TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

IINA LEHTOVIITA

APPLICATION OF MICROFABRICATION TECHNIQUES FOR TISSUE ENGINEERING A CARDIAC CONDUCTIVE DEVICE

Master of Science Thesis

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ABSTRACT

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Impairment of the atrioventricular electrical conduction (AV-block) is a major cause for the implantation of an electronic pacemaker device. Even though this is the standard treatment today, it has its disadvantages. One major problem is the implementation of long-term pacing therapy in pediatric patients owing to the restrictions imposed by a child's small size and their inevitable growth. Thus there is a genuine need for innovative therapies especially for children with cardiac rhythm disorders.

The aim of the Biopacer project is to develop an autologous conductive tissue device that will serve as an electrical conduit between the upper and lower chambers of the heart. The idea is to use pediatric cardiomyocytes together with a fibrin-based scaffold to produce a tissue construct that is completely autologous and has the ability to grow with the patient. Mimicking the complexity and highly organized structure of native cardiac tissue sets significant challenges for the engineering process. To address this challenge, bottom-up tissue engineering, in which structural components of different cell types with high-degree organization are fabricated separately and later assembled together, was applied. The aim of this thesis was to utilize different microfabrication techniques for the realization of the fibrin scaffold with different cell types to fabricate building blocks for the assembly of a conductive tissue device with a controllable, spatially organized tissue microarchitecture.

Two methods, micromolding in capillaries (MIMIC) and microfabrication of fibrin fibers by extrusion, were established and exploited in the fabrication of cell-seeded 3D fibrin tissue modules. Using the MIMIC technique, cells were spatially confined into a 3D fibrin gel structure and they were shown to align longitudinally in small diameter fibrin gels. In extruded fibrin fibers, endothelial cells were demonstrated to coalesce and bundle the surrounding fibrin into a core around which they arranged themselves resembling the phenomenon of tubulogenesis. The organization of these microfabricated tissue modules together to create a conductive tissue device consisting of separate functional units (e.g. aligned cardiomyocytes, vasculature, extracellular matrix) was additionally displayed. This way, all the structural components of native cardiac tissue are accounted for and highly organized in the engineered construct.

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Sydämen johtoratajärjestelmän häiriö eteisten ja kammioiden välillä on yleisin syy sydämen tahdistimen asentamiselle. Vaikka tahdistinhoito on hyvin yleinen ja luotettava hoitomuoto tänä päivänä, pitkäaikainen hoito erityisesti lapsipotilaiden keskuudessa on ongelmallista. Merkittävänä haasteena lasten tahdistinhoidossa on potilaan pieni koko ja lapsen väistämätön kasvu. Täten on aito tarve kehittää innovatiivisia hoitomuotoja sydämen rytmihäiriöistä kärsiville lapsille.

Biopacer projektin tavoitteena on kehittää autologinen kudosimplantti, joka toimii sähköimpulssien johtokanavana sydämen eteisten ja kammioiden välillä. Tarkoituksena on eristää sydänlihassoluja lapsipotilaan kudosnäytteestä ja käyttää niitä yhdessä fibriinipohjaisen skaffoldin kanssa valmistamaan täysin autologinen kudosimplantti, jolla on kyky kasvaa potilaan mukana. Sydänlihaskudoksen monimuotoisuus ja järjestyneisyys luovat haasteita kudoskorvikkeen valmistukseen laboratorio-olosuhteissa. Tässä opinnäytetyössä hyödynnettiin bottom-up -tekniikkaa, jossa kudosimplantti kootaan pienemmistä eri solutyyppien rakenneosista suuremmaksi kokonaisuudeksi. Työn tavoitteena oli soveltaa erilaisia mikrovalmistustekniikoita fibriinigeelirakenneosien valmistukseen ja myöhemmin koota nämä rakennuspalikat yhteen muodostamaan kolmiulotteinen, mikrotasolla hyvin järjestäytynyt ja sähköimpulsseja johtava kudosimplantti.

Fibriinipohjaisten mikrometrikokoluokan 3D kudosrakenteiden valmistamiseen sovellettiin kahta eri mikrovalmistustekniikkaa: muovaukseen perustuvaa pehmeän litografian MIMIC (micromolding in capillaries) -tekniikkaa sekä fibriinikuitujen ekstruusiota. MIMIC:n avulla muovatuissa fibriinigeelirakennelmissa solut järjestäytyivät pituussuunnassa muodostaen hyvin tiiviisti organisoitunutta kudosta. Ekstruusiolla valmistetuissa fibriinikuiduissa endoteelisolut siirtyivät kuitujen pinnalle ja muotoutuivat putkimaisesti fibriinigeelikuidun ytimen ympärille muistuttaen tubulogeneesi-ilmiötä. Kokoamalla nämä kudosrakenneosat yhteen voidaan valmistaa sydänkudosimplantti, joka koostuu useasta toiminnallisesta yksiköstä (esim. järjestäytyneet sydänlihassolut, verisuonitus). Käyttäen hyväksi bottom-up -kudosteknologiaa voidaan muodostaa järjestäytynyttä kudosta pienemmistä rakennepalikoista ja täten matkia natiivin sydänlihaskudoksen eri solutyyppien monimuotoisuutta ja järjestäytyneisyyttä.

PREFACE

This Master of Science thesis was carried out in the Tissue Engineering and Textile Implants group at the Institute of Applied Medical Engineering (AME), Helmholtz Institute of RWTH Aachen University and Hospital.

I would like to sincerely thank Professor Dr. Stefan Jockenhövel for giving me the opportunity to be a part of the group and to conduct my thesis in the fascinating field of cardiovascular tissue engineering. Also, I would like to thank Professor Minna Kellomäki for helping me at the start of my journey to Germany and for accepting to supervise this thesis.

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Tampere, January 2013

Iina Lehtoviita

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ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
4-HBA	4-hydroxy butyl acrylate
AV	Atrioventricular
BDM	2,3-Butanedione monoxime
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
CTD	Conductive tissue device
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco`s Phosphate Buffered Saline
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EME	Elektronenmikroskopische Einrichtung
Gtn-HPA	Gelatin-hydroxyphenylpropionic acid
HCl	Sodium chloride
HCN	Potassium/sodium hyperpolarization-activated cyclic nucleotide -gated channel
hEBs	Human embryoid bodies
hESC	Human embryonic stem cells
hMSC	Human mesenchymal stem cells
IZKF	Interdisziplinäre Zentrum für Klinische Forschung
ITA	Institut für Textiltechnik
KCl	Potassium chloride
Kir2	Inwardly rectifying potassium channel 2
KRP	Krebs buffer solution
Kv1	Potassium voltage-gated channel subfamily A
LED	Light-emitting diode
MeHA	Methacrylated hyaluronic acid
MIMIC	Micromolding in capillaries
NaOH	Sodiumhydroxide
PBS	Phosphate Buffered Saline
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PEGDA	Poly(ethylene glycol) diacrylate
PEO	Polyethylene oxide
PLGA	Poly(lactic-co-glycolic acid)
psi	Pressure per square inch
PVA	Polyvinyl alcohol
Ra	Arithmetical mean roughness

RWTH	Rheinisch-Westfaelische Technische Hochschule
SA	Sinoatrial
sccm	Standard cubic centimeters per second
SEM	Electron scanning microscopy
TBS	Trizma buffered saline
TE	Tissue engineering
TPLSM	Two-photon laser scanning microscope
UKA	University Hospital Aachen
UV	Ultraviolet
μCP	Microcontact printing
μTM	Microtransfer molding

1 INTRODUCTION

The impairment of atrioventricular electrical conduction (AV-block) in the heart due to congenital or acquired disorders is a major cause for pacemaker implantation. In AV-block, the transfer of electrical impulses from the natural pacemaker of the heart (sinoatrial node) to the ventricles is partially or completely blocked. This slows down the heart rate and decreases the heart's ability to pump blood to the rest of the body. In pacing therapy, an electrical pacemaker device is implanted in the chest cavity or abdomen. Electrical impulses are delivered to the heart through electrodes that are attached in the cardiac muscle to prompt the heart to beat at a normal rate. [1,2]

Even though pacemaker therapy is the standard treatment today, it has its disadvantages. Lead complications can cause severe problems for the patient and limited battery life leads to reoperations to replace the power supply. One major problem is the implementation of long-term pacing therapy in pediatric patients owing to the restrictions imposed by a child’s small size and their inevitable growth. [3,4] Thus there is a genuine need for innovative therapies as an alternative to conventional pacing therapy especially for children with cardiac rhythm disorders.

In responding to these challenges, efforts have been made to create a biological pacemaker as an alternative to conventional electronic pacemakers. Several research groups are focusing on the generation of pacemaker cells by using gene therapy or stem cell therapy. [5] Alternatively, developing an autologous biomedical implant using tissue engineering strategies could be used to restore conduction between the atria and the ventricles.

The aim of the entire Biopacer project is to develop an autologous conductive tissue device that will serve as an electrical conduit between the atria and the ventricles of the heart. Pediatric cardiomyocytes are isolated and used together with a fibrin-based scaffold to produce a tissue construct that is completely autologous and has the ability to grow with the patient. The anticipation is to use this type of engineered tissue to restore atrioventricular electrical conduction in children with complete heart block.

Mimicking the cellular complexity and highly organized structure of native cardiac tissue sets significant challenges for the engineering process. To address this challenge, bottom-up tissue engineering (TE), in which structural components of different cell types with high-degree organization are fabricated separately and later assembled together, is applied. This thesis is a part of the Biopacer project and focuses on different microfabrication techniques that can be exploited to fabricate fibrin-based structural tissue modules that can be used as building blocks to engineer a conductive tissue device. The aim is to establish and realize the techniques using fibrin gel, and further

exploit them in the fabrication of a conductive tissue device using the bottom-up approach. The idea is to utilize techniques of micromolding using soft lithography and fibrin fiber extrusion to fabricate cell-laden fibrin tissue modules of different cell types with a controllable, spatially organized tissue microarchitecture. Eventually, these modules could be assembled together and used as building blocks to create more complex 3D tissue constructs using the bottom-up approach.

The thesis is divided into two parts: a theoretical and an experimental part. The first section of the thesis, the theoretical part, provides background information about the structure and function of the conduction pathway of the heart and disorders associated with it. In addition, biological alternatives to conventional pacing therapy and different microfabrication techniques and their use in tissue engineering are described. In the experimental part of the thesis, the isolation of autologous pediatric cardiomyocytes is presented. Additionally, two microfabrication techniques are established and exploited for the fabrication of a conductive tissue device. Each technique is presented and discussed in a chapter of its own. Finally, the possibility of combining these techniques to produce tissue modules that can be brought together to create more complex tissue constructs using bottom-up tissue engineering is explored.

2 THEORETICAL BACKGROUND

2.1 The heart

The heart is a hollow, muscular organ that contracts continually and pumps blood throughout the rest of the body. It is a complex structure consisting mainly of cardiac muscle and connective tissue. The heart beats as a result of electrical impulses initiated and conducted in the cardiac muscle tissue. [6] In the following chapters, the structure and function of the heart specifically the cardiac conduction system is presented in more detail.

2.1.1 Anatomy of the heart

The heart is comprised of two halves, left and right, which function as separate pumping units. Both sides consist of two chambers: an atrium and a ventricle. A muscular partition, the septum, separates the two halves from each other. The atria receive blood returning to the heart as the ventricles pump it away from the heart. The right atrium receives oxygen-poor blood from the body and discharges it via the right ventricle into the lungs for oxygenation, whereas the left atrium of the heart receives oxygen-rich blood from the lungs and pumps it via the left ventricle to the rest of the body. [6]

The heart is a part of the complete circulatory system, which also includes the blood vessels and of course the blood itself. This system can be divided into two: the pulmonary and the systemic circulation. Both begin and end at the heart: the pulmonary circulation transfers blood between the heart and the lungs whereas the systemic circulation carries blood between the heart and the organ systems. [6]

2.1.2 Cardiac Tissue

At cellular level, the heart is composed mainly of cardiac muscle cells (cardiomyocytes), fibroblasts, endothelial cells, and smooth muscle cells. All cell types have a distinctive role in the structure and function of the cardiac tissue: cardiomyocytes play a role in the pumping of the heart, fibroblasts give structural support by synthesizing the extracellular matrix (ECM), and vascular cells, endothelial and smooth muscle cells, form the vascularization of the tissue. [7]

In terms of volume, cardiomyocytes make up the majority of the myocardial tissue. The rest are non-myocytes, mainly fibroblasts but also endothelial cells. [8] Even though in terms of volume endothelial cells and fibroblasts are the minority, they both outnumber cardiomyocytes. For example, capillaries formed by endothelial cells are found beside every cardiomyocyte to ensure sufficient supply of oxygen and nutrients to

the contracting cells. Also, fibroblasts are found surrounding cardiomyocytes providing structural support and bridging the spaces between the myocardial tissue layers. [9,10]

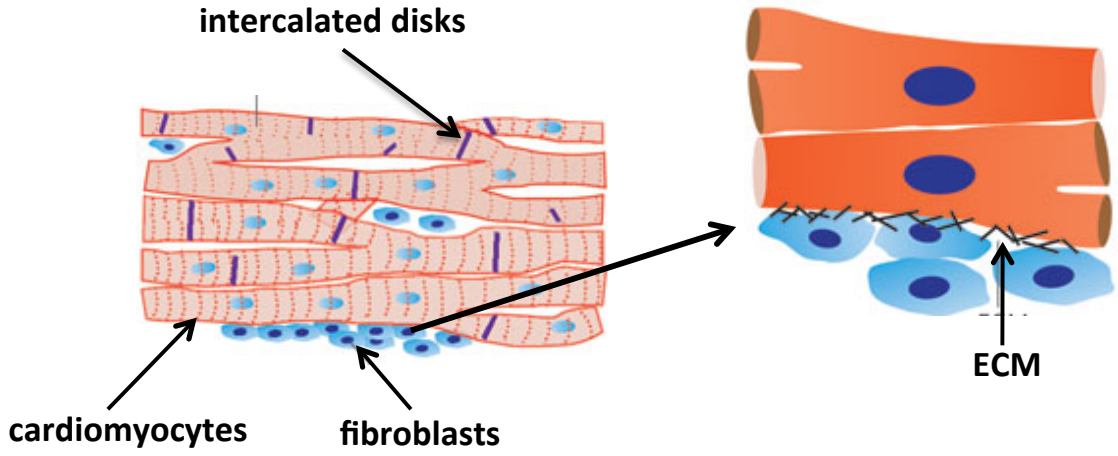

Figure 1. Cardiac tissue consists mainly of cardiomyocytes, fibroblasts, and endothelial cells (not displayed). Picture adapted from [11].

There are two types of cardiomyocytes: contractile cells and autorhythmic pacemaker cells. About 99% of the cells are contractile. These cells consist of contractile myofilaments (e.g. actin and myosin) that are organized close together to form striated myofibrils. They are responsible for the contraction force of the myocardium, but are not capable of generating action potentials. The rest 1% of cardiomyocytes are pacemaker cells, which are smaller in size and lack the organized myofilament structures. These cells are found along the electrical conduction system of the heart and their task is to initiate action potentials and thus set the pace for the heartbeat. [2,12]

A single cardiomyocyte, which contracts approximately 3 billion times in an average human lifespan, forms a synchronized beating unit with neighboring myocytes. This coordinated contraction activity creates the force for the heart to pump blood throughout the body. [12] The cardiomyocytes connect to each other through specified junctions at the end of the cells. These cell junctions are called intercalated disks (see figure 1) and consist of two components: desmosomes and gap junctions. The desmosomes give mechanical support to hold the cells together as the gap junctions allow electrical impulses to spread from one cell to another. Upon action potential initiation, the signal spreads to all other cells in the surrounding muscle tissue that are connected by gap junctions and leads to a coordinated, synchronized contraction of the cardiomyocytes. This network of cardiomyocytes connected through gap junction connections form a single contraction unit –a functional syncytium. Both the atria and the ventricles form their own functional syncytium. This ensures the synchronized contraction of alike chambers but separate contraction of the upper and lower part of the heart. [6]

2.1.3 Electrical conduction system of the heart

The heart pumps as a result of the generation and conduction of electrical impulses. Specific cells in the heart, pacemaker cells, initiate spontaneously action potentials which spread throughout the heart and causes rhythmical contraction of the heart muscle. The pathway of theses impulses is called the conduction system of the heart (figure 2). It is made up of several subcomponents, all which have a distinct role in the generation and the conduction of electrical signals. [2,6]

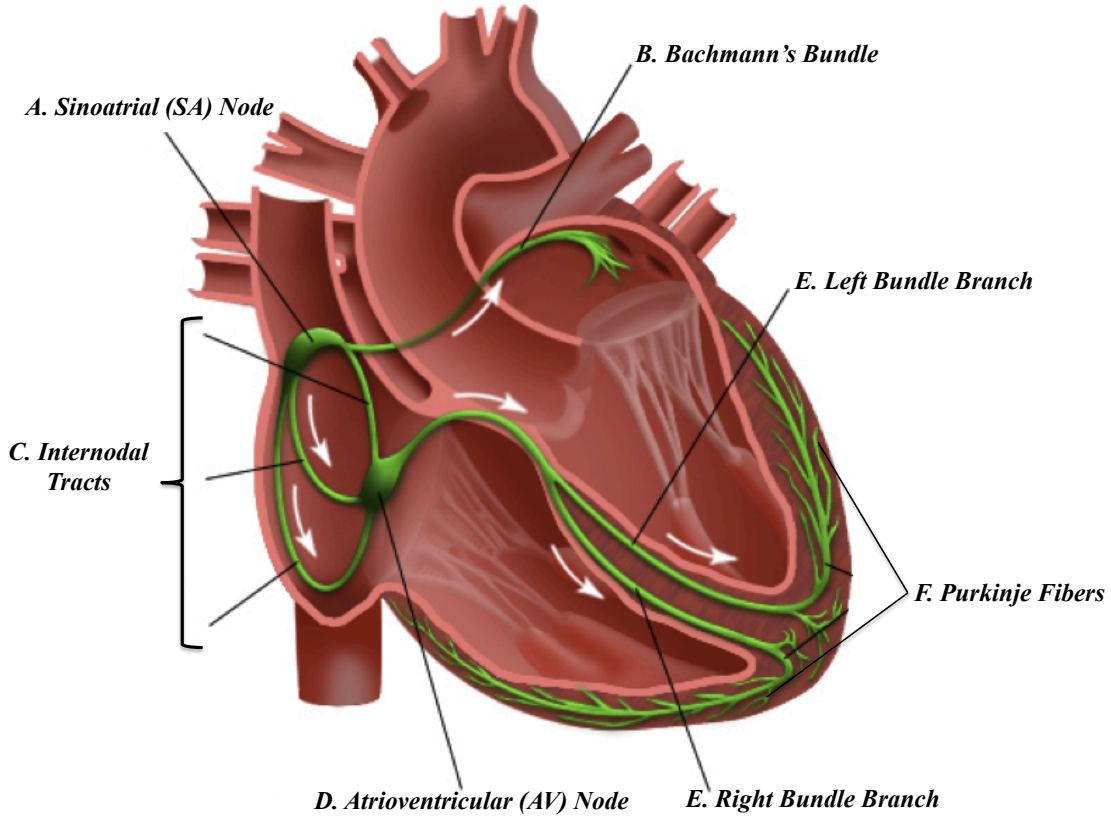

Figure 2. Electrical conduction system of the heart. Figure from [13].

