CZECH TECHNICAL UNIVERSITY IN PRAGUE

FACULTY OF BIOMEDICAL ENGINEERING

Department of Biomedical Technology

3D bioprinted Collagen I with incorporated stem cells

Bachelor Thesis

Study program: Biomedical and Clinical Technology

Study branch: Biomedical Technician

Bachelor thesis supervisor: Ing. Roman Matějka

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BACHELOR'S THESIS ASSIGNMENT

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II. BACHELOR'S THESIS DETAILS

Bachelor's thesis title in English:

3D bioprinted collagen I with incorporated stem cells

Bachelor's thesis title in Czech:

3D tisknutý kolagen I se zahrnutými kmenovými buňkami

Guidelines:

Validate various concentration of collagen I (porcine or bovine) solution and its ability to gelating with adipose derived stem cells. Test these solutions in syringe dispensers Cellink Inkredible+ bioprinter with various nozzles. Prepare 3D printed cylinder and ring shape from prepared gel with cells and compare it to the molded structure.

Bibliography / sources:

[1] Lanza, R., Langer, R., Vacanti, J., Principles of Tissue Engineering, ed. 3rd Edition , Elsevier Academic Press, 2007, ISBN 978-0123706157
[2] Lucie Bacakova, Martina Travnickova, Elena Filova, Roman Matejka, Jana Stepanovska, Jana Musilkova, Jana Zarubova and Martin Molitor, Muscle Cell and Tissue - Current Status of Research Field, ed. 1, kapitola Vascular Smooth Muscle Cells (VSMCs) in Blood Vessel Tissue Engineering: The Use of Differentiated Cells or Stem Cells as VSMC Precursors, 2018, IntechOpen
[3] Joseph D Bronzino, The Biomedical Engineering Handbook,, ed. I. ed, Boca Raton : CRC Press, c2000. , 2000, ISBN 0-8493-0461-X

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Assignment valid until: **19.09.2021**

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Dean's signature

DECLARATION

I hereby declare, that I have completed this thesis having the topic “3D bioprinted collagen I with incorporated stem cells” independently, and that I have attached an exhaustive list of citations of the employed sources.

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Anna Talakvadze

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ABSTRACT

3D bioprinted collagen I with incorporated stem cells

Nowadays, the significance of research in the field of engineering artificial tissue replacements can hardly be overestimated. This work is focused on helping in developing a new type of artificial tissue replacements using 3D bioprinting technology and its comparison with molding technology. Comparison is performed through creating cylinder- and toroid-shaped structures 15mm in diameter from hydrogel that consists of Collagen I in various concentrations and Adipose Derived Stem Cells. Then, structural stability, precision, and cell layer homogeneity are evaluated and compared. The molds are created through 3D modeling in Autodesk Inventor and printed from elastic resin and CNC milled polycarbonate. For bioprinting process optimization several different extrusion pressures are tested. Results show that molding creates a more uniform cell distribution, but when optimized, bioprinting creates structures that have a better structural stability, precision control, and homogeneity.

Key words

3D bioprinting, collagen-based scaffold, tissue replacements, stem cells

ABSTRACT

3D tisknutý kolagen I se zahrnutými kmenovými buňkami

Vývoj umělých tkáňových náhrad je aktuálním trendem. Tato práce je zaměřena na použití technologií 3D biotisku a odlévání hydrogelu a srovnání jejich vhodnosti právě pro aplikace umělých tkáňových náhrad. V rámci práce byly zvoleny dva tvary pro nosič – válec a toroid o průměru 15 mm na kterých byly srovnány tvarové přesnosti obou metod. Jako materiál pro přípravu těchto nosičů byl použit prasečí kolagen typu I, do kterého byly inkorporovány prasečí kmenové buňky z tukové tkáně. Odlévací formy byly vytvořeny z elastické pryskyřice pomocí 3D tisku a CNC obráběného polykarbonátu. Pro 3D biotisk byly testovány rozdílné extruzní tlaky. Obě metody poskytují vhodnou metodu pro přípravu 3D nosičů. 3D biotisk poskytuje přesnější stabilitu a jednodušší přípravu, ovšem dochází k nehomogenitám rozprostření buněk jež je možné kompenzovat nastavením software pro tvorbu ovládacího kódu tiskárny.

Key words

3D bioprint, kolagenové buněčné scaffoldy, tkáňové náhrady, kmenové buňky

Table of Contents

List of symbols

Symbol	Unit	Meaning
$V_{(cells)}$, V_c	[µl]	Volume of cells in hydrogel
$V_{(Col)}$	[µl]	Volume of collagen I in hydrogel
$V_{(bicarb)}$	[µl]	Volume of sodium bicarbonate in hydrogel
N	[# of cells]	Total amount of cells per structure
ρ_A	[cells/cm^2]	Cell density per area
A	[cm^2]	Area of structure
h	[mm]	Set height of structure
h_1	[mm]	Height of one layer of cells
ρ_V	[cells/ml]	Cell density per volume

List of abbreviations

Abbreviation	Meaning
CNC	Computer numerical control
SLA	Stereolithography
pADSC	Porcine adipose derived stem cell
PBS	Phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
DAPI	4′,6-diamidino-2-phenylindole

1 Introduction

Nowadays, the significance of research in the field of engineering artificial blood vessels can hardly be overestimated. Atherosclerosis is a second biggest cause of mortality and although ideally autologous vessels are used, that is not always attainable [1]. Moreover, the demand for transplants is rapidly outgrowing the capacity of the industry of organ transplantation, which makes progress in artificial tissue engineering even more anticipated [2]. Outstanding research has been done in the area of tissue engineering but so far the only structures that the scientists are able to fully recreate have fairly uncomplicated shapes, generally – flat and narrow configurations, because in order to make more volumetric and complex structures there emerges a need for small vessels that would provide nutrients to the tissue and dispose of the waste [3]. This project is aimed to assist in developing a new type of vascular protheses for medical applications using 3D bioprinting technology and compares it to the molding technology.

Three dimensional (3D) bioprinting technology is a part of tissue engineering that uses 3D printing technique to combine cells, growth factors and biomaterials into structures that imitate live tissues and organs. Instead of trivial ink this technology uses bio-ink – a specially developed living cell suspension – to create the structures layer by layer [4]. Bioink is a combination of live cells and a base that is compatible with it. It can be made of collagen, gelatin, hyaluronan, nanocellulose, and other materials. Base substance varies and provides a scaffold to grow on and nutrients to survive. The bioprinting technology plays a significant role in modern day bioengineering.

When attempting to print out a scaffold consisting of live organism structures on a 3D printer, several obstacles arise. The choice of material is strictly dependent on the compatibility of that material with living cells, and that factor matters after the bioprinting process as much as during it. The environment also plays a large role in the success of such experiments. It is necessary to choose structures that are permeable to nutrients so that the cells have the support to grow and reproduce [4]. Also, the structure should be stable enough and should not degrade as the time passes. With the new high-resolution bioprinting machines available it is possible to print microscopically fine 3D objects, but there is a need of liquids or gels that solidify at the right time, as the time window is extremely short.

One of the new approaches in tissue engineering uses preparation of suitable scaffolds and cell suspensions to produce artificial vessels, which would outmatch the synthetic vascular protheses that are being utilized in clinical practice now. It can be achieved by molding of a substrate, enabling creating custom sizes and shapes, and hence controlling the wall thickness and form. This approach will be compared with the bioprinting technology. Collagen is used for a scaffold material and adipose derived stem cells are used for a cell substrate.

2 Overview of the current state of the art

Cardiovascular diseases take on a very significant fraction of healthcare problems globally, and in industrial countries it is the number one cause of deaths. This class of diseases involving heart and blood vessels includes coronary (ischemic) diseases, stroke, heart failure, hypertensive heart disease, etc.

Initial treatment is similar with preventive care, and includes requirements of dietary improvements, increased physical activity, tobacco cessation, etc. Pharmacology and minimally invasive procedures sometimes are a better choice, but for urgent and more severe cases surgical interventions are necessary. For ischemic diseases the solution often includes reconstructing or replacing some parts of vascular system, for example coronary artery bypass surgery.

When surgery is necessary ideally patient's own vessels are used but often due to unavailability synthetic vascular protheses are necessary. They are successful for large-caliber replacements but have significant drawbacks for small-caliber applications – problematic healing and tissue regeneration, low long-term patency rates [5]. Other complications are susceptibility to infection, and lack of growth potential for pediatric patients with congenital heart problems.

Hence, there is a big focus on development of the new materials for vascular replacements. In the last 50 years tissue engineering has been developing and progressing, and so far, it seems achievable to develop partially or entirely living blood vessels.

2.1 Tissue engineering

When a new organ or a tissue replacement is necessary the main option is still organ transplantation – a medical procedure in which an organ or tissue is removed from the body of a donor and is placed in a recipient to replace damaged tissue, or a missing organ [6]. This procedure has two significant drawbacks: firstly, the demand for organs is drastically increasing with time, and the gap between the demand for and the supply of organs is inevitably becoming larger every year [2]. Secondly, various complications arise after the procedure because having a transplant from another body triggers immune responses

similarly to the way a body would respond to a foreign substance in order to protect the organism. The solution to these disadvantages is considered to lie within the concept of artificial tissue engineering which has been a big priority in medical research in the last decades.

Tissue engineering combines cells, scaffolds, and biologically active molecules into functional tissues [7]. This interdisciplinary field evolved from biomaterials development and is now a combination of engineering and life sciences the goal of which is development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [8]. As for now, engineered tissues are not often used in hospital practice regarding treatment of patients. Structures like trachea, supplemental bladders, small arteries, skin grafts, and cartilage have been implanted in patients, but such procedures are mostly considered experimental and are still very costly [7]. Although advancements in this field have been suitably beneficial in pharmacology – parts of generated tissues have been introduced as an innovative tool for drug testing. For example, it is used in the research for the attenuation of the central nervous system inflammatory response [9].

To be able to mimic native tissue it is crucial to understand how the native tissue is constructed. In human body, cells produce extracellular matrix – a network of extracellular macromolecules that supports the cells mechanically and biochemically. Hence, in artificial tissue engineering scaffolding is used as a temporary structure to influence physical, chemical, and biological environment surrounding a cell population [8]. It allows the cells to attach, proliferate, and regenerate new tissue. The scaffolding must be composed of biocompatible material and depending on the biological application numerous options are available. Materials that are often used include hydrogel, metal, ceramic, synthetic or natural polymers [3]. Growth factors can also be incorporated into the scaffold to use it for faster regeneration, as scaffold is used to deliver the growth factors to the repair site.

Generally, tissue engineering techniques follow several essential steps: cell extraction, cell incubation and proliferation, scaffold fabrication, tissue generation and implantation [8]. Cells can be autologous – derived directly from the patient, allogeneic – derived from a different person; and xenogeneic – derived from other species. Cell extraction can be performed either from liquid tissues such as blood, or from solid tissues. As scaffold plays an important part in cell growth and proliferation, the design and the choice of scaffold

material, as well as of growth factors, will greatly affect the outcome of tissue generation. After the cells are seeded onto the scaffold they are put to culture in a bioreactor – a device that supports biologically active environment. The main goals are to have uniform cell distribution and high cell viability in the sample.

2.1.1 Scaffold fabrication technologies

Various technologies have been used to fabricate scaffolds for tissue engineering, few of them being solvent casting/particulate leaching, gas foaming, freeze-drying, electrospinning, and bioprinting. As the latter is used in this research project it will be described in a separate chapter.

Solvent casting/particulate leaching method is used to produce polymer-based scaffolds for bone and cartilage tissue repair [10]. In order to mimic porous tissue structure, several steps are followed: at first, a polymer is dissolved in an organic solvent and salts – porogens – that have specific dimensions are added in. Then, the mixture is put in a 3D mold that has a geometry of the replacement tissue. The solvent is then evaporated, leaving only composite material inside the mold. After that the form is soaked in solvent specific to the porogen that dissolves the particles, leaving empty space and creating a porous structure. The advantage of such method is that the structure can be precisely controlled: the pore size, density and geometry can be adjusted by altering porogen's size, concentration, and geometry [11]. Existing litigations include residual solvent material that can have a harmful influence on live cells and low cell density after seeding [12]. The schematic description can be seen in Figure 2.1.

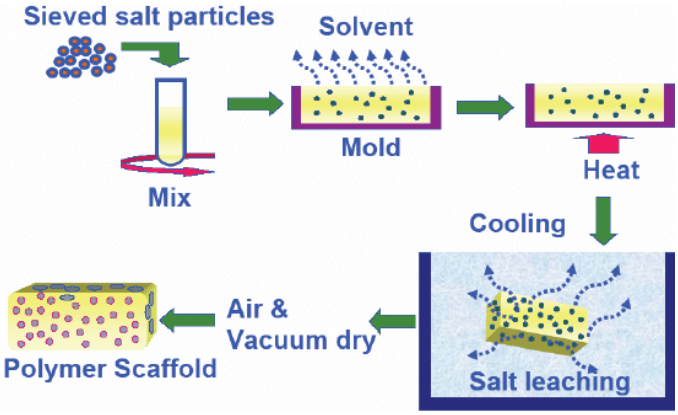

Figure 2.1 Schematic description of solvent casting/particulate leaching, [12]

Gas foaming method is used for creating synthetic matrices and suits well for incorporating sensitive molecules into matrices without severely decreasing its bioactivity through elimination of solvents in it. It involves the following steps: first, high temperature compression molding of polymer and salts into solid structure – semi-solidified polymer/salt complex is created. Then, the structure is put for a few days into a high-pressure carbon dioxide chamber to allow gas to infiltrate the polymer and create pores. The limitation of solvent casting/ particulate leaching – residual presence of solvent material – is therefore not an issue for this method, which enables incorporating sensitive bioactive molecules into such scaffold [11]. Figure 2.2 illustrates schematic description of this process.

