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Development of an innovative bioreactor system for human bone tissue engineering

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Abstract

In the last decades significant progress has been carried out leading to significant advances in the development of engineered tissues, thanks to taking into account three fundamental components: the cells to address tissue formation, a scaffold useful as substrate for tissue growth and development, growth factors and/or biomechanical stimuli to address the differentiation of cells within the scaffolds. In particular, mechanical stimuli are known to play a key role in bone tissue formation and mineralization.

Mechanical actuators, namely bioreactor systems, can be used to enhance *in vitro* culture steps in the overall cell-based tissue engineering strategy of expanding *in vitro* a stem cell source to be cultured and differentiated on a three-dimensional scaffold, aiming at implanting this scaffold *in vivo*.

The purpose of this study is thus to design a stand-alone perfusion/ compression bioreactor system. The developed prototypal system allows to apply physical stimuli mimicking native loading regimens. The results obtained in human bone marrow stem cells (hBMSCs) onboard of a 3D graphene/chitosan scaffold indicate that their exposure to a controlled dynamic environment is suitable to address bone tissue commitment.

Abstract

Undanfarana áratugi hefur orðið mikil framför á sviði vefjaverkfræði og frumumeðferða. Það má helst nefna þrjá þætti sem hafa leitt til þessara framfara: mikilvægar uppgötvanir í frumuræktunum, þróun og hönnun á lífstoðefnum sem nota má í burðarvirki meðal annars til að örva vefjamyndun í gegnum mekaníska og lífræna örvun. Mekaníska örvunin er talin vera sérstaklega mikilvæg þegar kemur að því að hanna og smíða beinvef, sérstaklega þegar kemur að steinefnamyndun í frumunum og myndun á utanfrumuefni sem gegnir lykilhlutverki í lífeðlisfræðilegri virkni beinvefs.

Tæki sem hægt er að nota til að örvar frumuvöxt og beinmyndun munu í framtíðinni gegna mikilvægu hlutverki í þróun beinvefjar sem ætlað er að gegna hlutverki í læknisfræðilegri meðferð.

Markmið þessarar doktorsritgerðar var að þróa og smíða tæki (bioreactor) sem getur líkt eftir þeim áhrifum sem frumur verða fyrir í beinmyndun. Um er að ræða tæki sem þarf ekki að staðsetja inni í frumuræktunarskáp og getur örvað vefjmyndunina bæði með mekanískum hætti og flæði sem tryggir næringu innan 3D burðarvirkisins. Heildarniðurstaða verkefnisins var að mesenkímal stofnfrumur fjölga sér og sérhæfast betur þegar þær eru ræktaðar á grafín/ kítósan burðarvirki í tækinu heldur en þegar þær eru ekki ræktaðar í slíku tæki. Um er að ræða tæki sem gæti nýst við rannsóknir á beinmyndun í framtíðinni.

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List of abbreviations

a-CaP amorphous Calcium Phosphate

ADSCs Adipose Stem Cells

ALP alkaline phosphate

BM-MSCs Bone Marrow Mesenchymal Stem Cells

BMP Bone Morphogenetic Protein

CAD Computer-Aided Drafting

CaP Calcium Phosphate

CHT Chitosan

CHT/G Chitosan Graphene

CU Control Unit

DBM decalcified bone matrix

DEX Dexamethasone

DMEM Dulbecco's Modified Eagle Medium

ES Embryonic Stem

FEM Finite Element Model

G gelatin

GUI Graphical Unit Interface

HA hydroxyapatite

hBMSCs human Bone Marrow Stromal Cells

hMSCs human Bone Marrow Mesenchymal Stem Cells

hMPCs human Mesenchymal Progenitor Cells

iPSCs induced Pluripotent Stem Cells

MMP-1 Matrix MetalloProteinase-1

PCL Polycaprolactone

PLA Poly Lactic Acid

PLGA Poly Lactic-co-Glycolic Acid

rMSC rat bone marrow derived stromal cells

SF Silk Fibroin

TCP Tricalcium Phosphate (PCL/TCP)

TPS tubular perfusion system

1. Introduction

Although survival expectancy of the European population has increased to a median age of 75 years for men and 82 years for women, inappropriate lifestyle factors lead to a sharp increase in obesity and poor physical activity triggering related bone disorders [1].

Bone repair and regeneration via tissue engineering strategies are thus considered a significant clinical options, given that a number of studies reported complications, shortcomings and limitations using autologous and allogenic functional bone grafts [2-8].

In fact, albeit autografts have the most important properties required for a bone graft material (histocompatible, non-immunogenic and endowed with fundamental elements to achieve osteoconduction (i.e. scaffolds), osteoinduction (i.e. specific growth, as BMPs) and osteogenesis (i.e. osteoprogenitor cells), they show a number of disadvantages. As an example, they require two operations, one to obtain the patient own bone tissue (normally extracted from the iliac crest) and one to implant the new tissue construct [9], which makes this kind of transplant expensive both from an economic point of view and for the donor site injury [10-12]. Furthermore, this treatment may be not useful in case of a large defect.

Allografts might be used to solve these limits, in particular as bone tissue is often obtained from a cadaver. However, allografts show disadvantages such as immunogenic reactions, risk of infection, limited osteoinduction, in addition to the requirement of a treatment to devitalize the cell component (i.e. freeze drying or irradiation) [13-15].

Other techniques, including the use of bone cement fillers, distraction osteogenesis or bone morphological proteins are also affording good results in terms of bone repair, although none possess all of the ideal characteristics: no size restrictions, biological safety, long shelf life, high osteoinductive and angiogenic potentials and reasonable costs.

In this regard, bone tissue engineering represents a novel treatment aiming at enhancing bone repair and regeneration [16] joining the skills of different specialists (i.e. engineers, surgeons, biologists) in order to define standard protocols useful for a clinical use.

In the past two decades significant progress was carried out in the field of biomaterials and cell therapy, leading to significant advances in the development of engineered tissues. To this aim, three fundamental components need to be included: appropriate cells to prime neotissue formation, a scaffold as a substrate for cell growth and development into a structured biological tissue, growth factors and/or biomechanical stimuli to address the cell phenotype within the scaffold [17,18].

The cells are the main responsible for tissue formation and to this aim, autologous primary cells are intended as the best choice. Bone marrow mesenchymal stem cells (BM-MSCs) [19-24], adipose stem cells (ADSCs) [25-27] and induced pluripotent stem cells (iPSCs) [28-32] in particular, are considered as suitable for the task.

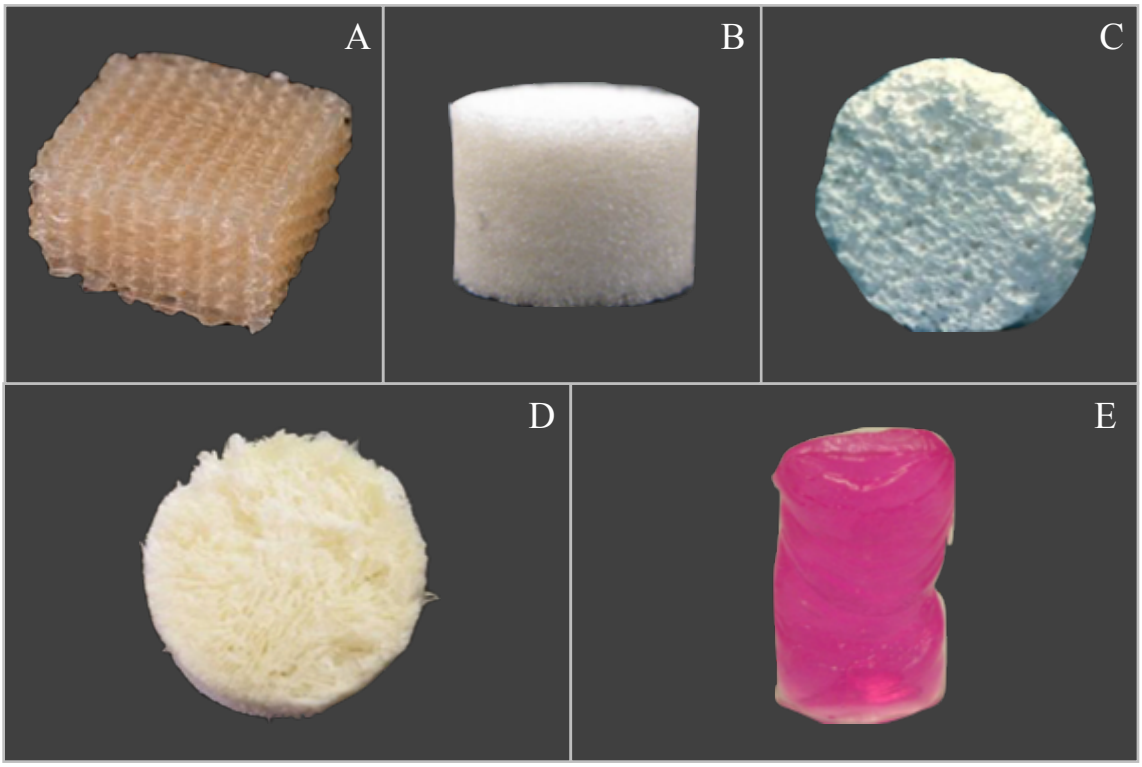

Figure 1.1 - Scaffolds for tissue engineering approach. A, PLGA; B, PLA; C, Calcium Phosphate; D, Chitosan; E, Alginate.

Most of the information about cell differentiation protocols was derived culturing these progenitors cells in monolayer static condition in the presence of differentiation media. However, to develop tissue constructs for potential *in vivo* implantation, porous scaffolds are needed as a support mimicking the 3D tissue properties [33]. Novel structures and fabrication techniques are continuously investigated in this respect. The use of synthetic polymers as poly lactic acid (PLA) [34-40] and poly lactic-co-glycolic acid (PLGA) [41-44], ceramic materials as calcium phosphate (CaP) [45-57], natural polymers as alginate [58-63] and chitosan [64-67] hydrogels, was widely reported in the literature. Bulk material properties and fabrication techniques are determining specific characteristics in terms of relevant properties of a scaffold, such as porosity, stiffness (Young's modulus) and biodegradability.

In addition to their supportive role, scaffolds might be used as functional structures useful to deliver biological active molecules, such as growth factors [68-69]. This configuration is however still missing of the biomechanical stimuli (i.e. dynamic condition), recognized as fundamental cues actives over natural tissues.

Indeed, human bone tissue is normally subjected to two forms of biomechanical stimuli: compression/tension loading generating fluid movement through lacunae (shear stress, 0.8 - 3 [Pa]) and physical deformation generating structural change (strain <2000 [με]).

In a way to reproduce these physiologic mechanical solicitations, actuators - namely bioreactor systems - were used *in vitro* to prime cell-based 3D tissue constructs, aiming at the following implant *in vivo* [70].

