Biocompatibility testing using advanced cultivation techniques

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I. Teoretická část
1. Zpracujte literární přehled týkající se podmínek kultivace buněk s důrazem na rozdíly mezi statickými a dynamickými podmínkami kultivace.

II. Praktická část
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[1] SNUSTAD, D.P., SIMMONS, M.J., RELICHOVÁ, J. et al. Genetika. Brno: Masarykova univerzita, 2009.
[2] ALBERTS B. et al. Molecular Biology of the Cell 5th ed. Garland Science.
[3] DAVID A.P. BIZIOS R. Biological Interactions on Material Surfaces. ISBN 978-0-387-98160-4.

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ABSTRAKT

Cílem této diplomové práce bylo prohloubit současné možnosnosti kultivace buněk za dynamických podmínek. Diplomová práce byla zpracována na Univerzitě Tomáše Bati ve Zlíně. *In vitro* dynamické kultivační techniky napodobují *in vivo* podmínky a poskytují tak přesnější informace o buněčném chování.

Teoretická část práce je zaměřena na rozdíly mezi statickou a dynamickou kultivací, na možnostech dynamické kultivace a také definuje dynamické síly běžně se vyskytující v lidském těle a jejich dopad na buňky. Praktická část popisuje design, výrobu a testování několika různých zařízeních pro dynamickou kultivaci. Je rozdělena do dvou částí. První část se zaměřuje na implementaci dynamické průtokové komory do každodenního využití v rámci laboratoře. Druhá část popisuje výrobu vlastního dynamického zařízení, také nazývaného jako Lab-on-chip. Dva ze tří designů byly úspěšně vyrobeny a mohou být využity při dalším testování.

Klíčová slova: statická kultivace, dynamická kultivace, Lab-on-chip, TiO_2 nanočástice, průtoková komora, elektrické pole

ABSTRACT

This Master‘s thesis aimed to deepen the current possibilities of cell cultivation under dynamic conditions. The thesis was processed at TBU in Zlín, precisely at the laboratory of cell biology at the Centre of Polymer Systems. The *in vitro* dynamic cultivation techniques efficiently mimic the *in vivo* conditions. Thus, it can provide more accurate information about cell behaviour.

The theoretical part of this thesis is focused on the main differences between static and dynamic cultivation, and the possibilities of dynamic cultivation. It also defines dynamic forces occurring in the human body and their effects on cells. The practical part describes the design, fabrication, and testing of few special devices for dynamic cell cultivation, followed by optimizing conditions for future testing. It is divided into two sections. The first part aims to implement the dynamic flow chamber into everyday use in the laboratory. The second section describes the creation and fabrication of microfluidic chambers called as well as Labs-on-chips. Three different designs were introduced in this thesis.

Keywords: static cultivation, dynamic cultivation, lab-on-chip, TiO_2 nanoparticles, flow chamber, electric field

Here I would like to thank prof. Ing. Humpolíček, Ph.D. for helpfulness and patience during the guidance of my Master's thesis. I would also like to thank Ing. Jakub Huba, Ph.D. His willingness and helpfulness enabled the creation of the Lab-on-chip device.

I hereby declare that the print version of my Master's thesis and the electronic version of my thesis deposited in the IS/STAG system are identical.

CONTENTS

INTRODUCTION

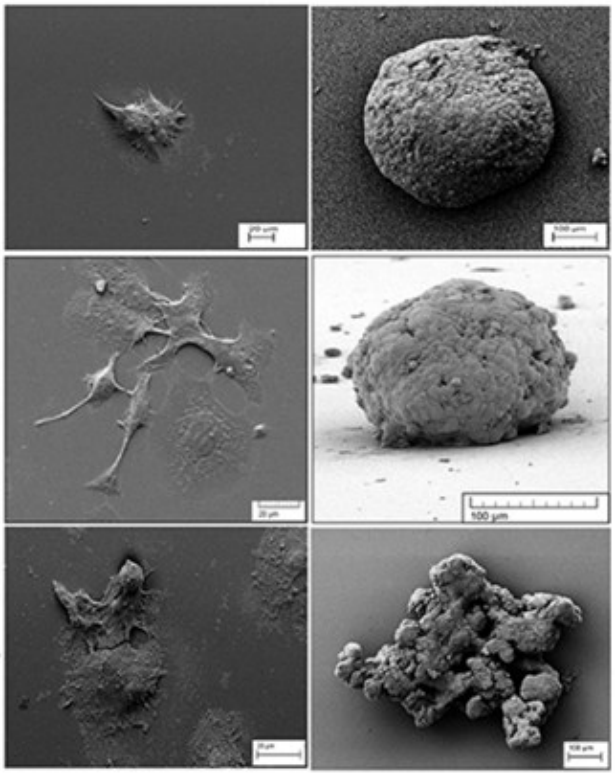
Since the techniques of *in vitro* cultivation of eukaryotic cells were developed, the static cultivation (C^{ST}) techniques were used. All cell culture laboratories and related researches are based on the principles of C^{ST}. C^{ST} is easily feasible from a practical point of view. It can be used as a tool for basic cytotoxicity testing, experiments in tissue engineering, or for getting further information about the behavior of stem cells (SC) [1]. However, results taken from C^{ST} give only a simplified image of cell biology. Thus, the image does not correspond with *in vivo* conditions in many directions. Due to its inaccuracy, scientists look for the ideal conditions and experimental set-ups of cell cultivation. Some imperfection of C^{ST} may be solved using the dynamic cultivation techniques (C^{DYN}) [2].

The idea of C^{DYN} is the creation of conditions, which resemble the environment in the human body. C^{DYC} devices differ in applied force, used cell lines, and in the final design [3]. Bioreactors are one of the types of equipment for C^{DYN}. They are commonly enriched with mechanical forces such as shear stress, hydrostatic pressure, or tension. Used mechanical forces are responsible for the improved similarity with *in vivo* conditions. However, bioreactors are multifactorial systems, and the evaluation of results may be complicated. Labs-On-Chips (LOCs) are another option for C^{DYN}. They are 2D systems providing the required dynamic conditions. Depending on their design, they can implement various dynamic forces and provide better communication between cells. Nowadays, they are widely used in biological laboratories [4].

This thesis aims to define the differences between static and dynamic cell cultivation. It focuses on finding new possibilities of biological testing, followed by their implementation into practice.

I. THEORY

1 STATIC *VS* DYNAMIC CULTIVATION OF CELLS



a) b)

Figure 1 SEM images of breast tumor cells a) tumor cells cultivated under static conditions, b) tumor cells cultivated under dynamic conditions [5].

Differences in construction and conditions during C^{ST} and C^{DYN} result in a change in cell morphology and physiology. Briefly, by using C^{ST}, cells are impacted only by biochemical factors (e.g. CO_2 concentration, humidity, temperature, the composition of the cultured medium, or concentration of waste product). However, cells are actually exposed to other factors including mechanical forces, shear stress, and intercellular interactions under *in vivo* conditions. C^{DYN} can deliver one of these conditions to improve similarity with *in vivo* or it can combine more factors together. Various devices can be used and the choice depends on the type of cultivated cells and aim of study or application [6].

At this point, it is in place to define differences between static and dynamic cultivation to understand why static cultivation is less accurate and what are the advantages of dynamic cultivation.

1.1 Static cultivation

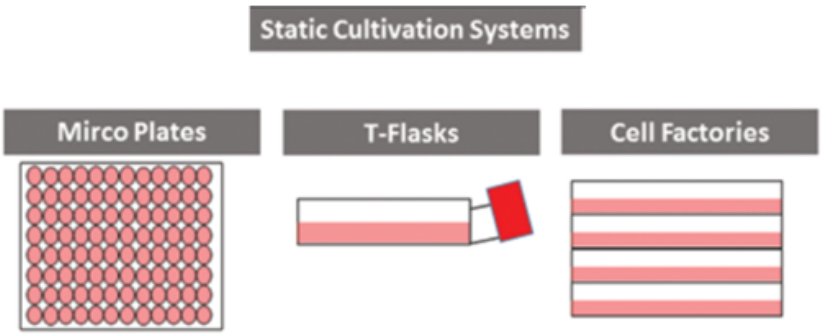


Figure 2 Different types of C^{ST} systems. These systems include micro plates (used for measuring cytotoxicity), T-flasks (for cells cultivation), or cell factories (used for extensive production of cells) [7].

C^{ST} is based on the cultivation of cells on special plastic dishes made of biocompatible materials, intended for use in biological laboratories. Despite some imperfections, it has great merit in knowledge about basic biological processes in living organisms. It is an achievable and easy method used in every biological laboratory. It allows quick defining of cytotoxicity, proliferation, changes in cell morphology on the surface of tested material, or cell adhesion to the surface.

Nevertheless, as previously mentioned, C^{ST} does not provide cues that occur *in vivo*. Firstly, the 2D environment is not natural for any cell. *In vivo,* cells are in the 3D environment. This fact has a huge impact on cell physiology and it is responsible e.g. the presence of stress fibers during C^{ST}. The multicellular organism is also a highly compact and connected system, where cells interact between themselves. The communication between cells from different tissues plays an important role in the retention of homeostasis, which is crucial for the survival of each individual. The connection and communication between cells are limited in C^{ST}. Another problem lies in the limited seeding efficiency and poor transport of nutrients, oxygen, and wastes, so it is less suitable for long-term testing [4].

1.2 Dynamic cultivation

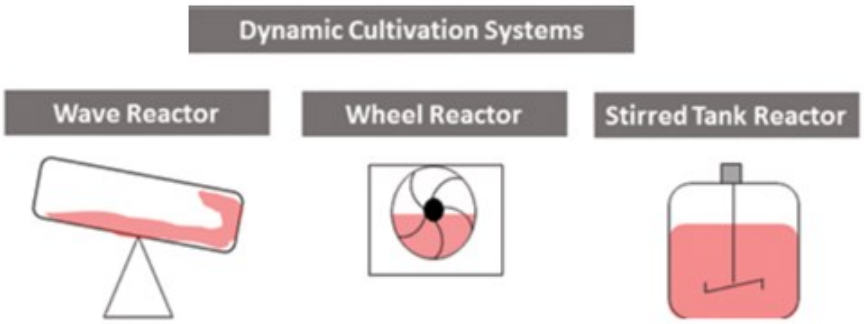


Figure 3 Examples of different types of C^{DYN} systems. The bioreactors in this example include wave reactor, wheel reactor, and stirred tank reactor [7].

Recently, C^{DYN} has started to spread out in biological research. In its elementary form, the C^{DYN} is characterized by the intentional active motion of the medium during cell cultivation. The active motion can be achieved either directly (agitation of culture medium) or indirectly (agitation of the culture vessels). The C^{DYN} provides better control of the cultivation process, monitoring, documentation, automation in computer-controlled systems, reduction of manual labour, improvement of reproducibility, and reliability of results. More advanced devices can mimic conditions presented in living tissue even better, by adding mechanic stimulation using compression, tension, or electrical field [8].

C^{DYN} can be preferential in the cultivation of stem cells (SCs). SCs are used in tissue engineering[1] (TE) or curing diseases, where they are administered to treat or prevent disease. C^{DYN} using, for example, 3D matrices combined with bioreactors allow the cultivation of SCs under resembling conditions as they occur in the human body. It may lead to better results in SCs differentiation and cultivation [1].

[1] Tissue engineering is defined as the application of principles of life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues.

2 SUBSTRATES USED FOR STATIC CULTIVATION

As it was mentioned previously, C^{ST} is still a predominant method for cell cultivation and biological research. There are many options for C^{ST}, including tissue plastic, film modification of the substrate surface, and 3D structures called scaffolds. All of these substrates may be also used in C^{DYN}. C^{DYN} is however enriched with other stimuli. A brief description of substrates for C^{ST} continues below.

2.1 Tissue plastic

Figure 4 Types of tissue plastic used for C^{ST}. [Figure was taken from: https://www.thermofisher.com/cz/en/home/lifescience/cell-culture/cell-culture-plastics.html]

One of the most often used tools for C^{ST} is tissue plastic made from polystyrene (PS). It has been used for more than 50 years. Due to its low production cost, optical clarity, and other properties, PS has largely replaced glass for work in biological laboratories.

Due to the hydrophobic nature of PS, its surface is usually modified. The modification provides enhancing of cell adhesion and proliferation [9]. The functionalization of the surface differs from one producer to another. It may be done through two methods: liquid treatment or plasma treatment. The liquid treatment is based on surface oxidation by using strong acids. However, using strong acids enhances the safety risk. Liquid treatment may be also responsible for the reduction of the optical clarity.

The plasma treatment is done by using ionized species of gas. Energized ions can interact with the surface and modify it. The treatment using plasma modifies the surface chemistry and breaks substrate surface bonds. Thus, plasma treatment enhances surface roughness, which improves cell growth [10].

2.2 Films

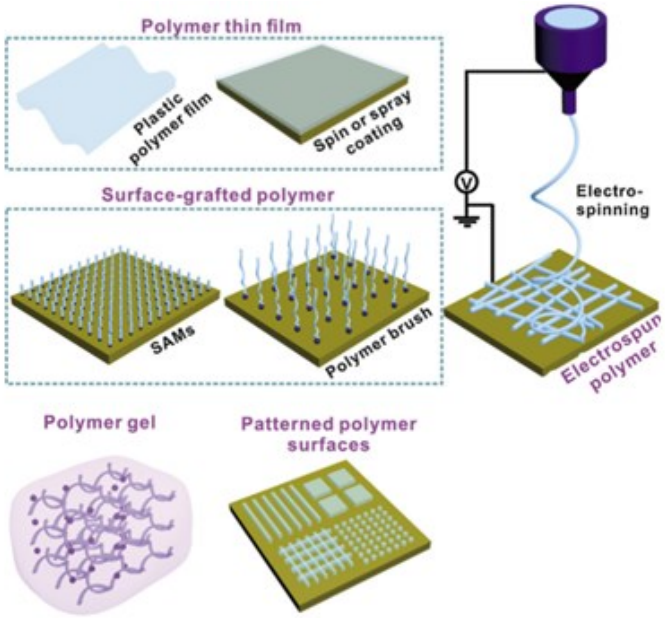


Figure 5 Surface modification by using polymer films. Surface modification can be done using polymer thin film (fabrication methods are electrospinning, spin or spray coating or polymer brush), and polymer gel [11].

