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The Use of Inkjet Printing and Thermal Phase Change Inks to Create Sacrificial Prevascular Networks

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Abstract

Within Tissue Engineering one of the greatest challenges is providing cells in an engineered tissue with nutrients and oxygen via blood or serum. The process, otherwise known as perfusion, brings these vital supplies to within 100-200 microns of all cells by means of the vascular network. If perfusion is insufficient cells begin to die (necrosis) which effectively self-regulates cell growth to within 150-200 micron of a diffusive oxygen source and limits the useful thickness of cellularised tissue that can be engineered in vitro. A spectrum of research approaches ranging from cell-based therapies that attempt to induce spontaneous budding of capillary structures to scaffold based therapies that provide a spatial prevascular structure to guide the formation of the vascular network are currently being investigated. This contribution discusses the research that has been undertaken to indirectly fabricate prevascular scaffolds. Inkjet printing is used to make sacrificial three-dimensional microstructures that are initially employed in the production of the biomimetic 3D microfluidic devices. The process is complimented by the use of low temperature thermal phase change materials e.g. Hexadecane and Octadecane, as inks that enable structurally sound three-dimensional microstructures to be constructed, and then sacrificed without damage to the biocompatible materials (gelatin with a variety of cross linking mechanisms) used for the prevascular scaffold.

Introduction

The term Tissue Engineering (T.E.), first used by Langer and Vacanti (1993) describes the use of a combination of cells, engineered scaffolds and suitable biochemical/ physiochemical cues to improve or replace impaired biological functions. Within T.E. one of the greatest challenges is providing cells in an engineered tissue with nutrients and oxygen via blood or serum (Auger et al. 2013). The process, otherwise known as 'perfusion', brings these vital supplies to within 100-200 microns of all cells by means of the vascular network. If 'perfusion' is insufficient cells begin to die (necrosis) which effectively self-regulates cell growth to within 150 – 200 micron of a diffusive oxygen source (Folkman. 1973) and limits the useful thickness of cellularised tissue that can be engineered in vitro.

To date applications for T.E. solutions have been limited to tissues in thin sheet-like formats for endothelia/ epithelia applications such as the cornea, or avascular tissues such as cartilage. A spectrum of research approaches ranging from cell-based therapies that attempt to induce spontaneous 'budding' of capillary structures to scaffold based therapies that provide a spatial 'prevascular' structure to guide the formation of the vascular network are currently being investigated (Novosel et al. 2011).

The complexity and micro-scale proportions required for prevascular scaffolds together with the probable need for frequent design changes and small production runs for experimental research naturally suggests the use of Additive Manufacturing technologies. However the direct fabrication of a prevascular scaffold (with spatial requirements for capillary (lumen) diameters as low as 10 micron and capillary wall thicknesses of 1 micron) is soon confronted by the technological limitations of resolution and accuracy for many Additive Manufacturing processes (for a comprehensive overview see Table 2, Melchels et al. 2012).

Indirect fabrication is the process of forming a material by means of a mould, which may then be removed or sacrificed to leave the desired structure in place. While indirect fabrication is frequently used in T.E. (particularly to create a desired level of porosity in a T.E. scaffold to enhance cell attachment and/or enable media to perfuse a structure) and Inkjet printing (I.J.P.), when considered collectively has demonstrated a wide range of competencies that are of particular relevance to T.E. (Wilson & Boland 2003; Boland et al. 2003; Boland et al. 2007; Chang et al. 2011; Roth et al. 2004) the use of I.J.P as an indirect fabrication technique for microvasculature T.E. has been infrequent.

Sachlos et al advanced the method of using I.J.P. to deposit a sacrificial structure of proprietary build and support materials in the shape of microchannels with a minimum feature resolution of approximately 100 micron to fabricate a porous collagen scaffold that could be perfused by means of those microchannels (Sachlos. 2003). Pataky et al have successfully utilised IJP for directly depositing alginate 'bio-inks' (biocompatible hydrogels with highly controlled spreading and setting behaviour) to form T.E. scaffolds that included a network of bifurcating lumen-like voids, for the subsequent perfusion of the hydrogel (Pataky et al. 2011). The minimum feature dimension for the lumen like voids was also approximately 100 micron. Further to the work of Pataky et al, Christensen et al fabricated alginate structures using calcium chloride as a cross-linking agent and a buoyant support material (to enable IJP of overhanging structures) (Christensen et al. 2015). Although the minimum feature resolution achieved was one millimetre (wall thickness) and lumen diameters ranged from 3mm to 5mm the construct was successfully seeded with fibroblasts and cell viability was reported as 92% after 24 hours.

