer cell line, 4T07, were maintained in the culture conditions described above for D1 cells.

To prepare cell-based bio-inks for printing, D1 and 4T07 cells were suspended in SF-DMEM at a density twice the desired final concentration. All cell suspensions were filtered using a 40 μm sterile cell strainer. Just prior to printing, 75 μl of the cell suspension was combined with 75 μl of HBSS containing 0.53, 1.06 or 2.12 mM EDTA, and was subsequently deposited into the HP26 cartridge well. Thus, the resulting 150 μl bio-ink consisted of D1 or 4T07 cells suspended in 50% SF-DMEM and 50% HBSS, with a final EDTA concentration of 0.27, 0.53 or 1.06 mM.

2.3. Cell viability and attachment

2.3.1. *LIVE/DEAD® assay for cell viability*

The viability of D1 cells printed in suspensions of the bio-inks described above was assessed qualitatively using a LIVE/DEAD viability kit (Molecular Probes, Eugene, OR, USA) with which live and dead cells fluoresce green and red, respectively. Additionally, cell morphology was observed to deduce whether the cells were attached to the TCPS. D1 cells were suspended in SF-DMEM at an initial concentration of 6×10^6 cells/ml and subsequently combined with HBSS containing EDTA. The final bio-ink solutions were comprised of 50% SF-DMEM and 50% HBSS, containing 3.0×10^6 cells/ml and 0.27, 0.53 or 1.06 mM EDTA. A 100% SF-DMEM solution, devoid of EDTA, was used as a control. D1 cells were fired from a single nozzle at a rate of 1000 drops/s for 240 s onto a glass microscope slide, after which 2 μl of the printed bio-ink was pipetted in triplicate ($n = 3$) into a 24-well plate. Wells were presoaked in a 50% solution of FBS in DMEM for at least 1 h prior to the addition of cells. D1 cells were allowed to attach in an incubator at 37 °C and 5% CO_2 for 30 min before adding 1 ml 10% serum-inclusive DMEM to each well containing cells. Cells were maintained for 24 h, after which the LIVE/DEAD assay was applied to the cultures according to the manufacturer's suggested protocol. Briefly, cells were washed in 1× Dulbecco's phosphate-buffered saline (DPBS) and incubated at room temperature for 45 min in LIVE/DEAD reagent comprised of 4 μM ethidium homodimer and 2 μM calceinAM in DPBS. Fluorescent images of the samples were captured using an inverted microscope (Carl Zeiss, Thornwood, NY, USA) and Image-Pro software.

2.3.2. *AlamarBlue® metabolic activity assay*

A quantitative AlamarBlue assay was conducted in order to assess relative effects of various EDTA concentrations on the metabolic activity of D1 cells. D1 cells were suspended in the bio-ink solutions, including a control with no EDTA. D1 cells were suspended in SF-DMEM at an initial concentration of 6×10^6 cells/ml and subsequently combined with HBSS containing EDTA. Cells remained suspended at room temperature (to simulate actual printing conditions) for 0, 15 or 30 min, after which they were pipetted into 24-well plates at 20% confluence; a sample size of four was used for each experimental and control group. Once cells were allowed to attach for 30 min, 1 ml 10% serum-inclusive DMEM was added to each well containing cells. The cells were maintained at 37 °C and 5% CO_2; the AlamarBlue metabolic activity assay was executed at both 4 and 24 h following cell seeding. A 10% solution of AlamarBlue dye in fresh culture medium was prepared and added to the appropriate wells after the treated medium was aspirated. Cells were incubated at 37 °C and 5% CO_2 for 2 h to allow for a colour change. Following incubation, 150 μl culture medium from each sample was transferred in triplicate (only the mean value was reported) to a black, Costar® 96-well plate (Corning Inc.), and the plate was read using a Fluroskan Ascent FL fluorescent plate reader (ThermoLabsystems, Franklin, MA, USA), using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Data were plotted and values within suspension time groups were analysed using factorial ANOVA with a significance level of $p < 0.05$ and a sample size of $n = 4$. Additionally, one-way ANOVA analyses were conducted in order to identify the significance of time in suspension and subsequently compare these values to solutions effects. Again, a significance level of $p < 0.05$ was selected with a sample size of $n = 16$.