The sinoatrial (SA) node is the main pacemaker of the heart and thus sets the beating rhythm of the rest of the heart. The SA-node lies in the right atrial wall and is composed of cells with the most rapid action potential initiation rate (figure 2a). The impulse then propagates to the left atrium via Bachmann's bundle (figure 2b) and through three intermodal tracts (figure 2c) to the atrioventricular (AV) node, which is located at the base of the right atrium close to the septum that divides the atria (figure 2d). It is the electrical connection of the atria and ventricles through a fibrous insulating layer of connective tissue. The main function of the AV-node is to delay the impulse to ensure enough time for the atria to contract and thus empty of blood before ventricle contraction. From the AV-node, the signal passes to the ventricular conduction network, which consists of the AV-bundle, bundle branches (figure 2e), and the Purkinje fibers (figure 2f). This network rapidly propagates the signal to the ventricles, which then contract synchronously. [2,6]

As mentioned earlier, the pacemaker cells are autorhythmic and non-contractile cells – their task is to initiate action potentials and propagate them rapidly along the conduction pathway. About 1% of the cardiac muscle cells are like this, the rest are contractile cells that take care of the mechanical contraction work. Autorhythmic cells are found along the conduction pathway in the SA-node, AV-node, AV-bundle, and Purkinje fibers. All these sites can thus act as pacemakers, but the rate at which action potentials can be initiated varies. [6]

The SA-node is the main pacemaker of the heart, consisting of cells with the fastest rate of action potential initiation, as the other components in the later stages of the conduction pathway are called latent pacemakers. The SA-node generates about 70-80 impulses per minute and thus drives the heart at this rate. If for some reason the SA-node fails to generate impulses, the AV-node takes over. It can produce about 40-60 action potentials a minute. The AV-bundle and the Purkinje fibers can also generate impulses (20-40 action potentials per minute) but at much lower rates. Abnormalities in the pacemaker action of the heart can lead to severe problems, as the heart is not able to pump blood properly to the rest of the body. [2,6]

2.1.4 Cardiac conduction disorders & conventional therapy

In cardiac conduction diseases, the pathway of the electrical impulses in the heart is damaged or completely blocked which can lead to life-threatening rhythm disorders. The damage can occur at any stage of the conduction pathway and can influence the impulse generation, conduction, or even both. [14]

A common conduction disorder, known as heart block, is the impairment or blockage of the conduction pathway between the atria and the ventricles. This leads to a decrease in heart rate and cardiac output. There are several levels of heart block according to the severity of the impairment. In first-degree AV-block, conduction of electrical impulses through the AV node occurs but the impulse propagation is abnormally prolonged. In second-degree block, conduction occurs only with some signals and in third-degree AV-block, the conduction pathway is completely blocked. [1]

In adults, the impairment of AV conduction is most often due to acquired conditions such as myocardial ischemia, age-related degeneration, or procedural complications. [14] In children, disorders can either be acquired (e.g. surgical complications, infectious disease) or congenital (e.g. maternal immune disease, structural abnormalities, long QT syndrome). The state-of-art treatment in conduction disorders, to restore heart rate and rhythm to normal, is the implantation of a pacemaker device. [4]

A pacemaker is an electrical medical device, consisting of a computerized generator, a battery, and wires with electrodes, that is used to regulate the heartbeat. It is implanted in the chest cavity or the abdomen, while the wires are guided to the heart. Electrical impulses are delivered to the heart through electrodes that are attached in the cardiac muscle to prompt the heart to beat at a normal rate. [15]

Even though implantation of an electrical pacemaker device is the standard treatment today, it has its disadvantages. Lead complications (e.g. dislocation, fracture, improper stimulation) can cause severe problems for the patient and limited battery life leads to reoperations to replace the power supply. [16] Especially in pediatric patients, the implementation of long-term pacing therapy is a major problem due to the small anatomical size of a child and their inevitable growth. Most often pacemaker leads need to be positioned epicardially rather than transvenously, which is much more invasive and increases the risk for complications. Re-operations are usually inevitable due to finite battery life and lead dislocations caused by size mismatch but lead to extensive scarring and high risk of infection. [3,4] Thus there is a genuine need for innovative therapies especially for children with cardiac rhythm disorders.

2.1.5 Biological pacemakers

Due to the drawbacks in conventional pacemaker therapy, alternative biological approaches are being explored. A number of research groups are focusing on the generation of pacemaker cells by using different cell manipulation techniques and various cell sources. The areas of interest can be roughly subdivided into three categories: gene therapy, cell therapy, and tissue engineering (figure 3). [3,17]

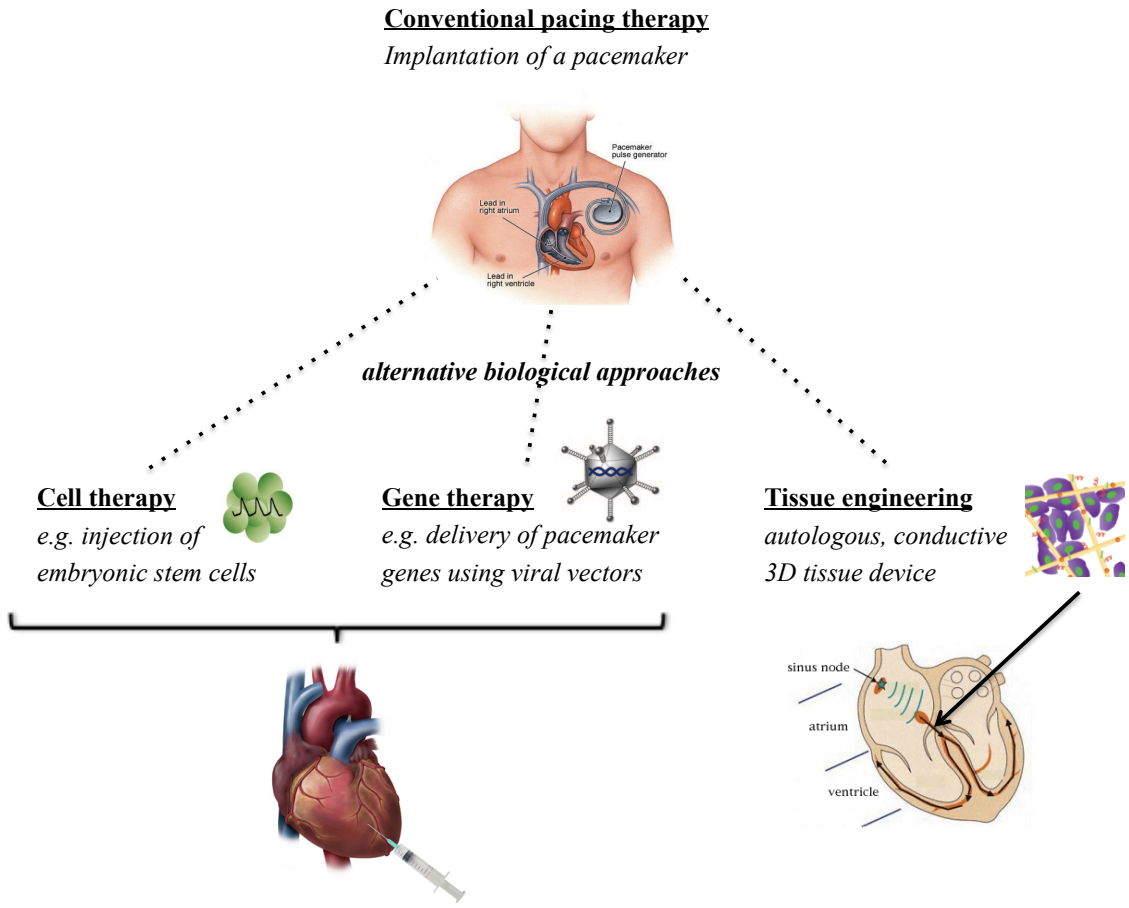

Figure 3. Biological alternatives to conventional pacing therapy. Pictures for schematic from [11,17-20].

In gene therapy, direct manipulation of so called “pacemaker genes” in contractile myocytes has been used to induce pacemaker activity in cells. For example, adenoviral vectors have been used to deliver certain ion channel encoding genes, e.g. potassium/sodium hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channel, into contractile myocytes in an attempt to overexpress distinguished pacemaker ion channels. The overexpression of HCN2 channels to elicit pacemaker activity has been displayed in a canine model. [21]

Miake et al. used viral gene transfer to suppress inwardly rectifying potassium 2 (Kir2) channels in ventricular myocytes to unleash pacemaker activity. Injection of these cells into the ventricle of guinea pigs resulted in spontaneous, rhythmic electrical activity in vivo. [22] Kashiwakura et al. manipulated a synthetic pacemaker channel based on potassium voltage-gated channel subfamily A (Kv1) ion channels by site-directed mutagenesis. Gene transfer of these synthetic pacemaker channels using a viral vector induced pacemaker activity in guinea pig adult ventricular myocardium. [23] Although these approaches have showed great potential in animal models, the use of viral vectors is not an option in clinical applications. [17]

A combination of gene and cell therapy has also been proposed. Human mesenchymal stem cells (hMSCs) have been used as vehicle for gene delivery to provide an alternative for viral vectors. [17, see 24] Human MSCs were manipulated to overexpress HCN2 channels through plasmid transfection and injected into the canine left ventricular wall. The idea was to impart pacemaker currents (I_f) to myocardium through electronic coupling of myocytes with the transfected hMSCs. This induced spontaneous rhythms of left-sided origin on chronic AV-block in the animals. The pacemaker activity persisted for approximately six weeks. [17, see 25] The use of hMSC compared to viral vectors as delivery vehicles in gene therapy is more attractive as they can be acquired in large quantities and are claimed to be immune-privileged. On the other hand, in order for this approach to function, high-degree of gap-junction coupling between the injected cells and the host myocytes is needed. The stability of these junctions is problematic and more research on this part is still needed. [17]

Plain cell therapy using human embryonic stem cells (hESCs) (as stand-alone with no genetic modification) as biological pacemakers has also been explored as an alternative. Spontaneously beating human embryoid bodies (EBs) formed by in vitro culture of hESCs were injected into the left ventricular wall of guinea pigs. Spontaneous action potentials were displayed from the left ventricular epicardium by ex vivo optical mapping. Controls (uninjected or saline-injected) showed no signs of action potentials. The major obstacle to overcome in using hESCs in clinical applications is host immune response and tumorigenesis. [26,27] Over all, even though many advances have been taken in the field of gene and cell therapy, there are many hurdles to overcome in both the use of hESCs and vector-mediated manipulation of human cells in pacing therapy before it can be considered clinically relevant. [17]

The use of tissue engineering (TE) as an alternative biological approach to conventional pacemaker therapy has also been explored [3]. Tissue engineering is "… an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function", as described by Langer and Vacanti in 1993 [28].

Steps have been taken into the direction to create a 3D conductive tissue implant using cells and different scaffold materials to restore AV conduction in the heart. An engineered 3D tissue implant would offer more precisely targeted delivery of the cells to the heart compared to cell injection-based methods described earlier. [3] Also by using autologous materials, immune host response could be prevented.

The idea of fabricating a conductive tissue construct using tissue engineering has been approached by Choi et al. They fabricated collagen-based semi-cylindrical 3D constructs containing fetal rat myoblasts from skeletal muscle. These constructs were implanted in the AV-groove of rat hearts to propose an alternative conduction pathway between the atria and the ventricles. Electrical coupling of the cells within the construct was demonstrated through expression and function of gap junction proteins. Also, the cells were shown to survive at the implantation site for the entire duration of the animal's natural life. However, their design resulted in nearly instantaneous atrial to ventricular depolarization, which is not consistent with the function of the delaying property of the AV-node. This could lead to undesired arrhythmias in the heart. Choi et al. proposed alternative cell sources with more desirable conduction properties should be explored. Nevertheless, this showed that it is possible to electrically connect the atria and the ventricles through engineered tissue constructs and thus steps are taken into the right direction in the developing of a tissue engineered biological alternative to conventional pacemaker devices. [3]

2.2 Engineering conductive tissue

In this chapter, the principle of tissue engineering (TE) is presented. The benefits of using hydrogels, especially fibrin gel, in TE are highlighted and the criteria for engineering conductive tissue for the heart presented.

2.2.1 Principle of tissue engineering

The principle of TE is presented in figure 4. The idea is use cells from a patient, combine them with a scaffold to create a 3D tissue construct, and implant it at the site where it is needed to replace or restore defective tissue. Autologous cells can be isolated from a tissue biopsy taken from the patient. The cells are amplified in vitro to achieve sufficient quantity and are combined with a scaffold. The construct is cultivated e.g. in a bioreactor for further tissue development. After this, the viable tissue construct is implanted in the patient as an autologous replacement. [28]

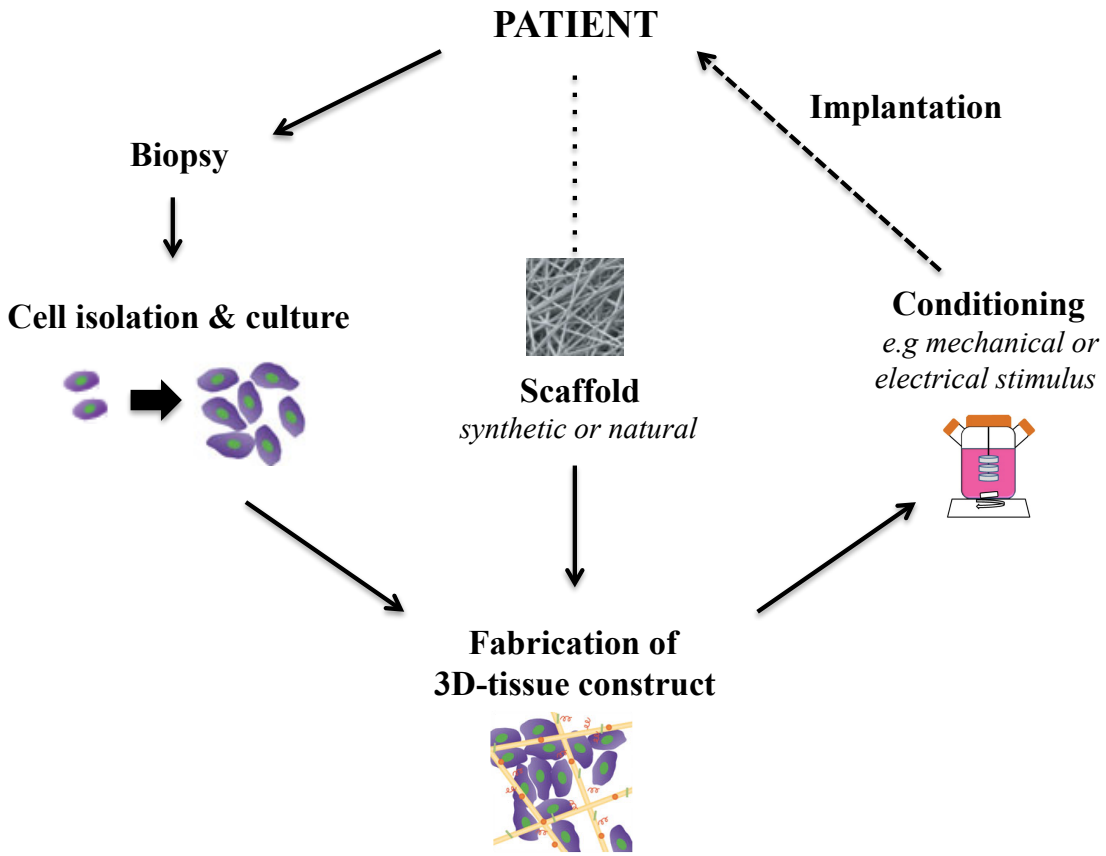

Figure 4. Principle of tissue engineering. Pictures for schematic from [11,29,30].

2.2.2 Fibrin-based tissue engineering

The scaffold is used in TE as a platform for the cells to organize themselves into a 3D architecture – in other words it serves as a synthetic ECM to the cells. A suitable scaffold for use in TE needs to meet some specific requirements. First of all, the scaffold needs to be completely autologous and neither the scaffold material nor its degradation products should cause any toxicity or inflammation in the body. Also, it should promote cell adhesion and proliferation, degrade controllably to adapt to the developing tissue, and allow homogenous cell distribution to facilitate uniform tissue formation. Easy fabrication of the material into 3D structures and high mechanical strength are also desirable characteristics. [29,31]

Hydrogels have received much attention in the field of TE for their structural similarities to the natural ECM. Hydrogels are 3D networks of cross-linked hydrophilic polymers and are thus highly hydrated materials. Several hydrogel materials, both synthetic and natural, have been used in TE applications. [32]

Fibrin gel is an attractive material for TE applications as it fulfills many of the criteria set for the scaffold. Fibrin is the major protein component involved in wound healing. It is converted from fibrinogen, a plasma protein, by the action of thrombin and calcium. The main components for the scaffold, fibrinogen and thrombin, can be autologously derived directly from the patient's blood, which prevents problems of immunogenicity. As the starting components of blood are in solution form, gels can be

easily molded into the desired construct e.g. through injection molding. Also, fibrin gel provides high cell seeding efficiency and homogenous cell distribution by gelation entrapment. [29] The degradation of the fibrin gel can be controlled by the use of a fibrinolysis inhibitor tranexamic acid [33].

2.2.3 Principle of the Biopacer

The idea of the Biopacer project is to develop an autologous conductive tissue device (CTD) that will serve as an electrical conduit between the atria and the ventricles of the heart. Pediatric cardiomyocytes are isolated and used together with a fibrin-based scaffold to produce a tissue construct that is completely autologous and has the ability to grow with the patient. The anticipation is to use this type of engineered tissue to restore atrioventricular electrical conduction in children with complete heart block.

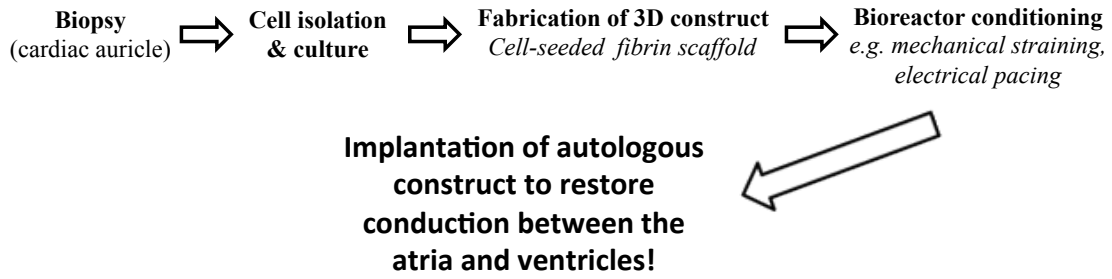

Figure 5. Principle of engineering a conductive tissue device (Biopacer).

2.2.4 Requirements for engineering a conductive tissue device

There are specific aspects, structural and functional, of the native AV node that need to be taken into account when engineering a CTD. First of all, the engineered tissue should consist of several cell populations (cardiomyocytes, fibroblasts, endothelial cells). [7] These cells should either rearrange themselves within the scaffold or be arranged by assembling functional tissue units separately to achieve the complex, highly organized native tissue-like architecture. [34] All these cell types have a specific role in the structure and function of the tissue. Cardiomyocytes make gap junctions with each other to form a functional syncytium to allow propagation of electrical impulses. Fibroblasts play a significant role in providing mechanical stability to the structure by synthesizing ECM. Endothelial cells, on the other hand, provide the vascularization to ensure sufficient oxygen and nutrition supply. [7] All these cell types need to be accounted for in order to fabricate a functional conductive tissue device. It is noteworthy to take into account that cardiomyocytes have a very limited or even nonexistent potential to proliferate. [7] This means, that the availability of the cell source plays a significant, usually limiting, role in engineering conductive tissue for the heart.

The engineered CTD should integrate into the implantation site in a way that the cells from the engineered tissue would electromechanically couple with the existing cells in the conduction pathway to allow the propagation of impulses from the atria to

the ventricles [7]. Also, the electrical impulses should propagate one-way at normal atrial activation thresholds and not give rise to the potential of arrhythmogenesis. Ideally, the engineered tissue device would also delay conduction as occurs in the native AV-node. To be applicable for clinical applications, the engineered tissue device should be autologously derived, easy to fabricate and implant, and it should have the potential to grow with the patient while maintaining conduction capability. [3] The use of autologous cells maximizes patient safety as it decreases the risk of immunogenic response. To be considered as long-term pacing therapy, it should function for the lifespan of the patient minimizing the need for reoperations.