The quality of cell cultivation during C^{ST} can be improved by modification of the PS surface by various films. These films enhance cell adhesion, control cell behavior, and may possess conductive properties. For modification, different kinds of polymers are used. There are six major types of polymer materials used in controlling cell behavior, including polymer thin films, surface-grafted polymer, electrospun polymer, polymer gel, and patterned polymer. The effect on cells then depends on the type of used polymer (polyethyleneglycol, polydimethylsiloxane, modified polyurethane,…), and method of fabrication. It is also tied with the way how films are attached to the substrate [11].

Natural polymers are frequently used for surface modification. Collagen, fibrin, or vinculin are very suitable for surface coating. Collagen is the fibrillar polymer constructing many tissues. It is cytocompatible, improves cell adhesion, and provides a native viscoelastic environment to resident cells. Collagen can be used in its amorphous form, well-known as gelatine [12].

Fibrin is a blood component involved in the blood clotting and wound healing processes. Fibrin is formed by the polymerization of fibrinogen in the presence of thrombin and $CaCl_2$. Films made from fibrin enhance cell adhesion to the substrate. It is also used to deliver various growth factors to improve cell growth [13].

Vinculin is the protein that mainly occurs in regions where cells are in contact with each other and are connected to the underlying substrate. It regulates adhesion by directly binding to actin, stimulating actin polymerization, and recruiting actin remodeling proteins. Therefore, vinculin is a very suitable polymer for coating the surface for cell cultivation. In the absence of vinculin, cell-matrix, and cell-cell adhesion are dramatically impaired [14].

2.3 Scaffolds

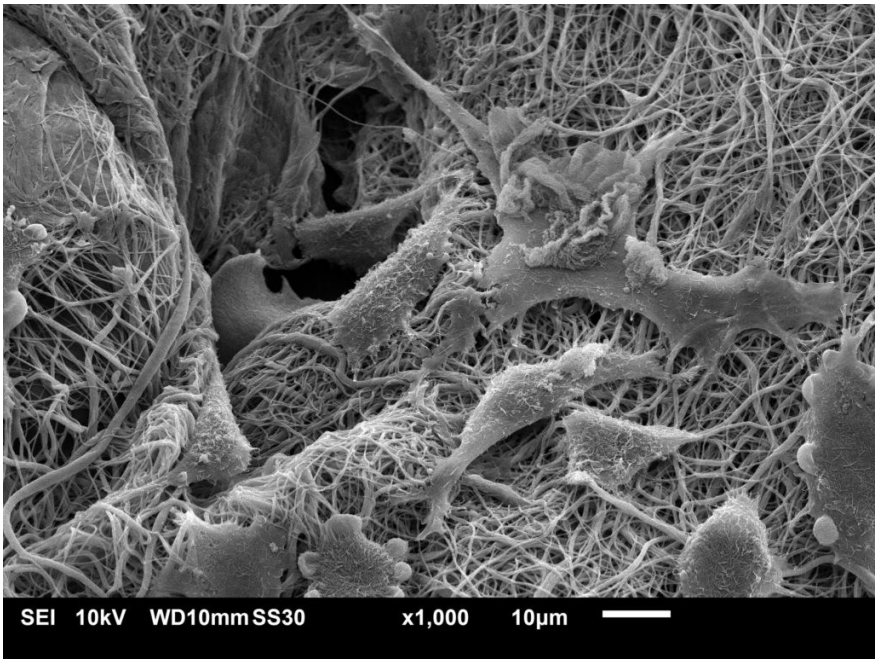


Figure 6 SEM image of stem cells grown on the nanofibrous scaffold. SEM was used to take Figure of human mesenchymal stem cells attached and grown on the surface of the scaffold [15].

Scaffolds are structures that act as cell carriers. They should mimic a suitable *in vivo* environment for cell growth and reproduction. The exact properties of scaffolds are crucial for their usage in TE. These properties include exact porous structures, pore size, pore distribution, degradation properties, surface properties, mechanical strength, and workability [16].

2.3.1 Properties of scaffolds

Scaffolds are used for guiding the adhesion, proliferation, and differentiation of the transplanted cells or for the native infiltrating cells into damaged tissue. The 3D scaffold is biocompatible, degradable, and resorbs at a rate corresponding to tissue growth. By using the scaffolds, it is possible to define the shape and function of the assimilated cell structure [17]. The biological activity of scaffolds is regulated by ligand density. Ligand density is determined by scaffold composition and porous fraction. Highly specified surface areas lead to good cell adhesion and anchorage. High pore volume fractions enable cell growth, migration, and effective transport of nutrients. The porous size depends on the type of cultivated cell tissue. For example, for bone tissue regeneration, the size of pores is about 100 μm and for new bone formation pores bigger than 300 μm are required. Vascular smooth muscles grow most easily in pores from 63 to 150 μm in size [18].

2.3.2 Materials used for scaffolds construction

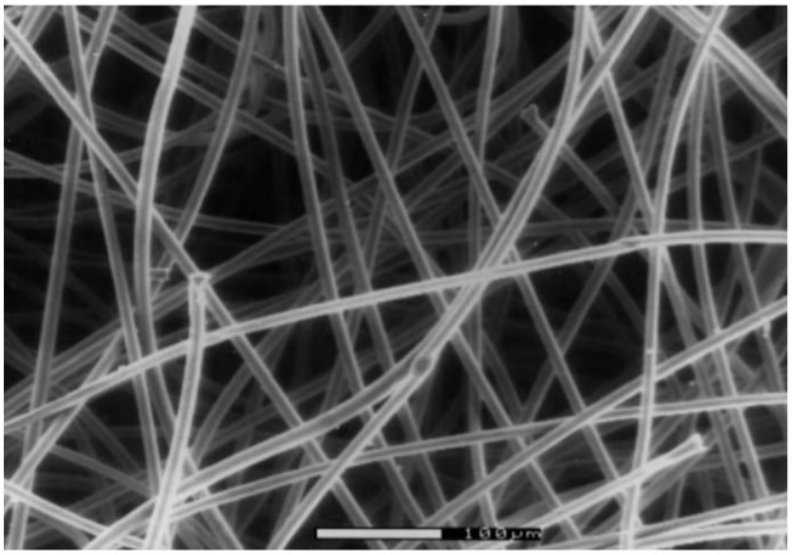


Figure 7 SEM of PGA scaffold. SEM picture of scaffold made from PGA with a fiber diameter of approximately 15 μm [19].

The scaffold may be constructed by using different materials. The materials include linear aliphatic polyesters, natural polymers, and certain inorganic substances. The group of linear aliphatic polyesters represents polyglycolic acid (PGA), polylactic acid (PLA), or polyhydroxy butyrate (PHB). Natural polymers such as collagen, silk, alginate or chitosan, possess useful biological properties desirable for TE applications. Inorganic substances can be categorized as porous bioactive glass and calcium phosphates. Inorganic materials have osteoconductive[2] properties [19].

2.3.3 Types of scaffolds

Types of scaffolds used in TE may be divided into three categories: fibrous, porous, and scaffolds made from hydrogels. Different types of scaffolds are suitable for different tissues. The choice of the right scaffold depends on resemblance with native ECM (extracellular matrix), e.g. porous ceramics are suitable for bone tissue, non-oriented fibers are used for endothelium, and so on.

The structure of fibrous materials may affect the behavior of cells. The size of the pores of the fibrous matrix regulates the organization and activity of cells. Fibrous scaffolds can easily mimic the native extracellular matrix, which consists of nanoscaled structural and adhesive protein fibers such as collagen or elastin. Nanoscaled polymeric fibers can be produced by different methods, e.g. electrospinning, self-assembly, or phase separation.

Porous scaffolds have shown success in growing cells into functional tissues. One of the benefits of porous materials is that they provide a large surface area for cell adhesion. Porosity also supports cell differentiation and proliferation. The porous scaffold may be fabricated using freeze-drying, particulate leaching, phase separation, and solid freeform fabrication.

Hydrogels can be natural or synthetic polymer chains that swell in aqueous solutions. They have tissue-like properties. They can encapsulate cells and create a proper environment for cell growth. Hydrogels provide high oxygen and nutrient permeability, and easy migration of cells from the tissue into scaffolds. Hydrogels are very useful in cartilage regeneration [20].

[2] The surface property supporting osteoblast‘s adhesion, growth, and differentiation. [56]

3 SYSTEMS USED FOR DYNAMIC CULTIVATION

On the market, a lot of commercial products are available for the C^{DYN}. As mentioned above, they differ from their application and many of them are tailor made. For purpose of this thesis, only two examples will be described.

3.1 Bioreactors

Bioreactors are devices simulating the *in vivo* physiological environment. They help with the promotion of cell growth and differentiation. Bioreactors may control temperature, oxygen concentration, carbon dioxide concentration and provide mechanical, chemical or electrical stimuli [17].

3.1.1 Types of bioreactors

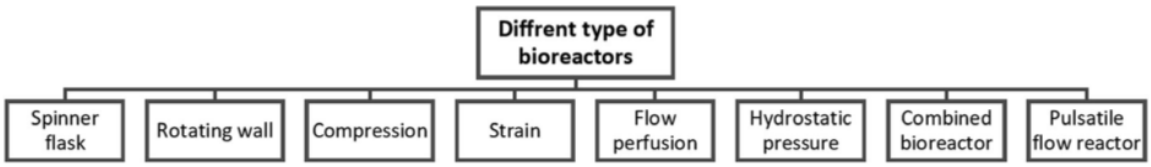


Figure 8 Different types of bioreactors may be used in TE. The bioreactors can induce compression, strain, hydrostatic pressure, pulsatile flow, and more [21].

Many different kinds of bioreactors exist, as can be seen in Figure 8 above. Their use depends mainly on the type of cultivated tissue. Lots of researches were made in the field of regenerative medicine successfully using bioreactors. For example, damaged osteochondral tissue may be replaced by tissue cultivated in bioreactors. The spinner flask bioreactor helps to achieve uniform distribution of cells seeded on the scaffold and helps with preventing cell aggregation. The spinner flask also promotes mass transport of nutrients between cells and medium, generates shear stress, increases differentiation of adipose stem cells into osteoblasts, and promotes osteoblast differentiation in osteochondral tissue [21].

3.2 Lab-on-chip

Lab-on-chip (LOC) is the 2D system enriched with dynamic forces influencing cells. Similar devices were designed and fabricated in the practical section of this thesis. Here is a brief description of what LOCs are and what are their main advantages.

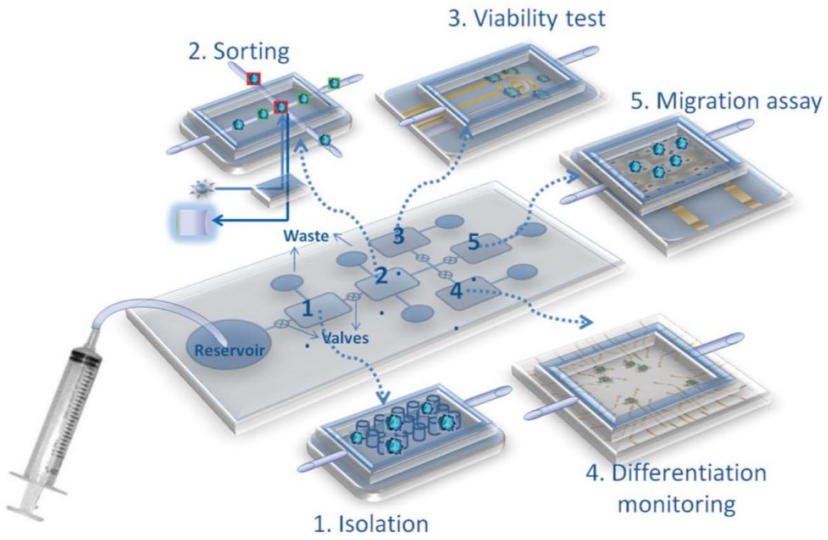


Figure 9 Example of a modular Lab-on-chip for stem cell studies. It consists of several microfluidic components and sensing modules that are coupled in one device, for cell isolation, sorting, viability testing, differentiation monitoring, or migration assay [22].

Lab-on-chip (LOC) is a biomolecular detection system based on microfluidics (therefore, it is known as a microfluidic device as well). Miniaturization of fluidic operations has a potential for analytic applications in the biomedical or environmental industry. [23] LOC devices provide a solid way for integration, automation, and parallelization of chemical processes. They carry out separations and detections with a high resolution and sensitivity. Their usage also decreases the consumption of expensive agents used for testing. The challenges with LOC usage are scaling capillary forces, surface roughness, channel clogging, and laminar flow-limiting reagent mixing to diffusion. Another issue relates to the mismatching volume of real-world samples and microfluidic components [24].

The biological cellular system is a complex physicochemical reactor, which can undergo adaptive changes towards external biophysical cues. To understand its behaviour, it is critical to develope a device that can closely mimic *in vivo* physiological conditions. Microfluidic systems (or LOCs) provide a platform for studying many of the intracellular processes along with cellular responses.

The foundation of microfluidic studies is based on cell culture techniques connected with cellular engineering. That means the ability to grow active cells outside the living system within a tailored device capable of simulating physiological conditions.

LOC systems may be very useful in regenerative medicine as they enhance the efficiency and stability of implants. This approach uses the cultivation of SCs isolated from human bone marrow. The velocity, quality, and suppression of immunogenic reactions of prepared tissue from SCs are crucial for their safe use [25].