2.4. Anti-aggregation of tumourigenic cells

EDTA was also tested for its ability to prevent aggregation of cells during the printing process. The 4T07 cell line was selected for this study because of its natural tendency, as a tumourigenic cell type, to aggregate. 4T07 cells were suspended in SF-DMEM at an initial concentration of 1.54×10^7 cells/ml and subsequently combined with HBSS containing EDTA in the concentrations described above. Final bio-ink solutions contained 7.7×10^6 cells/ml, which corresponds to approximately 1 cell/drop, estimated using a drop volume of 130 pl, as reported in Hewlett-Packard literature (Buskirk *et al*., 1988). Cells were printed in their respective bio-ink medium into 5 × 5 matrix (25 drops) patterns. Drops were separated by 430 μm to facilitate counting. Sample patterns ($n = 15$) were printed for each of the experimental groups, along with a control group, exclusive of EDTA. Cartridges were not changed between samples in the same experimental group because, in actual printing conditions, cells suspended in a cartridge well would be allowed to settle over time. Cell counts were recorded for each of the dots printed and data were plotted

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as frequency histograms, so that trends in changing cell counts could be deduced. Successful printing was defined as the ejection of one, two or three cells, while failure was defined as ejection of 0 cells or more than three cells.

2.5. Cartridge cleaning

At the completion of each bioprinting procedure, the HP26 cartridges were cleaned (i.e. unclogged) using a combination of chemical soaks and sonication. This combination addressed clogging resulting from protein adsorption, cellular debris and salt scaling. Cartridges were rinsed in distilled water to wash away any remaining bio-ink and subsequently agitated for 15 min at room temperature with a chemical rust and stain remover diluted 1 : 1 in distilled water (Weiman Products, LLC, Gurnee, IL, USA). The cartridges were rinsed again in distilled water and agitated for 30 min at room temperature with an instrument lubricant diluted 1 : 6 in distilled water (Weiman Products). Cartridges were then transferred to a distilled water bath and sonicated for 15 min.

3. Results

3.1. EDTA effectiveness

Each of the bio-ink solutions was fired from a single nozzle at 1000 Hz in successive test periods for a maximum of 25 min (1.5×10^6 total drops) or until the nozzle failed. A value less than 1.5×10^6 drops indicated that the nozzle failed during testing. Note that bio-inks containing 0.53 and 1.06 mM EDTA performed similarly and did not suffer nozzle failure during any of the trials. A serum-free cell culture medium with EDTA added at a concentration typically found in commercially available trypsin solutions (0.53 mM) could be fired from a nozzle at a rate of 1000 Hz for over 1.4 million drops without nozzle failure. Thus, reasonable levels of EDTA permit a nozzle to print over 1 million cells without failure. Table 1 lists the qualitative scores given to high-speed evacuation samples with various concentrations of EDTA added to prevent salt scaling. Figure 3 displays the approximate number of drops that could be printed before nozzle failure. Approximately 1.5×10^6 [standard error of the mean (SEM) = ± 0] drops could be printed with bio-inks containing 0.53 and 1.06 mM EDTA before failure occurred, while only 1.0×10^6 (SEM = $\pm 1 \times 10^5$) drops could be printed with 0.27 mM bio-ink and only 2.0×10^5 (SEM = $\pm 1.7 \times 10^4$) drops with 0 mM bio-ink.

3.2. Cell viability and attachment

3.2.1. LIVE/DEAD® assay for cell viability

A qualitative LIVE/DEAD assay for cell viability confirmed that the number of necrotic cells in each of the samples was negligible. Figure 4 depicts D1 cells, which were suspended in bio-ink containing (a) 0.53 mM EDTA and (b) 1.06 mM EDTA, printed, seeded in 24-well plates and maintained in culture for 24 h. Cells in all cases look healthy and viable, with no obvious differences between groups.

Table 1. Corresponding numerical values calculated from qualitative data

		0 mM EDTA	0.27 mM EDTA	0.53 mM EDTA	1.06 mM EDTA
Cumulative qualitative score	Trial 1	2.0	3.0	5.0	5.0
	Trial 2	1.5	3.0	5.0	5.0
	Trial 3	1.0	4.0	5.0	5.0
	TOTAL	4.5	10.0	15.0	15.0

Samples were assessed based on whether printing occurred, and qualitative scores were then given a corresponding value of yes = 1, yes with spatter = 0.5, and no = 0. Note that bio-inks containing 0.53 mM and 1.06 mM EDTA did not fail during any of the trials.