2.2.5 Bottom-up tissue engineering

Traditionally, tissue constructs are fabricated using a top-down (figure 6b) approach: cells and a scaffold material are combined and cultured with the aid of e.g. perfusion or mechanical stimulus until the cells remodel the support structure by producing the ECM. However, this requires the cells to rearrange themselves in complex tissue-like microarchitecture. For most tissues, having the right microarchitecture and well-defined cellular organization is crucial. For example cardiac tissue is highly organized and cardiomyocyte cell-cell interactions are extremely important to the functionality of the tissue. Thus trying to mimic the alignment and high-degree organization of native cardiac tissue is advantageous to engineering functional tissue constructs. [35]

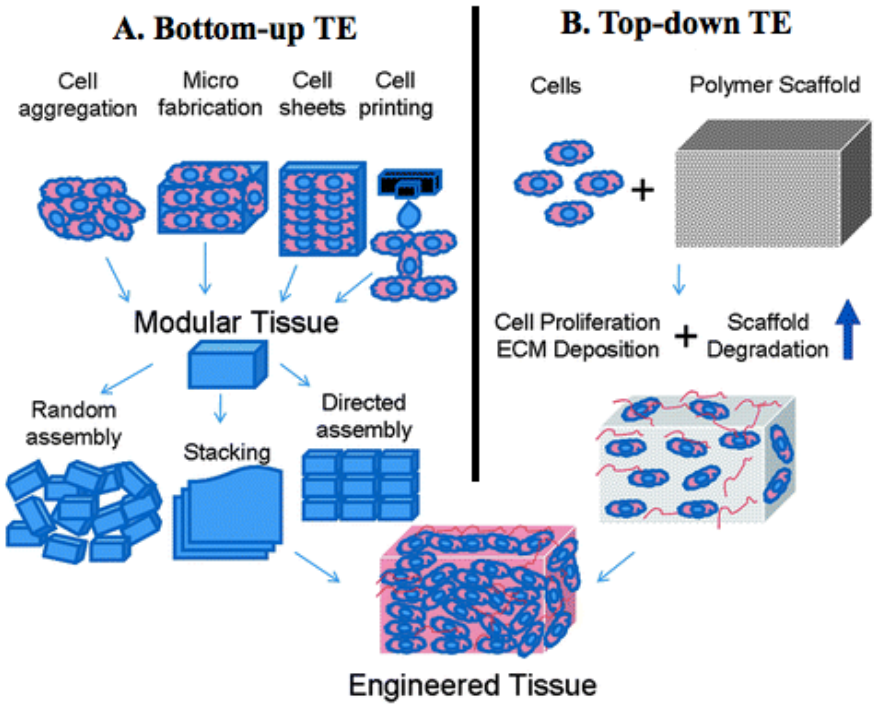

Figure 6. Bottom-up (A) and top-down (B) approaches in tissue engineering. Picture from [36].

This is the aim in bottom-up (figure 6a) tissue engineering, in which microscale structural features of tissues are fabricated as modules and combined together to create larger tissue constructs. In other words, tissue modules can be used as building blocks to create biomimetic hierarchical structures. Several microfabrication techniques have been used to create spatially organized tissue modules. [34,36] By fabricating structures in microscale, the cell-cell interactions can be evaluated better and the quantity of cells needed for fabrication can be minimized.

The group where this thesis was carried out has extensive experience and knowledge in cardiovascular tissue engineering. However, the techniques applied and established by the group have always been done using the top-down approach. In this thesis, microfabrication techniques that can be used to fabricate constructs using the bottom-up approach are established for the group. Two methods are presented and exploited to create cell-laden hydrogel modules that can be applied in the fabrication of a conductive tissue device.

Because of the complexity of the cardiac tissue with all the different cell types and the high-degree of organization, the application of microfabrication techniques is attractive in creating a CTD. Tissue modules of different cell types (e.g. cardiomyocytes, fibroblasts, and endothelial cells) can be microfabricated separately and later assembled together. This way, the organization and tissue formation of the separate components (e.g. vascularization, ECM) can be preformed and then combined to create a more complex but highly structured construct.

This thesis focuses on two fabrication techniques that can be used to create cell-laden hydrogel tissue modules and on how these components could be used as building blocks to further assemble larger scale CTD that would incorporate all the structural components (aligned cardiomyocytes, vasculature, ECM) present in native tissue. However, the microfabrication techniques established here can be transferred to various other TE applications and thus go far beyond the mere fabrication of a conductive tissue device. These methods could be used to tackle microvasculature insufficiency of engineered tissue, which is a major problem in TE. By preforming the structural outline for capillary formation using cell-laden hydrogel microstructures, vascular development could be induced and thus the problem of insufficient vascularization of engineered tissue constructs that exceed the diffusion limits of oxygen and nutrients could be confronted. Alternatively, the techniques could be used to fabricate multiple small-scale functional units, which could be stacked or assembled together to form tissue constructs consisting of repeating functional units. This could be advantageous for engineering tissue for example for the liver, which consists of repeating functional units (lobules).

2.3 Microfabrication techniques

Microfabrication technology is a generic term for techniques that allow the fabrication of miniature components and devices with micrometer resolution [37]. In tissue engineering, a number of techniques, e.g. emulsification, photolithography,

microfluidics, and micromolding, have been established to allow fabrication of microscale hydrogel tissue structures. These techniques can be used to engineering small, biologically relevant size structures with a controllable shape (spheres, rectangular blocks, fibers etc.). Fabrication of hydrogels in microscale with controlled 3D arrangement of cells can provide a way to mimic complex structures and architectures of biological tissues. [34,38]

In this thesis, two microfabrication techniques are used to fabricate fibrin hydrogel-based scaffolds for TE purposes: micromolding in capillaries (MIMIC) using soft lithography and fabrication of microfibers by extrusion.

2.3.1 Soft Lithography

Soft lithography is a group of techniques designed to fabricate microscale or nanoscale structures of soft materials (e.g. polymers, gels, and organic monolayers) based on printing, molding, and embossing. The methods are all based on using a patterned elastomeric polymer as a mask, stamp, or mold. These techniques came to life to provide a low-cost, simple, and cell compatible alternative to photolithography and electron-beam lithography. [35,39]

The main tool in soft lithography is the use of a polydimethylsiloxane (PDMS) layer with embossed structures on the surface. The PDMS is fabricated by replicating a master as shown in figure 7. PDMS pre-polymer is prepared by mixing two components, a base and a curing agent, together. It is then poured over the master and cured. Silicone hydride groups in the curing agent react with vinyl groups in the base resulting in a cross-linked elastomer. The PDMS can then be peeled off from the master completely intact due to low surface free energy and elasticity of the material. [40]

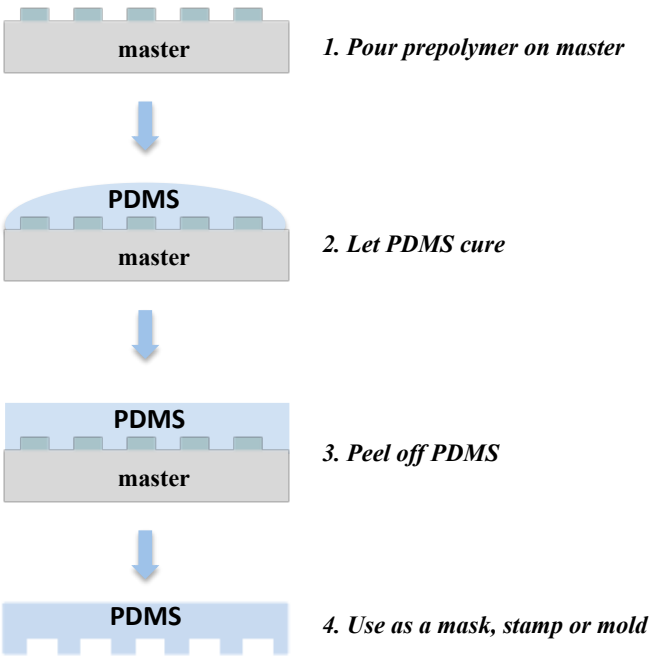

Figure 7. Replication of PDMS from a master.

2.3.2 Techniques in soft lithography

The patterned PDMS can be used in a number of different ways –as a mask, stamp or mold. The most common applications are presented figure 8 and shortly explained below.

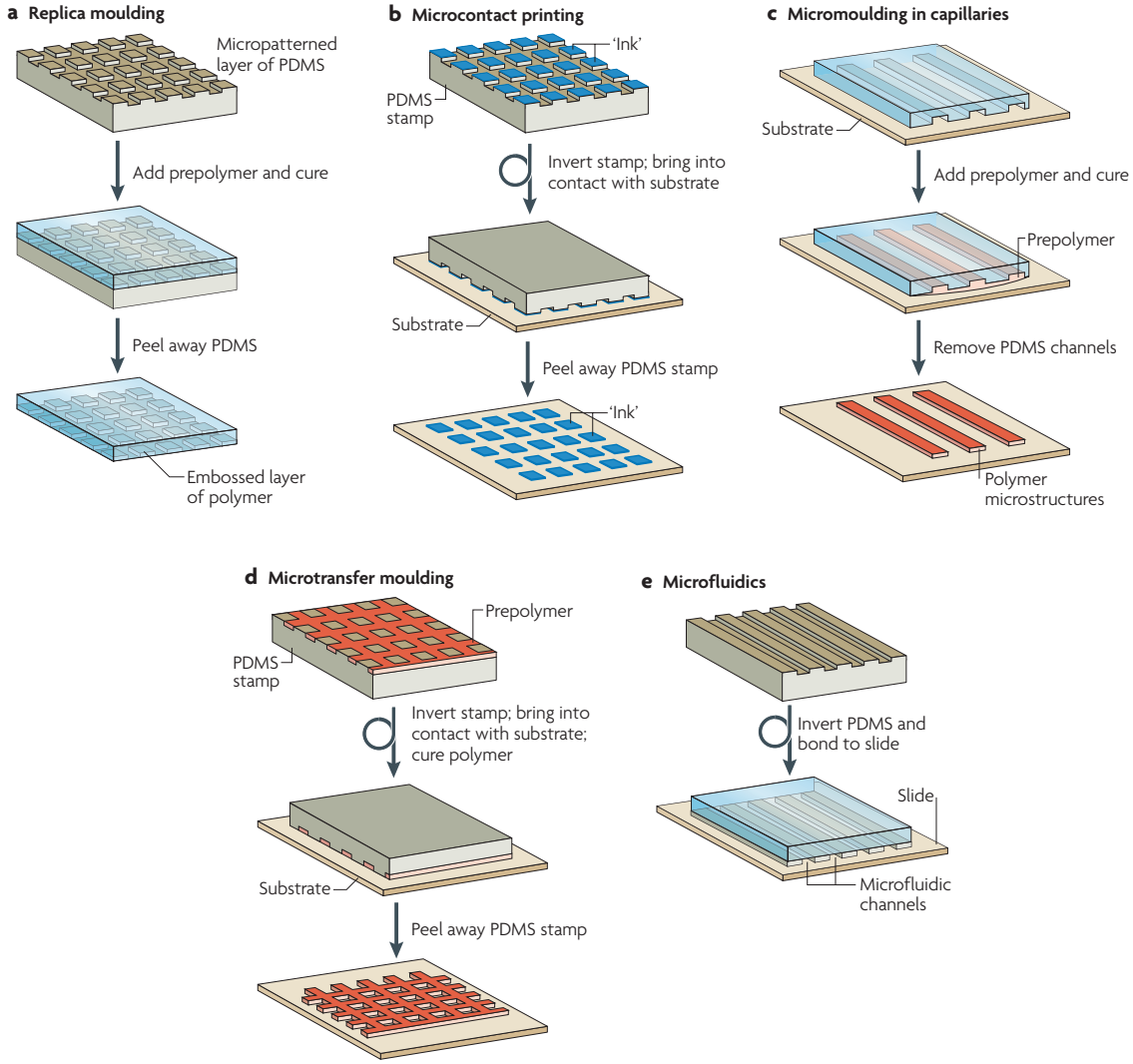

Figure 8. Techniques used in soft lithography: (A) replica molding, (B) microcontact printing (μCP), (C) micromolding in capillaries (MIMIC), (D) microtransfer molding (μTM), and (E) microfluidics. Picture from [35].

Replica molding (figure 8a) is a single step technique used to duplicate the topographical patterned surface structures of the PDMS master. It is the same concept used to fabricate the PDMS layer –a mold is covered with a liquid polymer, cured, and peeled off to form exact structures from the master. The exact dimensions of the patterns can be transferred to another polymer.

In microcontact printing (μCP) (figure 8b), the PDMS is used as a stamp to create patterns on surfaces. The stamp surface is submerged into a solution, and then pressed

onto the surface to transfer the pattern of the stamp to the substrate surface. This method has been used to create patterned layers of e.g. proteins and other small biomolecules.

PDMS can be patterned in a way that when brought together with a surface it forms microchannels. The PDMS is sealed to the surface of a solid substrate (e.g. glass slide) through van der Waals interactions, forming channels that can then be filled with pre-polymer. The filling can occur either by capillary action or by suction and after polymerization, the PDMS is carefully peeled off to reveal micropatterned lines left on the surface of the substrate. This technique is called micromolding in capillaries (MIMIC) (figure 8c).

In microtransfer molding (μTM) (figure 8d), the patterned PDMS layer is covered with a pre-polymer and the excess is scraped off. The pre-polymer-PDMS combination is then brought into contact with a surface. After polymerization, the PDMS is carefully peeled off revealing the patterned polymer structures on the surface of the substrate.

As previously mentioned, microfluidic channels can be formed by sealing a structured PDMS piece with a glass slide. The bond between these two substrates is tight enough to allow micromolding of e.g. hydrogels, but channels can leak if the fluid is put under pressure. A tighter bond between the two substrates can be made by exposing the two surfaces to oxygen plasma, which activates the surface and allows the covalent bonding of the PDMS to the glass. This way the microchannels can withhold pressures as high as 50 psi exerted from e.g. pumping fluid through the channels. With this technique, called microfluidics (figure 8e), a sealed microfluidic device is formed. [35,39]

2.3.3 PDMS properties

PDMS is a transparent, elastomeric silicon-based organic polymer. Liquid PDMS conforms to the shape of a mold and, due to low surface free energy and elastomeric behavior, it also releases easily from them. This makes it a very attractive material for use in soft lithography applications. The transparency of the material is good for visualization purposes. It is also impermeable to liquid but allows gases to pass through the bulk material. Most important, PDMS is non-toxic and inert which makes it compatible for use in TE approaches. [40]

PDMS contains repeating $–OSi(CH_3)_2$ units which makes it hydrophobic [41]. In micromolding and microfluidic applications, a hydrophilic surface is required to promote the filling of the channels and also to ease the sealing of the PDMS to the wanted surface. This can be achieved by treating the PDMS with oxygen plasma. [42]

PDMS seals easily to itself or other surfaces and it can be done reversibly or irreversibly depending on the wanted outcome. Reversible sealing enables the use of PDMS to pattern for example biomolecules by stamping or microfluidic molding. The PDMS can be easily peeled off the surface and even re-used if wanted. This reversible contact, induced by van der Waals contacts, can be made to clean, smooth surfaces without even plasma treating the PDMS. If the PDMS surface is first treated with oxygen plasma and immediately (<1 min) placed on a clean surface (e.g. PDMS or

glass), it will make a permanent bond. An even stronger bond can be made if both surfaces are oxidized and then immediately sealed together. [40] This is valuable when a microfluidic device is designed to be used under increased pressure e.g. as a flow chamber.

A noteworthy characteristic of microfluidic channels is the laminar flow of fluids; the velocity at a given spot in the channel is constant in time. The Reynolds number (Re) is a dimensionless value that describes the behavior of fluids. It is described by $Re = \rho u D_h/\mu$, in which ρ is the density of fluid (g per cm^3), u is the velocity of the fluid (cm s^{-1}), D_h is the hydraulic diameter (cm) of the channel, and μ is the viscosity of the fluid (g per cm per s). When the Re is less than ~2000, fluid is laminar. A Re over ~2000 correlates to a turbulent flow. The flow in microchannels, mainly due to the small dimensions, is typically laminar, as the Re does not exceed the cut-off value of 2000. This means that the flow rate at a given region of the channel is constant in time. [35]

In microscopic systems, the surface properties play an increasingly important role in the performance of the devices due to increased surface-to-volume ratios. [42] In soft lithography applications, special attention needs to be given to the surface properties of PDMS, especially to its hydrophobic nature, and how it interacts with different biological materials.

There are two major issues to overcome in using PDMS in micromolding applications with hydrogels. First, the hydrophobic nature of PDMS is a major obstacle as the filling of the small, microscale channels with aqueous solutions is almost impossible unless assisted with suction (e.g. using a Pasteur pipette). A hydrophilic surface is compulsory so that the channels can be filled with capillary forces with no external assistance needed. Also, a hydrophilic surface allows the PDMS to seal better to the wanted surface, in this case a glass slide. [42] During oxygen plasma treatment of PDMS, the methyl groups (Si-CH_3) are replaced with polar silanol (Si-OH) groups [41].

$$-O-\left[\begin{array}{c} | \\ Si-O \\ | \end{array}\right]_n- \xrightarrow{O_2 plasma} -O_n-\left[\begin{array}{c} OH \\ | \\ Si-O \\ | \\ OH \end{array}\right]_{n-4}- \qquad (1)$$

The PDMS surface retains its hydrophilic surface for some hours in air but due to mobile, low molecular weight monomers migrating from the bulk to the surface it will eventually turn hydrophobic again. It has been shown that the time of hydrophilicity is highly dependent on the storage conditions rather than the plasma parameters used. Kim et al. compared different solutions (e.g. PBS, air, and water) for storage after plasma treatment and discovered that water retained the surface hydrophilicity of the PDMS the longest. [43]

The second obstacle to overcome is the adhesion of hydrogels to the PDMS. The PDMS is used as a temporary mold to fabricate hydrogel lines using micromolding and is peeled off the glass substrate after polymerization. The intact demolding of the hydrogel lines is possible if the PDMS surface is modified to resist the nonspecific adsorption of proteins. [44] Hydrogels, e.g. fibrin gel that is a protein network formed by the crosslinking of fibrinogen and thrombin, adsorbs to the PDMS due to the hydrophobic interactions. To overcome this problem, the surface of the PDMS is to be made hydrophilic to prevent protein adsorption. Treating the surface with only oxygen plasma, as already mentioned before, makes the surface hydrophilic but it is not sufficient enough to prevent nonspecific protein adsorption. An additional protein-preventing polymer layer, e.g. PEG or PEO –based, is needed. [45]

Several groups have used Pluronic® as a surfactant to prevent the adhesion of fibrin to PDMS. [46,47] Pluronic® is a non-ionic surfactant used in many biotechnological applications to prepare protein-resistant biomaterial surfaces [48]. It is an amphipathic, triblock copolymer with a general structure of $(PEO)_m(PPO)_n(PEO)_m$. Pluronic® has low toxicity and immunogenic response making it suitable for use in medical applications. [49] In this thesis, Pluronic® F-127 ($m = 100$, $n = 65$) was used as a surfactant.

Pluronic® binds to the hydrophobic PDMS surface by the PPO segment as the PEO tails extend towards the aqueous solution. This conformation makes the surface of the PDMS hydrophilic and nonionic, which prevents the adhesion of the proteins to the surface. [48] If the PDMS is pre-treated with oxygen plasma, creating a hydrophilic surface, and then coated with Pluronic® the conformation of the adsorbed polymer is different. The Pluronic® adsorbs in a more flat conformation through hydrogen bonding of POE segments to the surface (see figure 9). [49] The PEO can then effectively prevent the adsorption of proteins by steric repulsion, as is shown by Amiji et al. with fibrinogen. [48]

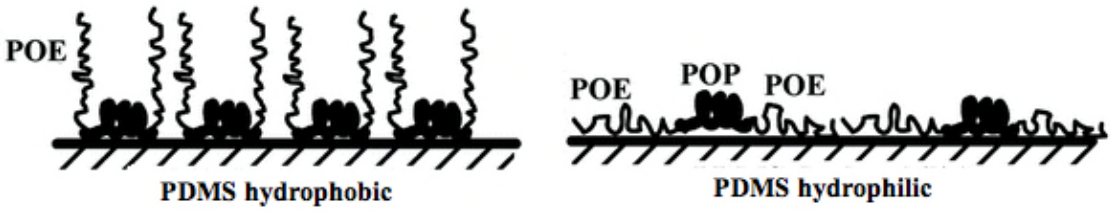

Figure 9. Adsorption of Pluronic F-108 onto non-treated (hydrophobic) PDMS (left) and oxygen plasma treated (hydrophilic) PDMS (right). Picture adapted from [49].