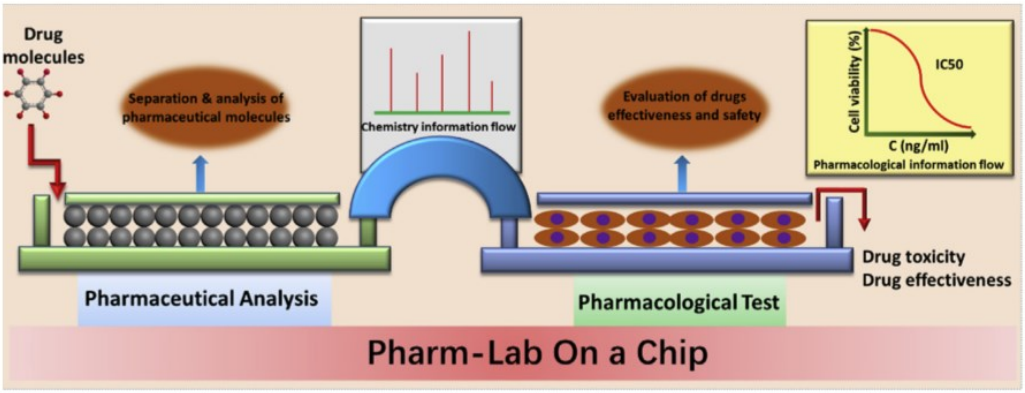


Figure 10 Scheme of the principle of Pharm-Lab-On-Chip device. The scheme represents a chip-based platform for the whole chain of pharmacy applications [26].

LOC has also attracted the great attention of the pharmacy community. It represents technology suitable to solve the old issues for the pharmacy industry, such as a long time consuming, laborious, high cost, and low productivity. LOC used in pharmacy is called Pharm-LOC. It is a chip-based platform for the whole chain of pharmacy applications from drug discovery to post-marketing product management. It may comprise all aspects of chip-based principles, techniques, and devices for pharmaceutical analysis, pharmacological/toxicological testing, and pharmaceutical production. Pharm-LOC may be used for pharmaceutical separation and analysis using electrophoresis chips, chromatography microchips, or microfluidic chips with sensitive and specific mass spectrometry-based detection.

For the fabrication of pharmacological/toxicological models organ-on-chip models, single organ-on-chip models, multi organ-on-chip or cell-on-chip models may be used. Cell-on-chip or organ-on-chip devices also can be used in the evaluation of the efficiency and safety of drugs [26].

4 CELLS UNDER DYNAMIC CONDITIONS

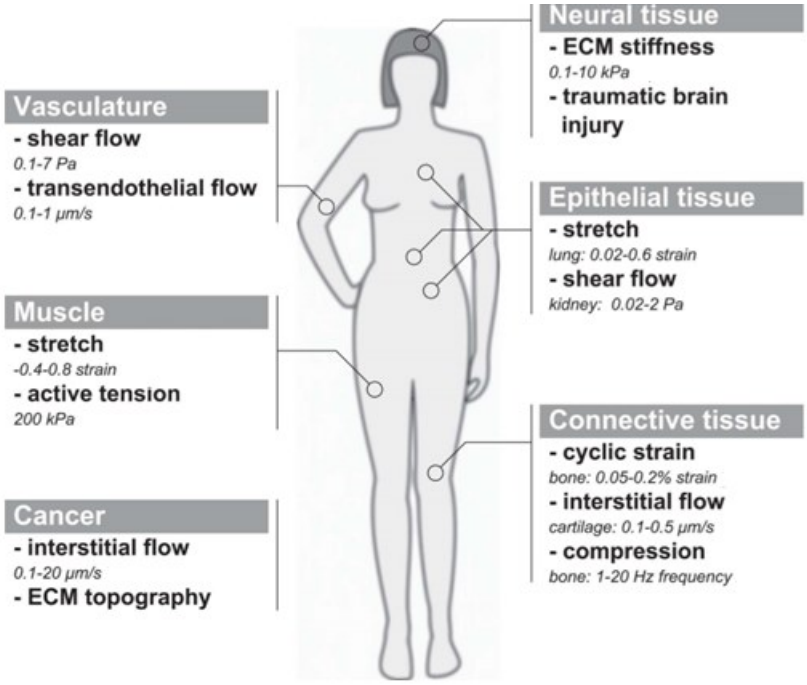


Figure 11 Illustration of mechanical stimuli and their magnitudes in the human body. Examples of various mechanical forces presented in the tissues, such as neural, epithelial, connective, vascular, or muscle tissue [27].

It is important to gain knowledge about dynamic stimuli presented in the human body, before their usage in TE. It has been shown that dynamic forces suitable for cell cultivation differ from various types of cells. Thus, it is crucial to bear in mind what kind of cells is cultivated, in order to use proper dynamic force and its right magnitude to enhance the quality of prepared tissue. Types of dynamic forces used in TE and their impact on cell growth are described below.

4.1 Compression

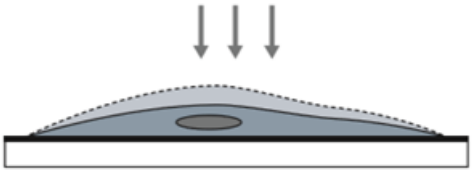


Figure 12 Cell under compression. Compression is one of the three main mechanical forces occurring in the human body. It is responsible for example for chondrogenic differentiation [8].

Compression is defined as the effect of balanced uniaxial forces on the surface of a material which leads to the reduction of the size in one or more directions [8]. In biology, compression of tissue occurs naturally during everyday movements such as walking down a stairway or rising from a chair. Tissues that are affected with compression the most are bone and cartilage tissue. The forces affect tissue from the top and that leads to the flattening of cells. It has been demonstrated that forces that imitate physiological compression induce chondrogenic differentiation. This state is related to the fact that mechanical stimulation of chemical signals plays an important role in the differentiation of mesenchymal stem cells into a chondrogenic or osteogenic phenotype [8]. In recent studies, it has been shown that the mechanical compression of 6.4 and 34 kPa affects the osteogenic differentiation abilities of stem cells [28].

4.2 Tension

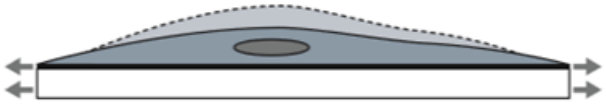


Figure 13 Cell under tension. With compression and shear stress, tension belongs to three main dynamic forces. It induces osteogenic, chondrogenic, and ligament differentiation [8].

Tension can be described as pulling forces applied axially on an object which results in an increase of size in one direction [8]. Tension or tensile strain occurs in human physiology typically in joints, tendons (tensile strength is around 80 $MN/1m^2$), bones (tensile

strength is around 180 MN/1m^2), cartilage (Young's modulus[3] in the range 5-20 MN/m^2), and muscles. It has been also shown that for example tendons can be stretched by about 10% of their size before breaking. On the opposite, bones may stretch only about 2% to 3% before they break. The easier breakage of bones is caused due to their different structure [29].

The ability of SCs to respond to mechanical tensile stimuli has been reviewed as well. Tension plays a significant role in regulating proliferation and differentiation pathways towards the osteogenic lineage [8]. Tension forces can be biaxial (loading in two directions) or multiaxial (loading in multiple directions). Multiaxial forces can help to induce ligament differentiation from mesenchymal progenitor cells [30].

In a study made by Ruixia Kuang et.al., the effect of cyclic mechanical stimulation coupled with stretching on dermal fibroblasts was observed. The study aimed to find out the effect of mechanical stimuli on dermal fibroblasts. Skin tissue was obtained during skin surgery from the scapular part of the upper back and medial side of the upper arm from 3 patients. From skin tissue, fibroblasts were isolated and seeded at a density of 3×10^4 cells/cm^2 into flexible-bottomed culture plates with 6 wells and sub-cultured at 37°C under 5% CO_2 in α-MEM medium containing 10% fetal bovine serum. When cells were grown enough for the experiment, 6 cultured plates were transferred onto a multi-channel tension-loading bioreactor and exposed to the uniaxial cyclic mechanical stress for 24, 36, and 48 hours, with a 10% stretching amplitude at a frequency of 0.1 Hz. The results demonstrate that applied mechanical stress increased the cell proliferation rate, and up-regulated the expression levels of production of substances crucial for cell growth and proliferation (integrin β1, cytoskeleton p130Cas, TGF β1 and type I collagen) in the stretched dermal fibroblast. They found out that there was a difference in the levels of responsiveness to applied mechanical forces between the fibroblasts from the scapular part of the upper back and fibroblasts from the medial side of the upper arm as well [31].

[3] Young's modulus describes the elastic properties of a solid undergoing tension or compression [55].

4.3 Shear stress

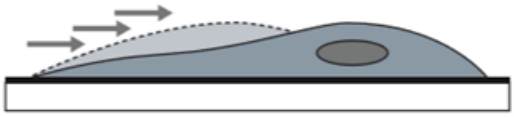


Figure 14 Cell impact by shear stress. It is the third main mechanical force affecting cells in the human body, where it arises from the movement of liquid [8].

Shear stress is observed when two parts of the materials slide over each other. Shear stress occurs parallel to the applied forces [32]. It can be induced between two solid materials. However, in living tissue, there is more likely to see fluid shear stress. Fluid shear stress is caused by the flow of fluid across the solid surface. The value of shear stress depends on the velocity of the surrounding fluid. Fluid shear stress for Newtonian liquid is defined as followed:

$$\tau(y) = \mu \cdot \frac{\partial u}{\partial y}$$

where,

- μ is the dynamic viscosity of the fluid
- u is the velocity of the fluid along the boundary
- y is the height above the boundary [33]

4.3.1 Shear stress and blood

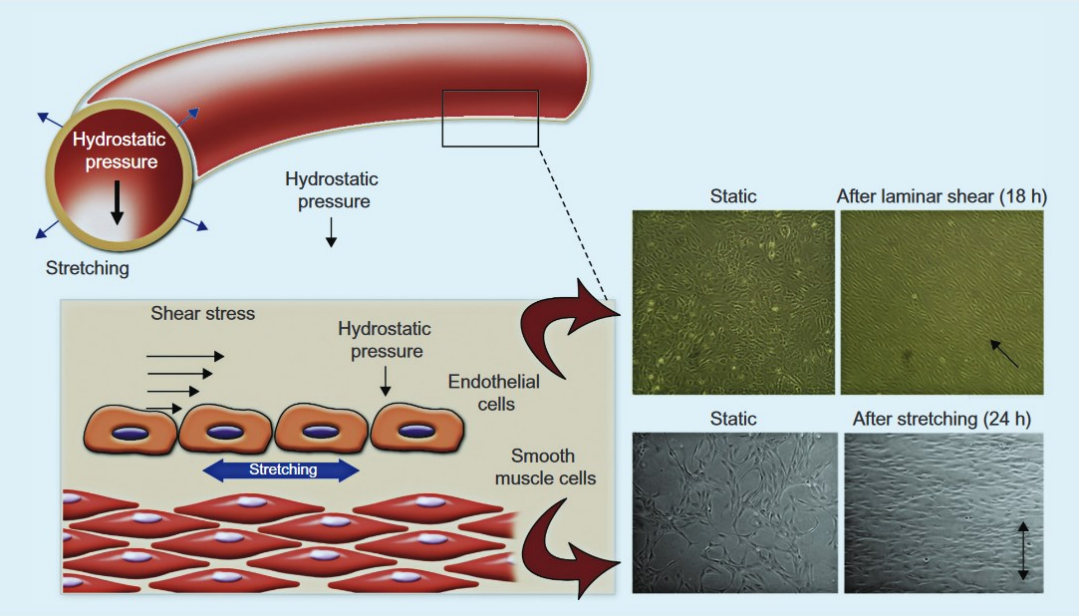


Figure 15 Blood cells affected by hydrostatic pressure, shear stress, and stretching, which resulted in a change in cell morphology and orientation [34].

Shear stress in the living organism is mostly represented as a part of hemodynamic forces, which regulate the structure and function of the blood vessel wall. The cells that are mostly affected by fluid shear stress are vascular endothelial cells, located at the interface between the circulating blood and the blood vessel. Shear stress is then expressed in the force-area unit (typically dynes[4]/ cm^2). In a straight artery, the regional blood flows occur without convective mass transference, thus the flow is described as laminar. In curves or arterial system bifurcations, the flow may show random movements and be described as oscillatory or turbulent [34]. The magnitude and pattern of the shear stress depend on blood flow, blood viscosity, and vascular geometry, which varies with the location in the vascular tree [35]. Shear stress patterns that occurred in the vascular system are mentioned in Figure 16 below.

[4] A dyne is a force that gives a mass of one gram an acceleration of one centimeter squared per second [54].

Arterial system			
Location		**Pattern**	**Shear stress 10–70 dynes/cm^2**
Great arteries	Straight regions	Laminar	15–30 dynes/cm^2
	Bifurcations/curves	Turbulent/oscillatory	±4 dynes/cm^2
Stenotic arteries		Turbulent/oscillatory	30–40 dynes/cm^2
Small arteries		Laminar	>12 dynes/cm^2
Venous system			1–6 dynes/cm^2

Figure 16 Shear stress in the arterial system. The magnitude of shear stress presented in the arterial system differs depending on the location, shape of arteries, and flow patterns [34].

4.3.2 Fluid shear stress and kidney

Blood cells and vessels are not only parts of the human body which are highly impacted by shear stress. Shear stress also affects kidney cells which are in the glomerulus and tubules continuously exposed to capillary flow and flow of filtrated urinary. Fluid shear stress of 1 dyn/cm^2 is approximately a glomerular filtration rate of 115 ml/min/1.73m^2. In general, every person is unique and the range of fluid shear stress may start from 0.7 to 1.2 dyn/cm^2 and can be up to 1.6 dyn/cm^2 in pathological conditions [36].

4.3.3 Shear stress and stem cells

Shear stress has also been used to stimulate SCs differentiation. The impact of shear stress was described at purified basal cell cultures from the adult olfactory epithelium. Cells were stimulated by mechanic shear stress to promote their neurogenesis. In a separate study, shear stress was used to enhance the concentration of nitric oxide and the production of prostaglandin in preosteoclast-like cells derived from bone marrow [30].

Moreover, due to the avascular nature of cartilage, shear stress participates in the cellular proliferation and growth of chondrocytes. It also enhances nutrient diffusion, elongates chondrocytes, and enhances proteoglycan and collagen synthesis [37].