3.2.2. AlamarBlue® metabolic activity assay

Figures 5a, b, display absolute AlamarBlue fluorescence values at the selected culture time points (see Table 2 for averages of absolute fluorescence and SEM). Following a 4 h culture period, data indicated that there were statistically significant differences at the 15 and 30 min suspension time points when cells that had been cultured in the presence of EDTA were compared to control cells. Following a 15 min suspension time, all three EDTA concentrations were significantly different from their respective controls, while at 30 min, only 0.53 and 1.06 mM were significantly different from the control. Following a 24 h culture period and 15 min in suspension, the effect of EDTA concentration, when compared with the control, is only significant between 0.53 and 1.06 mM. When D1 cells were kept in suspension with bio-inks for 30 min following 24 h culture, the solution effects ceased. When comparing 4 and 24 h culture times, it appears that EDTA concentration more significantly affected D1 metabolic activity following 4 h in culture.

3.3. Anti-aggregation of tumourigenic cells

EDTA was evaluated for its effectiveness in preventing cell aggregation during the bioprinting process. Histograms are depicted in Figures 6A–D, which represent the frequency of drops that contained a specified number of cells. Comparison of histograms indicates that a peak emerged in the lower cell-number range, when 0.27 and 0.53 mM EDTA was added to the 4T07 cell suspension. The proportions of successful and unsuccessful drops printed using each of the defined bio-inks are presented in Table 3. The greatest proportion of successful drops was printed using a 4T07 cell suspension containing 0.53 mM EDTA.

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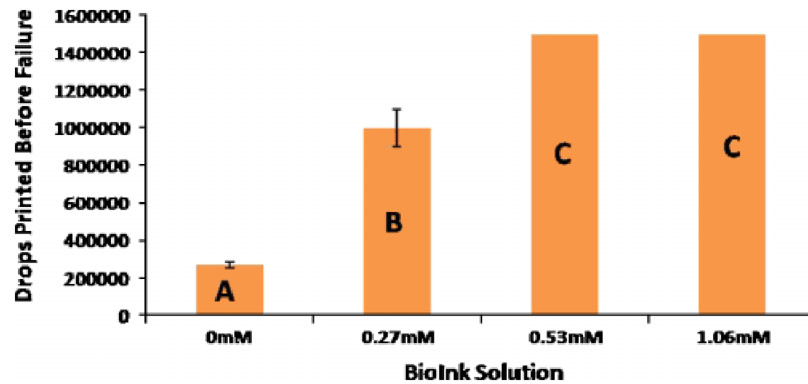

Figure 3. Approximate number of drops printed before failure occurred. A value of $<1.5 \times 10^6$ indicates that nozzle failure occurred. Bars with different letters are significantly different. Note that bio-inks containing 0.53 mM and 1.06 mM EDTA did not fail

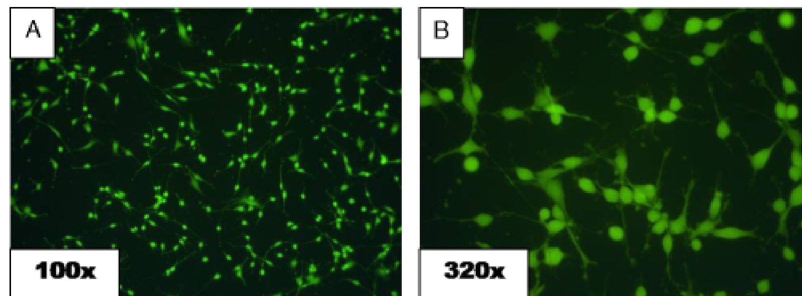

Figure 4. LIVE/DEAD® assay for cell viability. D1 cells were suspended in bio-inks containing EDTA and printed using a thermal inkjet printer and an HP26 cartridge. Cells were qualitatively assessed for verification of viability and attachment following 24 h in standard culture. (A) 0.53 mM EDTA. (B) 1.06 mM EDTA. Image was captured using only the FITC filter. The number of dead cells visible under the rhodamine filter was non-existent or negligible