2.3.4 Micromolding of 3D hydrogels using soft lithography

Microfabrication techniques using soft lithography have been widely used in tissue engineering. In this chapter, an overview is presented on how these techniques have been utilized to micromold 3D hydrogel constructs for TE applications.

Bian et al. fabricated muscle tissue constructs using PDMS tissue molds with mesoscopic-sized posts in order to form a muscle sheet with elliptical pores formed around the posts. A mixture of skeletal muscle cells and fibrin gel were cultured in the PDMS molds for two weeks. Due to gel anchorage at the end of the posts and tissue contraction, a strain field formed within the gel, which guided the cells to align along the pore boundaries. Evaluation of the tissue showed that the technique induced cell alignment in the 3D constructs. [46]

Trkov et al. studied vasculogenesis under spatially controlled conditions by using MIMIC. A mixture of endothelial cells and fibrin gel was molded in parallel microchannels to evaluate the interactions of the cells (e.g. sprouting) with each other as a function of distance over time. Endothelial cells of several different origins were used in the experiments. [50]

Raghavan et al. also spatially patterned endothelial cells within micromolded hydrogels in the attempt to evaluate the possibility to control endothelial tubulogenesis. They used microstructured PDMS with parallel grooves to form collagen gel tubes with cells embedded inside. The PDMS mold was faced grooves upwards, and the collagen-cell mixture was added on top. The excess was scraped so that only the grooves were filled. The tubes were then cultured and later the tubes were embedded with more collagen and removed from the PDMS mold. This method of organizing cells within channels filled with collagen created a novel platform to promote endothelial tubulogenesis. [51]

McGuigan et al. described a soft lithography technique to encapsulate cells in 3D gel modules of different shapes (e.g. pillars). A master that consisted of pillars with diameters ranging from 40 to 1000 μm was cast with PDMS to replicate a PDMS membrane with holes of the same dimensions. These holes were used as molds to fabricate round pillar-shaped 3D hydrogels (e.g. collagen, Matrigel™, and agarose) encapsulated with cells. The membrane was submerged into the gel-cell suspension. After incubation, the faces of the membrane were removed of excess gel and the modules embedded in the holes were gently shaken out into further cultured in suspension. This technique allowed the fabrication of 3D hydrogel modules of various different shapes. [52]

Yeh et al. micromolded cells encapsulated into methacrylated hyaluronic acid (MeHA) or poly(ethylene glycol) diacrylate (PEGDA) hydrogels using a PDMS stamp. Cells were suspended in pre-polymer solutions along with a photoinitiator and deposited onto a PDMS mold. A PDMS coverslide was placed on top and the polymer liquid was photopolymerized using ultraviolet (UV) light. After polymerization, the coverslide was

removed revealing the cell-encapsulated microgels which could be further cultivated. [53]

2.4 Fabrication of microfibers for tissue engineering applications

Microscale tubular tissue constructs, microfibers, have been fabricated mainly by utilizing microfluidics. The unique laminar-flow properties within microfluidic channels present an advantages approach to generating hydrogels with controlled features. Several materials, both synthetic and natural, have been explored as options in creating hydrogel-based microfibers for TE. [54]

Previously, hydrogel-based microfibers have been generated for TE purposes by using conventional spinning methods. Hu et al. used a triple-orifice spinneret to fabricate hollow and solid fibers of gelatin-hydroxyphenylpropionic acid (Gtn-HPA) [55]. A similar spinning method was used by Takei et al. to generate hollow alginate microfibers [56].

More recently, the fabrication of hydrogel microfibers has moved into the direction of utilizing microfluidic devices. Co-axial flow-based microfluidic devices embedded with a glass capillary tube have been used to fabricate microfibers of different shapes and sizes. [54] Shin et al. used this technique to form calcium alginate microfibers. By controlling the flow rate, the diameter of the fibers could be modulated. Cells were encapsulated into fibers successfully to demonstrate the possibility of the technique for tissue engineering purposes. [57] Other materials, such as poly(lactic-co-glycolic acid) (PLGA) and chitosan, have also been used to fabricate microfibers [54,58,59]. Hwang et al. fabricated PLGA microfibers of different diameters using a co-axial flow-based microfluidic device by altering the flow rate. They investigated the effects of the diameter of microfibers on cellular orientation by culturing fibroblasts on the surface of the PLGA fibers. They demonstrated that the orientation of the cells increased with decreased diameters. This provides a technique that can be applied to form aligned tissue. [59]. Chitosan microfibers have been fabricated using a cylindrical microchannel-based approach, without the use of glass capillary tubes. Kang et al. fabricated chitosan fibers of different compositions in multiple channels at a time scaling up the production process. [54,58] The flow-based microfluidic device has also been used to fabricate photocrosslinkable hydrogels, e.g. 4-hydroxy butyl acrylate (4-HBA) and polyvinyl alcohol (PVA), into fibers [59].

Shimoyama et al. used a plain capillary glass tube as a microfluidic channel to fabricate highly aligned fibrin microfibers. They seeded skeletal muscle cells on the surface of the fibers, which migrated into the bulk, aligned in the longitudinal direction, and formed multinucleus myotubes. This demonstrated a novel approach to fabricate highly aligned skeletal muscle fibers for TE. [60]

Cornwell et al. also used fibrin as a material to fabricate microfibers. Fibers were fabricated by coextruding fibrinogen and thrombin through polyethylene tubing. By

assembling microfibers together, larger scale scaffolds were formed either by bundling the fibers together without crosslinking or using UV light as a crosslinking agent. The uncrosslinked constructs were shown to support fibroblast proliferation and cellular alignment as the UV crosslinked constructs exhibited high mechanical properties but failed to support cell proliferation. The anticipation was to take advantage of the wound healing properties of fibrin by using the fibrin microfibers to help guide wound healing by directing cellular responses in the aligned system to the injury site to induce the regeneration. [61]

3 CARDIAC AURICLE AS A SOURCE FOR AUTOLOGOUS CARDIOMYOCYTES

A mixed population of cells for primary culture is obtained from the right auricle of pediatric patients two months to eleven years of age undergoing open-heart surgery. Consent is required from the parents for the use of the tissue for research purposes. The surgical procedures were carried out at the Department of Heart Surgery for Children and Adults with Congenital Heart Defects at the University Hospital Aachen (UKA).

The cardiac auricle is an ear-shaped appendage found on both atriums of the heart. The tissue contains a mixed population of cells, e.g. cardiomyocytes and fibroblasts. During open-heart surgery, a heart-lung machine is used to replace the function of the heart and lungs. When connecting the patient to the heart-lung machine, a cannula is inserted into the right atrium to draw blood from the body, which is then oxygenated, and brought back to the body. During the insertion of the cannula into the atrium, a small piece of tissue is obtained from the atrial appendage as “waste material”.

3.1 Materials and methods

3.1.1 Cell isolation

After surgery, the sample is transported in sterile conditions to the Helmholtz Institute Laboratory in a transport buffer, which is composed of calcium-free Krebs buffer solution (KRP) and 2,3-Butanedione monoxime (BDM) (appendix 1). The tissue sample is stored in the buffer at 4°C until the start of the isolation procedure.

Cells are isolated according to an enzymatic isolation protocol that has been previously used in the group. The complete isolation procedure is done sterile in a class II laminar flow bench (Heraeus HeraSafe HS 12). First, the connective tissue is removed and the sample is cut into small pieces using a scalpel (Feather Disposable Scalpel No. 23). The pieces are collected with tweezers and placed into a 50 ml falcon tube (BD Biosciences) with 45 ml of sterile KRP+BDM buffer. The falcon with the tissue pieces is gently mixed for five minutes and the pieces are left to settle at the bottom of the falcon. The supernatant is discarded through a cell sieve (BD Falcon Cell Strainer 100 µm Nylon, REF. 352360). This washing step is repeated twice.

The dissociation of the tissue is done using a two-step enzymatic digestion process. First, the tissue pieces are incubated with 5 ml of washing buffer (KRP with 6 U/ml proteinase 24, Sigma P-8038; 200 U/ml collagenase, Sigma C-9263) for 30 minutes at 37°C with constant mixing. The supernatant is discarded through a cell sieve, replaced

with a lysis buffer (KRP with 400 U/ml collagenase), and incubated as done previously. The solution is put through a sieve to remove any left over solid pieces. The final supernatant holds the released cells collected from the isolation.

The supernatant with the cells is centrifuged for 12 minutes at 200 G and re-suspended into 4 ml of medium (Gibco Dulbecco`s Modified Eagle Medium (DMEM); 10% Fetal Bovine Serum, PAA). The cell suspension is seeded out into a 75 cm^2 cell culture flask (Greiner Bio-One Tissue Culture Flask 250ml 75cm^2 with filter) and placed into an incubator (Binder CB 210) at 37 °C and 5 % CO_2 for four hours. This pre-plating procedure is carried out as a purification step to achieve a higher percentage of cardiomyocytes in the co-culture, as fibroblasts and endothelial cells adhere faster than the cardiomyocytes [62].

After four hours, the medium along with the non-adhered cells is carefully removed with a pipette. This contains the majority of cardiomyocytes that are obtained from the isolation, which should be immediately used for experiments as the cardiomyocytes have a limited proliferation capacity and do not benefit from being cultured in a flask.

If further cultured, the fibroblasts that are not eliminated by the pre-plating step would eventually take over the entire flask and the cardiomyocytes would be lost. Fresh medium is added to the pre-plating flask and the adhered cells, which are mainly fibroblasts, are placed in an incubator for further proliferation.

3.1.2 Immunocytochemistry

Cells are seeded into 96-well plates (Greiner Bio-One) straight from isolation for immunocytochemical staining. The cell culture medium is aspirated and -20°C methanol is added for 30 minutes for fixation. The cells are washed using PBS (Gibco Dulbecco`s Phosphate Buffered Saline) for 10 minutes and then incubated with 3% Bovine serum albumin (BSA, Sigma A-9647) in PBS for 30 minutes to block unspecific binding sites. The primary antibody diluted in 3% BSA is added and incubated at 37°C for 60 minutes. After washing with 3% BSA twice, samples are incubated with the second, fluorescent antibody, diluted in 3% BSA for 60 minutes at 37°C. Samples are washed again, now with PBS, and the cell nuclei are counterstained with DAPI (Molecular Probes) for five minutes at room temperature. Finally, the cells are washed three times with PBS and visualized using a microscope. Negative controls are treated as stated above except leaving out the primary antibody during the first incubation. Images were taken using an AxioImager microscope (Carl Zeiss) in combination with a mounted digital camera (Zeiss, AxioCam MRm).

Primary Antibody	Company	Dilution	Secondary Antibody
Anti-Sarcomeric alpha Actin	Thermo Scientific (MA1-21597)	Undiluted	Anti-mouse
Secondary Antibody	**Company**	**Dilution**	
Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L)	Molecular Probes® (A11059)	1:400	

Table 1. Primary and secondary antibodies used for immunocytochemical staining of cardiomyocytes.

3.2 Results and discussion

Cardiac auricle tissue consists of several cell types (cardiomyocytes, fibroblasts, and endothelial cells) and thus a co-culture is obtained from the isolation. In theory, all these cell types could be isolated from the cardiac auricle tissue biopsy, but in reality the implementation is much more difficult.

First, the availability of the cell source is very limited due to the fact that biopsies are obtained from pediatric patients undergoing open-heart surgery. It is not an everyday operation and also consent from the parents is needed. The tissue biopsies received are very small in size, which already indicates that large numbers of cells cannot be obtained.

Secondly, a majority of the cells are lost during the isolation procedure. This was observed by calculating the cells using a hemacytometer with trypan blue. Trypan blue is a dye that selectively colors dead cells blue making it easier to evaluate the relation between live and dead cells. After pre-plating, approximately 150 000-400 000 cells, are obtained from each isolation. This is a very small cell number to use in preliminary experimental studies.

Generally a very high density of cardiomyocytes is needed for engineering cardiac tissue due to the limited proliferation capacity of the cells. For example, Radisic et al. used a density of 50-100 million cells/cm^3 when engineering collagen-based constructs for cardiac TE[63]. This number corresponds to the cardiomyocyte density present in the native adult rat heart. Also, lower concentrations have also been used. Ye et al. encapsulated rat cardiomyocytes in fibrin gel constructs at a density of 5 million cells/ml of gel [47]. Thus the cell number obtained from the isolation is very low to be applied in TE approaches.

If the cells are seeded into flasks for further cultivation, keeping several cell types happy at once in a co-culture is very difficult. For example, endothelial cells are quickly lost, as the medium used for culture does not support growth and proliferation of these cells. Also, cardiomyocytes have a very limited or even nonexistent proliferation capacity thus the cell number cannot be amplified by further cultivation. As a result, if

the cells are seeded into culture flasks for further cultivation, cells that proliferate the fastest and receive a sufficient amount of nutrients, take over. This means that eventually the co-culture has turned into a culture of cardiac fibroblasts.

Even if the cells are used immediately for experiments, the percentage of cardiomyocytes obtained from the isolation is very low. Using the pre-plating step in the isolation procedure, some of the fast-adhering fibroblasts and endothelial cells can be eliminated which somewhat increases the cardiomyocyte purity. In figure 10, the immunocytochemical staining of the cells isolated from the cardiac auricle is displayed and shows that a co-culture of cells is obtained. Cardiomyocytes stain positive for alpha sarcomeric actin (green), a marker for alpha skeletal and alpha cardiac muscle actins. As the culture medium used does not support the growth and proliferation of endothelial cells, the cells negative for alpha sarcomeric actin can be concluded as cardiac fibroblasts.

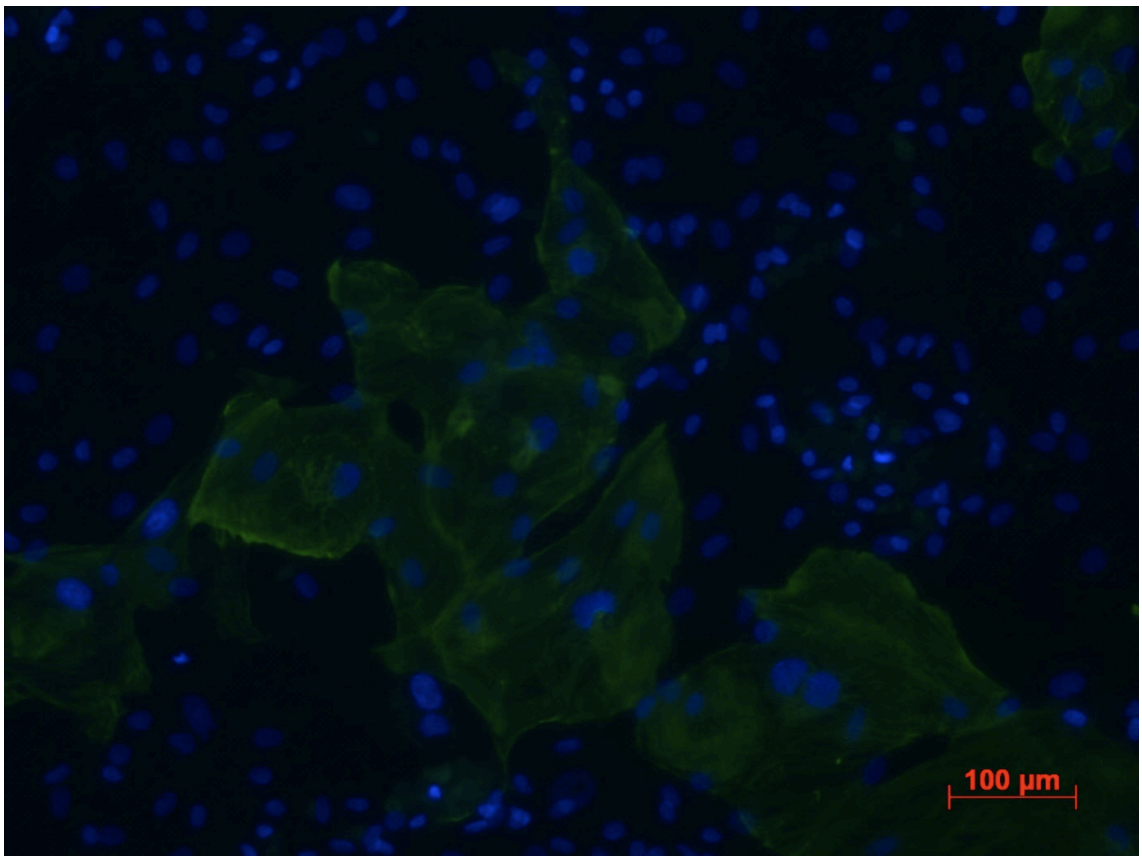

Figure 10. Immunocytochemical staining of cells isolated from a pediatric cardiac auricle tissue biopsy (day 42 of culture). Cardiomyocytes stain positive against alpha sarcomeric actin (green). Cell nuclei are stained using Dapi (blue).

As a result of the difficulty to obtain sufficient numbers of cardiomyocytes from the human cardiac auricle biopsy, the use of microfabrication techniques is explored to overcome the limitation of low cell numbers. By creating already aligned or at least high-density cardiomyocyte fibrin gel constructs in microscale, the cells can be

organized more efficiently compared to homogenously distributing the cells with a macro-scale construct using the top-down TE approach.

For microfabrication applications, smaller volumes of fibrin gel are needed, which means that it is easier to fabricate constructs with high cell densities. The volumes used in microstructuring can be expressed in microliters as opposed to most top-down TE fabricated constructs that require volumes measured in milliliters. As the cardiomyocytes have a very limited if not nonexistent potential to proliferate, they need to be seeded at very high densities into the constructs to allow the cells to form cell-cell connections via gap junctions. This is crucial to the functionality of an engineered conductive tissue device.

Even though the use of microfabrication techniques minimizes the cell number needed for the constructs, the number of cells obtained from each isolation is still not sufficient. As mentioned before, the rarity of the samples received and the small cell quantities obtained creates limitations. As a result, the cell source in not feasible for preliminary experiments to establish new techniques. Due to these reasons, the more abundant cardiac fibroblasts are used in this thesis. As the focus of the thesis is to realize microfabrication techniques of fibrin hydrogels for TE purposes, the techniques can be realized and established using other cells and then later proved using cardiomyocytes. Also, a very beneficial attribute of using cardiac fibroblasts is that they can be amplified in culture to reach a sufficient quantity and continuous supply of cells.

4 CELL-LADEN FIBRIN MICROSTRUCTURING USING SOFT LITHOGRAPHY

4.1 Concept

The soft lithography technique of micromolding in capillaries (MIMIC) is used to fabricate microscale fibrin gel constructs. The concept of MIMIC is displayed in figure 11. Shortly, a master is fabricated to replicate PDMS with the desired channel dimensions. The PDMS is then sealed to a cleaned and hydrophilized glass slide after which the channels can be filled with fibrin gel. After polymerization, the PDMS is peeled off depositing the fibrin lines onto the glass slide.

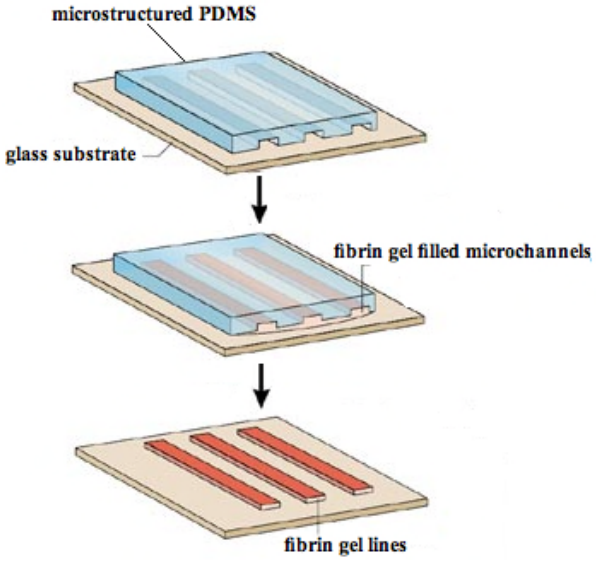

Figure 11. Concept for micromolding fibrin gel in capillaries (MIMIC). Picture adapted from [35].

