BT5130 - Tissue Engineering - End-semester Examination

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Question 1 - "Development of highly functional bioengineered human liver with perfusable vasculature" by Kim *et al.* [1]

(A) Describe the principle behind the process for developing the anti-CD31 aptamer that specifically binds to endothelial cells. How was the specificity confirmed?

In this paper, the authors try to establish a bioartificial liver from decellularized scaffolds. Liver, being a vascular organ, would require complete re-endothelialization in order to achieve the necessary vasculature. The anti-CD31 antibody (aptamer) was chosen so that it could bind to the ECs with high specificity, despite the presence of various coating agents that bind to EC with low-medium specificity. The principle behind the design of anti-CD31 aptamer is based on **SELEX** (systematic evolution of ligands by exponential enrichment). SELEX is a combinatorial technique that designs oligonucleotides (aptamers) that will specifically bind to ligand(s). An oligonucleotide library is first constructed with fully to partially random strands of certain length flanked by regions recognized for PCR amplification. This library of aptamers is incubated with the EC population. The unbound aptamers (not specific) were removed by washing while the bound aptamers were released and amplified by RT-PCR and this process is repeated. Over subsequent SELEX cycles, a population of aptamers with high specificity to the ligand (CD31 protein on ECs) will be generated [2]. Confirmation of specificity was done by two methods. First, incubation of the aptamers (at different concentrations) with hUVECs and other cell lines that do not express CD31, such as HepG2 cells and MSCs. It was proven that the aptamer did not bind with the latter group of cells even at higher concentration. Second, immunostaining analyses suggested that anti-CD31 aptamer always binds with CD31+hUVECs but not HepG2 cells or MSCs.

(B) What was the basis for the authors' hypothesis that aptamers would show an efficiency comparable to antibodies? Identify at least 1 application where this has been demonstrated earlier.

Nucleic acid aptamers are usually considered as alternatives to antibodies owing to their low immunogenicity and strong binding affinity for their conjugate proteins. The cost for production of/synthesizing these aptamers and modifications to these strands can also be achieved easily, hence proving themselves as candidates with efficiency comparable to that of native antibodies. This was also

proved when they tested the specificity of the aptamers (with antibodies as controls) to the CD31 protein and observed very similar results for the aptamers and antibodies. Since aptamers have such high specificity, they even replace antibodies in medical diagnosis and treatments. This has been demonstrated earlier by Shahdordizadeh et al. [3] where aptamers have been employed for targeted drug delivery systems in breast cancer. This review article illustrates the development of a variety of aptamers. Here are a few examples – (i) A DNA aptamer that binds specifically to HER2 (important biomarker and therapeutic target for breast cancer) was developed for imaging [4]. (ii) An RNA aptamer that binds to HER2 was designed. RNA aptamers are susceptible to RNAse digestion but can form a better 3D conformation than DNA aptamer. Hence functional groups were attached at the end of the RNA aptamer to stabilize it and safeguard from RNAse activity [5].

(C) Describe the experimental techniques used for recellularization of the decellularized rat liver scaffold. Also, explain how this recellularization was confirmed.

Two modes of cell delivery (parenchymal & non-parenchymal) are adopted in this study. Experimental techniques: After decellularization, HepG2 cells are delivered to the parenchymal space via bile duct and are maintained within the bioreactor. At day 2, HepG2 cells (same amount) are delivered to the parenchymal space and cultured until day 14. Until day 14, the constructs were cultured in DMEM high glucose media-FBS-antibiotics. At day 15, GFDA-labelled hUVECs and MSCs (ratio 2:1) are delivered to the vessel lumen via the portal vein while injecting LX2 cells via bile duct at the same time. The medium was replaced by EGM2-FBS-antibiotics. **Recellularization was confirmed by immunostaining.** At day 22, picrosirius red staining suggested that cells were spread across the construct. The CFA-positive ECs in anti-CD31 aptamer-coated VBHL constructs (APT-VBHL) were specifically aligned along the vascular channels while the ECs in VBHL constructs without coating (control) appeared as non-aligned structures. Immunostaining of albumin suggested that HepG2 cells in APT-VBHL constructs were distributed in the parenchyma and αSMA-positive cells (for MSCs) were present around the perivascular region of the anti-CD31 aptamer-coated vessels and interconnected with ECs, thus displaying liver-like recellularization.

(D) What is the Akt signalling pathway and how is it involved in angiogenesis?