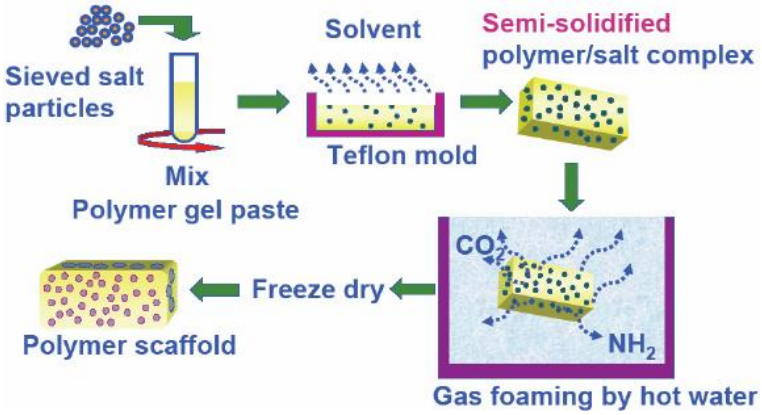

Figure 2.2 Schematic description of gas foaming, [12]

Freeze-drying is a method that creates pores in a polymer scaffold without the use of particulate leaching. A polymer is first dissolved into a solvent and then is mixed well with a liquid that is non-miscible to create emulsion mixture. This homogeneous mixture is then

poured into a mold and quenched either via liquid nitrogen or just using low temperatures. In the freeze-drying process both the solvent and the non-miscible liquid are removed. By varying the polymers and the different amounts of emulsifying liquid, the final polymer structure and porosity can be controlled. Advantages of this method are that the internal pores of such matrices are typically well connected which is good for nutrient supply, metabolic waste clearance, cellular ingrowth, and vascularization [13]; and that more than 90% porosity can be achieved. Although, processing parameters must be well controlled, and this method is highly sensitive [11].

These three above described conventional techniques are all suited for three-dimensional structure production using various biomaterials, though their limited reproducibility and versatility make bioprinting a better fit.

2.2 Bioprinting

Among available technologies in tissue engineering, bioprinting has been growing popular rapidly in the last decade. This method involves printing out biomaterials using a three-dimensional printer, however, instead of trivial ink, such as thermoplastic and resin, a special bio-ink is used. It is comprised of living cells, growth factors and is applied to a scaffold made of biomaterial to create structures that imitate live tissues and organs and their characteristics [4]. Bioprinting has grown rapidly into a promising field due to its advantages – biocompatibility, precision control through precise simultaneous 3D positioning of different cell types, easy manipulation. It also has favorable conditions for mass production due to automaticity [3].

Bioprinting is different from other methods in a way that usually cells are seeded in after the scaffold fabrication, while in this case scaffold fabrication and cell seeding are done simultaneously, by depositing the cells within the scaffold matrix. This is a big advantage, because with other methods it can be difficult to place the cells deep inside the pre-made scaffold and reach homogeneity [14]. Thus, bioprinting offers tissue reconstruction with homogenous cell distribution and high level of cell density. Printing must be performed in sterile conditions to optimize cell viability and printing resolution [4].

2.2.1 Bioprinting techniques

There are several different techniques in bioprinting, one of the most used are inkjet-based, extrusion-based, laser-based, and stereolithography bioprinting [3]. Simplified schematics are seen in Figure 2.3 [15].

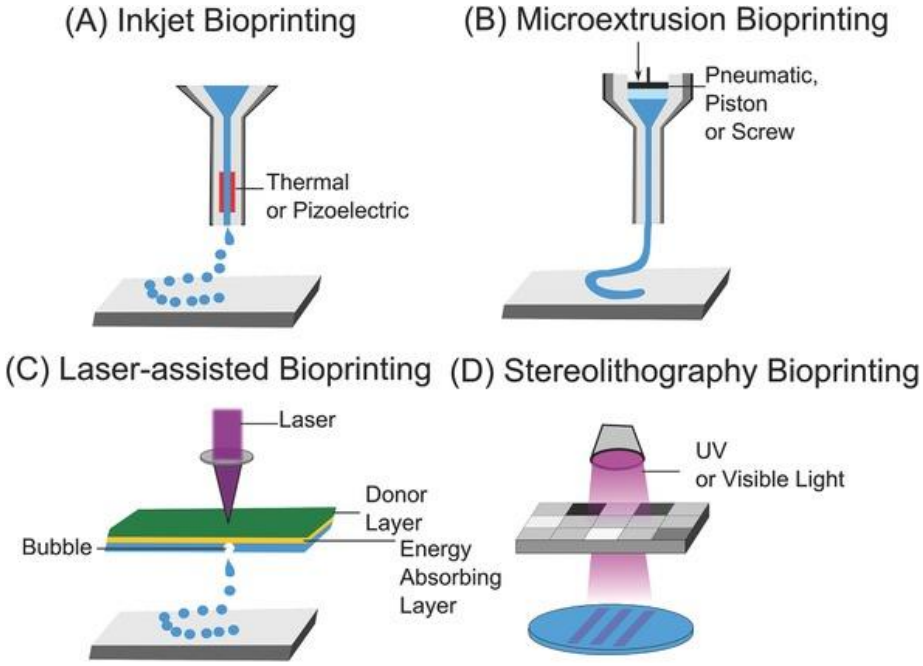

Figure 2.3 Examples of different bioprinting methods, [15]

Inkjet bioprinting creates small droplets of hydrogel to print tissue layer by layer either in a continuous way or by drop-on-demand. Diameter of a single droplet is bigger than the nozzle diameter due to surface tension and is around 50-300 µm. This method has been effective in skin and cartilage regeneration [15]. It is easy to implement with low-viscosity materials, has high printing speed and low cost. Although, there are some limitations: it has a reduced capacity to handle materials that are high in viscosity which limits production of materials with high cell density. Also, mechanical and thermal stress of the printer may damage live cells.

Micro-extrusion bioprinting deposits cell-containing hydrogel layer by layer using pneumatic or manual force. It is similar to inkjet-based bioprinting, but it can handle printing more viscous material, which allows to expect structures that are high in cell density. This parameter is very important when replicating highly cellular native tissues. The size of printed strand is around the size of the nozzle diameter. This method has been applied in

constructing aortic valve conduits, vascular grafts, cartilage constructs, among other structures [15]. The limitations of this method are mostly related to nozzle clogging and shear stress due to high viscosity of materials [3].

Laser-assisted bioprinting uses a laser to rapidly heat donor layer (see Figure 2.3) causing it to form bubbles which create pressure that patterns the cells on the substrate. This technique allows to achieve high resolution scaling in tenth of micrometers which makes it suitable to create structures in tissues that are approximately single cell sized. This allows precise control and since this method does not utilize nozzles the limitations such as clogging are avoided [15].

Stereolithography uses ultraviolet or visible light to selectively solidify bioink in a layer-by-layer manner. This method does not have as high resolution as laser-assisted bioprinting but has a higher printing speed than other methods.

In this project will be used micro-extrusion bioprinting method. In all the listed bioprinting techniques, however, the essential component is the bioink.

2.2.2 Bioinks

Although progress in bioprinting techniques have been significant in the last few decades, the research in bioinks is relatively underdeveloped, creating limitations in successfully applying these technologies. The bioinks must be appropriate specifically for 3D printing applications, as well as have the necessary bioactivity for different cell types.

Bioink is a solution of a certain biomaterial, or a mixture of few biomaterials, and it is used in the bioprinting process in a form of a hydrogel and typically containing necessary cells. Biomaterials for the bioink can be chosen from natural sources or they can be synthetically derived. It can also be a blend of the two materials. During or right after the printing process bioink can be cross-linked – stabilized – to reach the final form of the structure. Ideally, mechanical, biological, chemical, and rheological properties of the desired tissue should be manifested in the bioink to establish the tissue's expected functionality. Thus, the selection of the material is a crucial step in the bioprinting process [16].

In this work, collagen-I will be used as a base for the bio-ink. It is the main structural protein of the extracellular matrix (ECM) for mammals, and hence, its physical and chemical properties closely match those of natural tissue [16]. It is also easily obtainable, very

biocompatible, and its stiffness and gelating can be modified by physical or chemical crosslinking of the bio-ink [17]. Molding such material enables creating complex shapes such as aortic valves. Meanwhile, bioprinting it allows high precision and mass production [18]. As a cell substrate adipose derived stem cells (ADSC's) are used. They are mesenchymal stem cells that are extracted from abundant adipose tissue. Such cells have broad differentiation abilities and are easy to isolate. Porcine cells are used which are a popular choice for preclinical studies due to similarities in anatomy and physiology with the human organism. [19]

2.2.3 Steps in bioprinting

There are three steps in the process of bioprinting: preparation, printing, and maturation. For the first step of the process it is necessary to prepare a digital three-dimensional model of the scaffold shape and using appropriate software such as Slic3r to create g-code containing the sliced layer-by-layer path of the printer, and such parameters as pressure of extrusion and speed of printing based on the set optimization instructions. Meanwhile, the cell should be prepared and multiplied.

After choosing the appropriate nozzle size and placing the bioink in the cartridge the printer produces viscous structure that need to be processed after printing in order to achieve more solid consistency. That involves maturation – the process of reaching dimensional stability through chemically and mechanically stimulating the printed material to crosslink [4]. This can be reached by applying ultraviolet light, certain chemicals, or heat. Chemicals work through sending the cells signals to regulate growth and remodeling. To speed up the process of maturation the material is put to bioreactor. This provides cells with additional physiological environment which also facilitates tissue vascularization and viability [17].

3 Aims

The main aim of this work is to compare collagen structures that have been bioprinted to those that have been molded and see which of the methods is better for potential engineering of blood vessels. This can be achieved through the following steps:

- to make molds and prepare for bioprinting;
- to validate various concentrations of porcine collagen I solution;
- to test these solutions in the 3D printer and in the molds with added porcine adipose derived stem cells.

The general schematic depiction of the methods of this work can be seen in Figure 3.1.

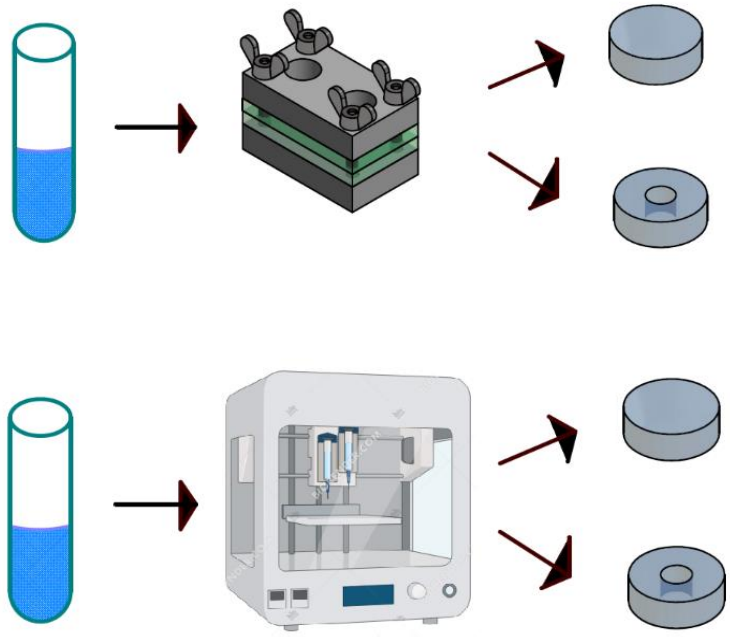

Figure 3.1 General scheme of the work

For the first step completion, making of the molds includes creating 3D models of the molds for a cylinder and for a toroid, and printing all mold forms using a 3D printer. Preparation for bioprinting includes 3D syringe bioprinter *Cellink Inkredible+* calibration and testing various extrusion pressures and nozzles using commercially available hydrogel.

For the second step it is necessary to test and validate various concentrations of porcine collagen I solutions and to test its ability to gelating with porcine adipose derived stem cells.

The third step includes testing the abovementioned solutions firstly by dispensing them in prepared molds thus creating molded structures, and secondly by bioprinting them using extrusion technology thus creating bioprinted structures; and then comparing the two methods.

4 Methods

4.1 Mold design

To achieve the aims of this project at first it was necessary to create two molds: one that would accommodate two cylindrical shapes, and one for two toroid shapes. Using such structure rather than the one that would accommodate only one shape per mold was a measure to increase throughput of the process and decrease the overall time consumption. The desired resulting shapes in details are presented as basic 3D models including dimensions in Figure 4.1, the cylinder shape on the left and the toroid on the right. Resultant structures would be 15 mm in diameter and up to 5 mm in height.

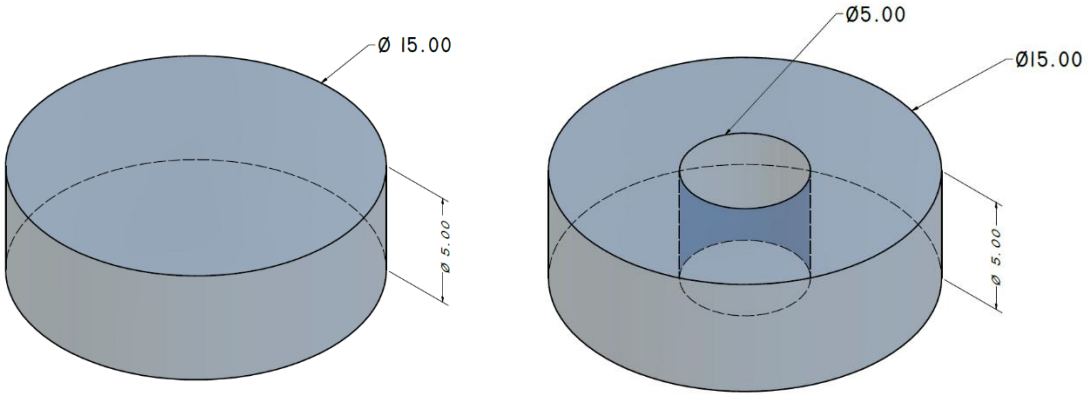

Figure 4.1 3D models of final shapes, dimensions given in [mm]

Several structures were made separately to comprise a complete mold when combined. Each mold had four layers: top and bottom layers for both cylinder and toroid shapes were comprised of polycarbonate platforms and were used to provide support and compression to the two middle parts which were made of elastic resin and provided directly the desired shape. The whole structure is then held by four screws in combination with four butterfly nuts to provide regulation of compression.

