A Bioreactor for chondrocyte differentiation: design, modelling and prototyping

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Abstract

Articular cartilage is a tissue located in diarthrodial joints and responsible for transmission of loads and lubrication of these structures. Current therapeutic methods for this tissue are limited by the quality of the neocartilage and its ability to withstand the physiological loads, long therm. The need for Articular cartilage tissue engineering arises from these limitations. Mechanical stimulation is considered important for the correct differentiation of Mesenchymal stromal cells (MSC) and for maintenance of phenotype of cultured chondrocytes. The most common forms of stimulation are hydrostatic pressure (HP), direct compression (DP) and shear stress. In this work, ideation and development of a bioreactor design is done, considering the mechanical stimulation of articular cartilage tissue engineering constructs. The final bioreactor here proposed is chamber perfused and includes two independent chambers associated with each side of the construct, for osteochondral differentiation. The mechanical stimulation apparatus proposed administers a combination of direct compression with contact shear. This design was called cam for the resemblance with the mechanical part with the same name. The various evolutions of the design were modelled in 3D with the Computer-aided design program Solidworks. This program's computer aided engineering tools were also used for computational fluid dynamics simulation of the perfusion chamber and scaffold, it was concluded that for a typical porous scaffold the interstitial velocity felt by the cells is in the relevant range for MSC differentiation. The bioreactor was partially prototyped, with most parts being constructed by 3D printing. Additionally, a proof-of-concept circuit for driving/ controlling the mechanical stimulation was constructed.

Key words: Bioreactor, joint, cartilage, MSC, chondrocyte, compression, shear

1. Introduction

1.1. Articular cartilage tissue overview

Articular cartilage (AC) is a connective tissue that is mainly found in diarthrodial joints, also classified as synovial joints[1], these joints are a common structure in animals, they are located between skeletal segments and allow the relative motion between such segments. AC is a form of hyaline cartilage located in synovial joints. This tissue's main function is to offer a coating surface with low friction for bones and a medium for mechanical load transfer in the joints[2].

AC is composed of a dense extracellular matrix (ECM) and a relatively sparse population of cells, the chondrocytes [3]. Chondrocytes are essential for the formation and maintenance of

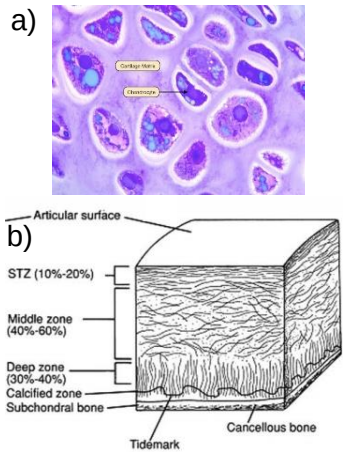

Figure 1 - a) Microscopy image of chondrocyte after toluidine blue staining; b) cross section representation of the collagen fibre architecture in healthy articular cartilage; adapted from Fox, et al (2009) [6]

the tissue, possessing high metabolic activity and synthesizing the ECM components[2]. Chondrocytes are known to differentiate from mesenchymal stem/stromal cell origin[4], an adult stem cell multipotent population that is known to give origin to cells of the skeletal system as well as others.

Articular cartilage structure can be divided in four different zones, distributed along an axis from the surface to the underlying bone: superficial zone, the middle zone, the deep zone, and the calcified zone. These zones/regions are defined by different ECM compositions, ultrastructure and, as mentioned, chondrocyte density[3]. The zones of Articular cartilage are schematically represented in figure 1 b). The ECM of articular cartilage is mostly composed of water, collagen, and proteoglycans (mostly proteoglycan). Collagen (≥90% type II) is the most abundant biomolecule of the ECM being two thirds of the dry mass of tissue [5].

Articular cartilage is an avascular tissue, which means that there is inherently limited mass transfer from and to this tissue. This limits the oxygen availability in the tissue limiting the chondrocyte metabolism. Notoriously, the chondrocyte metabolism, and consequently, the composition of the ECM, are very responsive to the chemical and physical environment[6]. This is observed both *in vivo* and for tissue engineering approaches. This response to the environment allows the maintenance of the ECM homeostasis[2] in response to stimuli. An important part of chondrocyte metabolism regulation is mechanotransduction[7], i.e. the ability of chondrocytes to sense the mechanical properties and forces acting in the cellular environment.

Biomechanical studies of articular cartilage have been mainly motivated by the interaction between mechanical stimulus and chondrocyte phenotype but also by the unusually high loads that this tissue is able to withstand. The high loads experienced in a rotating joint and, to a smaller degree, in resting have implied high contact stress. To counteract these stresses and prevent friction, the tissue is capable of lubricating to a high degree[8].

Ploughing friction, which working principles are schematically shown in figure 2[8], is a main mechanical phenomenon occurring in articular cartilage; it consists of the combination of a load with motion in an orthogonal direction. Joint cartilage is a relatively soft tissue allowing this type of mechanical phenomenon. Ploughing friction is a main stress in articular cartilage and the lubrication response to this is theorized the Biphasic lubrication theory. This theory is based on the assumption that when under strain the water entrapped in the tissue's molecular sized pores flows outwards producing a lubricating effect[9]. The low permeability of the tissue 10^{-15} m^4/Ns [8] is thought to, with the fluid flow, induce a high drag force that combined with the fluid pressurization allow for energy dissipation and load support[8].

