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ÚSTAV BIOMEDICÍNSKÉHO INŽENÝRSTVÍ

CELL TRANSPLANTATION METHODS IN CARDIOLOGY
METODY PŘÍPRAVY BUNĚČNÝCH TRANSPLANTÁTŮ V KARDIOLOGII

MASTER'S THESIS
DIPLOMOVÁ PRÁCE

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Master's Thesis

Master's study field **Biomedical and Ecological Engineering**
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TITLE OF THESIS:

Cell transplantation methods in cardiology

INSTRUCTION:

1) Literature search on the topic of PDMS polymer and adhesion of cells. 2) Literature search on the topic of "cell sheet" and "cell layer on scaffold" and their stability in vitro and experimental transplantation. 3) Selection of polymer surface for cell culture, cell sheet and planar cell layer preparation (cardiomyocytes or fibroblasts), analysis of factors for induction of isotropic structure. 4) Co-culture of two types of cardiac cells on polymer and scaffolds, microscopy analysis of cells co-culture. 5) Practical tests and evaluation of isotropicity by modern micrscopical methods and test of cell sheet and cell layer transfer from in vitro plates. 6) Statistical conclusion.

RECOMMENDED LITERATURE:

[1] UTTAYRAT P. et al. Topographic guidance of endothelial cells on silicone surfaces with micro to nanogrooves: Orientation of actin filaments and focal adhesions. Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials, 2005, 75.3: 668-680.

[2] SHIMIZU T. Cell sheet-based tissue engineering for fabricating 3-dimensional heart tissues. Circulation Journal, 2014, 78.11: 2594-2603.

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Supervisor: Mgr. Josef Skopalík, Ph.D.
Consultant:

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Subject Council chairman

ABSTRACT

This master's thesis is devoted to tissue engineering, notably to creation homogeneous, isotropic and planar cardiac muscle cell layer using two technologies scaffold-based and scaffold-free. Firstly it describes the histology of heart wall, cardiac muscle cells, and cells cultures. This is followed by a description of tissue engineering, which includes cell sheet technology and scaffold-based tissue engineering. And at the end of the theoretical part application of tissue engineering and cell visualisation assay are described. The practical part is dedicated to the creation of a planar cell layer from cardiomyocytes and fibroblasts, using information from the theoretical part.

KEYWORDS

Tissue engineering, cell sheet, scaffold-based tissue engineering, scaffold-free tissue engineering, calcein AM, PKH26, CellMask, PCL, PDMS, PNIPAAm.

ABSTRAKT

Tato diplomová práce se zabývá tkáňovým inženýrstvím, zejména tvorbou homogenní, izotropní a planární vrstvy buněk srdečního svalu pomocí dvou technologii:"scaffold-based" a "scaffold-free". Nejprve popsaný histologie srdeční stěny, buňky srdečního svalu a buněčné kultury. Následuje popis tkáňového inženýrství, který zahrnuje technologii "cell sheet" a tkáňové inženýrství na bázi scaffoldů. Na konci teoretické části je popsána aplikace tkáňového inženýrství a buněčná vizualizace. Praktická část věnovaná tvorbě planární buněčné vrstvy z kardiomyocytů a fibroblastů s využitím informací z teoretické části.

KLÍČOVÁ SLOVA

Tkáňové inženýrství, scaffold-free tkáňové inženýrství, scaffold-based tkáňové inženýrství, calcein AM, PKH26, CellMask, PCL, PDMS, PNIPAAm.

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DECLARATION

I declare that I have written my master's thesis on the theme of "Cell transplantation methods in cardiology" independently, under the guidance of the master's thesis supervisor and using the technical literature and other sources of information which are all cited in the thesis and detailed in the list of literature at the end of the thesis. As the author of the master's thesis I furthermore declare that, as regards the creation of this master's thesis, I have not infringed any copyright. In particular, I have not unlawfully encroached on anyone's personal and/or ownership rights and I am fully aware of the consequences in the case of breaking Regulation S 11 and the following of the Copyright Act No 121/2000 Sb. of the Czech Republic, and of the rights related to intellectual property right and changes in some Acts (Intellectual Property Act) and formulated in later regulations, inclusive of the possible consequences resulting from the provisions of Criminal Act No 40/2009 Sb. of the Czech Republic, Section 2, Head VI, Part 4.

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I would like to thank and recognize the supervisor of this project Mgr. Josef Skopalík, Ph.D., for continued support and guidance throughout the completion of this project.

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Introduction

The concept of tissue engineering (TE) was offered by Professor Robert Langer, a chemical engineer, and Drs. Joseph and Charles Vacanti, two medical doctors, in 1993 [1]. Tissue engineering was developed for the purpose to restore damaged tissues and organs by implanting artificial tissue constructs assembled from primary or cultured cells and extracellular matrix. The goal of biotechnology is the development of artificial or synthetic organs because traditional methods such as transplantation organs of another human (most often post mortem) are not sufficient. Millions of patients are waiting for organs from donors, the necessity of the organ transplant waiting lists is very hopeless and the main idea of TE is to overcome this problem in the near future.

Two major limitations of traditional organ transplantation are not only a critical lack of donors but also the high risk of graft rejection; to overcome these disadvantages the intensive research was started in order to develop live tissue and whole organs in vitro. The main aim of tissue engineering is to develop clinical technology to treat diseases that could not be handled by conventional medicine methods. Almost 30 years have passed since the term “tissue engineering” was created, but there are still many challenges that TE techniques face and that need to be solved such as cell growth [2], vascularization [3, 4], biodegradation rate and stress-shielding caused by scaffolds, cellular phenotype changes, biomaterial’s immunogenicity [5], and many more. [6]

The presented thesis will discuss and will focused on experimental testing of some methods of cell culture, cell “co-culture”, assembling of extracellular matrix and cells, and final preparation of product which mimicking the real tissue.

The thesis contains mainly cardiac cells culture techniques, and tissue engineering. In spite of the fact that many breakthroughs have been made in cardiovascular surgery and medicine heart attacks and heart failure still remain a great danger for human life [7]. The main cause is that adult heart muscle cells (cardiomyocytes) cannot divide to replace injured cells, which means that the heart cannot repair itself by native processes. Appeared scar tissue is able to keep the organ intact but it cannot contract with efficient power. Clinical intervention can avoid scar formation or replace formed scar tissue with functional cardiac tissue. One of the possible ways is to use cells (so-called stem cells or progenitor cells) injections into damaged areas. However, studies have achieved limited success because of cell death, the exit of cells from the heart, and poor cellular integration with the receiving heart tissue (see chapter 3.2).

Cells have defined requirements for their survival in an environment such as the nutrients, oxygen and some types of cells, including cardiomyocytes, need to be attached to a suitable supporting surface. This is the aim of TE, to create a viable cellular environment by using biologically acceptable materials [1]. The approach is that transplantable cells can be contained and organized in so-called engineered scaffolds. Such scaffolds with cells can then be used in treating or replacement a part of the body [8]. Another method is the application of the scaffold-free approach for creating a cell sheet.

This master's thesis is dedicated to the formation of the cellular confluent monolayer that consists of cardiac muscle cells and fibroblast, using both scaffold-based, and scaffold-free approaches. Also, this thesis presents information about the histology of cardiovascular system and cells that can be used in TE; provides a description of basic methods of tissue engineering and their application; and methods of cells visualization. The practical part of the thesis is focused on the implementation of theoretical knowledge in order to create cellular confluent monolayer using different types of scaffold, evaluation of biocompatibility of scaffolds, evaluation of cell-scaffold and cell-cell adhesion and testing the experimental prototype of cell sheet technology.

1. The histology of the cardiovascular system

The heart is a muscular pump that propels blood at high pressure around the body through the blood vessels. The heart contracts rhythmically and autonomously. Contractions begin at the apex of the heart and spread through to the postero-basal region. The cardiovascular system consists of the heart, arteries, microvascular bed, veins, and lymphatic vessels, all these components are crucial for exchanging oxygen, carbon dioxide, metabolites, salts, and metabolic waste products. The heart wall has three layers, as shown in Figure 1 below: epicardium (outer layer), myocardium (middle layer) and endocardium (inner layer). [9]

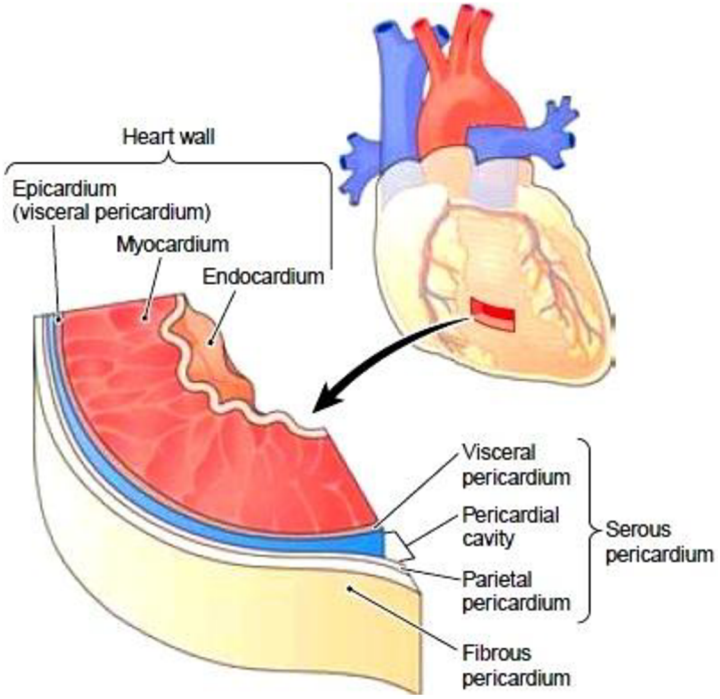

Figure 1: The layers of the heart wall. [10]

Epicardium is the outer layer of the heart wall. It is also known as visceral pericardium as it forms the inner layer of the pericardium. The epicardium is composed primarily of connective tissue, including elastic fibres and adipose tissue, and a single layer of mesothelial cells. The epicardium functions to protect the inner heart layers and also assists in the production of pericardial fluid. This fluid fills the pericardial cavity and helps to reduce friction between pericardial membranes. In this heart layer there are also found coronary blood vessels, which supply the heart wall with blood. The inner layer of the epicardium is in direct contact with the myocardium. [9, 11, 12]

The myocardium is the middle layer of the heart wall. It is composed of cardiac muscle fibres, which enable heart contractions. The myocardium is the thickest layer of the

heart wall, with its thickness varying in different parts of the heart. The myocardium of the left ventricle is the thickest as this ventricle is responsible for generating the power needed to pump oxygenated blood from the heart to the rest of the body. Cardiac muscle contractions are under the control of the peripheral nervous system, which controls involuntary functions including heart rate. Cardiac conduction is made possible by specialized myocardial muscle fibres. These fibre bundles, consisting of the atrioventricular bundle and Purkinje fibres, carry electrical impulses down the centre of the heart to the ventricles. These impulses trigger the muscle fibres in the ventricles to contract. [9, 11, 12]

The endocardium is the thin inner layer of the heart wall. This layer lines the inner heart chambers, covers heart valves and is continuous with the endothelium of large blood vessels. The endocardium of heart atria consists of smooth muscle, as well as elastic fibres. [9, 12]

2. Cardiac muscle cells *in vivo* and *in vitro*

The adult mammalian heart is composed of many cell types cardiomyocytes represent only 20%-30% of the total cell number of; the remaining 70-80% are occupied by so-called non-myocytes. Non-myocytes in the heart are represented by such types of cells as fibroblasts, endothelial cells, and vascular smooth muscle cells, with the majority being fibroblasts. They are organized in a complex 3-dimensional tissue that includes mechanically and electrically connected cardiomyocytes intimately coupled to other cell types. [13]

There is a confusion with cardiac fibroblasts in the literature; they have various terms that vary depending on the author. Among them are fibrocyte, telocyte, myofibroblast, protomyofibroblast, mesenchymal cell and stromal cell. [14]

Cardiomyocyte can be found only in heart muscle. They are very similar to skeletal muscle, but unlike it, they are shorter, organized into sarcomeres and each cardiac muscle cell contains a single (or more) nucleus in the centre of the cell (see Figure 2). Cardiac muscle cells have a branched shape and connected with nearby cells via intercalated disc, which allows contracting in a wave-like pattern. [15]

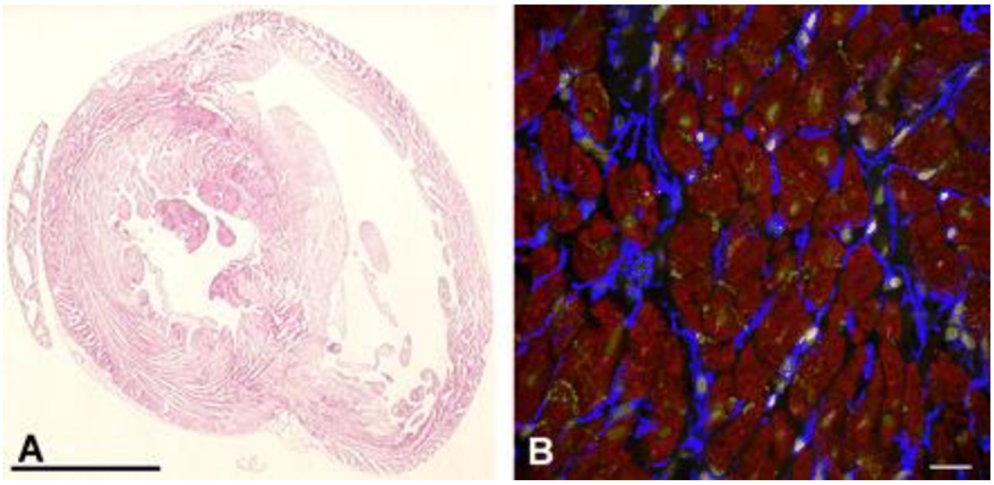

Figure 2: Macroscopic cross-section of heart and microscopic details of tissue with specific staining of cardiomyocytes and fibroblasts. (A) Hematoxylin and Eosin staining of cardiac muscle cells (pink), filled with (un-labelled) non-myocytes. (B) Immuno-stained myocytes (red) (labelled with anti-myomesin, anti-vimentin to mark fibroblasts (blue), anti-connexin43 (bright green dots) and with DAPI (4'-6-Diamidino-2-phenylindole) to label nuclei (pale yellow-green patches), confocal microscopy tissue cross-section. Scale bars in A and B: 5 mm and 20 µm, respectively. [16]

2.1. Isolated cardiomyocyte culture

There are several problems with isolated cardiomyocytes. The first problem is that cells are not multiplying in culture, because after birth they are not able to divide. Another problem lies in the fact that intercalated disks become distracted after isolation, which is manifested in the risk of overloading the cells with Ca^{2+} and subsequent hyper contracture and cell death. [17]

Neonatal cardiomyocyte culture

This type of cardiomyocytes is received from the rat heart one or two days after birth. Isolated cells lose their rectangular shape but keep the ability to contract when stimulated. [17, 18]

Adult cardiomyocyte culture

Connection of cardiomyocytes via gap junctions is disrupted through the isolation process and as a result cells are partially permeable. Therefore, adult cardiomyocyte have to be digested and maintained in a medium which free from Ca^{2+} as long as the cell membrane is restored. Isolated cardiomyocytes remain their structure but without stimulation they gradually lose it day by day. Nuclei of cardiomyocytes are not visible under light or phase-contrast microscopy. [17, 19]

HL-1 cells

The HL-1 cells derived from a mouse AT-1 cardiomyocytes are new cardiac muscle cells. In this line, cells were transformed via cardiac-directed expression of the SV40 large T antigen. HL-1 cells continuously divide and spontaneously contracts while maintaining a differentiated cardiac phenotype. They are widely used in laboratories as a good cellular model that mimics the electrophysiology of real cardiomyocytes, as well as partly cell morphology and metabolism. [17, 20]

2.2. Co-culture of cardiomyocytes and fibroblasts

As noted in chapter 2, the mammalian heart consists not only of cardiomyocytes but also of endothelial cells, fibroblasts, and smooth muscle cells. Fibroblasts are the largest cell population among non-myocytes in the myocardium [21], they are located in the whole heart alongside individual myocytes and between layers of the myocardium (see Figure 3) [22]. Accordingly, encapsulation of cardiomyocytes and cardiac fibroblasts is used to develop *in vivo*-like myocardium. The main functions of cardiac fibroblasts are the mechanotransduction and mechanical support of cardiomyocytes (strong connection of cells with the elasticity and flexibility of the tissue at every moment of the heartbeat), synthesis or degradation of ECM components such as fibrin and collagen [16], and partially electrical conductivity through the heart [21, 23] (however, fibroblasts are not excitable cells), in addition, cardiac fibroblasts affect the phenotype of cardiomyocytes

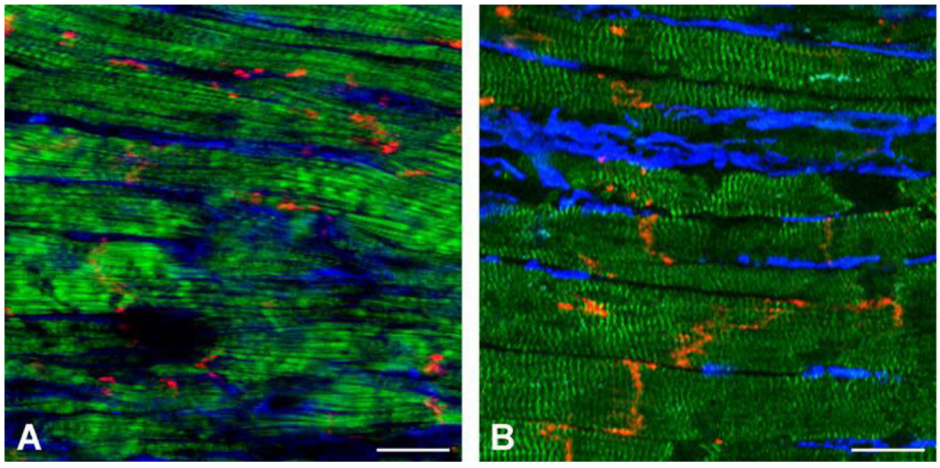

Figure 3: Fibroblast myocyte interrelation in cardiac tissue. (A) Fibroblasts labelled with anti-DDR2 (blue) separate phalloidin-labelled myocytes (green) in mouse ventricular tissue (red label: connexin43). (B) Fibroblasts labelled with anti-vimentin (blue) form layers between anti-myomesin labelled myocytes (green) in sheep ventricular tissue (red: connexin43). Scale bar: 20 µm. [16]

The assembly of fibroblasts and cardiomyocytes into a specific structure that mimics real tissue is not easy. Several studies have used the co-culture of fibroblasts and cardiomyocytes with engineered tissue constructs [22, 24- 27]. These studies demonstrate that the co-culture of cardiomyocytes and cardiac fibroblasts is significant for promoting the maturity of engineered tissues. For instance, Camelliti et al. [22] presented 2D *in vitro* model of cardiac myocyte–fibroblast interrelation. The outcome of this study demonstrated that structured co-cultures of cardiomyocytes and fibroblasts mimic *in vivo* ventricular tissue organization and provide a novel tool for *in vitro* research into cardiac electromechanical function. Similarly, in another study [28] there were developed 3D cardiac microtissues embedded with the co-culture of cardiomyocytes and cardiac fibroblasts using GelMA hydrogel. In comparison with monoculture condition, enhanced cytoskeletal organization, specific cardiac protein expression, and synchronous contraction of the co-culture of the cells was observed. Also, this study demonstrated that for an optimized cell-cell ratio, decreasing the geometrical features of the constructs adversely affected the synchronous contraction of the cells.

3. Tissue engineering

Tissue engineering is one of the important technologies of regenerative medicine. It is a combination of cells, engineering, materials methods, suitable biochemical and physicochemical factors to improve or replace biological tissues. Although, increasing interest in the regenerative medicine and tissue engineering appeared in the last 10-15 years cell-based therapies have been clinically used for 40 years. [29, 30]

Traditional tissue engineering methods have two strategies: either the injection of isolated cell suspensions or application of the cells, which are in solid or semi-solid scaffolds (mostly biodegradable scaffolds to support tissue formation or additive grow factors for final cell maturation). However, there is a relatively new technology, originating in 1990 [31], called “cell sheet technology“.