To create such mold, it was needed to make a 3D model for each of the structures first, and then to print it out with a 3D printer. To accommodate 3D modeling, *Autodesk Inventor 2020* software was used. A scheme for one of the shapes – the toroid – with the

relevant captions is displayed in Figure 4.2. Detailed drawing for each of the utilized mold forms can be found in the Appendix A; schemes for assembled molds are placed in Appendix B.

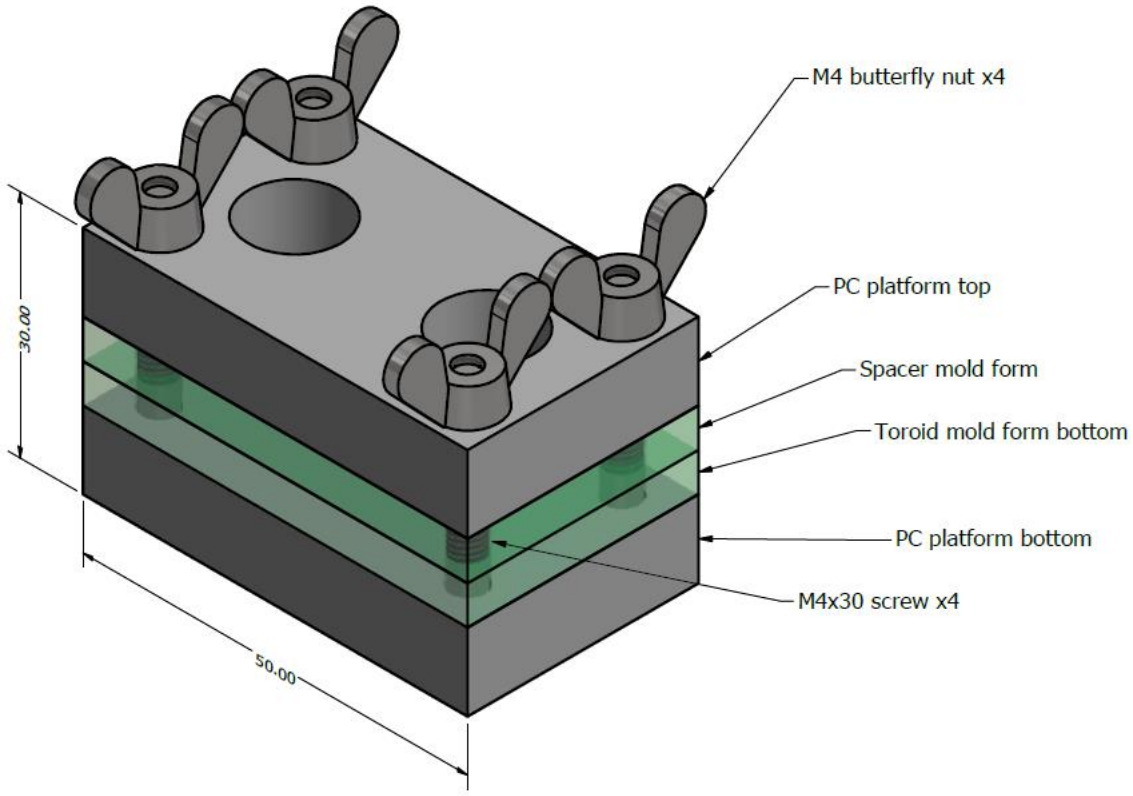

Figure 4.2 3D model of a mold for toroid shapes

For the mold production two materials have been used: Polycarbonate sheet by *Makrolon, Germany* and Elastic Resin for soft flexible parts by *Formlabs, USA*.

Polycarbonate sheet is biocompatible, nonstick, abrasion resistant, and has a high impact strength. It is resistant to variety of cleaners, solvents, and other corrosive elements that can attack standard polycarbonate sheet [20]. It can withstand such chemicals as acetone, ethylene dichloride, and sodium hydroxide for more than 24 hours. Hence, it can be subjected to sterilization, which is crucial for tissue engineering applications.

Elastic resin material is suitable for prototyping parts normally produced with silicone and is appropriate for medical models and devices. This material bends, stretches, compresses, and holds up to repeated cycles without tearing [21]. It has less than one percent size gain after being immersed for such chemicals as acetic acid, hydrogen peroxide or sodium hydroxide for 24 hours.

To mill the mold parts made of polycarbonate was used three-axis CNC 6090 router (*ELO6090, BlueElephant, Taiwan*). HPTec 698 flat end mill with 3,125 diameter was used (*model 698, HPTec, Germany*). G-code for controlling CNC machine was generated based on 3D models with Autodesk Inventor CAM (formely HSM). Spindle speed was set to 15000 RPM, federate 800 mm/min with 2mm roughing step. No coolant was used.

To print out the mold parts made of elastic resin a *Formlabs* 3D printer *Form 2* was used. It utilizes stereolithography (SLA) technology, has a laser spot size of 140 microns, an automated resin fill system and self-heating resin tank that heats to 35 ˚C [22]. Mold forms with scaffolding prepared for SLA printing of two middle layers for both shapes – 4 structures in total – with elastic resin can be seen in Figure 4.3. Table 4.1 includes basic information about the printing of these structures.

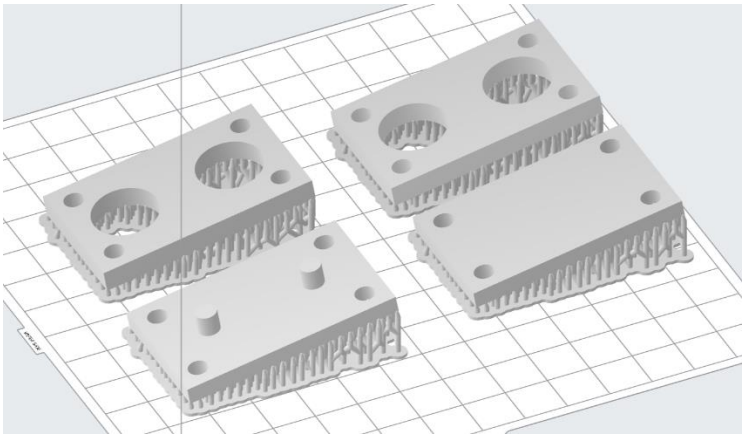

Figure 4.3 Mold forms with scaffolding prepared for SLA printing

Table 4.1 Basic information on the 3D printing process of the structures from Figure 4.3.

Material	Elastic resin
Printing time	4 h 15 min
Volume	40.81 ml
Layer thickness	0.1 mm

Appearance of the finished 3D printed molds together with the screws and nuts in the disassembled state can be seen in Figure 4.4. The assembled molds in their final form can be seen in Figure 4.5.

Figure 4.4 3D printed mold forms with nuts and screws, disassembled

Figure 4.5 Assembled molds

4.2 Preparation of cells

To achieve the consistency of a hydrogel that would enable successful bioprinting, different concentrations of collagen I were tested. In this work the cell that have been used are porcine Adipose Derived Stem Cells (pASCs). The experiments with cells were conducted in sterile conditions to avoid contamination.

At first, the cells have been washed in phosphate-buffered saline (PBS) to eliminate dead cells and cellular debris. PBS is used because it is isotonic and non-toxic to most cells.

Then, a solution of 0.05% trypsin in 0.53mM EDTA was added (*Sigma-Aldrich, Missouri, USA*). Trypsin is an enzyme that breaks down proteins and EDTA (Ethylenediaminetetraacetic acid) is added to remove the calcium and magnesium from the cell surface which allows trypsin to hydrolyze specific peptide bonds. This removes cell to cell adhesion and separates cells from the dish. To inhibit trypsin a culture media was used. Finally, the cell suspension was centrifugated at 300g acceleration for five minutes to concentrate the cells at the bottom of a vessel. Then, the pellet is resuspended by the appropriate density in the same cell medium that has been described above.

Culture media that was used is DMEM and F-12 in a ratio 1:1 (*Sigma-Aldrich, Missouri, USA*) with 10% FBS (*Gibco, California, USA*) and 10 ng/ml FGF-2 (FGF-basic, 154aa) (*Genscript, New Jersey, USA*). DMEM – Dulbecco's Modified Eagle Medium – is a widely used basal medium for supporting the growth of many different mammalian cells [23]. A 1:1 mixture of DMEM and Ham's F-12 combines DMEM's high concentrations of glucose, amino acids, and vitamins with F-12's wide variety of components. FBS – fetal bovine serum – is the most widely used serum-supplement for the in vitro cell culture of eukaryotic cells. It has a very low level of antibodies and contains more growth factors, allowing various cell culture applications [24]. FGF-2, or Fibroblast Growth Factor-basic, is produced by a variety of cells, including cardiomyocytes, fibroblasts, and vascular cells. It regulates a variety of processes including cell proliferation, differentiation, survival, adhesion, motility, apoptosis, limb formation and wound healing [25].

In Figure 4.6 is displayed a phase contrast microscope image of porcine adipose derived stem cells in confluency. The microscope used is *Leica DMi8* with N-plan objectives.

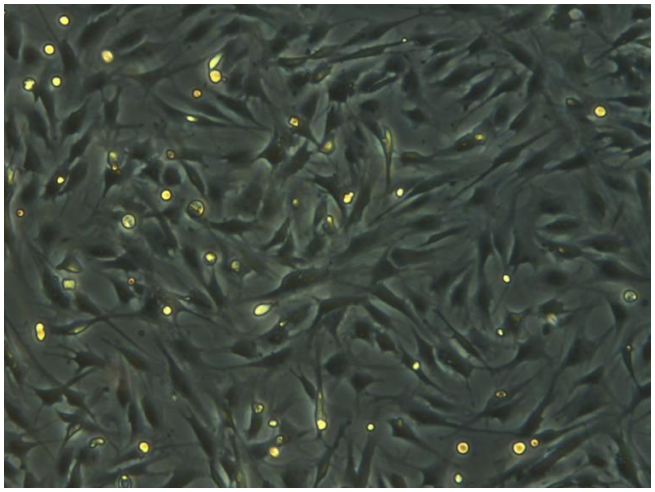

Figure 4.6 Microscope image (phase contrast) of porcine adipose derived stem cells in confluency

After three days of cultivation, the 3D scaffolds with cells were washed using PBS and fixed in 4% paraformaldehyde solution for 20 minutes. Afterwards these samples were stained using Phalloidin and DAPI (both from *Sigma-Aldrich*) to visualize fibrous actin cytoskeleton and cell nuclei.

4.3 Hydrogel preparation for mold

To prepare a suitable hydrogel for the molded structures, it was necessary to test various combinations of concentrations of collagen. Two stock concentration of collagen I have been tested: at first 5 mg/ml, then 10 mg/ml.

Hydrogel for molding is comprised of three components: collagen I, cell suspension, and sodium bicarbonate. The three ingredients are mixed in a ratio 0.60: 0.39: 0.01, respectively [26]. Sodium bicarbonate addition is needed because collagen itself is a relatively acidic protein, so this helps to change the pH and hence facilitate fiber formation and gelating.

The volume of hydrogel that is supposed to be put into a mold is calculated based on the desired height and the known surface areas of the structures. For this experiment four different heights were taken, and volumes calculated for three different shapes: toroid, cylinder, and for a well of a 24 well plate – to enable preliminary testing of the material before placing it to molds. To aid and accelerate all the calculations for this part of the work three Excel sheets were created that are attached in Appendix C.

The heights that were chosen for testing are 0.5, 1, 2, and 4 millimeters. After computing the volumes for each height for each mold shape, it was necessary to compute individual volumes for each of the hydrogel's components, based on the abovementioned ratio. Tables 4.2 and 4.3 show sections of the excel sheet that contains the distribution of the final volume into three separate portions for a toroid and for a cylinder, respectively. V_1 to V_4 represent each of the four volumes computed based on the four heights.

Table 4.2 Volume distribution computation for each component of hydrogel based on a set ratio, for toroid shape.

Volume of each component based on ratio [µl]				
	$V_{(cells)}$	$V_{(col)}$	$V_{(bicarb)}$	Sum (=V)
V_1	30.6	47.1	0.8	78.5
V_2	61.3	94.2	1.6	157.1
V_3	122.5	188.5	3.1	314.2
V_4	245.0	377.0	6.3	628.3

Table 4.3 Volume distribution for each component of hydrogel based on a set ratio, for cylindrical shape.

Volume of each component based on ratio [µl]				
	$V_{(cells)}$	$V_{(col)}$	$V_{(bicarb)}$	Sum (=V)
V_1	34.5	53.0	0.9	88.4
V_2	68.9	106.0	1.8	176.7
V_3	137.8	212.1	3.5	353.4
V_4	275.7	424.1	7.1	706.9

The optimum cell density to achieve a confluent layer in two dimensions is 75,000 cells per square centimeter. The aim is to compute three-dimensional cell density value, in cells per milliliters. Considering that one cell layer takes up around 50 microns of height, desired total amount of cells per each structure is computed using equation 1.

$$N = \frac{\rho_A \times A \times h}{h_1} \qquad (1)$$

where N is the total amount of cells per structure, [number of cells]; ρ_A is desired cell density per area, [cells/cm^2]; A is area of structure, [cm^2]; h is set height of the structure, [mm]; h_1 is height of one layer of cells, [mm].