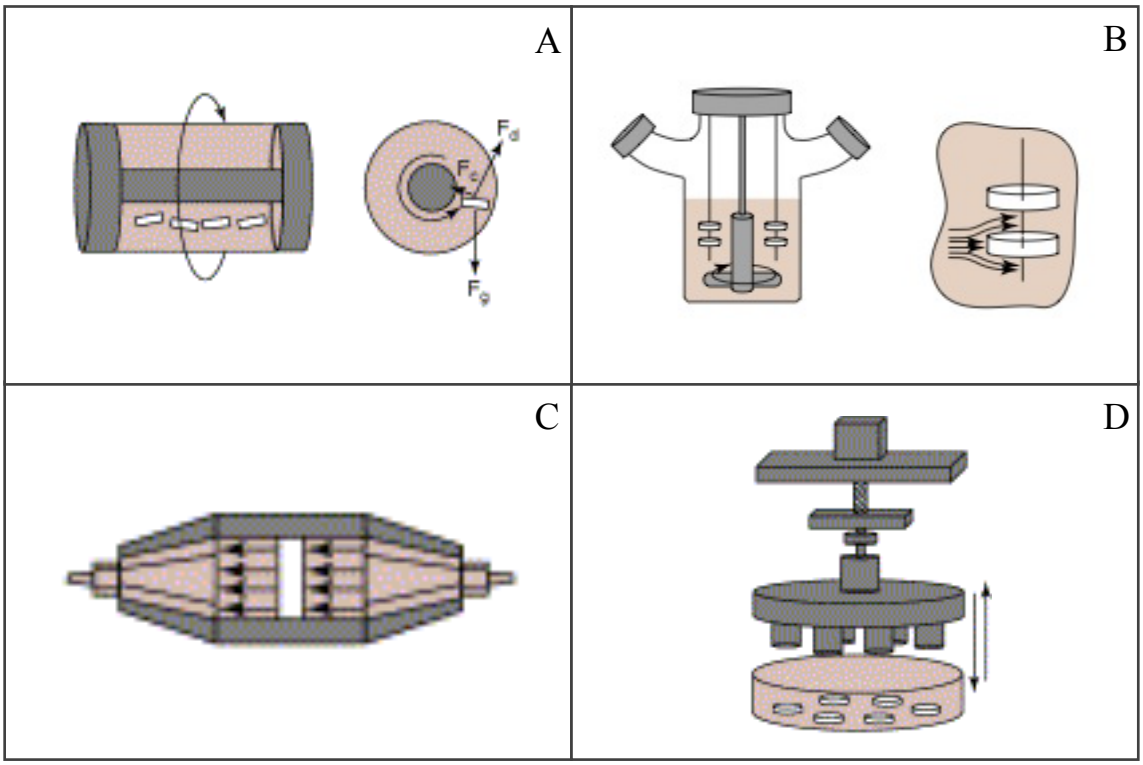

Figure 1.2 - Example of bioreactors for tissue engineering approaches. A, Rotating wall; B, magnetic stirring; C, perfusion; D, compression. [71]

Several configuration were designed for different kind of stimuli. Most diffused bioreactor systems are: rotating wall systems, spinner flask systems, perfusion system, compression and strain systems [72].

Different studies have demonstrated that a combination of "dynamic culture" enriched with proper mechanical stimulation may promote efficient progenitor cell expansion and differentiation *in vitro* [73-79].

The recent scientific literature about this paradigm is reviewed in the following, overviewing the proposed bioreactor design, the specific physical stimulus applied and the performed osteogenic differentiation evaluation (Table 1.1).

Author	Year	Cell Type	Scaffold	Physical stimuli	Amount	Stand-Alone Apparatus	Biological effects
Ding et al. [80]	2016	Sheep BMSCs	Mineral	Perfusion	0.25 mL/min	No, it requires an incubator	10 days of culture are enough to promote bone formation
Nguyen et al. [81]	2016	hMPCs	Alginate	Perfusion	3 mL/min	No, it requires an incubator	Improving differentiation of hMPCs into osteoblasts
Sinlapabodin et al. [82]	2016	rMSC	Thai silk fibroin/gelatin/ hydroxyapatite	Perfusion	1, 3, 5 mL/min	No, it requires an incubator	1 mL/min more cells, good differentiation 3 mL/min less cells, high differentiation 5 mL/min less cells, low differentiation

Table 1.1 - Recent (last 5 years) scientific literature overviewing published bioreactor systems for bone tissue engineering. Effect of the perfusion applied mechanical stimulus.

Author	Year	Cell Type	Scaffold	Physical stimuli	Amount	Stand-Alone Apparatus	Biological effects
Brunelli et al. [83]	2017	hES-MPs	PCL/ Collagen	Compression	5% strain.	No, it requires an incubator	Cyclic stimulation: is a trigger for delayed proliferative response of cells; play an important role in the mineralization processes.
Maeda et al. [84]	2017	Immature bone tissues	Immature bone tissues	Compression	1-2% strain, 3-4 cycles/min.	Yes	Increasing in the elastic moduli and calcified area
Revichandran et al. [85]	2016	hMSCs	PCL-TCP	Compression	0.22% strain, 1 Hz frequency	No, it requires an incubator	After 14 days mechanical stimulation enhances: osteogenic gene expression, ALP activity levels, mineralized matrix production.

Table 1.1 - Recent (last 5 years) scientific literature overviewing published bioreactor systems for bone tissue engineering. Effect of the compression applied mechanical stimulus.

Author	Year	Cell Type	Scaffold	Physical stimuli	Amount	Stand-Alone Apparatus	Biological effects
Teng et al. [86]	2016	hBMSCs	Polyurethane	Perfusion and compression	Perfusion: 1-10 mL/min Compression: 10 % strain, 0.5-5 Hz frequency	No, it requires an incubator	Perfusion enhances cell proliferation; high frequency cyclic compression inhibits the proliferation and osteogenic differentiation; low frequency cyclic compression is more effective.
Baumgartner et al. [87]	2015	hASCs	PLGA/a-CaP	Perfusion and compression	Perfusion: 0.3, 0.5, 2 mL/min Compression: 5 % strain, 1 Hz frequency	No, it requires an incubator	Perfusion and compression allowed the generation of linear cell density gradients and beginning osteogenic differentiation.

Table 1.1 - Recent (last 5 years) scientific literature overviewing published bioreactor systems for bone tissue engineering. Effect of the perfusion and compression applied mechanical stimuli.

Author	Year	Cell Type	Scaffold	Physical stimuli	Amount	Stand-Alone Apparatus	Biological effects
Li et al. [88]	2014	BMSCs	Bovine decalcified bone matrix	Perfusion and compression	Perfusion: 0.5 mL/min Compression: 10 % strain, 0.5 Hz frequency	Yes	Device does not require manual interference for 21 days. Cell proliferation increasing and higher ALP and calcium levels were detected in dynamic vs. static condition.
Petersen et al. [89]	2012	Primary dermal human fibroblasts	Porcine collagen-I	Perfusion and compression	Perfusion: 5 mL/min Compression: 10 % strain, 1 Hz frequency	No, it requires an incubator	The results suggest that scaffold stiffness has only a temporary effect on cell behavior, while the impact of mechanical loading is preserved over time.

Table 1.1 - Recent (last 5 years) scientific literature overviewing published bioreactor systems for bone tissue engineering. Effect of the perfusion and compression applied mechanical stimuli.

Bioreactors used for bone tissue engineering were classified by applied physical stimuli.

Inasmuch as perfusion is concerned, Ding et al. 2016 developed a computer-controlled perfusion bioreactor aiming to automate: initial filling, cell seeding and prolonged cell/tissue culture. Chemo-optic micro-sensors were included to monitor non-invasively the levels of oxygen and pH during the culture period. A standard incubator was used to maintain standard culture condition. Ovine bone marrow stromal cells were seeded onto porous mineral scaffold. A perfusion flow rate of 0.25 [mL/min] was maintained during the dynamic differentiation protocol. After 10 days of incubation, the mineral scaffolds was implanted *in vivo* for 4 weeks. Bone formation was observed in bioreactor cultured scaffolds compare to control, revealing the capability of activation larger viable bone graft material, even after shorter incubation time of graft material.

Nguyen et al. 2016 explored the influence of applied flow in the tubular perfusion system (TPS) bioreactor on the osteogenic differentiation of human mesenchymal progenitor cells (hMPCs) onboard alginate scaffolds. During the dynamic culture the TPS bioreactor was placed into an incubator and a flow rate of 3 [mL/min] was applied. The results demonstrated enhanced expression of osteogenic markers in cells cultured under perfusion flow and the addition of exogenous growth factors.

Sinlapabodin et al. 2016 used: a perfusion bioreactor realizing an uniform axial distribution; Thai silk fibroin (SF)/gelatin (G)/hydroxyapatite (HA) scaffolds as a tool to evaluate the suitable perfusion flow rate; rat bone marrow derived stromal cells (rMSC) were used in during osteogenic differentiation. The bioreactor was placed inside a CO2 incubator for cell

culture, thus, it can't be considered a stand-alone system. For the dynamic culture different perfusion flow rates (1, 3 and 5 [mL/min]) were tested. The perfusion flow rate of 3 ml/min gave the highest rMSC osteogenic differentiation on a SF/G/HA scaffold than other flow rates, as observed from the significantly highest number of alkaline phosphate (ALP) enzyme activity and the calcium content without any significant cell growth.

Among devices applying only compression, Brunelli et al. 2017 proposed the use of a BOSE Bioreactor in combination with 3D hybrid (polycaprolactone/Collagen) scaffolds and human embryonic mesodermal progenitor cells (hES-MPs). As stimulus a 5% strain ramp followed by peak-to-peak 1% strain sinewaves at 1 Hz for 15 min were applied. Cell viability, DNA content and osteocalcin expression were tested. Samples were further stained with 1% osmium tetroxide in order to investigate tissue growth and mineral deposition by micro-computed tomography (μCT). The results suggest how cyclic stimulation: is a trigger for delayed proliferative response of cells; play an important role in the mineralization processes.

Maeda et al. 2017 designed a custom-made bioreactor system for cyclic compression loading. An *ex vivo* scaffold (thickness of 3 [mm]) was obtained from the tibias of 0-day-old chick. Cyclic compression (3-4 [cycles/min]), with an amplitude of 0.3 N corresponding to 1 to 2% compressive strain was applied to immature bone specimen during a 3-day culture period. Stress-strain relationship was evaluated before and after the dynamic culture. ALP, cell viability and tissue calcification were also verified. The elastic moduli of bone slices were significantly elevated at the end of the 3-day culture in the presence of cyclic compression; no

significant changes in the moduli were observed in the absence of cyclic compression. The increases in the moduli were coincided with the increase in calcified area in the bone samples. It was confirmed that immature bone can respond to compressive loading in vitro and demonstrate the growth of bone matrix, similar to natural, *in vivo* maturation. The elevation of the elastic moduli was attributable to the increased calcified area and the realignment of collagen fibers parallel to the loading direction.

Revichandran et al. 2016 present a compression bioreactor system that applies cyclic compression Polycaprolactone-β/Tricalcium Phosphate (PCL/TCP) scaffolds seeded with Mesenchymal Stem Cells (MSC). A compression stimulus at physiological strain value of 0.22 [%] and a frequency of 1 [Hz] was applied for 4 weeks for 4 h per day. Osteonectin, COL1A1 (7 days) and ALP activity (14 days) were evaluated revealing an increased expression in dynamic condition compare to static group.

When both perfusion and compression were applied, Teng et al. 2016 investigated the effects of cyclic compression, perfusion, dexamethasone (DEX) and bone morphogenetic protein-7 (BMP-7) on the proliferation and differentiation of human bone marrow stromal cells (hBMSCs) in polyurethane scaffolds. Dynamic culture was performed mixing six different conditions: 10% Cyclic compression at 0.5 and 5 Hz; 10 ml/min perfusion; 100 nM DEX; 100 ng/ml BMP-7; and 1 ml/min perfusion without mechanical and biochemical stimulation (control). On days 7 and 14 cell proliferation, Runx2, COL1A1 and osteocalcin, osteocalcin content, calcium deposition, and the equilibrium modulus of the tissue specimen were evaluated. The obtained results suggest that BMP-7 and perfusion

enhance cell proliferation, whereas high frequency cyclic compression inhibits the proliferation and osteogenic differentiation of hBMSCs. Low frequency cyclic compression is more effective than DEX, but less effective compared with BMP-7 on the osteogenic differentiation of hBMSCs seeded on polyurethane scaffolds. Baumgartner et al. 2015 observed the effect of a bioreactor realizing perfusion and uniaxial cyclic compression on electrospun nanocomposite scaffolds of poly-lactic-co-glycolic acid and amorphous calcium phosphate nanoparticles (PLGA/a-CaP) seeded with human adipose derived stem cells (ASCs). A perfusion flow rate of 0.3, 0.5, 2 [mL/min] was applied in combination with a cyclic compression of 5 [%] of strain and at 1 [Hz] of frequency. Osteogenesis was analyzed on the protein level (osteopontin). The obtained results revealing how perfusion and compression allow the generation of linear cell density gradients and begin osteogenic differentiation of the stem cells.
Li et al. 2014 designed a prototype of a bioreactor system including perfusion, cyclic compression, automatic substance exchange and feedback control of pH and PO_2. The effect of dynamic culture was evaluated on mouse bone-marrow mesenchymal stem cells seeded in decalcified bone matrix (DBM). A perfusion flow rate of 10 [mL/min] was applied in combination with a cyclic compression of 5 [%] of strain and at 1 [Hz] of frequency. The results shown higher cell densities, proliferation, phosphatase activity and calcium content in scaffolds maintained in dynamic culture compared to those in static culture. Furthermore, because its innovative features the bioreactor can be considered as a stand-alone system with a strong potential for long-term tissue culture.