3.1. Cell sheet technology

Cell sheet tissue engineering is a scaffold-free approach to regenerating damaged tissue cells. Cell sheet technique was created to overcome the limitations of conventional tissue engineering methods such as cell suspensions, scaffolds, and growth factors. Cell sheet is represented by a complete cell layer with cell-connections, nexin, ion channels, extracellular matrix (ECM) and growth factor and a lot of other significant cell proteins in the microenvironment of cells. In cell sheet technology, sheets are collected without breaking, as an intact cell layer, without the use of proteolytic enzyme treatment or the use of mechanical scraping to detach cells from the culture dish. [29]

According to [32], cell survival rate after cardiac cell sheet transplantation was higher compared with the injection of "suspension of single cells". Also, the variant of the cell sheet caused higher improvement in cardiac function than the suspension of the single cells. In addition to cardiac applications, cell sheet technology can be used for the treatment of a large number of diseases with encouraging results.

For example, for patients with bilateral total limbal stem-cell deficiency, epithelial cell sheets were harvested for transplantation onto the ocular surface. Besides, it is possible to stabilize it to the host eye without sutures due to the presence of ECM in the cell sheet that provided sufficient adhesion. Also, cell sheet technology (using different cell types) can be used to treat oesophageal cancer, periodontitis, bone fractures, liver disease, and diabetes. [33]

Another example of the advantages of cell sheet engineering is the reconstruction of smooth muscle (see Figure 5). It is crucial to create engineered constructs consisting of cells that are intimately connected, forming a single continuous tissue similar to native smooth muscle. Firstly, see 5A, smooth muscle cells are harvested as intact sheets cultured using temperature-responsive dishes. Secondly, cultured smooth muscle cells were layered to two (see Figure 5B) and five sheet structure (see Figure 5C). Later, after the cell sheet was transplanted (see Figure 5D) which took only five minutes due to the existence of deposited ECM on the basal sheet surface. According to the authors of the article [6], a week after transplantation resected tissues demonstrates the presence of the layered smooth muscle sheets. Also, at the implant area site is forming a viable cell-dense structure (see Figure 4A), also authors indicate, that sutures on the Figure 5D are used only to mark the borders of the transplanted constructs. By comparison, after injection of the isolated smooth muscle cells suspension, small island like aggregates with significant cell loss was detected what is represented in Figure 4B. [6]

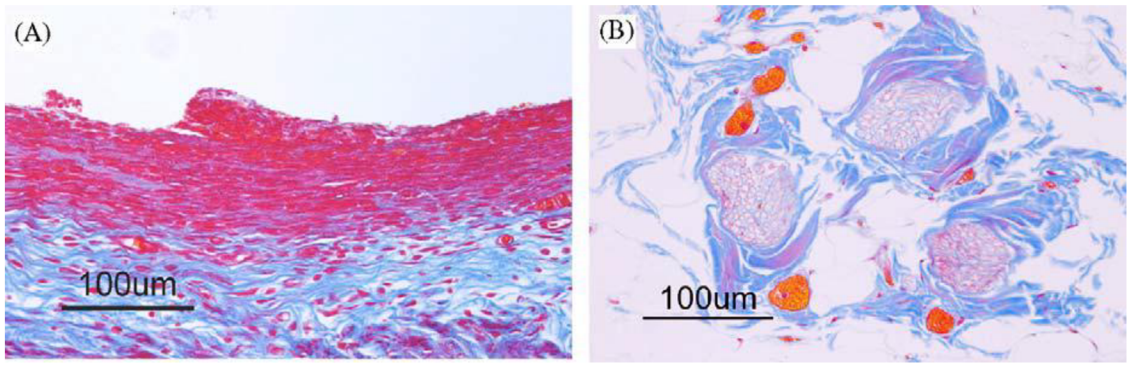

Figure 4: Transplanted smooth muscle cells. (A) Cell sheet one week after surgery, azan staining. (B) The injection of a smooth muscle cell suspension. [6]

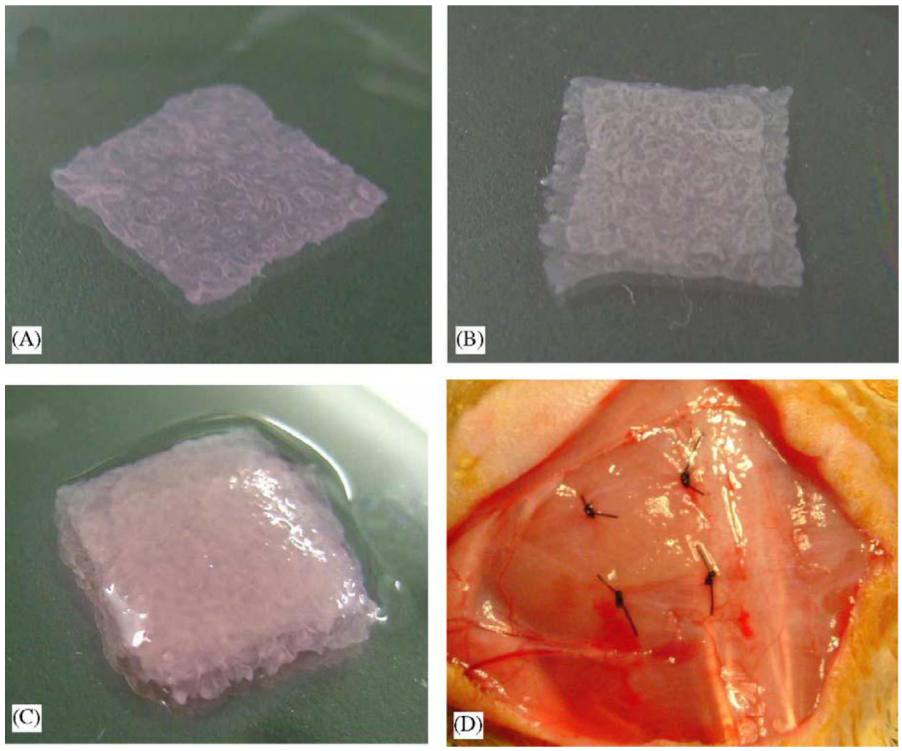

Figure 5: Smooth muscle cell sheets. (A) A sheet of smooth muscle cells. (B) Two or (C) five smooth muscle cell sheets. (D)Transplanted five-layer smooth muscle cell. [6]

Cell sheet technology also has some disadvantages, for example, it shows poor mechanical properties, which makes it difficult to handle. To address this problem, plunger-like devices were created, which allow manipulating with cell sheet without damages [29]. But this method requires highly skilled and experienced users, it is expensive and time-consuming. Also, while using more than one cell sheet they can be overlapped, this may lead to necrosis in the middle of the cell sheets due to poor nutrition or hypoxia, and furthermore ischemia can limit cell sheet survival. [5]

3.1.1. Thermoresponsive polymer surfaces for cell sheet engineering

Thermoresponsive coatings and surfaces were established especially for cell culture and are based primarily on poly(N-isopropylacrylamide) (PNIPAAm). For the first time, the temperature was used to detach confluent cell sheets, without applying enzymatic treatments, in 1990 by Takezawa et al. [34]. In this study, human dermal fibroblasts were cultured at 37°C on the dish, consisting a blend of PNIPAAm and collagen until cells created confluent monolayer. After the temperature was decreased to 15°C, fibroblasts sheet starts to detach from the dish. The biophysical principal of the used thermoresponsive polymer for isolation of intact monolayer can be described as: "adhesive forces and connectivity to basis pad must be cut before any destruction of intracellular connection".

Temperature-responsive cell culture surface is an essential technology for the preparation of cell sheets. The surface of tissue culture polystyrene (TCPS) dish is modified with a thin layer of temperature-responsive polymer, PNIPAAm, which is the most studied thermoresponsive polymer [35]. This polymer consists of a hydrocarbon backbone, hydrophilic groups in the form of adjacent carbonyl and amide groups and hydrophobic moieties in the form of isopropyl groups [36]. In pure water, PNIPAAm changes its physical properties across its lower critical solution temperature (LCST) of approximately 32 °C [37]. At this temperature occurs sharp and reversible hydrophilic (T < LCST) to hydrophobic (T > LCST) transition (Figure 6). That means that cells can be cultured at temperature when T > LCST and can be detached by reducing the temperature to T< LCST (polymer coating becomes hydrophilic). Actually, these polymers are popular in biomedical applications due to the fact that transition occurs in a physiologically relevant temperature range.

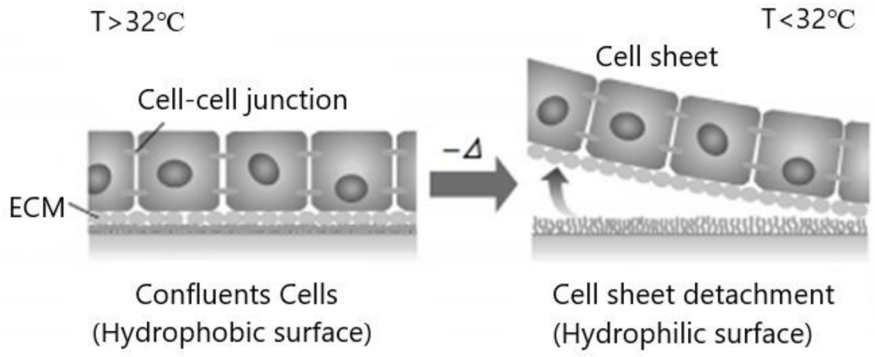

Figure 6: Cell sheet with preserved cell-cell junctions and associated extracellular matrix without the use of enzymatic treatment. Adopted from [37]

Mammalian cells in their natural environment do not exist in solution but interact with ECM for the development, organization, and growth of the tissues. Therefore, cells adhere to solid substrates to maintenance. Consequently, attached cells proliferate and produce ECM, forming confluent cell monolayers. [38]

Conventional cell detachment methods use enzymatic digestion, usually trypsin, or mechanical scraping, that results in the disruption of the cellular membrane and the cleavage of important transmembrane proteins and may lead to compromised function. Also, during proteolysis cell to cell and cell to ECM junctions are lost and as a result, it is impossible to retain a solid cell sheet which can be used in TE. In contrast, cells which were detached from thermoresponsive surfaces do not suffer such damage. Using simple temperature control allows the detachment of cells, with cell to cell junctions and residual ECM maintained, from a thermoresponsive surface (see Figure 7). [36]

Nowadays, there are different ways to obtain PNIPPAm surface. Nevertheless, some PNIPAAm-based surfaces do not support cell adhesion that makes them inappropriate for this purpose. The most commonly used techniques are radiation cross-linking, atom transfer radical polymerization, electron-beam processing, oxygen plasma-treatment and UV light irradiation [39]. As part of this work, oxygen plasma-treatment will be used. Plasma treatment is an approach to improve cell adhesiveness. Oxygen-plasma treatment of the PNIPAAm film can modify the surface of the film and therefore enhance the attachment of cells. Shimizu et al. [40] subjected PNIPAAm film to high-intensity plasma treatment. The treated films showed increased cell adhesion and proliferation.

The two most used parameters for the characterization of thermoresponsive surfaces are contact angle and thickness. The contact angle is one of the common ways to measure the wettability of a surface. The contact angle is 0° in the case of complete wetting. If contact angle is between 0° and 90°, the solid is wettable and above 90° it is not wettable (see Figure 8). Wilhelmy method or sessile drop method, for example, can be used for measurement of contact angle. Usually, the contact angle is determined to both temperatures T > LCST and T < LCST.

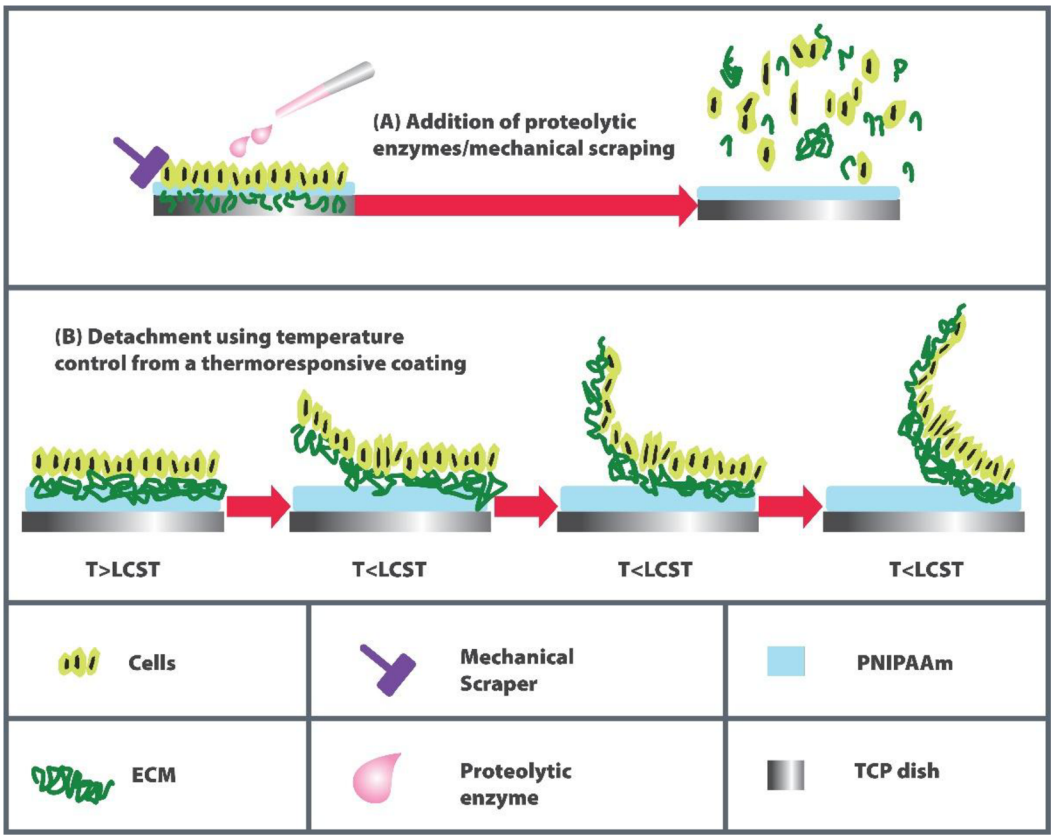

Figure 7: (A) Schematic illustrating the detachment of cells via conventional processes via the addition of proteolytic enzymes or mechanical scraping. (B) Schematic illustrating the detachment of cells from a thermoresponsive surface using temperature control. Adopted from [36]

Another significant parameter is the thickness of the PNIPAAm coatings. Temperature-controlled cell adhesion and detachment are achieved only if the thickness is selected correctly. The difficulty, however, is that all fabrication methods, with the exception of plasma polymerization, require different thickness to achieve cell adhesion and detachment.

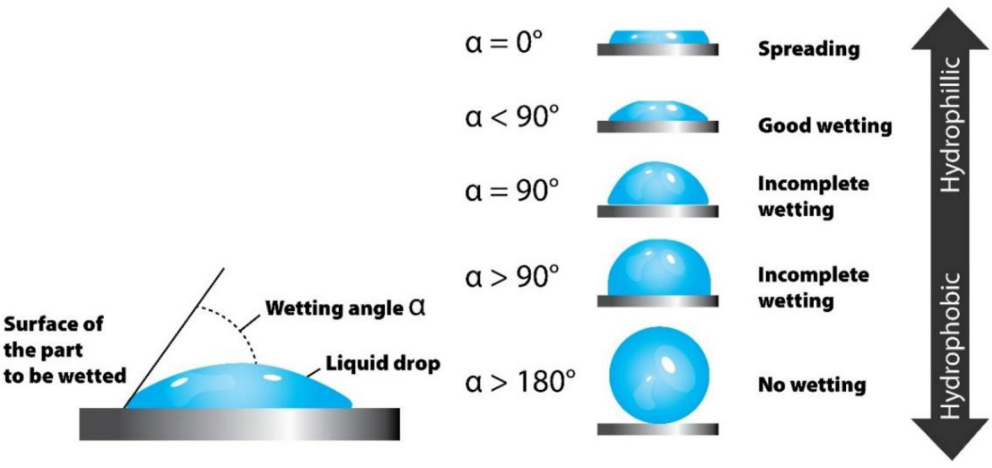

Figure 8: Contact angle on different materials. Adopted from [41]

3.2. Scaffold-based tissue engineering

Using biodegradable scaffolds is a method in TE that stay between transplantation of cell sheet and injection of single cells. The polymer scaffolds for organ reconstruction using polymers with various properties are applied for the seeding of different cell types, according to requirements. Biodegradable scaffolds are used in TE for temporary replacement of ECM during the regeneration and remodelling period, whereupon scaffold should be degraded and substituted by the new tissue. The main objective for the tissue engineering scaffolds is to create a biomimetic scaffold that allows step-by-step remodelling of the surrounding tissues as well as emerging ECM. The aim is to develop such scaffold that will resemble the natural extracellular matrix after the removal of the bioactive molecules during the process of their degradation, meanwhile ensuring mechanical stability. [42, 43]

Scaffold-based TE can be used for treatment or regeneration of various tissues such as skin, lung, trachea, intestine, liver, kidney, heart, blood vessels, nervous system, thyroid, pancreas and musculoskeletal tissues [44]. All scaffolds used in tissue engineering shall comply with general scaffold design requirements. Materials used in the production of scaffolds should be highly biocompatible, that means that they do not elicit an immune response after implantation. Another essential requirement is porosity because with changing the size of pore and porosity it is possible to create conditions for different tissue types. Also, scaffolds used in tissue engineering have to be developed to provide attachment, migration, proliferation, and differentiation of cells. To obtain orientated adsorption of molecules to the surface of the scaffold the macrotopography, microtopography and chemical properties of the scaffold can be changed [45]. Besides, at the time of implantation, the mechanical properties of the scaffold should match the characteristics of the tissue as much as possible. [43]

Nevertheless, the application of scaffold-based structures has limitations. The first disadvantage involves scaffold degradation because after degradation the spaces previously filled with the polymer scaffolds often become occupied by large amounts of deposited ECM. Thus, in the reconstitution of cell-sparse structures such as cartilage or bone, artificial structures can look like natural tissues, with relatively few cells and significant amounts of ECM. But on the other side, in situations which require cell-dense structures such as in the heart or liver tissues, the result can differ from native tissue and sometimes this can result in pathological cases of fibrosis. The second problem, in case of bigger construction, it is often remarked that cells on the periphery of the scaffolds are generally healthy and very similar to native tissues, but cells at the centre are necrotic. These methods often result in unsatisfactory

tissue regeneration due to the limits on passive diffusion, both the delivery of nutrients and the removal of metabolic wastes are limited and lead to the observed loss of cell viability. [6, 43]

3.2.1. Scaffold materials

The human body consists of tissues which constitute a complex of organized and well-defined 3D structure. Scaffolds are used in tissue engineering as an artificial extracellular matrix, which means that they should possess all the features that have real ECM. [46]

The major reason for the application of biodegradable scaffolds in TE is temporarily substituting the extracellular matrix and its biological functions during the regeneration and remodelling period. Whenever the remodelling and regeneration periods are over, the scaffold is degraded and replaced by new tissue. In this thesis, two types of scaffold were used: polycaprolactone scaffold and polydimethylsiloxane scaffold.

Polycaprolactone

Poly(ε-caprolactone) (PCL) is widely used in biomedical applications due to its properties. PCL is a biodegradable and biocompatible polymer, which makes it to be an appropriate material for scaffold-based tissue engineering. Furthermore, PCL has been approved by the Food and Drug Administration (FDA) [47]. The cells live in a complex mixture of pores, ridges and fibres of ECM and electrospinning have proved to be an effective technique for the production of nanofibers with these characteristics.