Then, the cell density in three dimensions is calculated using equation 2.

$$\rho_V = \frac{N}{V_c} \qquad (2)$$

where ρ_V is cell density per volume, [cells/ml]; N is the total amount of cells per structure, [number of cells]; V_c is volume of cell suspension in a structure [ml]. Aimed cell density for a hydrogel that is to be molded composed 38.5 million cells per milliliter.

4.4 Bioprinting

Hydrogel composition that was used for molding differed from the hydrogel used for bioprinting. The hydrogel that has been used for the molds needed higher temperature and lower pH to facilitate crosslinking and fiber creation. To achieve that, sodium bicarbonate was added to change pH to more basic, and the molds were put into incubator for several minutes after dispatching the hydrogel to expose them to a higher than room temperature. Some of the advantages for such hydrogel are possibility to create complex shapes and to use a substrate.

In the bioprinting experiment, the collagen was modified through addition of a photo-activator, because bioprinter enables crosslinking facilitation to achieve gelating through ultraviolet light. Hence, addition of sodium bicarbonate was not necessary. Although, the chemicals added to the hydrogel can be cytotoxic.

4.4.1 The 3D printer and its properties

To conduct the experiment a 3D printer *Inkredible+* by *Cellink* was used. It has dual printheads which enables printing with two different materials at the same time without having to change cartridges and pause the process [27]. It is possible to create sterile conditions inside of the printer which is crucial in order to avoid contamination when working with living cells. An ultraviolet crosslinking system hardens the structure allowing it to be moved without losing shape.

This printer uses pneumatic-based microextrusion system, which applies pressurized air to extrude material through a printhead and works in three dimensions – x, y, and z. Some technical specifications of the printer are included in Table 4.4. Software used to work with this printer is *Cellink HeartWare* that contains *Slic3r* for g-code creation. Commercially available hydrogel was utilized due to its consistency before conducting main experiments for a better optimization of the printer's settings.

Table 4.4 Technical specifications of Inkredible+ bioprinter by Cellink [27].

Printhead temperature range	Room temperature to 130 C˚
Printbed temperature range	Room temperature to 65 C˚

Build volume	130*80*70 mm
Positioning precision	XY axes: 10 µm Z axis: 2.5 µm
Layer resolution	100 µm
Gel's viscosity range	0.001 – 250 Pa.S
Maximum operating pressure	700 kPa

4.4.2 Bioprinting optimization

To optimize the bioprinting process it was necessary to test different nozzles and extrusion pressures. Nozzles for the bioprinter used in this work come in three colors, each a different size, see Table 4.5. According to the manufacturer's recommendation, the blue nozzle was used.

Table 4.5 Colors and respective values for nozzle sizes for bioprinter Inkredible+ by Cellink.

Color	Nozzle size, [µm]
Clear	200
Red	250
Blue	410

As the extrusion printer uses pneumatic force to distribute the material, the volume of the material dispensed into a structure depends on the extrusion pressure set on the printer. To achieve desired result, several pressures were tested, and the final structures were then compared between each other. The tested volumes can be seen in table 4.6.

Table 4.6 Values for extrusion pressure that have been tested.

	Pressure, [kPa]
p_1	35
p_2	25
p_3	20
p_4	18
p_5	15

5 Results

5.1 Molding

The preliminary testing showed that the structures smaller than 2 millimeter in height are not suitable for molding because large portion of the material appears on the sides of mold due to surface tension. Hence, two heights were used: 2 and 4 millimeters. Two concentrations of stock solution were tested: 5 and 10 mg/ml.

5.1.1 Collagen concentration 5 mg/ml

For the first concentration the 2 mm toroid molding was unsuccessful due to surface tension. It can be seen in Figure 5.1 that the volume of hydrogel in the toroid mold was not enough to produce a structure.

Figure 5.2 Unsuccessful test for 2 mm height due to surface tension

The result for the cylinder is displayed in Figure 5.2. The magnified images were taken using a digital microscope *Dino-Lite AM7915MZT*. It is seen that the structure is not consistent, has clumps, and is very thin. The radius of the structure is 7.20 mm, height 0.89 mm.

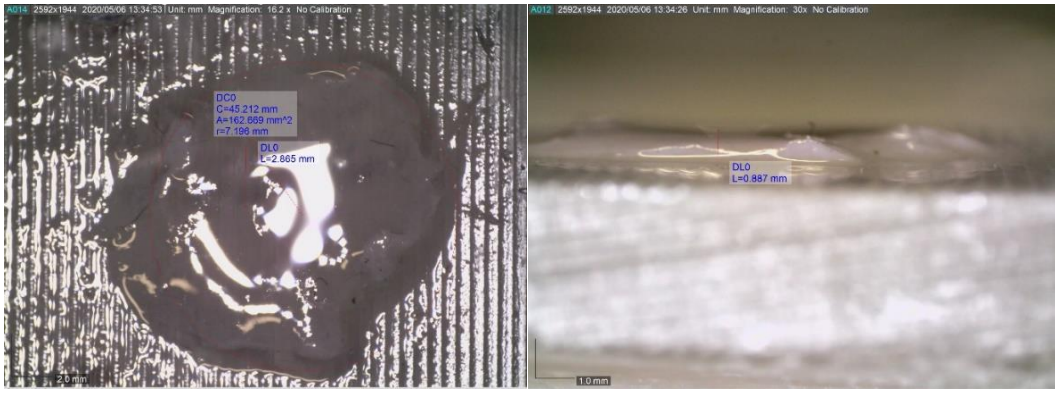

Figure 5.3 Cylinder shape, 2 mm height

After this test it was chosen to continue only with structures of 4 mm height. In Figures 5.3 and 5.4 a toroid and cylinder shapes of that height are displayed on a glass. For the toroid, the outer radius comprised 7.57 mm, inner – 2.04 mm, height – 3.90 mm. For the cylinder the radius comprised 7.57 mm, height – 3.18 mm.

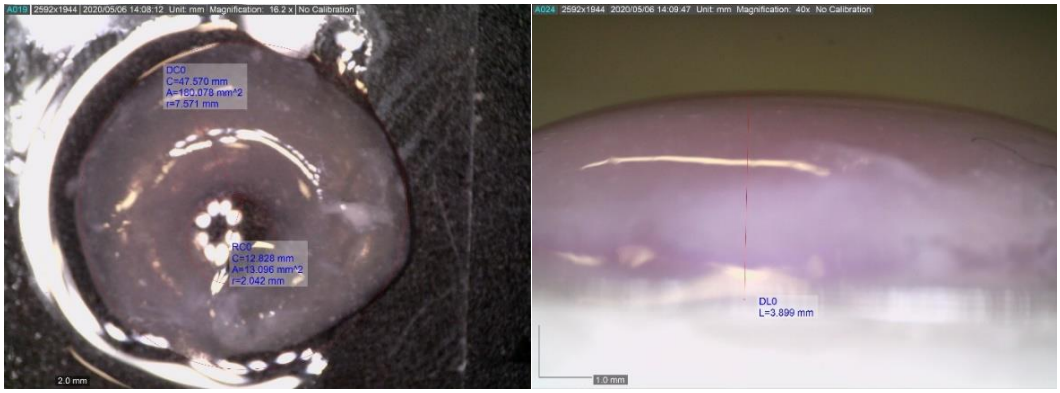

Figure 5.4 Toroid shape, 4 mm height

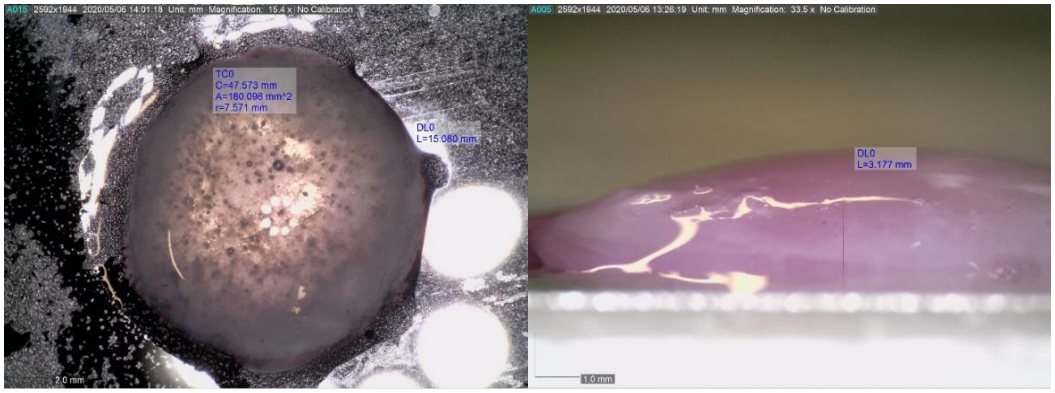

Figure 5.5 Cylinder shape, 4 mm height

In the next experiment a hydrophobic cloth was put beneath the hydrogel into the cylindrical mold to see if the result would be affected by it. The achieved structure is displayed in Figure 5.5. For 4 mm of height the radius of 8.20 mm was attained. A side by side comparison of a structure on glass to the structure on cloth is displayed in Figure 5.6.

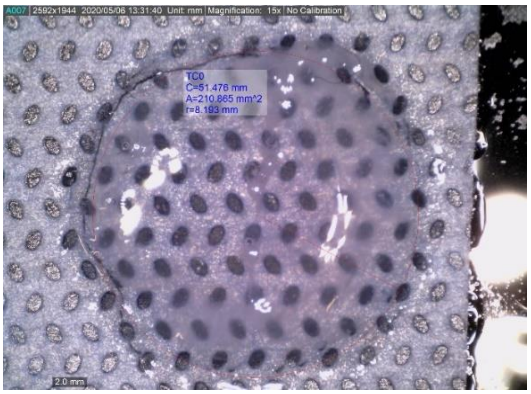

Figure 5.6 Cylinder shape on hydrophobic cloth, 4 mm height

Figure 5.7 Side by side comparison of structures on glass and on cloth

The results for all experiments with a hydrogel with 5 mg/ml collagen concentration are summarized in table 5.1 for toroid shape and table 5.2 for cylinder shapes.

Table 5.1 Summary of results for 5 mg/ml concentration for toroid shapes, [mm].

	Outer radius	**Inner radius**	**Height**
2 mm	-	-	-
4 mm	7.57	2.04	3.90

Table 5.2 Summary of results for 5 mg/ml concentration for cylinder shapes, [mm]

	Radius	**Height**
2 mm	7.20	0.89
4 mm	7.57	3.18
4 mm with class	8.19	-

5.1.2 Collagen concentration 10 mg/ml

For further experiments the concentration of collagen was increased twice and made up 10 mg/ml, which made the hydrogel mode dense. Experiments were conducted again with 2 and 4 mm of height. In figure 5.7 is shown a molded toroid structure of 2 mm. Its outer radius comprised 6.90 mm, height – 1.47 mm. Compared to 5 mg/ml concentration this structure appears more consistent and homogeneous.

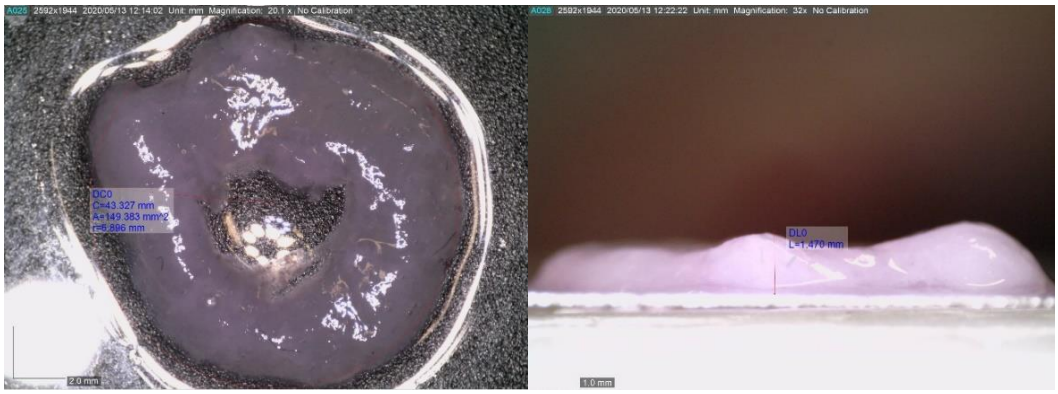

Figure 5.8 Molded toroid shape, 2 mm height

However, the results for the cylindrical structure appear to be worse than for the 5 mg/ml concentration. It is shown in Figure 5.8 that the shape is not round, thin, and some clumps can be observed. The radius comprised 4.93 mm, height – 0.98 mm.

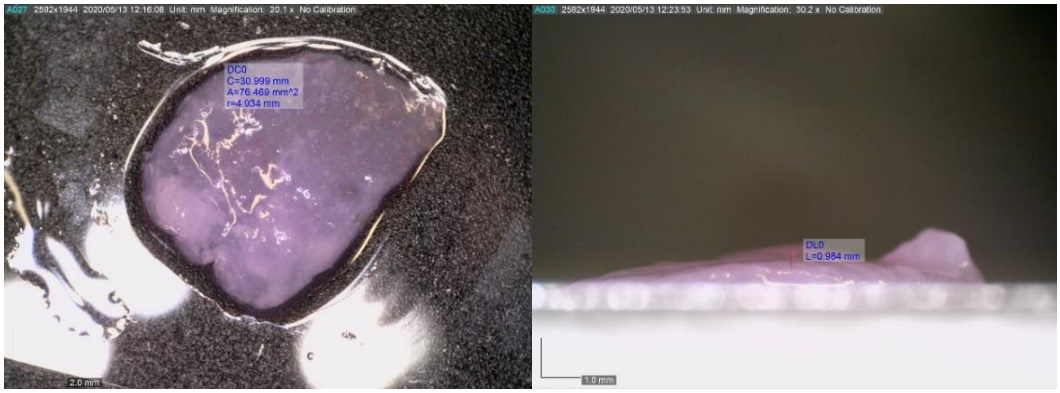

Figure 5.9 Molded cylinder shape, 2 mm height

Then, 4 mm toroid was molded and had 7.29 mm in outer radius and 1.89 mm in height (see Figure 5.9).