4.4 Hydrostatic pressure

Hydrostatic pressure is defined as the pressure exerted on a fluid at equilibrium at any point of time due to the force of gravity. It is proportional to the depth measured from the surface as the weight of the fluid increases when a downward force is applied.
Hydrostatic pressure is defined as followed:

$$p = \rho \cdot g \cdot h$$

where,

- p is the pressure exerted by the liquid in $N.m^{-2}$, Pa
- ρ is the density of the liquid in $kg.m^{-3}$
- g is the acceleration due to gravity taken as 9,81 $m.s^{-2}$
- h is the height of the fluid column in meters [38]

4.4.1 Hydrostatic pressure in the human body

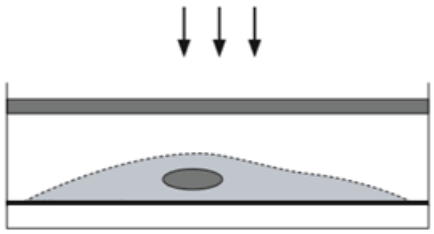


Figure 17 Impact of hydrostatic pressure on cell. The hydrostatic pressure is a compressive force that is applied on the liquid in a closed space [8].

Hydrostatic pressure is responsible for inducing uniform stress throughout the cell's body. It causes no tissue deformation due to the incompressibility of water and the extracellular matrix. Despite that, hydrostatic pressure may influence the production of different components typical for specific tissue in the human body. In relationship to chondrocytes, hydrostatic pressure supports protein synthesis, gene expression, production of GAG (glucosaminoglycans), and collagen. The amount of impact of hydrostatic pressure on tissue depends on its frequency, magnitude, regimens, and application time [39].

4.4.2 Hydrostatic pressure and blood

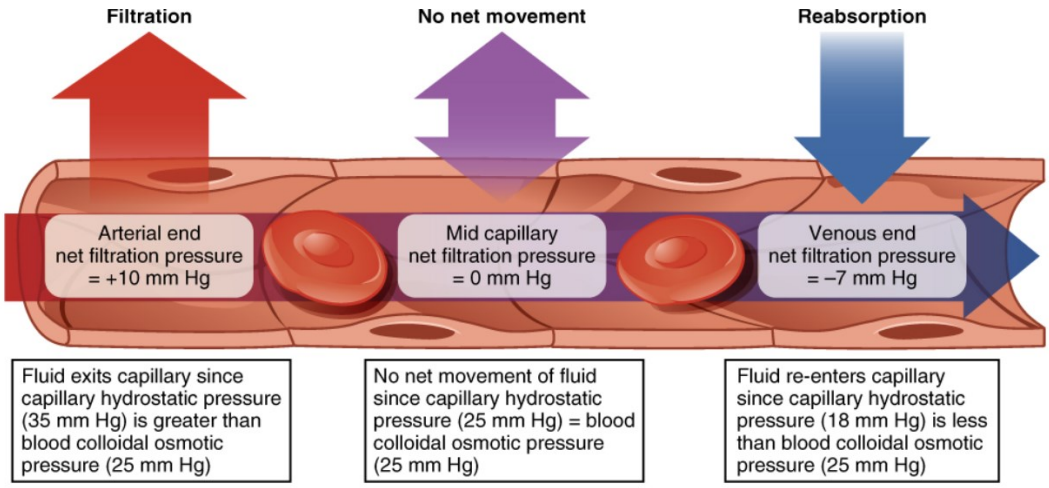


Figure 18 Capillary exchange and amount of present pressure. The net filtration occurs when the capillary hydrostatic pressure (CHP) is higher than blood colloidal osmotic pressure (BCOP). When the CHP and the BCOP equal, no net filtration appears. If the CHP is lower than BCOP, net reabsorption occurs [40].

As hydrostatic pressure may be explained as the force exerted on a fluid against a wall, which results in the movement of fluid between compartments. Thus it has a major impact on blood flow and the exchange of nutrients. The hydrostatic pressure of blood is the pressure exerted on blood against the walls of the blood vessels by the pumping action of the heart. In capillaries, hydrostatic pressure is higher than the opposing colloid osmotic pressure in blood at the arteriolar end of the capillary. This pressure forces plasma and nutrients out of the capillaries and into surrounding tissues. Fluid and the cellular wastes in the tissues enter the capillaries at the venule end, where the hydrostatic pressure is lower than the osmotic pressure in the vessel. Filtration pressure squeezes fluid from the plasma in the blood to the interstitial fluid surrounding the tissue cells [40].

4.5 Torsion

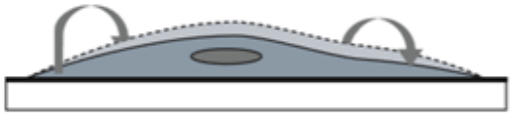


Figure 19 Impact of torsion on cell [8]

Torsion can be defined as twisting one end of material while the other is turned in the opposite direction. Twisted structures are highly represented in a human body, they may contain structures from DNA and RNA to microtubules, etc. Torsion forces take place in morphogenesis and cell differentiation [41].

4.6 Bending

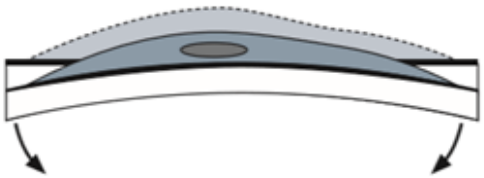


Figure 20 Bending of cell [8]

Bending is a combination of tension and compression stresses [32]. The main structures located in cells responsible for cell bending are microtubules (MTs). They are part of the eukaryotic-cell cytoskeleton. They connect the cell physically and biochemically to the external environment, generate coordinated forces that enable the cell to move, change shape, support movement of chromosomes during mitosis, and so on. Mechanical and chemical stimuli may trigger changes in the MTs network. For example, increasing tension on neurons promotes the assembly of MTs and a high local curvature would result in the breaking of MTs. Bending cells using dynamic forces may have a key role in exploring their biological functions because remodeling of MTs structure often launches disease processes [42], [43].

4.7 Electrical field

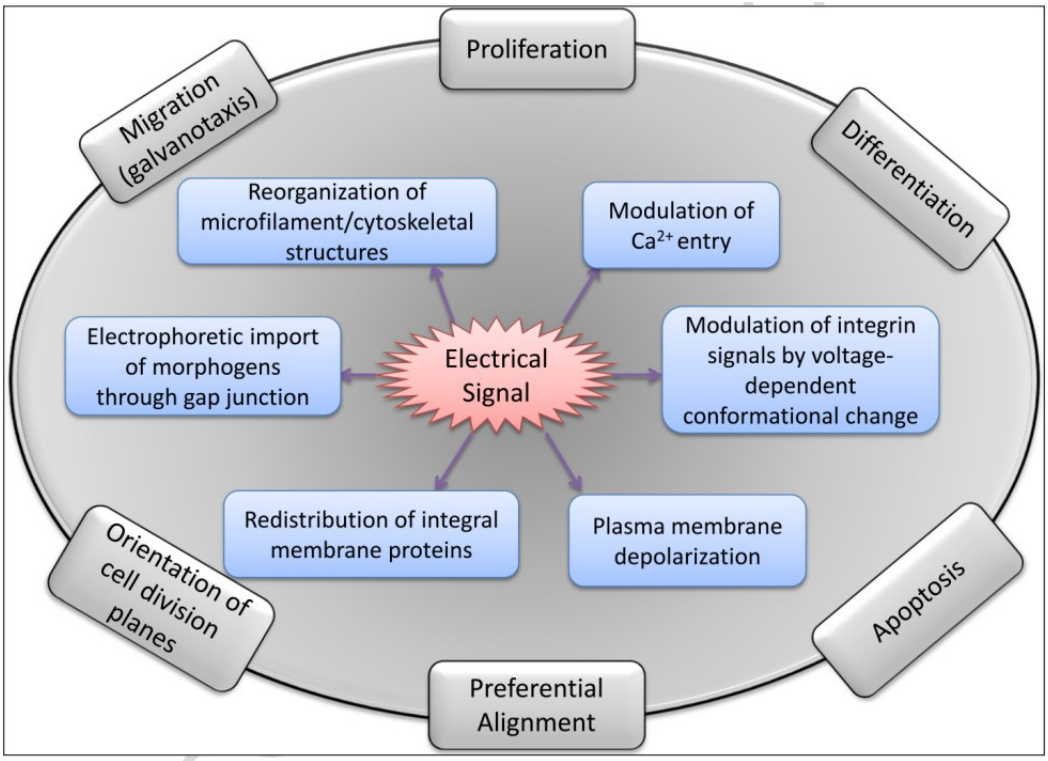


Figure 21 Bioelectrical control mechanisms at the cellular level. Electrical signals trigger various bioelectrical mechanisms which then influence cell life on the level of proliferation, differentiation, migration, and apoptosis [44].

4.7.1 Electrical field *in vivo*

Electrical field (EF) is another force, which normally occurs in a living organism and is missing in C^{ST}. It is important in the physiology and right function of the majority of all human tissues. The cells are the main source of electricity *in vivo*. Due to the constant pumping of ion channels in the cell membrane, the cells produce a voltage gradient across their membranes and the electrochemical gradient appears [45]. The electrochemical gradient is an important source of energy for cells used for transmitting information, the transport of various molecules, etc [46]. Other phenomenons in cells that are driven by electrical signals are represented in Figure 21. Electric signals are involved in migration or galvanotaxis by the reorganization of cytoskeletal structures in the cell. They are also responsible for the redistribution of integral membrane proteins. The redistribution of proteins precedes cell division, especially it causes the orientation of the cell division plane. Electrical signals are in charge of modulation of Ca^{2+} ions and in plasma membrane depolarization. They impact phenomenons such as proliferation, differentiation, or programmed death of cells, called apoptosis. The endogenously generated bioelectric current plays a crucial role in important biological processes such as normal growth

of organisms, embryogenesis, wound healing, tissue repair, tissue remodelation, etc. The endogenous electrical field exists in the cytoplasm as well as in the extracellular space and can vary from a few mV/mm to hundreds of mV/mm. The electrical field has been used in clinical settings to repair damaged tissue in the neuromuscular system or to accelerate the healing of injured musculoskeletal tissue [44].

4.7.2 Electrical field *in vitro*

The effect of electrical field on live cells is well known for a long time. However, the understanding of the mechanism behind cell-electrical field interaction is not fully clarified yet. The regulatory system of EF on various cell functions has been observed using *in vitro* techniques [47]. The electrical stimulation *in vitro* may be done through various systems. The most basic one is to apply an electrical field using DC voltage, simply generated by batteries. The more sophisticated systems apply EF through sinusoidal or square wave signals, pulse bursts, or using a continuous flow of electricity. These signals can be provided by simulator chips, signal generators, or an appropriate therapeutic system [45].

4.7.3 Using EF in tissue engineering and regenerative medicine

EF is considered as a useful tool in TE and regenerative medicine. Exogenously applied EF can be used to control cell behavior and tissue growth. Electric signals may be a guidance for cell migration during wound healing. It has been shown that keratinocytes and epithelial cells move toward the cathode if EF is about ~25mV/mm. The positive effect of EF on the cells is also observed during angiogenesis. Angiogenesis means a recovery of damaged capillaries. Applying EF induces producing vascular endothelial growth factor, stimulates cell migration, and cell elongation [48].

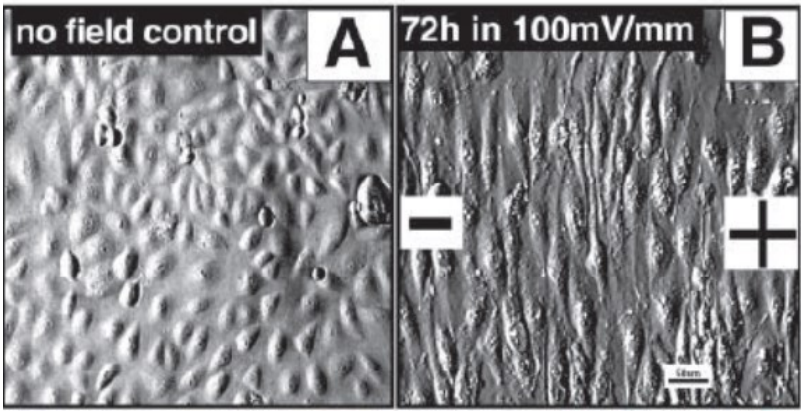


Figure 22 Differences in cell elongation between no field and application of EF for 3 days. a) Endothelial cells were not exposed to EF, b) cells after EF exposition for 72 hours, in 100 mV/mm, elongation and orientation of cells were observed in comparison to the control [48].

EF properties are also used to manipulate stem cells to optimize outcomes in regenerative medicine. The ultimate aim is to apply exogenous chemical and/or electrical stimuli to regulate stem cell function and behaviors. The EF can increase, decrease, and entirely block cell proliferation, differentiation, migration, alignment, and adherence to scaffold materials. The effect of EF depends on the type of SCs, the time for which EF was applied, on used voltage, and on the power of EF [49].

II. ANALYSIS

The practical part aims to design and fabricate the novel dynamic cultivation devices. These devices enable applying various external stimuli mimicking the *in vivo* conditions. Cells exposed to dynamic environments behave differently than under static cultivation conditions. The cell behaviour depends especially on cell lineage and a type of applied external stimuli (e.g. dynamic forces, shear stress, and electrical field). The practical part is based on work with the dynamic flow chamber and the creation of a simple Lab-on-chip device.

The design and fabrication of dynamic cultivation chambers and Lab-on-chip were realized thanks to the collaboration with colleagues on TBU, precisely with colleagues from the Department of Physics and Material Engineering and Department of Production Engineering. Due to their willingness and helpfulness, it was possible to create a dynamic chamber and microfluidic device and extend the knowledge about advanced cell cultivation.