Device for electrospinning is dived into four basic parts such as a syringe, a spinneret, fibre collector, and a high-voltage power supply (see Figure 9). The syringe is a reservoir for the PCL solution, the syringe is connected to the spinneret. PCL solution is fed through the spinneret, the feeding rate is controlled by a syringe pump. Underneath the spinneret, the fibre collector is located. To convert PCL solution into an electrified polymer jet high voltage/low current is required. Voltage (up to 30 kV) is applied across the spinneret and the grounded collector during electrospinning. [46]

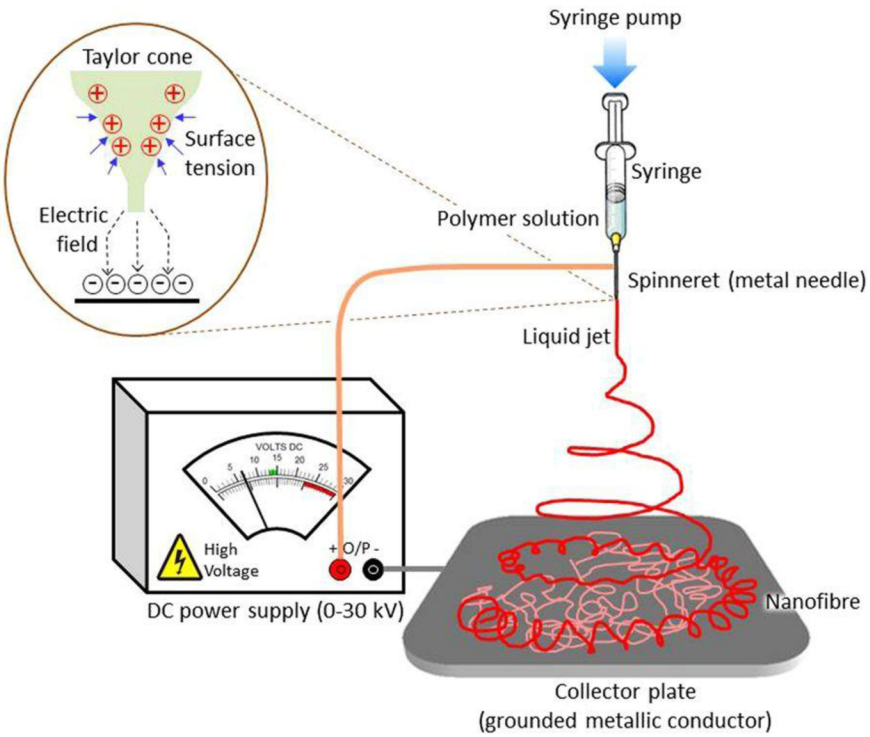

Figure 9: Scheme of production of PCL nanofiber using electrospinning. [48]

Polydimethylsiloxane scaffold

Polydimethylsiloxane (PDMS) is used in biomedical applications because of its advantages such as biocompatibility, low toxicity, chemical inertness, mechanical flexibility, durability and because it is elastomeric, it can contact non-planar surfaces.

As the master for PDMS substrate was used silicon. To increase photoresist adhesion on master, it should be hydrophobic (for example silicon can be coated with HMDS). Spin coat photoresist S1813 on silicon to create a 2 μm thick layer. Bake photoresist at the hot plate at 115 °C for 60 s. Expose pattern by direct writing by DWL with power 4.8 mW. Rinse master with patterned photoresist in deionized water and dry by N_2 gas. Again coat master by HMDS vapors in N_2 gas at 100 °C for 60 s (to easily peel off PDMS from master). Prepare PMDS by mixing silicone elastomer and a silicone elastomer curing agent at a ratio of 10:1 and pour on HMDS treated master and cure at 80 °C at the oven for 90 minutes. Then peel off PDMS substrate and cut to the desired shape. PDMS substrates used in the thesis has lamellae 3 μm tall, 3 μm wide with the distance 5 μm, 10 μm and 20 μm. Process of creating of PDMS scaffold using the previous description is represented in Figure 10.

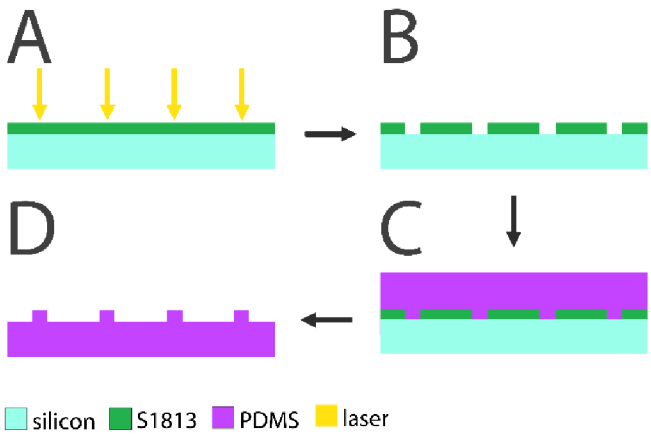

Figure 10: Scheme of fabrication of PDMS scaffold.

4. Molecular theory of cellular adhesion

Cell adhesion molecules (CAM) help to a single cell to stick to another cell or an ECM. Cell adhesion is crucial for cell communication and regulation. Interactions between cells to each other and their extracellular matrix ensure a stable environment for cell growth, differentiation, and migration. There are four groups of adhesion molecules: integrins, selectins, cadherins, and members of the immunoglobulin superfamily (see Figure 11).

Cell adhesion molecules can be divided into three parts: an intracellular domain, a transmembrane domain, and an extracellular domain. In essence, CAMs can be described as an extracellular domain anchored to the surface membrane of the cell by a short, hydrophobic transmembrane domain that is followed by a cytoplasmic tail of variable length, depending on the receptor. They are serving as "molecular bridges" which connect molecules outside of the cell with a cytoskeletal and signal-transducing mechanism within the cell. [49-52]

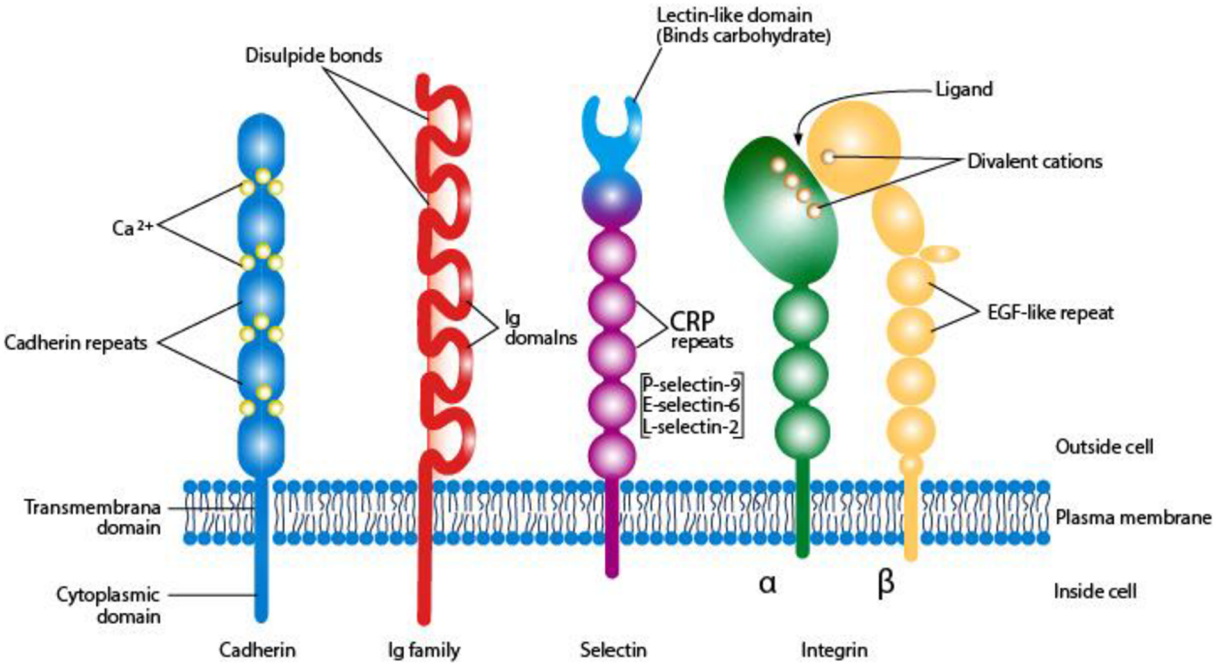

Figure 11: Schematic representation of the major families of cell adhesion receptors.

4.1. Cell-cell and cell-ECM adhesion

Cells in multicellular populations are mechanically connected both to the ECM and neighbouring cells. Physical interactions are mostly guided by adhesions between neighbouring cells and between cells and the ECM [53, 54]. For the purposes of this thesis cell-cell and cell-ECM adhesions are important.

The integrins

Integrins are heterodimeric cell surface receptors that consist of one alpha (α) and one beta (β) chains. Integrins primary serve as mediators of cell-ECM adhesion, and they also serve as one of many families of molecules active in cell-cell adhesion. The subunits have a head region, which forms the main binding site for extracellular matrix proteins, such as fibronectin, fibrinogen, and collagen. There are about 18 different alpha subunits and 8 subunits beta [55]. The chains expressed in myocytes are： $\alpha_1, \alpha_3, \alpha_5, \alpha_6, \alpha_7, \alpha_9$, and α_{10} [56]. Cardiac fibroblasts express similar subunits like cardiomyocyte, but they do not express α_6, and α_7, because cells have no laminin-containing basement membrane. And meanwhile α_V and the collagen-specific α_2 subunit are expressed only by the cardiac fibroblasts, but not by cardiac myocytes [56].

Various combinations of alpha and beta chains determine the particular ligands bound. Some alfa subunits can bind to more than one β subunit, for example α_V associates with five different β subunits. At the same time, a complex of α and β subunits can bind to one or several ligands and in addition, several different integrins can serve as receptors for the same ligand (see Table 1). For example, $\beta_1\alpha_3$ and $\beta_1\alpha_5$ are integrins expressed on cardiac myocytes, and while $\beta_1\alpha_3$ bind to fibronectin, collagen I, and laminin, $\beta_1\alpha_5$ serves exclusively as a fibronectin-binding integrin. Also, cardiomyocyte integrins $\beta_1\alpha_1, \beta_1\alpha_3$ and $\beta_1\alpha_7$ are laminin-binding, but fibroblast integrins such as $\beta_1\alpha_5$ and $\beta_1\alpha_V$ are binding fibronectin. [51]

Table 1: Integrin subunit associations. [57- 59]

Beta chain	Alpha chain	Ligands
β_1	α_1	Collagen, laminin
	α_2	Collagen, laminin, E-cadherin
	α_3	Fibronectin, laminin, collagen
	α_4	Fibronectin, VCAM-1, MAdCAM-1, TSP
	α_5	Fibronectin, TSP, osteoponin, fibrillin
	α_6	Laminin, TSP, CYR61
	α_7	Laminin
	α_8	Fibronectin, vitronectin, tenascin
	α_9	Tenascin, osteoponin, VCAM-1,plasmin, VEGF-A, VEGF-C, VEGF-D, HGF
	α_{10}	Collagen
	α_{11}	Collagen
	α_V	Fibronectin, vitronectin, collagen, fibrinogen, VWF, laminin
β_2	α_L	ICAM-1, ICAM-2, ICAM-3, ICAM-4, ICAM-5
	α_M	Fibrinogen, fibronectin, iC3b, ICAM-1, ICAM-2, factor X, glucan, heparin
	α_X	Fibrinogen, IC3b, ICAM-1,factor X, heparin
	α_D	VCAM-1, vitronectin, plasmonogen, ICAM-3
β_3	α_{IIb}	Fibrinogen, fibronectin, VWF,TSP
	α_V	Fibrinogen, osteoponin, VWF, PE-CAM-1, vitronectin, fibrinogen, TSP, collagen, entacin, tenoscin, VEFG-A, fibrillin-1
β_4	α_6	Laminin
β_5	α_V	Vitronectin, fibronectin, fibrinogen, osteoponin, TSP
β_6	α_V	Fibronectin fibrinogen, vitronectin, tenoscin, fibrillin-1
β_7	α_4	MadCAM-1,fibronectin
	α_E	E-cadherin
β_8	α_V	Fibronectin, vitronectin

After ligand binding, the integrin receptors become like nano- or microdomains on the cell membrane, called focal adhesions. In focal adhesions, the integrins communicate with structural and signalling molecules. They are represented by focal adhesion proteins, such as talin, α-actinin, filamin, paxillin or vinculin [60, 61]. These proteins are the linkers between the integrin receptors and the cytoplasmatic actin cytoskeleton and they are represented by cytohesin-1, focal adhesion kinase (FAK), integrin-linked kinase (ILK), mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), β_3-endonexin, integrin cytoplasmic domain-associated protein-1 (ICAP-1), receptor for activated protein kinase C (Rack-1), calcium- and integrin-binding protein (CIB) or small GTPases. [62]

The cadherins

Cadherins represent another class of CAMs. They create homophilic interactions between cells (cell-cell adhesion), forming intercellular connections also known as adherens junctions, these junctions are critical for cell-cell connection, multicellular groups, and larger tissues. [63]. Cadherins are monomeric receptors, unlike the integrins (see Figure 11). Also, they are calcium-dependent receptors which means that the cadherin molecule on one cell binds to the identical cadherin molecule on the other cell only in the presence of calcium. Cadherin-mediated cell adhesion plays a critical role in heart, for example, N-cadherins form part of the intercalated discs that link myocytes. [51]

5. Modern transplantation of cells in scaffolds and cells in the form of the pure cell sheet

Organ transplantation is the main medical treatment for patients with organ failure. However, the significant lack of donor organs and transplant rejection make it difficult. It is expected that regenerative medicine will become an alternative to organ transplantation and will solve these two issues.

5.1. Cell sheet tissue engineering

Cell sheet tissue engineering involves single cell sheet implantation, cell sheets combined with scaffolds implantation and 3D (multilayer) cell sheet implantation. Single cell sheet implantation is used to treat periodontal ligament, skin, corneal epithelium and bladder

epithelium. Cell sheet combined with scaffolds is used to construct bone and cartilage. Multilayer cell sheet implantation is used mainly for myocardium, smooth muscle, liver, and other 3D tissues.

Heart

The heart is a crucial organ in the body and can have plenty of complications for example heart failure. The cardiomyocytes lose the ability to proliferate shortly after birth, therefore, the regeneration of cardiomyocytes is very complicated [64, 65]. The specific mixture of several cell types was used in combination, such as adipose tissue mesenchymal stem cells from adipose tissue [66, 67], cardiomyocytes [64] and skeletal myoblasts [68, 69] to simulate real heart tissue.

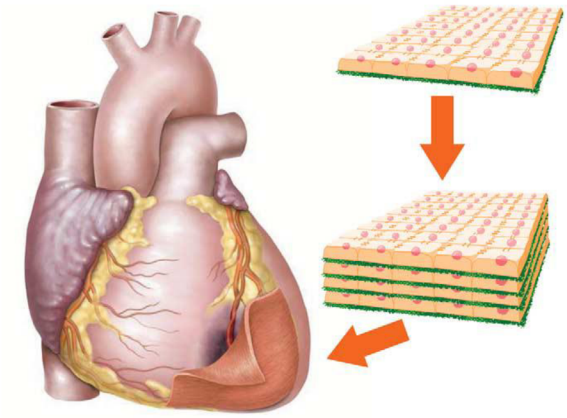

Figure 12: Cardiac myocyte sheets. [70]

To create cardiac tissue, cell sheets are layered with multiple sheets creating 3D structure as shown in Figure 12 [6, 70]. In cardiac cell sheet, cell-to-cell communication is highly important because the sheets are thicker and, therefore, are needed to work at an optimal level. Another important part of the cell-to-cell connections that improve the connection between the layers of cells is ECM [3]. This method allows cell sheets to be directly layered on top of each other and link to the host directly also cell sheet will create gap junctions through the whole cell sheet, which is not possible when the scaffold is used [6].

Cornea

The damaged cornea can impair the vision of a person, this damage can be from abrasions or other harmful substances in the eye. The oral mucosal epithelial cells are used for cell sheet of the cornea because it is a better representation of the native corneal epithelium [6, 71]. The

used technic of preparing cell sheet is implemented with a single cell sheet layer that allows attaching to the surface of the host cornea quickly without the use of sutures [6, 29, 72]. This approach allows making the cell sheet less fragile because it is held with cell-to-cell junction and ECM proteins [73], and avoids the need for carrier substrates [6]. Besides, this technic does not require to use the proteases or scaffolds and without that, the cell density is still high [73].

Liver

The liver is important because it takes part in protein synthesis, metabolism, and detoxification [64]. The liver cell sheet is created by the same approach as heart cell sheet namely multilayer, which formed a 3D structure. In this technic hepatocytes and high oxygen demanding cells are used, these cells can undergo ischemia more often than other types of cells [64, 74-76].

Bladder

The bladder is a hollow and contractible muscular organ. The bladder cell sheet for the implantation is constructed of urothelial cells, these cells are able to attach spontaneously and have adhesive properties, therefore there is no need for suturing or fixing [70, 77]. To create a urothelial cell sheet a biopsy is taken from the bladder and grown in culture. After implantation of the cells, the problems with the side effects of lithiasis, urinary tract infection and electrolyte imbalance may occur [70].

Oesophagus

Regeneration of injured or resected oesophagus is a relatively new method in comparison with heart, liver or cornea tissue regeneration. Nowadays the main way to treat oesophageal cancer is a surgical resection. After resection some patients suffer from ulcerations and loss of function [78]. Using cell sheet engineering technology enables to improve patients' quality of life by preventing stricture formation after endoscopic submucosal dissection. Cell sheet transplants from autologous oral mucosal epithelium allow avoiding stenosis and inflammation, and help with the reconstruction of the oesophageal surface. [79]

5.2. Scaffold-based tissue engineering

Nowadays biomaterials and tissue-engineered technologies are constantly evolving. In tissue engineering scaffolds are used as an artificial ECM that guide cells during the development of new tissues (see chapter 3.2). Scaffold-based TE can be used for tissue regeneration of some targeted organs such as cornea, skin, heart, trachea, blood vessels [80].

Heart

Polymeric scaffolds can be used as degradable heart patches that provide cellular growth. Three-dimensional cardiac patches constructed from biodegradable materials are mostly used in cardiac tissue engineering. In cardiac tissue engineering are used synthetic polymers with suitable biodegradable and mechanical properties. There are polyglycerol sebacate, polyethylene glycol, polyglycolic acid, poly-L-lactide, polyvinyl alcohol, PCL, polyurethanes, and PNIPAAm among them. For example, Pok et al. investigated multi-layered scaffold of PCL multilayered in gelatin–chitosan hydrogel [81]. This scaffold can be used in cardiac patch applications because of its ability to support cardiomyocyte viability.

Cornea

The cornea is located in the front part of the eye and constitutes a transparent layer that focuses light as it enters the eye. In developing of corneal tissue natural and synthetic polymers have been explored. Madden et al. grew an endothelial layer on a collagen-coated fibroin substratum. I was the first successful growth of primary human corneal endothelial cells on coated fibroin. Also, biodegradable chitosan–PEG hydrogel films, chitosan, PDMS, or PCL with excellent biocompatibility can be used as a scaffold for the regeneration and transplantation of corneal endothelial cells. For example, Klenkler et al. used a PDMS scaffold that was modified with epidermal growth factor to improve the growth of corneal epithelial cells. [80, 82, 83]

Skin

Skin is the largest organ of the body it has a surface area between 1.5-2.0 square metres, representing approximately 12-15% of the human body weight. The most frequently, damage appears as a result of surgery, burns, traumatic and chemical injuries. Skin is composed of three layers: the epidermis, the dermis and the hypodermis. Skin interacts with the environment and protects the body against any external physical, chemical and biological

impacts, prevents excess water loss from the body and thermoregulates. A large number of materials have been designed and fabricated as a tissue engineering scaffolds that have to promote wound healing. Among them there are collagen gels/sponges, alginates, polypeptides, glycosaminoglycans, hyaluronan and fibronectin to synthetic materials, such as polyvinyl chloride, polylactate/glycolate fabrics, etc. [80]

Trachea

The trachea is a part of the breathing system represented by the tubular air pipe. Even if, the method that can be used to replace damaged tissue of trachea does not currently exist, many biomedical engineers and clinicians are focused on tracheal regeneration. For example, Lin et al. developed a scaffold-based bioreactor system for the creating tissue-engineered trachea under the influence of fluid flow. They used chondrocytes that were seeded on the scaffold from the poly(3-caprolactone)-type II collagen and grew under controlled rotational speed/fluid flow. Rotation increased cell proliferation and collagen content in the constructs compared to static culture. Also, hematoxylin and eosin staining showed the formation of neocartilage and the alignment of chondrocytes along the flow direction. [80]

Blood vessels

Numerous studies have been focused on the creation of biodegradable scaffold that can temporarily substitute the blood vessel. Synthetic polymers or natural materials like collagen have been used to develop vascular graft. However, characteristics of these materials such as unsuitable rates of degradation, the lack of suitable mechanical properties and the poor capacity to create an optimal microenvironment for cell adhesion and differentiation did not fit for further application in the human body. But in spite of it all, some methods were developed to prepare vascular grafts. For instance, Shin'oka et al. developed a PCL-based scaffold to engineer venous blood vessels. The PCL/PLA copolymer was seeded with autologous smooth muscle and endothelial cells. After 10 days, the construct was implanted as a pulmonary bypass graft into a 4-year-old child. [80]

6. Fluorescent dyes for cells visualization

Cells, all types of solid constructs and cell suspensions, that are used for transplantation, should be monitored during the process of preparation in vitro, as well as in the time after transplantation (in vivo, in tissue), and mostly also in the time post mortem (macroscopic or

micro-histological checking of transplantation effectivity on the experimental animal after euthanasia). For this purpose fluorescent dyes can be used.