4.2 Materials and methods

4.2.1 Self-made master fabrication

Low-cost wafers are fabricated using tape, a glass slide, and aluminium foil as previously explained by Shrirao & Perez-Castillejos [64]. This fabrication technique is very simple and the materials needed are basic, inexpensive, and found in almost every laboratory. The fabrication process is displayed below in figure 12.

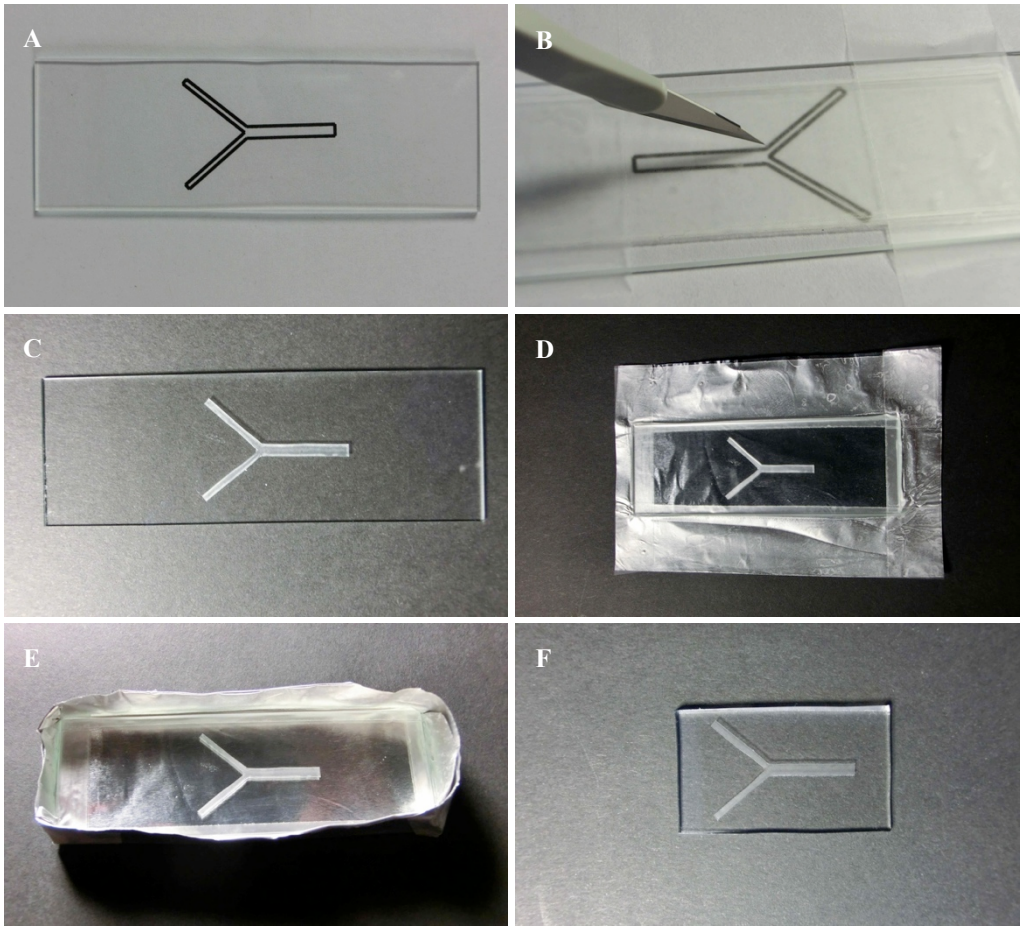

Figure 12. Design and fabrication of a simple microstructured PDMS layer: A design for the wanted structure is placed under a glass slide (A). Layers of tape are added and the structure is cut out according to the design (B & C). Aluminium foil is fixed around the glass slide (D) and folded (E) to create walls for the wafer. PDMS is added, cured, and peeled off from the wafer, giving rise to the designed microchannel (F).

A design of the desired microstructure is printed out on a piece of paper, positioned under the glass slide and fixed with more tape from the corners of the slide (figure 12a). Either opaque Scotch tape (Scotch® Magic™ Tape) or transparent Tesa tape (tesafilm® transparent) is used to create the structures. Four (Scotch tape) or five (Tesa tape) layers of tape are attached to the glass slide (76mmx26mmx1mm, Geputzt / Gebrauchsfertig, Engelbrecht Medizin und Labortechnik GmbH). A scalpel is used to cut out the designed structure (figure 12b), following the outlines of the layout, and the excess tape surrounding the designed pattern is removed (figure 12c). It is then placed into an oven at 65°C for three minutes to improve the adhesion of the tape to each other and also to the glass. The glass slide is then fixed from the sides with tape to a layer of aluminium foil (figure 12d), which was folded from the sides to create walls for the mold (figure 12e). The mold can then be filled with PDMS, cured in the oven, and peeled off giving rise to the PDMS microchannel designed (figure 12f).

The thickness of the tape will determine the height of the microchannel and can be easily altered by changing the number of tape layers. The thickness of the tape was measured with a 3D laser-scanning microscope (Violet Laser Scanning Microscope;

Keyence VK-9710) in order to determine the thickness of the final microchannel. The height was measured to be approximately 50 μm for both tapes, thus the microfluidic channels used in this thesis have a height of approximately 200-250 μm.

4.2.2 PDMS molding

PDMS (Sylgard® 184 Silicone, Dow Corning) is prepared by mixing the silicone elastomer base with the curing agent in a 10:1 ratio by weight. The mixture is let stand for a few minutes until most of the air bubbles are cleared and then it is cast into the desired mold. Left over air bubbles are carefully punched with a needle (Magellan™ Safety Needle, Kendall) and then the PDMS is cured in a heating oven (Binder FD 115) at 60°C for approximately one hour. A scalpel is used to cut out the PDMS from the side of the wafer wall to release it from the mold. The PDMS is stored in a plastic petri dishes (100 mm ∅) sealed with parafilm in a clean and dry environment until use.

4.2.3 Surface treatment of PDMS and glass

The PDMS is treated with oxygen plasma (Plasma Line 415, Tegal Corporation; 160 W, 160 sccm, 12 seconds) to create a hydrophilic surface that will promote the filling of the channels. After plasma treatment, the plastic petri dish is immediately closed and sealed with parafilm. The PDMS pieces are covered with 70% ethanol for 30 minutes and then rinsed with milliQ™ three times.

One of the two surfaces, depending on where the fibrin gel lines should be deposited, is coated with Pluronic® F-127 (Sigma P-2443) to prevent the adhesion of the fibrin gel to the unwanted surface (glass or PDMS). Incubation is done in room temperature for approximately two hours. After the Pluronic® coating, the PDMS/glass slide is washed three times with milliQ and dried.

Microscope cover glasses (20 mm ∅, REF 0111600, Marienfeld) are sterilized at 200°C for four hours. The glass slides are then cleaned and hydrophilized using 5% NaOH (Sigma), rinsed with milliQ™, and finally submerged in 70% ethanol for a few seconds and let air-dry.

4.2.4 Cardiac fibroblast cultivation

Cardiac fibroblasts are cultured in cell culture flasks using DMEM at 37 °C and 5 % CO_2. The medium is changed twice a week to remove waste and supply nutrients to the cells. When the cells reach confluence, they are either harvested for use in experiments or passaged and seeded into new flasks to increase the surface area for the cells. During passaging, the medium is first aspirated from the flask and the cells are washed with 7 ml of PBS, which is followed by incubation with 3 ml of 0.05% Trypsin EDTA (PAA, L11-660) for 5 minutes at 37°C and 5% CO_2 to detach the cells from the surface. The flask is gently hit to help detach all the cells. A double volume of medium is quickly added to cancel out the effect of the trypsin before it starts to damage the cells. The cell

suspension can then be transferred to e.g. multiple 75 cm^2 cell culture flasks or one 175 cm^2 cell culture flask, and later on in 500 cm^2 Triple flasks (VWR, 734-2001) depending on when cells will be used.

The same procedure is carried out when cells are harvested from the culture flask to be used for experiments. Cells are trypsinized, as explained above, and transferred to a 50 ml Falcon tube and centrifuged at 200 G for 10 minutes. The supernatant is aspirated and the cell pellet is re-suspended in the required volume of Trizma buffered saline (TBS: 4l MilliQ^{TM}; 4.91 g/l Trizma HCl (Sigma T-3253); 0.72 g/l Trizma Base (Sigma T-6066); 9.00 g/l NaCl (Sigma S-9625); 0.23 g/l KCl (Merck 1.04936.0500); pH adjusted to 7.40) for fibrin gel fabrication. Cardiac fibroblasts were used for experiments at passage 2-5.

When cells are embedded in fibrin gel and the constructs are further cultivated, several supplements are added to the normal cell culture medium. DMEM-based tissue culture medium, which is used for cardiac fibroblasts embedded in fibrin gel, consists of 10 % fetal bovine serum, 1 % antibiotic-antimycotic solution (Gibco, 15240-026), 888 µg tranexamic acid (Pfizer Cyklokapron®) and 160 mg of L-ascorbic acid (Sigma A8960-5G) per 0.5 liters of DMEM.

4.2.5 Fibrin synthesis

Lyophilised fibrinogen from human plasma (Calbiochem Cat. #341576) is dissolved in purified water (MilliQ^{TM} Millipore). After a few hours the solution is transferred into a dialysis tubing (Spectrum, Spectra Por No 132655, Pore size 6000 – 8000 MW) for dialysis against four liters of TBS overnight. The concentration is determined using a photo spectrometer (ThermoSpectronic Genesys 6) at a wavelength of 280 nm using the following equation:

$$c_{fibrinogen} = \frac{absorption_{280nm}}{1.55} \cdot dilution \qquad (2)$$

In this thesis a fibrin gel concentration of 10 mg/ml is used. Fibrin gel consists of 50% Fibrinogen solution, 35% TBS-cell suspension, 7.5% 50 mM $CaCl_2$ ($CaCl_2$: 735 mg $CaCl_2$ (Sigma C-3881) in 100 ml TBS) and 7.5% 40 U/ml Thrombin (Sigma T-4648 in TBS) (see figure 13). TBS-cell suspension, thrombin and $CaCl_2$ are mixed together as one component, which is then brought together with the fibrinogen.

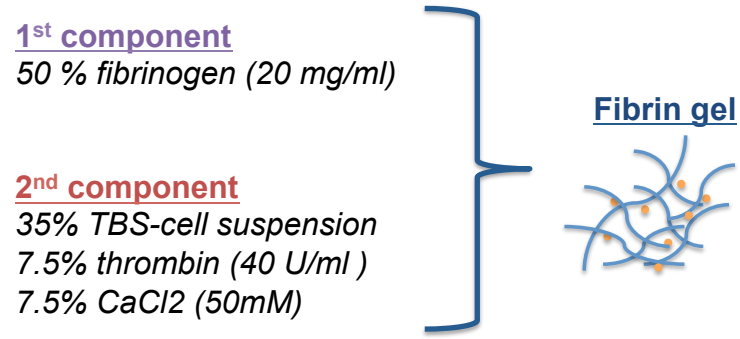

Figure 13. Components needed for fibrin gel fabrication.

All components can be sterile filtered using a syringe filter (Corning; Part No. 431219) or TBS and $CaCl_2$ can alternatively be sterilized in an autoclave (Systec 2540El). The final cell concentration used in experiments was approximately 500 000 cells per ml of fibrin gel.

4.2.6 Fibrin micromolding

Thrombin, TBS, and $CaCl_2$ are mixed together and 20 µl of the solution is put in an eppi. In unsterile experiments, food coloring (Schwartau) is used as a visualization aid. The same amount of fibrinogen is taken and while the solution is still in pipette, the volume of the pipette is set to 30 µl. The fibrinogen solution is carefully released into the eppi, the two solutions are quickly mixed, and injected into the microchannel. After 5 minutes, TBS is carefully added to the entrance and the exit of the channels to ensure that the gel does not dry out. The gel is left to further polymerize for about 30 minutes. Then the PDMS is carefully peeled off.

4.2.7 Microscopy

A light microscope (Axiovert 25, Zeiss) connected with a camera (JVC, KYF75U) is used to evaluate cell behavior in the molded fibrin microstructures. A laser-scanning microscope was used to determine surface roughness and thickness of the tapes used to fabricate wafers for PDMS replication.

4.3 Results and discussion

4.3.1 Device fabrication

A microfluidic device is constructed using microstructured PDMS and a glass slide. The two are sealed together to form a channel that can be used as a mold. The technique used for the fabrication of the self-made master for the PDMS replication gives a simple, fast, and low-cost alternative to the conventional master fabrication using rapid prototyping with a photolithography process. The fabrication of a photolithographically prepared master is done in a cleanroom, which can be a limiting factor for most laboratories. Additionally, the design and fabrication of the master requires sufficient

knowledge and skills from the person. The self-made master fabrication technique is ideal for preliminary studies where medium size microstructures are needed. The accuracy can be further increased and smaller structures can be obtained by using laser cutting instead of a scalpel for the cutting. However, fabricating a master from tape and a glass slide also has its downsides. As masters are hand-made, every single one is unique and thus differs in dimensions from the previous one. Also, the masters have a limited lifespan because the lines of tape tend to peel of with the PDMS.

To prove that the surface treatment of the PDMS with oxygen plasma and Pluronic® truly increase the hydrophilicity of the surface; a homemade contact angle measurement was done using a camera. The hydrophilicity of the PDMS was evaluated before and after surface treatment to see the effect of the surface treatment on the hydrophilicity of the surface. Even though proper contact angle measurements were not carried out due to lack of equipment, the pictures below (figure 14) clearly display that surface treatment, either plain oxygen plasma or both oxygen plasma and Pluronic® coating, truly increase the hydrophilicity of the PDMS surface.

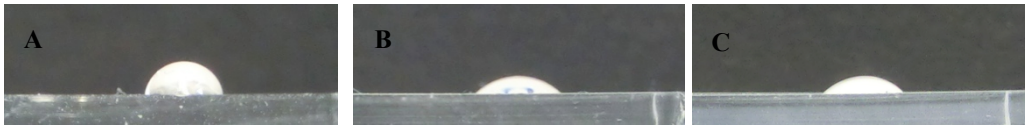

Figure 14. Hydrophilicity of PDMS: without treatment (A), with oxygen plasma treatment (B), and with oxygen plasma treatment and Pluronic® coating (C).

The PDMS can be sealed permanently or temporarily to the glass slide depending on the use of the device. In the case of micromolding, the PDMS is sealed temporarily to the glass slide so that it can be removed after the polymerization of the gel. If the device is used under increased pressure e.g. as a flow chamber, the PDMS should be sealed to the glass permanently. A microfluidic device is fabricated and presented in the figure below.

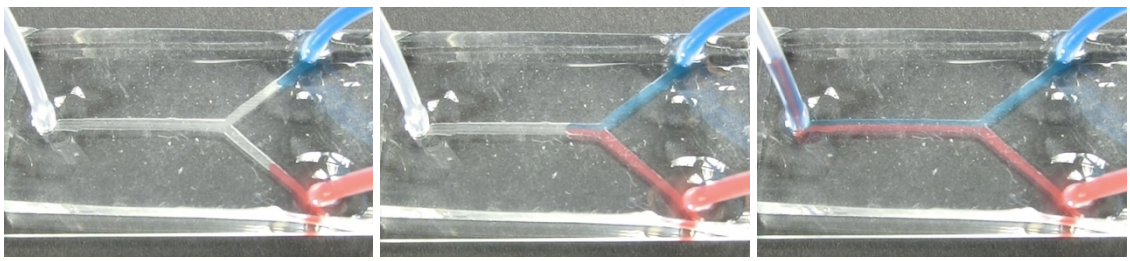

Figure 15. Filling of a permanently bonded PDMS to glass slide microfluidic device.

A noteworthy characteristic of microfluidic channels is the laminar flow of fluids; the velocity at a given spot in the channel is constant in time. This is the reason the two adjacent streams of different color liquids are flowing side-by-side in the microfluidic device presented below (figure 15) –mixing of the two colors is only by diffusion at the interface. [35]

As stated in chapter 3, isolating large numbers of cardiomyocytes from the cardiac auricle tissue biopsy is challenging. Because of the limited amounts of cardiomyocytes that would be available for the micromolding application, it is better to make very simple microstructure design for the molding procedure. Also, it is best to exploit the capillary filling of the structure with practically the exact volume to fill the channels to avoid any dead volume. For example in the device presented in figure 15, there is a lot of dead volume that needs to be filled. To prevent this, a master is created with simple parallel stripes of tape (approximately 1 mm x 1 cm) and replicated to form channels with a height of approximately 250 µm. When cells are used, a single channel is replicated into PDMS to even further decrease the amount cells needed. Figure 16 presents the PDMS channel replicated from the self-made master.

4.3.2 Optimal tape for master design

Two different tapes, Scotch and Tesa, were evaluated for use in master fabrication. The Scotch tape has a white, matte appearance but when glued on a surface it is transparent. The adhesive side is not as sticky as in normal office tape, which is ideal as there are no glue residues. During replication, the white surface of the Scotch tape is transferred to the PDMS thus resulting in opaque microchannels (figure 16a). This is a great disadvantage if the PDMS is to be visualized under the microscope e.g. if cells are cultured on microchannels created using this tape.

The Tesa tape is see-through and results in transparent PDMS microchannels (figure 16b). This is a great advantage if the PDMS wants to be visualized under the microscope. The downside of using office tape is that the adhesive side is stickier compared to the Scotch tape and thus at times results in glue residues at the edges of the lines of tape These are then of course replicated to the PDMS creating additional patterning around the microchannel.

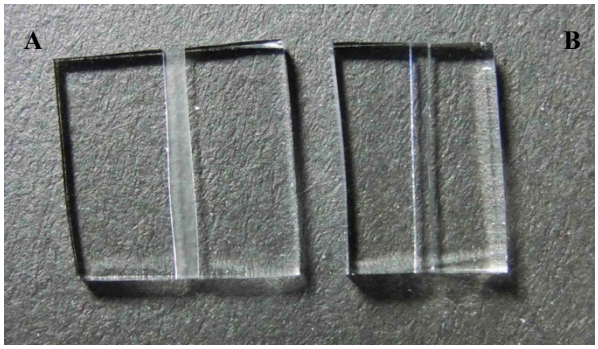

Figure 16. Comparison of PDMS replicated from a master made with opaque Scotch tape (A) and with transparent Tesa tape (B).

As the surface properties play a significant role in microfluidics, the surface of the tape used for the master fabrication is evaluated. A strip of tape (both Scotch and Tesa) was sealed to a glass slide and the surface roughness was measured using a laser-scanning microscope. The figure below presents the area of measurement (highlighted

in blue). As it can be seen from the figure without any other measurement data, the Scotch tape (figure 17a) appears to have much higher surface roughness compared to the Tesa tape (figure 17b).

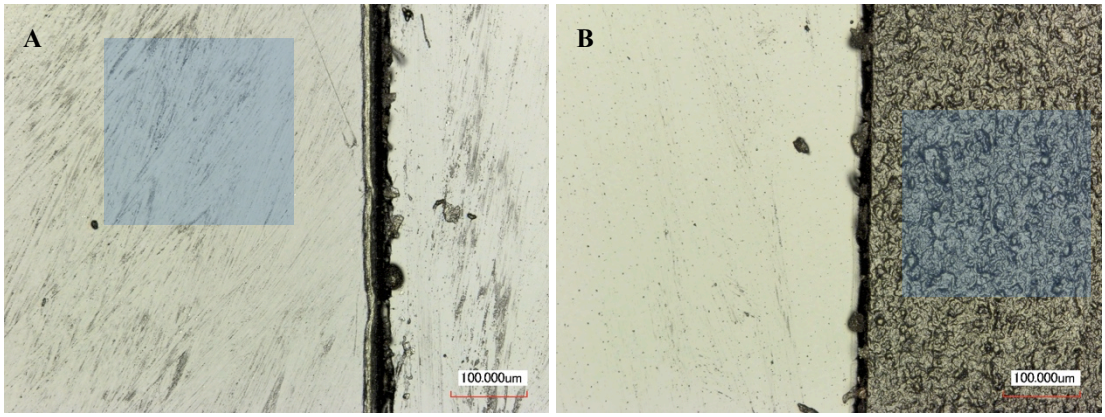

Figure 17. Surface roughness analysis of Scotch (A) and Tesa (B) tape. The blue square represents the area of measurement (tape).