In spite of the sparsity of literature, I.J.P. is of particular interest for the fabrication of sacrificial pseudo-microvascular structures when it is considered that the feature resolution range for drop-on-demand Inkjet printing (20 -100 micron) corresponds well with the lumen sizes present in microvasculature (10 – 100 micron). Furthermore, inkjet output is circular, which is ideal for lumen-like sacrificial microstructures. In this case I.J.P. technology is used in conjunction with thermal phase change inks to make sacrificial lumen-like three-dimensional microstructures that are sacrificed once encased within a suitable biomaterial, thus leaving capillary sized and shaped voids within the prevascular scaffolds. Although the long-term objective of the research is centred on providing an engineered spatial prevascular scaffold for microvasculature for inclusion within T. E. organ scaffolds,

in the interim more immediate research goals have been identified.

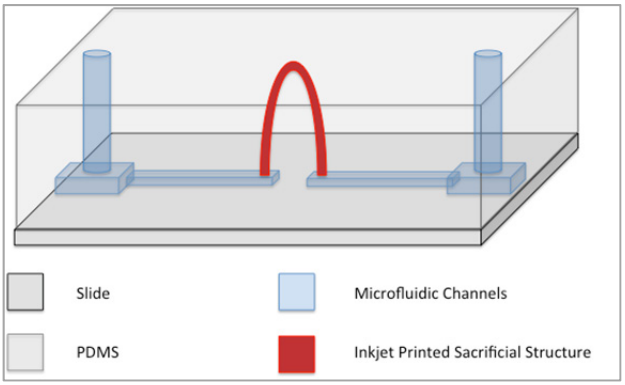

Figure 1: *Microfluidic device with IJP sacrificial structure*

The initial focus is on establishing the appropriate I.J.P. setup and printing parameters to create three-dimensional sacrificial microstructures. In the first instance these 3D structures are combined with basic 2.5D microfluidic channels (see Figure 1 above), then embedded in Polydimethylsiloxane (PDMS). The resulting microfluidic device represents an improvement on current microfluidic technology in terms of the minimum conduit diameters that can be achieved in 3D as well as the degree of tortuosity that may be achieved within those conduits (Hasan, A. et al, 2014). This advance may prove useful in analysing aspects of flow through conduits that reproduce both the dimensions and tortuosity found in microvasculature.

Further research will progress to embedding the sacrificial microstructures within cross-linked gelatin (a biocompatible material) to enable flow to be assessed in conjunction with a material that can be 'tuned' (by manipulation of the proportions of gelatin and cross-linking material) to match the mechanical properties of the relevant human tissue that the pseudo-microvasculature is flowing through.

Materials and Methods

Inkjet printing setup

Initial work to establish the appropriate I.J.P. setup and printing parameters was conducted using a MicroFab "Drop on Demand" printer with JetLab software (MicroFab Technologies, Version 6.3, build 4.0.18.3011). This was combined with the MicroFab MJ-SF style drop-on-demand heated single jet-dispensing device that maintains thermal phase change inks at suitable temperatures prior to dispensing, and complimented with a PH-04a Polymer Jet™ high-temperature, drop-on-demand print head (60 micron aperture). For hexadecane, background pressure on the ink reservoir was adjusted to provide sufficient pressure so that a stable hemisphere of 'ink' was visible at the head of the inkjet nozzle. 'Dwell' and 'echo' settings of 70 V and -20 V caused sufficiently sized and properly timed pressure waves via contraction of the piezo electric crystal in the ink jet nozzle to cause well defined single droplets of ink to be ejected from the ink jet aperture.

A Peltier thermoelectric cooler module heatsink and fan assembly controlled with a TEC temperature controller (Electron Dynamics Ltd, Southampton, U.K.) unit was fitted to the printing platform of the MicroFab printer to enhance the degree of undercooling at the substrate level so that the printed phase change ink droplets would solidify on contact with the substrate.

