Inkjet Printing of Microvascular Networks
Fei Zheng, Jason Wong* and Brian Derby
1School of Materials, University of Manchester, M13 9PL, UK;
2Division of Cell Matrix Biology & Regenerative Medicine, University of Manchester, M13 9PL, UK
Fei.zheng-3@postgrad.manchester.ac.uk

Introduction
Fabrication of viable vascular networks within engineered tissue remains one of the greatest challenges facing the fields of biomaterials and tissue engineering. Although many methods derived from microfluidic and bioprinting technologies have been developed, none combine the resolution, repeatability and hydrogel compatibility required to replicate and mimic biomimetic microvascular constructs incorporating hierarchical and bifurcating features. In this study, we present a novel fabrication method using high resolution inkjet printing to create high precision microvascular replicates embedded within a hydrogel matrix.

Methods
Two hydrogels with distinctive thermosensitivity were used as sacrificial and permanent materials, respectively. Pluronic F127, a synthetic triblock copolymer that liquefies when cooled below a critical temperature, was chosen to provide a temporary support before removal to generate hollow channels. Gelatin methacrylate (GelMA), a crosslinkable biomaterial, was used as a permanent matrix material and mixed with living cells at physiological temperature to cast around the printed patterns. By altering nozzle size, jetting voltage and drop spacing, channels with sizes ranging from 5 μm to 50 μm can be achieved. The final construct was injected with cell culture medium and endothelialized by seeding human umbilical vein endothelial cells (HUVECs).

Results and Discussion
Unlike traditional drop-on-demand (DOD) inkjet printing that uses acoustic force to eject ink, high resolution inkjet printing uses the electrostatic Taylor cone to generate droplets with volumes at the femtolitre scale (droplet diameters close to 1 μm). Electrostatic drop generation is compatible with highly concentrated inks of high viscosity (>10,000 cP), which is a particular advantage over traditional DOD technique especially when working with hydrogels. By regulating printing conditions and concentrations of the Pluronic aqueous solution, printed fluidic networks can be printed with dimensions similar to the capillary scale in living tissue. Figure 1 shows an optical image of hexagonal networks with feature size = 20 μm. Methyl blue solution was perfused in microchannels for visualization and size measurement. Further in vitro experiments with HUVECs and fibroblasts have been carried out with different channel arrangements.

Figure 1. Methyl blue solution filled hexagonal networks within crosslinked GelMA matrix with feature size = 20 μm.

Conclusions
In summary, we developed a novel approach for creating microvascular constructs with high spatial resolution on the capillary scale. Electrostatic inkjet printing has been used with two distinct thermosensitive hydrogels. This approach provides a possibility to fabricate sophisticated microvasculature at a scale similar to real capillaries for further researches in biofabrication and tissue engineering.
Multi-Nozzle Industrial Inojet toward high-throughput Bioprinting for 3D Tissue Engineering

R. Wenger, M. Mauron, F. Bircher, R. Nussbaumer
iPrint Institute, School of Engineering and Architecture of Fribourg, Member of the University of Applied Sciences of Western Switzerland, Switzerland

Introduction
Bioprinting, thanks to the unique flexibility and spatial accuracy it offers, has a promising future for the deposition of cells and extracellular matrices (ECM) toward the fabrication of physiologically relevant 3D tissue models. Most bioprinting platforms offer switching single-nozzle printheads. Such deposition systems are not best suited for the large-scale production of tissue models for pharmaceutical screening or grafting since their throughput is strongly limited. As experts in multi-nozzle inkjet technology, we (iPrint) hypothesized that industrial printheads allow for a reliable, fast, and therefore cost-effective production of 3D tissue models leading bioprinting toward industrialization. Industrial inkjet offers the control of hundreds of nozzles in parallel, allowing the individual tuning of each of them in order to jet drops in physiologically relevant dimensions (20-200pl). To test our hypothesis, we are investigating the reliability of the printing process, its throughput and its impact on cells.