AKT signalling is an intracellular pathway that acts based on extracellular signals and is necessary for metabolism, cell growth, proliferation, vascular tone and especially potentiates angiogenesis. This pathway involves various key protein kinases such as RTKs, ATKs (or protein kinase B), PI3K and

complex phosphate molecules such as PIP2 and PIP3. The AKT signalling pathway is involved in angiogenesis by regulating the NO signalling in ECs [6]. NO synthesis is regulated by the enzyme NOS. eNOS is an isoform of NOS and it is crucial in VEGF-induced angiogenesis and vascular permeability. VEGF can induce NO production, which is attenuated by PI3K inhibitors via phosphorylating eNOS by AKT. Increased NO or VEGF (both dependent on Akt signalling) subsequently induces angiogenesis. In this study, shear stress in ECs activates the β1 and β3 subunits of integrin which binds to the ECM and consequently integrin-associated adhesion induces phosphorylation of AKT at Ser473. This further activates the AKT signalling pathways which initiates angiogenesis (even under mechanical stress).

(E) What is the principle of qRT-PCR? How was this tool used in this paper?

Quantitative Reverse transcription Polymerase Chain Reaction (qRT-PCR) is a technique that quantifies the amount of nucleic acid and works by combining the process of reverse transcription and the conventional PCR. Reverse transcription is the process of converting RNA to cDNA with the aid of the enzyme reverse transcriptase. This process is conventionally used by certain viruses to replicate their genome content, once inside the host body. The quantification of the amplification during PCR is monitored by fluorescently labelling which enables the collection of data. PCR works under the principle that the dsDNA strand gets denatured at high temperature and anneals based on the Watson-Crick pairing when the temperature is lowered. qRT-PCR can either be done in one-step or a two-step assay. The one-step assay combines reverse transcription and PCR in a single tube while in a two-step assay RT and PCR are done in separate tubes with optimized conditions for each separately [7]. In this article, qRT-PCR was used as a tool for the quantification of various mRNAs in different conditions.

- *ITGB1, ITGB3*, Integrin β3 and *NOS3, VE-Cadherin, CLDN5* (Higher when shear stress is applied to APT and Abt-coated groups than static and uncoated HUVECs)
- Fibrosis related genes (mRNA) - *αSma, Vimentin, Tgf-β1* and *Timp1* (Decrease in the APT implanted group vs Sham (only surgery) in the recipient rat livers.)

(F) How was the functionality of the bioengineered liver evaluated? Describe the in vitro and in vivo studies used for confirming this.

The vascularity of the bioengineered liver tissues is measured to check if the functionality is restored.

In vitro – The vascular structural integrity is tested by the FITC-Dextran perfusion Assay where a significant increase was observed in intravascular dextran levels in the APT-coated constructs.

Upregulation of junctional marker genes and ZO-1 expression indicating tightly connected vascular structures and restoration of endothelial barrier function. Quantification of angiogenic molecules (secreted NO, VEGF, NO3 expression). Upregulation of hepatocyte markers (albumin, CYP2E1, CK19 and AFP) which can be confirmed with histological analysis, Periodic acid-schiff (PAS) staining which reports the presence of hepatocytes in high levels, ELISA analysis which reports secretion of albumin and urea (typical features of hepatocytes). It was also ensured that APT-VBHL constructs showed increase in viability of cells and decreased apoptosis with respect to other cells, confirming that the bioengineered system is stable and active.

In vivo (**Before transplantation** of the constructs, to check for reperfusion) – The APT-VBHL constructs exhibited intense blood flow without clot formation while the other groups performed poorly. Immunostaining analysis suggested that integrin αIIb-positive cells were non-significant in APT transplanted groups suggesting that this construct prevents platelet aggregation. RT-PCR analysis showed that expression of genes involved in platelet activation was well-reduced in the APT transplant groups suggesting that the bioengineered system can protect from vascular thrombosis after transplantation.

In vivo (**After transplantation** of the constructs to the injured rats, and post induction with TAA the following analyses was performed to confirm the in vivo functioning of the liver) – Histological analysis with H&E staining and picrosirius red staining suggested no septal thickening or adverse conditions in the case of APT-VBHL construct implants. qRT-PCR analysis for analysing the gene expression patterns on fibrosis-related genes (*αSma, Vimentin, Tgf-β1* and *Timp1)* showed overall reduction of these genes in APT-VBHL construct implants. No significant elevation of ALT or serum AST levels in APT implanted groups suggesting the paracrine effect of these constructs regulating liver homeostasis. Rats that received APT constructs exhibited amelioration of hepatic cirrhosis and restoration of liver functions, confirming the functioning of bioengineered livers.

Question 2 - "Toward a neurospheroid niche model optimizing embedded 3D bioprinting for fabrication of neurospheroid brain-like co-culture constructs" by Li *et al.* [8]

(A) What is the overall goal of this work?