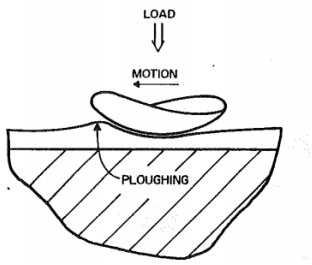

Figure 2 - Illustration of the ploughing effect present in a moving joint, adapted from Mow et al (1993) [8]

Works like those of Sutter et al (2014)[10] and Chan et al (2016)[11] measure the deformation strain of AC in response to dynamic activity with results showing strains on the range of 5% up to 10% on the knee. The forces acting on the knee measured using instrumented tibia prothesis by Kutzner et al (2010)[12] are in the range of 100% up to 350% of bodyweight.

1.2. Articular Cartilage tissue engineering

The structural complexity of articular cartilage and the high prevalence of cartilage pathologies combined with the inherent low repair ability of the tissue and inefficiency of the current available clinical methods are the motivation for the need for novel articular cartilage tissue engineering strategies. The cell sources to produce tissue engineering constructs are mostly chondrocytes and mesenchymal/ stromal cells. Chondrocytes have been used in established therapeutic strategies such ACI, currently there is also success in chondrocyte use to produce

engineered cartilage constructs. Major limitations regarding the use of chondrocytes is their low availability and reduced potential for cell expansion. MSC represent a good alternative to chondrocytes because of their superior availability, expansion capacity and ability to differentiate into cells of the osteoskeletal system including chondrocytes.

The current paradigm in articular cartilage tissue engineering field is that in order to obtain hyaline-like tissue it is required to incorporate cells with biocompatible scaffolds and to recreate a biomimetic microenvironment in terms of chemical and mechanical cues that allow for the differentiation of these cells into the appropriate chondrocyte phenotype[13]. The most commonly used scaffold type in articular cartilage regeneration settings is hydrogels, which are physiologically relevant due to their high water content that mimics the native cartilage [13]. The main hydrogel materials include collagen types I and II, fibrin[14], hyaluronic acid[15], chondroitin sulfate[16], polyethylene glycol (PEG)[17], alginate, and agarose[18].

1.3. Mechanical Stimulation of AC tissue engineering constructs

The use of mechanical stimulation with physiological level forces has various advantages in general it enhances the convection of nutrients and waste products through the tissue[19], it also allows the maintenance of chondrocyte function[20] and modulates chondrogenesis of MSCs[21].

The main forms of mechanical stimulus applied to AC tissue engineering are shear, hydrostatic pressure and direct compression (DC).

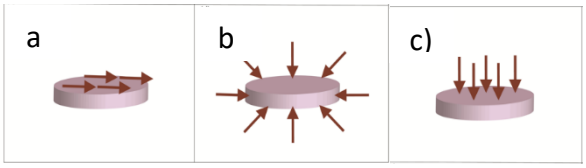

Figure 3 – Schematic representation of the three most common forms of mechanical stimulation used in articular cartilage tissue engineering, the red arrows represent the direction of the forces applied to the tissue engineering constructs; a) shear; b) hydrostatic pressure; c) direct compression; image adapted from[28].

The physiological stress varies in most movement types from 3 to 10MPa[22] but can be as high as 18 MPa in the hip joint[22]. These stresses are translated to hydrostatic pressure as described by the biphasic theory. Hydrostatic pressure is generally applied by injection of a compressed gas into the culture chamber[23] or by the action of a piston [24]. The stimulus can be applied in a dynamic or static regime, when dynamic is used . the frequency is typically 1 Hz the human walking cadence [25]. The magnitude of the stimulus is generally in the physiological range, magnitudes around 5 to 10 MPa [26,27] have been shown to produce good resulting tissue constructs.

AC experiences shear stress during physiological movement moreover fluid shear is a main part of the load absorption in the joint. Frequencies under 1Hz and magnitudes of stress smaller than 0.5 Pa has been found to favor chondrogenic differentiation[28,29]. *In vitro*, generally using purpose-built bioreactors, shear stress is applied in one of two forms: fluid flow over or within the AC construct or through direct contact of the construct surface with an actuating object[30]. Another option that is relatively common is low shear mixing solutions. Rotating microgravity bioreactors are commonly used to apply this type of stimulus[31].

Under physiological conditions, the dynamic mechanical environment includes various transient multi-axial compressive strains[32]. In general, strains lower than 10% have been described as beneficial in static and dynamic compression. Bioreactor designs for static DC are simple. Considering passive DC, the system generally consists of weights placed over the AC constructs, in which the weights are calibrated to produce the desired compressive stress to generate the strain required. The weights are removed during media change and otherwise kept acting over the AC construct[33]. Bioreactors designed for dynamic loading generally use pistons, springs or linear actuators to dynamically load and unload the constructs.