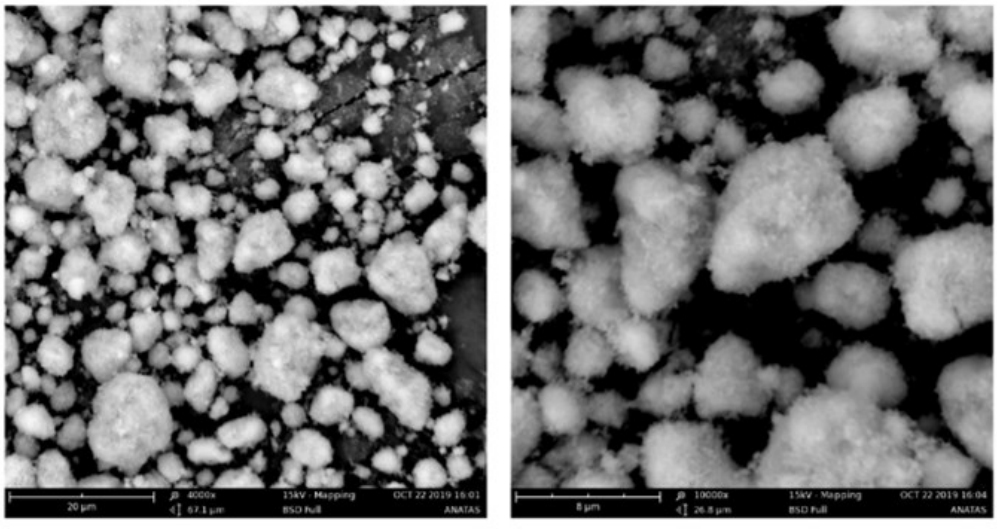
5 MATERIALS

5.1 Cell line

The mouse embryonic fibroblast cell line (NIH/3T3) was used. NIH/3T3 cell line is suitable for various biological testing. According to ISO standard, NIH/3T3 line is defined as a line used for determining cytotoxicity. The advantages of the NIH/3T3 cell line are rapid growth rate, easy accessibility, and undemanding cultivation [50].

5.2 TiO_2 nanoparticles

The TiO_2 nanoparticles were used to determine the cytotoxicity under different conditions (static/dynamic cultivation techniques). The TiO_2 nanoparticles (Cat No. 634662-25G) were purchased from Sigma Aldrich, Germany. A mixture of anatase and rutile was used. For the determination of the cytotoxicity in static conditions, the initial suspension of TiO_2 was at concentration 10000 µg/ml and then was diluted to required concentrations (5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, and 250 µg/ml). The concentration of nanoparticles used during dynamic testing was 3000 µg/ml. The nanoparticles were dispersed in a cultivation medium and sonicated for 30 minutes before each experiment.



a) b)

Figure 23 Characterisation of TiO_2 nanoparticles using SEM, a) magnification – 4000x, b) magnification 10000x (Figures were taken from Bachelor's thesis focused on physicochemical and biological properties of (nano)particles for cosmetic applications, done by Dita Moricová) [51].

5.3 Polydimethylsiloxane

For the fabrication of the LOC devices, PDMS was used. Different types of polymer materials may be used, but the majority of articles focused on the fabrication of LOC suggested PDMS. PDMS is suitable due to its biocompatibility, easy curing and polymerization process, transparency, and availability. The PDMS kit used in this thesis was purchased from Sigma Aldrich, and it was the Sylgard 184 clip-pack. The pack included non-polymerized PDMS and a curing agent already prepared in an adequate ratio.

5.4 Cultivation medium and other reagents

Cells were cultivated in Dulbecco's Modified Eagle Medium (Biosera), enriched with calf serum (Biosera) and penicillin-streptomycin (Biosera). For cell's passaging, phosphate-buffered saline (PBS, Biosera) and trypsin were used. The determination of cytotoxicity was done by using MTT (Duchefa biochemie) and DMSO (dimethylsulfoxide, Duchefa biochemie). For fluorescent microscopy, 4% formaldehyde (Penta chemicals), 10% Triton (Sigma Aldrich), PBS, and fluorescent colour (Hoechst, Sigma Aldrich) were used.

6 METHODS

6.1 Cell cultivation

NIH/3T3 cells were cultivated in the incubator at 37°C in 5% CO_2 in humidified air. When cell density was about $2x10^7$ cells in a 75 ml cultivation flask, they were ready to be used in testing. The cultivation medium was removed out from the flask, cells were washed with 15 ml of PBS, and 3.5 ml of trypsin was added to cells for maximum 15 minutes. Then, trypsin was inactivated by adding the aliquot volume of the medium. The mixture was put into a 15 ml test tube and centrifuged for 3 minutes at 37°C 1100 rpm. The medium with trypsin was carefully removed from the test tube, and 1 ml of pure medium was added. Suspension of cells was then diluted to the required concentration ($2x10^5$ cells/ml).

6.2 Determination of cytotoxicity

The cytotoxicity was determined by using the MTT assay according to ISO 10993-5. Before each testing, cells were seeded into a 96-well plate in the concentration of $2x10^5$ cells per ml of medium and were incubated for 24 hours. Simultaneously, the extracts of tested samples were prepared, according to the type of testing material. On the second day, the pure cultivation medium was replaced with extract (PDMS extract) or samples (TiO_2 suspension) in required concentrations, and cells were left in an incubator for another 24 hours. On the third day, the MTT assay followed. The solution of MTT and deionized water was prepared in a concentration of 5 mg of MTT per 1 ml of deionized water. The extracts or samples were replaced with 90 µl of fresh medium and 10 µl of MTT solution was added. The 96-well plates were returned to the incubator and left there for 4 hours. After 4 hours, the violet crystals of the formazan were diluted using DMSO. The measurement of absorbance followed. The used wavelength was 570 nm and 690 nm as the reference wavelength.

6.3 Determination of cell proliferation

The proliferation was determined using fluorescent microscopy. Cells were seeded on the surface of the sterile sample in concentration $2x10^5$/ml in total volume 200 µl of cell suspension. Cells were left to incubate for 24 hours. After 24 hours, cells were fixed. The first step of fixation was to add 200 µl of 0.5% formaldehyde for 15 minutes. Then, formaldehyde was removed and cells were washed with PBS. 10% solution of Triton

was then added and left with cells for the other 5 minutes. After 5 minutes, Triton was removed and cells were washed three times with PBS. Then 200 µl of pure PBS was added and 2.5 µl of Hoechst (stains cell's nuclei) stain was added. The cells on the sample and reference were left for 30 minutes in the dark. After 30 minutes, PBS with stains was removed and replaced by pure PBS. The cell compartments were observed under a fluorescent microscope.

6.4 Conditions used for dynamic cell cultivation

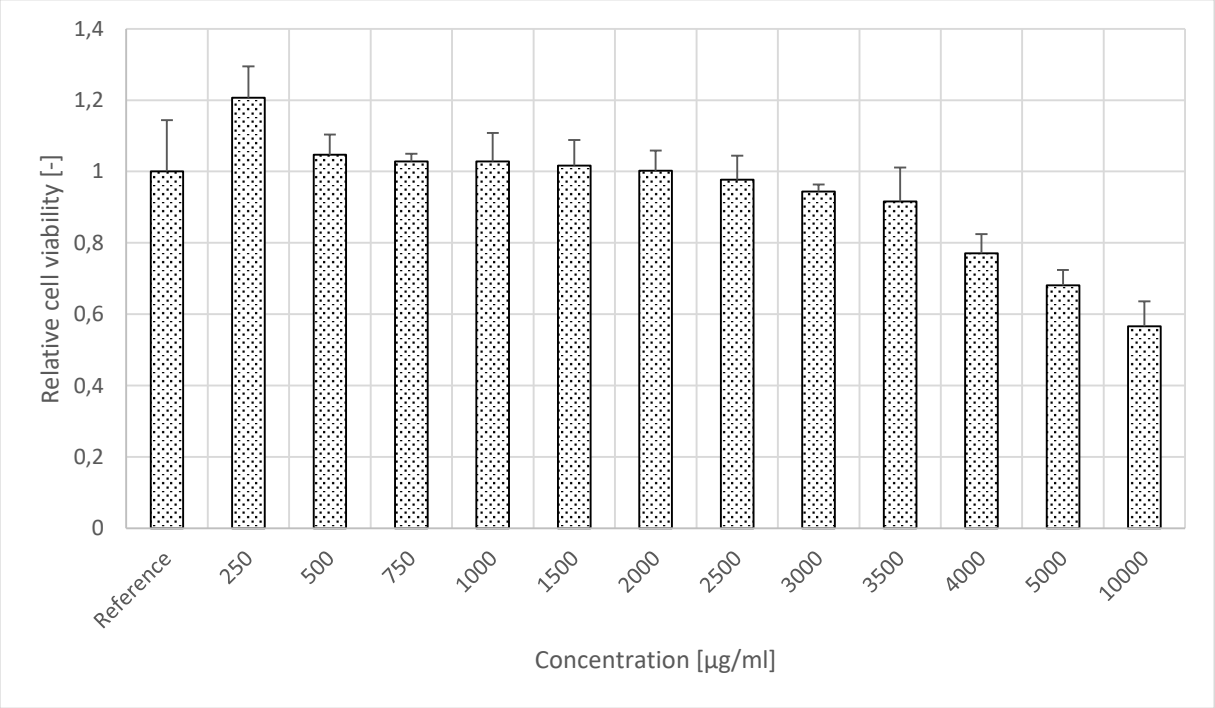
The cells during dynamic cultivation were exposed to the flow at 4200 µl/min using the first type of pump. The flow was adjusted after replacing the pump. The adequate flow was then considered to be 1800 µl/min. The concentration of nanoparticles was 3000 µg/ml and the total volume of the used suspension was 100 ml. The duration of the experiment was 24 hours. The cells were cultivated at 37°C in 5% CO_2 in humidified air. The conditions of testing were changed few times to achieve appropriate results.

7 RESULTS

7.1 Cytotoxicity of TiO_2 nanoparticles under static conditions

Cytotoxicity of TiO_2 nanoparticles was first determined using the static cultivation technique evaluated with the MTT method. The static method was used to determine the first nontoxic concentration of nanoparticles. Then, this concentration was used in dynamic cultivation. The prediction was, that the nontoxic concentration of nanoparticles will be toxic for them in dynamic conditions, due to the shear stress induced by the flow of medium with nanoparticles.

The initial suspension has a concentration of 10000 µg nanoparticles per ml of medium. After its sonification, it was diluted to the required concentrations: 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500 and, 250 µg/ml. Suspensions were added to the cells, which were prepared the day before. On the third day, MTT assay was applied as described previously. After the addition of DMSO, the supernatant from each well was carefully removed to the new 96 well-plate to avoid misinterpretation of results due to the presence of sedimented nanoparticles. The measurement of absorbance followed. The results are shown in Graph 1 below.



Graph 1 Results of cytotoxicity of TiO_2 nanoparticles under static cultivation conditions.

The results were evaluated according to ISO 10993-5 Tests for *in vitro* cytotoxicity. In ISO standard, the relative cell viability less than 0.7 indicates the cytotoxic effect. The cell viability in the presence of concentrations 10000 and 5000 μg of nanoparticles per 1 ml was lower than 0.7. Therefore, these two concentrations were evaluated as toxic. The viability at 4000 μg/ml concentration was slightly higher than 0.7, so it was considered to be nontoxic. However, to ensure that nanoparticles do not induce cytotoxicity, the concentration 4000 μg/ml was considered as non safe, as well as the following concentration (3500 μg/ml). The concentration of 3000 μg/ml nanoparticles was then used for dynamic cultivation.

7.2 Implementation of the dynamic chamber into experiments

The flow chamber was made by colleagues from the Department of Physics and Materials Engineering of FT TBU using 3D print according to our proposal. The challenge was to make a chamber that possesses various properties that make the chamber useful in biological testing. These properties include:

- biocompatibility of the material
- transparency
- removable inserts (cells can be observed during testing)
- simple sterilization
- reusability

Figure 24 The dynamic flow chamber. The device was made by colleagues at TBU using the 3D print method.

The final design is shown in Figure 24. The chamber contained two removable parts, which were put in the one-piece base, one input and output for connection the chamber to oxygenator and medium reservoir. To avoid leakage of the fluid, a simple seal was added to removable parts and then they were secured to the chamber by bolts and nuts. The chamber was then connected to the reservoir bottle, oxygenator, and pump by tubes. The assembled system is presented in Figure 25 below.

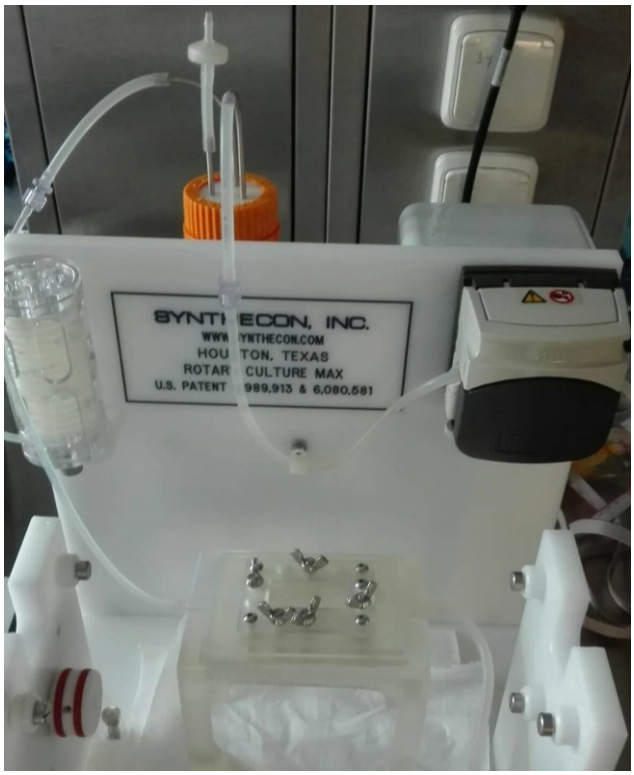


Figure 25 The dynamic flow chamber. The chamber is assambled together with pump, reservoir bottle and oxygenator.

Before the experiment, both removable parts and the whole chamber was sterilized using 70% ethanol for a few minutes. They were left in the flow box overnight to dry completely. Tubes with oxygenator and bottle for medium were sterilized using an autoclave.

After all parts of the chamber were sterilized, cells were seeded onto the surface of the bottom removable part covered with 0.1% gelatine solution. The surface modification was done to ensure the proper attachment of cells. The cell suspension was at a concentration of $2x10^5$ and 500 µl of suspension was poured onto the bottom insert. Cells were cultivated for 24 hours in the incubator under normal static conditions.