4. Discussion

An average adult human organ might contain several hundred million functional units (i.e. cells of the parenchyma) in addition to supportive stromal tissue. Nozzle failure after several hundred thousand firings is thus a very serious roadblock on the path to creating tissue test systems, and an even greater impediment in engineering entire organs. The recognition of salt scaling as a new obstacle in bioprinting applications arose from our development of a high-resolution bioprinter, which we achieved via the reverse engineering process of an HP 520C series inkjet printer and HP26 ink cartridge. Successful printing of various cellular and acellular solutions using the HP26 cartridge is described in the literature (Xu *et al*., 2005); some, e.g. DMEM, calcium chloride and DPBS, largely contain inorganic salts, while others like fibrinogen and laminin solutions contain low concentrations of proteins. Prior to the construction of a custom bioprinting system with which single-nozzle control could be achieved, it was hypothesized that most nozzle failure, i.e. failure to eject a drop of liquid when the nozzle is fired, occurred as a result of clogging due to high cell densities and 'sticky' proteins. Cartridge failure due to salt scaling likely went unrecognized by bioprinting researchers because previous literature had focused on creation of only small-scale patterns. However, when we assessed EDTA effectiveness in acellular solutions, SF-DMEM caused nozzle failure after approximately 3.0×10^5 drops. Therefore, as bioprinting procedures become more advanced and larger tissue samples are constructed, the use of SF-DMEM as a bio-ink would likely very quickly lead to large pattern disruptions and, ultimately, complete nozzle failure.

EDTA is a synthetic amino acid, and it was selected for this study because of its widespread use in cell culture and *in vivo* therapeutic applications. It was hypothesized that EDTA, in concentrations that would not compromise cell viability, could prevent salt scaling by acting as a chelating agent to prevent crystallization of ions during evaporation at the printer cartridge surface. Furthermore, addition of EDTA to a cell suspension did not change the fluid viscosity, a parameter which could compromise the performance of the thermal inkjet mechanism (Sen and Darabi, 2007). The starting point for selection of EDTA concentrations was 0.53 mM, which is the concentration found in commercially available trypsin solution. It was demonstrated that a concentration of 0.27 mM EDTA in bio-ink reduced but did not eliminate nozzle failure within the 25 min testing period. With increased EDTA concentrations of 0.53 and 1.06 mM, the nozzles ejected drops reliably without spatter for the entire 25 min testing period. Through experience, spatter was determined to be a sign of approaching failure and, as shown in Figure 1, spatter results in significant pattern disruptions.

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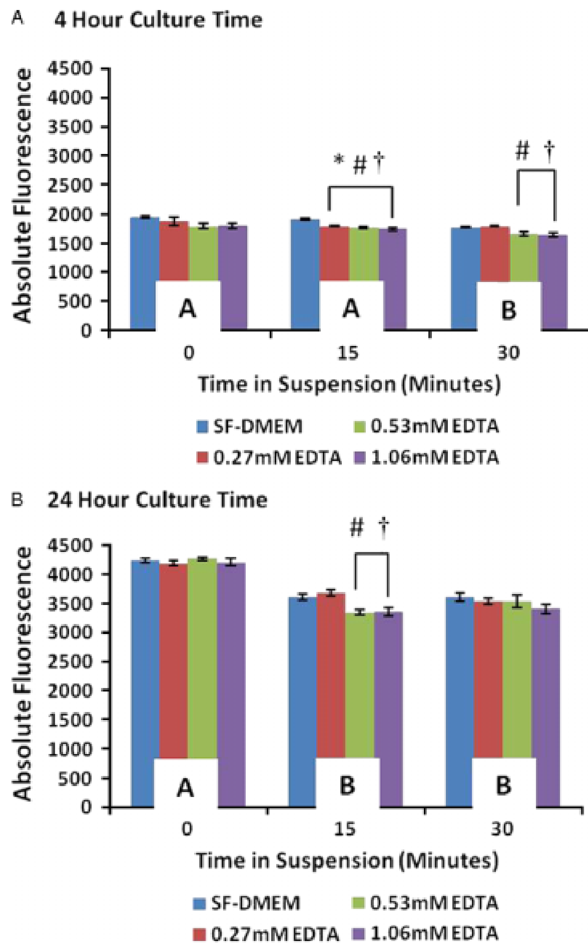