Results from the first experimental series using hexadecane showed that the range of suitable printing parameters was limited (particularly with regard to frequency and attainable height of the microstructures), in order to mitigate this problem a bespoke heated jet-dispensing device was designed and manufactured. These details appear in the results and discussion section below.

Thermal phase change inks

Paraffin wax (Sigma-Aldrich) has frequently been utilised as phase change ink for I.J.P., and is considered to be non-cytotoxic in the presence of cells (but prevents cell adhesion). Although sacrificial structures fabricated from Paraffin wax are suitable for immersion in PDMS, the melting point of paraffin wax of between 53 ^{0}C and 80 ^{0}C required for the removal of the structure is not suitable for all biocompatible polymers that may be considered for a vascular scaffold. Other wax–like substances have melting points that are more conducive for the use of the full range of biopolymers. As the material being considered for the T.E. vascular scaffold is gelatin cross-linked with either glutaraldehyde or genipin, Hexadecane and Octadecane (with melting points of 18^0 C and 28^0 C respectively, supplied by Sigma-Aldrich) were also considered suitable candidates for the phase change inks to be used in the I.J.P. process. All inks were filtered with a 5-micron filter prior to use.

Thermal gradient measurements

Temperature measurements were taken using a Signstek 6802 II dual channel digital thermometer with a 2 K-type thermocouple sensor probe (Signstek Ltd U.K.). At each measurement point the sensor was allowed to acclimatise for two minute, then the temperature range was observed for one minute. The median point of the range was then selected and recorded.

Fabrication of microfluidic devices

The 2.5 D basic sacrificial microfluidic channels were designed with 'Inkscape' software (open-source vector graphics editor), then converted into '*dxg.*' files and fabricated with a 'Graphtec Craft Robo Pro' vinyl cutter (Graphtec Corp. California U.S.A.) using KPMF 50 micron film thickness cast matt self-adhesive vinyl (KPMF Ltd. Newport, U.K.).

The 'negatives' of the microfluidic channels were removed from the backing film and mounted on glass slides. The 3D pseudo-vascular sacrificial microstructures were then printed in paraffin wax to connect the terminals of the microfluidic channels. The complete structure was then placed in a petri dish, and PDMS was carefully poured into the dish until the structure was submerged. The PDMS was then allowed to cure at room temperature for 48 hours. Once cured the PDMS was removed from the petri dish and glass slide (complete with the sacrificial vinyl microfluidic channels) before being trimmed to match the dimensions of the glass slide.

The PDMS structure and a secondary glass slide were then plasma treated using a 'Zepto' plasma system (Diener Electronic, Ebhausen, Germany). After treatment the PDMS was then mounted on the glass slide. Holes (1mm diameter) were punched into the end terminals of the microfluidic channels and matching diameter tubing was attached. In order to remove the residual paraffin wax structure from the PDMS, the complete microfluidic device was heated to 80 ^{0}C and hot water of a similar temperature was then forced through the microfluidic channels of the device by means of syringe under gentle manual pressure. On completion of the process, once the microfluidic device had cooled to room temperature coloured water was then applied to the microchannels by means of a

syringe under manual pressure. The microfluidic device was then examined using a stereo microscope (Model W30663-230, 3B Scientific, U.K.).

Results and Discussion

The seminal work of Gao and Sonin provides a comprehensive theoretical analysis for the dynamic and thermal aspects of deposition and solidification process of thermal phase change droplets in three dimensions (Gao & Sonin 1994). Although the materials used for their investigation were hard waxes it is posited that the resultant theory can be generalised to other phase change materials. Consequently the immediate objective of the initial experimental work was to identify an IJP setup and a set of printing parameters that would enable the timely and consistent fabrication of sacrificial wax structures that replicate three-dimensional bifurcating capillary structures.