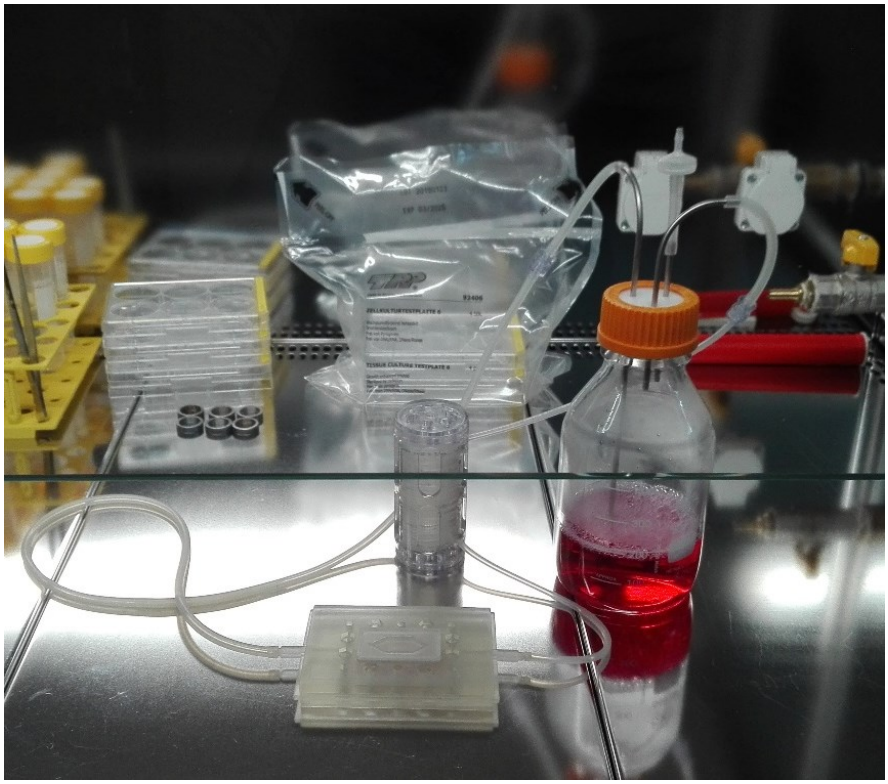


Figure 26 The dynamic flow chamber before placing it into the incubator. The dynamic chamber already contained an insert with seeded cells, attached to an oxygenator and reservoir bottle with cultivation medium.

After 24 hours of cell cultivation, the chamber was assembled. The bottom insert with cells was carefully attached to the base with sealing as well as the upper insert. Everything was secured with bolts and nuts and attached to the reservoir bottle and oxygenator in the flow box. The last step was to connect the chamber to the pump, which was placed into the incubator. The pump was switched on and the dynamic chamber was left in the incubator overnight. The next day, cells should be fixed, stained, and observed under fluorescent microscopy. However, it was not possible to observe cells neither with light microscopy, because the material of the inserts reflected light (Figure 27).

Figure 27 The dynamic flow chamber on sunlight. The material from which was chamber made reflected the sunlight and was not suitable to observe directly under the microscope.

This was solved by using insert cut from tissue plastic. It was put on the bottom removable part of the chamber. The rest of the testing was done in the same way as in the first attempt. After 24 hours of dynamic cultivation, insert from tissue plastic was removed from the chamber, and cells were stained using fluorescent dyes. The fluorescent microscopy followed, but no cells were presented on the surface. The experiment was repeated with a prolonged time of cell adhesion, but no change occurred. The flow was too high (4200 µl/min) and it washed cells completely from the surface of the insert. The optimization of the layout of this experiment followed.

7.3 Optimization of conditions for using the chamber

Figure 28 New pump. To solve the problem with the high flow of the medium, a new pump was purchased and implemented into experiments.

As the original set-up did not work properly, adjustments followed to optimize the conditions. The first step was to replace the old pump with a newer model. To evade the reflection of light and to enable observing cells under light and fluorescent microscope, the insert from tissue plastic was cut and put onto the bottom removable part of the chamber. The insert was also covered using a 0.1% solution of gelatine to enhance the adhesion of cells on the surface. The new pump also enabled an easier and more precise setting of the flow. A series of measurements was done to find the optimal flow, which did not remove cells from the surface of cut inserts. The optimal flow was considered to be 1800 µl/min.

7.4 Cytotoxicity of TiO_2 nanoparticles under dynamic conditions

7.4.1 Initial experiments

After optimization of the testing conditions, the effect of TiO_2 nanoparticles on cells in the dynamic chamber was tested. Two sterile inserts from tissue plastic (one as a reference, the second was placed into the chamber) were coated with 0,1% solution of gelatine. Gelatine was left to cure for another 30 minutes. The residues of gelatine were removed and cells were seeded in the concentration of $2x10^5$ cells per 1 ml on the surface of inserts. The inserts with 200 µl of cell suspension were placed into the incubator and left

there until the next day. The next day, the mixture of TiO_2 (anatase and rutile) and the medium was prepared in concentration 3000 µg/ml. The final volume of suspension was 100 ml. The suspension was sonicated for 30 minutes before using, in the same way as it was done in the testing of cytotoxicity using static cultivation.

Figure 29 The assambly of the system including with medium and TiO_2 suspension.

The TiO_2 suspension was placed in a sterile glass bottle, connected to the chamber and pump and the whole system was put into the incubator. The flow was set to 1800 µl/min and the pump was switched on. After 24 hours, the pump was switched off, the chamber was disconnected from the reservoir bottle. The insert from plastic was carefully removed from the chamber and placed into a Petri's dish and was compared with the reference.

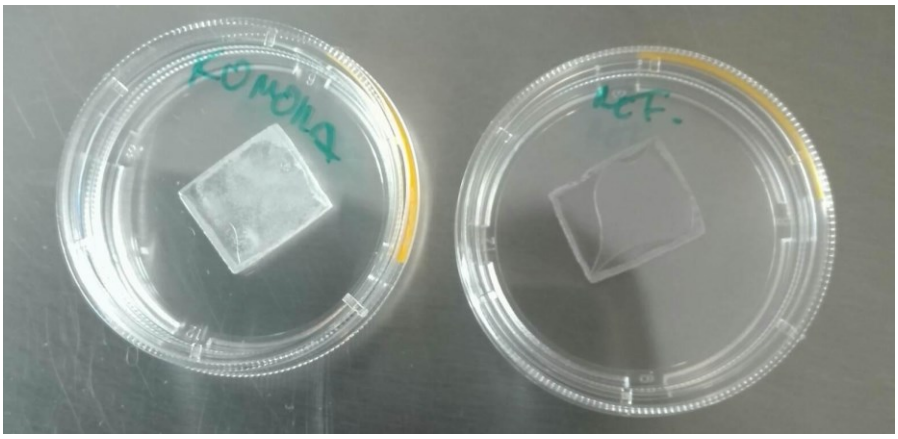


Figure 30 Insert from flow chamber (Petri's dish on the left) vs reference (Petri's dish on the right)

The experiment has shown that after 24 hours of suspension flowing, the nanoparticles sedimented and covered the surface of the plastic insert completely. There was an attempt to wash the insert very carefully with PBS to not wash out the cells. However, the attempt was not sufficient, because the layer of nanoparticles was too robust. Therefore, it was not possible to remove it just with a very gentle rinse. The inserts were then observed using light microscopy to find out if there is a presence of cells before fluorescent microscopy.

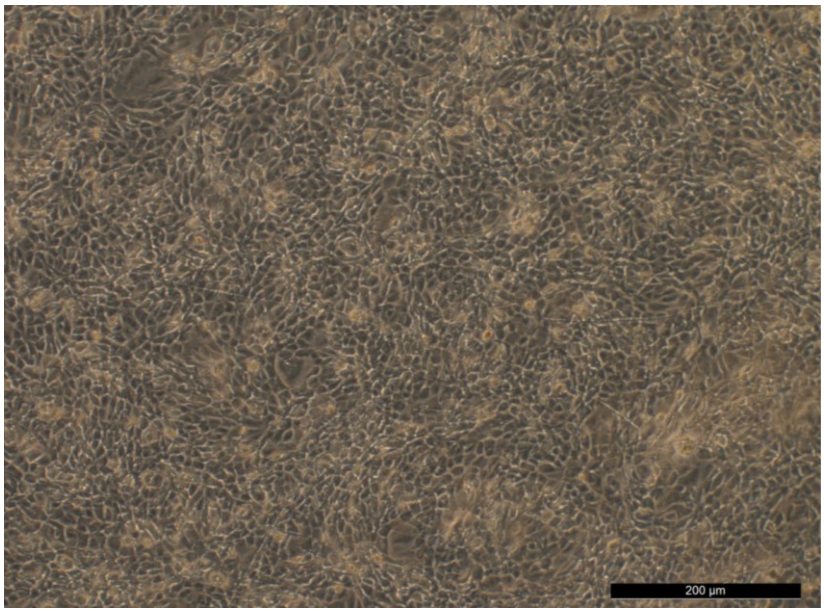


Figure 31 Reference. The cells grown on the reference (tissue plastic) under static cultivation conditions after 24 hours.

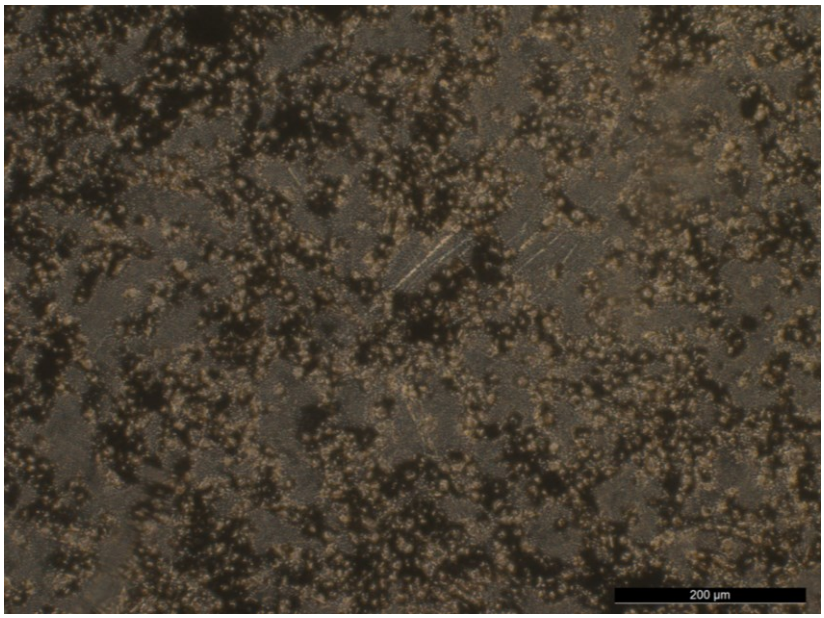


Figure 32 The surface of the plastic insert is covered with a layer of TiO_2 nanoparticles. Plastic insert removed from the dynamic chamber after 24 hours of incubation.

After using light microscopy, no cells were observedto under a layer of nanoparticles exposed to flow at the same time. Sets of adjusting followed to try to avoid the nanoparticles sedimentation.

7.4.2 Adjusting the experimental set-up to avoid nanoparticles sedimentation

Figure 33 Reservoir bottle on an orbital shaker in the incubator. To prevent the sedimentation of nanoparticles, a reservoir bottle with suspension was put on to orbital shaker in the incubator.

Due to the fact, that no cells were observed, the layout of the experiment was changed slightly. The flow was increased at 2400 µl per minute. The reservoir bottle and chamber were placed on an orbital shaker, to prevent sedimentation of nanoparticles. The rest of the experimental layout (the type of nanoparticles, their concentration, preparation of cells…) was the same as in the first try. The experiment ran another 24 hours, with a gentle shake. After the ending of the experiment, the system was deconstructed, and the insert was observed again.

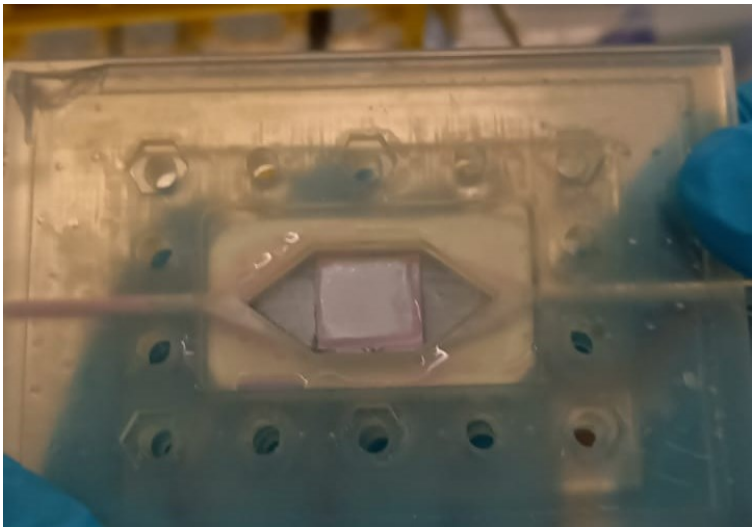


Figure 34 Insert after incubation on orbital shaker. The dynamic flow chamber with the plastic insert was covered with TiO_2 nanoparticles after the incubation with gentle shake.

As can be seen from Figure 34, the nanoparticles sedimented again despite the shaking and higher flow. The reason might be a combination of the high weight of nanoparticles and insufficient flow rate. The flow rate was not high enough to keep nanoparticles well distributed in the whole volume of the medium.

The third try was to remove the reservoir bottle and create a closed system, where the whole volume of liquid is flowing through the chamber. The scheme is shown in the Figure 35 below.

Figure 35 Closed system. To avoid sedimentation, the reservoir bottle was removed from the scheme, so the full volume of liquid was moving.

The concentration of nanoparticles was decreased to 20 µl/ml. The lack of nanoparticles was meant to be solved with a longer time of incubation. This layout was tried without cells first. The system was left overnight in the laboratory. The flow was set to 2400 µl per minute and ran for 24 hours, as the previous tries.

Figure 36 The removable part of the chamber with residues of nanoparticles after dynamic cultivation.

Despite the low concentration and closing of the whole system, the nanoparticles sedimented again. As can be seen from Figure 36.