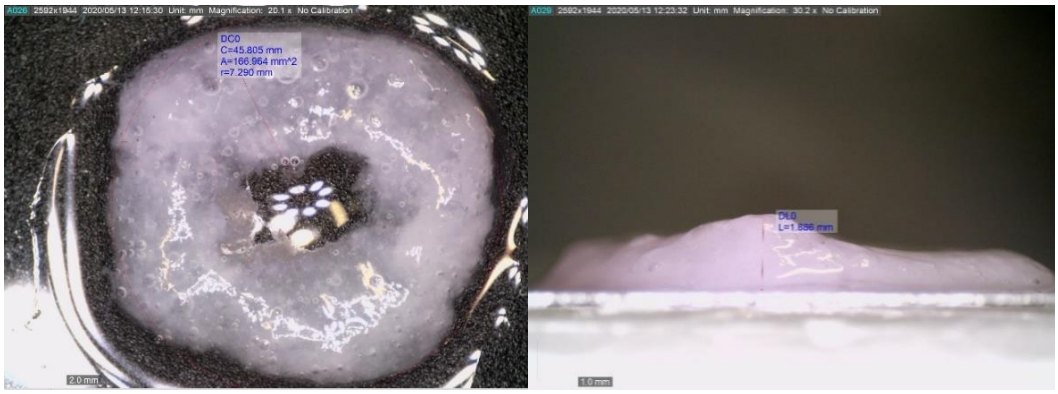

Figure 5.10 Molded toroid shape, 4 mm height

The results for the experiments with a hydrogel with 10 mg/ml collagen concentration are summarized in table 5.3 for both shapes.

Table 5.3 Summary of results for 10 mg/ml concentration, [mm].

	Toroid		**Cylinder**	
Set height	Outer radius	Height	Radius	Height
2 mm	6.90	1.47	4.93	0.98
4 mm	-	-	7.29	1.89

5.2 Bioprinting

For the experiments with bioprinted structures few pressures were tested to then choose a structure which shape is the closest to the desired shape.

The biggest tested pressure of extrusion – 35 kilopascals (kPa) resulted in structures that were very large in volume. Front and side views of the toroid structure can be seen in Figure 5.10. Its outer radius comprised 11.90 millimeters that was compared to the anticipated 7.5 mm, and the height comprised 3.71 mm contrasted with aimed 5 mm due to the hydrogel' spread.

Figure 5.11 Toroid shape bioprinted at 35 kPa

Front and side views of the cylindrical structure can be seen in Figure 5.11. Its radius comprised 12.04 millimeters, and the height 3.86 mm.

Figure 5.12 Cylinder shape bioprinted at 35 kPa

Then, a smaller pressure of 25 kPa was tested. It showed a big improvement with the outer radius of toroid of 9.42 mm (see Figure 5.12) and the radius of the cylinder 9.39 mm (see Figure 5.13).

Figure 5.13 Toroid shape bioprinted at 25 kPa

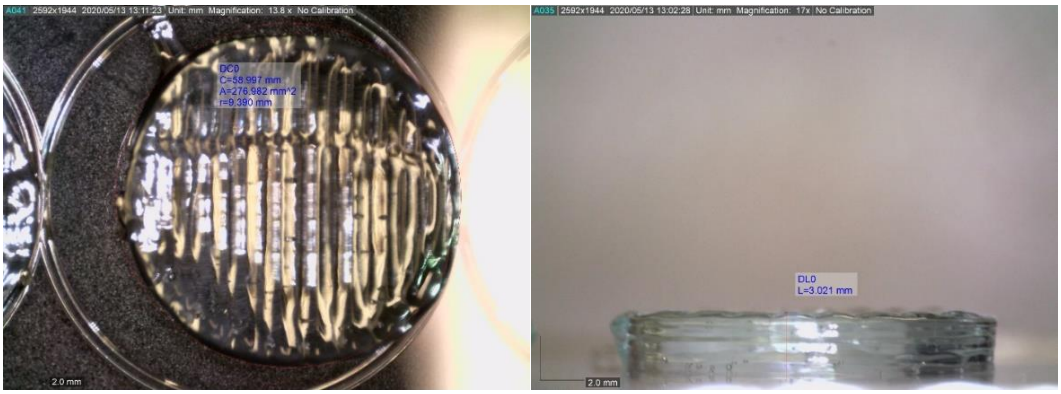

Figure 5.14 Cylinder shape bioprinted at 25 kPa

Then, extrusion pressure of 20 kPa was tested, with a resulting outer radius of a toroid of 9.52 mm (see Figure 5.14). and radius of a cylinder 8.74 mm (see Figure 5.15).

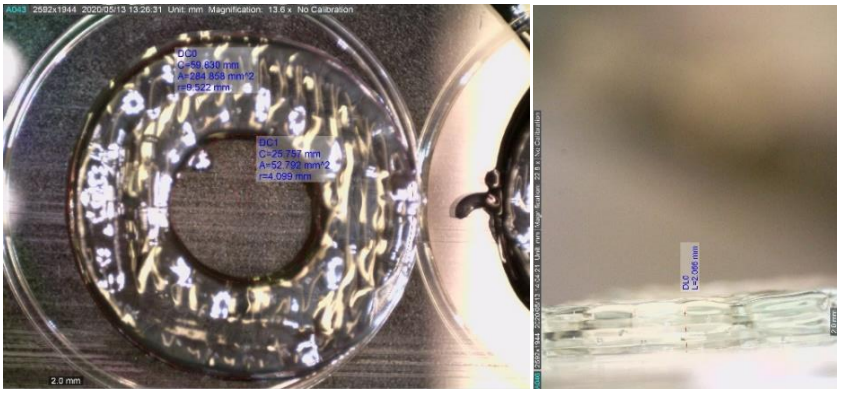

Figure 5.15 Toroid shape bioprinted at 20 kPa

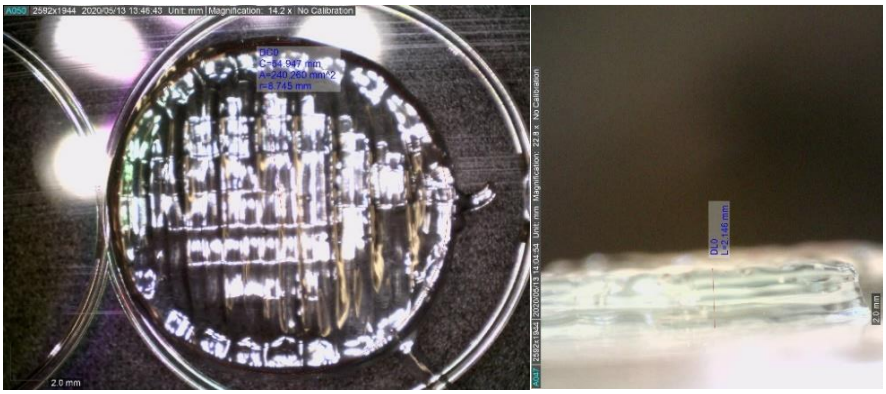

Figure 5.16 Cylinder shape bioprinted at 25 kPa

The result of a 20 kPa extrusion was close to the original shape, but it was decided to experiment further and test the pressure of 15 kPa. As a result, it was seen that such pneumatic extrusion is not sufficient for structure creation from a hydrogel of such viscosity. The

structure was not consistent and had clumps of hydrogel that were not homogeneous. Resultant toroid and cylindric shapes are displayed in Figure 5.16.

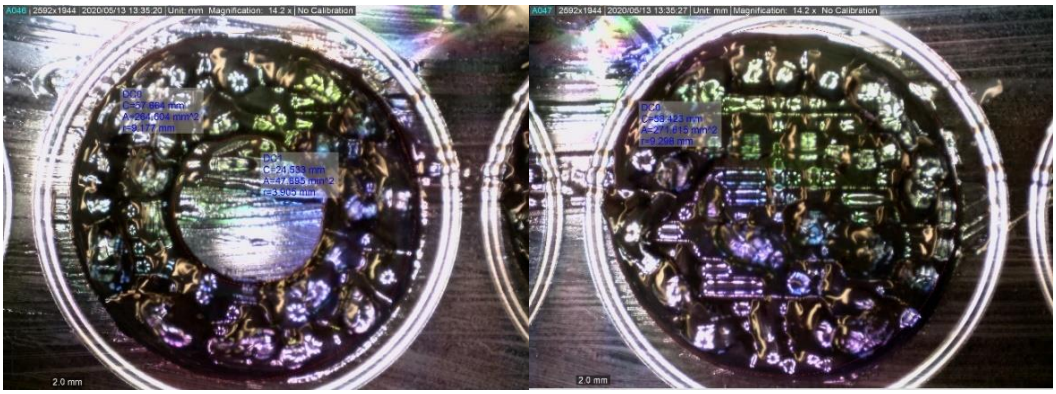

Figure 5.17 Toroid and cylinder shapes bioprinted at 15 kPa

Finally, an extrusion pressure of 18 kPa was chosen to create a structure that would be smaller than the structure printed out at 20 kPa but would have a consistent form, as opposed to the structure printed out at 15 kPa. The result is displayed in Figure 5.17 for the toroid shape, in Figure 5.18 for the cylinder. These structures resembled the goals the most: the outer radius for toroid of 8.98 mm, inner radius of 4.08 mm; 8.90 mm radius for the cylinder.

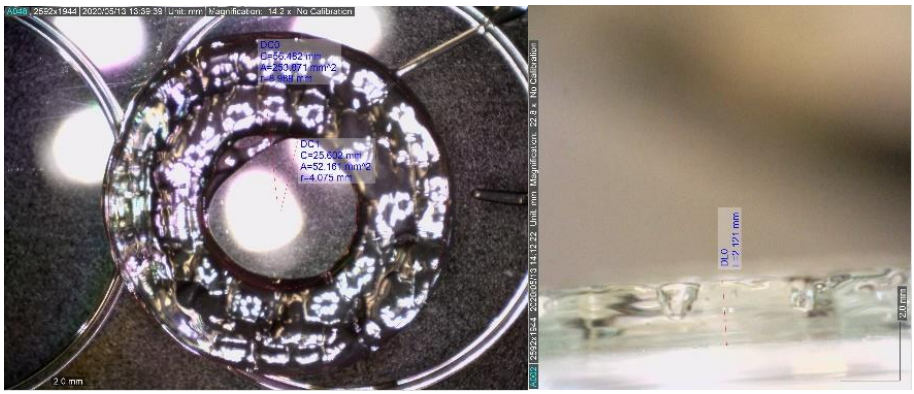

Figure 5.18 Toroid shape bioprinted at 18 kPa

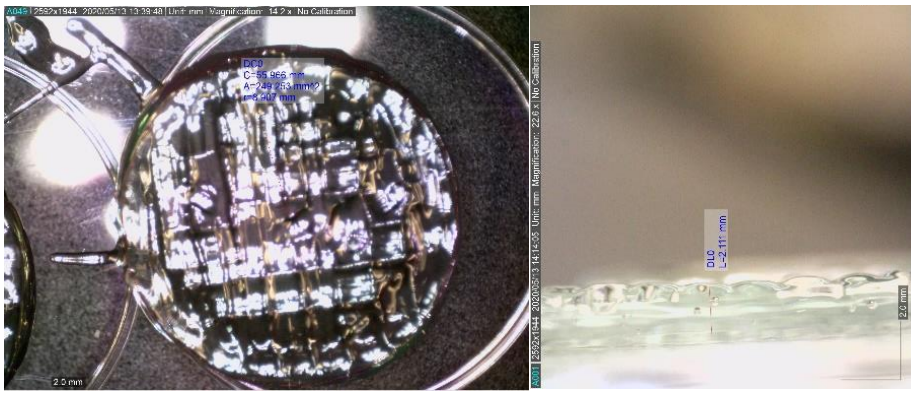

Figure 5.19 Cylinder shape bioprinted at 18 kPa

All results for bioprinting experiments are summarized in Table 5.4.

Table 2.4 Summary of results for bioprinted structures.

	Toroid			**Cylinder**	
Extrusion pressure [kPa]	Outer radius [mm]	Inner radius [mm]	Height [mm]	Radius [mm]	Height [mm]
35	11.90	2.10	3.71	12.04	3.86
25	9.42	3.88	3.24	9.39	3.02
20	9.52	4.10	2.07	8.74	2.15
18	8.98	4.08	2.12	8.90	2.11
15	9.12	3.91	-	9.30	-

5.3 Fluorescence microscopy of scaffolds

A microscopy image was taken after the samples were stained for fibrous actin cytoskeleton and cell nuclei visualization and it is displayed in Figure 5.20. Images on the left correspond to the molded hydrogel, on the right – to the bioprinted. Fibrous actin is stained red, cell nuclei – with blue color. Different structure and morphology of cells can be seen.