Materials and Methods
A 3D Bioprinting platform, compatible with various printheads, was internally developed and set up. Multi-nozzle piezoelectric industrial printheads were selected based on their compliance with cell-loaded bioink deposition (nozzle diameter >30um, low dead volume, 128-256 nozzles, aqueous ink compatible). For this first series of tests, a Xaar 128/80L printhead was used. An ink supply to digitally control the purge and the meniscus pressure, while featuring a minimal dead volume, was set up. Dropoutwatching was implemented to visualize drops formation, characteristics (velocity, volume, shape) and stability of the jetting process. Bioinks based on culture media with viscosity modifiers were prepared and characterized (viscosity, density, dynamic surface tension) to achieve stable cell suspension while remaining liquid enough for inkjet printing (<20mPa*s). Human lung epithelial type II cells (A549) were cultivated and suspended in bioinks (3×10^6 cells/mL = 100% concentration) and their sedimentation rate was measured. 7mL of the cell-loaded bioinks were fed into the ink system and were kept there for up to 35min of alternating sequences of printing and settling at room temperature. Prior to each printing step, a short purge was implemented to free the nozzles from clogging. Batches of cell-loaded bioinks were produced by printing 75'000 lines (128 nozzles per line) at 1kHz as one big drop in a petri dish that was subsequently divided into n=3 wells for culture. To quantify the reliability of the process, cell concentration was measured after each batch printing. To measure the impact of the process on the cells, a viability test (Trypan Blue exclusion assays) was performed after 4 days in culture.

Results and Discussion
When using DMEM with 10%FBS as a bioink, suspended cell concentration in the printed batches dropped to 31% after 5min settling. Furthermore, large aggregates of cells were observed in the product from the purges. This indicates that the cells sedimented in the printhead during the settling time and were mostly ejected during the purge prior to printing. Increasing the viscosity of the bioink by adding 5% Ficoll PM400 led to an increase of the cell concentration to 81% after 5 minutes settling. This clearly indicates that the rheological properties of the bioink have a direct impact on the overall stability of the printing process. To assess the impact of the printing process on the cells, viability was measured after 4 days in culture. Trypan Blue exclusion assays were performed on the printed batches and compared to the “Gold Standard” of deposition by pipetting (Figure 1). All viabilities were over 85%, suggesting that the time spent in the printing system at room temperature does not have a great impact on the cell. The viability of the printed cells tends to be lower than the ones deposited by pipetting while remaining in an acceptable range. The addition of Ficoll also tends to slightly reduce the viability.

![Cell viability (%) vs. Deposition process and settling time](image)

Figure 1: Viability (n=3) of human lung epithelial type II cells (A549) suspended in bioinks after 4 days in culture as a function of the deposition process and time spent in the ink system

Conclusions
We showed that by tuning the rheological properties of the bioinks, the cell concentration over settling time could be increased. The cells printed with the industrial inkjet printhead showed viabilities of over 85% even after prolonged settling times in the ink system. Our study demonstrates that this technology indeed has the potential for a reliable cell deposition and has a low impact on their viability. These encouraging preliminary results support our hypothesis and provide a solid claim for a more thorough investigation of industrial inkjet for bioprinting. Further tests using different cell types, viscosity modifiers, surfactants, hydrogels and printheads will be performed to verify our hypothesis.
Fabrication of Acellular Biodegradable Small-Diameter Vascular Graft

Z. Liu, T. Gao, W. Sun
Bio-manufacturing Center, Department of Mechanical Engineering, Tsinghua University, Beijing, China

Introduction: Small-diameter vascular graft (OD<6mm) has a great clinical need and at present there are various approaches to generate tissue-engineered vascular graft. The acellular biodegradable vascular graft is one of the best solutions, where the material and the structure of the scaffold take a very important place. In this study, in order to make the scaffolds have better mechanical and biocompatibility properties which are almost the same as real blood vessels, suitable material was applied and the structure that mimic real three layer blood vessels was designed through tissue engineering. After the implantation of vascular graft, the scaffold would replace damaged blood vessels and help to generate new blood vessels.