6.1. Calcein AM

Calcein-acetoxymethyl ester (Calcein AM) is a non-fluorescent, hydrophobic, cell-permeant compound, also, due to hydrophobic compound, Calcein AM easily permeates intact, live cells. In live cells the non-fluorescent Calcein AM is converted to Calcein, after the hydrolysis by intracellular esterases, resulting in green fluorescence (see Figure 13). Received Calcein is a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. If the cell is apoptotic or dead the cell membrane cannot retain Calcein, as a result, these cells do not fluoresce. [84-86]

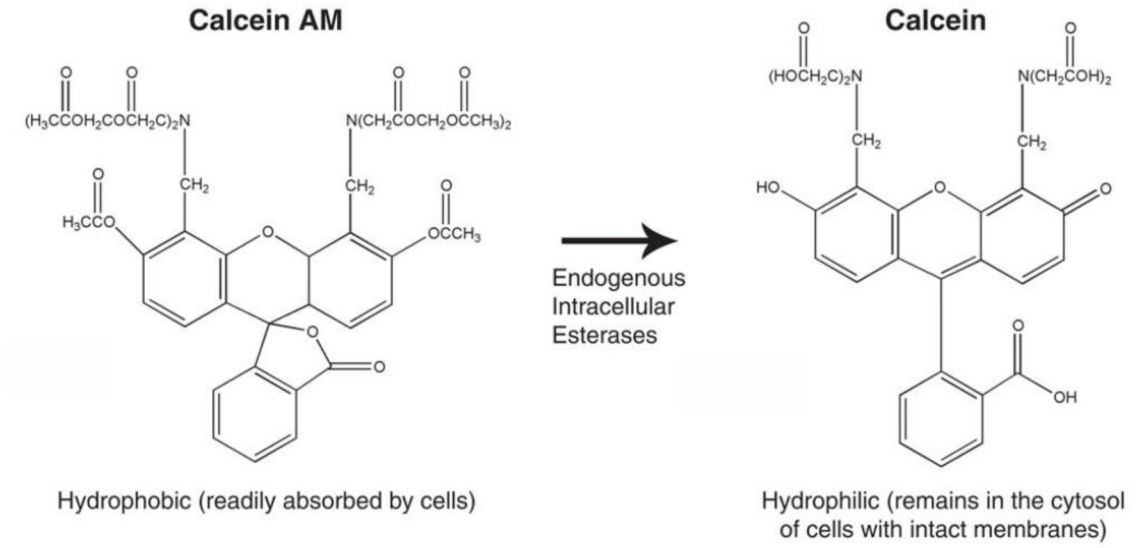

Figure 13: After Calcein-AM is taken up into the cell, it is converted by esterases into calcein. [85]

As Calcein AM is responsible for providing morphological and functional information about living cells it is used in microscopy and fluorometry. Calcein has an excitation peak of 495 nm and an emission peak of 515 nm (see Figure 14).

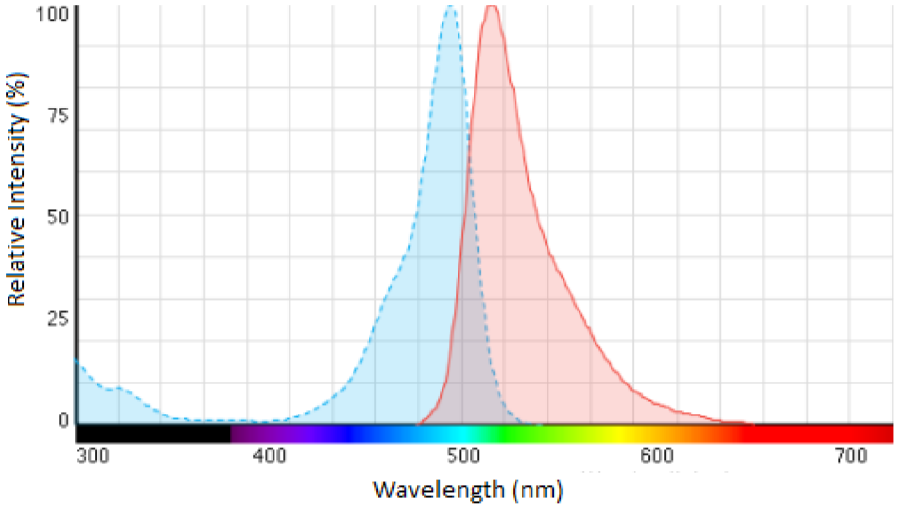

Figure 14: Absorption and emission spectra of calcein. Adapted from [87]

6.2. PKH26

PKH26 is a fluorescent dye with molecules that can be incorporated into the cell membrane bilayer, and, as a consequence, labelled cells fluoresce in the yellow-orange region of the spectrum. PKH26 has an excitation peak of 551 nm and an emission peak of 567 nm (see Figure 15). Due to the absence of obvious cytotoxicity, various types of cell can be labelled with this dye, however, the negative influence of this lipophilic dye to cell connection and another cell membrane process is still not fully described in the literature. An important point is that the original function and cell viability in cells labelled with PKH26 are kept, also this dye is not moving from labelled to non-labelled cells and vice versa. PKH26 is often used in proliferation analysis based on dye dilution and can be useful for monitoring viruses, platelets, and other nanoparticles; cell-cell membrane transfer; phagocytosis; antigen presentation; adhesion. [88, 89]

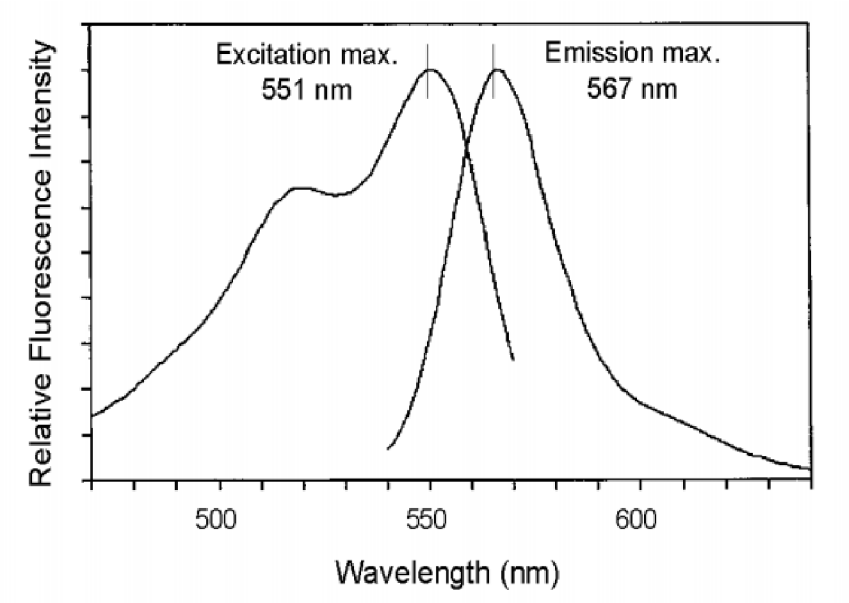

Figure 15: Absorption and emission spectra of PKH26. [88]

6.3. CellMask plasma membrane stains

The CellMask plasma membrane stains are a patended family of biocompatible low-molecular stains, which have an affinity to the lipid cell membrane. They can be used in histology for marking cell boundaries. CellMask plasma membrane stains are represented in three colours: CellMask Orange, CellMask Deep Red, and CellMask Green. These dyes can be used across a wide variety of mammalian cell types. Also, these plasma membrane stains provide rapid (30-90 minutes), robust and flexible staining in living cells that can be useful for fluorescent microscopy applications, automated imaging, and analysis. As noted before, there are three types of CellMask plasma membrane stains and each of them has different fluorescence excitation/emission maxima: CellMask Orange: 554/567 nm (see Figure 16A); CellMask Deep Red: 649/666 nm (see Figure 16B); CellMask Green: 522/535 nm (see Figure 16C). [90]

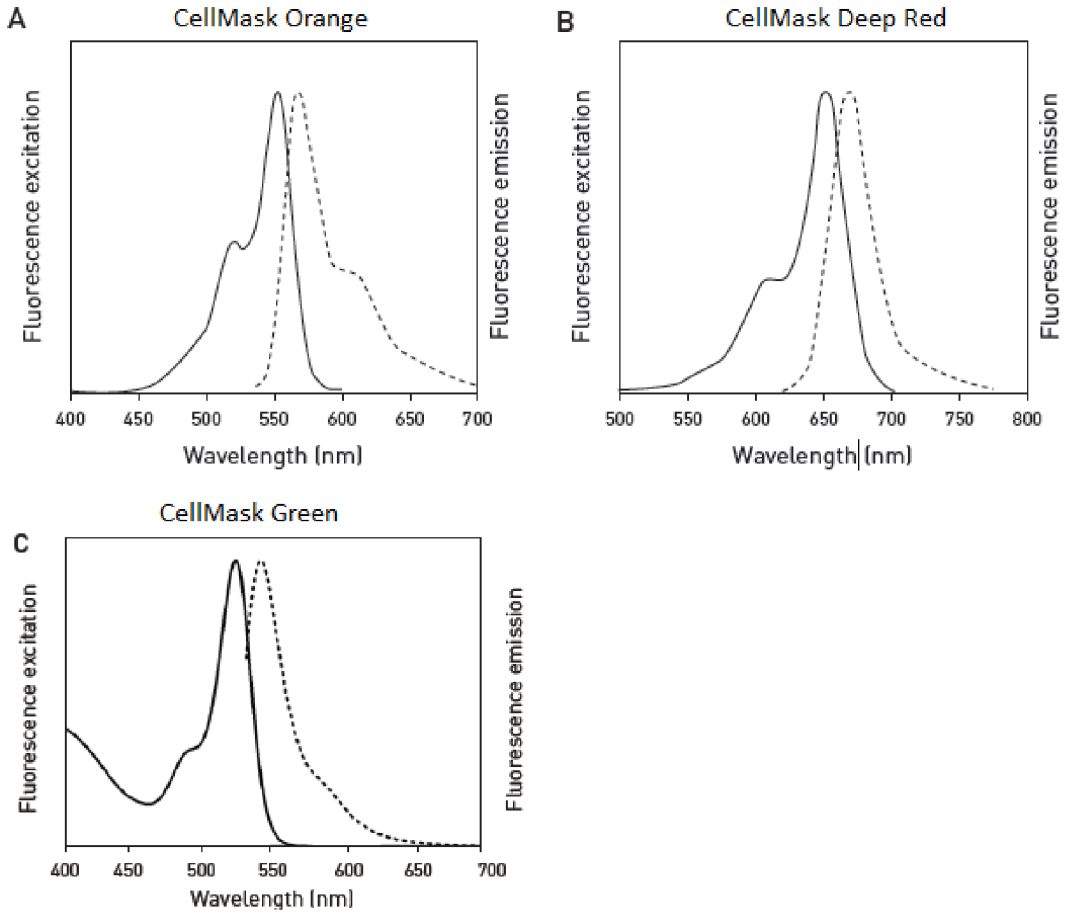

Figure 16: Absorption and emission spectra of CellMask plasma membrane stains. A: CellMask Orange; B: CellMask Deep Red; C: CellMask Green. Adapted from [90]

7. Practical work

The practical part of the thesis is devoted to the in vitro experiments with HL-1 cells, adult cardiomyocytes, fibroblasts and cultivation of these cells on different surfaces; cell visualization via Calcein AM, PKH26 and CellMask plasma membrane stain orange fluorescent dyes and analysing of received results. Also, practical part contain series of experiments.

In chapter 7.1 cell cultivation and cell visualization are described . Specifically, this chapter contains a description of the procedure of cell cultivation and cell labelling via Calcein AM, PKH26 and CellMask plasma membrane stain orange and at the end, the test of cells surviving in vivo was carried out. These manipulations with cells will be used in further experiments.

Chapter 7.2 describes the experiments where the PCL scaffold was used. Scaffold is

used as a base for the creation and growth of planar, confluent cell layer. PCL is a nanofiber that is used in tissue engineering because it is biodegradable and biocompatible polymer. This scaffold can be implanted and it will temporarily substitute the extracellular matrix during the regeneration and remodelling period, but after the scaffold is degraded will be replaced by new tissue.

Chapter 7.3 provide information about experiments with scaffold-free technic that involves using thermoresponsive polymer, PNIPAAm. This polymer represents a new technology which makes it possible to harvest confluent cell layer using simple temperature control and allows the detachment of cells without using proteolytic enzymes.

Chapter 7.4 is focused on another scaffold that made from PDMS that used in biomedical applications because of its biocompatibility, low toxicity, chemical inertness and mechanical flexibility. Also, it is easier to monitor cells proliferation process because PDMS is a transparent material.

Chapter 7.5 includes information about cell orientation. Cell orientation is an important parameter in experiments aimed to developing in vitro tissues that would resemble, in their structure, tissue in a living heart.

Chapter 7.6 contains information about experiments devoted to the cultivation of co-culture of cardiomyocytes and fibroblasts. These cells are cultured together to create tissue that resembles real heart tissue.

In the practical work, inverted fluorescence microscope Leica DMi8 and LAS-X Leica software were used for cell visualization. All manipulations with cells were carried out in laminar flow cabinet. Also, an incubator was used to grow and maintain cell cultures under optimal temperature, humidity and other conditions such as the CO_2 and oxygen. Moreover, the microscope incubation chamber was used during the experiments with PNIPAAm polymer in order to avoid temperature decrease and therefore cells detachment. Plasma generator was used for the treatment of the PNIPAAm with oxygen plasma.

For statistical analysis GraphPad Prism (GraphPad Software, La Jolla, CA) was used. Comparisons of parameters among the groups were made using T-test. Cell counting was performed using four fields of view and then the number of cells was converted to the square centimetre.

7.1. Basic tests of cell culture and surviving in vitro

7.1.1. Basic methodology of cells cultivation

In the practical part of this thesis three types of cells were used: adult rat cardiomyocyte (isolated from heart, source: Department of Physiology - Masaryk University), HL-1 cardiac muscle cell line (MERCK, SCC065) and rat fibroblasts (Sigma-Aldrich, NIH 3T3 Cell Line).

Every experiment starts with the preparation of cells. Firstly, if cells are previously digested or frozen they should be placed in fresh medium. The medium consists of DMEM (Sigma-Aldrich, D6046) augmented by 10% FBS (Sigma, F6178), in case of adult cardiomyocyte or HL-1 cells it is better to use low glucose DMEM, 10000 units/ml penicillin and 10 mg/ml streptomycin (P4333-100ML). Cells are seeded to the traditional plastic chamber. To prevent contamination of cells all manipulations carried out in laminar flow cabinet (Aura Mini, LV30000). Then, cells were placed into an incubator (Memmert, INCO 153) at 37˚C under a humidified atmosphere of 5% CO_2 and 95% air.

The second important process is continual maintaining of culture media in the culture chamber, control of surviving, and passaging the cells when cells reach the high confluence. A medium change was performed twice a week. The passaging means removing the adherent cells from the plate by the aid of accutase (type 6964, Sigma-Aldrich), collecting by centrifugation (1000 rpm, 5 min) and seeding to a new chamber. The cells preparation process is displayed in Figure 17.

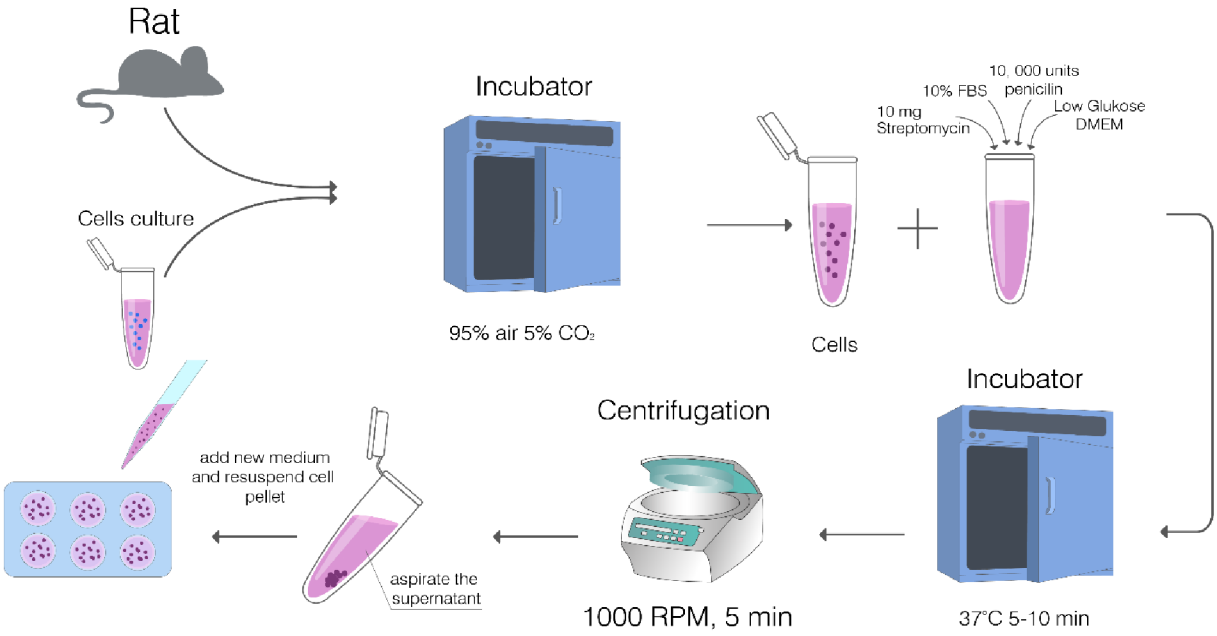

Figure 17: Cell cultivation process step-by-step.

7.1.2. The basic test of cell growth on traditional culture well

Before cell testing on new scaffolds and modern surfaces for the production of cell sheet and cell layer, the evaluation of cell viability on standard culture chamber had to be done. The main goal of this experiment is to test cells surviving and cells shape development (HL-1, adult myocytes, fibroblast) on traditional culture well.

Firstly, cells were prepared according to the procedure described in chapter 7.1.1. Then cells were seeded in a well of a 24-well cell culture plate (one cell type in one well) and placed into the incubator in the conditions of 37 °C, 95% air and 5% CO_2. The culture medium was replaced every two days and during two weeks the growth of cells was controlled. First time cells were checked in three days after seeding. Some adult cardiomyocyte and HL-1 cells were spontaneously contracting. HL-1 cells and fibroblasts had typical spindle shape morphology, adult cardiomyocytes had a typical rectangular shape in the first week. Details about viability are described in chapter 7.1.3.

During the next verification process cells were developing and growing. However, if we compare the number of alive cells on the third and on the fourteenth days, a higher number of alive cells was observed on the third day.

Regardless, this experiment has demonstrated that cells are able to develop and grow on the plastic surface during a long period of time, which means that these cells can be used in the following experiments.

7.1.3. Cell visualization and viability assay

Calcein AM

It is crucial to monitor cell viability. Cell viability determination is based on the detection of intracellular enzyme activity, which is monitored by selective labelling with fluorescence probes. Calcein AM easily penetrates the membrane and it is well maintained in living cells, where after being changed under the influence of esterases it begins to radiate the intense green light at a wavelength of 515 nm (see chapter 6.1). In the case of a dead cell having impaired membrane integrity, the dye is not retained in the intracellular environment and the cell remains unlabelled.

Before the cell labelling procedure starts, the Calcein AM (Molecular Probes, L-3224) must be removed from the refrigerator and warmed to the room temperature (incubator

can be used for this purpose). Calcein AM was diluted in PBS in the ratio of 1:500 and then injected into the medium in the culture dish with cells. After cell labelling with Calcein AM, the medium was aspirated and cells were washed, then labelled cells were left in the incubator for 20 minutes.

Visualization of stained cells is done by fluorescence microscopy (490 /520 nm). Typical results of calcein staining of HL-1, fibroblasts and adult cardiomyocytes are selected in Figure 18.