From the measurement data, the arithmetical mean roughness (Ra) values of the two tapes were compared. The Ra is the arithmetical mean of all the sums of all profile values. The Ra for the Scotch tape was 1.599 µm and for the Tesa tape 0.226 µm, which demonstrates that the surface roughness of the Scotch tape was much higher than that of the Tesa tape.

In the end, both tapes had its advantages and disadvantages. In this thesis, Tesa tape was used for applications where the transfer of the fibrin was done onto the PDMS. This is because the Tesa tape is transparent, so the cells embedded in the fibrin could be visualized with the microscope. On the other hand, Scotch tape was used for applications where the PDMS was used just as a temporary molding device. In this case, the surface properties in terms of visualization did not matter.

4.3.3 Proof of principle

The first experiments are done without cells to demonstrate that the concept designed is feasible. The microstructured PDMS and the glass slide are sealed together to create microchannels and filled as previously explained. Blue food coloring is used as a visualization aid and it is dissolved into the TBS before it is mixed with the other components.

The filling of the channels needs to be done very quickly so that the gel does not polymerize before it has a chance to fill the channel completely. If the mixing in the eppi takes too long, the fibrin gel can already be too polymerized and will not even enter into the channels. This is the most crucial phase of the entire micromolding process.

As the PDMS and the glass slide are both made hydrophilic, the fibrinogen solution fills the channels very easily. The unpolymerized fibrin gel mixture is introduced to the

entrance of the channel, and due to capillary forces it fills the channel (see figure 18). No external pressure is needed.

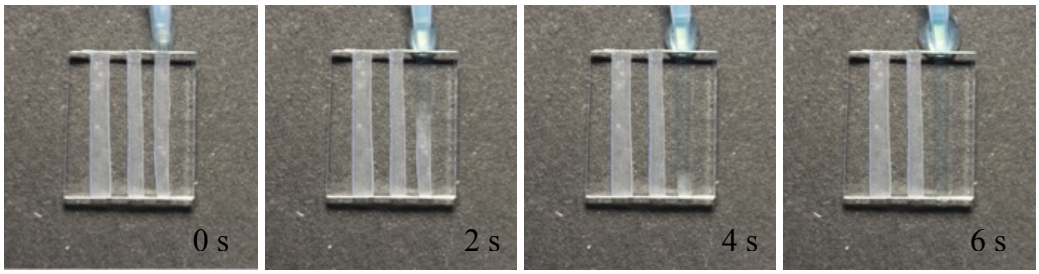

Figure 18. The filling of the channel by capillary forces. Time represented in seconds.

After 5 minutes, TBS is carefully added to the entrance and the exit of the channels to ensure that the gel does not dry out. The gel is left to further polymerize for about 30 minutes. Then the PDMS is carefully peeled off. The micromolded lines of fibrin gel are deposited on either the glass (figure 19a) or the PDMS (figure 19b) depending on which surface is coated with Pluronic®.

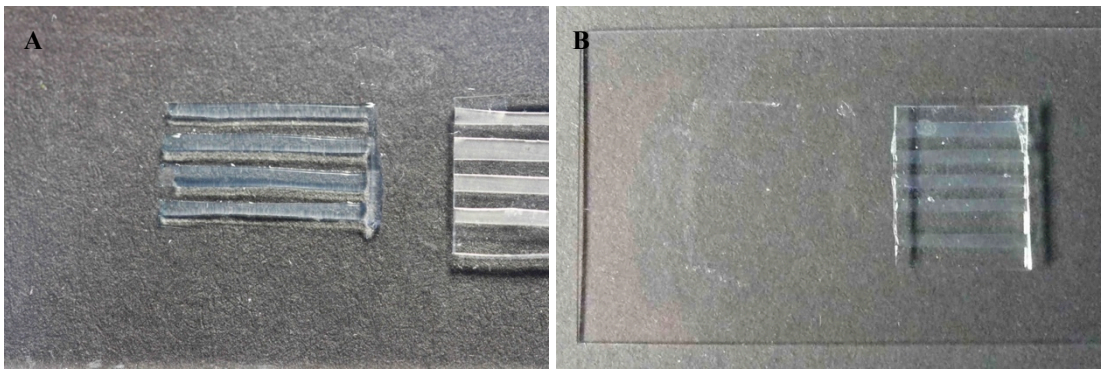

Figure 19. Micromolding of fibrin in capillaries. A: PDMS is coated with Pluronic® thus the deposition of fibrin gel is on the glass. B: Glass slide is coated with Pluronic® thus the fibrin gel stays in the PDMS.

Two fibrin gel concentrations were compared to find the optimal one for use in micromolding. In the group the standard fibrin gel concentration used is 5 mg/ml of gel. This was attempted in the first trials but resulted in a very watery gel (see figure 20).

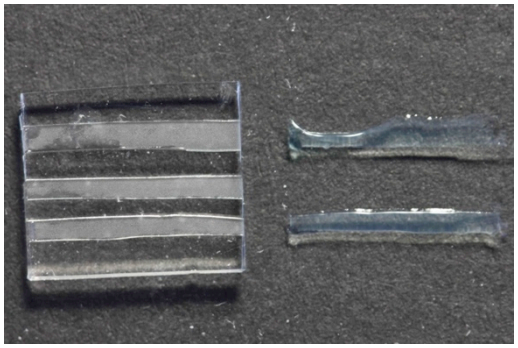

Figure 20. Comparison of fibrin gel concentration: 5 mg/ml (top) and 10 mg/ml (bottom).

It seemed that the gel did not polymerize properly in such a small, confined space. A double concentration was tested and it resulted in a stiffer, stronger, and less watery gel. The gel structure was much easier to handle and it could even be peeled off from the glass slide completely intact. A downside of using the higher fibrin gel concentration is that it polymerizes quicker and thus the filling of the channels needs to be done swiftly.

4.3.4 Micromolding cell-laden fibrin gels

After demonstrating the concept of micromolding fibrin in capillaries is functional, cells are incorporated into the molding process. The behavior of the cells in the confined geometry is evaluated using phase contrast microscopy. Both deposition of fibrin onto PDMS and onto glass are displayed.

The 10 mg/ml fibrin gel supported proliferation of the cardiac fibroblasts and cells were distributed homogenously within the gel during the molding process. Figure 21 displays the deposition of fibrin gel with cells onto glass using MIMIC. The cells aligned in the longitudinal direction. The diameter of the microchannel used to mold the fibrin gel seemed to have an effect on the degree of orientation of the cells. As the width of the channel decreased, higher cell alignment was observed (figure 21b, 21d and 21f).

As a downside, when cells proliferated they started migrating out of the fibrin and started growing is 2D on the glass. In the future, this could be possibly prevented by e.g. coating the glass with a cell-repellent.

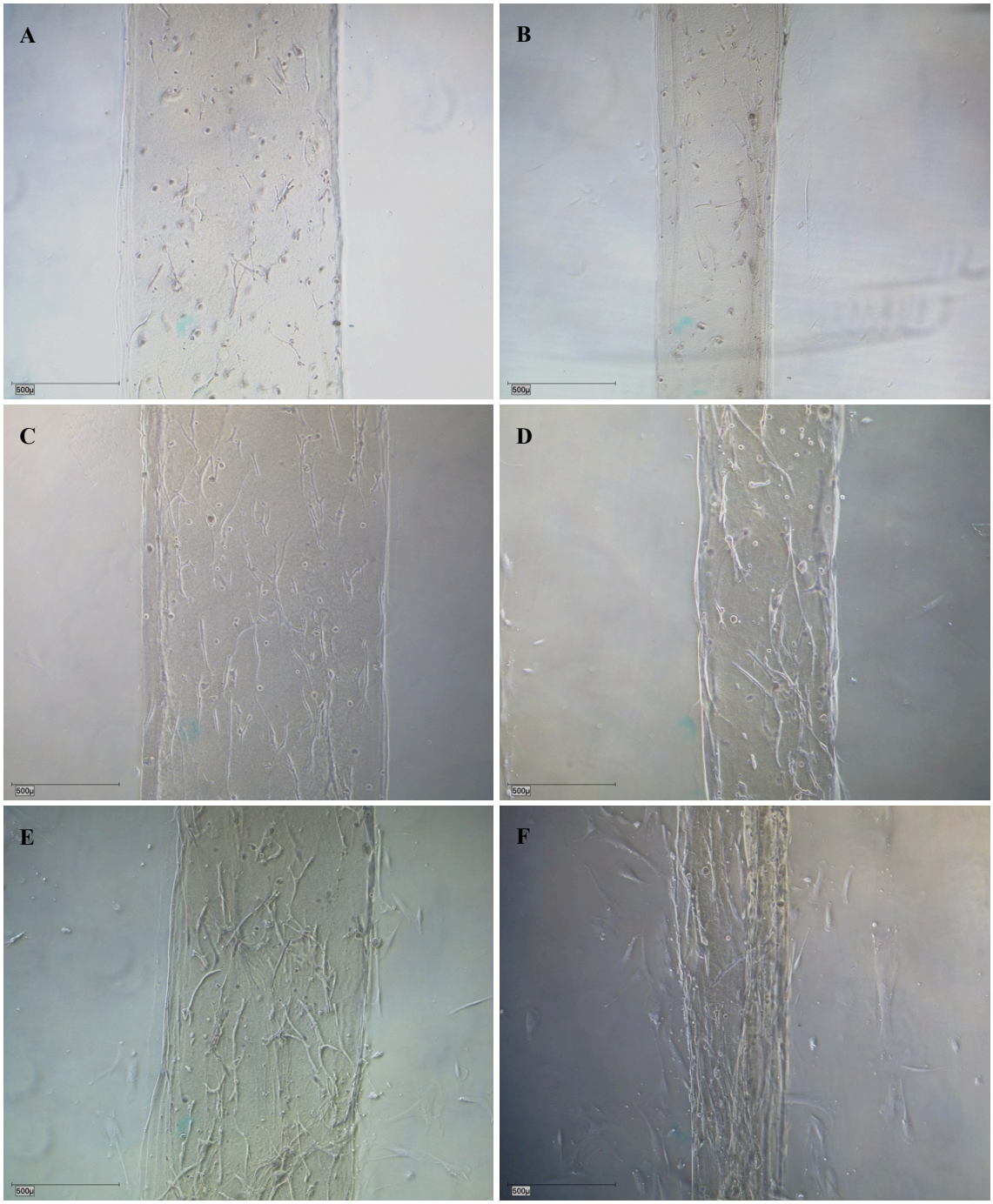

Figure 21. Fibrin gel with embedded cardiac fibroblasts (passage 2) molded using MIMIC technique onto glass. A & B: Day 3, C & D: Day 6, E & F: Day 12. Scale 500 µm.

Overall, cell behavior between different batches was observed. This could be affected by the age and also the type of cardiac disorder the patient had. The cells behaved differently already in culture as some cells did not grow at all.

When observing the arrangement of the cells in different depths of the molded fibrin, cells demonstrated alignment mainly at the top of the 3D gel line, not at the bottom. The figure below (figure 22) presents the different alignment properties of the cells at different depths in the 3D gel. When focusing near the glass slide, the cells

appear to be randomly oriented with very little longitudinal orientation (figure 22a). On the other hand, when focusing to the top of the gel near the surface, alignment of cells can be seem (figure 22b). This could be due to different surface properties of the glass and the PDMS. As previously explained, the surface of the Scotch tape used to fabricate the channels is rather rough which replicates to the roof of the PDMS microchannel. This means the top and the bottom surfaces of the microchannel possess different surface properties. When the channel is filled with fibrin, the different surface properties can effect the polymerization of the fibrin gel. This could then reflect straight on the cell behavior.

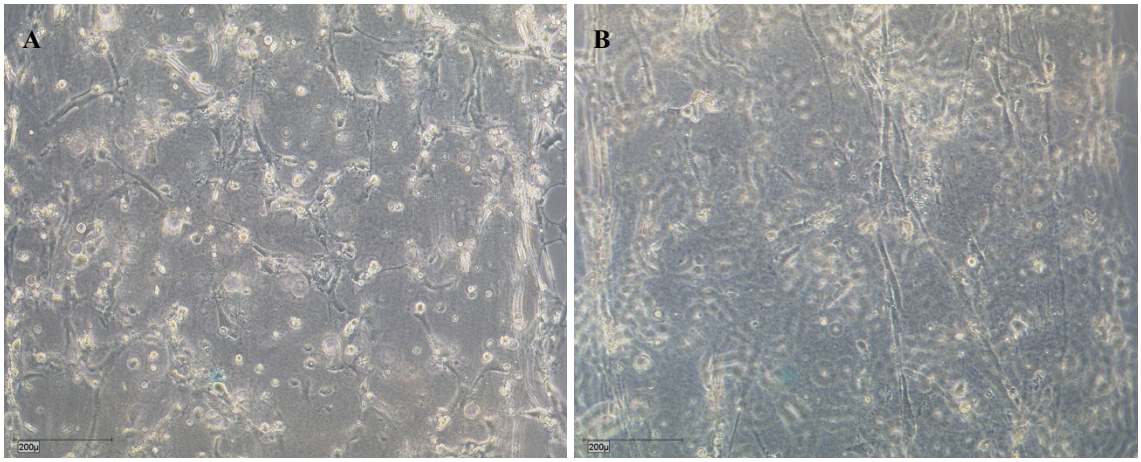

Figure 22. Fibrin gel with embedded cardiac fibroblasts (passage 5) molded using MIMIC technique onto glass. Focus is set near the glass surface (A) and at the top near the surface of the fibrin line (B). Pictures taken after approximately one month of culture. Scale 500 µm.

Figure 23 demonstrates the deposition of fibrin gel with cells onto PDMS using MIMIC. As the cells proliferated, they started to migrate out of the fibrin gel embedded in the PDMS grooves. This shows that the PDMS surface is appealing to the cells as they also grow there. As mentioned before, using a cell-repellent coating on the PDMS interspaces could possibly prevent this. The cells did not demonstrate alignment in the construct. The width of the channels used was probably too big, thus the use of smaller channels could create higher cell alignment.

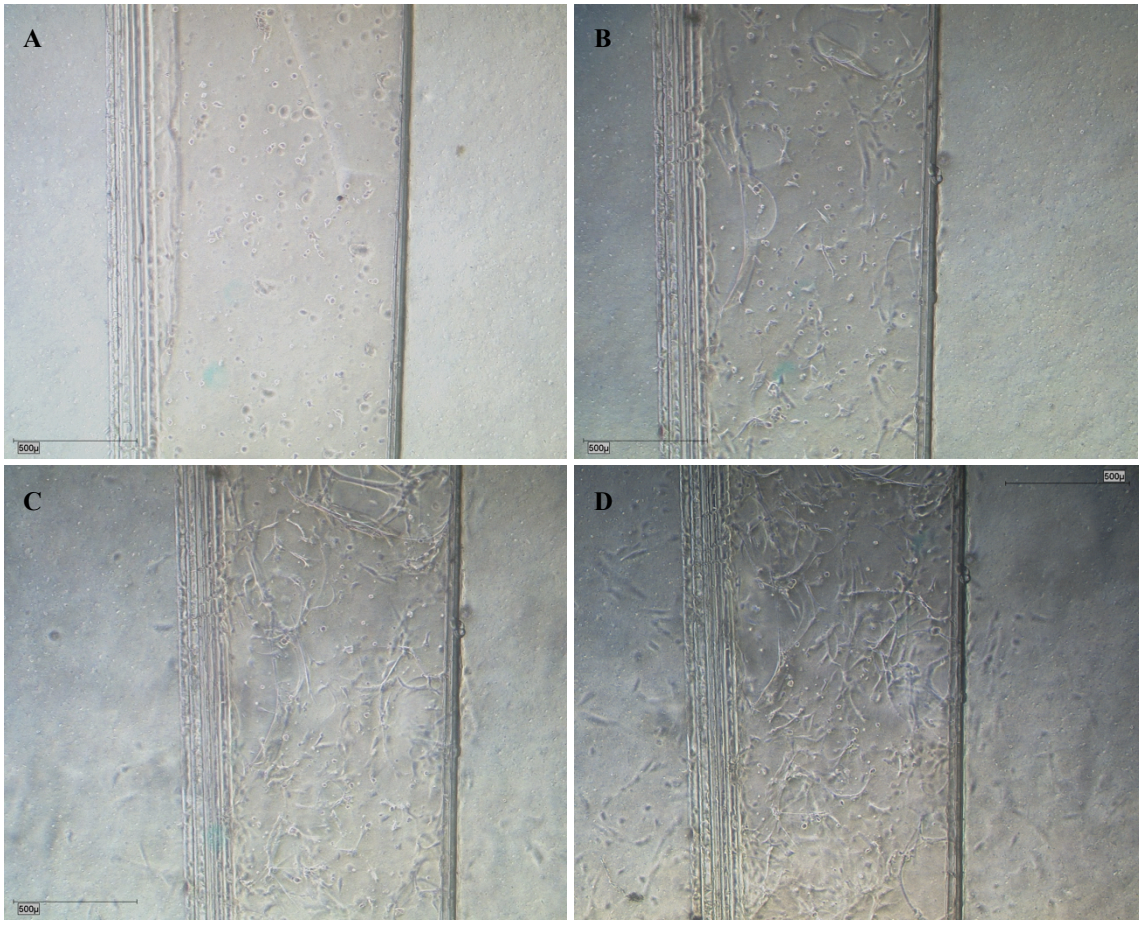

Figure 23. Fibrin gel with embedded cardiac fibroblasts (passage 3) molded using MIMIC technique onto PDMS. A. Day 1; B. Day 5; C. Day 8; D. Day 10. Scale 500 µm.

The manipulation of one molding surface with Pluronic® provides the freedom to decide on which surface the deposition of the gel is wanted. This creates different possibilities of how the fibrin lines can be used, for example how they can be incorporated into a larger scale construct for TE applications. If the molded lines of fibrin are to be used as separate units and need to be peeled off from the mold, the deposition of the fibrin gel needs to done on glass. This way, the fibrin lines can be peeled off without damaging them. This cannot be done from the PDMS substrate as the adhesion of the gel is too tight and the fibrin line breaks fairly easily. As an example, the peeled off fibrin lines can be incorporated into a thin layer of additional fibrin gel which can then be manipulated e.g. by rolling to form a final tissue construct. Alternatively, additional fibrin gel can be added on top of the microfabricated lines on the glass or on PDMS to embed the lines into more fibrin gel. The microfabricated fibrin lines and the additional layer can be encapsulated with different cell types to allow the fabrication of a construct with several cell types in an organized manner.

5 MICROFABRICATION OF FIBRIN FIBERS

5.1 Concept

Fibrin hydrogel microfibers are fabricated by coextruding the two liquid solutions, fibrinogen and thrombin, through small diameter tubing. A syringe pump is used to set a continuous flow in the system. The idea is to extrude microscale tubular hydrogel fibers that can be applied in TE applications. This fibrin extrusion concept was demonstrated previously by Cornwell et al. in 2006. [61] In this thesis, the same principle is applied but with alterations. Endothelial cells (ECs) are incorporated into the molding process to fabricate fibers with spatially patterned cells that could be further used as preformed vascularization modules and possibly incorporated into larger scale tissue constructs. The concept is presented in the schematic below (figure 24).