Figure 5. AlamarBlue® metabolic activity assay of D1 cells maintained in culture for 4 and 24 h. Cells were suspended in bio-inks containing EDTA, pipetted into 24 wells and allowed to attach for 30 min. Values within groups were analysed using factorial ANOVA with a significance level of $p < 0.05$ and a sample size of $n = 16$. Groups labelled with different letters were significantly different. Bars that were significantly different from their respective controls are labelled 0.27 mM (*), 0.53 mM (#) and 1.06 mM (†)

Data generated to show the effect of EDTA on D1 cells in culture suggest that the variable indicating total amount of time in suspension has a stronger effect on metabolic activity than does solution effects. The more significant effect of EDTA concentration on D1 metabolic activity following 4 h in culture is likely a result of slowed cell attachment in the presence of EDTA, which no longer delayed metabolic activity at the later time point, once cells had fully attached. We have demonstrated the attachment of cells and the preservation of a 2D μm-scale pattern over a 5 day period (Parzel *et al.*, 2009); thus, the presence of EDTA in the culture medium should not prevent creation of a 3D construct, especially when integrin-binding materials (i.e. collagens) are introduced into the construct. It will be important to incorporate this information into the bioprinting process to ensure that, regardless of the bio-ink solution, cells should be left in solution for a minimal amount of time before being injected into the well of the printer cartridge. Future work will involve the assessment of cell viability within 3D constructs, following the addition of EDTA to a bio-ink. Regardless of the results, the prevention of salt-scaling and cell aggregation at the nozzle surface will remain an important milestone for high-throughput 2D culture.

The shapes of the frequency histograms for 4T07 studies show that a peak in the low cell-number range began to form while printing with 0.27 and 0.53 mM EDTA. However, when EDTA concentration was increased to 1.06 mM EDTA, the shape of the histogram began to revert back to the shape portrayed by the SF-DMEM samples. The data further suggest that cells in toxic environments will begin to aggregate and thus, although the 1.06 mM EDTA solution prevents clogging, it is not an optimal solution to achieve the most successful cell ejection frequency.

The novel cartridge cleaning protocol was implemented after observing that sonication alone was not an effective tool for unclogging nozzles. Ultrasonic cleaning methods were employed in previous studies because it was believed that clogging resulted primarily from protein adsorption and cell aggregation. The commercially available chemicals (rust remover and instrument lubricant) utilized in the new cleaning procedure were intended to address nozzle clogging due to salt scaling. Additionally, the instrument lubricant may help prevent contamination, as it remains bacteriostatic for approximately 1 month after use. There was a noticeable increase in the effectiveness of this cleaning procedure for unclogging nozzles when compared to sonication alone, although no formal studies have been completed to confirm these observations statistically.

The ultimate goal of this research is to apply 3D cell-printing techniques toward the development of *in vitro* tissue test systems in which we can infuse heterogeneous cell populations and design an appropriate layout of biomaterial components. With this tool, we can expand our knowledge toward understanding why disease-related cellular abnormalities occur (including those related to metabolic activity, migration, growth factor release and mode of cell death) and then employ therapeutic

Table 2. Alamar blue® metabolic activity assay

Culture time	Time (min)	Absolute fluorescence value (SEM) SF-DMEM	0.27 mM EDTA	0.53 mM EDTA	1.06 mM EDTA
4 h	0	1962.4 (±18.8)	1886.6 (±65.2)	1801.5 (±41.3)	1810.7 (±46.3)
	15	1920.1 (±20.6)	1801.5 (±8.8)	1780.5 (±17.1)	1753.1 (±30.0)
	30	1784.7 (±15.4)	1802.8 (±14.3)	1660.2 (±35.8)	1654.6 (±39.1)
24 h	0	4242.8 (±37.7)	4194.9 (±47.9)	4276.3 (±28.3)	4212.3 (±60.0)
	15	3615.5 (±59.8)	3681.2 (±53.1)	3351.0 (±44.0)	3363.2 (±72.2)
	30	3608.9 (±71.0)	3542.1 (±55.6)	3542.7 (±109.7)	3411.0 (±83.1)

Numerical values of absolute fluorescence followed by standard error of the mean (SEM). Values are presented in graphical form in Figure 5A, B.