The sedimentation occurred, probably because of the size and aglomeration of nanoparticles and low flow rate. There is a limitation that did not allow to increase the flow rate too much. From the set of tests which preceded the actual testing of nanoparticles, it resulted that cells were washed away from the surface when the flow was 3000 µl/min and higher. These experiments were however not provided due to the Covid-19 related restrictions.

The implementation of the flow chamber into biological testing was successful. Despite the fact that, this setting was not suitable for testing cytotoxicity of nanoparticles, it can be very useful for testing structured surfaces with SCs under shear stress. Nevertheless, the focus should be put on improving evaluation of results in further testing. The results from the flow chamber were compared to the reference grown on plastic tissue

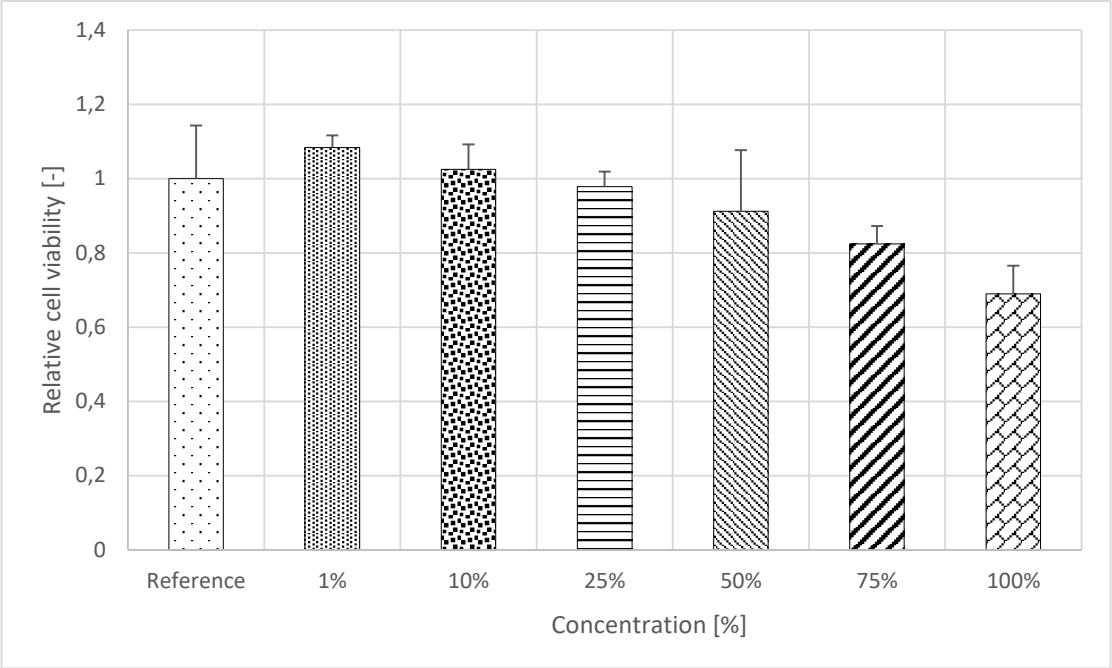
under standard cultivation conditions. This can lead to misinterpretation of results. With such a setting, it is hard to distinguish if the changes in cell structure are made due to the presence of nanoparticles, structured surface, or due to presence of shear stress, induced by the flow of the medium. The ideal setting would be represented using two parallel chambers. One supplemented with testing substance or surface, the second just with cells and pure medium. Then, with the reference grown on tissue plastic under static cultivation, it will be possible to distinguish between the effect of shear stress and tested substances or structured surfaces.

7.5 Determination of PDMS cytocompatibility

As the PDMS was used to fabricate LOC, the cytotoxicity of PDMS extract was studied to ensure, that cells can grow onto its surface and that the cured PDMS does not release any toxic compounds. It was also important to ensure, that the final product is transparent and does not interfere with light, so it can be observed easily by microscope.

7.5.1 Cytotoxicity of PDMS extract using MTT

The extract from polymerized PDMS was made in the concentration of 0.02 g of material per 1 ml of medium. The PDMS was cut into smaller pieces and put into a plastic tube. The medium was added and the tube was left in the Incucell at 37 °C for 24 hours while gentle shaking. On the same day, cells were seeded into 96-well plates at a concentration of $2x10^5$ cells in ml. After 24 hours, the extract was filtered and cells were grown enough to be used in testing. The medium in wells was replaced with extract, using six different concentrations: 100%, 75%, 50%, 25%, 10% and 1% extract. The plates were put into an incubator for another 24 hours. On the third day, the cytotoxicity was measured using the MTT method described earlier. The results can be seen in Graph 2 below.



Graph 2 Cytotoxicity of PDMS extracts

The results were evaluated using the same ISO 10993-5 standard as the cytotoxicity of TiO_2 nanoparticles. The cell viability at the presence of 100% extract was 0.69 that according to ISO standard, determines cytotoxicity. However, the cytotoxic effect was so negligible that the PDMS was considered suitable for future testing. The following concentrations were nontoxic. Since the PDMS was supposed to create the LOC, the most substantial factor was cell growth on the PDMS surface. Thus, the determination of proliferation of cells on the surface of PDMS followed.

7.5.2 Proliferation and adhesion of cells on the PDMS surface

The proliferation was determined by using fluorescent microscopy, observing cell compartments dyed with fluorescence stains. Cells were seeded on a sterile surface of polymerized PDMS. After 24 hours of incubation, they were fixed, stained, and observed under a fluorescent microscope. Results were compared to the cells seeded onto the surface of tissue plastic.

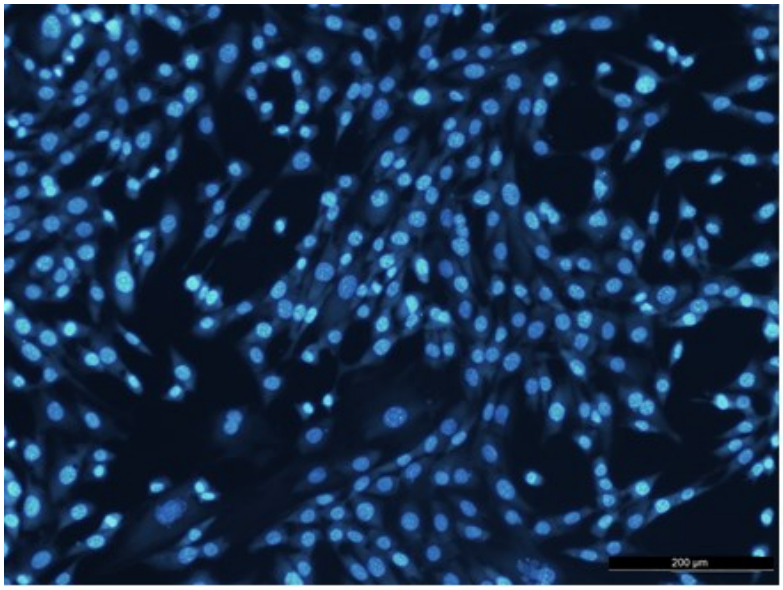


Figure 37 Reference. Nuclei (DNA counterstained by Hoechst) of the NIH/3T3 cells were used as reference.

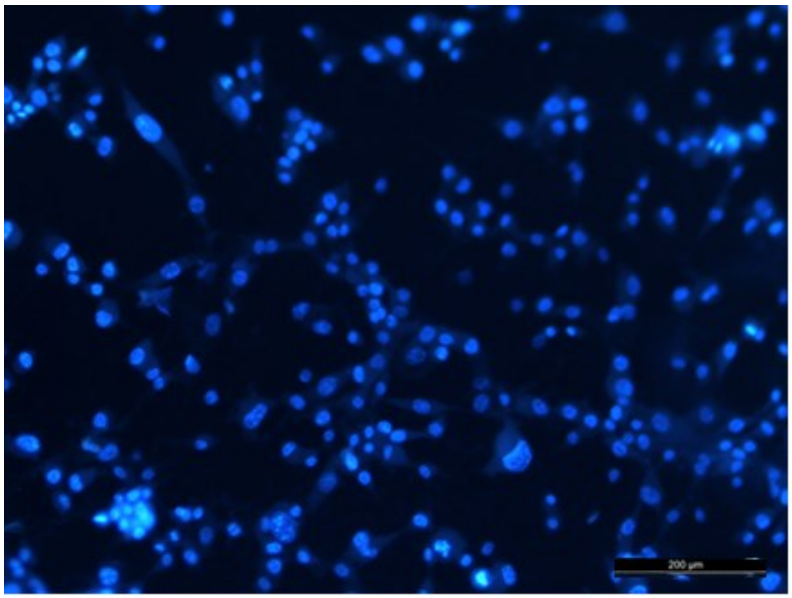


Figure 38 Nuclei (DNA counterstained by Hoechst) of the NIH/3T3 cells cultivated on the surface of the LOC made of PDMS.

As can be seen in Figure 38, cells were able to adhere, grow and divide on the surface of PDMS. It did not interfere with fluorescent light and it was possible to focus on cells easily. In comparison to the reference, there are fewer cells on the surface of PDMS. This problem can be solved with a coating of the surface of PDMS with gelatine to make the surface more likeable for cells. The stress filaments were seen poorly, but this problem can be improved by gelatine as well.

7.6 Design of LOC

The second part of the thesis was focused on the creation of a simple Lab-on-chip. The benefits of LOC are described earlier in this thesis. The process began with an extensive study of many scientific articles pursued on the LOC theme. Two articles [52]; [53] were chosen, both using the electrical field as a dynamic force. The last design is a simple LOC observing the effect of studied substances impacting cells.

7.6.1 Reusable LOC with implemented electrodes

The first article used as an inspiration for LOC design was 3D-Printed reusable cell culture chamber with integrated electrodes [52]. The article aimed to observe the effects of electric field on cells generally. Based on this article, the form for PDMS with incorporated electrodes was developed. It was a square-shaped form with space for two electrodes located on the opposite sides of the form. The areas for electrodes were 1 mm high and 15 mm wide with space in the middle of the LOC for cell growth.

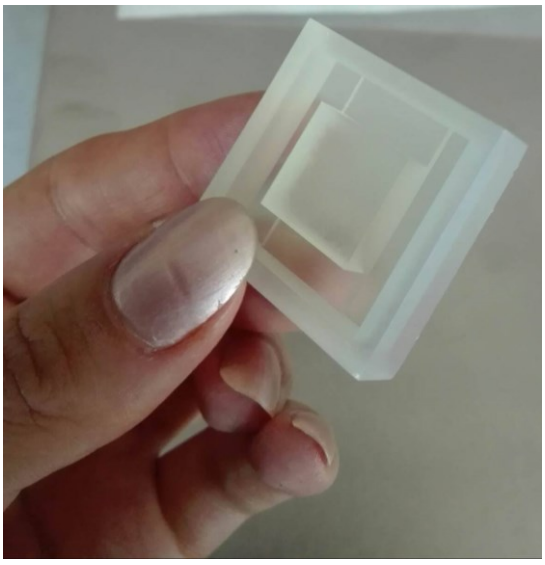


Figure 39 Form for LOC. The first design for LOC was made by 3D print with space for two electrodes and a middle part for cell cultivation.

The PDSM was used to cast the LOC. About 3 ml of PDSM mixture was poured into the form, which was earlier treated with the solution of detergent and water to prevent the sticking of PDMS to the surface of the form. The PDMS was then left to solidify at room temperature for three days. After three days, solidified PDMS was carefully removed from the form and placed on a microscopic slide.

The electrodes were then added to the device. The remaining liquid PDSM was used as glue to attach electrodes to the device and then the whole device was put on the microscopic slide. The next step was to seed cells in the middle part of devices and implementing the electrical field.

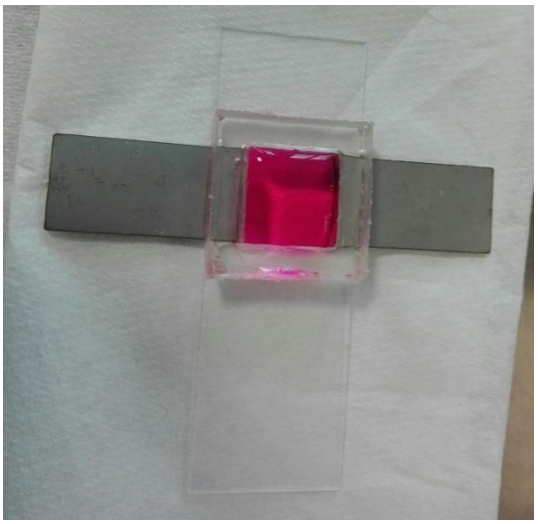


Figure 40 Fabricated LOC with attached electrodes and cells seeded in the middle part of LOC.

The LOC was sterilized using 70% ethanol before seeding of cells. It was left to dry completely in the flow box. Cells were seeded in the middle part of sterilized LOC. One ml of cell suspension at concentration $2x10^5$ was poured in the middle part of the device and the whole system was put into the incubator at 37°C in 5% CO_2 in humidified air for 24 hours. After 24 hours, the system was checked. Despite that a small part of the medium flowed out of the system and residues of PDSM, cells were able to grow.
The area for cell growth was washed properly because of the resence of PDMS residues. Rubber bands were added to provide perfect adhesion between the device with electrodes and microscopic slide. At this point, the experiment was ended because of time deficiency and restrictions related to Covid-19.

7.6.2 Design of LOC for studying effects of EF on neurogenesis of stem cells

The second article observed the impact of electricity on stem cell differentiation in neuronal tissue [53]. The design of LOC was modified to better fit the possibilities of production and to simplify its manipulation. The model of LOC is shown in Figure 41 below.