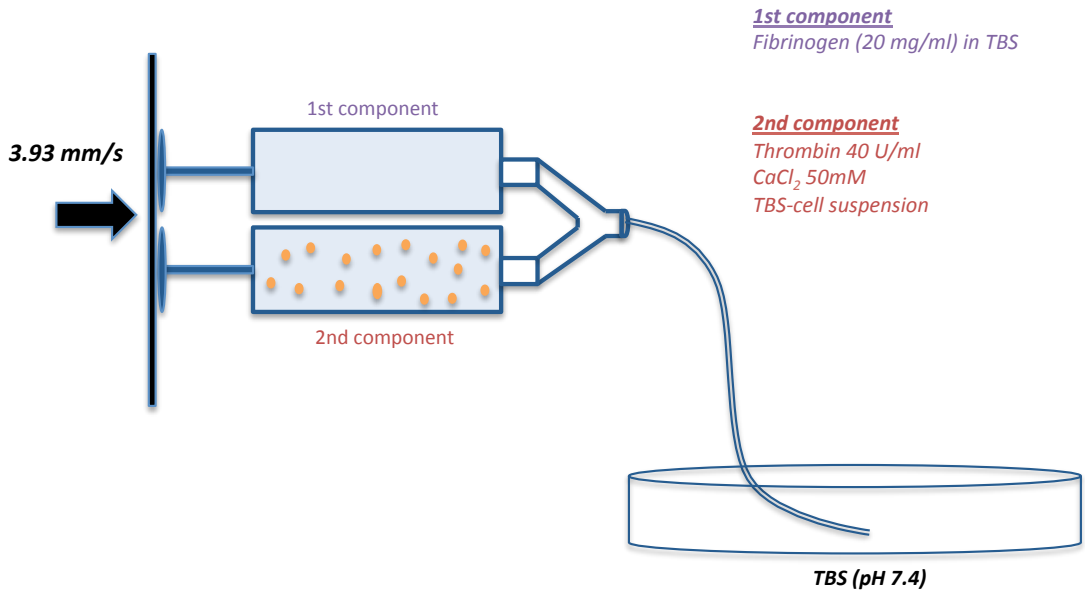

Figure 24. A schematic drawing of the concept used to extrude fibrin fibers.

5.2 Materials and methods

5.2.1 Endothelial cell cultivation

Human vein endothelial cells, a kind gift from a colleague, are cultured in 75 cm^2 cell culture flasks using Endothelial Cell (EC) Growth Medium (Basal medium C-22010; Supplements C-39215, PromoCell). The flasks are first coated with 0.02% Gelatin (Bovine Skin, Type B, Sigma G-9391 in MilliQ) for cell adherence. Gelatin is steam

sterilized in the autoclave, aliquoted and stored at 4°C. Upon use, it is heated to 37°C and each flask is filled with 5 ml while the excess is aspirated. The flasks are let dry under the laminar flow bench for approximately one hour before cell seeding.

The cells are cultured in an incubator at 37°C and 5% CO_2. The culture medium is changed twice a week. Upon confluence, the cells are passaged as described for cardiac fibroblasts in chapter 4.2.4 and re-seeded into gelatin-coated 175 cm^2 cell culture flasks. Cell harvesting is done as also described for cardiac fibroblasts with the exception that the cells are centrifuged at 500 G for 5 minutes. Endothelial cells were used for experiments at passage five.

When cells are embedded in fibrin gel and the constructs are further cultivated, several supplements are added to the normal cell culture medium. The EC-medium is supplemented with 1 % antibiotic-antimycotic solution and 888 µg tranexamic acid per 0.5 liters of medium.

5.2.2 Fibrin synthesis

Fibrin gel synthesis is done as described previously in chapter 4.2.5. The final cell concentration used in experiments was approximately 2.5 million cells per ml of fibrin gel.

5.2.3 Fibrin fiber extrusion

Fibrin fibers are extruded into a glass petri dish filled with either TBS or EC culture medium using a syringe pump (Perfusor® Compact S, Braun). Two syringes (Injekt-F / Omnifix®-F 1 ml, Braun) containing the fibrinogen solution and the thrombin solution are fixed to a DUPLOJECT double syringe holder (Tisseel, Baxter) and the syringes are connected together with a dual-lumen spray head, which upon coextrusion combines the two solutions together and ensures profound mixing at the tip. The tip of a 50 ml syringe (Original-Perfusor® Syringe, Braun) is cut off and the entire DUPLOJECT system is then assembled and fixed tightly inside it by using surgical tape and the solutions are coextruded through perfusor line tubing (Original Perfusor® Line 50 cm, Braun) with an inner diameter of 1 mm. Both the syringe holder and the spray head can be sterilized in an autoclave.

The perfusor line is attached to the spray head and the line cut to a length of about 25 cm. In unsterile experiments, food coloring (Schwartau) is used as visualization aids. On the other hand, in sterile conditions an LED-light is used to aid visualization, which can be sterilized by hydrogen peroxide (H_2O_2) plasma.

5.2.4 Immunohistochemistry

The immunohistochemical staining procedure for the two-photon microscopy is explained here shortly. The tissue piece is fixed with -20°C methanol for 30 minutes at room temperature. Then, the sample is washed with PBS consisting 1% gentamycin and 0,32% tranexamic acid over night. After that, the sample was incubated with 4',6-

diamidino-2-phenylindole (DAPI, Sigma D-9564) to mark the cell nuclei for 30 minutes. The sample is stored in PBS consisting 1% gentamycin and 0,32% tranexamic acid at 4°C.

5.2.5 Microscopy

A two-photon microscope is used to evaluate cell orientation in the 3D fibrin fibers produced. For image acquisition the two-photon laser scanning microscope (TPLSM) of the IZKF Aachen was used consisting of an Olympus FluoView 1000MPE with a 25x water objective NA1.05 (Tokyo, Japan), a mode-locked MaiTai DeepSee Titanium-Sapphire Laser (Spectra-Physics, Stahnsdorf, Germany) and the acquisition software FluoView FV 10 2.0.

Scanning electron microscopy (SEM) is used to evaluate fiber alignment of the microfabricated fibrin fibers. The preparation of the fibrin gel samples for scanning electron microscopy (SEM) was done at the University Hospital Aachen (Elektronenmikroskopische Einrichtung (EME)). Shortly, samples were fixed by immersion in 2.5% glutaraldehyde in 0.1M Sorenson's buffer (pH 7.4) for 12 h. Dehydration was done in rising acetone concentrations. The samples were then dried using critical point drying (E-300 Critical Point dryer; Polaron Equipment, Watford, United Kingdom). The samples were transported in a vacuum desiccator to the Institut für Textiltechnik (ITA) at RWTH Aachen for gold-palladium spattering and imaging. The images were acquired at 15 kV.

5.3 Results and discussion

5.3.1 Proof of principle

The first experiments are done without cells to demonstrate that the concept designed is feasible. The complete procedure is executed in a way that is then reproducible under sterile conditions. Thrombin, TBS, and $CaCl_2$ are mixed together in a 24-well plate and 0.5 mL is filled into a syringe. An equal amount of fibrinogen is placed in another syringe and both syringes are fastened into a double-syringe holder (figure 25a). Removal of air bubbles from the syringes is extremely important as this can result in holes or even complete gaps in the fibers. The syringes are connected to the syringe pump (figure 25b) as explained in the previous chapter. By using a syringe pump, the entrance of the components into the tubing occurs simultaneously and also the formed fiber is pushed out continuously, which minimizes blockage at the exit. It is crucial that the double syringe holder is placed very tightly, exactly in the middle of the larger syringe to ensure continuous flow and even distribution of solutions from both syringes.

A LED-light source is placed around the glass petri dish to allow better visualization of the formed fiber. Also, blue food coloring, which is dissolved in TBS, is used as a visualization aid.

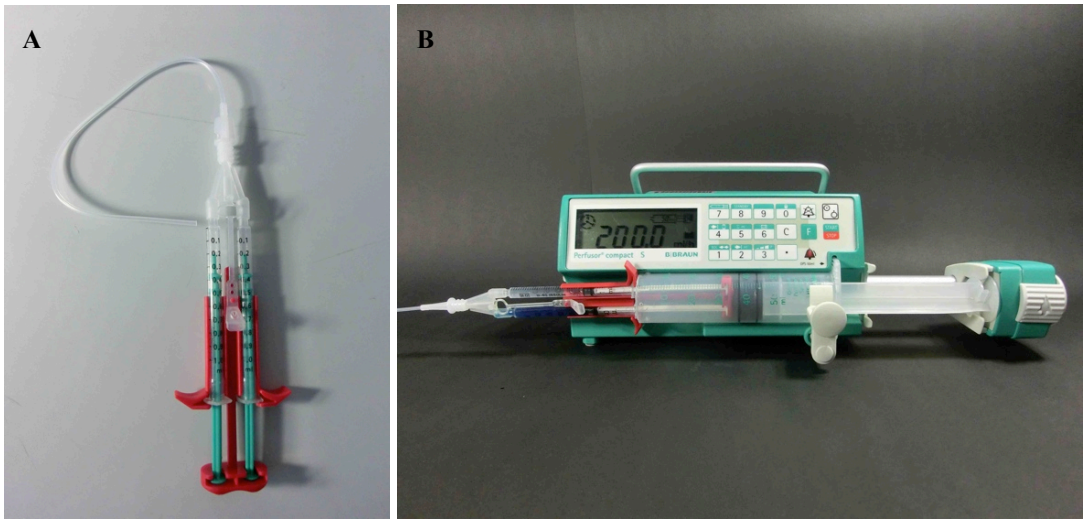

Figure 25. Components used for fiber extrusion.

The syringe pump is set to 200 ml/hour (3.33 ml/min). The priming of the tubing takes about 1-2 minutes. The fiber formed at a rate on 3.93 mm/s. The tubing is slowly dragged by hand vertically at the bottom of the glass petri dish through the bath as the fiber started to form. The dragging of the tubing tip is done at approximately the same rate as the polymerizing fibrin moves in the tubing. This eased the exit of the fiber into the bath as it had a surface to attach to. Also, the movement on the tip of the tubing is crucial to the outflow of the fiber so that it would not form clumps.

The length of the tubing and the speed of the pump were adjusted so that the fiber was polymerized as it exited the tubing. Fibers were left in the bath for further crosslinking for approximately 10-15 minutes. Then they were carefully detached from the bottom of the petri dish with tweezers and placed in a smaller petri dish with TBS.

The resulted fibers are displayed in figure 26a in a petri dish. The diameter of the formed fibers in TBS was evaluated using light microscopy. As the dragging of the tubing during fiber processing was done by hand, the speed was not consistent and thus resulted at times to non-uniform fibers. If the tip was pulled too fast, the fiber stretched and became thinner. Other the other hand, if the dragging of the tip was too slow the fibrin would start to collect together and form a bundle. At most parts of the formed fiber, the diameter was measured to be about 1 mm (see figure 26b), which is equal to the inner diameter of the tubing.

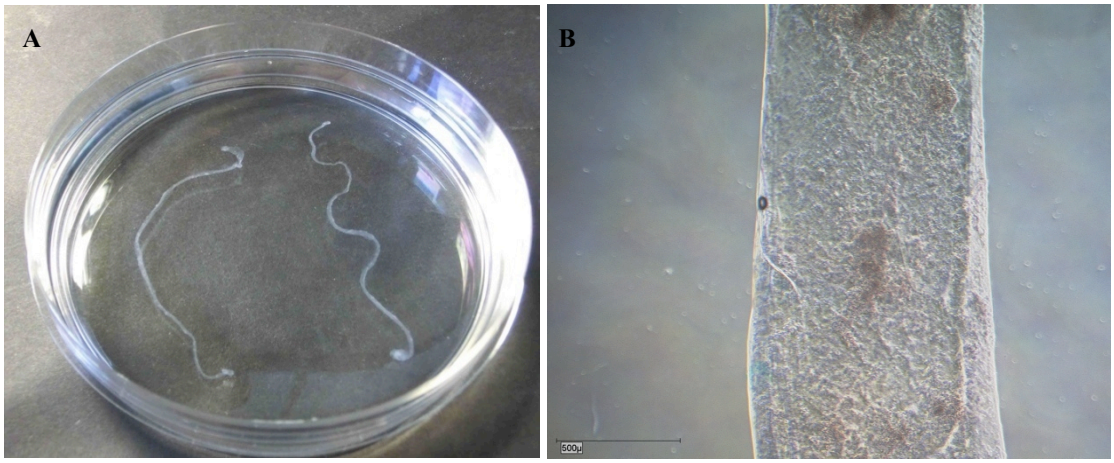

Figure 26. A. Extruded fibrin fibers in a plastic petri dish using (100 µm ∅) B. Light microscope picture of an extruded fibrin fiber, with a diameter of approximately 1 mm, in TBS.

In an attempt to create smaller diameter fibers, a pipette tip (TipOne® 200 µl, Starlab) is attached to the end of the tubing as displayed in figure 27a. This resulted in fibers with a diameter of approximately 400-500 µm (see figure 27b). The fabrication was more difficult compared the larger diameter fibers mainly due to the small size. Also, the fibers were much weaker and more difficult to handle. Additionally, the tip was difficult to secure tightly onto the tubing, which caused some leakage.

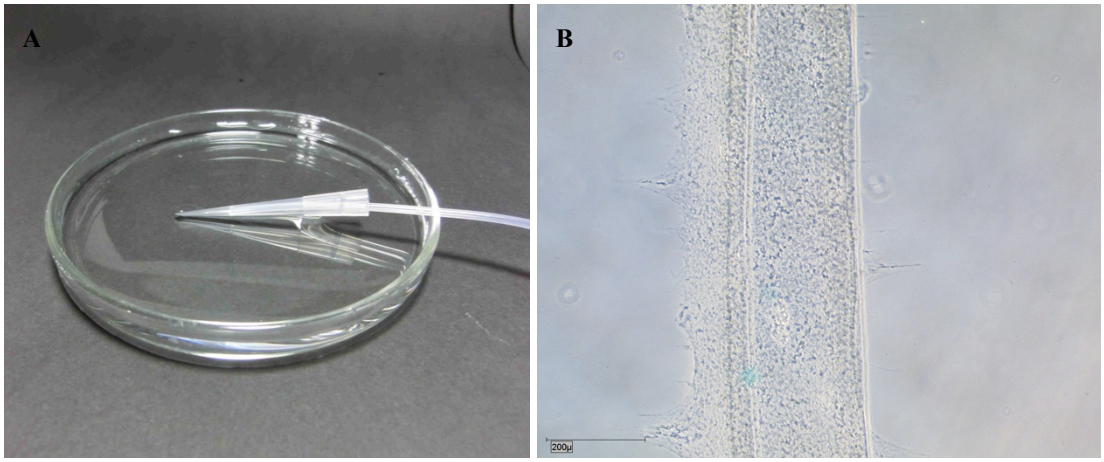

Figure 27. A. A pipette tip (200 µl) was added to the end of the tubing to allow fabrication of smaller diameter fibers. B. Light microscope picture of an extruded fibrin fiber, with a diameter of approximately 400-500 µm, in TBS.

The effect of flow on fibrin fiber alignment during extrusion was analyzed by SEM. The image below (figure 28) indicates that flow during polymerization of the gel increases the orientation of the fibers in the direction of the extrusion. This could be advantageous for applications where cells want to be aligned in 3D hydrogel constructs.

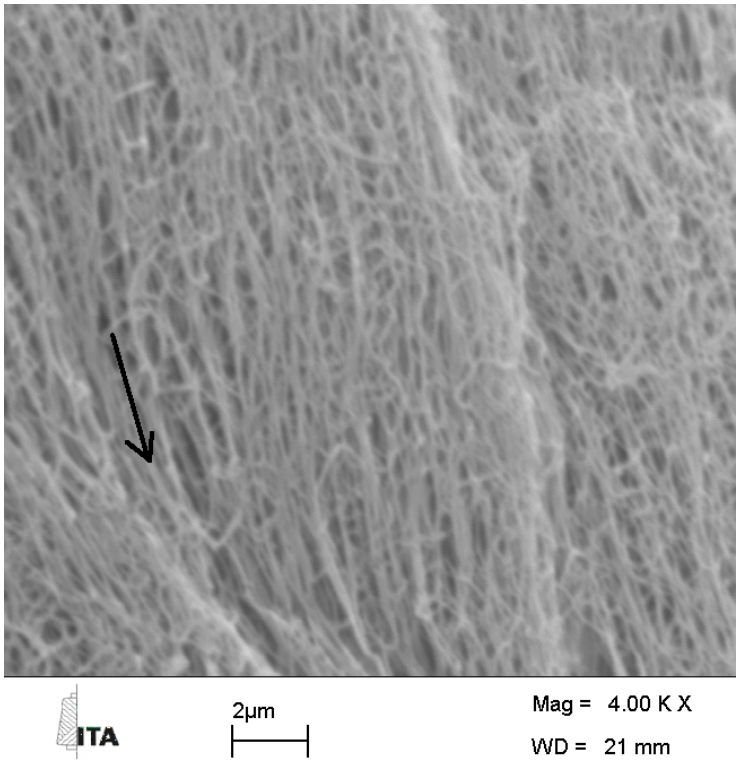

Figure 28. SEM image of an extruded fibrin fiber with the direction of the flow being indicated by the arrow.

5.3.2 Extrusion of cell-laden fibrin fibers

After proving the concept of fibrin fiber extrusion is functional, cells are incorporated into the molding process. Instead of using a TBS bath, the fibers are extruded into a bath of EC medium. This way the time that the cells are left without nutrients is minimized. The length of a single fiber was intentionally kept under 4 cm to prevent tangling of the fiber during moving of the well plate e.g. to the incubator. The forming fiber was cut using a small spatula upon extrusion at the tip of the tubing. After polymerization, the fibers are carefully transferred into a 6-well plate with fresh EC medium and placed in an incubator for further cultivation. The fibers were cultivated for 8 days and the behavior of the cells was evaluated by light microscopy.

The distribution of cells in the formed gel was homogeneous in the fibers formed at the beginning of the extrusion process. Towards the end, the cell number decreased as the cells started to clot in the syringe. This indicates that the entire extrusion process needs to be executed quickly while the cells are still evenly distributed in the solution.

The fibrin gel concentration of 10 mg/ml was shown to support EC proliferation and migration. Over time, the endothelial cells appeared to coalesce and bundle the surrounding fibrin into a core around which they arranged themselves. This resembles the process of tubulogenesis, the early development of vascularization, in which individual cells connect to form a loose network and further organize into multicellular cords by subsequent lumen formation. The phenomenon is consistent with previous data demonstrating that ECs organize into tubes by bundling the collagen into the core and forming a cylinder around it. [51] Raghavan et al. used this idea to create constructs of

endothelial tubules by micromolding collagen gel with endothelial cells in microchannels using soft lithography. As discussed by the authors, the presence of collagen within the lumen of the endothelial tubule is potentially a limitation due to the fact that the collagen will not disappear by degradation. The use of fibrin gel is more advantageous as it will over time degrade and thus could have the potential to create an open lumen for the fibers.

This tubulogenesis phenomenon can be observed in microscope pictures taken of the extruded fibrin fibers. The cells appear to migrate to the surface of the fiber (see figure 29) to form a monolayer of cells, which is consistent with the behavior of these cells in nature. On day one, the cells are embedded within the matrix (figure 29a). With time, they appear to coalesce and arrange themselves around the fiber by bundling the fibrin into the core of the fiber as the cells form a cylinder around it. On day 4 (figure 29b), some cells can be seen at the surface (at the edges) of the fiber. By day 6, the cells have almost all the cells have migrated to the surface. In figure 29c and 29d, which are taken on day 8, the focus is set the bottom surface of the 3D fiber.