Petersen et al. 2012 developed a perfusion/compression bioreactor system working with a perfusion flow rate of 5 mL/min and cyclic mechanical loading of 10 [%] of strain at 1 [Hz] of frequency. A macroporous scaffold made of porcine collagen-I was seeded with primary dermal human fibroblasts. While mechanical loading resulted in a clear upregulation of procollagen-I and fibronectin production, scaffold stiffness showed to primarily influence matrix metalloproteinase-1 (MMP-1) secretion and cell-induced scaffold contraction. The results suggest that scaffold stiffness has only a temporary effect on cell behavior, while the impact of mechanical loading is preserved over time.

The proposed review highlights recent promising results about engineered bone tissue construct obtained within mechanical actuation devices. Different kind of cells and 3D supports were used. The examined works suggest that perfusion and compression are biomechanical stimuli relevant for a proper tissue proliferation/differentiation. Particularly, it is noteworthy to observe that a perfusion flow rate plays an important role for cell proliferation in a range of 1-3 mL/min. Cell proliferation is inhibited when low frequency compression is applied, i.e.: high compression frequencies are preferred. Only two of the proposed devices might be defined stand-alone; no bioreactor allows real-time analysis.

2. AIM

Bioreactor systems were widely used, in tissue engineering to improve the proliferative and differentiate efficiency of *in vitro* cell cultures.

Recent scientific literature suggest that perfusion and compression stimuli are fundamental cues to address a proper osteogenic differentiation.

The purpose of this study is to show a stand-alone perfusion/compression bioreactor, originally developed as a synthesis of previous promising results obtained with mechanical actuation devices reported in the literature.

Namely, perfusion will be applied to increase diffusive exchange and waste removal during a cell culture, thus, to increase cell survival within the core area of 3D engineered scaffolds.

Compression stimulus will play a fundamental role for a proper osteogenic differentiation mimicking the physiological load bearing bone function.

The device will be released as a stand-alone apparatus for incubator independent cell culture with the added value of a potential real-time/in-line analysis of the tissue engineered bone construct.

3. Material and Methods

The presented work was carried out within an agreement for the co-direction of a PhD research/study between the University of Bologna (Italy) and the Reykjavik University (Iceland).

The bioreactor system was designed, built and developed at the "Institute for Biomedical and Neural Engineering/Biomedical Technology Centre" (Reykjavik University, Reykjavik, Iceland) under the supervision of Professor Paolo Gargiulo providing facilities and training; the wet-lab procedures were carried out at the Bloodbanki (Landspitali Hospital, Reykjavik, Iceland) under the supervision of Professor Ólafur E. Sigurjónsson, providing facilities, training, reagents, cells; the analytical analysis were performed at the Mol & Cell Eng Lab (ICM) (University of Bologna, Cesena, Italy) under the supervision of Professor Emanuele D. Giordano providing facilities and training.

Partial subvention to grant a bursary to the PhD candidate was provided by all the three mentioned Institutions.

3.1 The bioreactor system

A stand-alone perfusion/compression bioreactor system was designed and built up with the aim of improving proliferation, growth and differentiation of stem cells seeded onboard of scaffolds of interest.

Sensors were incorporated to monitor parameters such as pH and temperature in the cell culture environment. A Control Unit (CU) with a Graphical Unit Interface (GUI) was implemented in order to control the device and to acquire data from sensors in such a way to monitor these variables during the cell culture and to correct any of these if needed.

3.1.1 A stand-alone apparatus

A system of automatic replacement of the media was incorporated in order to facilitate the replacements of the media and also prevent contamination.

For this purpose, two little peristaltic pump (Figure 3.1 - Welco WPX1) were used, one to feed fresh media into the bioreactor circuit, the other one to remove the waste media from the bioreactor circuit. This operation is completely automatized thanks to the CU present in the device, thus no manual operation is required by the operator.

Furthermore, 3 heating pads (Figure 3.2) were placed in the bioreactor to maintain the temperature of the perfusion circuit at 37°C.

Figure 3.1 - Welco WPX1 peristaltic pump used for the automatic media replacement system.

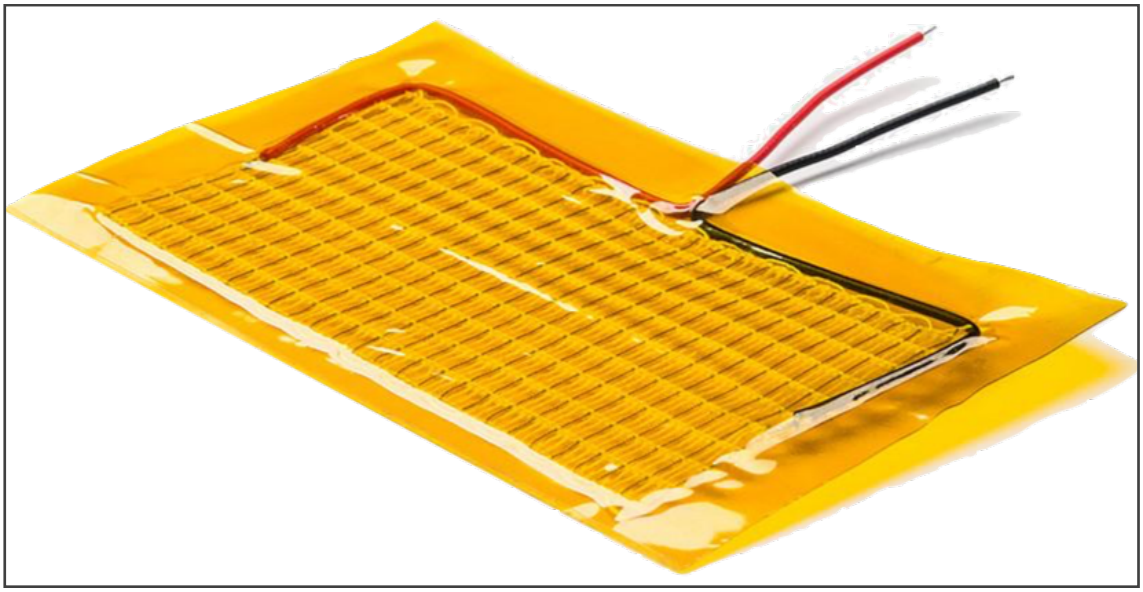

Figure 3.2 - Heating pad used to maintain the temperature of the perfusion circuit at 37°C.

The temperature was constantly monitored and eventually corrected through the use of a temperature sensor (Figure 3.3, [A] - Sparkfun Electronics, DS18B20) within the media.

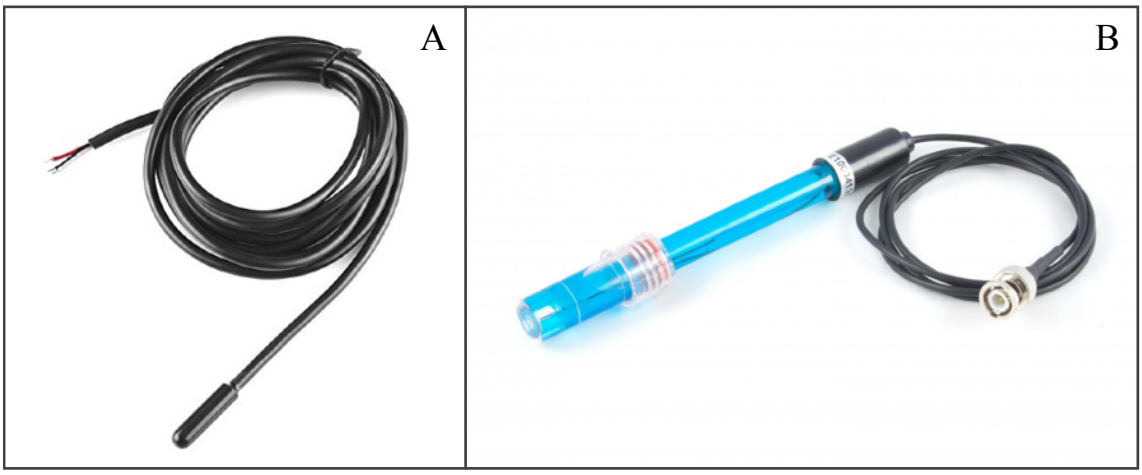

Figure 3.3 - Sensors used to monitor cell culture parameters. A, Temperature sensor; B, pH sensor.

Finally, a tank containing (5% CO_2, 20% O_2, 75% N) was used to guarantee the correct gas exchange; pH level of the media was constantly monitored by a pH probe (Figure 2.3, [B] - Phidgets, BNC pH Lab Electrode).

3.1.3 A mechanical loading actuator

To promote nutrient exchange and waste removal a perfusion apparatus ,based on a peristaltic pump (Figure 3.4, [A] - Welco WP10), was implemented allowing to tune flow rate in a range of 0.16 ÷ 5 mL/min for each one of the six chambers.

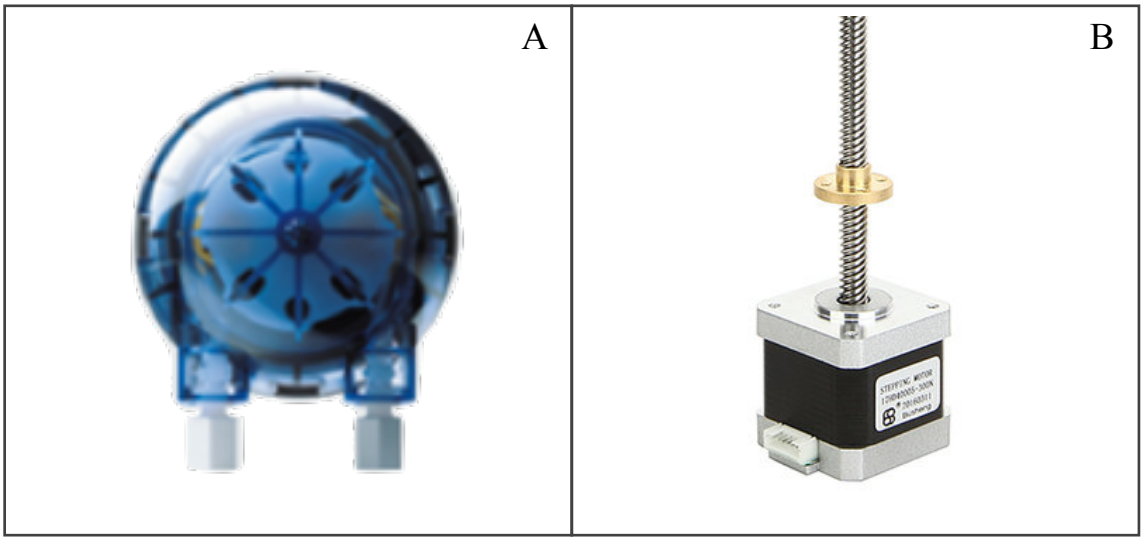

Figure 3.4 - Actuators. A, peristaltic pump used to apply perfusion; B, stepper motor with lead screw.