This study aims at constructing a 3D model of brain tissue that can be used for analysing various neurological patterns, disorders, neural regeneration and for drug development purposes. The 3D model will faithfully include the complex cell-cell interactions and communications between various different cell types found in brain tissue, such as neurons, glia, astrocytes and so on. The scope of studying the aforementioned patterns is very limited in the case of 2-dimensional tissue culture in petri dishes. However, these limitations are overcome with the use of 3D neurospheroids and organoids which can recapitulate the complex functional and behavioural aspects of the brain. This study, along with incorporating intricate details of the brain, also aims at understanding the activation pattern of neural stem cell niche and how it interacts with the astrocytes to support in neuronal differentiation. Therefore, in this work, the authors use an embedded 3D bioprinting mechanism to develop a soft tissue consisting of astrocytes and neurospheroids resembling the brain's composition, within a scaffold that provides the mechanical stiffness of the brain. This study also optimizes the conditions for fabrication of the brain tissue. With specific measures to control the biophysical properties of the material and their organization, the study aims at reproducing a brain-like tissue with a robust neural stem cell niche.

(B) What was the basis of choosing the bioink composition for this work?

In this study, the authors have used a neural stem cell laden bioink to prepare the neurospheroids. This bioink is used inside an astrocyte-laden thermal-healing supporting gel which will direct the organization and differentiation of the neurospheroids. The neuron-laden bioink is made up of GelMA, gelatin, alginate, and laminin mixture (GGA bioink). GGA bioinks can also be altered to finetune the viscosity, porosity and neuron-compatible stiffness, the GelMA material provides cell adhesion surfaces and the gelatin in the bioink improves printability. It has been proved through previous studies that embedded bioprinting with GelMA materials often recapitulates soft tissues very well. The Ca2+ ions in the pre-polymer bath crosslinks the alginate in the bioink which offers mechanical advantages like well-defined boundaries for the microfibres. The materials present in the bioink have a lower Young's Modulus which mimics the brain tissue well. Studies have also reported that GelMA and alginate together in a bioink offers neural differentiation from stem cells. Therefore, considering all the above factors, GGA bioink was chosen.

(C) Explain the principle behind UV-crosslinking used for curing the bioink.

The ink and supporting hydrogel are exposed to UV irradiation which crosslinks them. The UV light excites the free radicals present in the curing bioink. This serves as a photo-initiator which creates covalent bonds between the matrix (hydrogel) and the bioink [9]. Since the irradiation is basically exposure to harmful UV wavelength, the duration of exposure to the UV source should be minimised. Depending on the duration of the UV exposure, the formed hydrogel might have very different biophysical properties, including effects on viability on high exposures. In this project, the bioink had full crosslinking in the case of high GelMA concentration and high UV exposure time. Insufficient crosslinking due to lesser UV irradiation can lead to undesired degradation of hydrogel during the incubation period which comes prior to the maturation of cell-laden.

(D) What rheological characterizations were performed? Explain the results

1. **Yield stress analysis –** A linear ramp shear rate is exerted on the biomaterial and over a continuous flow, the response is measured. Here the yield stress is obtained by fitting the stress/strain curve to the conventional Bingham, Casson or Herschel-Bulkley models. This experiment is not performed under steady state conditions and consequently the results are very dependent on the experimental set up.
2. **Yield stress analysis by Oscillatory sweep test –** This test is performed either using a plate-cone or plate-plate viscometer. At a frequency of 10 Hz, strain is applied on the material between low (1%) and high (250%) strains, every 2 minutes. Through this test the ability of the bath and the bioink to recover in the presence and absence of shear is measured.
    a. **Bioink -** It was observed that when high strain is applied, there was a rapid shift from gel-like properties to fluid-like properties, which is a required ability of the bioink. This was also indicated by G'' surpassing G'. On the contrary, during the periods of low strain, the material was able to quickly move from fluid-like material to a solid-like material. This indicates the shear-thinning characteristics and fluid-behaviour of the bioink that is required for extrusion based bioprinting.
    b. **Bath –** The oscillatory model of the bath showed a higher storage modulus for the bath with respect to the bioink. The variations in the storage modulus with changing strains suggested thermal-healing properties of the bath. This also reports the ability of the supporting hydrogel to rapidly recover after needle sweep without causing cracks or distortions.

(E) What is different information gathered from fluorescence microscopy and confocal microscopy? How have these tools been used in this paper?