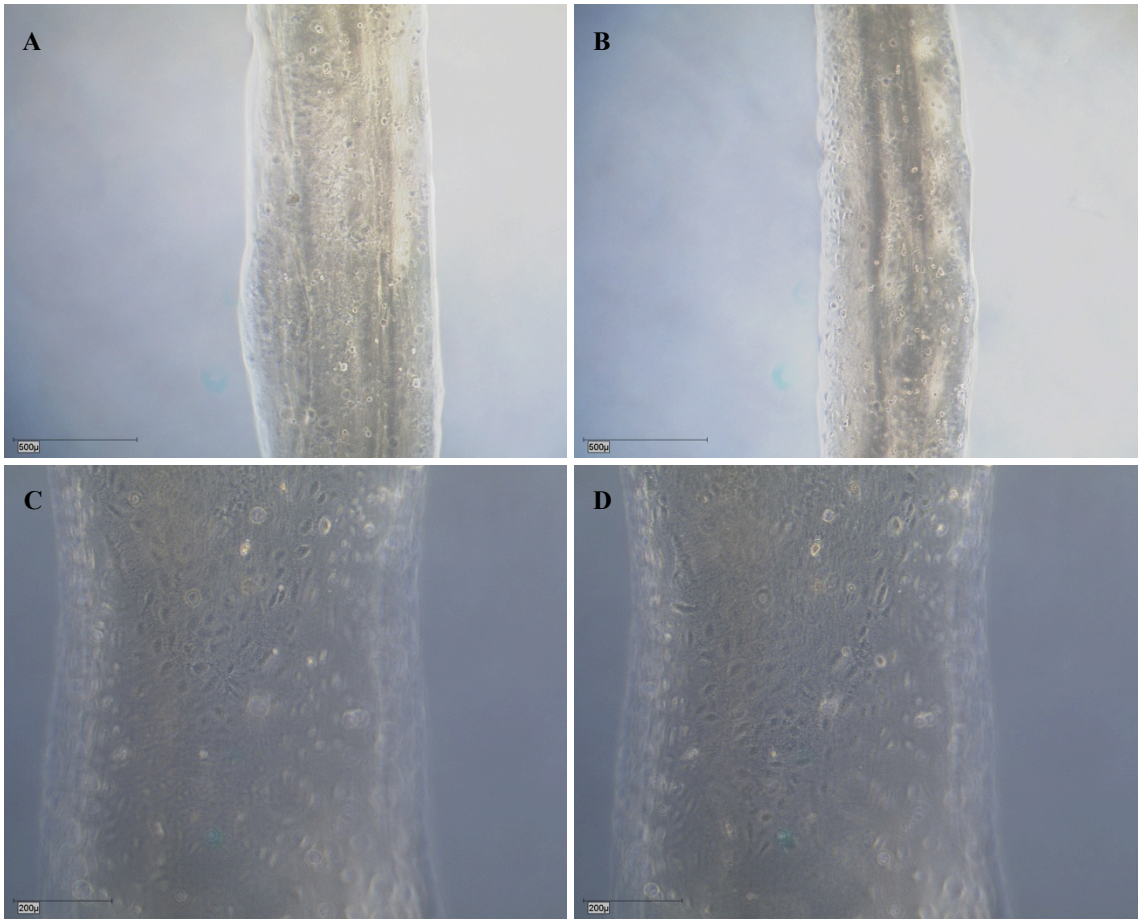

Figure 29. Tubulogenesis of endothelial cells in fibrin fibers. On day 2 (A), cells are embedded homogenously inside the fibrin matrix. On day 4 (B), some cells have migrated to the surface and by day 6 almost all the cells are at the surface. Scale bar 500 µm. Pictures C and D are taken on day 8 from the lower surface at two planes of focus at a short distance to prove that the whole surface was covered. Scale bar 200 µm.

As the 2D pictures taken with the phase contrast microscope cannot give a full evaluation of the position of the cells in 3D space, the nuclei of the cells are stained with a fluorescent dye (DAPI) and visualized using a two-photon microscope to confirm the results obtained from the phase contrast microscopy pictures. Figure 30 below demonstrates the pictures obtained by the two-photon microscope, which clearly confirms that the cells have migrated to the surface of the fibrin fiber and are arranged in a tubular orientation around the core.

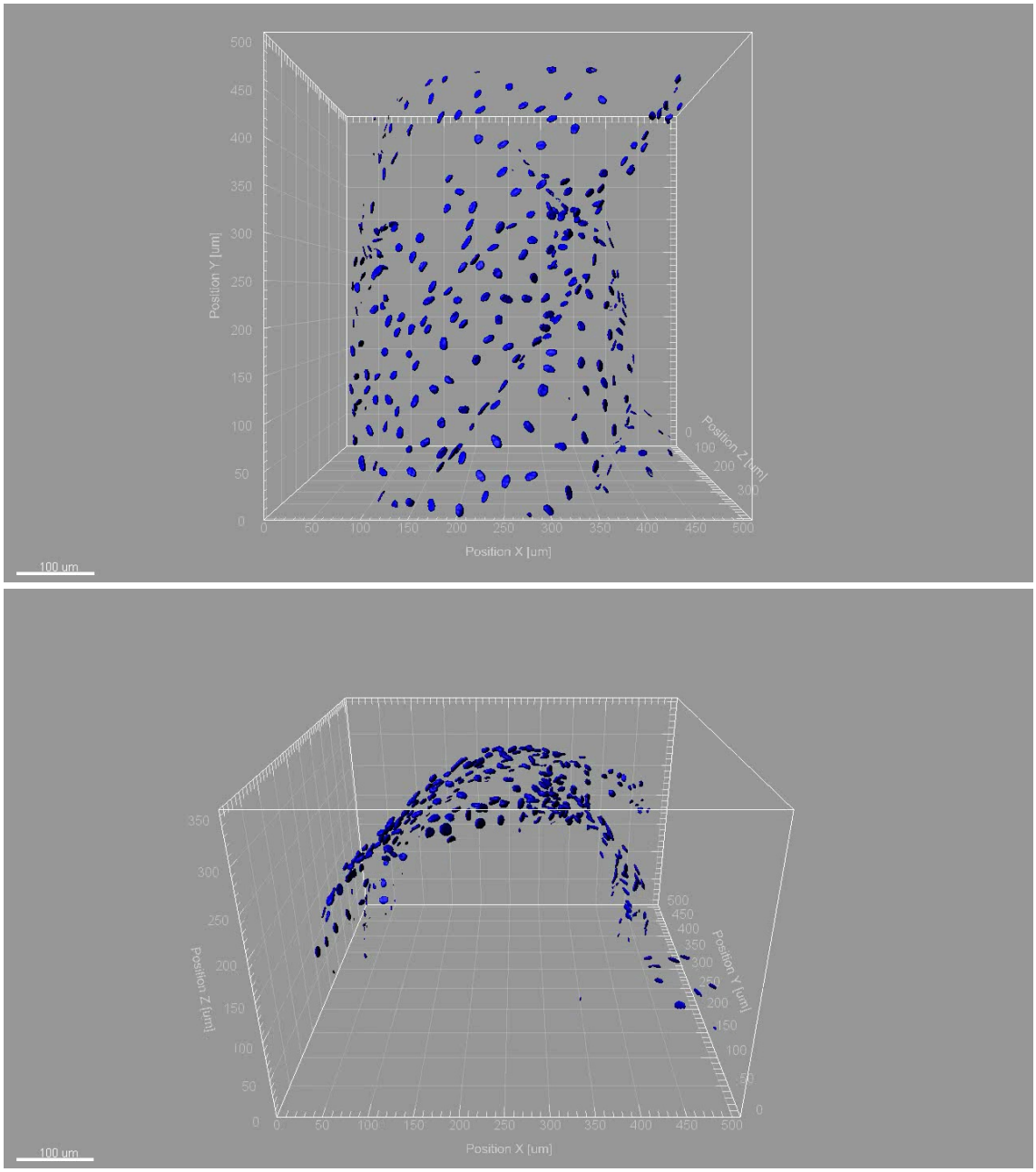

Figure 30. Two-photon microscopy pictures of fibrin fibers embedded with endothelial cells with cell nuclei displayed in blue: top figure presents the top surface of the fiber as the bottom figure shows the side view with cells deposited on the surface of the fiber.

The technique of coextruding fibrin fibers has been previously published and patented by Cornwell et al. [61,65] In this thesis, as a difference to the methods used by Cornwell et al., cells were extruded with the fibrin gel components to homogeneously embed the cells within the fibers as opposed to seeding them on the surface. Thus in this thesis, a novel fabrication process to create cell encapsulated fibrin gel fibers is presented. In addition, Cornwell et al. used more than a three-fold higher concentration of fibrin gel to fabricate smaller diameter fibers of high mechanical strengths. Most likely, this high concentration would not support cell proliferation and migration of cells embedded within the fibrin matrix. On the other hand, stiffer gel fibers are much easier to handle which is beneficial for e.g. collecting the fibers.

The hydrogel extrusion technique presented can be applied in many ways in TE applications. The endothelial cell-laden fibrin fibers can be used to form functional patterned vascular network within a tissue-engineered construct. Alternatively, other cell types can also be used for molding microscale tubular tissue constructs. The extrusion technique is a way to fabricate long, thin tissue constructs with a degree of orientation. As demonstrated in chapter 5.3.1, polymerization under flow increases fibrin fiber alignment, which has been demonstrated previously by several groups [60,66]. This nanoscale alignment of fibers the fibers in the gel can also guide cells to orient in the same direction, and thus create highly aligned 3D tubular tissue constructs. [60]

6 BOTTOM-UP TISSUE ENGINEERING OF A CARDIAC CONDUCTIVE DEVICE

6.1 Concept

Microfabrication of small, biologically relevant size structures with controllable shape and combining them together is a promising approach to create complex, highly organized tissue constructs. In this thesis two techniques for fibrin micromolding are presented with the intention to provide a highly controllable three-dimensional environment that can mediate cell differentiation and functional assembly.

The micromolded tissue constructs demonstrated in this thesis could be used as functional tissue units and further assembled in a larger scale tissue construct using the strategies of bottom-up tissue engineering. Many tissues, e.g. cardiac tissue, are constructed of several cell types that form functional tissue units that are further assembled together to create the complex tissue formation. For example, cardiac tissue is made up of tightly packed aligned myofibrils with dense supporting vasculature and ECM. Even though cardiomyocytes are the major functional component, the supporting role of fibroblasts and endothelial cells is vital. Especially without sufficient vasculature, the energy consuming cardiomyocytes are deprived of oxygen and other nutrients. These components (cardiac myofibrils, supporting ECM, and capillary network) could be fabricated as separate tissue units using the microfabrication techniques presented and later combined to create a construct with all the valuable building blocks.

As previously described, the MIMIC technique could be used to orient cardiomyocytes longitudinally in 3D fibrin gel constructs to allow the cells to form gap junctions with each other to form a functional syncytium that would allow the conduction of the electrical impulses. The extruded fibrin fibers with endothelial cells could be applied to preform the vascularization to provide sufficient nutrition to the cells, especially to the cardiomyocytes, which cannot tolerate hypoxia for long time periods. The third missing component of the cardiac tissue is the ECM. Fibroblasts produce the ECM, which provides the mechanical support for the tissue.

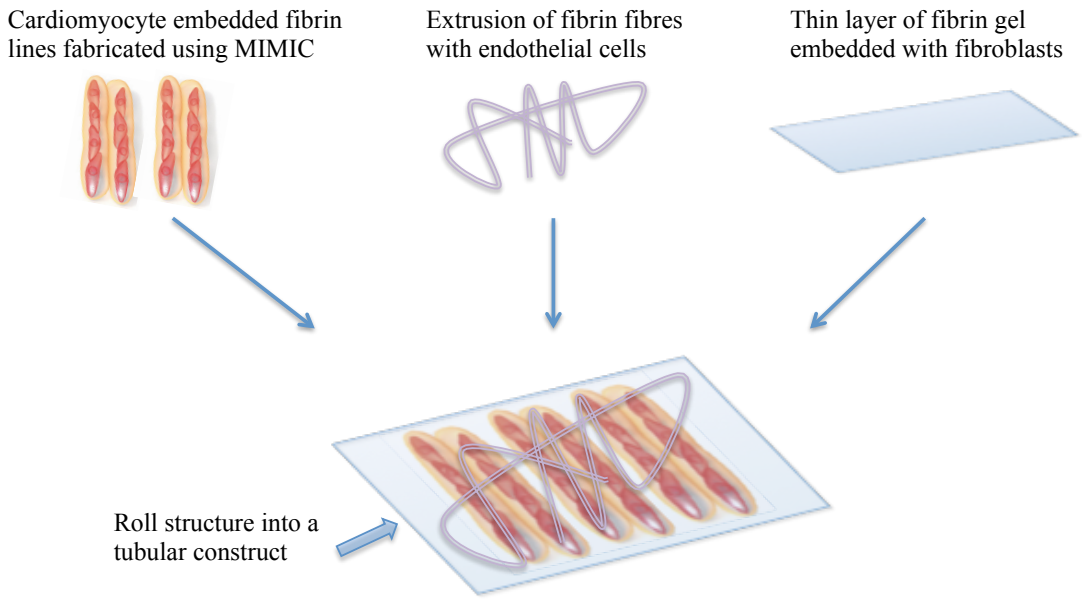

Figure 31. Schematic of the bottom-up tissue engineering approach for the Biopacer.

The idea of using bottom-up TE for the fabrication of a conductive tissue device is displayed in figure 31. The idea is to fabricate a thin layer of fibrin gel with fibroblasts encapsulated homogenously inside. Before it fully polymerizes, the other two building blocks, the fibers with endothelial cells and the aligned cardiomyocyte lines, are assembled in the desired orientation into the thin layer. After polymerization, this thin layer could be rolled tightly enclosing the added components within the construct giving rise to a TE conductive tissue device fabricated using the bottom-up TE approach containing all cellular components.

6.2 Proof of principle

A proof of principle of how the micromolded tissue constructs fabricated in this thesis could be assembled together to form a conductive tissue device is presented in this chapter. The demonstration is done unsterile using food coloring as a visualization aid. Cardiomyocytes could be molded into fibrin gel lines using MIMIC (figure 32a). These lines could be further cultured to allow formation of cell-cell junctions and the guided organization of the cells in the longitudinal direction. These lines can then be embedded into a thin layer of fibrin gel (figure 32b), which can be rolled (figure 32c), in this case with a suture line, to create an organized tubular large-scale 3D tissue construct (figure 32d). Supportive cells, e.g. fibroblasts, can be incorporated into the thin fibrin layer, which could eventually start to form ECM around the aligned cardiomyocyte lines.

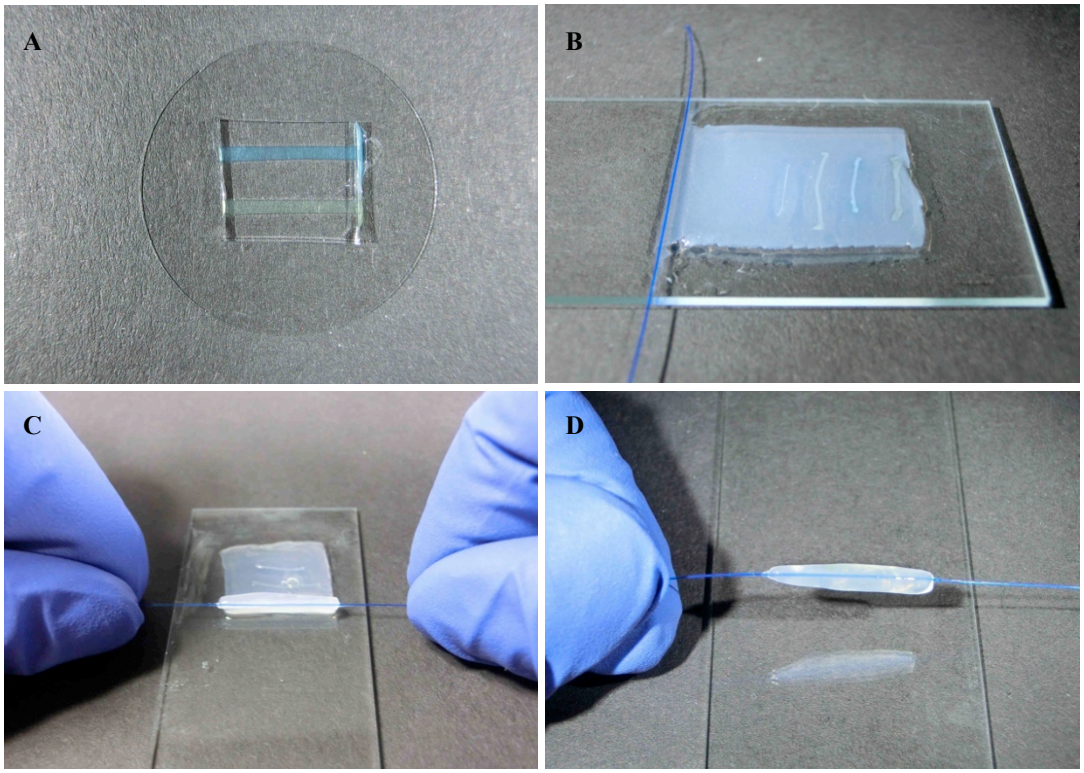

Figure 32. The fabrication of a 3D tubular tissue construct by using a bottom-up TE approach with micromolded fibrin tissue units as building blocks.

Additionally, extruded tubular fibers of fibrin gel with endothelial cells could be incorporated into the construct in the same way. These fibers could be embedded randomly along with the strings of cardiomyocytes fabricated using MIMIC in the thin fibrin layer before rolling the complete construct into a tubular 3D structure with the idea that the fibers would provide a preformed basis for the vasculature. Additional sprouting of the endothelial cells away from the fibers could possibly accommodate for further capillary formation. By preforming the structural outline for capillary formation, it could induce vascular development and thus tackle with the problem of the inability to vascularize tissue constructs that exceed the diffusion limits of oxygen and nutrients.

7 CONCLUSION

Applying microfabrication techniques to create hydrogel constructs with controlled 3D arrangement of cells can provide a way to mimic the high-degree organization of native tissues. Microfabrication techniques can be used to create functional tissue modules to use as building blocks to engineer larger scale 3D tissue constructs. This way a spatially organized, native-like tissue microarchitecture could be created.

In this thesis, two microfabrication techniques were established and exploited for engineering a biological pacemaker –a conductive tissue device. The techniques were realized with the use of fibrin gel and human cells. The proof of principle for molding fibrin gel in capillaries (MIMIC) using PDMS replicated from a self-made wafer was demonstrated. By coating one surface (glass or PDMS) of the closed mold with a protein-repellent (Pluronic®), fibrin gel deposition could be selectively carried out onto either surface. Longitudinal orientation of cells was observed in small diameter fibrin gel lines, demonstrating that the technique can be used to engineering aligned tissue constructs in microscale. Due to the difficulties of obtaining sufficient quantities of cardiomyocytes for the experiments, the MIMIC technique was realized in this thesis using an alternative cell type. Eventually, after further developing the cell isolation procedure to obtain higher quantities of cardiomyocytes, the technique can be realized also using cardiomyocytes.

Additionally, a novel technique for extruding viable fibrin fibers for use in TE was established. Endothelial cells were incorporated into the process to demonstrate a technique for extruding cells homogenously within the hydrogel fibers. SEM images taken of the fibers indicated that polymerization of the gel under laminar flow induced the alignment of the fibrin fibers. The endothelial cells extruded within the fibers were shown to coalesce and bundle the surrounding fibrin into a core around which they arranged themselves resembling the process of tubulogenesis, the early development of vascularization.

Overall, the techniques presented in this thesis demonstrate two prosperous methods for engineering spatially organized tissue constructs in microscale for TE applications. The possibility of using these microfabricated fibrin gel tissue modules as building blocks to create a conductive tissue device using bottom-up tissue engineering was also described. This way the functional units of the tissue, including highly organized cardiomyocytes, vasculature and ECM, could be engineered separately and later assembled together to give rise to a construct of high cellular complexity and organized tissue microarchitecture. All in all, the techniques established and realized in this thesis are not solely applicable for engineering a conductive tissue device but can also be

transferred into other TE applications. In the future, the techniques should be further developed to fabricate even smaller microscale tissue structures to better mimic the native microarchitecture of complex tissues. Additionally, the handling of the micromolded hydrogel structures and especially the process of assembling the tissue building blocks together require further improvements.

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APPENDIX 1: BUFFERS USED FOR CELL ISOLATION

Calcium-free Krebs buffer (KRP)

Name of chemical	Chemical formula	Amount per 1,5 Liter milliQ
KRP basic buffer		
Sodium chloride	NaCl	3,0681 g
Potassium chloride	KCl	0,5312 g
Potassium dihydrogen phosphate	KH_2PO_4	0,2430 g
Monosodium phosphate	NaH_2PO_4	2,8800 g
Sodium bicarbonate	$NaHCO_3$	3,1504 g
D-Glucose	$C_6H_{12}O_6$	2,7024 g
Saccharose	$C_{12}H_{22}O_{11}$	68,8023 g
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)	$C_8H_{18}N_2O_4S$	5,7193 g

Calcium-free Krebs buffer (KRP) + 2,3-Butanedione monoxime (KRP+BDM)

KRP+BDM		
2,3-Butanedione monoxime (BDM)	$C_4H_7NO_2$	4,5500 g

1. The chemicals are dissolved into 1.5 liters of purified water (MilliQ™ Millipore)
2. The pH is set to 7.4.
3. The buffer is sterilized using a sterile filter (Steritop™ Filter Unit) and stored at room temperature.

APPENDIX 2: LIST OF FIGURES AND TABLES