Materials and Methods: A certain type of polyurethane (PU) was chosen as our final scaffold material from several biomaterials according to the results of mechanical test, cell cultivation experiment, blood compatibility experiment and platelet adhesion experiment. The PU was fabricated, which has soft segments of PCL-PEG-PCL specific block, hard segments of MDI and a certain component ratio, in order to get suitable mechanical property and degradability. In order to mimic the structure of three-layer real vessels, a kind of three-layer tube was designed whose each layer has different structures. Water-soluble PVA sticks were printed with a diameter of 5mm and were coated with PU solution layer by layer. After the solution dried, the PVA sticks were dissolved and scaffolds were obtained. In order to estimate whether the vascular grafts could be applied on the animal experiment, these scaffolds were tested by different criteria like tensile property, radial compliance, bursting strength and suture retention strength.

Results and Discussion: PU scaffolds with high quality and uniform thickness were successfully fabricated. The radial compliance decreases as the wall thickness increases and become stable in the end. The vascular grafts with a wall thickness of 0.45-0.55mm were chose to be used in the animal experiment. The radial compliance is around 1%80-120mmHg, the suture retention strength is beyond 6000N/cm² and its burst strength is beyond 200KPa, far more than the human body blood pressure. All the mechanical properties satisfied the requirements of vascular graft. 6 vascular grafts have been applied in the animal experiment, only one of them was blocked within 1 month and 3 of them maintained good condition at 6 month after implantation.

Conclusion: In this study, the biodegradable PU was chosen and fabricated to be used as the material in the fabrication of three-layer blood vessel scaffold. The vascular grafts have been successfully fabricated and have good mechanical properties like radial compliance, bursting strength and suture retention strength, which meet the requirements of the clinic. These vascular grafts have been applied on the animal experiment, which has good results 6 months after implantation and shows that our vascular grafts are suitable for implantation.
3D Bio-printing Method for geometry-controllable film as artificial biosynthetic cornea

Qian Xue\textsuperscript{a,b}, Hanyi Hu\textsuperscript{a,d}, Bin Zhang\textsuperscript{b}, Liang Ma\textsuperscript{b}, Yichen Luo\textsuperscript{b}, Lei Gao\textsuperscript{b}, Huayong Yang\textsuperscript{a,b,*}
a. State Key Laboratory of Fluid Power & Mechatronic Systems, Zhejiang University, Hangzhou (310058), People’s Republic of China
b. College of Mechanical Engineering, Zhejiang University, Hangzhou (310058), People’s Republic of China
c. College of Medicine, Zhejiang University, Hangzhou (310058), People’s Republic of China
d. Department of Ophthalmology, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou (310058), People’s Republic of China

Contact email address: yhy@zju.edu.cn

Statement of Purpose

The cornea acts as an optical interface to contribute the vast majority of the total refractive power. The only treatment for visual restoration of corneal blind patients is corneal transplantation[1,2]. However, the shortage of donor cornea is a global issue. There is estimated 12.7 million patients in the waiting list\textsuperscript{[3]} which promotes the R&D of alternative solutions. The existing corneal substitutes mainly rely on conventional tissue engineering manufacturing methods, such as molding, dry casting, thermo responsive cell culture, etc that only support the preparation of flat or curved films with uncontrollable curvature which fail to reconstruct the corneal optical function. We proposed to construct curved films with designed geometric features to achieve a certain optical functional restoration of corneal substitute with 3D bioprinting. In order to obtain geometry-controllable films, the study of 3D bioprinted film with different materials proportion and printing process was performed.