Viability and activity can be studied using fluorescence microscopy and confocal microscopy by immunostaining procedures. These analyses would give us information regarding the presence of certain proteins qualitatively and quantitatively. A primary antibody allows visualization of the protein when it is bound to a secondary antibody with attached fluorophore. In a fluorescence microscope the entire material is placed evenly under the light and all the parts get excited equally to fluoresce. In contrast, a confocal microscope is based on point illumination and light produced by fluorescence that is close to the focal plane can only be detected. However, this upper-hand with resolution is at the cost of decreased signal intensity in case of confocal microscopy. In summary, confocal microscopy is a specific type of fluorescence microscopy that allows for reconstructing 3D images of the sample with better resolution.

In this paper, these tools were used to observe astrocytes and neuron networks by staining and analysing various molecules - F-actin, GFAP, DAPI, βIII tubulin and FUJ1.

(F) How were the printing parameters optimized? What factors had to be considered for this optimization?

The rheological properties of the biomaterials were analysed and optimised based on various tests as mentioned above. The bioink possessed shear thinning properties and fluidic behaviour that supports extrusion-bioprinting and the bath showed rapid recovery. The bioink's **extrusion parameters** such as printed fibre diameter, nozzle speed, and the flow rate of the bioink impacts the size and quality of the fibre that is printed. These parameters were optimized by printing the ink on the bath and adjusting as necessary. The nozzle speed is controlled to print fibres continually. The diameter of the fibre is inversely related to the nozzle speed and the flow rate when the nozzle's diameter is fixed. The **mechanical properties** of the biomaterial were tested by varying GelMA concentrations. They can also be optimized by changing UV exposure time, chemical cross-linking ratio and so on. It was also important for the authors to consider the *in vivo* set up which might have an impact on the biomaterials. Hence a small section of the rostral migratory system, which guides the neuroblasts from the stem cell niche to their destinations, was printed with extensive branches. Several versions of this system were printed by varying the number of branches and the respective angles in which they are placed. This way the printing parameters were optimized for the specific problem at hand.

(G) What was the role of the microenvironment on developing the neurospheroid niche model?

As stated in the aim of this project, the intricate microenvironment present in the brain dictates a lot of parameters for various functions, which includes the meshwork-like glial tunnel that guides immature neuron cells from niche along the rostral migratory system to their destination. Hence, faithful abstraction of this microenvironment in the neurospheroid model is very important for developing a 3D brain model which can be used to study neural regeneration, degeneration, and for drug developmental purposes. The co-culture model as adopted in this study, provides a common media for growth and differentiation of different types of cells. The neurospheroid model also controls the neurons from attaining excitotoxicity which happens due to larger production of glutamate in the tissue. This is controlled by astrocytes that take up and recycle glutamate into glutamine. This phenomenon would not be possible in the neurospheroid without the intrinsic microenvironment with astrocytes as well. Further, optimizing the microenvironment and its biomaterials provide an effective interface between glial population and the neurospheroids thus modelling a neural stem cell niche perfectly.

Question 3 - "Wnt-modified materials mediate asymmetric stem cell division to direct human osteogenic tissue formation for bone repair" by Okuchi *et al.* [10]

Introduction and Overview of the paper

This paper establishes a novel methodology to treat bone repairs. There have been a variety of reported studies that suggest the upcoming of cell-based therapies for bone repair involving human skeletal stem cells (hSSCs). However, the survival rate is rather low which has in turn motivated the authors to investigate the underlying molecular mechanisms that direct bone repair and the event involvement of hSSCs in the processes.

It is well known that the self-renewal of hSSCs is dependent on external factors, such as Wnt/ β-catenin signalling. Although, it is only the Wnt molecules that are covered in lipid micelles that contribute to the induced signalling pathway and therefore bone repair. Thus, there is a need for a proper delivery of the Wnt molecules to eventually repair damages to the bone. The authors had recently established a Wnt3a-platform where microbeads bound to Wnt3a molecules are delivered at the site of injury. In this work, Okuchi et al. have improvised on the platform by the introduction of 3 dimensional osteogenic structures, the 3D WIOTM model (Wnt-induced human osteogenic tissue model). The WIOTM on collagen type1 (COL1) gel was proven to recapitulate 3D physiological human bone niche in vitro, via observing mineralized nodules in the upper part of the WIOTM. This study aims at establishing an osteogenic included Wnt3 delivery mode for bone repair, which can potentially be taken forward to clinical applications.