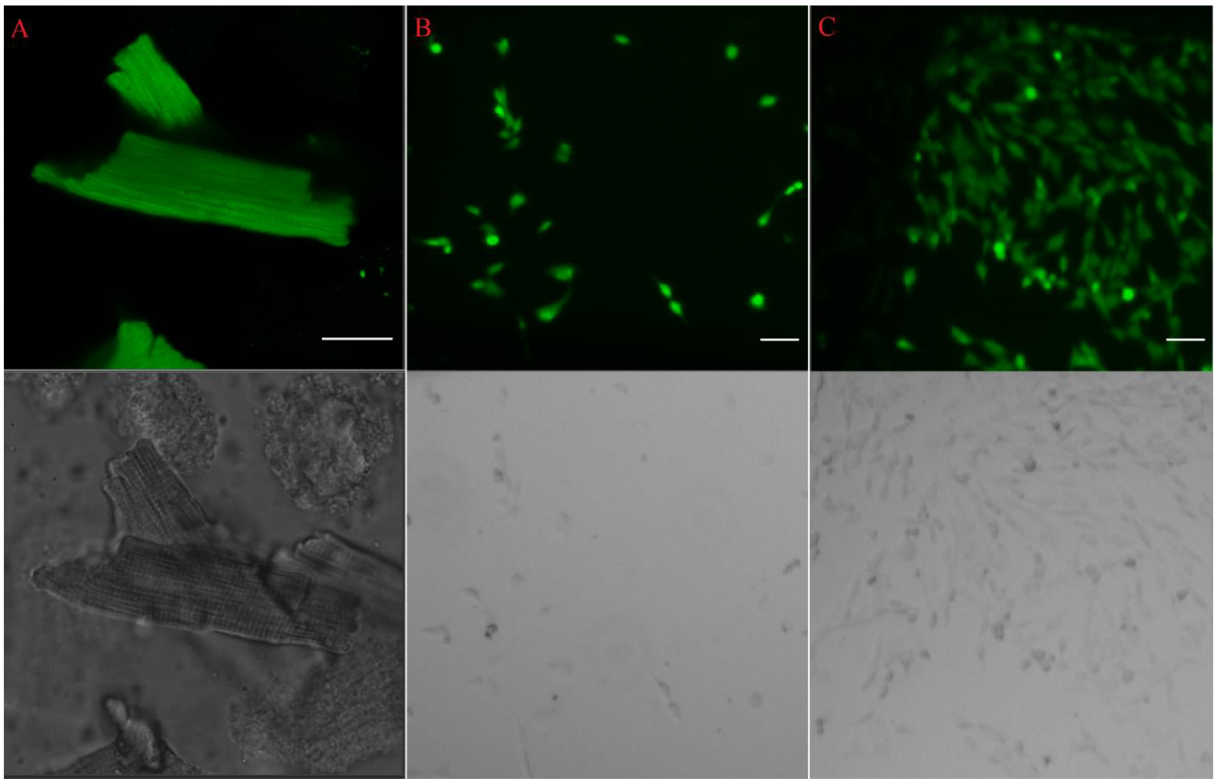

Figure 18: Calcein staining of adult cardiomyocytes (A), HL-1 cells (B) and fibroblasts (C). Leica DMi8. Scale bar 50µm.

PKH26

Another cell labelling dye used in this thesis is PKH26 (Sigma-Aldrich, PKH26GL-1KT). Fluorescence occurs in the red region of the spectrum (see Figure 19), the excitation maximum is at 551 nm and emission maximum is at 567 nm. The cells suspension (after trypsinization) must be washed by the medium without FBS (FBS has a negative effect on PKH staining effectivity) and centrifuged (12000 RPM). After centrifugation, the supernatant should be carefully aspirated, in order not to remove any cells but leave no more than 25 µl of supernatant.

The 1 ml of diluent C was added to the cells and resuspended with gentle pipetting to ensure complete dispersion. Afterwards immediately prior to labelling, 4×10^{-6} M

solution of PKH26 in the diluent C in a new dish was prepared, by adding 4 µl of the PKH26 ethanolic dye solution to 1 ml of diluent C and mixed well to disperse. Then, 1 ml of PKH26 solution to 1 ml of cells was added and mixed immediately after that cells were incubated with dye suspension for 1–5 minutes with periodic mixing.

Staining was stopped by adding 2 ml of medium with FBS. The cells were centrifuged for 10 minutes at 20-25 °C and the supernatant was carefully removed, being sure not to remove cells. Cells were resuspended in 10 ml of medium, transferred to a fresh tube, centrifuged for 5 minutes at 20-25 °C, thereafter cells were washed several more times with 10 ml of medium to ensure removal of unbound dye. After the final wash cells were resuspended in 10 ml of medium for assessment of cell recovery, cell viability, and fluorescence intensity. To achieve desired final concentration of viable cells, they should be centrifuged and resuspended one more time.

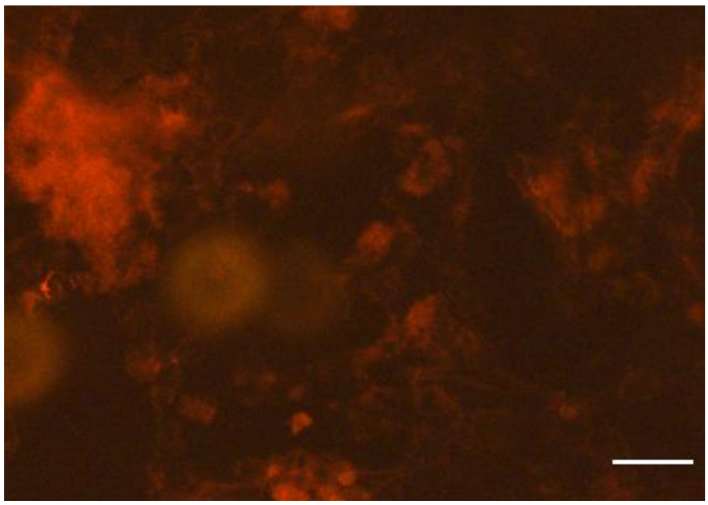

Figure 19: Fibroblasts labelled with PKH26. Leica DMi8, x20. Scale bar 50µm.

CellMask plasma membrane stain Orange

To determine cell surface area a plasma membrane labelling agent, CellMask orange plasma membrane stain (C10045, Thermo Fisher) was used, according to manufacturer instructions. Firstly, cells were washed with PBS and incubated with a solution of 2 µl CellMask in 2 ml DMEM without serum for 5 minutes, at 37˚C. After that suspension was washed three times with PBS. Images were collected immediately afterwards using a Leica DMi8 confocal microscope system (see Figure 20).

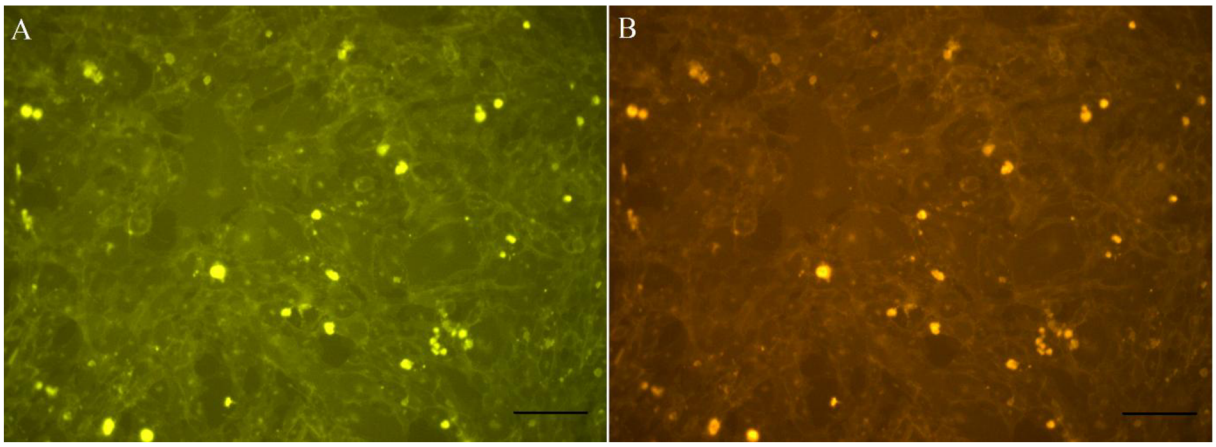

Figure 20: HL-1 cells labelled via CellMask plasma membrane stain Orange (541-550/567-640 nm). A: RHOD filter. B: DSRED filter. Leica DMi8, x20. Scale bar 75 µm.

7.2. Experiments using PCL scaffold

The previous experiment (chapter 7.1.2) described development and growth of adult cardiomyocytes, HL-1 cells, and rat fibroblasts on the traditional plastic surface. It was proved, that such kind of cells is able to stay alive within two weeks. The experiment in this chapter is focused on testing cell survival on PCL scaffolds. The process of manufacturing PCL nanofiber is described in chapter 3.2.1.

7.2.1. The basic methodology for using PCL scaffold

HL-1 cells were prepared according to the description in the chapter7.1.1. Then, PCL scaffold was cut by a sterile scalpel to small pieces (square - $1cm^2$) and the pieces were spread to the bottom of the plastic culture chamber. After that, HL-1 cells were seeded on the top of scaffold. This experiment is schematically presented in Figure 21. Moreover, in order to monitor cell viability, cells were labelled via Calcein AM fluorescent dye, according to the description in the chapter 7.1.3. Thereafter, cells were placed into the incubator in the conditions of 37 ˚C, 95% air and 5% CO_2. The culture medium was replaced every 2 days and the growth of cells was controlled.

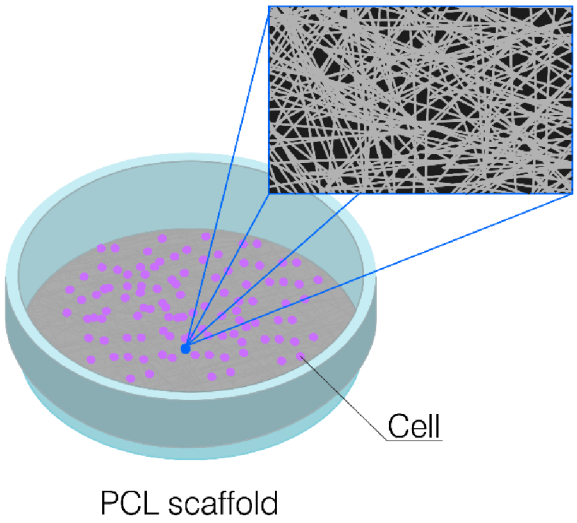

Figure 21: Cultivation of cells on the PCL nanofiber scaffold.

7.2.2. Results

Three days after seeding the cells were checked. Firstly, cell growth was controlled. Cells were scanned using a Leica DMi8 fluorescent microscope, using parallel plane imaging (different z-planes) with a distance of 5 µm. The green area (calcein positive cells) and also free space under cells were visualized using LAS-X Leica software. In Figure 22, it is visible that cells are developing and growing.

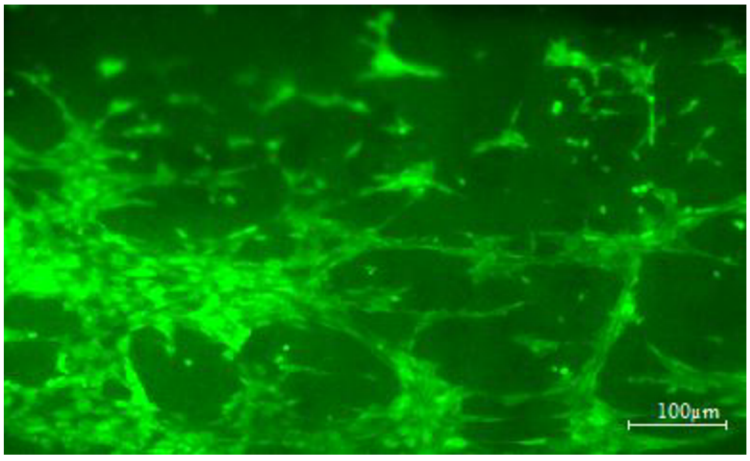

Figure 22: HL-1 cells on PCL scaffold, 3 days after seeding. Microscope Leica DMi8, x20. Scale bar 100.

It is crucial to be cautious during the process of cells seeding on this scaffold because cells can move from the nanofiber to the plastic bottom of the plate, this case is represented schematically in Figure 23. A cell cannot keep up to adhere to the scaffold surface and jet can move cells from the scaffold, another problem is that PCL nanofiber is very thin and light and even small jet can move it or medium (with cells) can leak under the nanofiber

and thereby lift it. As a result, some cells can be found not only on the PCL nanofiber scaffold but also on the plastic bottom of the plate. Consequently, some problems can occur.

The first problem is that all cells can be moved from the scaffold and if there are no cells that can develop and grow, it is not possible to get a cell layer on the nanofiber. The second problem, if only several cells were moved to the plate bottom and, as a result, there will be two layers of cells one layer on the nanofiber surface and another on the plate bottom. However, in general, this is not such a serious problem as compared with the previous, but during the cell control with the microscope, it will be required to check if the microscope focused on a proper cell layer.

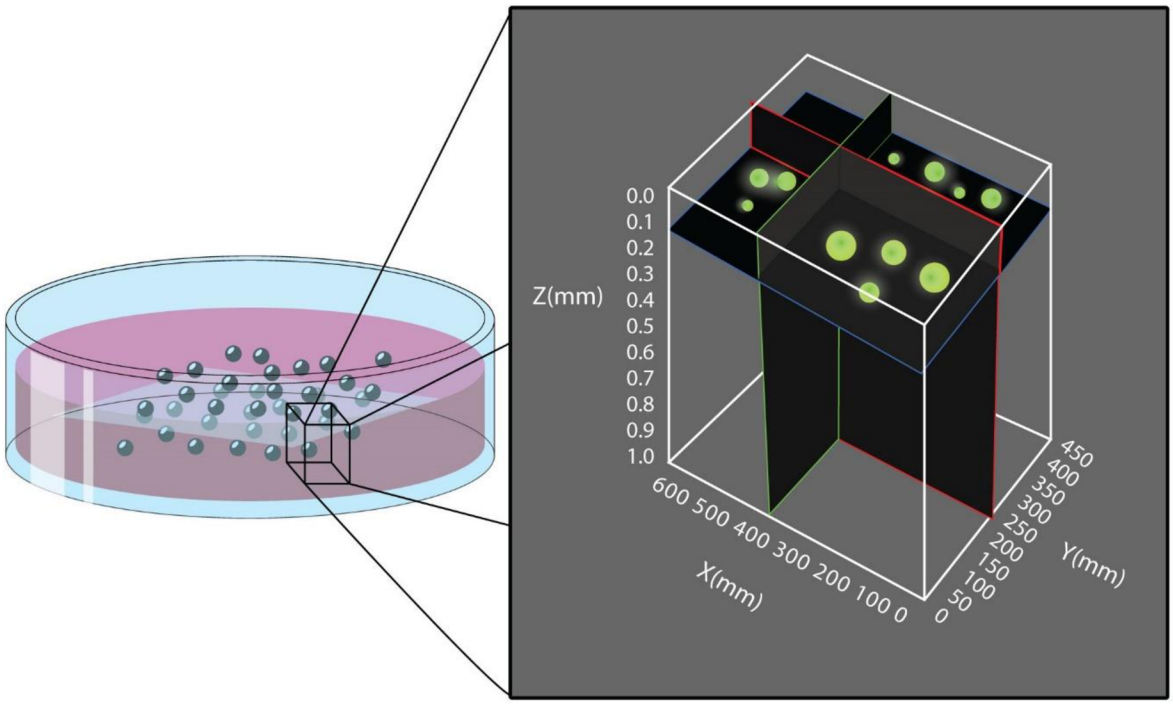

Figure 23: Cultivation dish containing PCL nanofiber (white sheet) placed in a medium with cells (black balls) on the scaffold and on the dish bottom including representation of the scanning area.

So, to be sure that cells are on the scaffold, confocal microscope Leica DMi8 was used. The culture plate was scanned from the plate bottom to the nanofiber surface.

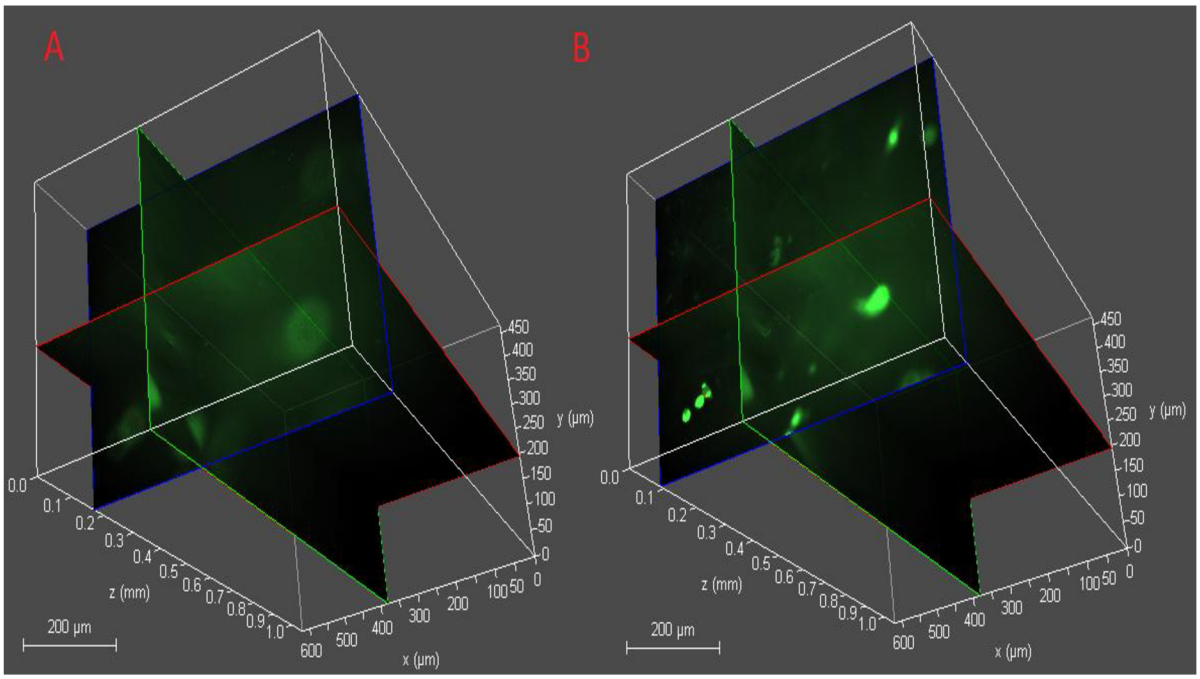

Figure 24: 3D scanning of the HL-1 cells cultured on the PCL scaffold. A: bottom of the plate. B: the surface of the nanofiber with HL-1 cells (green marks). Microscope Leica DMi8. Scale bar 200 µm.

Figure 24 illustrated scanned layers of the plate with HL-1 cells, it is visible that there is 0.1 mm distance between bottom and layer in which cells are placed. That means that cells are not on the plate bottom. To obtain more precise results another mode of 3D scanning was used (see Figure 25).

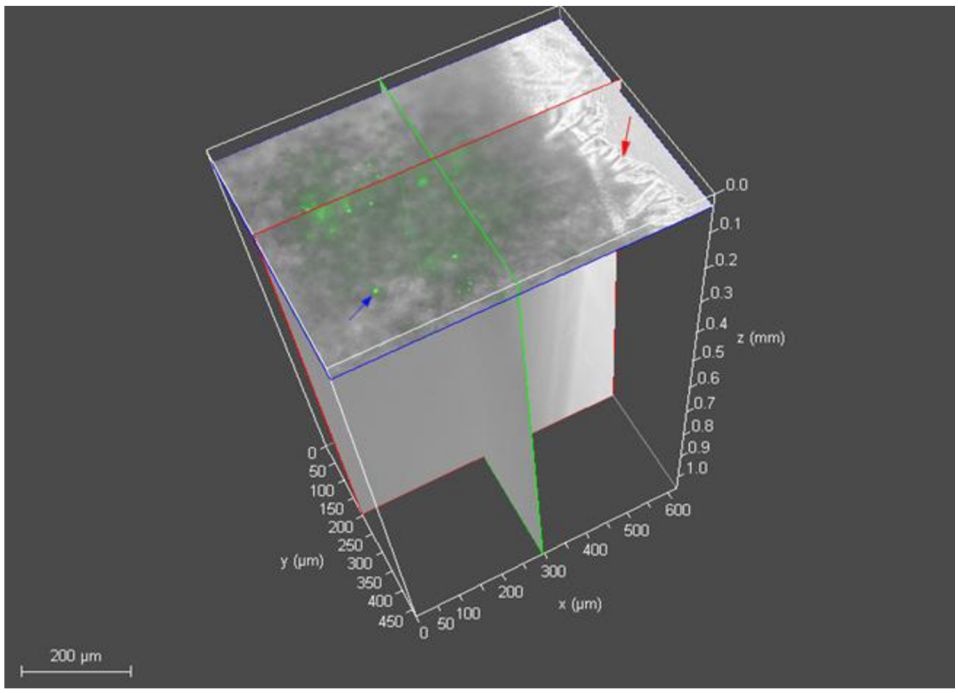

Figure 25: 3D scanning of the HL-1 cells labelled via Calcein AM (green dots) on the PCL nanofiber. The red arrow shows the edge of the PCL nanofiber and blue arrow show HL-1 cell labelled with Calcein AM. Microscope Leica DMi8. Scale bar 200µm.