Materials and Methods

The key geometric parameters and other dominant factors of maintaining corneal visual function and physiology were studied by building mathematical model of cornea. According to the topography of natural cornea, accurately customized artificial cornea was modelled through computer-aided design. The film for corneal substitute was prepared by combining digital light processing (DLP) and extrusion bioprinting. By changing the percent of calcium alginate in gelatin-calcium alginate hydrogel, the quality and integrity of printed film was improved. The printing process was designed and optimized based on the model. The calcium alginate-gelatin hydrogel combined with human corneal epithelial cells (HCECs) was prepared as bioink. The 3D live/dead fluorescence imaging was obtained with confocal microscope through scanning the z-axis of the printed film at 3 h. The 3D printed HCECs-laden film with a certain thickness and curvature as pre-regeneration scaffold for refractive purposes was manufactured by combined 3D bioprinting method we developed.

Results and Discussion

In the \textit{in vitro} study, the geometric, mechanical and optical properties of the printed curved film were evaluated. The surface quality and structural integrity was improved significantly by adjusting material proportion and printing process. There are remarkable improvements on the formability of the printed film through modifying the printing platform and motion trajectory of the extrusion nozzle. The centre thickness of the films in equilibrium hydrated state was evaluated around 180-200\textmu m. SEM analysis of the printed acellular film demonstrated the integrity and good surface roughness as a smooth optical interface without visible step pattern or crack. The image under the film is clearly visible. The 3D live/dead stain images demonstrated that the HCECs distributed uniformly in the printed film with extraordinary high survival rates of more than 90 % at 3h after printing. The curved geometric characteristic of the film is clear and smooth. The results make the printed films suitable candidates using as a corneal equivalent.

Conclusions

Our studies demonstrate that, owing to the structural controllability and other advantages, the 3D bioprinting is a powerful tool for geometry-controllable artificial biosynthetic cornea with refractive power and could serve as pre-scaffold for corneal regeneration and replacement as donor cornea.

![Image](image_url)

Figure 1. (a, b) Examples of 3D printed calcium alginate-gelatin acellular film in equilibrium hydrated state. (scale bar, 5mm) (c) The thickness of printed film was measured in equilibrium hydrated state using scanning acoustic microscope system (the horizontal scale bar, 3mm); the vertical scale unit: transit time. (d, e, f) Micrograph of the film with different printing parameters (scale bar, 500 \textmu m) (g) Printed film with good transparency (scale bar, 5mm). (h) Top surface view on SEM image of printed film (scale bar, 250 \textmu m). (i, j) Representative 3D live/dead stained confocal images of printed HCECs-laden film.

Reference:

Novel Gelatin Methacrylate-Hyaluronic Acid-Chondroitin Sulfate and Polycaprolactone Composites for Meniscus Regeneration

C.A. Murphy1, G.M. Cummiskey2, A.K. Garg3, M.N. Collins1

1Stokes Laboratories, Bernal Institute, School of Engineering, University of Limerick
23D Printing Center of Excellence, Global Supply Chain, Johnson and Johnson, 152-160 Pearse Street, Trinity College Dublin
3Manufacturing Technology and Innovation Global Supply Chain, Johnson & Johnson, 430, Route 22 East Bridgewater, NJ 08007

Introduction: The meniscus is a complex cartilaginous structure located within the knee joint and plays a vital role in knee joint functionality associated with load distribution and shock absorption. The meniscus tissue lacks a blood supply to support repair once damaged. This poor healing capability commonly leads to progressive degeneration resulting in the early onset of osteoarthritis. Therefore, there is a pressing need to develop novel approaches for meniscus regeneration to restore the functionality. To date, tissue engineered scaffolds typically lack the complex architecture of the native tissue and are yet to yield long-term reliable results [1]. The aim of this study is twofold, firstly to create a 3D scaffold with an architecture replicating the native tissue organisation displaying mechanical properties similar to the native meniscus. To achieve this goal, bioprinting technology has been utilised to produce high-resolution polycaprolactone (PCL) scaffolds with a complex tailored internal 3D microstructure. The second aim is to develop a bioink which is capable of being printed within the support scaffold. It is expected that the bioink will mimic the natural extracellular matrix of cartilage allowing the promotion of cell ingrowth and proliferation. This novel bioink is composed of photo-crosslinkable gelatin methacrylate (GeMA) blended with chondroitin sulfate (CS) and hyaluronic acid (HA).