1.4. Bioreactors for AC

Bioreactors are particularly valuable for articular cartilage tissue engineering because they enable the application of different types or physical stimuli, but are also important for enhancing the diffusion to and from the tissue constructs[34]. **Spinner flasks** have been commonly used to expand MSCs in adherent culture systems (e.g., microcarriers), resulting in enhanced mass transport which leads to higher cell densities[35]. **Rotating wall vessels** are composed of a hollow cylinder with a detached support for scaffolds, in which the cylinder is filled with growth media and rotates in its radial axis[36].

The perfusion bioreactor architecture is generally composed by the abovementioned chamber designed to fit the geometry of the scaffold, a media reservoir and a waste container. Another possibility is the use of a closed loop system avoiding the use of a waste reservoir[37]. Perfusion can be done in a direct regime (trough the construct) or chamber perfusion.

Bioreactors for **Hydrostatic pressure** can be continuous or discontinuous depending on the capability co culture the constructs in place[38], the culture can be perfused or static in both cases. Bioreactors employing hydrostatic pressure stimulation have been shown to have a significant effect in inducing cartilage formation. Studies with monolayer chondrocytes have shown improved results with dynamic stimulation[26], Bioreactors for **direct compression** commonly are composed of a vessel containing culture media, an apparatus for fixing scaffolds within the vessel and an actuator.

The current trend in the development of bioreactors for AC tissue engineering employs the use of various forms of mechanical stimulation in simultaneous to mimic as closely as possible the mechanical environment of the synovial joint and native articular cartilage tissue.

The combination of compression and shear stress has been targeted in several studies, including the works of Shahin and Doran[39] and Gharravi et al[40], which are early representatives of this tendency combining direct shear and fluid induced shear, respectively, with direct compression. Another design possibility that has been pursued is the use of a compression piston for compression in the z axis while moving the actuator in the x axis to produce a direct shear stimulation.

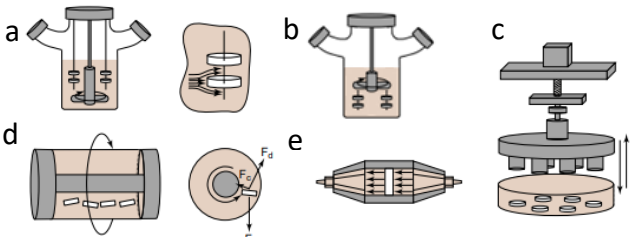

Figure 4 – Schematic representation of traditional bioreactors used for articular cartilage tissue engineering; a) spinner flask; b) rotating bead bioreactor; c) Uniaxial compression apparatus; d) rotating wall vessel; e) perfusion bioreactor. Adapted from Martin et al (2004) [203]

2. Materials and methods

2.1. CAD design

The software used to produce the 3D models was Solidworks 2018 student edition. This software is a solid modelling CAD (computer-aided design) software that includes also CAE (computer aided engineering capabilities). The workflow of producing a 3D model in Solidworks consists in first producing a 2D sketch and extruding this design's features into a 3D space, this extrusion can be done linearly, rotationally around a chosen axis or along a previously established 3D profile. The other main form of modifying the 3D model is to produce a 2D sketch and extrude from this a cut of an already modelled 3D part.

2.2. Computational Fluid Dynamics

As discussed in the previous section Solidworks is not exclusively a CAD program but also includes CAE tools, within these tools is a Fluid simulation plugin. This plugin was used for simulating the flow within a culture chamber of the bioreactor. The first step is to create a new simulation in the Fluid flow tab of the program, for this the wizard option was used. The unit system used was centimeter, gram, dyne (cgd) because it better fits the scale of the problem, the wall roughness was defined as 25 μm[41], Finally, temperature was defined as 37° C, the culture temperature and 1 atm of pressure. The mesh and fluid domain were chosen automatically by the program and were

verified by the tools of this program to fit the domain of interest. The number of fluid cells created is 30799.

Table 1 – Synthesis of the Boundary conditions and number of iterations by CFD assay

Boundary Conditions	Number of iterations
Q_{in}= 2 mL Q_{out}= 2 mL $P_{at\ the\ fluid\ surface}$= 1 atm	150
Q_{in}= 1 mL Q_{out}= 1 mL $P_{at\ the\ fluid\ surface}$= 1 atm	118
Q_{in}= 0.5 mL Q_{out}= 0.5 mL $P_{at\ the\ fluid\ surface}$= 1 atm	118

2.3. Prototyping

For the prototyping of the bioreactor additive manufacturing technique[42] was employed. Construction of the bioreactor components was done by Fused deposition modelling, 3D printing.

The 3D printer used in this work was the Makerbot model Replicator 2x. The filament used was white ABS or Acrylonitrile butadiene styrene of 1.75 mm diameter and density 1.03 g/cm^3 of the brand Velleman.

This thermoplastic, ABS, is appealing for cell culture applications because it is chemically inert and not affected by biological agents [43] and can be suitably sterilized using ethanol[44]. To solve clogging problems, it was decided to operate the machine in colder extruder temperature, at 210° C a value bellow the recommended by the manufacturer for ABS (220° C to 270° C).