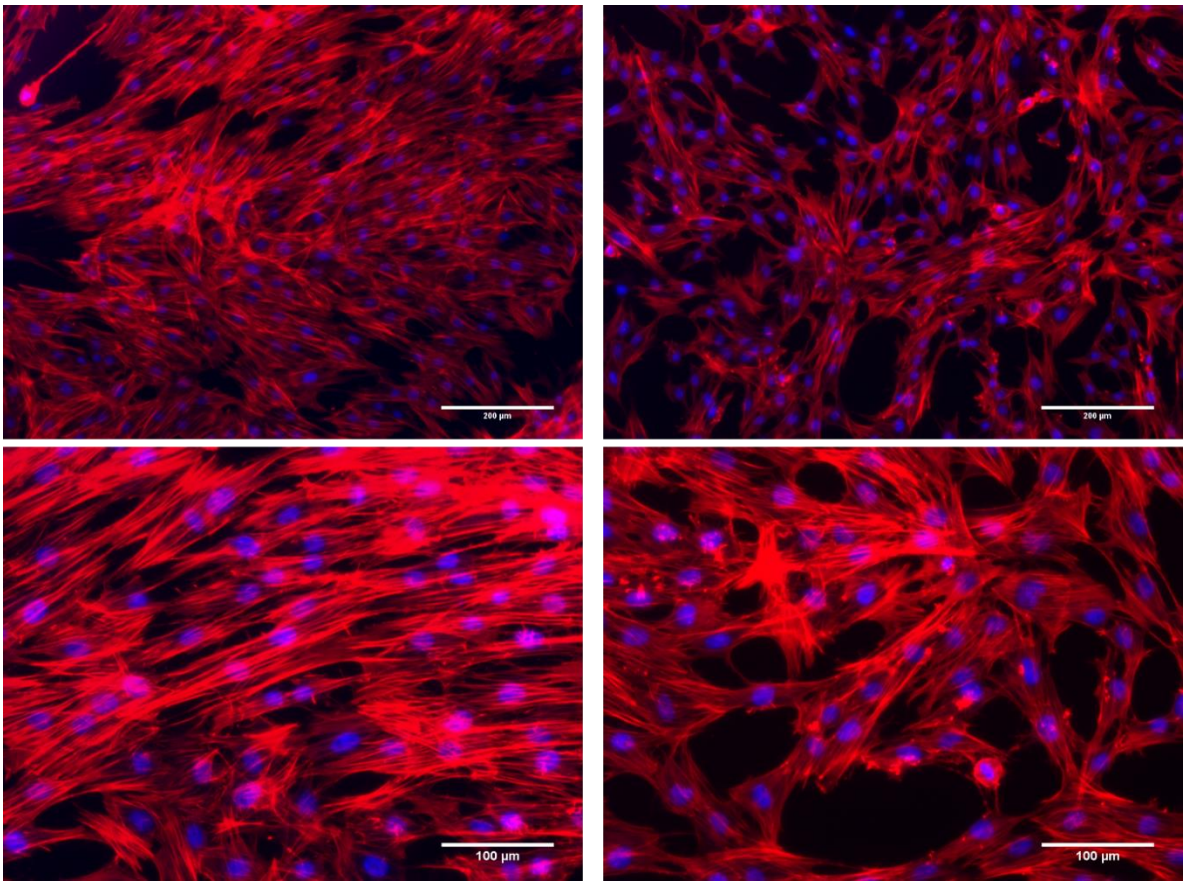

Figure 5.20 Comparison of molds (left) and bioprinted scaffolds (right). Fluorescence staining of F-actin (red) and cell nuclei (blue)

6 Discussion

The results demonstrated that generally the bioprinted structures have better structural stability and consistency than the molded structures. Although the initial plan for molded structures was to test four different heights, there is a limitation that involves the issue of surface tension. Particularly, the less concentrated hydrogel exposed that there needs to be a bigger volume of hydrogel to achieve a stable structure. Generally, the structures of bigger volume showed a better structural stability.

Another potential limitation is the surface of the 3D printed elastic resin mold parts. Image 5.2 was made without transferring the structure onto a glass, and the ridged surface of the resin can be seen up close. This obstructed transferring the structure out of the mold for analysis and could potentially be affecting the result. Although, generally, molded structures can be easily transferred from the mold, as opposed to the bioprinted parts due to the hydrogel material differences.

The experiments with using a small cloth under the hydrogel to aid the transferring of the structure after gelating demonstrated no solid evidence of improvement (see Figure 5.6 for a side by side comparison). Moreover, after some time hydrogel was starting to seep into the cloth material.

The results of bioprinted structures generally showed that appropriate 3D bioprinter optimization is crucial for achieving desired results. Comparisons of the dimensions of molded structures and bioprinted structures for which the pressure of extrusion was too big revealed that in such case a molded structure would be a better fit. Such comparison for the cylindrical structure is displayed in Table 6.1.

Table 6.1 Comparison between radius sizes of molded cylinder to unoptimized bioprinted cylinder.

Average value of radii of molded cylinders (4 mm), [mm]	**Radius of a cylinder printed at 35 kPa, [mm]**
7.68	12.04

However, when comparing structures printed with properly optimized extrusion pressure to molded ones, the difference in quality is substantial. Overall, bioprinted structures

have a better appearance and structural properties. The process of setting an exact shape for a 3D bioprinter to recreate makes this method significantly more precise.

Regarding the cell structure and morphology after seeding, it is shown on fluorescence images in Figure 5.20. In molded structures the cells were spread more homogeneously over overall volume of hydrogel. This was probably caused by more homogenous mixing of prepared solutions of collagen and cell suspension.

In bioprinted scaffolds the cells were creating some clusters. This can be caused by two factors. Firstly, since the composition of hydrogel contains agents for starting gelation (methacrylated compounds and photo-activator) that can be slightly cytotoxic. Secondly, the extrusion of hydrogel generates small lines of hydrogel that are placed each to each resulting in volumetric shape. The orientation of these lines is affected by slicing software. The pathway produced for creating cylindrical or toroid shape was not following concentric manner but was creating a grid. Based on such settings of slicing software, these can lead to creating non-homogeneous cell spreading.

To sum up, a molding technology is a less costly option that allows to create more complex shapes, and has a better cell spread and homogeneity; nonetheless, bioprinting shows to be a significantly better substitute when the process is suitably optimized. It allows precise hydrogel distribution, has better structural shape, and is more automated, overcoming the limitation of having to utilize special machinery for mold creation.

7 Conclusion

In this work, 3D bioprinting technology of artificial tissue engineering was compared to a more conventional molding technique. For that, the molds for two different shapes were made from a hydrogel consisting of collagen I, cell suspension, and sodium bicarbonate. Hydrogel made from collagen with some photo-activators was 3D bioprinted using Inkredible+ bioprinter by Cellink in the same two shapes.

The mold preparation consisted of 3D modeling in Autodesk Inventor and printing mold forms from two materials – polycarbonate for outer layers and elastic resin for the inner layers. Cell suspension preparation involved adipose derived stem cells culture in sterile laboratory conditions. To make a suitable hydrogel several concentrations were tested, with volumes and cell densities computed with the aid of an Excel sheet. To optimize the bioprinting process several different extrusion pressures were used and the resultant structures have been compared between each other and with the molded structures.

The results showed that with a good printing optimization, which is crucial in a bioprinter, the printed structures have a better precision control, structural stability, and homogeneity. The molding has advantages of enabling creation of more complex structures and creating a more uniform cell distribution, but a bioprinter can be used for mass production due to automaticity and does not require special mold production. This work adds to a growing corpus of research showing that 3D bioprinting is a promising technology and shows why it has gained increasing popularity in the recent years.

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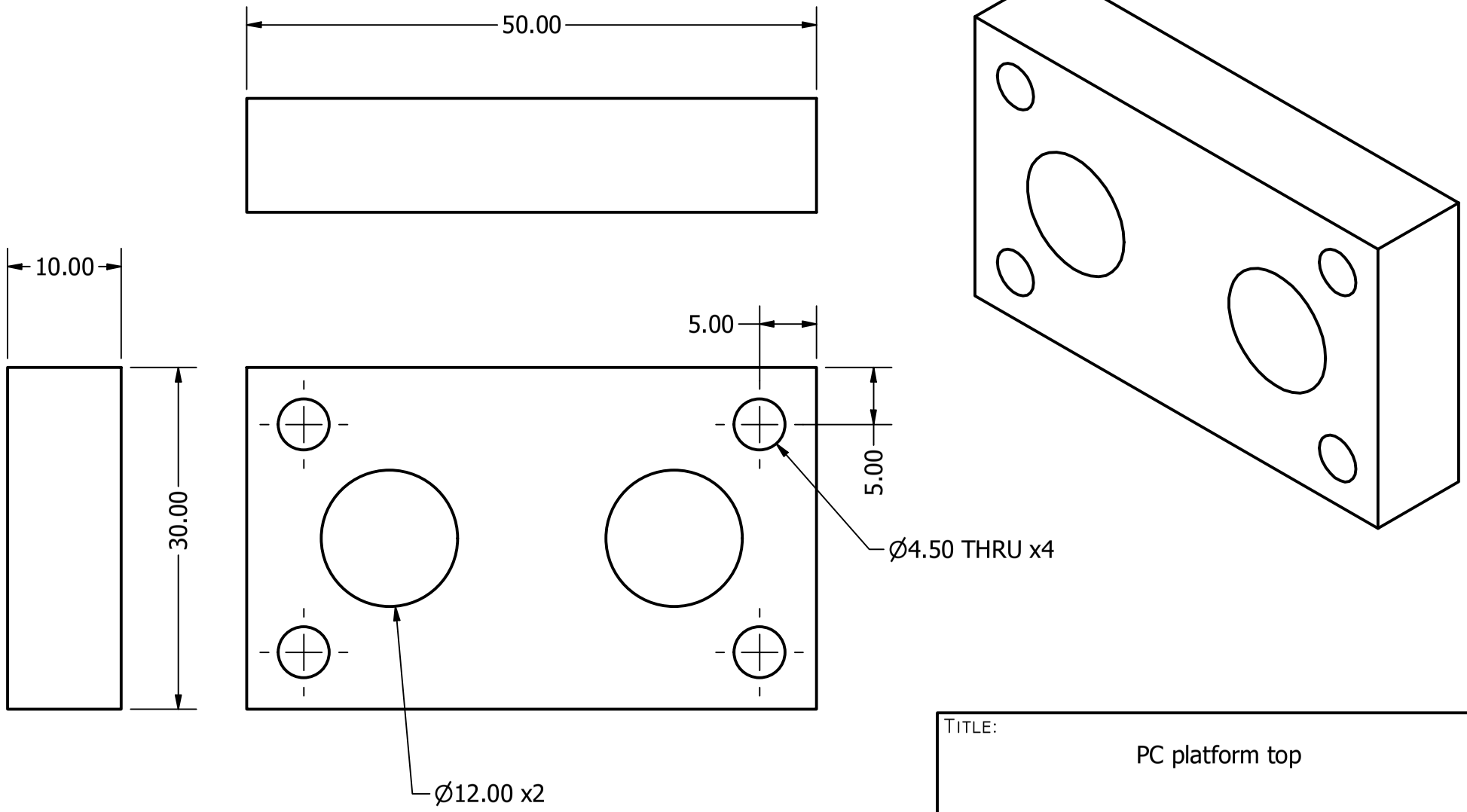
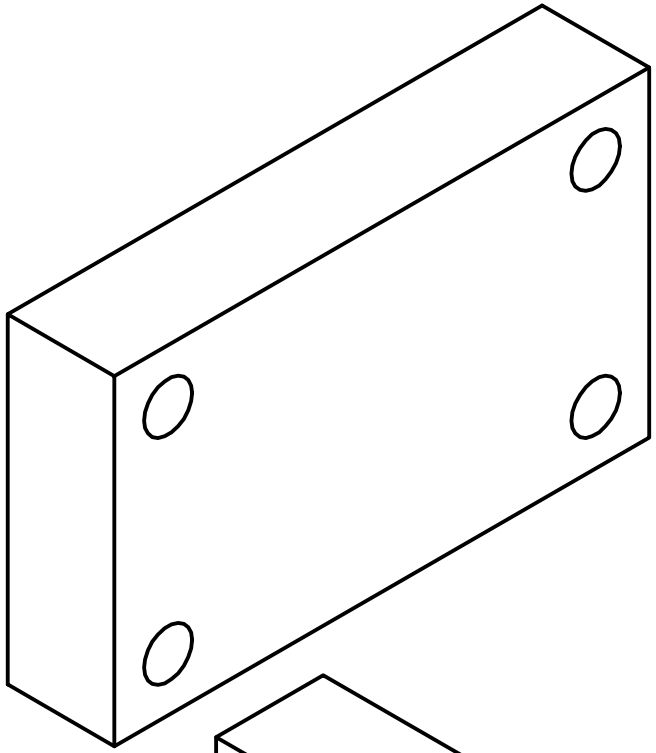
Appendix A: Drawings of parts

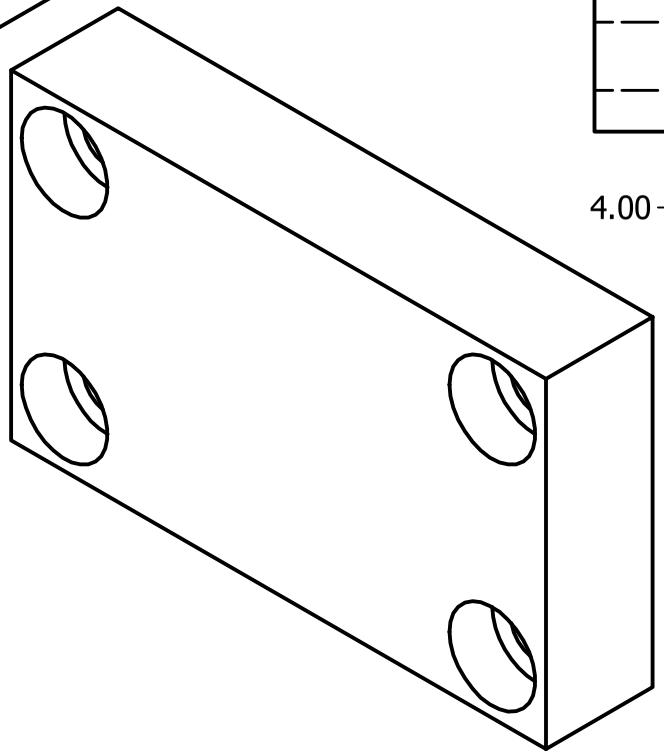
Autodesk Inventor 2020 drawings of mold parts