In the following experiment cell adhesion to such scaffold was tested. HL-1 cells were seeded on the scaffold, just as in the previous experiment. Also, to compare results, the same amount (50 000 $cells/cm^2$) of cells was seeded to the polystyrene and glass surface. Moreover, cells were labelled via CellMask plasma membrane stain orange.

In 30 hours cells were scanned using Leica DMi8 fluorescent microscope. 33 000 cells per square centimetre were counted on the scaffold, and approximately 15 000 cm^2 were found on the glass under the scaffold (see Figure 26).

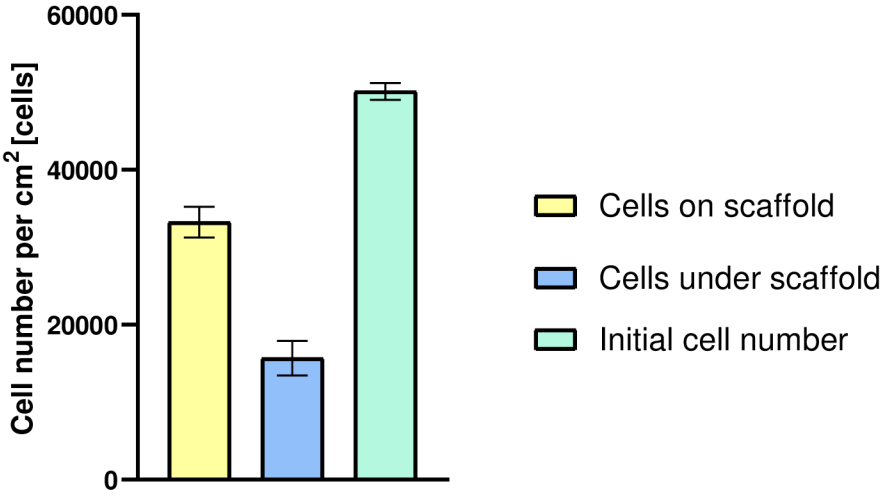

Figure 26: Comparison of cell number on the PCL scaffold and on the glass under the scaffold, 24 hours after seeding.

7.3. Cell culture experiments using temperature-responsive culture dishes

7.3.1. Basic methodology for using thermoresponsive polymer

In order to get a homogeneous hydrogel, 0.1 g of Poly(N-isopropylacrylamide) (PNIPAAm; Polysciences, Inc., Warrington, PA) was dissolved in 1 ml of the dehydrated ethanol (71250-11000, Penta). The obtained solution was spread evenly over the interior of each confocal glass bottom dish and dried under an ethanol atmosphere for more than 16 hours. The resulting PNIPAAm film was treated with oxygen plasma generated by the oxygen plasma generator (PlasmaPro 100 RIE, Oxford Instruments), with the applied power of 40W and 100W and the treating time of 2 min. Power range was selected according to the article [40]. Figure 27 shows schematically the placement of cells and materials during this experiment.

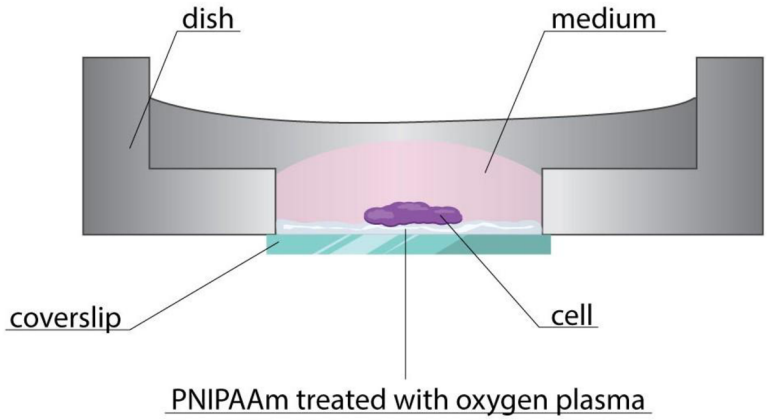

Figure 27: Section of the confocal glass bottom dish that was covered with PNIPAAm polymer and treated with oxygen plasma.

Rat fibroblasts and HL-1cells were cultured in growth medium according to the procedure described in chapter 7.1.1, at 37˚C, in a humidified atmosphere of 5% CO_2 and 95% air. And furthermore, culture dish with cells was placed in a box with distilled water to maintain humidity. A medium change was performed every second day. Also, to visualize cells cultured on the PNIPAAm, cells were stained with CellMask plasma membrane stain orange before transfer to the gel (staining process is described in chapter 7.1.3).

7.3.2. Results

Stained rat fibroblasts in growth medium were seeded at 50 000 $cells/cm^2$ onto the PNIPAAm film in a well of a 24-well cell culture plate and placed into incubator. In 48 hours the cell number was estimated. The medium was kept at 37˚C during the experiment to prevent the dissolution of the PNIPAAm film.

In 48 hours cells were scanned using Leica DMi8 fluorescent microscope. In Figure 28 fibroblasts labelled with CellMask plasma membrane stain orange. In the beginning, fibroblasts were seeded in the number of 50 000 $cells/cm^2$ and in 48 hours the cell number was estimated. It has been calculated that the number of adhered cells per square centimetre is 12 000 $cells/cm^2$.

Although the fibroblasts could not attach to the surface of PNIPAAm without oxygen plasma treatment [34], they attached to the PNIPAAm surface treated with oxygen plasma generated at 100W for 2 min. When cells were rinsed with fresh medium some cells remained on that surface (see Figure 28), suggesting that the surface was suitable as a cell culture substrate.

The aim of this experiment was to develop temperature-responsive culture dishes using PNIPAAm and to test cell adhesion and proliferation on such surface. Also, based on the results it can be concluded, although they are not perfect, that this polymer can be used in further experiments.

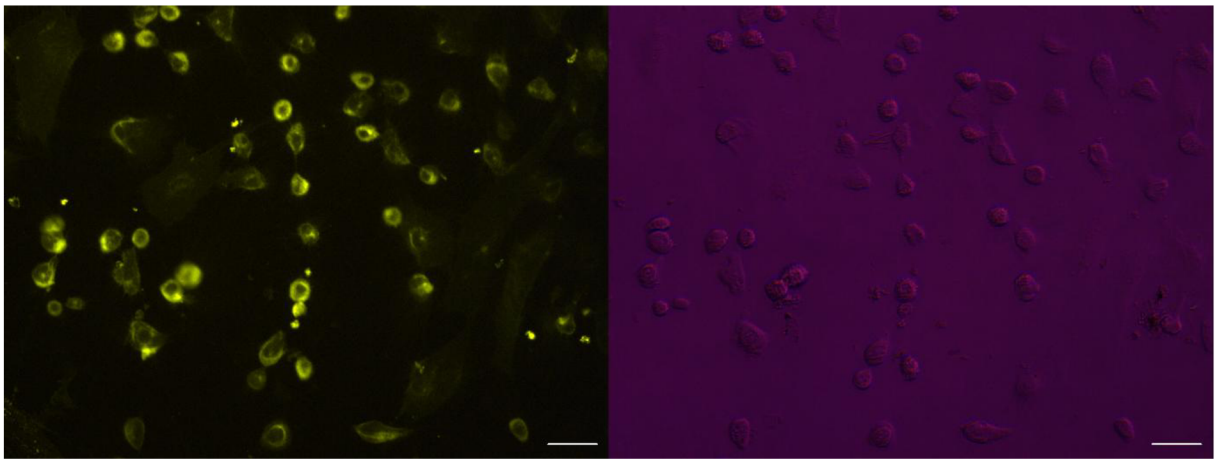

Figure 28: Rat fibroblasts stained via CellMask plasma membrane stain orange and harvested on the PNIPAAm-coated dish for 48 hours. Microscope Leica DMi8, x20. Scale bar 50 µm.

In the following experiment, three different culture dishes were used to compare cell development on usual surfaces and on thermoresponsive polymer. The first dish is a usual polystyrene culture dish (TCPS), the second one is a confocal glass bottom dish, and the third one is a confocal glass bottom dish that was coated with PNIPAAm. Coated culture dish was prepared and treated with oxygen plasma in the same way like in the previous experiment.

The cells were seeded in the same amount (50 000 $cells/cm^2$) into these three dishes. In 30 hours cells were rinsed with the fresh medium and the number of adhered cells was estimated. Only 47% cells were adhered to the polystyrene culture dish. From the initial cell number 58% cells were adhered to the confocal glass bottom dish and 7% to the thermoresponsive gel. The measurement results are illustrated in the Figure 29.

This experiment revealed that during the short time (about 5 minutes), when the cells were controlled under the microscope, PNIPAAm film changed its physical properties from hydrophobic to hydrophilic due to the temperature changes from 37˚C (in incubator) to the room temperature (23-25°C). As a result the number of cells that adhere to the thermoresponsive polymer is dramatically reduced. Then, it was decided to use microscope incubation chamber (H301-EC-LG-UP-1x35, Okolab) that is illustrated in Figure 30, also, every time when cells are taken out of the incubator they should be in the box (in which they are stored in incubator) to minimize temperature changes.

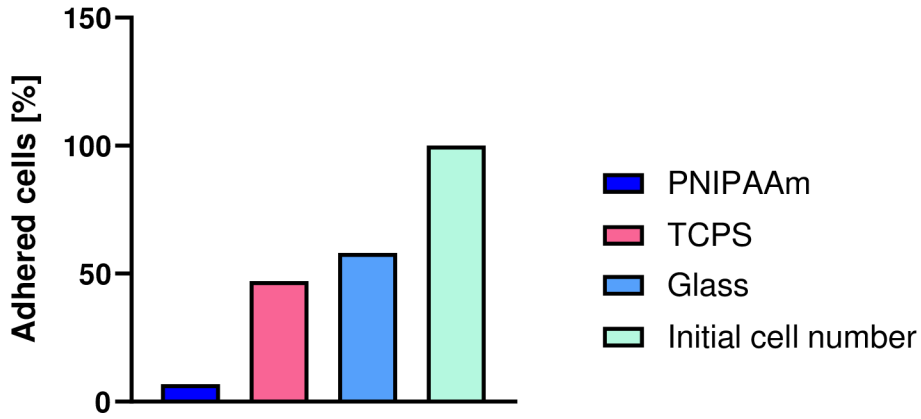

Figure 29: The number of adhered cells to different surfaces (PNIPAAm, TCPS, glass) in percentage.

The microscope incubation chamber or stage top chamber creates a small environment right on the microscope stage with superior control of temperature, humidity, carbon dioxide, and oxygen concentration. Feedback to the controller is provided by the temperature sensor embedded into the chamber main body.

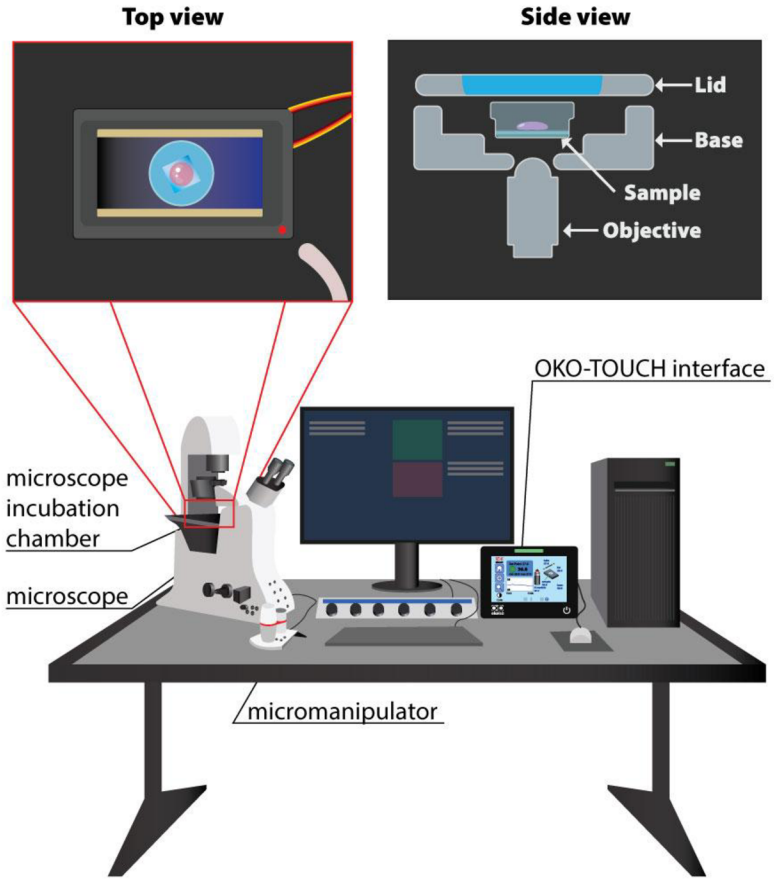

Figure 30: The illustration of emplacement of the microscope incubation chamber on the microscope stage.

The next experiment is dedicated to the testing how power of the oxygen plasma generator can influence cell adhesion. One culture dish was coated with PNIPAAm polymer and treated with oxygen plasma generated at 100W, another dish was treated with oxygen plasma generated at 40W. HL-1 cells were seeded in the amount of $50\,000\ cells/cm^2$ onto resulting culture dishes. Also, before seeding, the cells were labelled using CellMask plasma membrane stain orange, according to the description in chapter 7.1.3.

In 30 hours the cells were scanned using Leica DMi8 fluorescent microscope. Figure 31 shows adhered HL-1 cells on the PNIPAAm surface treated with oxygen plasma generated at 40, and 100W in 30 hours after cell seeding. The results were compared with the results obtained on more traditional surfaces like polystyrene and glass dish. Only 6% of cells were adhered to the polymer for 40W it was 10% for 100W it was 15% for glass surface and 30% for polystyrene.

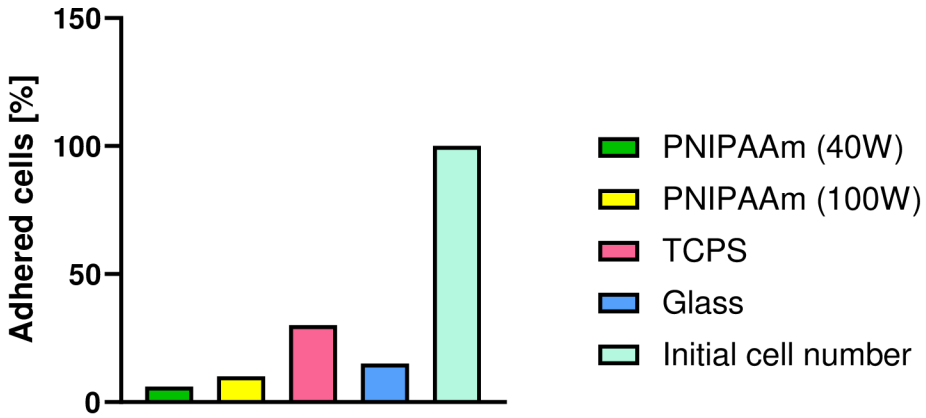

Figure 31: The number of adhered cells to different surfaces (TCPS, glass) compared to the adhered cells to PNIPAAm treated using different power (40W and 100W) in percentage.

As it can be visible in Figure 31, the number of attached cells on PNIPAA treated with oxygen plasma generated at 100 W is slightly more, compared to 40 watts. At the same time, the number of attached cells to glass is not much more than on PNIPAA treated with oxygen plasma generated at 100 W.

Later, in order to obtain a cell sheet, more experiments were carried out. However, only in one experiment the cellular sheet was obtained. As in the previous experiments, PNIPAAm was treated with 100 W oxygen plasma. Also, fibroblasts were labelled with CellMask plasma membrane stain orange and placed on the treated polymer. During the cell check, it was found that most of the cells on the polymer were not adhered, except for a small area in the centre. After the temperature was lowered below 32°C, this small piece of cells

detached from the surface of the polymer, while the connections between the cells remained (see Figure 32).

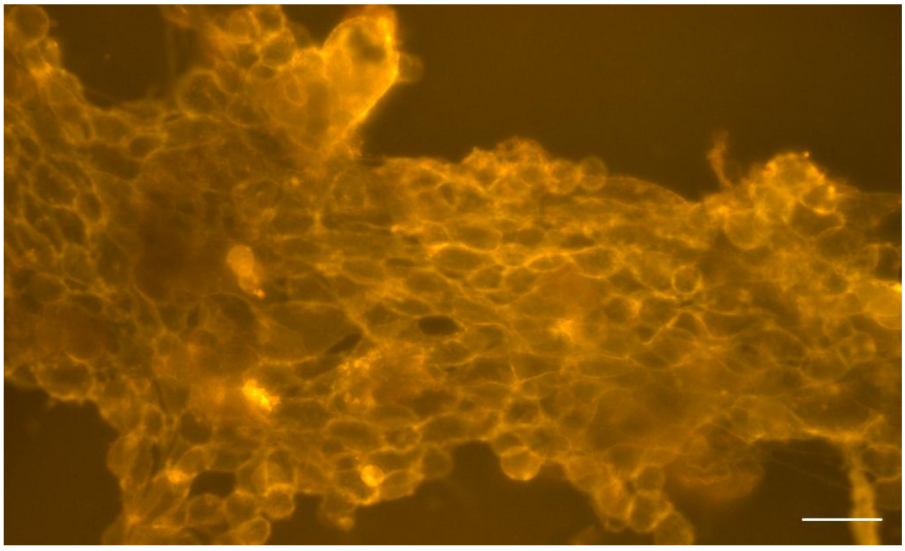

Figure 32: Cell sheet obtained from fibroblasts labelled with CellMask plasma membrane stain orange and cultured on the PNIPAAm treated with oxygen plasma (100W). The detachment was performed by temperature lowering below 32°C. Microscope Leica DMi8, x20. Scale bar 50 µm.

Although during these experiments it was not possible to create a confluent cell sheet from the whole culture dish, it was proved that this material can be used for these purposes, however, an improvement of the method is required to enhance cell adhesion.

7.4. Experiments using PDMS scaffold

Cell suspensions containing either mainly fibroblasts, mainly myocytes, or a mixture of myocytes and fibroblasts were cultured on gelatin-coated surface and on fibronectin-coated surface. For comparison purposes, the same cell suspensions were cultured under the same conditions but on non-patterned substrates.

7.4.1. The basic methodology for using PDMS scaffold

HL-1 cells or alternatively fibroblasts were prepared according to the description in chapter 7.1.1. Then, on the bottom of the plate, PDMS scaffold with the micropatterned surface was placed (see Figure 33). After that, the cells were seeded on the top of the scaffold. Also, the cells were labelled via Calcein AM or CellMask plasma membrane stain Orange, according to the description in chapter 7.1.3, to distinguish cell types. Thereafter, received PDMS model was placed into the incubator in the conditions of 37˚C, 95% air and 5% CO_2. The culture

medium was replaced every 2 days and the growth of cells was controlled. The process of manufacturing PDMS is described in chapter 3.2.1.

This PDMS scaffold consists of parallel lamellae 3 µm tall, 3 µm wide with distance of 5 µm, 10 µm or 20 µm. The lines are designed to mimic morphology and orientation in the myocardium. This system mimics important aspects of *in vivo* architecture of myocardium, such as the highly organized striation patterns of cardiomyocytes, both within individual and between neighbouring cells, as well as the intimate interrelation of fibroblasts with the densely packed myocyte strands (for more information see chapter 2.2).

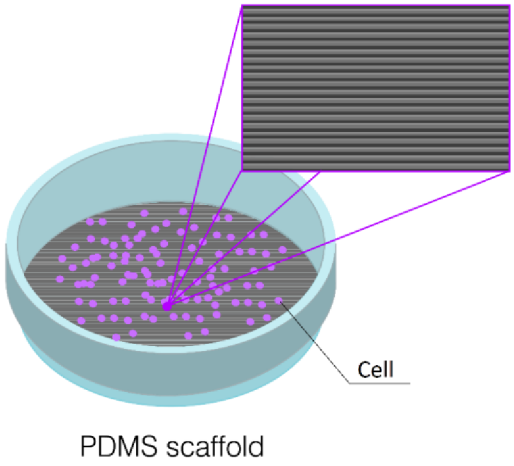

Figure 33: Cultivation of the cells placed on the PDMS scaffold that is represented by parallel lamellae.