Table 2 - 3D printing parameters

Material	Acrylonitrile butadiene styrene (ABS)
Printing Infill	10%
Layer height	0.20 mm
Extrusion head temperature	210° C
Build plate Temperature	100° C
Speed while extruding	40 mm/s outlines; 90 mm/s infill, insets, top and bottom

2.4. Actuation Control/ Drive

For the construction of the actuator control and driving system the materials used were: Arduino mega 2560 microcontroller board, USB cable to connect the board, power source VT-20250 from V-TAC, a A4988 stepper motor driver, a model MT-1704HS168A stepper motor from Motech motor, jumper cables and a 50 µF capacitor. The code for the microcontroller was created in the Arduino specific programing environment Arduino IDE, that includes the Arduino specific C++ programing libraries.

3. Results and Discussion

3.1. Design Objectives

The objective of this thesis work is to propose, design and prototype a bioreactor for articular cartilage tissue engineering. The overall goal for the bioreactor here presented is to produce tissue with hyaline like properties, with clinical and research relevance. The design objectives are based on the literature review briefly presented in the introduction. The first objective is to design a system optimized for mechanical stimulation of AC constructs. Secondly the bioreactor should be able to maintain the optimal culture atmosphere conditions. The Bioreactor should allow the culture of clinically relevant constructs in terms of size. The bioreactor should comply as much as possible with the good manufacturing practices, in particular in terms of avoiding microbiological contaminations. The bioreactor should be capable of parallel integration. Finally, the preferred method of construction is 3D printing.

3.2. Ideation for the bioreactor design

The choice of perfusion is a starting point for the design of the bioreactor. All the suggested designs present some form of prefusion. The advantages of perfusion have been discussed previously. Most important improvement of mass transfer, savings of media volume needed to fill a chamber (in comparison to agitated vessel bioreactor designs), lower need of operator interaction (in comparison to static culture) and introduces fluid induced shear stimulation.

The other forms of stimulation that are proposed are Hydrostatic pressure, direct compression and contact induced shear.

Various initial design options were first explored, in terms of architectures capable of integration of either hydrostatic pressure or direct compression, utilizing more traditional strategies, like the use of pistons (linear actuators) or compressed fluids (hydrostatic pressure). HP and DC were considered redundant stimulus, this is based on the way DC manifests in a porous media, pressurizing the interstitial fluid. For this reason, these two forms of stimulation were not incorporated in any design simultaneously.

The first design that was considered promising and novel enough to be CAD designed was the design presented in Figure 5.

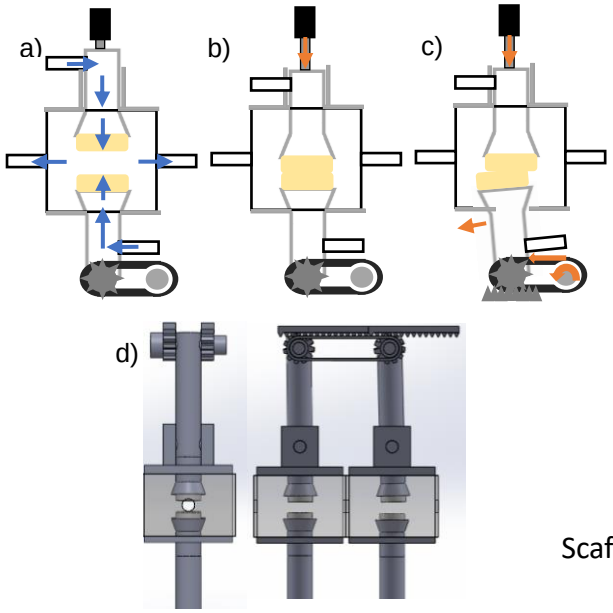

Figure 5 – Cut schematic representation of the joint mimicking bioreactor concept; a) direct perfusion operation similar to the one presented for the previous design concept, in the state without compression and contact shear; b) bioreactor state where the linear actuator is causing compression of both cell-scaffold constructs; c) stimulation state where an electric step motor is activated to trough a belt rotate a gear that is associated with the lower scaffold older, this gear travels through a gear rack that produces a linear motion that is transmitted to the scaffold holder that moves in a pendulum like motion producing surface drag between the two constructs; d) Two different profile views of the 3D model of the joint mimicking bioreactor concept design, produced in Solidworks; the → (blue arrow) represents the direction of flux, the yellow box represents the scaffold, grey represents the mechanically actuating parts, ; the → (orange arrow) represents the direction of movement of a component,

This design is inspired by type of movement provided by a pendulum that has the potential to mediate contact shear using simpler mechanisms for creating a rotating motion, mechanically simpler.

The compression stimulation is produced by the movement of a linear actuator that pushes the top scaffold holder against the lower scaffold and the lower scaffold holder rotates in a pendulum like movement sliding over the other scaffold producing contact shear. This design contains two scaffolds in the chamber, that act as mutual actuators and are under direct perfusion. This design is capable of multiple stimulus (direct compression, fluid induced shear, contact shear), but is still possible to do parallel integration of various chambers. This design was not pursued further because of practical considerations about the design of the scaffold holder and the sealing of the gap for the movement of the actuators. Using two scaffolds is interesting for recapitulating the joint anatomy but is not necessarily useful for clinical application, this is because GMP do not allow the culture of two different patient's constructs in the same chamber.