Droplet frequency

Hexadecane was heated to 30 ^{0}C (melting point of 18 ^{0}C) and maintained at that temperature in the reservoir of the MicroFab MJ-SF style drop-on-demand heated single jet-dispensing device. The Peltier was set at 5 ^{0}C, two degrees above the dew point to prevent moisture formation at the substrate level, giving a theoretical maximum undercooling for the Hexadecane of 13 ^{0}C. The offset between the print head and the substrate on the z-axis was set at 5 mm. 30 droplet columns with 1mm spacing (giving a known distance for subsequent calculations) between column centres were fabricated with droplet deposition frequencies of 100, 10 and 1 Hz were tested (see Figure 2 below).

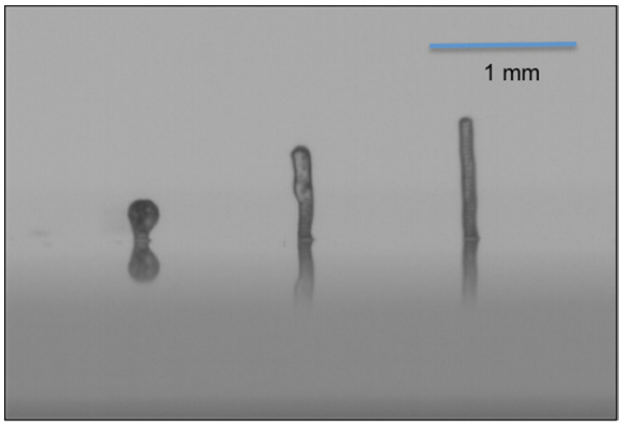

Figure 2: *30 droplet columns at 100, 10 and 1 Hz (left to right) at 1 mm spacing*

On contact with the temperature-controlled substrate, the droplet spread and hardened, generating a cylindrical disk. This was measured at 90 micron in diameter and 25 micron in height. The droplet volume was calculated at 160 pL giving a droplet diameter of 67 micron. The droplet size is consistent with the use of a 60-micron print nozzle. It was found that a rate of 1 Hz enabled the hexadecane droplet to solidify and cool sufficiently to absorb the thermal impact of the next droplet without distortion, absorbing the thermal energy of the arriving droplet without melting the substrate droplet, which lead to consistent column formation. Subsequent experiments examined the range of frequencies between 1 and 10 Hz and it was found that the maximum frequency associated with consistent column formation was 2 Hz.

Column Height

At this point Octadecane was selected in preference to Hexadecane due to its more favourable lab-handling characteristics. The ink was heated to 40 ^{0}C and columns of 10, 20, 40, 80 and 160 droplets each were printed on glass slides at room temperature (creating a 8 ^{0}C degree of undercooling) at a frequency of 1 Hz. at 1 mm spacing between column centres.

The results of the column height experiment (see Figure 3) indicated that a thermal gradient existed between the print head and substrate, which ensured that the degree of undercooling on an individual droplet decreased as proximity to the heated print head increased, resulting in the formation of bulbous heads at the top of the printed columns. Furthermore, as the offset between the substrate and print head was increased the accuracy of droplet deposition became less consistent due to the decrease in velocity of the droplet over the course of its flight, making it more susceptible to airflows within the IJP cabinet.

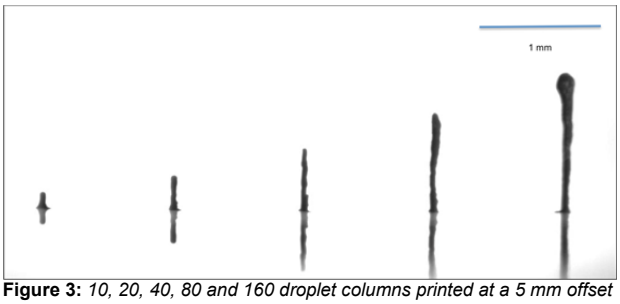

Figure 3: *10, 20, 40, 80 and 160 droplet columns printed at a 5 mm offset*

In order to address this issue, a script designed to maintain a constant offset between the top of the increasing printed column and the print head was written for the IJP setup. In theory, this would ensure that the deposited droplet was exposed to a constant degree of undercooling. However, limitations on the continuity of z-axis motion for the IJP meant that the frequency of droplet deposition became inconsistent, so that while a droplet was exposed to a constant degree of undercooling the duration of that undercooling (prior to the impact of the next arriving droplet) was significantly greater than expected.