To apply a proper mechanical stimulus, onto the tissue construct, a mechanical loading actuator was designed; this actuator, based on a stepper motor (Figure 3.4, [B] - NEMA 17-size hybrid bipolar stepping motor), allows to obtain a controlled compression tunable in a strain range of 1 ÷ 5 % at different level of frequency 0.5 ÷ 5 Hz.

3.1.2 A real-time/in-line monitor

Taking into account the desired scaffold geometry and dimension, a custom chamber, based on the single well dimension, i.e. a 300 µL volume, of a standard 96 multiwell plate, was designed to allow to apply perfusion and compression over the 3D scaffold. Biocompatible material was used to 3D

print (Materialise, Belgium) six chambers used to realize the core of the perfusion circuit of the bioreactor.

In a way to perform a real time/in line analysis (i.e.: the analysis of the very same tissue construct at different time points) mobile units were conceived.

For this reason, the chambers were connected to the perfusion circuit by Spyros/MicroClave devices (ICUMed, USA), realizing a simple detachable system (i.e.: the mobile unit) useful to prevent leakage and infection risk when real-time/in-line analysis (e.g.: spectrofluorometric analysis, fluorescence analysis; X-ray μCT for 3D imaging analysis) have to be performed. In order to evaluate the monitor, a specific culture was set-up. One CHT/G scaffold, hosting onboard human mesenchymal stem cells (hMSCs), was placed within a mobile unit cultured for 10 days in static traditional condition (i.e.: within a cell culture incubator). At day 10th, the mobile unit was moved into the bioreactor where perfusion and compression were transferred with the same regimens used in the previous experiment. After 24h (i.e.: day 11th), the mobile unit was transferred from the bioreactor system, processed by X-ray μCT (phoenix|x-ray Systems, nanotom s, GE) and then reconnected to the bioreactor. At the end of the test (i.e.: day 14th) the unit, thus the same scaffold, were processed for the second time by X-ray μCT. Data acquired at day 11th (sample-pre) and day 14th (sample-post) were elaborated aiming to obtain the respective images, thus, an histogram of their gradient values distribution.

3.2 Finite Element Modeling of stress distribution

A Finite Element Model (FEM) was implemented to investigate the stress distribution occurred, onto the scaffolds fibers, during the cell culture, when perfusion and/or compression stimuli are applied. The model was realized by using COMSOL Software; all the components were obtained using primitive geometry and boolean operation.

3.2.1 Model culture unit

Three-dimensional model of the culture unit was generated using cylindric geometries and boolean operations (Figure 3.5, [A]). The chamber unit dimension were 30 mm in length and 11 mm in height. The inlet/outlet diameter was 1.6 mm. The inner volume (Figure 3.5, [B]) was modeled as water.

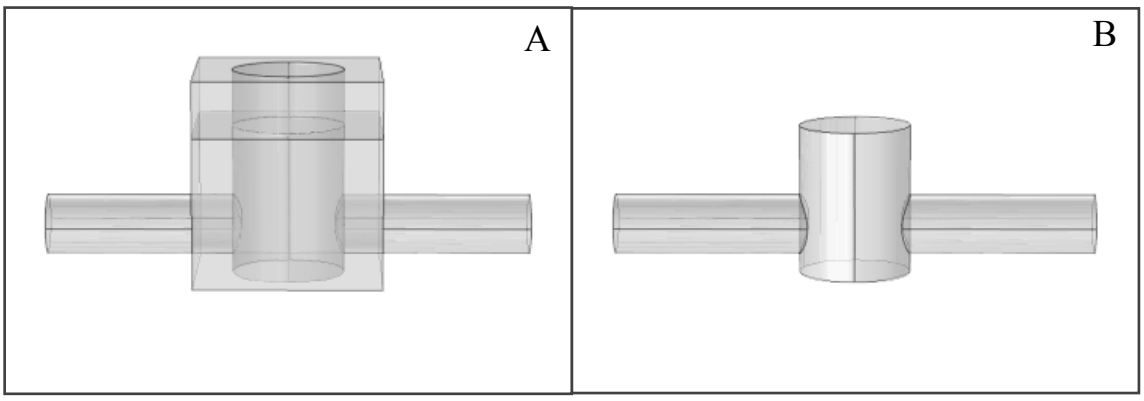

Figure 3.5 - Three-dimensional modeling of the culture unit. A, whole geometry; B, inner volume.

3.2.2 Model scaffold

A 3D model of the scaffold was generated using cylindric geometry, as shown in the following figure:

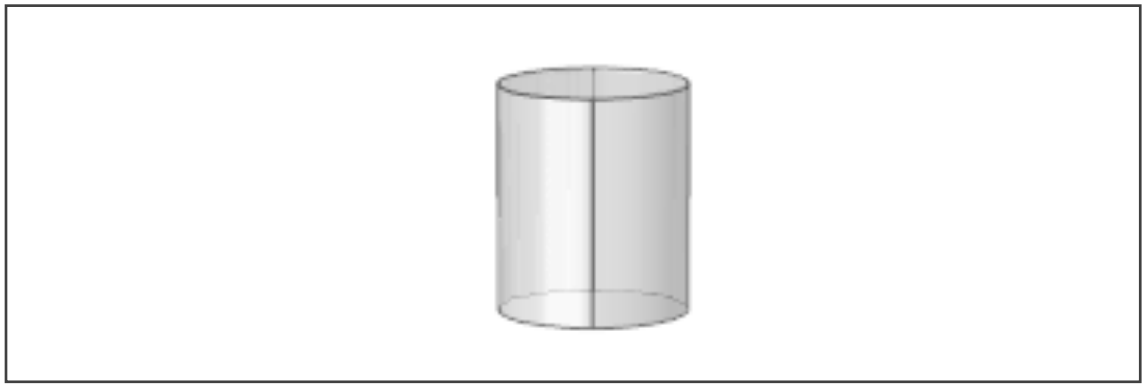

Figure 3.6 - Three-dimensional modeling of the scaffold.

The scaffold dimensions were 5 mm in diameter and 6 mm in height. It was defined as a poroelastic material and initiated by specifying a random pore location. Materials properties were defined as follow:

Parameters	Values	Units
Density	35	[Kg/m^3]
Young's Modulus	9E+03	[Pa]
Permeability	7.27E-11	[m^2]
Porosity	0.85	[-]
Poisson's coefficient	0.8	[-]

Table 3.1 - Scaffolds material parameters.

All the requested parameters were chosen by Podichetty et al. 2013 [90].

3.2.3 Model physics

Laminar flow, based on the Darcy's Law, and solids mechanics were set up as physics. To not affect the results, a sensitivity studies of the mesh was performed (Figure 3.7), in order to obtain the most computationally efficient mesh.

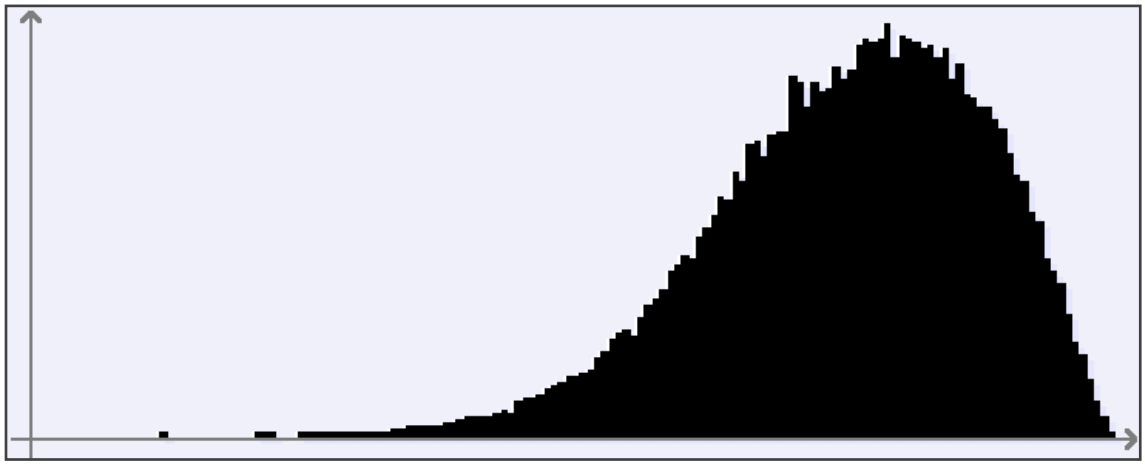

Figure 3.7 - Histogram of the elements quality.

For the computational analysis different conditions were applied: only perfusion with a flow amount of 0.5, 1, 2, 5 mL/min, only compression with a deformation equal to 1, 2, 3, 5 % of the total scaffold volume, perfusion and compression with a values among those mentioned.

3.3 human Mesenchymal Stem Cells harvesting and culture

The data presented in this manuscript were obtained using primary human bone marrow derived Mesenchymal Stem Cells (hMSCs) from a single donor (age 39) for a single experiment including a dynamic and a control static cell cultures. Cells were used at passage number two. Cells were acquired from Lonza inc (Allendale, NJ, USA) They were expanded in a monolayer in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) containing 0.1% penicillin/ streptomycin (Life Technologies, Carlsbad, CA, USA), 0.1 mM non-essential amino acids (Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA). After two passages, hMSCs were trypsinized (Life Technologies, Carlsbad, CA, USA) and counted in an hemocytometer using Trypan Blue staining to evaluate the number of necrotic cells.

3.4 Cell Media

An osteogenic culture medium was formulated containing: high glucose DMEM, 40 mg/mL proline (Sigma-Aldrich, St. Louis, MO, USA), 0.1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA), 0.1% sodium pyruvate (Life Technologies), 50 mg/mL ascorbate 2-phosphate (Sigma-Aldrich, St. Louis, MO), 0.1 mM dexamethasone, 1% ITS + premix (BD Biosciences, Bedford, MA), and 10 ng/mL TGF-b3 treatment (R&D systems, Minneapolis, MN).

3.5 Chitosan-graphene scaffolds

Graphene oxide was supplied by National Institute for Research and Development in Microtechnologies (Romania), prepared according to Hummers procedure [91]. Acetic acid (99.7%) and chitosan from crab shells were purchased from Sigma-Aldrich (St. Louis, MO, USA). All materials were used without further purification and the water used in this work was double distilled water.

2.5 g of Chitosan (CHT) were mixed with 250 ml acetic acid solution (10% by weight in water) at 50°C in order to form a homogeneous viscous solution. Further, different contents of graphene oxide (0; 0.5 and 3 % (wt/ vol) were added into CHT solution and mixed by ultrasonication for 1 h at room temperature. The homogeneous solutions were casted onto transparent glass Petri dish, then frozen overnight at -70°C and freeze-dried for 2 days at -50°C (0.040 mbar). After sublimation of ice crystals by freeze-drying, the polymer structure became porous. The 3D dried materials were thermally treated in vacuum, according to the following procedure: 50°C for 30 min, 70°C for 30 min and overnight at 90°C. The obtained samples were then subjected to advanced characterization of the bulk surface and to in vitro biocompatibility assessment [92].

3.6 3D cell culture

Chitosan-graphene (CHT/G) scaffolds were seeded with hMSCs (1 x 10^6 cells/mL) into a standard multi-well plate. After 1 hour some scaffolds were

placed into the bioreactor chambers. Starting from the 12 chitosan graphene scaffolds hosting hMSCs 2 groups were created: 6 scaffolds were placed in a petri dish for a static culture (control) while the others 6 scaffolds were placed in the six chambers of the bioreactor for a dynamic culture. The same osteogenic media was used and changed every 3 days in both cultures.

The bioreactor was set up as follow: flow was driven by a peristaltic pump (WELCO WP10) at a flow rate of 1 mL/min for each chamber; the compression stimulus was applied at a frequency of 1 Hz and a displacement of 1 %. All the 6 dynamically cultured CHT/G scaffolds with hMSCs inside were exposed to perfusion flow for 14 days and to compression 2 times/day for 30 min each time, with a 1 % strain at 1 Hz. The whole system was maintained at 37°C and 5% CO_2.