The next design seriously pursued was the rotor bioreactor design. This design was developed from the interest of exploring new geometries for the bioreactor. This concept is presented in Figure 6.

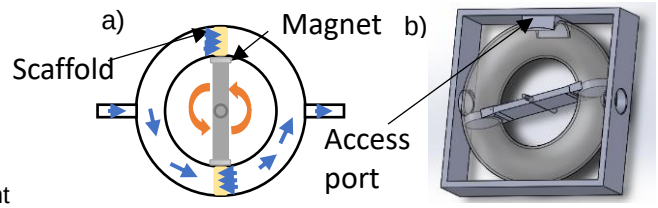

Figure 6 – The rotor bioreactor concept, a perfusion system; a) schematic cut representation of the rotor bioreactor concept , it is composed of a donut shaped culture chamber, a rotor with two "arms" in the centre of the donut, this arms are magnetically clamped to magnetic scaffold holder, this rotor rotating makes the scaffold travel in the culture chamber in a circular pattern; b) profile of the bioreactor. → (blue arrow) represents the direction of flux, the yellow box represents the scaffold, grey represents the mechanically actuating parts, ; the → (orange arrow) represents the direction of movement of a component

This bioreactor includes exclusively perfusion, that means only fluid induced shear stimulus are applied. This is a step back compared to the previous design. In this design

the scaffolds move around in the media and are latched to the revolving rotor by a magnetic latching system. This system has the advantages of being self-pumped avoiding the use of external pumps, that are potential contamination sources, parallel integration is also very simple. The design nevertheless as the disadvantage in variety of mechanical stimuli and the magnetic latching system is a difficult design problem. For this reason, the next design will build on the ideas presented in the previous designs but will be a different approach.

The next and final design concept is the cam bioreactor. The inspiration of this design is the pendulum motion already referred. It was noted that the deformation of the scaffold follows a circular profile. For this reason, a mathematical description was developed of this phenomenon, the conclusion was that for a scaffold 4 mm thick and with maximum 10% strain the pendulum arm would need to be 10 cm to achieve an average strain of 90% the maximum.

The pendulum is modified to be a cam, the cam is a mechanical part used in applications of mechanical engineering like the internal combustion engine, in this application it is used for periodically actuating over another part, which is essentially the objective of this design. In general, the cam rotates over the scaffold creating a combination of compression and contact shear stimulus simultaneously. The design followed an iterative process and the final state is presented in Figure 7. One of the culture chambers inundated with media is located at the bottom of the structure containing the cam actuator. This design has the main feature of including another culture chamber. This second chamber is associated to the bottom side of the AC construct, with a separate culture media circuit, this allows for the use of chondrogenic and osteogenic media respectively to produce osteochondral constructs. The construct is fixed to the lower culture chamber, this culture chamber is associated with a system of screws and gears that allows fine tuning of the position of the scaffold in relation to the actuator. This design includes places for the attachment of tubing fittings that can be associated to an external pump or a future purpose-built pump.

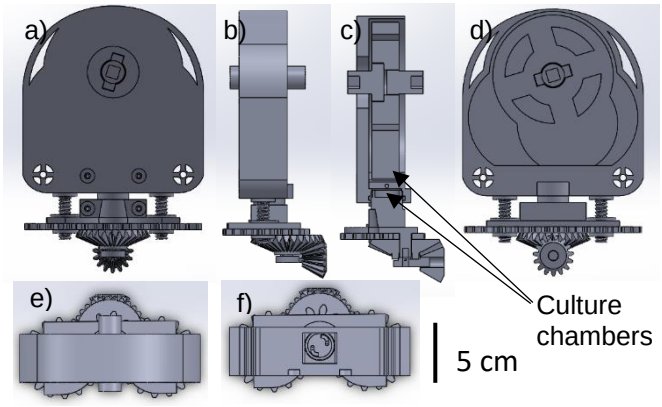

Figure 7- 3D models of the final bioreactor design; a) back view of the bioreactor showing the spot for entrance and exit tube fitting fixing on each of the chambers; b) lateral view of the bioreactor; c) cut of the lateral view of the bioreactor, on the top it shows the detail on the fixing of the actuator to the axel, lower the cut of the culture chambers is visible; d) front view of the bioreactor; e) top view of the bioreactor; f) top view cut of the bioreactor exposing the view of the culture chamber

3.3. Computer Fluid dynamics analysis

To obtain a preview of the effects of various volumetric flows of perfusion in the bioreactor CFD or computational fluid dynamics modelling was done. This allows to, in some degree, have a beforehand idea of the effects of the fluid perfusion on the cell fate. The Frontier conditions are presented in Table 1, the fluid mesh included 30799 cells and the stated goals for the calculation were dynamic pressure in the fluid, average velocity, total forces on walls, shear stress and localized in the scaffold surface the velocity, force felt and shear stress. The results of velocity profile in a cut are presented in figure 8.