Materials and Methods: PCL architectures were produced using a Biobot 2 printer (Allevi, USA). Printing parameters were optimised and constructs were mechanically characterised varying fibre spacing (n=5) and number of offsets (n=3) to maximise mechanical properties. PCL scaffolds were printed with optimal fibre spacing to achieve the complex circumferential architecture of the native meniscus and to provide a structural support for the bioink. To assess the effects of the addition of HA and CS to GelMA, the following groups were analysed GelMA 10% (w/v), GelMA 10%-HA 2%, GelMA 10%-CS 1% and GelMA 10%-HA 2%-CS 1%. Firstly, the rheological properties of each group were analysed to access the bioink’s printability (n=3). Furthermore, a photo initiator lithiumphenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was added to each group at a concentration of 0.5% w/v. The blends were cast into custom made Teflon moulds (6mm x 2mm) and cross-linked under blue light. The gels were subsequently analysed for swell ratio (n=4) and compressive properties (n=4). Each gel combination was seeded with fibroblast cells and cell viability and proliferation were analysed utilising Alamar Blue (n=4), DNA assay (Hoechst, 33258) (n=4) and LIVE/DEAD viability assay (n=4). Finally, composite constructs were printed with GelMA-HA-CS alternated with PCL. Channels were left between the GeMA-HA-CS and PCL fibres to allow cells to infiltrate the scaffold once implanted.

Results and Discussion: Characterisation of the bovine meniscus tissue elucidated the collagen fibre orientation of the native meniscus (Fig 1B), which allowed for the subsequent design of a 3D scaffold with predominant circumferential fibers and radial tie fibers replicating the tissue organisation of the native meniscus (Fig 1D).

![Figure 1](image)

Figure 1: Printed circumferential PCL scaffold inspired by the native structure A) Illustration of the native meniscus internal structure, B) native collagen fiber orientation under polarized light microscopy, C) 3D design of scaffold with circumferential fibers (orange arrow) and radial tie fibers (green arrow), D) 3D printed circumferential scaffold under optical microscope.

With regards the bioink, it was found that the incorporation of HA to GelMA allowed for the controlled deposition of the hydrogel strands by increasing the viscosity of the bioink. The addition of both HA and CS is expected to lead to increased cell viability compared to GelMA alone, displaying a higher DNA content after 7 days in culture. Overall the addition of HA and CS to GelMA is predicted to increase the swell ratio of the bioink and slightly decreased the compressive modulus prior to cell seeding. However, in terms of cell behaviour the optimum hydrogel combination is expected to be GelMA-HA-CS.

Conclusion: From this study a promising strategy for meniscus tissue engineering will be demonstrated, as the scaffold will provide a novel biomimetic architecture and environment to promote regeneration of functional components of the meniscus extracellular matrix.

Acknowledgements: Johnson and Johnson, 3D Printing Center of Excellence and the Irish Research Council (Project ID- EPSPG/2015/93).

Bioprinting of Cartilage Tissue with an Advanced Enzymatically Crosslinkable Bioink

Philipp Fisch1, Nicolas Broguiere1, Sergio Finkielstien2, Thomas Linder1, Marcy Zenobi-Wong1
1 Tissue Engineering & Biofabrication Laboratory, Institute for Biomechanics, ETH Zürich, 2 Marine Polymer Technologies, Inc., 3 HNO Luzerner Kantonspszital
philipp.fisch@hest.ethz.ch