Results

The molecular mechanism underlying the generation of WIOTM is first established, by investigating if there are noticeable changes in cell division's orientation which might lead to asymmetric presentation on one side of the hSSCs. The hSSCs were cultured in an osteogenic media with COL1-gel, on immobilized Wnt3a platform. On an average, cells either arranged themselves perpendicularly (more so often when placed on a platform) or parallelly to the platform and the former groups displayed a variation in a variety of cell markers such as aPKCζ (polarity protein), β-catenin and APC. These markers directed the orientation of spindle formation during cell division causing an asymmetrical cell division (ACD), thus generating WIOTMs. Several markers for hSSCs were profiled using immunofluorescence techniques to identify patterns during cell division that might direct hSSCs' fate. It was observed that osteogenic

differentiation factors (OPN and OCN) were found in the distal-hSSC from the platform while stem cell markers (CDH13 and PLXNA2) were found in the proximal-hSSC from the platform thus preserving stem cell identity. Therefore, the *in vitro* studies suggested that localized Wnt3a orients hSSC divisions to generate the WIOTM and also segregates cell fate markers in dividing hSSCs.

The next step was to test whether the WIOTM would recapitulate characteristics of the periosteum which is found in healing bones. A biocompatible bandage-model of the WIOTM was designed using PCL polymer with a O_2/APTES[1] treatment. A series of procedures involving serial washing with buffers were performed to prepare the WIOTM-bandage. The TCF-Luciferase reporter cell line (LS/L) assay provides information regarding the β-catenin signalling pathway and it displays higher Wnt3a-binding efficiency of the WIOTM bandage. Further experiments to test for healing is done on several control models, including non-signalling BSA-PCL, inactivated Wnt3a platform on PCL (iWnt3a-PCL), along with the activated Wnt3a-bandage that would deliver WIOTM upon transplantation. A 13-week-old SCID mice model was induced with a calvarial bone defect to test the hypothesis. It was observed that 8 weeks after implantation of the models, the WIOTM-bandage displayed the highest levels of healing compared to other groups. There was de novo formation of bone, mineralised regions in the newly forming bone, small evidence of vascular cell formation in the transplanted cell, and even fusion of the existing and the new bones.

Following successful transplantation, the authors proceeded to elaborate on how the WIOTM-bandage offers enhanced performance over the Wnt3a-bandage. Immunohistochemical analyses suggested that SSC markers were present in comparatively higher quantities in the WIOTM area. Differentiation markers such as OPN and OCN were also seen to be present in the implanted WIOTM area indicating matrix deposition and bone expansion by osteocytes and osteoblasts, which display tissue-level organization comparable to that in WIOTM-bandage. The contribution of WIOTM to the newly forming bones were measured by quantifying the levels of Sclerostin (SOST)[2] in the injured area and by observing the lineage of those cells. Human origin SOST+ cells (hSSCs) were found to be ~36% of the total SOST cells and

[1] This method is performed by exposing the scaffolds' surface with Oxygen plasma followed by incubation with aminopropyl-triethoxysilane (APTES). This treatment facilitates Wnt3a conjugation and is better than the previously established HMDA approach.

[2] Bone cells positive for Sclerostin (SOST) had an equivalent density to healthy calvarial bone. SOST is a marker expressed in mature osteocytes in advanced mineralization and a Wnt/β-catenin signalling antagonist critical for osteoblast–osteocyte transition.

human cells were not detected in the neighbouring bone area. The result of iWnt3a-bandage was significantly low suggesting that the implantation of a model that includes an osteogenic structure is crucial in bone repair. The CDH13+ and PLXNA2+ profiles were analysed and were shown to have higher concentration in WIOTM-bandages. Overall, this proved that Wnt3a-bandages increase the number of cells that express stem cell markers in the connective-like tissue. The WIOTM-bandage shows enhanced performance by increasing the supply of human cells in tissue.

Strengths of the study

There are numerous strengths for all the experiments done in this study. First, several cell markers have been included to verify all the hypotheses of this paper, including multiple cell markers to confirm the same result. Second, various assays and sophisticated tools - the TCF-Luciferase reporter cell line (LS/L) assay for confirming the activity of Wnt3a in β-catenin signalling pathway, assay to test the activity of O2/APTES-PCL-Wnt3a films using hSSCs that contains the 7xTCF-eGFP/SV40-mCherry reporter (eGFP is under the 7xTCF binding sites and mCherry is constitutively expressed in all hSSCs), the micro-computed tomography analysis for screening the defect site in the brain and to observe the healing, H&E staining and Movat's pentachrome staining to display the mineralised regions in newly upcoming bones, have all been performed throughout this study demonstrating the extensive research that has been done. Third, various control groups have been incorporated and extensive comparisons within these groups (as in Fig 3d) suggest that the final WIOTM-bandage is indeed more efficient than any of the other models. Fourth, the steps to also confirm that the bone regeneration is indeed from the hSSCs have also been experimentally tested out in detail. Finally, the authors have also scaled up the in vitro designing to in vivo application to prove the efficacy of this novel methodology.