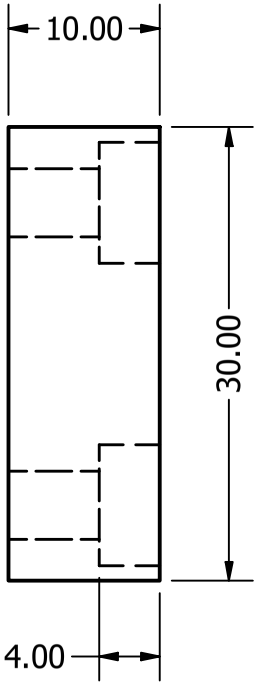
1. PC platform top
2. PC platform bottom
3. Toroid mold form bottom
4. Spacer mold form
5. Cylinder mold form bottom

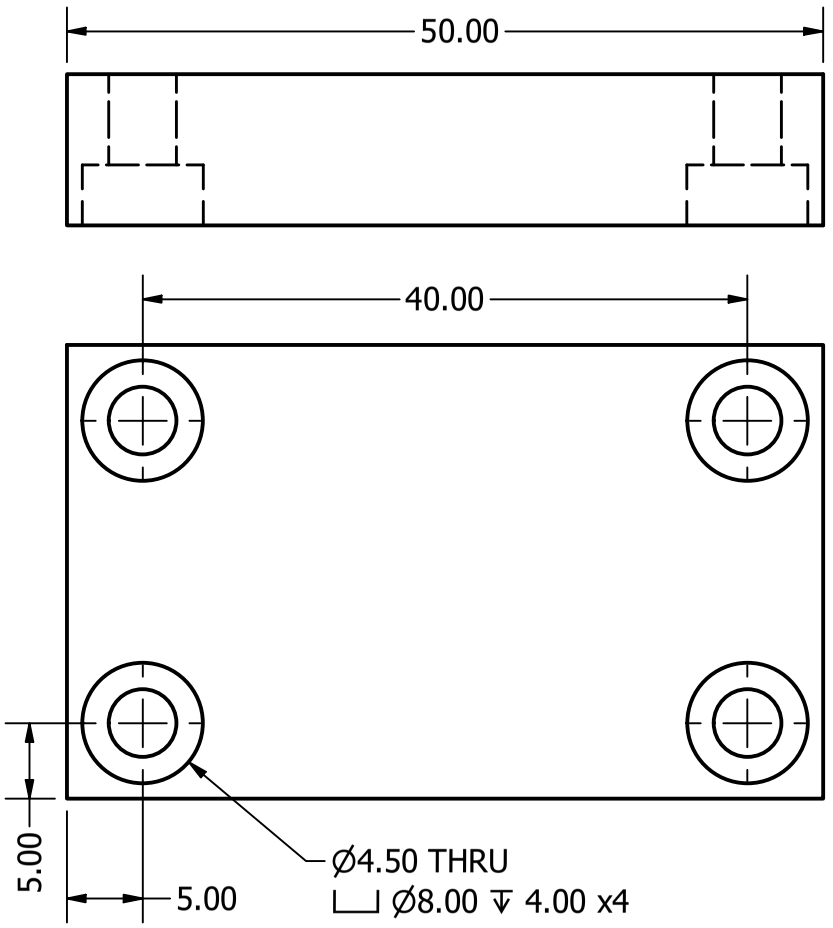


TITLE: PC platform top	
PART NUMBER: 1	MATERIAL: Polycarbonate
SIZE: A4	AUTHOR: A. Talakvadze
SCALE: 2:1	DATE: 04-Mar-20

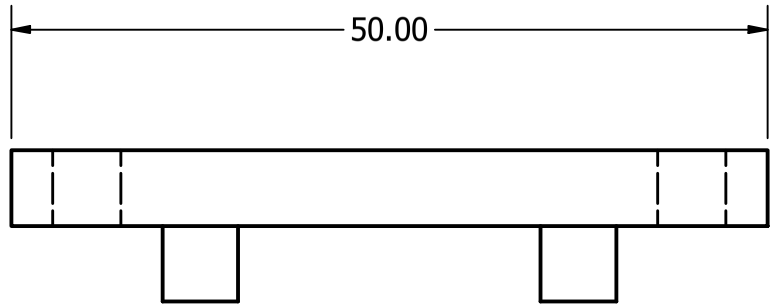


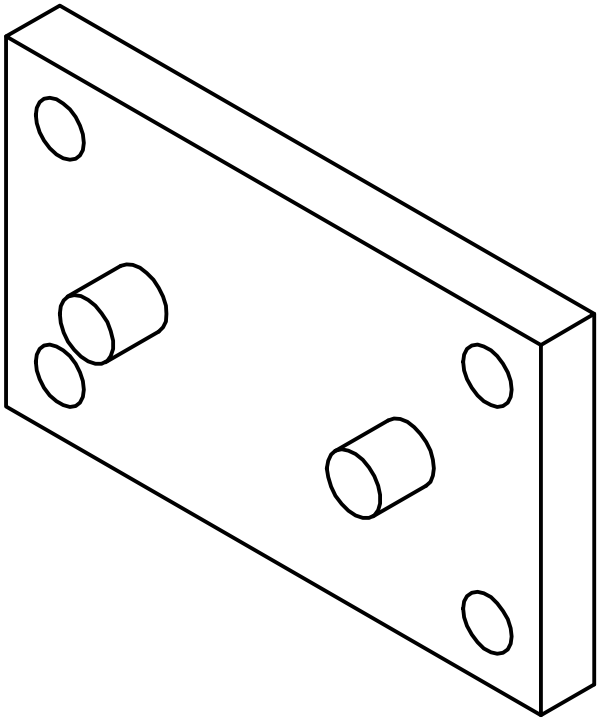


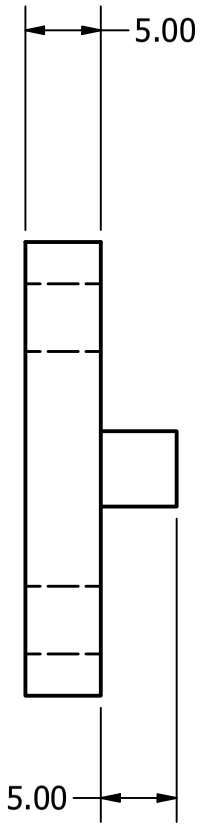


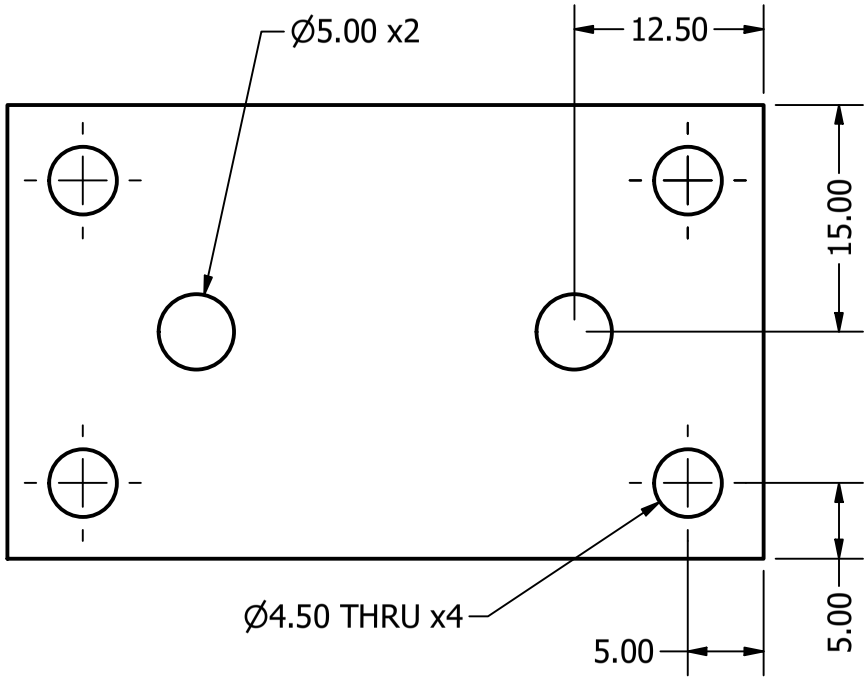


TITLE: PC platform bottom	
PART NUMBER: 2	MATERIAL: Polycarbonate
SIZE: A4	AUTHOR: A. Talakvadze
SCALE: 2:1	DATE: 04-Mar-20