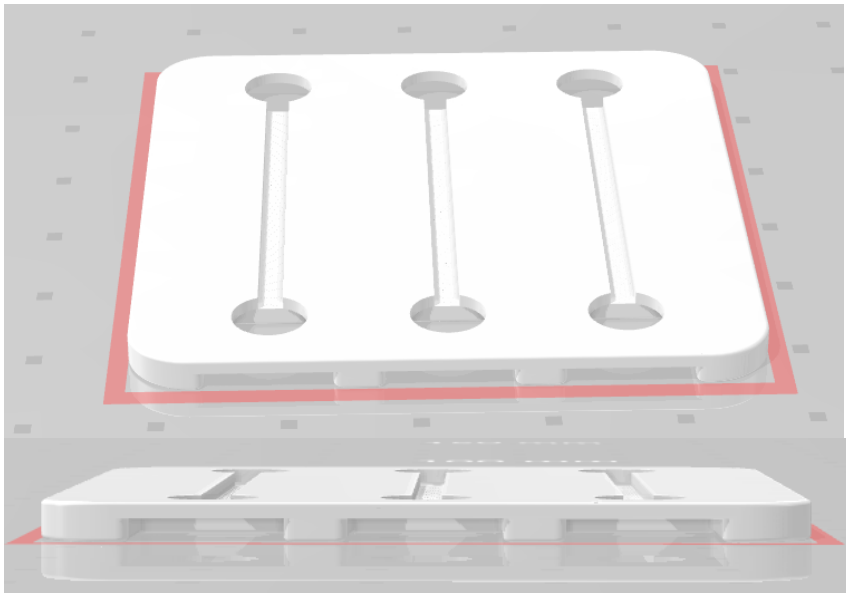


Figure 41 Desing of the second LOC. This Figure illustrates the design of LOC with three implemented electrodes for observing the effect of EF on neuronal differentiation of stem cells.

The LOC contains 3 parallel channels, 35 mm in length, ended on both sides with an area for electrodes. These areas are circular and have 5 mm in diameter. From the bottom of LOC, inputs for electrodes are seen, and they are 1 mm high and 10x10 mm wide. The channels for cell growth are 2 mm wide and 1 mm high. The surface of each channel contains grooves to enhance cell attachment. The prototype of LOC (Figure 42) was 3D printed, however, the material did not possess the required properties for using it in further testing.

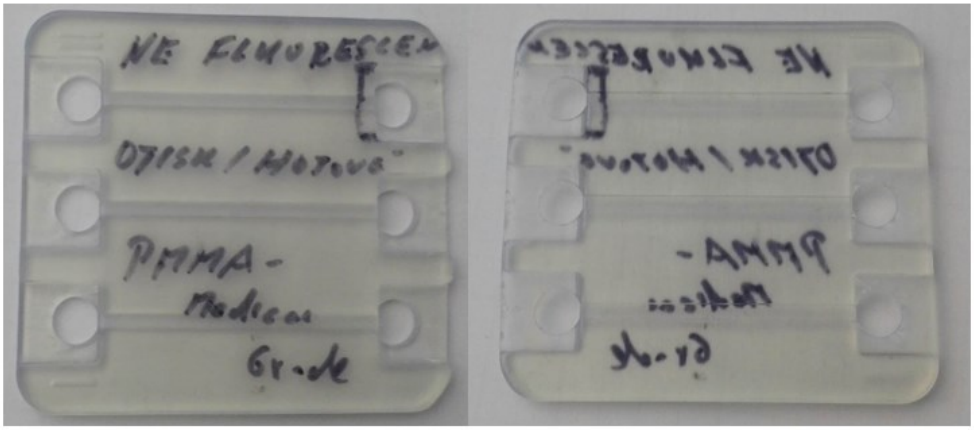


Figure 42 3D printed prototype of LOC. The prototype of the second design of LOC fabricated using a 3D.

The problem with the material was solved by using PDMS as well. Thus, it was needed to create a mold, from which LOC could be molded. The mold was 3D printed by colleagues at the Faculty of Technology. The mixture of PDMS and curing agent was poured into the mold, secured by two plastic clips to ensure that PDMS would not flow out of the mold. The PDMS was left to set up for a week at room temperature before the mold was opened.

Figure 43 Mold with PDSM secured with clips to protect the PDMS from leaking out.

Even though the producer of PDMS clips declares that the polymer should be cured under room temperature, PDMS locked in the mold was not solidified in a whole week. The higher temperature may solve this problem, however, there was a possibility that the plastic clips would not handle the high temperature in an oven. Therefore, the third type of mold was made from stainless steel by colleagues from the Department of Production Engineering using a different design.

7.6.3 Design of LOC for testing cytotoxicity of different chemicals simultaneously

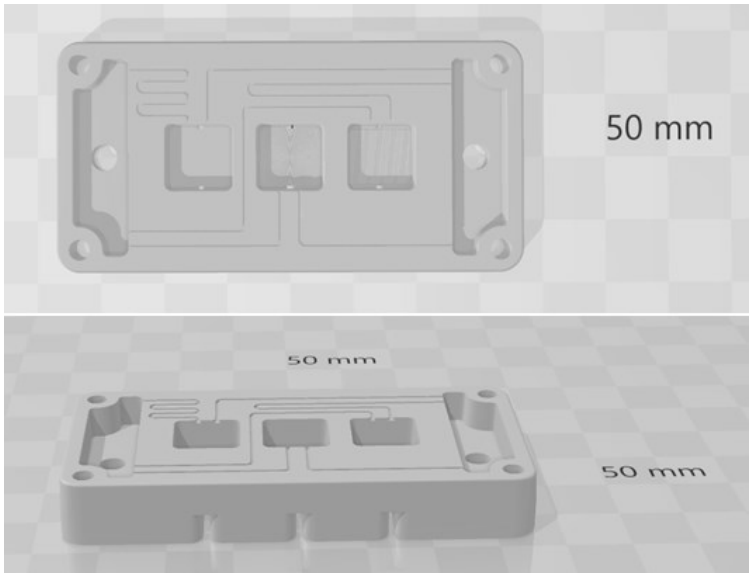


Figure 44 Design of LOC for cytotoxicity testing of several chemicals at the same time.

The third LOC was made according to own design for testing cytotoxicity of more substances simultaneously. The substance should diffuse from two reservoirs placed on the sides of LOC. Reservoirs are connected using channels with three chambers where cells are seeded. The two of the chamber have structured surfaces. The middle chamber is supplemented by grooves in concentric circles. In the lateral chamber, the grooves are linear.

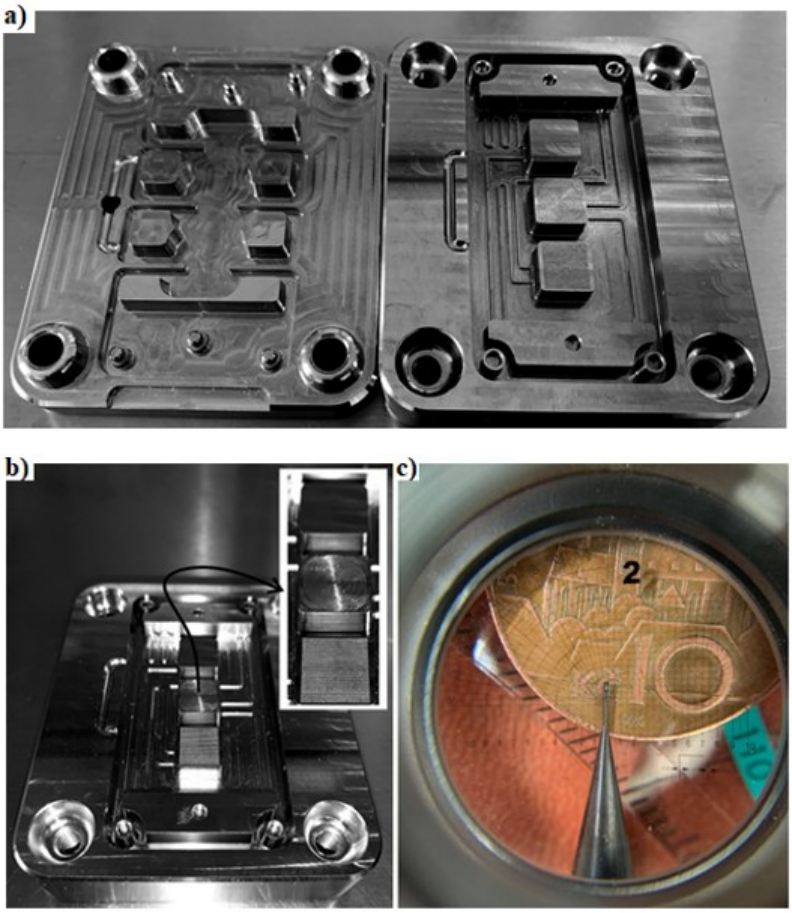


Figure 45 The stainless steel mold for LOC a) upper and lower part, b) details of grooves, c) cutter (Figures were taken by Ing. Jakub Huba, Ph.D.)

The mold was made from stainless steel using milling cutters (Figure 45c) with different sizes. When the mold was made, it was secured with bolts, and injection of PDMS followed. The system was put into the oven, which was preheated to 80°C for 3 hours. The mold was left to cool down in the oven until the next day. The next day, bolts were carefully taken out and the lid was removed. The PDMS was not polymerized properly, so for the second try, mold with PDMS was left in the oven at 100°C, for 45 minutes and left in the oven overnight. The final LOC (Figure 46) missed the bottom of the lower part due to the lack of material and possessed bubbles, which formed during polymerization. However, these imperfections might be easily improved by using a sufficiet amount of PDMS and using a vacuum evaporator to avoid bubbles. The experiments ended here because of the lack of PDMS and time deficiency related to Covid-19 restrictions.

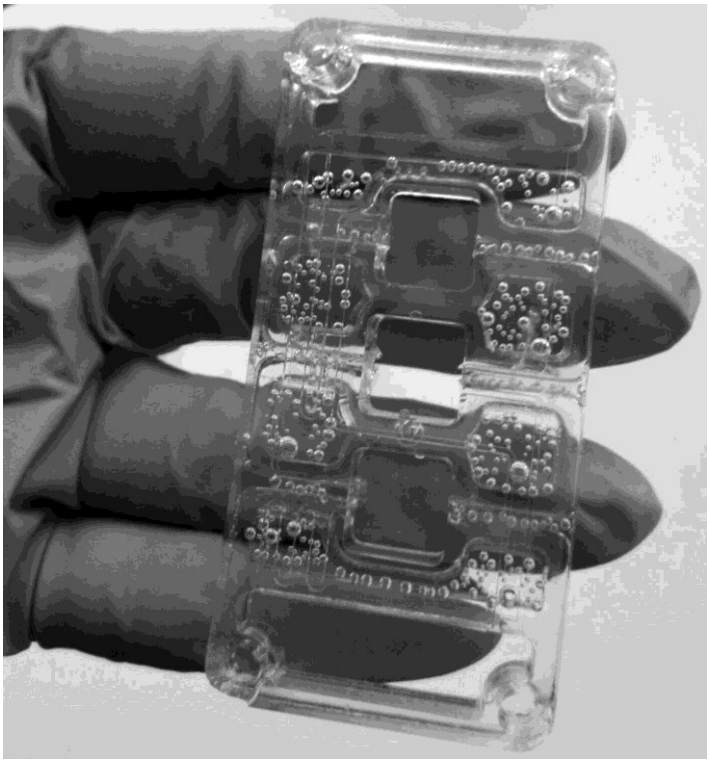


Figure 46 LOC made from PDMS. LOC casted using PDMS, carefully peeled off from the stainless steel mold.

7.7 Future use of LOC

Two types of LOC were successfully designed. The reusable LOC with implemented electrodes can be used in the following testing, connecting to the source of EF, and the impact of EF on different kinds of cells (cardiac cells, muscle cells, or neurons) can be observed. Successfully fabricated was also the third type of LOC, made from PDMS using the stainless steel form. The last step is to avoid the bubbles and to fill the mold enough with PDMS, so the bottom layer will occur. The LOC for studying neurogenesis needs more work before it can be used. There are two ways how to improve LOC. The first is to use a different kind of material for the 3D print, which does not reflect light and thus could be observed under a microscope. Secondly, the mold could be made again but using stainless steel or other heatproof material which can be closed properly and the plastic clips will not be necessary.

CONCLUSION

The theoretical part aimed to define and understand the differences between static and dynamic cell cultivation. The intention was to define possible ways of introducing dynamic conditions into cell cultivation. The possibilities are extensive and vary from the purpose of the testing. Bioreactors are one of the most extended facilities used to provide dynamic conditions. Different bioreactors use a different type of dynamic force and cell lines. The microfluidic devices also called Labs-on-chip are another option for providing dynamic cultivation conditions. Results taken from LOC systems are more accurate. Their use also decreases the number of used chemicals for testing as well as the final waste. Last but not least chapter of the theoretical part was focused on exploring and understanding how cells in the human body are faced with dynamic forces everyday, and how these forces affect their function, growth, division, and differentiation.

The practical part was focused on implementing some of the dynamic cultivation techniques in biological testing. Firstly, the flow chamber was optimized for laboratory use. As the conditions were improved, the effect of TiO_2 nanoparticles on cells was tested. Despite that the flow chamber worked well, there was a problem with the sedimentation of nanoparticles. There is also room for improvement in case of evaluation the results. However, the chamber can be used to observe the effect of structured surfaces and shear stress on cell growth.

The second part of the practical section was focused on the design of the LOC. Two designs worked well and now they can be used in experiments, just with slight adjustment during the casting process. The third design needs more work to accomplish its proper fabrication.

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LIST OF ABBREVIATIONS

BCOP – Blood colloidal hydrostatic pressure

C^{DYN} – Dynamic cultivation

C^{ST} – Static cultivation

CHP – Capillary hydrostatic pressure

CO_2 – Carbon dioxide

DC – Direct current

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

ECM – Extracellular matrix

EF – Electric field

ESC – Embryonal stem cells

GAG – Glucosaminoglycans

kPa – kilopascal

LOC – Lab-on-chip

MTs – Microtubules

MTT – 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide

NIH/3T3 – Mouse embryonic fibroblast cell line

PBS – Phosphate buffered saline

PDMS – Polydimethylsiloxane

PGA – Polyglycolic acid

PHB – Polyhydroxybutyrate

PLA – Polylactic acid

PS – Polystyrene

SCs – Stem cells

SEM – Scanning electron microscope

TBU – Thomas Bata University

TE – Tissue engineering

TiO_2 – Titanium dioxide

UV – Ultra violet light

LIST OF FIGURES

LIST OF GRAPHS