3.7 Cell viability assay

A working solution of approximately 2 μM calcein AM and 4 μM EthD-1 was obtained adding 20 μL of 2 mM EthD-1 and 5 μL of the supplied 4 mM calcein AM solution to 10 mL of sterile, tissue culture–grade PBS.

A scaffolds slice was placed on the top of a 22 mm square coverslip and covered with 100 μL of the working solution, so that all cells were totally covered. Incubation was performed in a covered petri dish, to prevent contamination or drying of the samples, for 1h at room temperature and darkness. Following incubation, the sample was washed with PBS.

Using fine-tipped forceps, the wet coverslip was inverted and mounted over a microscope slide. To prevent evaporation, the coverslip was sealed to the glass slide with clear fingernail polish. Each sample obtained was analyzed under a fluorescence microscope.

3.8 Von Kossa staining analysis

In order to assess mineralization of extracellular matix (ECM), scaffolds maintained in both static and dynamic condition were stained and analyzed by optical microscope.

To this aim, a standard Von Kossa staining protocol [93] was used to quantify the calcium/calcium salts deposition occurred during the ECM mineralization.

CHT/G scaffolds were sectioned and, from each of those, two slices were collected and fixed, on top of a microscope slides, in cold methanol for 15-20 min. After three rinses, the slides were incubated with 5% silver nitrate solution under a strong UV light. Thereby the calcium was visualized as metallic silver nodules as black spots.

Von Kossa staining analysis was performed by acquiring 4 images from each slice treated and then comparing control group (static) vs dynamic group (bioreactor) data; to perform these measurements, qualitative and quantitative data were assessed. The images were acquired by using a Nikon TE 2000U optical microscope and processed, using the ImageJ software in order to: (i) detect the black spots obtained, for qualitative

analysis; (ii) calculate the amount of area covered from those spots, for quantitative analysis.

Both the data were obtained using the max entropy segmentation, a method that allows to distinguish between the objectives (black spots) and background (scaffolds).

Particularly, to quantify the amount of area covered (A) by the black spots the following formula was used:

$$A = np \cdot pd^2$$

where np is the number of pixels detected and pd is the pixel dimension.

3.9 Statistical analysis

A Shapiro-Wilk Normality test was performed to confirm that data display a normal distribution. Statistical evaluation was performed using a Student's t-test to determine significant differences among groups. The significance level was set at $P < 0.05$.

All results are presented as mean ± standard deviation.

4. Results

The bioreactor system detailed in this manuscript is a novel stand-alone device that allows culturing 3D cell constructs within a controlled environment where tightly regulated compression and perfusion are administered, intended to address undifferentiated cells towards an osteogenic phenotype. Perfusion is aimed at improving diffusive exchanges, i.e. nutrient uptake and waste product removal. On the other hand, compression is applied to exert a mechanical strain over the cell onboard the scaffold, with the aim of addressing their phenotype commitment.

Normally, when a new device has to be developed, three questions need to be answered: (*idea*) Which is the idea behind the device? (main *function*) Which functions the device has to satisfy? (*novelty*) There are already similar device on the market? Compared to devices present on the market, which might be the features that has to be included to define the device as "novel"?

To answer to those questions, a series of steps is implemented building the device: create its design including all the required features; manufacture a prototype; test if it works as expected and, in case, debug it.

Taking back these concepts to the bioreactor device development, the following list of answer were obtained: (*idea*) obtaining a device useful for bone tissue engineering; (main *function*) applying perfusion and compression aiming to reply the biomechanical stimuli physiologically related to the bone functioning; (*novelty*) compare to the bioreactor systems

present in the last 5 years literature, focusing on three main features: stand-alone device, automatic media replacement option, real-time analysis of the tissue construct allowed.

4.1 Design of the bioreactor system v 1.0

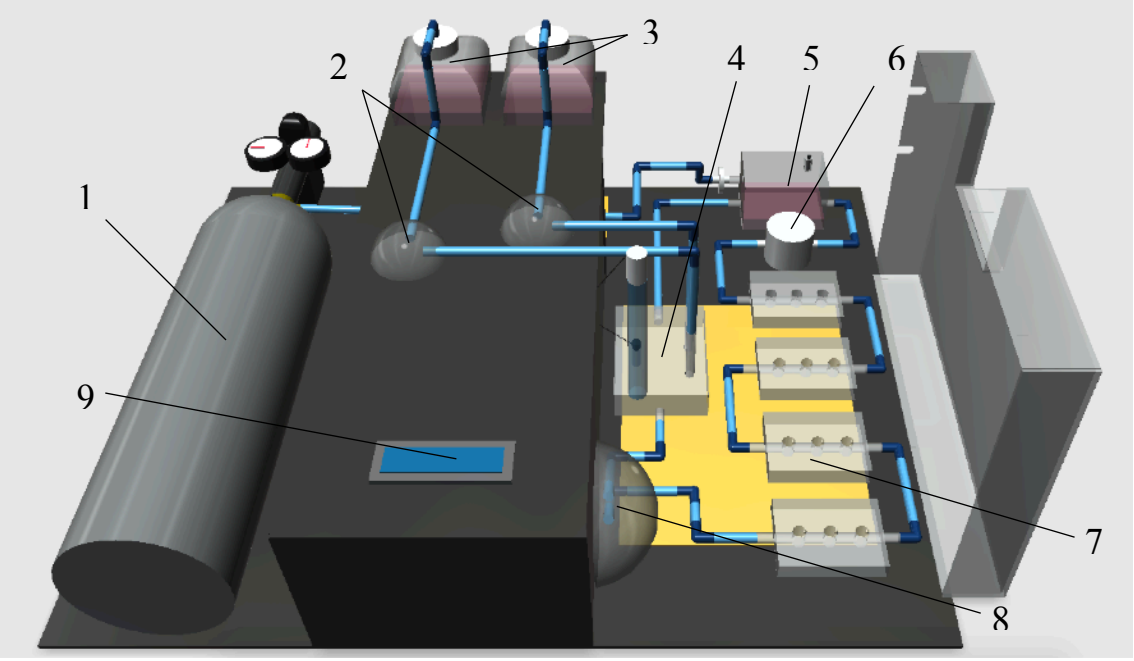

Figure 4.1 - Bioreactor system layout (v 1.0). Sketch front view of the bioreactor system set-up. 1, CO_2 tank; 2, Automatic media replacement peristaltic pumps; 3, Fresh and waste media bottles; 4, Measurement chamber: temperature and pH sensors inside, heating pad (in yellow) below; 5, Gas exchange chamber; 6, Flow meter sensor; 7, Scaffolds units; 8, Perfusion peristaltic pump; 9, device display unit.

Taking into account the requested innovative features the design of the bioreactor system, initially sketched as the version (v 1.0), is shown in the Figure 4.1.

The layout illustrates a range (n=4) of rectangular culture units (Figure 4.1, [7]) each one hosting 3 scaffolds. All the culture units are under a continuous medium flow monitored by the flow meter sensor (Figure 3.1, [6]). Medium temperature and pH are constantly monitored and adjusted to 37°C and 7.4, respectively, without the need of a dedicated cell culture incubator (i.e. the device can be located over a standard lab benchtop). Automatic culture medium replacement was operated by additional peristaltic pumps (Figure 4.1, [2]) from a fresh to a waste media bottles (Figure 4.1, [3]). A display unit (Figure 4.1, [9]) allows to set and monitor continuously all the culture parameter.

An *in silico* evaluation was carried out to estimate the velocity profile occurring when perfusion is applied. Figure 4.2 shows a finite elements model (FEM) used for this assessment where geometries and material properties of the culture chamber were reproduced with the velocity profile occurring into the culture unit when a perfusion of 1 mL/min is applied. A laminar velocity profile with a mean velocity value of 0.04 m/s was calculated. No turbulences are apparent.

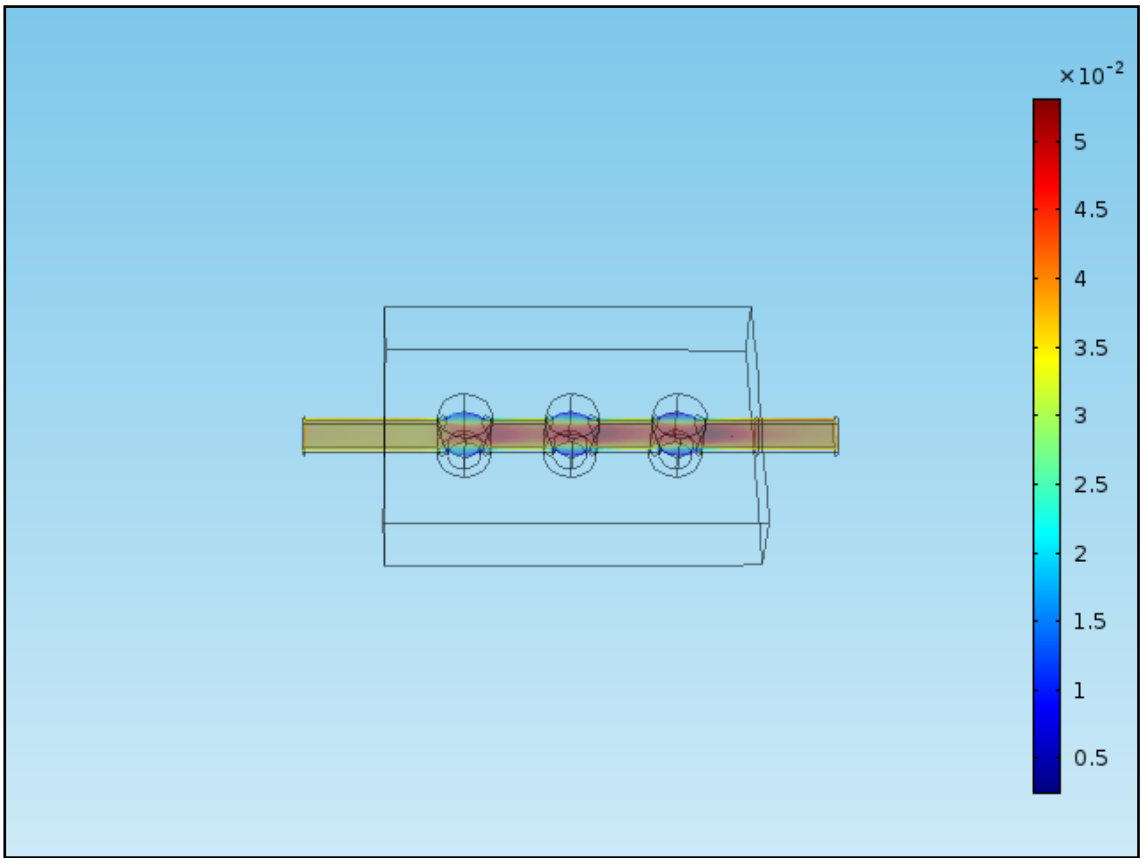

Figure 4.2 - Computational fluid dynamics analysis. Velocity profile obtained within a scaffolds culture unit under continuous perfusion flow.

4.2 Design of the bioreactor system v 2.0

In order to address the bone tissue phenotype commitment the perfusion, aimed at improving diffusive exchanges, was coupled with compression intended to exert a mechanical strain over the cell onboard the scaffold. To administer the compressive stimulus, a changes to v 1.0 was performed. A new prototype versione was sketched as shown in the following figures.