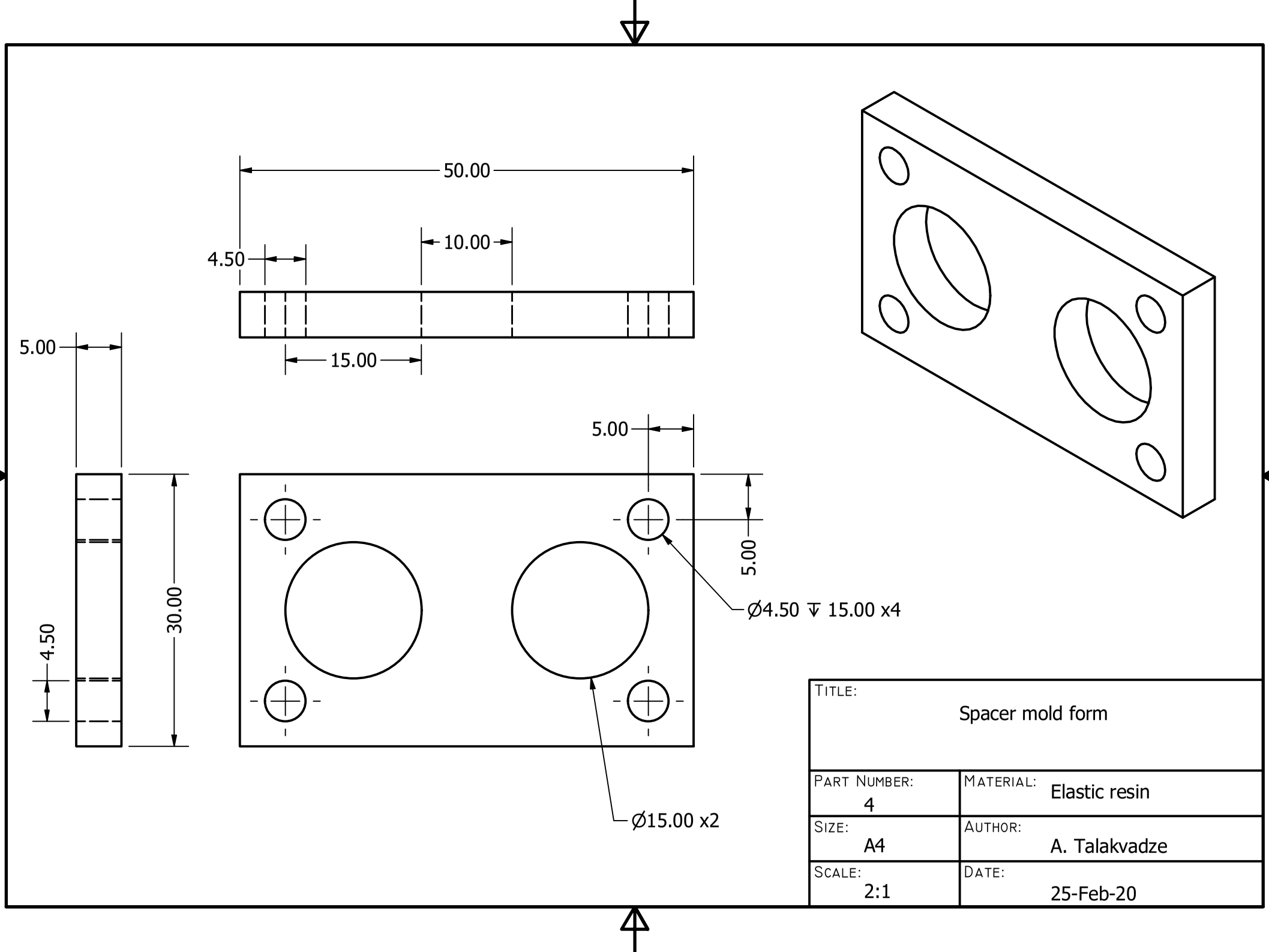




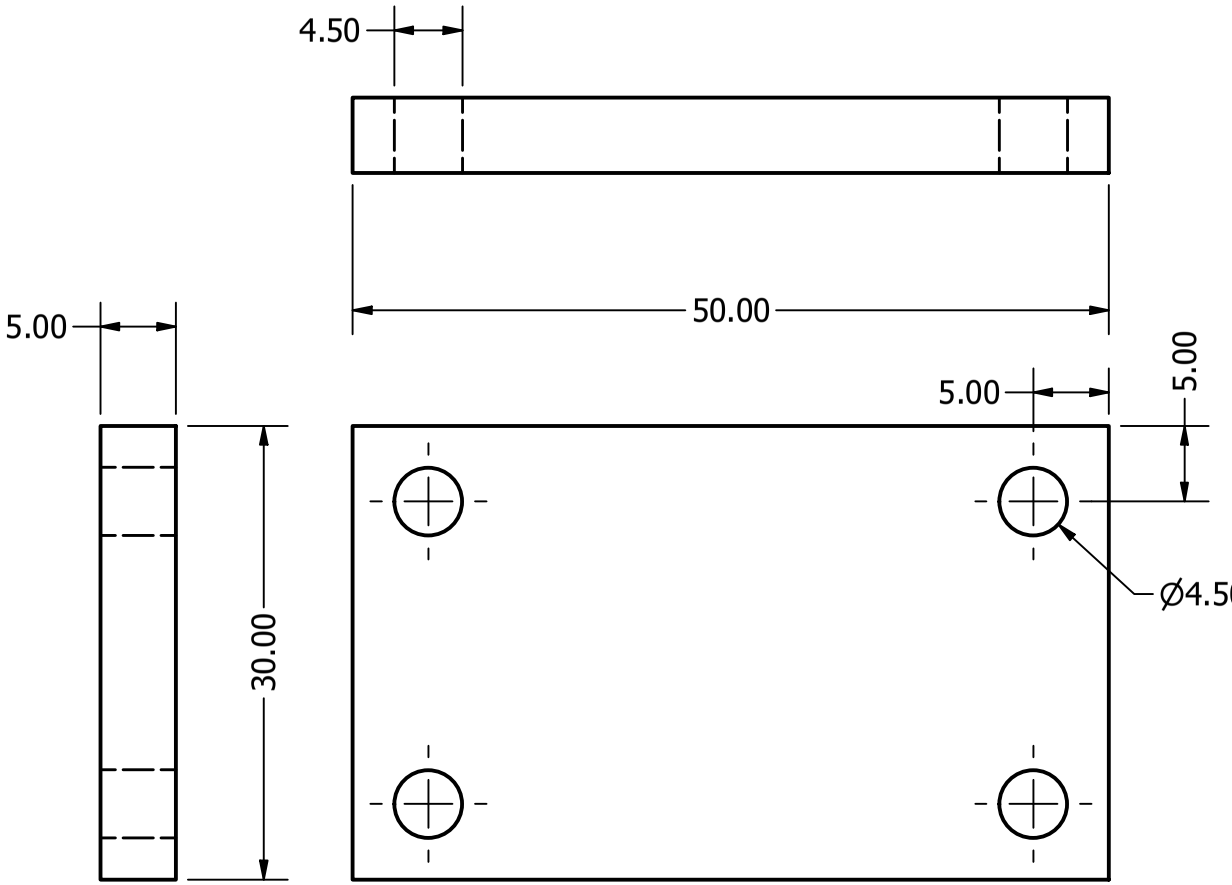


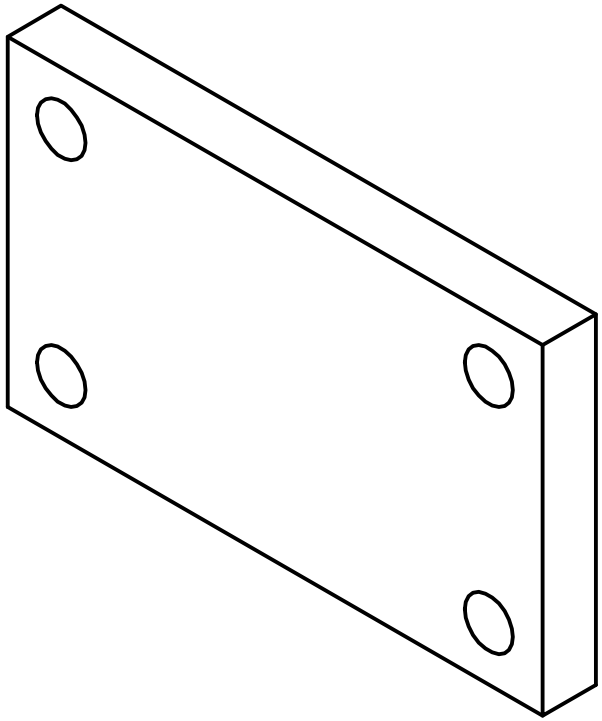


TITLE: Toroid mold form bottom	
PART NUMBER: 3	MATERIAL: Elastic resin
SIZE: A4	AUTHOR: A. Talakvadze
SCALE: 2:1	DATE: 04-Mar-20



TITLE: Spacer mold form	
PART NUMBER: 4	MATERIAL: Elastic resin
SIZE: A4	AUTHOR: A. Talakvadze
SCALE: 2:1	DATE: 25-Feb-20



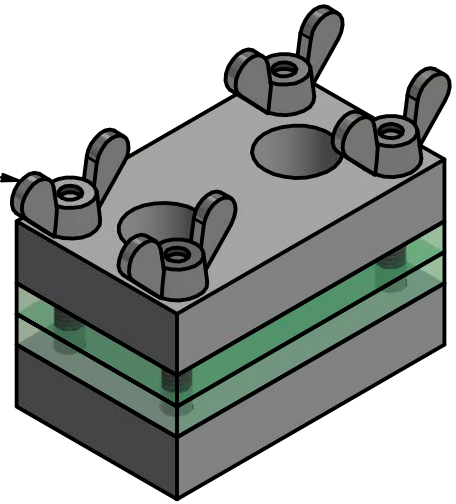


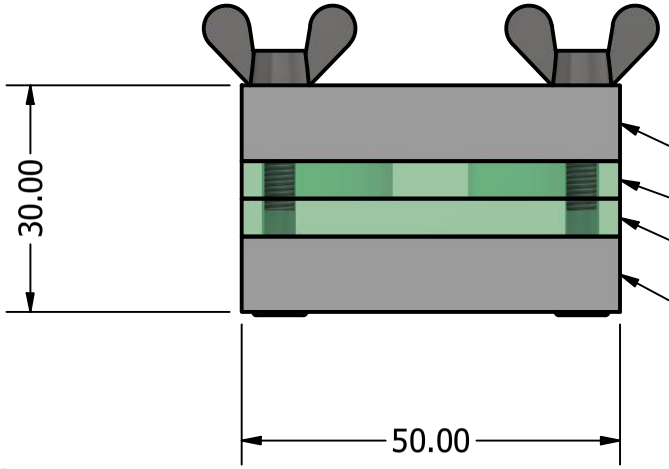
TITLE: Cylinder mold form bottom	
PART NUMBER: 5	MATERIAL: Elastic resin
SIZE: A4	AUTHOR: A. Talakvadze
SCALE: 2:1	DATE: 04-Mar-20

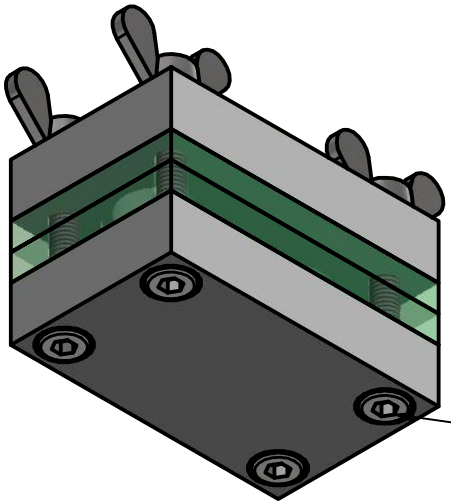
Appendix B: Drawings of assemblies

Autodesk Inventor 2020 drawings of the molds comprised of combined parts, with captions:

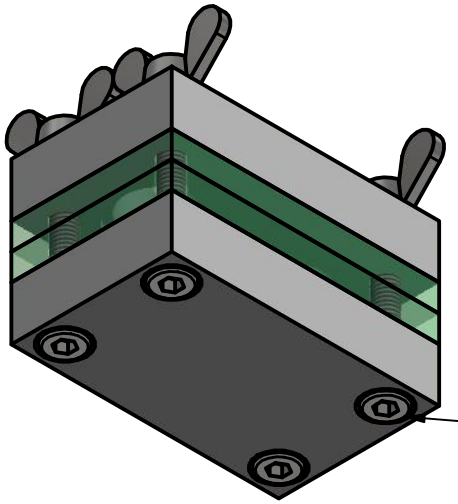
1. Cylinder assembly
2. Toroid assembly

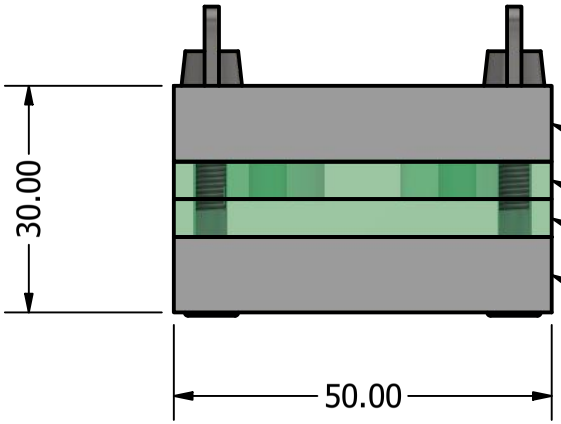


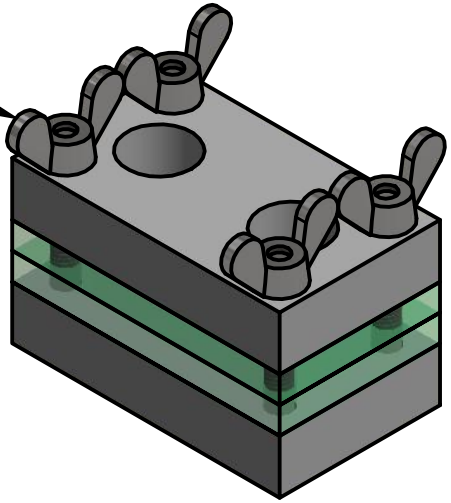




TITLE: Mold for two cylinders	
ASSEMBLY NUMBER: 1	MATERIAL: Polycarbonate, rubber, metal
SIZE: A4	AUTHOR: A. Talakvadze
SCALE: 1:1	DATE: 04-Mar-20







M4 butterfly nut x4

PC platform top

Spacer mold form

Toroid mold form bottom

PC platform bottom

M4x30 screw x4

TITLE: Mold for two toroids	
ASSEMBLY NUMBER: 2	MATERIAL: Polycarbonate, rubber, metal
SIZE: A4	AUTHOR: A. Talakvadze
SCALE: 1:1	DATE: 04-Mar-20

Appendix C: Hydrogel compositions

Excel sheets for hydrogel compositions

1. Well plate

Area of one well [mm^2]	
d =	18.00
r =	9.00
A =	254.47

Height [mm]	
h_1	0.5
h_2	1.0
h_3	2.0

Total volume [mm^3 = µl]	
V_1	127.23
V_2	254.47
V_3	508.94

Calculation of total № of cells per structure, N			
Desired cell density:	75000 [cells/cm^2]		
Size of 1 cells layer:	0.05 [mm]		
	h_1	h_2	h_3
N [number of cells]	1.9 mil	3.8 mil	7.6 mil

Volume ratio	
Cells	0.39
Collagen I	0.60
$NaHCO_3$	0.01
Sum (=1)	**1.00**

ρ, [cells/ml]	38.5 mil

Volume of each component based on ratio [µl]				
	$V_{(cells)}$	$V_{(col)}$	$V_{(bicarb)}$	**Sum (=V)**
V_1	49.6	76.3	1.3	**127.2**
V_2	99.2	152.7	2.5	**254.5**
V_3	198.5	305.4	5.1	**508.9**

2. Toroid

Area of the toroid [mm^2]	
D	15.0
d	5.0
R	7.5
r	2.5
A	157.1

Height [mm]	
h_1	0.5
h_2	1.0
h_3	2.0
h_4	4.0

Total volume [mm^3 = µl]	
V_1	78.5
V_2	157.1
V_3	314.2
V_4	628.3

Total № of cells per structure, N				
Desired cell density:	75000 [cells/cm^2]			
Size of 1 cells layer:	0.05 [mm]			
	h_1	h_2	h_3	h_4
N [number of cells]	1.2 mil	2.4 mil	4.7 mil	9.4 mil

Given volume ratio	
Cells	0.39
Collagen I	0.60
$NaHCO_3$	0.01
Sum (=1)	**1.00**

ρ, [cells/ml]	38.5 mil

Volume of each component based on ratio [µl]				
	$V_{(cells)}$	$V_{(col)}$	$V_{(bicarb)}$	**Sum (=V)**
V_1	30.6	47.1	0.8	**78.5**
V_2	61.3	94.2	1.6	**157.1**
V_3	122.5	188.5	3.1	**314.2**
V_4	245.0	377.0	6.3	**628.3**

3. Cylinder

Area of the cylinder [mm^2]	
d =	15.00
r =	7.50
A =	176.71

Height [mm]	
h_1	0.5
h_2	1.0
h_3	2.0
h_4	4.0

Total volume [mm^3 = µl]	
V_1	88.4
V_2	176.7
V_3	353.4
V_4	706.9

Calculation of total № of cells per structure, N				
Desired cell density:			75000	[cells/cm^2]
Size of 1 cells layer:			0.05	[mm]
	h_1	h_2	h_3	h_4
N [number of cells]	1.3 mil	2.7 mil	5.3 mil	10.6 mil

Volume ratio	
Cells	0.39
Collagen I	0.60
$NaHCO_3$	0.01
Sum (=1)	**1.00**

ρ, [cells/ml]	38.5 mil

Volume of each component based on ratio [µl]				
	$V_{(cells)}$	$V_{(col)}$	$V_{(bicarb)}$	**Sum (=V)**
V_1	34.5	53.0	0.9	**88.4**
V_2	68.9	106.0	1.8	**176.7**
V_3	137.8	212.1	3.5	**353.4**
V_4	275.7	424.1	7.1	**706.9**

Appendix D: Content of the enclosed CD

- Key words
- Abstract in Czech
- Abstract in English
- Scan of the assignment of the topic of the diploma thesis
- The complete diploma thesis