Thermal gradient

The thermal gradient between the print head and substrate was then assessed at an offset of 5 mm, which had proven to be the greatest offset that provided acceptable consistency in the accuracy of droplet deposition. Temperatures of 5 ^{0}C for the substrate and 40 ^{0}C for the print head were selected in order to represent the maximum operational thermal range .The existence of the thermal gradient is clearly evident in Figure 4 below; indeed the effect of the heated print head on the thermal gradient extends to the surface of the substrate. However a zone where a degree of relatively consistent undercooling can be observed between x and y. which serves as a limitation on the height of microstructures that can be reliably fabricated.

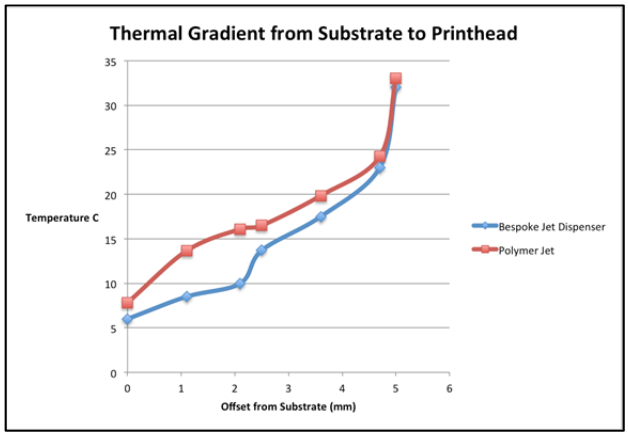

Figure 4: *Thermal gradient between substrate and print head*

As heat transfer by convection can be described as '$q = h\,A\,(T\,a - T\,b)$' (where q is the heat transferred per unit time, A is the area of the object, h is the heat transfer coefficient T_a is the object's surface temperature and Tb is the fluid temperature), it was hypothesised that a reduction in the convective surface area of the MicroFab MJ-SF style heated single jet-dispensing device would decrease the intensity of the thermal gradient (particularly near the print head) and consequently extend the zone of constant undercooling.

Design of revised jet dispensing device

The MicroFab MJ-SF has a total surface area of 0.036 m^2, whilst the conical area around the print head, which is the primary convective force on the thermal gradient, has a surface area of 0.004 m^2. The relatively low melting temperatures of the phase change inks being used enabled the use of an internal disposable cylindrical cartridge as a reservoir, which could be connected to a standard 60 micron MJ-A drop on demand print head by means of a standard 'luer' connection.

The introduction of reduced internal reservoir dimensions then allowed reduction in the dimensions of the encasing thermal jacket. Total surface area was reduced 64.2% to 0.013 m^2 and the conical surface area around the print head was reduced 23.6% to 0.003 m^2. Furthermore the angle of the conical area was increased thereby reducing the aggregate proximity of the convective face to the thermal gradient. The thermal gradient was assessed and is shown in Figure 4 above. A clear reduction in the effect of the heated print head on the thermal gradient is observable as is an extension in the zone where constant undercooling may be achieved, which enables larger microstructures to be consistently fabricated.

The bespoke heated single jet-dispensing device was then used to fabricate 3D arches of approximately 70-micron diameter (representative of capillary loops shown in Figure 5 below) onto 2.5 D microfluidic channels, which was then submerged in PDMS (see Materials and Methods). The ink-filled arches within the resulting microfluidic device are shown in Figure 6 below.