7.4.2. Results

As well as in the previous experiment, the cells were checked in three days after seeding. This experiment showed, that PDMS scaffold is acceptable for cells growing. After the medium changing, about 10% of cells were attached to the surface of the scaffold (see Figure 34).

HL-1 cells growing on such scaffold were tested in the previous experiment. Also, it showed that PDMS material is harmless for cells growing and developing. In the following experiment the same type of scaffold was used. It consists of parallel lines on the PDMS surface, with areas that have different lines width 5 µm (area A), 10 µm (area B) and 20 µm (area C). Fibroblasts in growth medium were seeded onto the PDMS scaffold in cell culture plate and placed into incubator.

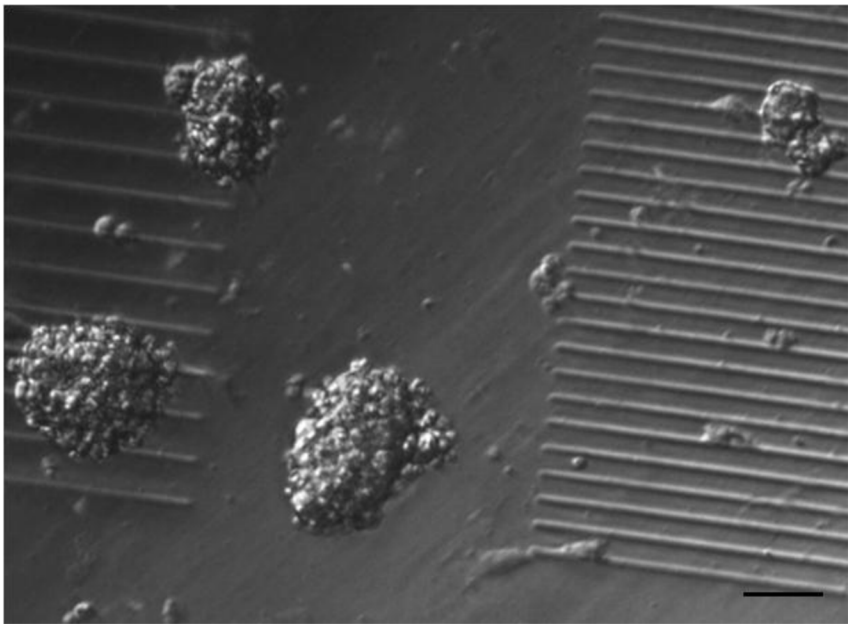

Figure 34: HL-1 cell on PDMS scaffold with different width (20 µm on the left and 5 µm on the right side). Microscope Leica DMi8. Scale bar 50µm.

In 48 hours cells were scanned using Leica DMi8 fluorescent microscope. Initially, fibroblasts were seeded in the number of 20 000 $cells/cm^2$. Considering that this scaffold has lines with three different widths between lines, this areas (A, B and C) were separated from each other. The purpose was to identify differences in cell adhesion and orientation. The same amount of cells was seeded on the three areas and cell number was determined. Moreover, in order to improve cell adhesion PDMS was coated with gelatin from porcine skin (G2500, Sigma-Aldrich).

In 48 hours 4 000 $cells/cm^2$ were detected in the area A (5 µm) and 20% of cells were stretched parallel to scaffold lines. The best results were achieved in the area B (10 µm) where about 8 000 cells were detected and 35% of them were attached to the scaffold in 48 hours after seeding. In case of the area C (20 µm) in 48 hours approximately 7 000 $cells/cm^2$ were detected. In addition, the number of cells, which mimic the lines structure was counted. Approximately 30% of the cells were stretched parallel to scaffold lines (see Figure 35).

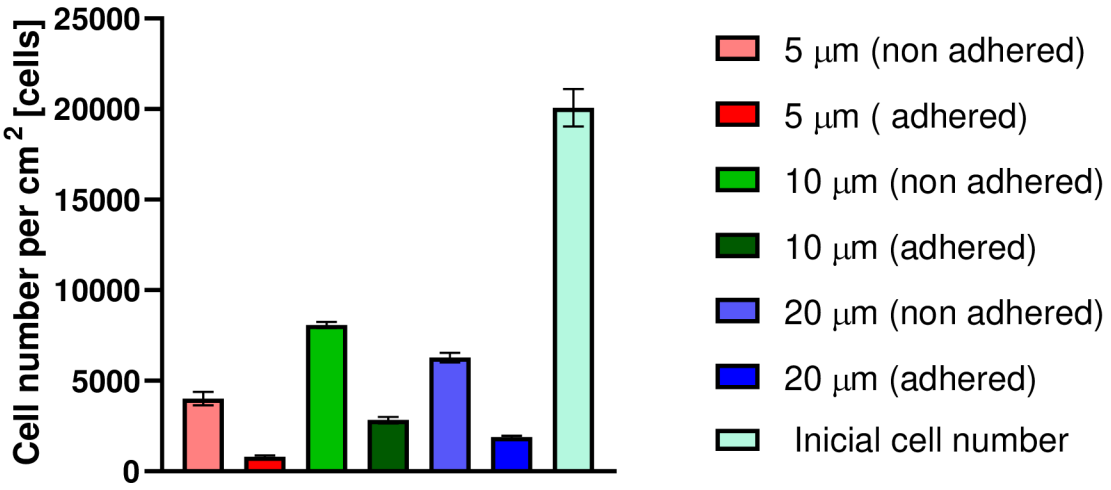

Figure 35: Comparison of cell number on lamellae with different distances (5 μm and 20 μm) 48 hours after seeding.

The results from the previous experiment indicate that gelatine slightly improves cell attachment in comparison with bare PDMS surface but a large number of cells were washed away during the rinsing. The remaining cells did not represent a confluent layer but only individual cells distant from each other, some were stretched along *in vitro* model.

In order to provide greater adhesion for cells fibronectin was used. Micropatterned PDMS model was coated with fibronectin (Gibco) and placed into incubator for 1 hour, for drying and after that the excess fibronectin was removed by aspiration. Fibroblasts cells were prepared according to the description in chapter 7.1.1 and cell suspension was injected on the coated PDMS scaffold. For comparison purposes, the same amount of cell suspension was cultured under the same conditions but on non-coated substrate.

Figure 36 represents fibroblasts cultured on the fibronectin coated PDMS surface with lamellae (with the distance of 10 μm between the lines) and without them. From Figure 36 it is visible that on the surface with lamellae the cells are stretched along them thereby the cells are oriented in one direction. In case of flat surface the cells have different orientations. Also, it should be noted that the total number of adherent cells exceeds the number of non-adherent cells in both cases.

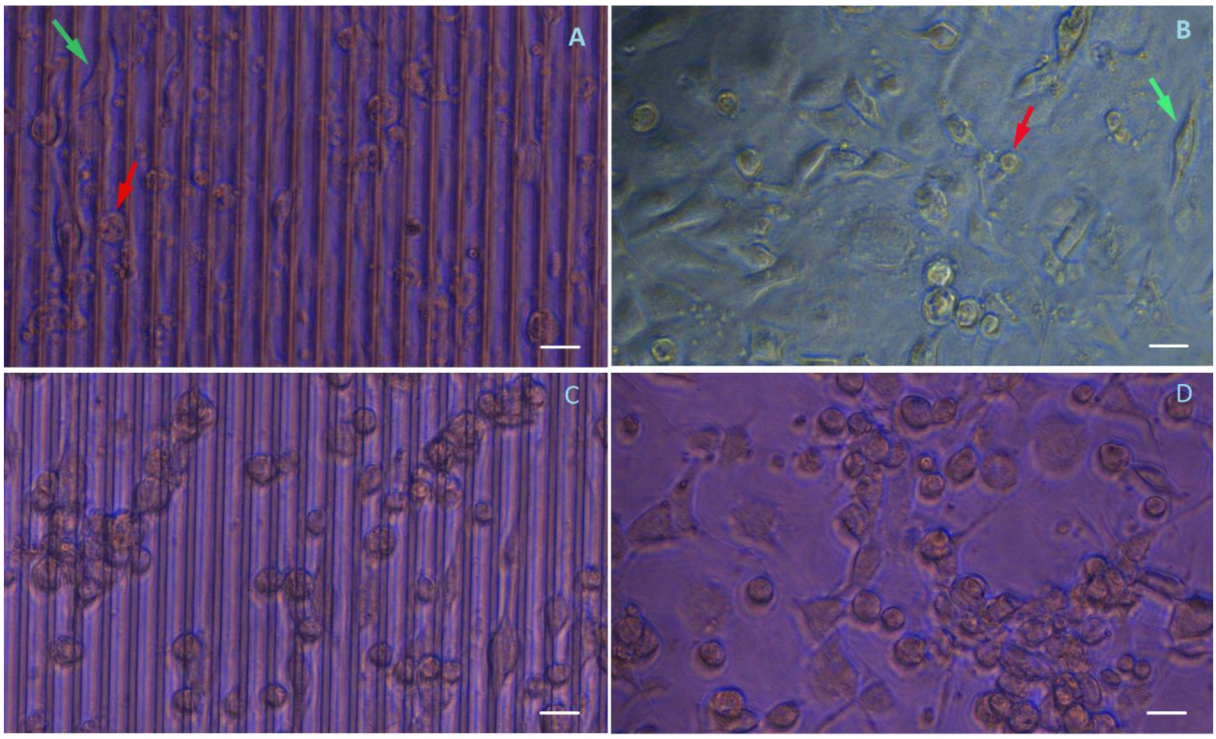

Figure 36: Fibroblasts cultured on the fibronectin coated PDMS (before rinsing) and on the non-coated PDMS scaffold, the red arrows show an example of cells that are not attached to the surface and green show an example of cells that attached to the surface. A: Fibronectin coated surface with lamellae. B: Fibronectin coated surface without lamellae. C: Non-coated lamellae. D: Non-coated surface without lamellae. Microscope Leica DMi8, x10. Scale bar 20 µm.

Initially, cells were seeded in the amount of 95 000 $cells/cm^2$. In 24 hours it has been calculated that there are about 70 000 $cells/cm^2$ attached cells on the surface coated with fibronectin and only 10 000 $cells/cm^2$ on non-coated surface (see Figure 37).

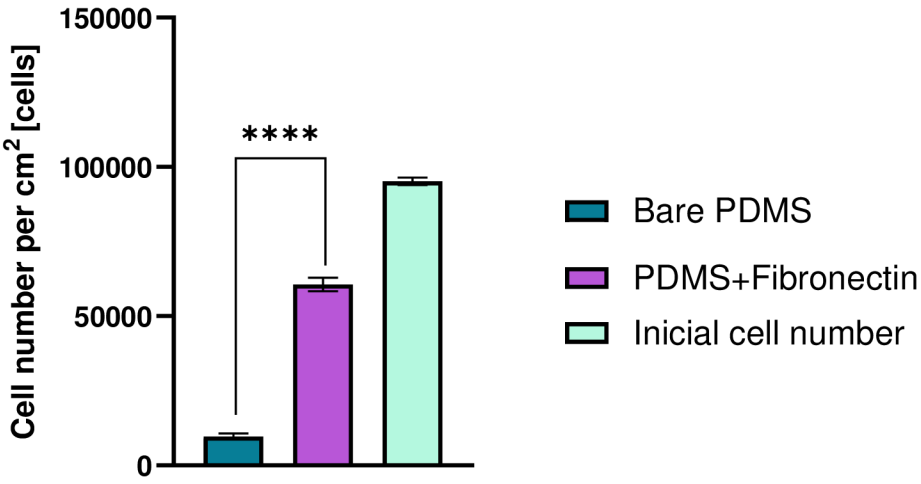

Figure 37: Comparison of the number of fibroblasts cultured on the fibronectin coated surface and non-coated PDMS surface. The difference is statistically significant the P-value is <0.0001.

This experiment showed that fibronectin significantly increased the adherence of cells to PDMS. Also, it was also found out that the width between the lamellae of 10 µm is optimal

for fibroblasts, they fall into the space between the lamellae and thus most of them are oriented in the same direction.

The following experiment focuses on the cultivation of cardiomyocytes. HL-1 cells were seeded approximately in the same amount as in the previous experiment (about 93 000 $cells/cm^2$) on the fibronectin coated PDMS with lamellae (with the distance of 10 µm between the lines) and on the bare PDMS scaffold.

In 24 hours cells were labelled via calcein AM according to the description in chapter 7.1.3. In Figure 38 it is visible, as in the case of fibroblasts, that HL-1 cells cultured on the surface coated with fibronectin are oriented along the lines of scaffold and cell adhesion is much better compared to non-coated PDMS.

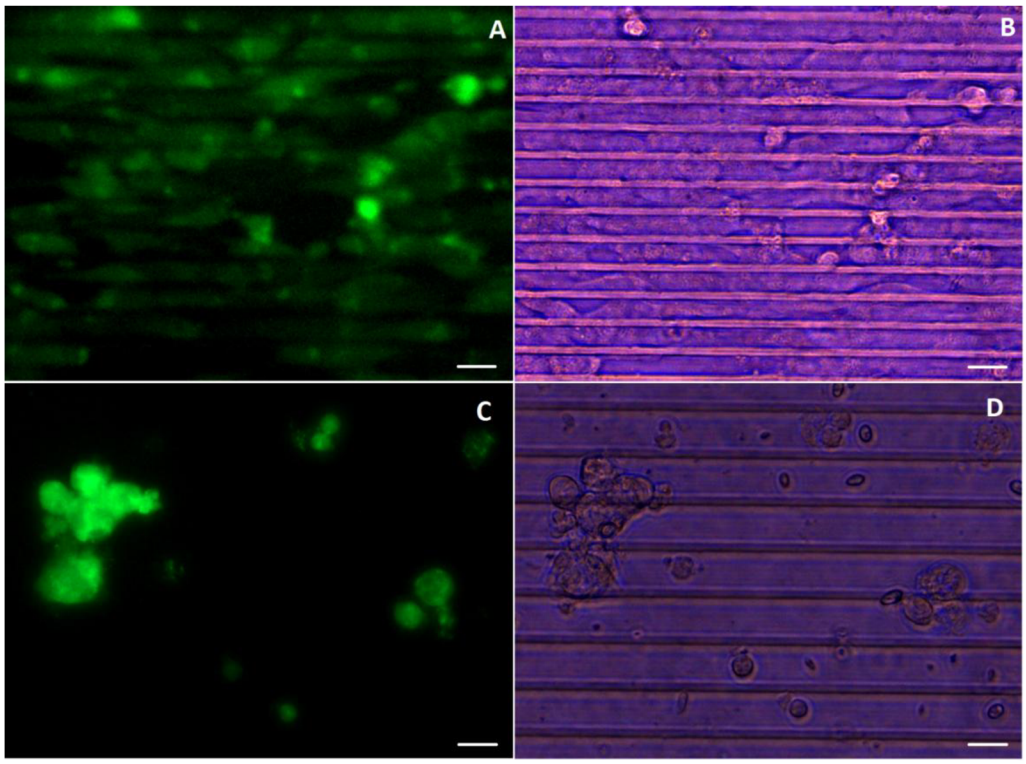

Figure 38: HL-1 cells cultured on fibronectin coated PDMS (A, B) and non-coated PDMS scaffold (C, D). Cells labelled via calcein AM. Microscope Leica DMi8, x10. Scale bar 20 µm.

It was counted approximately 54 000 $cells/cm^2$ on the fibronectin coated surface and less than 8 000 $cells/cm^2$ on the non-coated PDMS surface (see Figure 39). It indicates that fibronectin coating significantly improves adhesion not only of fibroblasts but also of cardiomyocytes. In the case when fibroblasts were cultivated on the surface covered with fibronectin, cell adhesion increased by 53% compared to bare PDMS. Similarly, in the case of cardiomyocytes, adhesion increased by 49%.

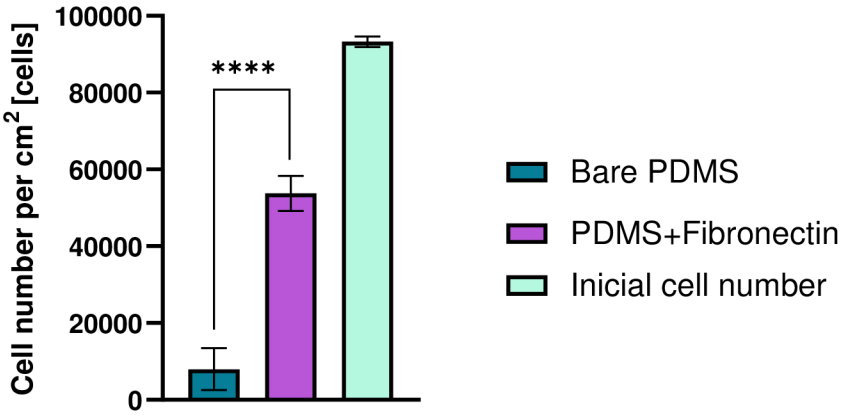

Figure 39: Comparison of the number of HL-1 cells cultured on the fibronectin coated surface and non-coated PDMS surface. The difference is statistically significant the P-value is <0.0001.

During the control of the HL-1 cells, 24 hours after seeding, it was also found that a greater number of stretched cells was found on the bare PDMS before rinsing than after rinsing. This may indicate that the cells can attach to PDMS that is not covered with ECM proteins (like fibronectin), however, the adhesion strength is so small that during rinsing with the fresh medium the jet is able to detach cells from the surface. An example of this situation is shown in Figure 40.

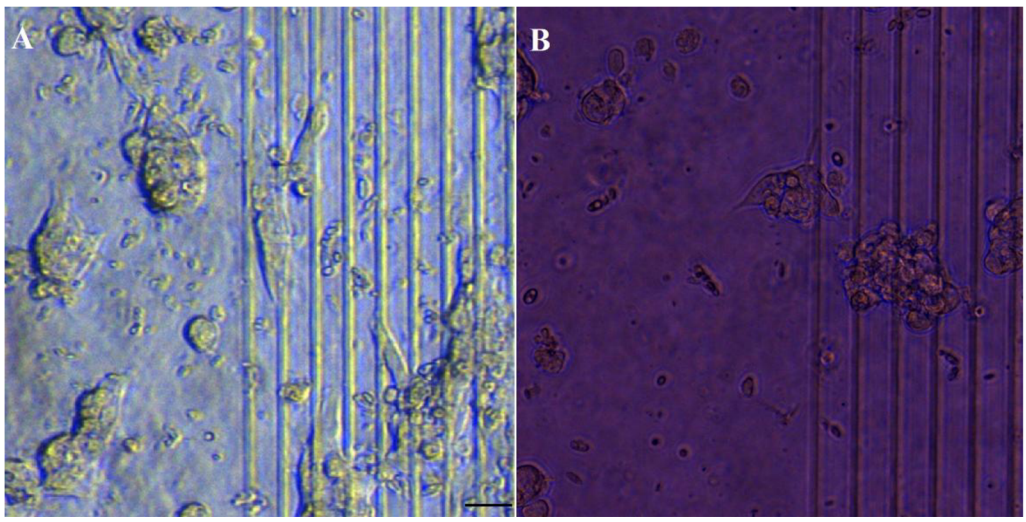

Figure 40: HL-1 cells cultured 24 hours on the bare PDMS. A: HL-1 cells before rinsing. B: HL-1 cells after rinsing. Microscope Leica DMi8, x10. Scale bar 20 µm.

7.5. Cell orientation on different materials and ability of creating of parallel elongated cell structure

In the previous experiments, various tissue engineering materials were tested. When using

PCL nanofiber, a sufficiently good cell adhesion was obtained, however, the cells were randomly oriented. In case of the next tested material, cell adhesion was low, but based on the fact that the structure of the PNIPAAm polymer was flat and it was not processed in any way to obtain a surface with a uniform structure, the same cell orientation was not expected. The latter material (PDMS) was chosen specifically for the purpose of creating a cell layer in which the cells have the isotropic orientation.

Quantification of the isotropic orientation of HL-1 cells on PDMS (in variants without lamellae and with lamellae) was the final aim of this work. The method is based on semi-automatic analysis, the main axes of the cells were indicated and estimated automatically by the algorithm implemented in MATLAB R2014a. Images were generated by Leica DMi8 with objective 10 and five fields of view were scanned, received images were exported from LAS-X software and MS Paint software was used for manual indicating of the main axis of the cells (Figure 41A). Pictures with green axis on cells were saved and imported to MATLAB, where the quantification of the cell angle was done, the output of the algorithm is the histogram. The histogram represents the cell angle distribution. The illustrative results, in 48 hours after cells seeding on the flat PDMS scaffold and on the PDMS with lamellae, are represented in Figure 41B. This is evidence, that the cells without lamellae did not generate isotropic cell culture, the cell on the surface with lamellae did generate.