To investigate the velocity felt by cells within a porous scaffold the simulations were repeated but the model for the scaffold was updated with 200 µm pours added. The velocity of fluid within the pours was calculated as: for 2 mL/s around $3x10^{-5}$ mm/s, for 1 mL/s was $2x10^{-5}$ mm/s and for 0.5 mL/s approximately $1x10^{-5}$ mm/s. According to the model of Pendergast et al (1997)[45] modified by Stops et al (2010)[46] velocity in a 10% strain operating bioreactor needs to be from 0 to $6x10^{-4}$ mm/s, to be in chondrogenic conditions, for this reason all the volumetric flow conditions tested should contribute fluid induced shear in the correct range for chondrogenesis of MSC.

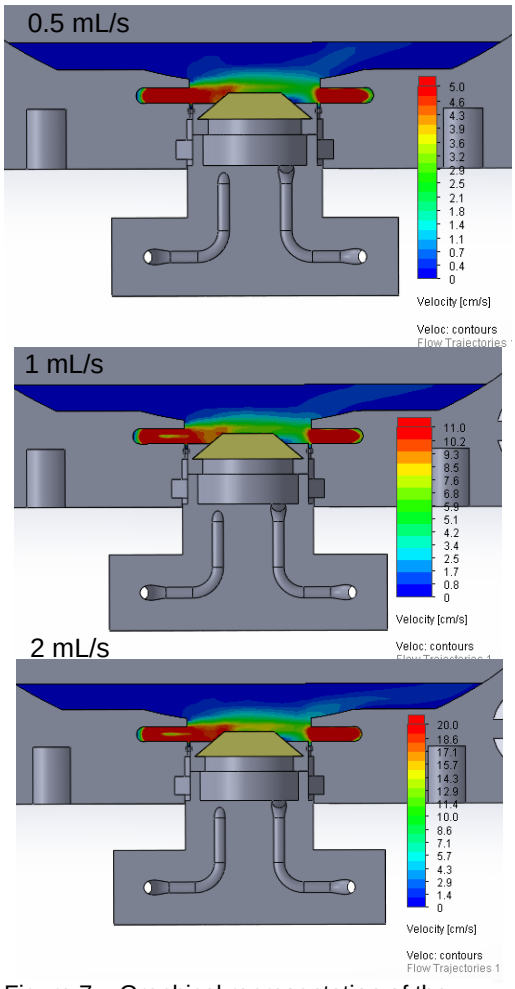

Figure 7 – Graphical representation of the results of CFD, velocity profile in a cut

3.4. Prototyping

Prototyping of the bioreactor design was done by 3D printing of the parts designed in Solidworks, and according to the final design presented in the ideation section. In general, good results were obtained in the 3D printed parts, many of them required various attempts. The parts were tested for leaks an none were found, which was one of the most likely points of failure of 3D printing. The main errors done in the process were not taking into account the tolerances needed due to the inherent error associated with the printing and printing errors, most importantly of the taller and finer parts. The prototype was not completed lacking in the association of the mechanical actuation system with the control system, and a chamber cover was also not prototyped. The control system for the actuation was done at a proof of concept level with the use of Arduino based circuit, the circuit was based on a similar circuit schematics[47] and the movement was programed in the Arduino IDE program.

Figure 8 - Final Bioreactor prototype

4. Conclusions and future work

The ideation process was successful as the final design proposed is capable and has some novelty value and has no direct comparison is available in the literature. Possibly the most comparable design are the ones done by Vainieri et al[48] (2018), Shahin et al.[39] (2012), Bilgen et al[49] (2013), all of these works incorporate direct compression and contact shear. This design as the advantage comparing to these published designs of incorporating perfusion and having a simple and inexpensive construction. Additionally, the incorporation of two chambers for osteochondral construct culture and stimulation is a major novelty factor. The prototyping was not fully completed due to the time constraints and the time spent in the ideation process that was longer than expected.

Future work would consist of finishing the prototyping of the drive and control system of the actuator, confirmation of the mechanical forces applied on a scaffold by the actuation system by finite elements simulation and finally testing of the bioreactor with a cell-scaffold construct.