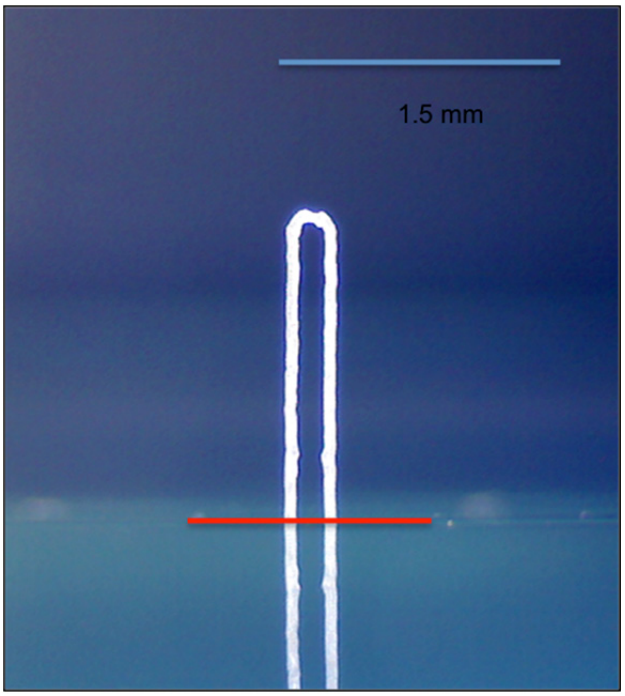

Figure 5: *IJP Pseudo-capillary arch*

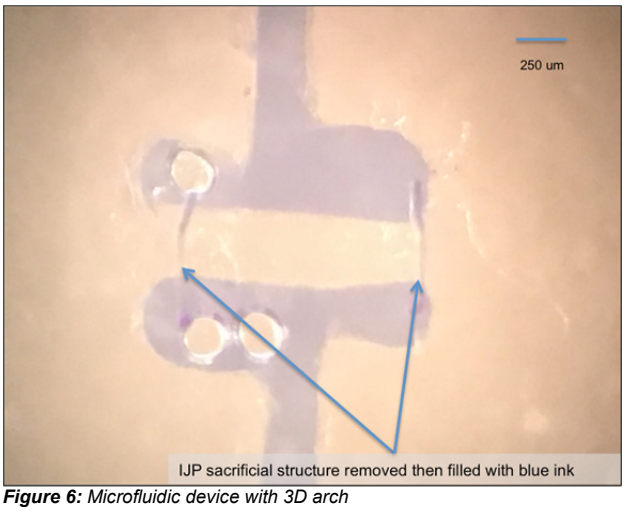

Figure 6: *Microfluidic device with 3D arch*

Conclusions

I.J.P. using phase change inks is shown to be a novel method for the fabrication of sacrificial pseudo-microvascular structures for use in microfluidic devices, whilst further research may also demonstrate its suitability for use in conjunction with T.E. scaffolds. This is of particular relevance when it is considered that the feature resolution range for drop-on-demand I.J.P. (20 -100 micron) corresponds well with the lumen sizes present in microvasculature (10 – 100 micron) enabling biomimetic microvascular scaffolds to be fabricated. Furthermore the phase change temperatures of the inks under consideration are compatible with many biopolymers.

Results from the first experimental series to establish the appropriate I.J.P. setup and printing parameters showed that the MJ-SF style drop-on-demand heated single jet-dispensing device was having a significant effect on the thermal gradient between the substrate and the heated print head. Consequently each ink droplet being dispensed was being exposed to a wide variation in undercooling (and subsequent phase change behaviour), which resulted in variable microstructure formation.

In order to mitigate the thermal effects of the MJ-SF heated dispenser on the thermal gradient a bespoke heated single jet-dispensing device that significantly reduced the total convective area of the device was designed and manufactured. Measurements of the thermal gradient showed a reduction of the effect of the heated print head on the thermal gradient and an extension in the zone where constant undercooling of the dispensed droplets may be achieved.

Use of the bespoke heated single jet-dispensing device facilitated the fabrication of 3D arches onto 2.5 D microfluidic channels, thereby providing the basis for a novel 3D microfluidic platform with 70-micron diameter conduits using a 60-micron aperture print head. As standard print heads are available with aperture sizes to a minimum of 20 micron are available, further reductions in conduit diameter may be achieved. Such a microfluidic platform may prove useful in analysing aspects of flow through conduits that reproduce both the dimensions and tortuosity found in human microvasculature.

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Leon Edney completed his Masters of Engineering in Industrial Design at Queen Mary University of London in 2010. He is currently working toward the completion of his PhD in Tissue Engineering and Regenerative Medicine within the group of Dr. Patrick J. Smith at the University Of Sheffield. His project focuses on the indirect manufacture of prevascular scaffolds for incorporation in dermal substitutes for wound and ulcer repair, and more specifically on the fabrication of three-dimensional (sacrificial) pseudo vascular microstructures for incorporation in biocompatible scaffolds.