Limitations in the study and Critical comments

Although the study covers extensive research and a broad range of experiments, there are a few limitations of this study. First, the choice of the model system is very critical for the outcomes of any study and the subsequent conclusions drawn from it. In this experiment, the authors have chosen the immuno-deficient SCID mice for testing whether the WIOTM badge works well in vivo. Although the immuno-deficiency might be an advantage for the reduced effects after implantation, it seems very uncertain on whether this study can be taken up to the next level without more complications. The innate immune response is a very common effect in higher order mammals and must be carefully considered for scaling up. Therefore, it is

important to translate these studies to healthy mice with calvarial bone defect before considering clinical implications on human beings. Second, the paper constantly supports the efficiency of Wnt3a-bandage although it does not perform as equally as the WIOTM bandage, in terms of bone regrowth. Albeit their explanation on why an osteogenic model is better and necessary, they continue to support the working of Wnt3a-bandages without clear explanation. Finally, the authors have not characterized the biophysical, mechanical and chemical properties of these materials such as Wnt3a-bandage and WIOTM-bandage before implantation. Such analyses are necessary for establishment and optimization of new materials.

In **conclusion**, the study aims at establishing novel and conducive conditions for bone repair which involves transplanting osteogenic material along with the stem cells. Albeit the novelty, the study needs to address a few more concerns regarding the immune response and characterization before taking it forward.

Question 4 - Identify an ongoing project on "Engineering a biomimetic cardiac tissue". Discuss the lacuna that is being addressed in the proposed work. Identify one publication that has come out of this project and describe the approach to address the problem identified. Identify another paper from the literature that addresses the same problem with a different approach. Compare the two strategies and the results obtained.

Project - Rapid 3D bioprinting of biomimetic vascularized tissue constructs

Principal Investigator – Shaochen Chen

Project number - 5R01EB021857-03

Project Start Date & End Date – 1 July 2016 – 31 March 2020

Description of the project and the lacuna that is addressed

The field of cardiac tissue engineering is yet to experience a lot of advancements before we can achieve patient-specific treatment. The fabrication of highly organized tissues with intricate micro architecture is still a challenge in the field of tissue engineering and regenerative medicine, with respect to almost all the physiological organs. The heart holds a very important role in an organism's living as it ensures the supply of blood to almost every other organ within the organism. Hence repairing a damage in the cardiac tissue can be extremely fragile and complicated to handle, especially given the fact that there are different kinds of cells stacked up in a specific architecture for its functionality. Towards this goal of fabricating intricate microarchitecture, different prototyping strategies have been established based on stereolithography and bioprinting. The key challenges that one would face with these advancements are – (a) To creating clinically relevant models which mimics disease condition, a longer duration for the sustainability of the cells need to be ensured, but viability of cells on such time-scales has not been established, (b) Tissue constructs are usually made of a multitude of other cells and the bioprinting of such a sophisticated architecture is very difficult.

Through this project, Chen's group plans on developing a Rapid, Multimaterial Bioprinting (RMB) technology. The RMB technology will allow for printing multicomponent complex structures and is faster than conventional bioprinting. RMB would also allow for using diverse cell-laden biomaterials continually which would in turn allow the construction of biomimetic tissues in vitro. The group would specifically

try to achieve the in vitro bioprinting of pre-vascularized heart tissues and blood vessels of different architecture. The aim is to integrate a programmable microfluidic system with an optical printing method which would deliver different cells (iPSC-derived human cardiomyocytes -iCMs and endothelial cells -ECs) and biomaterials to mimic the characteristics and composition of cardiac tissue. The printed material will be assessed in vitro followed by implantation in rats to test their performance.

Publication: "Direct 3D bioprinting of cardiac microtissues mimicking native myocardium." [11]

In this study, the group demonstrates a novel method of cell encapsulation in 3D methacrylated gelatin (GelMA) scaffolds that are patterned using Microscale Continuous Optical Printing (μCOP). μCOP can micropattern cells onto a photocrosslinkable hydrogel. The cells used in this study are neonatal mouse ventricular cardiomyocytes (NMVCMs). These encapsulated cardiomyocyte cells preferentially order themselves to mimic the myocardium in vivo. Conventional topographical patterning will only provide cues for the cardiomyocytes to arrange themselves on a 2-dimensional platform, which isn't of much physiological relevance. As an improvement, several groups attempted 3D mimicking of cardiac tissue by seeding cells on prefabricated scaffolds. But this often gave confounded results due to non-uniform distribution of cells along the wells and channels. Therefore, to overcome this limitation, Chen et al. have attempted 3D printing of the scaffold using the μCOP technique which offers high precision and faster results. Complex patterning of cells forces the cardiomyocytes to mis-align and contract along the main axis of the scaffold which reduces contractile forces on the tissue.