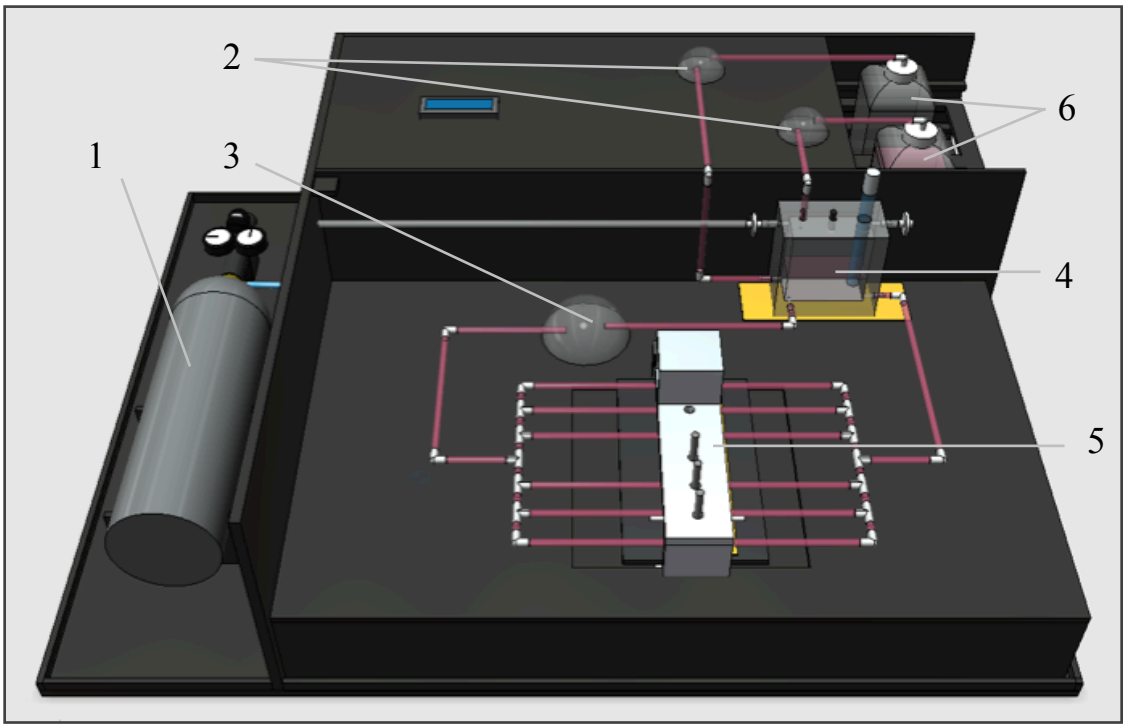

Figure 4.3 - Bioreactor system layout (v2.0). Sketch front view of the bioreactor system set-up. 1, CO_2 tank; 2, Automatic media replacement peristaltic pumps; 3, Perfusion peristaltic pump; 4, Measurement chamber: temperature and pH sensors inside, heating pad below; 5, Mechanical loading unit; 6, Fresh and waste media bottles.

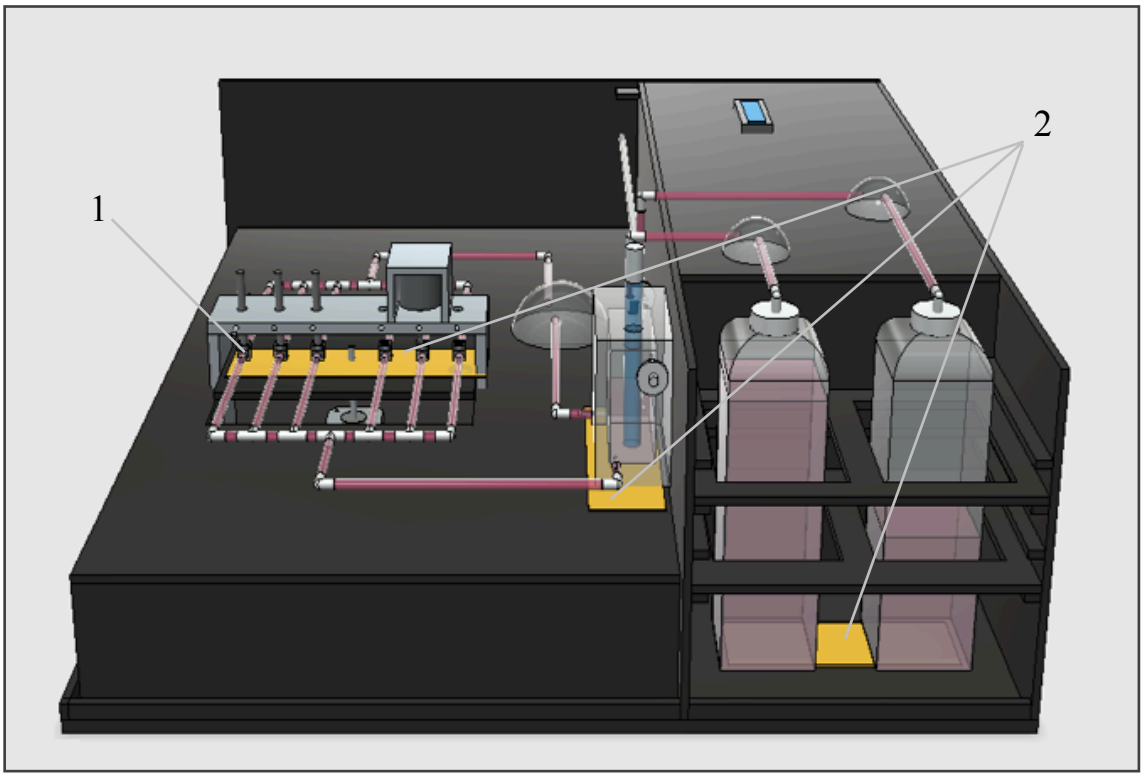

Figure 4.4 - Bioreactor system layout (v2.0). Sketch side view of the bioreactor system set-up. 1, Scaffolds units; 2, Heating Pads.

The device dimensions are 80L, 60W and 25H (cm). Suitable biocompatible materials (i.e. silicon Tygon® tubing and TuskT® culture units) were used for the cell interfacing environment, while polycarbonate, silicon, aluminum, or stainless steel were used for the other components of the device.

To perform the perfusion a peristaltic pump (Figure 4.3, [3]) was included in a way to regulate the desired perfusion regimen through the bioreactor perfusion circuit. Although perfusion was described as a stimulus for phenotype commitment in several published bioreactor systems [80-82,94], we assumed that compressive load is the main physical cue in natural bone.

The perfusion flow was not intended to pass through the entire scaffold, but to flow over it, in a way to increase the diffusive exchange s from/to the culture medium (see *in silico* evaluation).

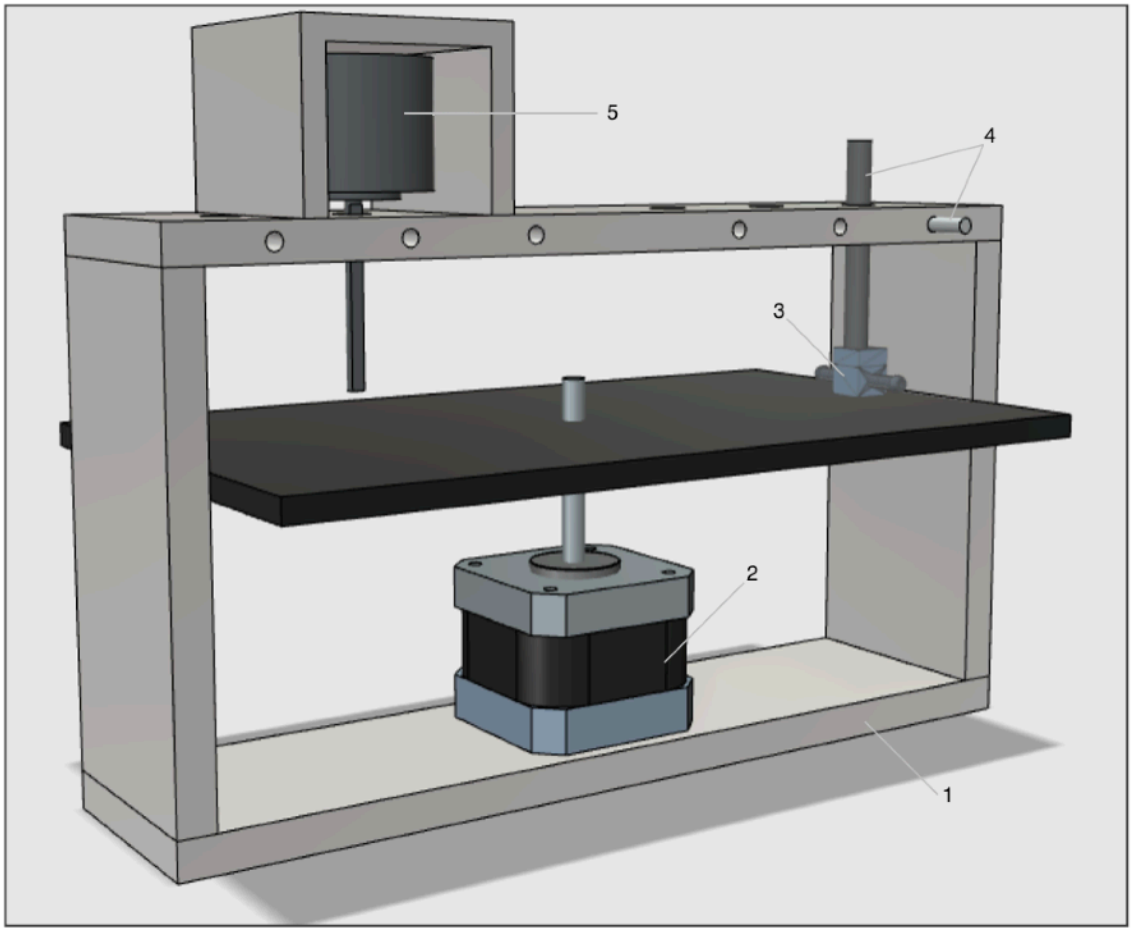

Figure 4.5 - Mechanical loading unit. Layout of the unit useful to apply mechanical compression onto seeded scaffolds hosted within the custom made chambers. 1, Aluminium case; 2, Stepper motor driving the compression plate screwed on it; 3, Custom made chamber; 4, Piston and it locker; 5, Load cell.

To this aim a custom made mechanical loading unit (Figure 4.3, [5]; Figure 4.5) was used to compress the scaffolds, delivering a mechanical strain controlled in frequency and displacement. An aluminum case (Figure 4.5, [1]) was used as supporting structure for a stepper motor including a lead screw (Figure 4.5, [2]), useful to drive the vertical movement of an integral plate. The top of the plate hosts up to six culture units (Figure 4.5, [3]) in contact with adjustable pistons (Figure 4.5, [4]) able to compress the scaffolds within the chambers along with the plate strokes. The design includes the option of a load cell (Figure 4.5, [5]).

As mentioned above a range of (n=6) culture units (Figure 4.6, [A]) were included in parallel alignment. The culture chamber (Figure 4.4, [1]; Figure 4.5, [3]; Figure 4.6, [B]), which reproduces the volume of a single well of a standard 96-well plate, was modeled on the chosen design of our culture units. It is worth to note that the chamber design is a custom option, potentially adaptable to different scaffold dimensions, materials and shapes. Each culture unit allows to: host a single 3D scaffold and to maintain it under a continuous medium flow and a cyclic compression; easily connect/ disconnect a culture chamber from the perfusion line, thanks to the use of Spyros®/MicroClave® connectors avoiding risk of leakage from the fluid circuit. Each single unit is thus a mobile component (Figure 4.6, [A]).

This approach allows to maintain, in the same *in vitro* culture condition, different small scaffolds (i.e.: ~270 mm^3), which are expected to be "sintered" at subsequent time of a potential *in vivo* implantation in a larger volume (i.e.: ~1.5 cm^3) promoting the vascularization process, thus, avoiding internal necrosis of the engineered tissue construct.

Furthermore, within the device medium temperature and pH are constantly monitored and adjusted to 37°C and 7.4, respectively, without the need of a dedicated cell culture incubator in a way to locate the device over a standard lab benchtop.