Statement of Purpose: We developed a novel bioink based on an enzymatically crosslinkable hydrogel and thereby introduced a novel way to crosslink bioinks. The bioink is based on the modification of hyaluronan (HA) with transglutaminase (TG) substrate peptides, referred to as HA-TG.[1] Crosslinking of HA-TG occurs in the presence of activated factor XIII (FXIII), an enzyme belonging to the transglutaminase family, being responsible for blood coagulation. As FXIII is naturally represented in the body, the crosslinking mechanism is fully biocompatible.[2] The material has been used as an injectable system and here we describe its translation to the field of bioprinting and in particular to the field of cartilage tissue engineering. To be able to crosslink the material post-printing, we developed a protocol to activate the enzyme post-printing: FXIII is converted from itszymogen to its enzymatic form by the addition of thrombin which cleaves the activation peptide of FXIII. Then, through the addition of calcium ions (Ca2+), the two β subunits of FXIII dissociate from the two α subunits of FXIII (activated FXIII) enabling the enzyme to covalently crosslink glutamine and lysine residues.[3] To render HATG printable, first we show that HATG mixed with thrombin and FXIII does not crosslink without the addition of Ca2+ and that the crosslinking can be triggered by the external addition of Ca2+. Secondly, to render HATG into a shear thinning bioink with good printability, nanofibers were added to the bioink composition. Nanofibers, such as cellulose nanofibers (CNF), have been shown to make polymeric solutions printable while maintaining cell viability.[4] To evaluate the potential of this novel bioink for cartilage engineering applications, human auricular chondrocytes (hAUR) were encapsulated and bioprinted with it. Development of these samples was followed up for 2 months performing viability, compression and indentation tests as well as histological analysis. Samples were analyzed extensively in vitro and are currently being evaluated in vivo in a subcutaneous nude rat model.

Methods: HATG was synthesized using high molecular weight HA and a degree of substitution of approximately 10% was achieved. Cellulose nanofibers (CNF, Cellink) and poly-(N-acetyl glucosamine) nanofibers (sNAG, Marine Polymer Technologies) were combined with HA-TG. Rheological measurements were performed using an Anton Paar MCR 301 rheometer with a PP20 probe on which crosslinking, shear thinning, and shear recovery tests were performed. Bioprinting was performed utilizing the Biofactory bioprinter (regenHU). 10 million hAUR/mL, passage 2 for casting and passage 3 for bioprinting, were mixed with the bioinks. In total 4 different donors were used. Viability was assessed at day 1, 7 and 21 using Calcein AM and propidium iodide. Samples were imaged under a two-photon microscope (Leica SP8 MP). Compression tests were performed at day 1, 7, 21 and 63 using a TA.XTPlus system (Stable Micro Systems). Histological analysis and Indentation tests (Bioindenter, UNITH Bi, Anton Paar) were performed after 21 and 63 days and compared to acellular samples. Bioprinted samples were implanted in the back of 9 weeks old nude rats (RNU rat, Charles River) and will be analyzed after 1, 3 and 6 months and compared to in vitro samples.

Results: Crosslinking kinetics of HATG-FXIII-thrombin showed no change in storage modulus in the absence of Ca2+, but a strong increase in storage modulus was observed upon addition of external Ca2+ proving the working principle of the developed protocol. Two bioinks could be identified based on shear thinning and shear recovery tests, one supplemented with CNF fibrils, the other with sNAG fibrils. Printing trials showed good printability without clogging due to crosslinking of the bioink. Crosslinked samples were flexible, soft and could be handled well (Figure 1). Viability in casted samples was above 90% for all time points and both bioinks. In bioprinted samples viability was at 70% for the CNF and 80% for the sNAG bioink at day 1 which increased to 95% after 7 days. Compression tests revealed a significant increase in compressive modulus in casted samples from 2 kPa to above 100 kPa after 21 and above 200 kPa after 63 days in culture. A similar trend was observed in bioprinted sNAG samples. In contrast, bioprinted CNF samples did not show such an increase. Along with the strengthening process, histological results revealed a significant increase in glycosaminoglycan content over the timeframe of 21 and 63 days. Nude rats tolerated the implantation of bioprinted samples well and did not show any rejection of the grafts.

Figure 1. External ear printed with the two developed bioinks. Left: post printing, right: post crosslinking.

Conclusion: HATG is a fully biocompatible hydrogel applicable to the field of bioprinting. The bioinks developed here showed an outstanding increase in compressive strength and matrix deposition over time, making them ideal candidates for cartilage engineering applications.