Upon seeding the cells onto glass (control group) and GelMA μCOP -printed scaffold, the basic compatibility of NMVCMs to the GelMA material was tested by analysing the formation of striated α-actinin fibres in both cases. NMVCMs preferred GelMA material as their ability to align in the patterned gel was enhanced. The patterned gel was further optimized for encapsulation and it was observed that NMVCMs that are encapsulated in 3D scaffolds showed improved alignment, similar to what would be observed in mature myocardium. This was observed by staining sarcomeres and analysing under a confocal microscope. Further, the μCOP printed scaffolds allowed rapid tailor of the structure for force measurement using the mechanical testing instrument "Microsquisher". The encapsulated scaffold's functionality is compared with seeded scaffolds by staining for α-actinin and it was observed that there was higher compaction of cells in the former case as compared to the latter case. Cells within the encapsulated scaffolds showed higher displacement and behaved similar to in vivo conditions. The

micropatterning was changed from parallel lines to other geometries such as slab, grid, dispersion and random structure to study the ability of cardiomyocytes to remodel their environment. The microenvironment in turn had a major role to play in the level of displacement that was observed in the NMVCMs, with the line shape displaying highest displacement and the slab shape displaying the lowest displacement.

The μCOP system has proven to be extremely supportive for cardiac tissue regeneration by specifying the microarchitecture for the cardiomyocytes to migrate and grow on. The μCOP system can also be tailored specific to the tissue that is being printed. For instance, by decreasing material concentration to decrease overall stiffness, tissues made up of human embryonic stem cell cardiomyocytes or human iPSC-derived cardiomyocytes can be printed. This is the first study that directly prints 3D microstructures on cellular alignment, and their tissue mechanics have been effectively measured. The pre-patterning of cells in specific geometries would also be very effective in developing disease models such as that of hypertrophic cardiomyopathy, where the underlying cardiomyocytes expand in size but not in number. Along with the ability to mimic disease models, it was also observed that encapsulated cells from the μCOP system showed longer survival (>30 days) than conventional cell seeding techniques. The microarchitecture and the viability of cells will definitely support drug discovery in diseases such as cardiomyopathies and even the underlying theory on how cells interact and so on.

Another article from the literature which also tries to recapitulate the complex microarchitecture of cardiac tissues is as follows – **"Biomimetic engineering of the cardiac tissue through processing, functionalization, and biological characterization of polyester urethanes" – Vozzi *et al.*** [12]

This article is also based on constructing a scaffold with the innate micro-structure of cardiac tissue so that it can be used for drug testing purposes. This study starts with the same ideas about how a 2-dimensional scaffold would not capture all the intricate biology regarding a tissue and they establish a need for a 3-dimensional scaffold which would indeed consist of the necessary porosity and composition required to mimic the physiological conditions. However, there are different ways of constructing a 3D model – hydrogels made of ECM proteins, self-organized cell aggregates i.e., organoids and prefabricated scaffolds with specific microarchitecture, and this study adopts the final methodology which is to create a scaffold representing the complex patterning of the cardiac tissue using conducive biomaterial.

Comparing the strategies –

In the case of cardiac tissue, this biomaterial should express elastomeric properties and anisotropy. The first level of difference is introduced by **difference in underlying biomaterial in both the studies**. The former employs GelMA (3D methacrylated gelatin) and in this study the scaffold is made up of polyester urethanes (PURs). While GelMA is well characterized and is employed in several tissue engineering applications, this study is among the firsts to employ PUR scaffolds owing to their low Young's modulus and elastomeric behaviour. The second level of variation is brought in by the **different strategies for constructing the scaffold**. In the former study, GelMA is bio-printed by the uCOP system, and in this study, to induce better porosity, Thermally Induced Phase Separation (TIPS) is employed to fabricate the scaffolds. 3D bioprinting offers the users the ability to control and govern the microarchitecture, including the pores. TIPS and electrospinning also offer good control over the pore size and morphology tuning, and this can be tuned by several parameters such as polymer concentration, thermal gradient, temperature, and so on. In this study, plasma surface modification and fibronectin immobilization (to mimic ECM) were conducted to alter the surface properties of the scaffold and not affect bulk properties. Functionalization was not performed in the former study. The cells that are encapsulated/seeded are very similar in both the studies, i.e., neonatal cardiomyocytes. The fibre alignment along the electrospun PUR scaffold allows the cardiomyocyte cells to generate tissue-like structure as observed in myocardium. Vozzi et al. also maintain a control group of cardiomyocytes seeded on tissue culture plates to compare the results. **Scaffold preparation and characterization -** In the former study, mechanical characterization was performed using a Cellscale microsquisher which measured displacement and force exerted by the developed tissue/scaffold. The uCOP system employed UV polymerization in a layer-by-layer fashion for crosslinking. In the PUR scaffolds, biomaterial and scaffold characterization was done by ATR-FTIR spectroscopy, XPS and SEC analysis. Morphological properties were analysed using confocal laser microscopy and the contact angle measurement suggested that the surface of PUR scaffolds were hydrophobic. The authors reason that the hydrophobicity is indeed due to the porosity in the structure while it does not affect cell adhesion. The properties of the PUR scaffold were optimized such that they mimic the myocardial tissue.