An automatic culture medium replacement is operated by additional peristaltic pumps (Figure 4.3, [2]) from a fresh to a waste media bottles (Figure 4.3, [6]). In detail, at day 1st the bioreactor is filled with the cell media; then, every 3 days, the medium is replaced removing 80% of the exhausted medium (gathered into the waste media bottle) and adding the same quantity of fresh volume (collected from the fresh media bottle). In fact, preventing and minimize the infection risk is an important aspect to be maintained during a cell culture, so limiting the interaction with an external operator was considered an important feature for this device. In addition, an automatic media replacement protocol might allow to progressively (e.g. 1mL/hr) replace the media, avoiding to administer to the cell culture the stress that is typically occurring with sheer media changes within traditional subculture procedures [95].

Finally, an *in silico* evaluation was carried out to estimate the stress affecting the scaffolds, therefore the cells onboard, when perfusion and/or compression are applied.

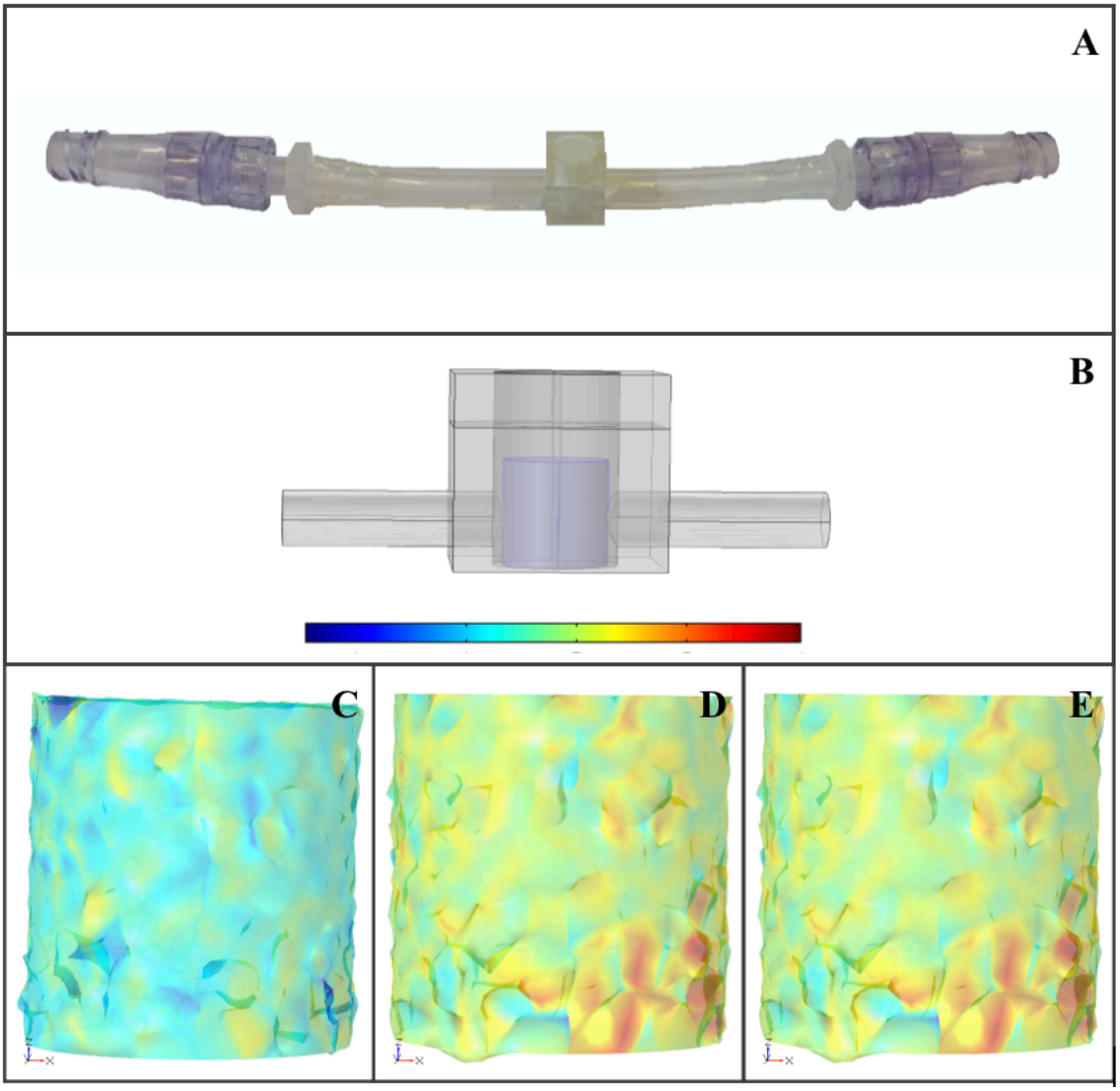

Figure 4.6 - Perfusion/compression culture chamber hosting a 3D scaffold. A, Removable culture unit; B, FEM modeled chamber (gray) and scaffold (blue); Stress impacting over the scaffold when perfusion (C), compression (D) and perfusion/compression (E) are applied.

Figure 4.6, [B] shows the finite elements model (FEM) used for this assessment where geometries and material properties of the culture chamber (gray) and of the scaffold (blue) were reproduced. The chitosan/ graphene (CHT/G) mechanical properties were used to model the scaffold.

Figure 4.6, [C, D, E] shows how perfusion, compression or perfusion/ compression together respectively affect the scaffold. The colorbar shows a relative range of stress values from lower (blue) to higher (red) figures. Figure 4.6, [C] shows the stress values occurring, onto scaffolds fibers, when only perfusion (flow amount 1 mL/min]) was applied; light blue color identifies low stress average values of about 10^{-5} Pa. On the other hand, Figure 4.6, [D] [E] show stress values, occurring onto scaffolds fibers, when only compression (1% of the total scaffold height) or either perfusion/compression (above-mentioned conditions) were applied; in both cases the same yellow/orange color distribution identifies higher stress values (average of about 10^{-1} Pa) when compared to those elicited when only perfusion was applied. This result allows to consider the perfusion stimulus as negligible when compared to the compression stimulus, supporting the view that perfusion alone is a weak determinant to induce the cell towards the desired phenotype commitment.

The *in silico* model allows to evaluate how perfusion and compression contribute during the cell proliferation/differentiation. In detail, as also reported in the reviewed literature: perfusion alone, with a flow rate of 1 ml/min, is able to administer a stimulus useful to increase nutrient diffusion, thus the cell proliferation, but are negligible for inducing differentiation; compression alone, with a frequency of 1 Hz and 1 % of strain, procures stresses that are enough to address the cells towards the differentiation fate, but inhibits their proliferation. For this reason, for a proper tissue growth, both stimuli are required.

4.3 Operation of the bioreactor system

Starting from the proposed design (v2.0), the prototype bioreactor system shown in the following figure was built.

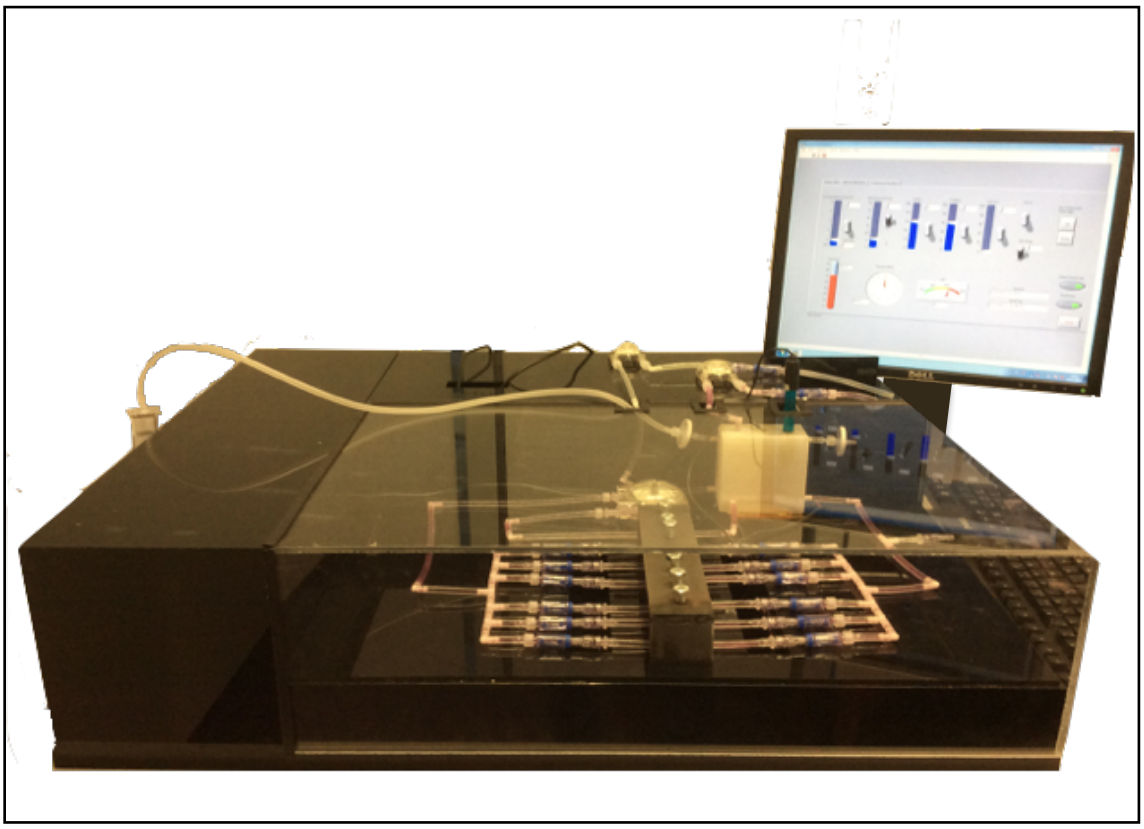

Figure 4.7 - Bioreactor layout based on the proposed design (v2.0).

A detail of the compression/perfusion unit is shown in the following figure:

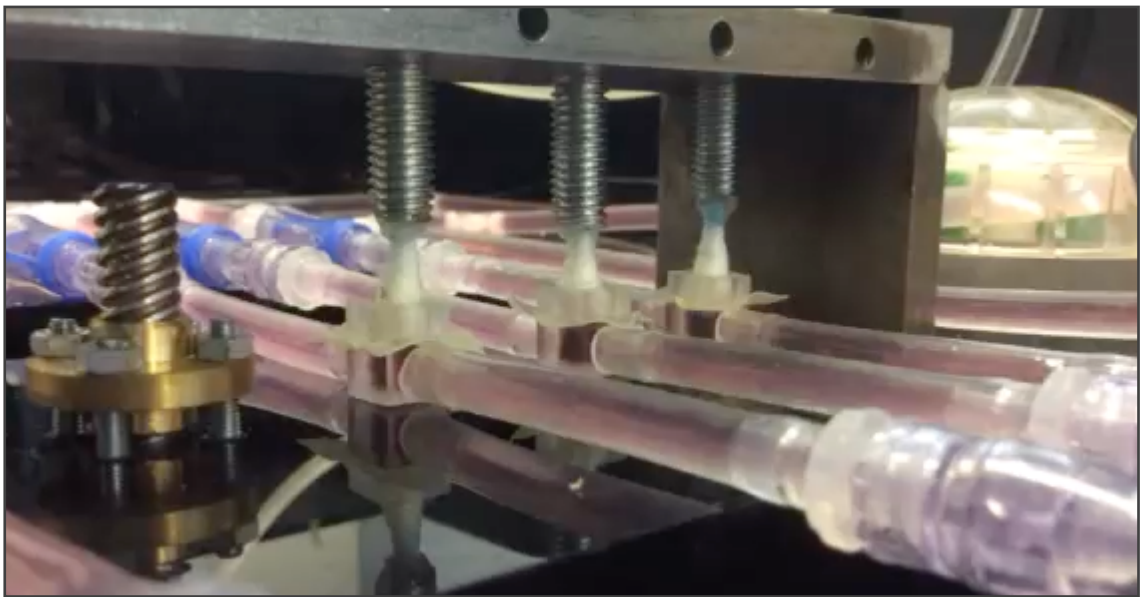

Figure 4.8 - Detail of the cell culture perfusion/compression unit.