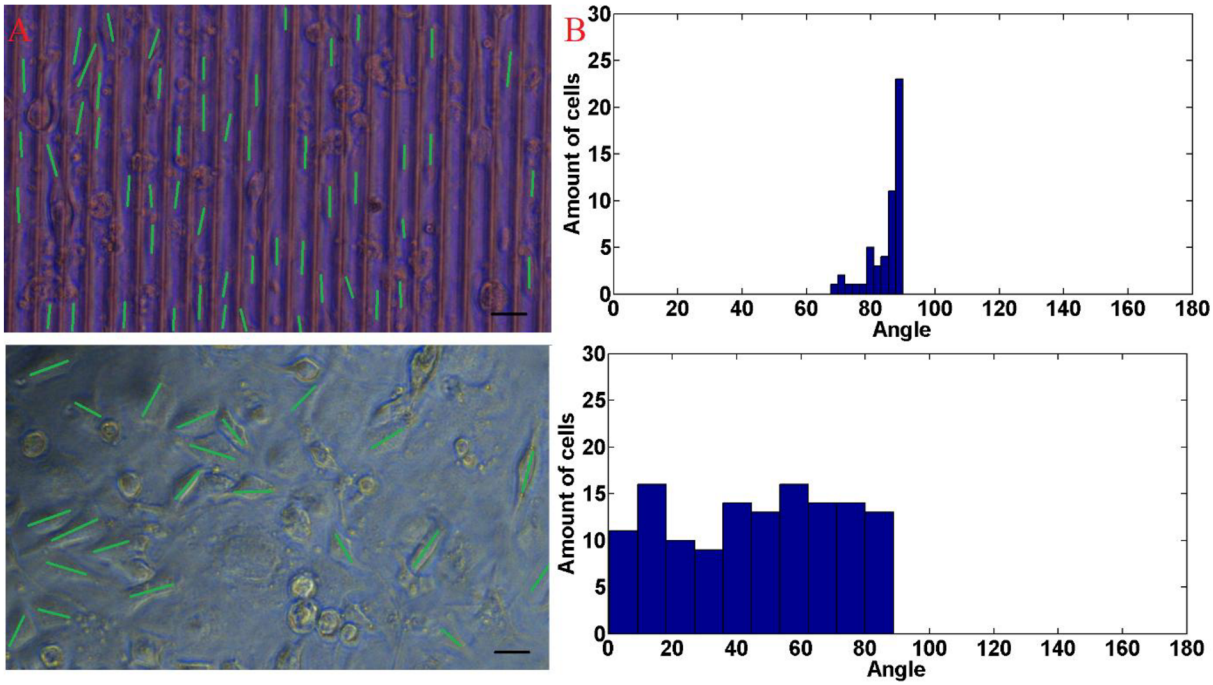

Figure 41: Comparison of cell orientation on the surface with lamellae and on a flat PDMS. A: main axis of cells (picture on top: PDMS with lamellae, bottom picture: flat PDMS). B: Cell angles computed in Matlab (histogram on top: PDMS with lamellae, bottom histogram: flat PDMS). Microscope Leica DMi8, x10.

7.6. Co-culture of fibroblast and cardiomyocytes on micropatterned PDMS surface

In previous chapters, various surfaces were tested, including the cultivation of the fibroblasts or cardiomyocytes on their surface. The final experiments were dedicated to the cultivation of the cardiomyocytes and fibroblasts together. The same cell seeding concentration and cell seeding conditions were used as in chapter 7.4. The cardiomyocytes were stained by calcein AM (green) and the fibroblasts by CellMask Orange. The final culture was scanned 12 hours after seeding (Figure 42), the adhesion and prolongation of the cardiomyocytes are excellent, the prolongation of fibroblasts is not perfect, however longer cultivation period should improve these shape imperfection in the future.

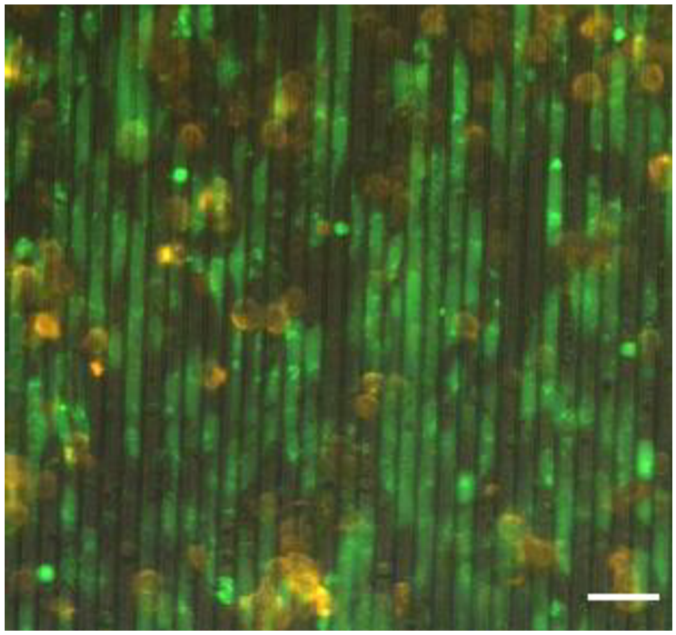

Figure 42: PDMS surface with lamellae containing two types of cells: cardiomyocytes stained by calcein AM (green) and the fibroblasts stained by CellMask Orange (orange). Microscope Leica DMi8, x10. Scale bar 50 µm.

Discussion

Cell co-culture and fabrication of system consisting of cells and extracellular matrix are important issues for biomedical research and development of workplaces today, their aim is mimicking real tissue. These in vitro constructs mimicking real tissue should be used for in vitro experiments in tissue biology and for testing of curing intervention in preclinical phase, and what is more, these construct are also optimised for near-future use as “artificial tissue patch” in transplantation medicine.

In the present thesis, two methods for fabricating of planar cell layers were tested. The first and the second method involves using scaffolds that support cells assembling to planar construct and long-time surviving of these cells. As a scaffold, two different solid polymer materials were used, in the first method: PCL nanofiber, in the second method: micro-patterned PDMS. The third method involves the use of a thermoresponsive polymer, this layer should play role only of a time-limited supporting "bottom-layer" and the separation of cells should give the intact cell sheet (planar artificial tissue with distance of millimetres and not including any synthetic polymer). Overview of all three methods in Figure 43.

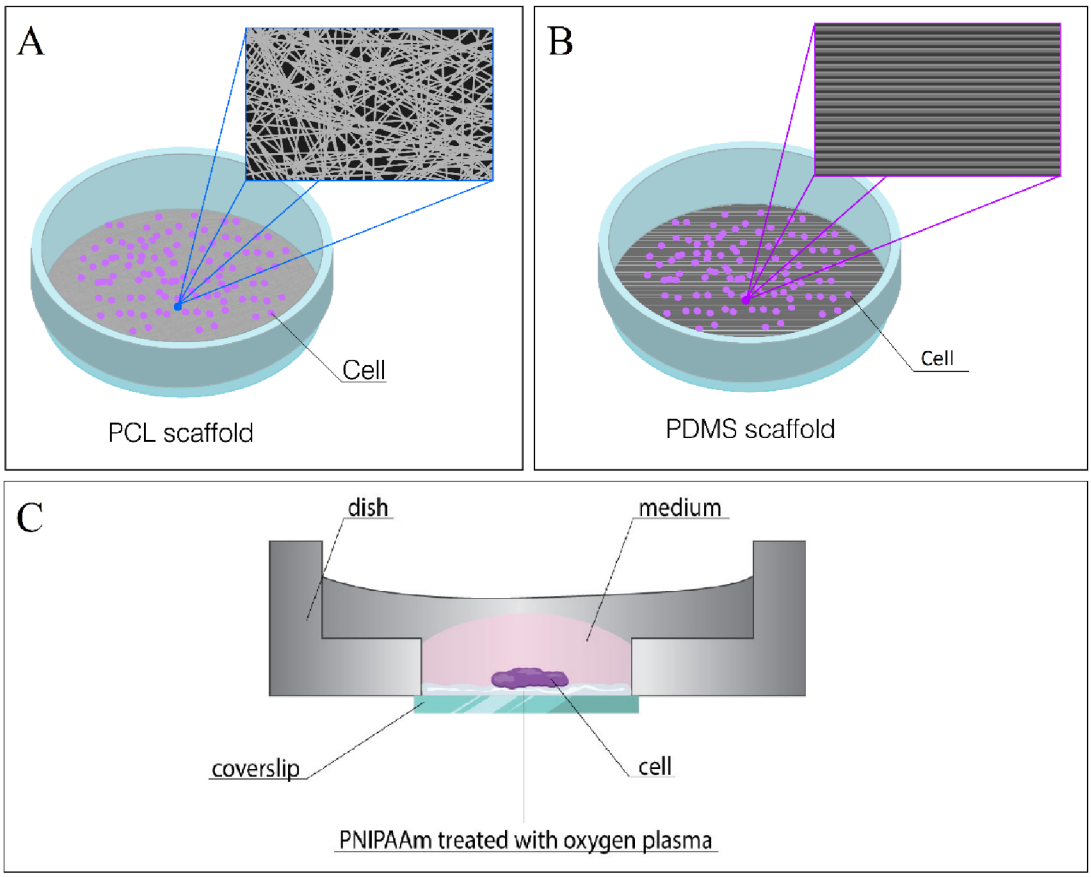

Figure 43: Concepts of used methods in the practical part. A: The concept of seeding cells on the PCL scaffold. B: The concept of seeding cells on the PDMS scaffold with the micropatterned surface. C: The concept of seeding cells on the PNIPAAm polymer.

The tests and results described in this thesis have the following order:

- the first tested material is PCL;
- the second material is thermoresponsive polymer PNIPAAm;
- the third is PDMS with the micropatterned surface.

The first used material was a PCL nanofiber (see chapter 7.2). Scaffolds from PCL nanofiber can be used for transplantation of the damaged cardiac tissue with cell layer on it,

because it is biodegradable, which mean that sometime after transplantation nanofiber will degrade and replaced by a new tissue, and also it is biocompatible and there will be no immune response to it [47]. During experiments it was observed that because of PCL nanofiber is not transparent, but only semi-transparent it is harder to visualize cells using an inverted microscope, it is necessary to label cells, for example using some fluorescent dye. This material has proven to be good for cell adhesion, experiments show that about 66% of the cells were found on the scaffold in 24 hours after seeding (see Figure 26). Also, due to the fact that this material is biocompatible, the cells had a good survival rate on this material and after three days they were able to create an almost confluent cell layer.

The second material was a thermoresponsive polymer (see chapter 7.3). This material was firstly described in publication in 1990 by Takezawa et al. [34]. The covering of the culture dishes with PNIPAAm is widely used. To improve cell adhesion to PNIPAAm, a large range of different methods can be used to fabricate thermoresponsive surfaces, such as radiation cross-linking, atom transfer radical polymerization, electron-beam processing, oxygen plasma-treatment and UV light irradiation or the use of ECM proteins [38, 39]. Since the authors of the article [35] have reported that the use of ECM proteins cannot provide high stability in long-term storage and low cost, it was decided to choose another way to improve cell adhesion. In this work, in order to improve cell adhesion, it was decided to use oxygen plasma treatment. The authors of the article [35] state that cell adhesion to the surface increases after plasma treatment. In this paper, oxygen plasma treatment was tested at 40 W and 100W.

Cell adhesion and detachment should be controlled by changing the temperature. The great advantage of this material is that it is transparent, it simplifies the control of cells during their development and growth. However, experiments do not show perfect results, problems with the initial cell adhesion were found, using both 40W and 100W oxygen plasma treatment (see Figure 31).

It was not possible to obtain a cell sheet from the entire surface of the culture dish, but using PNIPAAm treated with 100 W oxygen plasma, a small piece of the cell sheet was obtained. In addition, it was possible to separate this small cell sheet from the surface of the polymer using changes in temperature conditions. This is evidenced in Figure 32.

The third material was a PDMS with the micropatterned surface. This material is mostly used for specific cell culturing in vitro [22]. Scaffold used in this thesis was not prepared as a standard flat construct, but a very original PDMS construct containing surface lamellae (parallel lines that allow the cells to be oriented in the desired direction) was

prepared. Thus there was hypothesis that these lamellae could initiate the isotropic cell assembling and bring better mimicking of native tissue. As in the previous case, it is easy to control cells as the material is transparent. During the first experiment, this scaffold did not come up as a material suitable for creating a cell layer, however, after it was coated with fibronectin, cell adhesion significantly increased. Once the scaffold was improved by covering with fibronectin, about 53% of fibroblasts and 49% of cardiomyocytes were found attached to the scaffold in 24 hours after seeding (see Figure 37 and Figure 39). Moreover, three days after seeding, the cells were able to create an almost confluent layer.

Although these materials are very different they all have advantages and disadvantages. The undisputed advantage of PCL nanofiber is that it is easy to manipulate with. Since it is biodegradable, it can be transplanted along with the cells to the heart and will disintegrate over time. PCL showed itself as a scaffold with a fairly good adhesion. However, since the structure of its fibres is chaotic, it was not possible to obtain the same orientation of the cells on the surface of this scaffold. One can try to achieve this by creating a nanofiber with a uniform fibre structure.

A distinctive feature of PNIPAAm from two other materials is that it helps to obtain a cell sheet that can be used for transplantation without using any other materials. Based on the information obtained from the article, PNIPAAm was prepared and treated with oxygen plasma. However, a small cell sheet was obtained only once. This method of creating a surface for obtaining a cell sheet has to be refined and improved. As in the previous case, this material does not allow to obtain a structured tissue as in a living heart, in view of this it is necessary to combine it with other methods such as PDMS with a micropatterned surface.

Of all tested materials, PDMS was the only one that made it possible to control the orientation of cells on the surface of the scaffold. Tests have shown that it can be used to grow cells with a structure that resembles their placement in a living heart. Moreover, in the case of this scaffold, different widths between the lines (5, 10 and 20 μm) were tested (see Figure 35). Tests have shown that a distance of 10 μm is optimal for both cardiomyocytes and fibroblasts in spite of differences in their size. The cells fell in the gaps between the lines and, during adhesion, stretched along the length of these lines, hence creating a continuous cell line. However, its great disadvantage is that it cannot be transplanted into the heart, such as the PCL scaffold, but can only be applied to the heart muscle, due to the fact that it is not biodegradable.

In order to test how PDMS with lamellae affects the cell orientation, a small algorithm in Matlab was created. This algorithm allows to measure the angle of inclination of the cells,

however, the main axis of the cells must be pre-marked manually. The result of the algorithm is a histogram containing information about cell angles (see Figure 41). The histograms indicate that the use of PDMS with lamellae allows to obtain an isotropic cell structure, in contrast to a flat PDMS.

Important aspects of all three used material are safety and possibility of the certification via the rule of the EMA (European medical agency in EU) and FDA (Food and Drug Administration in the USA). PCL and PDMS were certified by FDA or EMA for specific applications used in the human body (for example surgical suture or contact lenses in ophthalmology), however there are also new running preclinical studies on different muscle and dermal disorders and there is high probability that nontoxic and sterile variant of PCL and PDMS will be certified also for introduction to the muscle tissue [91]. PNIPAAm has not been mentioned till today in any certificating protocol or clinical study [91].

Table 2: Certification via the rule of EMA and FDA.

Type of surface	**Cell adhesion**	**Cell confluency**	**Possibility of continual "cell sheet" detachment and transfer**	**Mentioned in clinical testing and EMA or FDA protocols**
Glass (in vitro chamber)	Yes	Yes	No	Yes
Polystyrene (in vitro chamber)	Yes	Yes	No	Yes
PDMS	Yes (better with fibronectin coating)	Yes	No	Yes
Polycaprolactone	Yes	Not tested	No	Yes
Poly(N-isopropylacrylamide) (PNIPAAm)	Yes (very poor and depends on plasma treatment)	No (but some publications describe confluency)	Yes	No

Conclusion

In this thesis materials for fabrication of planar confluent cell layer using cardiomyocytes and fibroblasts were tested. Two materials are used as scaffolds and one refers to the scaffold-free technology.

The purpose of this thesis is to provide a brief summary of publications in the field of TE in cardiology. The first chapter is devoted to the histology of the cardiovascular system in which the main layers of the cardiac tissue are described, including their composition and function.

The next chapter describes the cellular composition of the heart and the cultures of cardiomyocytes used in the experiments. This chapter also contains a description of the co-culture of cardiomyocytes and fibroblasts. Since these cells are predominant, both in volume and in number, their combination use can be a significant step in growing a tissue that will resemble real heart tissue as much as possible.

The next chapter is devoted to a description of tissue engineering. This chapter is divided into two parts. The first part contains a description of the technology of "cell sheet" which is scaffold-free. It consists in the use of a thermoresponsive polymer in which the hydrophilic and hydrophobic properties change according to the temperature conditions. In this way, cell adhesion and detachment can be controlled, resulting in a cell sheet in which the connections between neighbouring cells are preserved. The next part of the chapter contains information related to scaffold-based tissue engineering. Two different materials that can be used as a scaffold are described here. The first one is a PCL nanofiber, which is biodegradable. The second one is made of PDMS and contains a micropattern that can be used to obtain a tissue with the desired texture.

The next chapter describes the principle of cell adhesion and the molecules that participate in it. The integrins that are important in cell-ECM adhesion and the cadherins that participate in cell-cell adhesion are described in more details.

The following chapter contains information about tissues that can be developed using scaffold-based and scaffold-free technologies. The last chapter of the theoretical part contains information on fluorescent dyes. Three types of dyes such as calcein AM, PKH26 and cell mask plasma membrane stains are described here. Dyes help to control cells when it impossible with a passing light on a microscope. In the final experiments only Calcein AM and CellMask Orange were used, since they proved to be the most suitable.

The practical part describes the main stages of cell cultivation and passage. These procedures are very important in any experiments with cells and they are preceding all experiments. The next important step in the preparation of cells is their staining. Staining is used both to control cells and to distinguish between them. The following is a description of the experiments in which cells were placed on different materials such as PCL, PDMS and PNIPAAm and their growth and development were monitored. Cell growth was analysed and the results showed that the best cell adhesion was found on the PCL and PDMS scaffolds, on the contrary, in the case of PNIPAAm, the initial cell adhesion was poor and the method needed improvement. Also, in the case of PDMS, an assessment was made of the success of using a scaffold with a micropatterned surface, the angle of inclination of the cells was measured. At the end, an experiment was carried out in which fibroblasts and cardiomyocytes were grown together on a PDMS scaffold with a micropattern.

At the end of work, after the tests of selected cell types on different surfaces, the co-culture experiment was carried out to test cell adhesion and cell composition when two types of cells were seeded on the PDMS culture surface. Final culture displays high ratio of isotropy, distribution of all types of cells was determined by lamellae, all cells are planar and adherent and do not create any nonplanar aggregation. Final co-culture consisting of the two types of cells demonstrates propinquity to the structure of real heart tissue, however, future culture experiments are required for easier setting of the ratio of cell types and for evaluation of co-culture stability, intracellular communication, the possibility of transplantation and long-time macroscopical functions of “artificial tissue”.

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List of abbreviations

AC	Adult cardiomyocyte
AT	Atrial tumor
Calcein-AM	Calcein-acetoxymethylester
CAM	Cell adhesion molecule
CIB	Calcium-and integrin binding protein
CYR61	Cysteine-rich angiogenic inducer 61
DMEM	Dulbecco's modified eagles media
ECM	Extracellular matrix
EMA	European medical agency
ERK	Extracellular signal-regulated kinase
FAK	Fokal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and drug administration
HGF	Hepatocyte growth factor
HMDS	Hexamethyldisilazane
ICAP	Cytoplasmic domain-associated protein
ILK	Integrin regulated kinase
LCST	Lower critical solution temperature
MAdCAM	Mucosal vascular addressin cell adhesion molecule
MAPK	Mitogen-activated protein kinase

PE-CAM	Platelet endothelial cell adhesion molecule
PCL	Polycaprolactone
PDMS	Polydimethylsiloxane
PNIPAAm	Poly(N-isopropylacrylamide)
RACK	Receptor for activated protein kinase
TCPS	Tissue culture polystyrene dish
TE	Tissue engineering
TSP	Thrombospondin
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VEGFA	Vascular endothelial growth factor
VWF	Von Willebrand's factor
3D	Three-dimensional

List of attachments on CD

A. The electronic version of the thesis in the pdf format.

B. The program for calculating the angles of the cells *CellAngle.m.*

C. Figures used to test the program test_1 and test_2.