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References

1. Anatomy & physiology. *OpenStax College* (2013).
2. Sophia Fox, A. J., Bedi, A. & Rodeo, S. A. The basic science of articular cartilage: Structure, composition, and function. *Sports Health* (2009) doi:10.1177/1941738109350438.
3. Stockwell, R. A. Chondrocytes. *Journal of clinical pathology. Supplement (Royal College of Pathologists)* (1978) doi:10.1385/1-59259-018-7:267.
4. Alford, J. W. & Cole, B. J. Cartilage restoration, part 1: Basic science, historical perspective, patient evaluation, and treatment options. *American Journal of Sports Medicine* (2005) doi:10.1177/0363546504273510.
5. Eyre, D. Articular cartilage and changes in Arthritis: Collagen of articular cartilage. *Arthritis Res* (2002) doi:10.1186/ar380.
6. Coates, E. E. & Fisher, J. P. Phenotypic variations in chondrocyte subpopulations and their response to in vitro culture and external stimuli. *Annals of Biomedical Engineering* (2010) doi:10.1007/s10439-010-0096-1.
7. Wang, N. Review of cellular mechanotransduction. *Journal of Physics D: Applied Physics* (2017) doi:10.1088/1361-6463/aa6e18.
8. Mow, V. C., Ateshian, G. A. & Spilker, R. L. Biomechanics of diarthrodial joints: A review of twenty years of progress. *Journal of Biomechanical Engineering* (1993) doi:10.1115/1.2895525.
9. Katta, J., Jin, Z., Ingham, E. & Fisher, J. Biotribology of articular cartilage-A review of the recent advances. *Med. Eng. Phys.* (2008) doi:10.1016/j.medengphy.2008.09.004.
10. Sutter, E. G. *et al.* In Vivo Measurement of Localized Tibiofemoral Cartilage Strains in Response to Dynamic Activity. *Am. J. Sports Med.* (2015) doi:10.1177/0363546514559821.
11. Chan, D. D. *et al.* In vivo articular cartilage deformation: Noninvasive quantification of intratissue strain during joint contact in the human knee. *Sci. Rep.* (2016) doi:10.1038/srep19220.
12. Kutzner, I. *et al.* Loading of the knee joint during activities of daily living measured in vivo in five subjects. *J. Biomech.* (2010) doi:10.1016/j.jbiomech.2010.03.046.
13. Tan, A. R. & Hung, C. T. Concise review: Mesenchymal stem cells for functional cartilage tissue engineering: Taking cues from chondrocyte-based constructs. *Stem Cells Translational Medicine* (2017) doi:10.1002/sctm.16-0271.
14. Deponti, D. *et al.* Collagen scaffold for cartilage tissue engineering: The benefit of fibrin glue and the proper culture time in an infant cartilage model. *Tissue Eng. - Part A* (2014) doi:10.1089/ten.tea.2013.0171.
15. Zhu, M., Feng, Q., Sun, Y., Li, G. & Bian, L. Effect of cartilaginous matrix components on the chondrogenesis and hypertrophy of mesenchymal stem cells in hyaluronic acid hydrogels. *J. Biomed. Mater. Res. - Part B Appl. Biomater.* (2017) doi:10.1002/jbm.b.33760.
16. Varghese, S. *et al.* Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells. *Matrix Biol.* (2008) doi:10.1016/j.matbio.2007.07.002.
17. Bryant, S. J. & Anseth, K. S. Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels. *J. Biomed. Mater. Res.* (2002) doi:10.1002/jbm.1217.
18. Awad, H. A., Wickham, M. Q., Leddy, H. A., Gimble, J. M. & Guilak, F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* (2004) doi:10.1016/j.biomaterials.2003.10.045.
19. Albro, M. B. *et al.* Dynamic loading of deformable porous media can induce active solute transport. *J. Biomech.* (2008) doi:10.1016/j.jbiomech.2008.08.023.
20. Buckwalter, J. A. & Mankin, H. J. Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instructional course lectures* (1998).
21. Panadero, J. A., Lanceros-Mendez, S. & Ribelles, J. L. G. Differentiation of mesenchymal stem cells for cartilage tissue engineering: Individual and synergetic effects of three-dimensional environment and mechanical loading. *Acta Biomaterialia* (2016) doi:10.1016/j.actbio.2016.01.037.
22. Hodge, W. A. *et al.* Contact pressures from an instrumented hip endoprosthesis. *J. Bone Jt. Surg. - Ser. A* (1989) doi:10.2106/00004623-198971090-00015.
23. Urban, J. P. G. The chondrocyte: A cell under pressure. *Rheumatology* (1994) doi:10.1093/rheumatology/33.10.901.
24. Mizuno, S., Watanabe, S. & Takagi, T. Hydrostatic fluid pressure promotes cellularity and proliferation of human dermal fibroblasts in a three-dimensional collagen gel/sponge. *Biochem. Eng. J.* (2004)