Comparing the results –

In the former study by Chen et al., all the experiments (force measurement and so on) were performed within a month and the cardiomyocytes started synchronously beating around the third day and survived in culture for about ~30 days. However, in this study by Vozzi et al., the cardiomyocytes colonize the scaffold and remain adherent for about ~50 days and the cell viability remains stable until day 14. They also report that the cardiomyocytes present in the tissue, upon stimulation display a synchronous beating activity for ~50 days. Next, the gene expression of the PUR scaffold displayed upregulation of hypertrophy related genes and glucose metabolism and no significant difference in the fatty acid metabolism. (Gene expression profiling was not mentioned by Chen et al.) These observations suggested that the neonatal cardiomyocytes were maturing but still displayed fetal-like phenotype, thereby ensuring cellular wellness. Finally, both the groups provide a novel technique to construct 3D scaffolds for mimicking the cardiac tissue which can further be used for various purposes including drug development.

References -

[1] D.-H. Kim *et al.*, "Development of highly functional bioengineered human liver with perfusable vasculature," *Biomaterials*, vol. 265, p. 120417, Jan. 2021, doi: 10.1016/j.biomaterials.2020.120417.

[2] H. Markides *et al.*, "5.09 - Isolation of Mesenchymal Stem Cells From Bone Marrow Aspirate☆," in *Comprehensive Biotechnology (Third Edition)*, M. Moo-Young, Ed. Oxford: Pergamon, 2019, pp. 137–148.

[3] M. Shahdordizadeh, R. Yazdian-Robati, M. Ramezani, K. Abnous, and S. M. Taghdisi, "Aptamer application in targeted delivery systems for diagnosis and treatment of breast cancer," *J. Mater. Chem. B*, vol. 4, no. 48, pp. 7766–7778, Dec. 2016, doi: 10.1039/C6TB02564A.

[4] M. Chu *et al.*, "Evaluation of human epidermal growth factor receptor 2 in breast cancer with a novel specific aptamer," *Cell. Mol. Immunol.*, vol. 14, no. 4, Art. no. 4, Apr. 2017, doi: 10.1038/cmi.2015.31.

[5] S. Shigdar *et al.*, "Aptamers as theranostic agents: modifications, serum stability and functionalisation," *Sensors*, vol. 13, no. 10, pp. 13624–13637, Oct. 2013, doi: 10.3390/s131013624.

[6] J. Karar and A. Maity, "PI3K/AKT/mTOR Pathway in Angiogenesis," *Front. Mol. Neurosci.*, vol. 4, Dec. 2011, doi: 10.3389/fnmol.2011.00051.

[7] "Basic Principles of RT-qPCR - IN."

//www.thermofisher.com/in/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/spotlight-articles/basic-principles-rt-qpcr.html

[8] Y.-C. E. Li *et al.*, "Toward a neurospheroid niche model: optimizing embedded 3D bioprinting for fabrication of neurospheroid brain-like co-culture constructs," *Biofabrication*, vol. 13, no. 1, p. 015014, Nov. 2020, doi: 10.1088/1758-5090/abc1be.

[9] "A Guide to Matrix Bioinks," *Allevi*, Jan. 24, 2020. https://www.allevi3d.com/a-guide-to-matrix-bioinks/

[10] Y. Okuchi *et al.*, "Wnt-modified materials mediate asymmetric stem cell division to direct human osteogenic tissue formation for bone repair," *Nat. Mater.*, vol. 20, no. 1, Art. no. 1, Jan. 2021, doi: 10.1038/s41563-020-0786-5.

[11] J. Liu *et al.*, "Direct 3D bioprinting of cardiac micro-tissues mimicking native myocardium," *Biomaterials*, vol. 256, p. 120204, Oct. 2020, doi: 10.1016/j.biomaterials.2020.120204.

[12] F. Vozzi *et al.*, "Biomimetic engineering of the cardiac tissue through processing, functionalization, and biological characterization of polyester urethanes," *Biomed. Mater. Bristol Engl.*, vol. 13, no. 5, p. 055006, Jul. 2018, doi: 10.1088/1748-605X/aaca5b.