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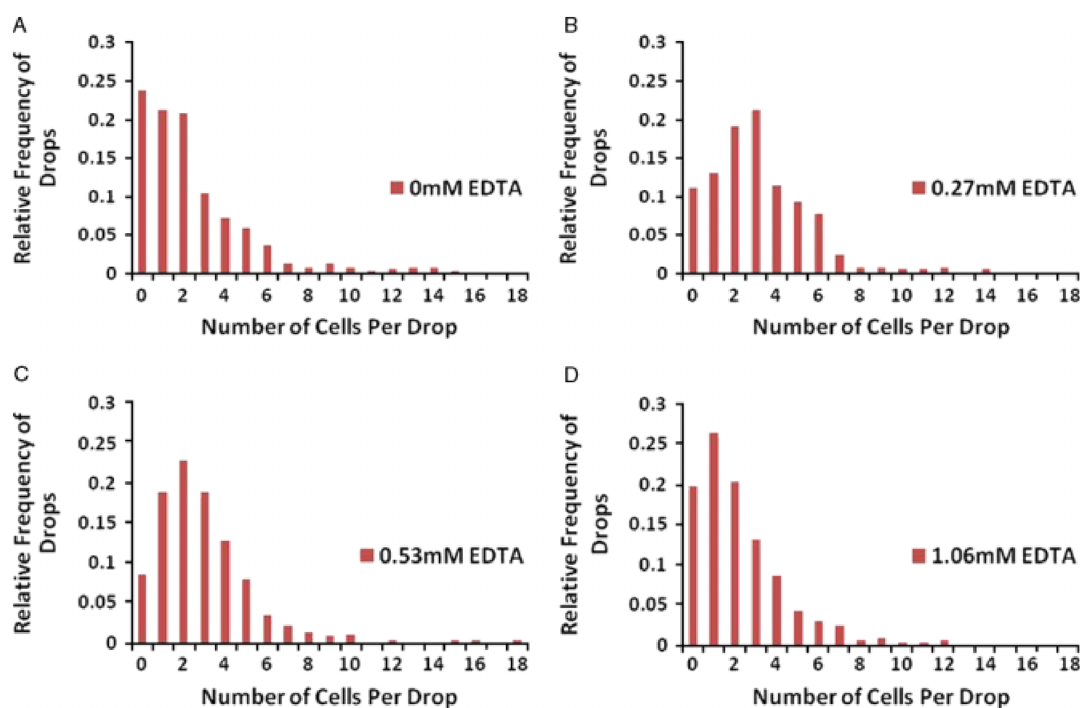

Figure 6. Frequency histograms illustrating the number of 4TO7 cells per drop using the defined bio-inks. Histograms were assessed for the presence of trends that could suggest more homogeneous printing in the presence of EDTA

Table 3. Proportion of successful and unsuccessful drops printed using each of the bio-ink cell suspensions

Solution	Success	Failure
0 mM EDTA	197/375 = 0.53	178/375 = 0.47
0.27 mM EDTA	201/375 = 0.54	174/375 = 0.46
0.53 mM EDTA	228/375 = 0.61	147/375 = 0.39
1.06 mM EDTA	224/375 = 0.60	151/375 = 0.40

Success was defined as 1, 2 or 3 cells/drop, while failure was defined as 0 cells or >3 cells/drop. The greater proportion of successful drops was printed using a 4T07 cell suspension containing 0.53 mM EDTA.

treatments to our 3D systems of interest. However, in order to use inkjet printing to move closer to this goal, the printer must be able to eject millions of cells out of a single nozzle without failing.

5. Conclusions

The salts in standard cell culture media create deposits inside a printhead, which causes the printhead to fail prematurely, well short of printing 1 million cells. The results presented in this paper show that EDTA may be added to cell culture media in order to prevent nozzle failure and to facilitate the high throughput production of 3D complex tissues. The 0.53 mM solution of EDTA was the best solution tested, because it prevented nozzle failure for the duration of the study, had the greatest proportion of successful drops printed in the cell ejection study, and had no statistically significant toxic effects when cells were suspended in bio-ink solutions for zero minutes and maintained in culture for 24 h. An approximate concentration of 0.53 mM EDTA in HBSS should be incorporated into all bioprinting cell suspension solutions that could trigger salt crystal formation leading to printhead failure. Far more emphasis must be placed on bioprinting details and methodologies in order for it to be a clinically relevant tool, capable of fabricating 3D complex tissues in high throughput. The nozzle fouling that can limit the ejection of high volumes of cells is one such detail.

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