doi:10.1016/j.bej.2003.09.019.
25. Waters, R. L., Lunsford, B. R., Perry, J. & Byrd, R. Energy-speed relationship of walking: Standard tables. *J. Orthop. Res.* (1988) doi:10.1002/jor.1100060208.
26. Duraine, G. D. & Athanasiou, K. A. ERK activation is required for hydrostatic pressure-induced tensile changes in engineered articular cartilage. *J. Tissue Eng. Regen. Med.* (2015) doi:10.1002/term.1678.
27. Toyoda, T. *et al.* Hydrostatic Pressure Modulates Proteoglycan Metabolism in Chondrocytes Seeded in Agarose. *Arthritis Rheum.* (2003) doi:10.1002/art.11250.
28. Salinas, E. Y., Hu, J. C. & Athanasiou, K. A Guide for Using Mechanical Stimulation to Enhance Tissue-Engineered Articular Cartilage Properties. *Tissue Eng. - Part B Rev.* (2018) doi:10.1089/ten.teb.2018.0006.
29. Gemmiti, C. V. & Guldberg, R. E. Shear stress magnitude and duration modulates matrix composition and tensile mechanical properties in engineered cartilaginous tissue. *Biotechnol. Bioeng.* (2009) doi:10.1002/bit.22440.
30. sharifi, N. & Gharravi, A. M. Shear bioreactors stimulating chondrocyte regeneration, a systematic review. *Inflamm. Regen.* (2019) doi:10.1186/s41232-019-0105-1.
31. Mellor, L. F., Baker, T. L., Brown, R. J., Catlin, L. W. & Oxford, J. T. Optimal 3D culture of primary articular chondrocytes for use in the rotating wall vessel bioreactor. *Aviat. Sp. Environ. Med.* (2014) doi:10.3357/ASEM.3905.2014.
32. Anderson, D. E. & Johnstone, B. Dynamic mechanical compression of chondrocytes for tissue engineering: A critical review. *Frontiers in Bioengineering and Biotechnology* (2017) doi:10.3389/fbioe.2017.00076.
33. Elder, B. D. & Athanasiou, K. A. Effects of confinement on the mechanical properties of self-assembled articular cartilage constructs in the direction orthogonal to the confinement surface. *J. Orthop. Res.* (2008) doi:10.1002/jor.20480.
34. Martin, I., Obradovic, B., Freed, L. E. & Vunjak-Novakovic, G. Method for Quantitative Analysis of Glycosaminoglycan Distribution in Cultured Natural and Engineered Cartilage. *Ann. Biomed. Eng.* (1999) doi:10.1114/1.205.
35. Ferrari, C. *et al.* Investigation of growth conditions for the expansion of porcine mesenchymal stem cells on microcarriers in stirred cultures. *Appl. Biochem. Biotechnol.* (2014) doi:10.1007/s12010-013-0586-3.
36. Brunelli, M., Perrault, C. & Lacroix, D. A Review of Bioreactors and Mechanical Stimuli. in (2019). doi:10.1007/978-981-10-8075-3_1.
37. Mabvuure, N., Hindocha, S. & S. Khan, W. The Role of Bioreactors in Cartilage Tissue Engineering. *Curr. Stem Cell Res. Ther.* (2012) doi:10.2174/157488812800793018.
38. Zvicer, J. & Obradovic, B. Bioreactors with hydrostatic pressures imitating physiological environments in intervertebral discs. *Journal of Tissue Engineering and Regenerative Medicine* (2018) doi:10.1002/term.2533.
39. Shahin, K. & Doran, P. M. Tissue engineering of cartilage using a mechanobioreactor exerting simultaneous mechanical shear and compression to simulate the rolling action of articular joints. *Biotechnol. Bioeng.* (2012) doi:10.1002/bit.24372.
40. Gharravi, A. M. *et al.* Design and fabrication of anatomical bioreactor systems containing alginate scaffolds for cartilage tissue engineering. *Avicenna J. Med. Biotechnol.* (2012).
41. Hartcher-O'Brien, J., Evers, J. & Tempelman, E. Surface roughness of 3D printed materials: Comparing physical measurements and human perception. *Mater. Today Commun.* (2019) doi:10.1016/j.mtcomm.2019.01.008.
42. Wong, K. V. & Hernandez, A. A Review of Additive Manufacturing. *ISRN Mech. Eng.* (2012) doi:10.5402/2012/208760.
43. Su, C. K., Yen, S. C., Li, T. W. & Sun, Y. C. Enzyme-Immobilized 3D-Printed Reactors for Online Monitoring of Rat Brain Extracellular Glucose and Lactate. *Anal. Chem.* (2016) doi:10.1021/acs.analchem.6b00272.
44. Neches, R. Y., Flynn, K. J., Zaman, L., Tung, E. & Pudlo, N. On the intrinsic sterility of 3D printing. *PeerJ* (2016) doi:10.7717/peerj.2661.
45. Prendergast, P. J., Huiskes, R. & Søballe, K. Biophysical stimuli on cells during tissue differentiation at implant interfaces. *J. Biomech.* (1997) doi:10.1016/S0021-9290(96)00140-6.
46. Stops, A. J. F., Heraty, K. B., Browne, M., O'Brien, F. J. & McHugh, P. E. A prediction of cell differentiation and proliferation within a collagen-glycosaminoglycan scaffold subjected to mechanical strain and perfusive fluid flow. *J. Biomech.* (2010) doi:10.1016/j.jbiomech.2009.10.037.
47. Bakker, B. de. How to control a stepper motor with A4988 driver and Arduino. *Makerguides.com* https://www.makerguides.com/a4988-stepper-motor-driver-arduino-tutorial/.
48. Vainieri, M. L., Wahl, D., Alini, M., van Osch, G. J. V. M. & Grad, S. Mechanically stimulated osteochondral organ culture for evaluation of biomaterials in cartilage repair studies. *Acta Biomater.* (2018) doi:10.1016/j.actbio.2018.09.058.
49. Bilgen, B., Chu, D., Stefani, R. & Aaron, R. K. Design of a biaxial mechanical loading bioreactor for tissue engineering. *J. Vis. Exp.* (2013) doi:10.3791/50387.