Aiming to manage easily the device, a Control Unit (CU) was built up and a Graphical Unit Interface (GUI) was implemented. Input to the actuators and data from sensors are constantly exchanged, in such a way to monitor these variables during the cell culture and to correct any of these if needed.

4.3.1 CU design

The CU of the bioreactor can be considered the functional core of the device. A prototype board was realized aiming to test each electrical/ electronic component. Its layout is shown in the following figure.

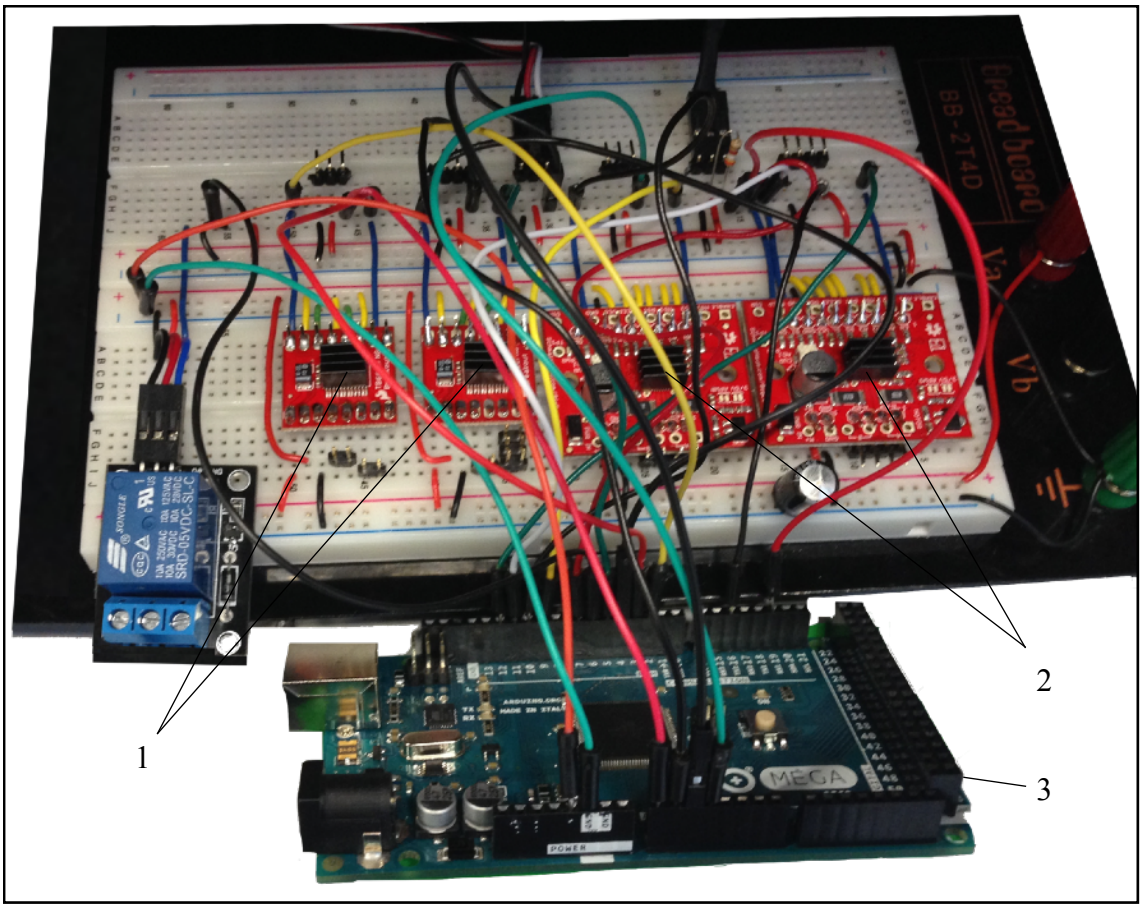

Figure 4.9 - Control Unit prototype board.

The board includes two drivers (Sparkfun EasyDriver® - Figure 3.10, [1]) useful respectively to control the peristaltic pumps involved in the automatic media replacement and the heating pads required to constantly maintain the cell culture at 37°C. Two others drivers (Sparkfun BigEasyDriver® - Figure 4.9, [2]) are used respectively to control the perfusion peristaltic pump and compression unit motor. A controller (Arduino Mega 2560® - Figure 4.9, [3]) is the processor unit employed to send, receive and elaborate data acquired during the bioreactor operation. A

series of capacitors and resistors were used into the configuration. A bench top power supply is used to power all the components.

After testing, because of the presence of different wires and not stable elements, aiming to obtain a fixed and steady board, a customized electronic shield was realized, as shown in the following figure.

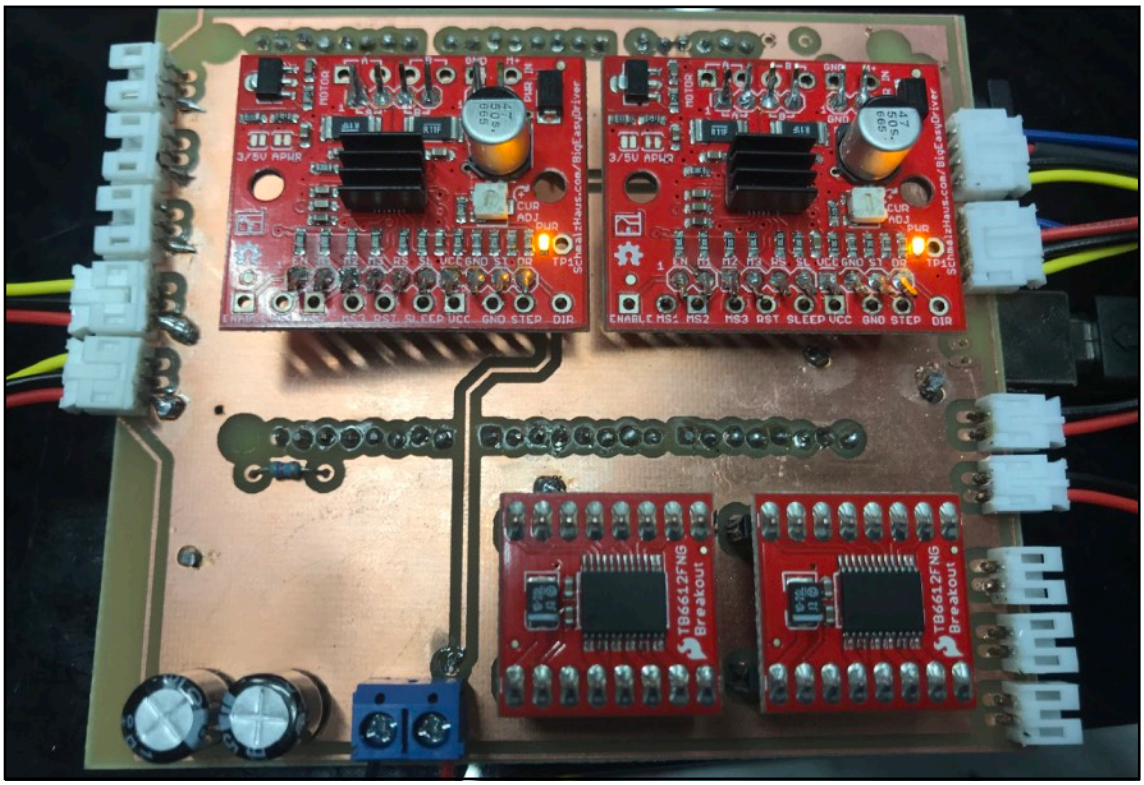

Figure 4.10 - Layout of the electronic shield.

In the final version layout, all the components are welded to the shield. This is plugged on top of the Arduino. Compared with the prototype board, a series of plug and play connectors are used to connect and disconnect easily each actuator/sensor. All the drivers are plugged onto the shield through a series of female stacking headers, in a way to have the possibility

to replace them easily in case of break. A commercial power supply (12V, 5A) is used to power each component.

4.3.2 GUI

With the aim to provide a system usable by anyone working in a tissue engineering laboratory, a user friendly graphical unit interface (GUI) was designed, as shown in the following figure:

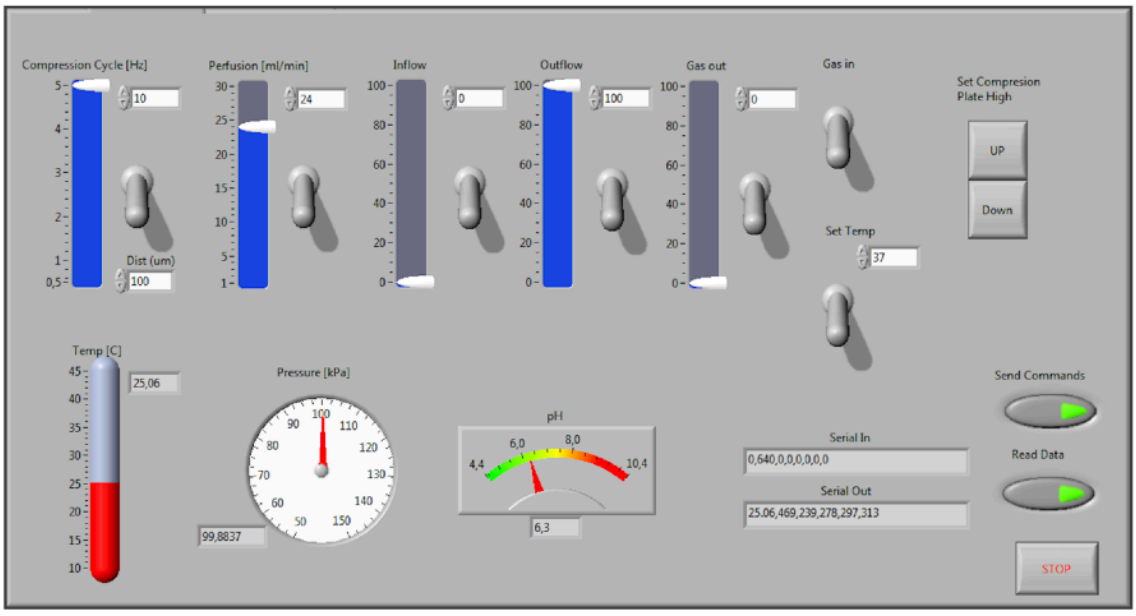

Figure 4.11 - Graphical Unit Interface. Interface useful to tune all the actuators and to check all the operative parameters deriving from the sensors.

The interface allows to control easily the actuators through a series of virtual on/off switches and adjustable bars, simultaneously allowing the constant monitoring of each sensor.

Compression stimulus can be performed setting the displacement [μm] and the frequency [Hz] of the compression plate integrated within the compression unit; two buttons (Up, Down) can be used to align the compression plate, rather, to allow the contact between the cylindrical scaffolds units and the pistons of the compression unit. Perfusion can be tuned changing the flow intensity [ml/min] related to the dedicated peristaltic pump. Automatic media replacement can be performed setting the “inflow” and “outflow" parameters [a.u.] related to the dedicated peristaltic pumps. In case of gas manual adjustment, within the measurement chamber, an air pump can be connected to the CU and the gas can be added/removed using respectively the “Gas in” “Gas out” switches. The desired culture temperature can be set through the “Set Temp” graphic element. Temperature [°C] , Pressure [kPa] and pH [-] can be monitored by dedicated scalebar. Two arrays (“Serial In”, “Serial Out”) are useful to check constantly if the right input/output are correctly sent/received to/from the CU. A “STOP” button is present to allow the interruption of all operations.

4.4 Effect of bioreactor system over cell viability and matrix mineralization

The cells were cultured during 14 days either under a dynamic or a static (control) condition. In order to evaluate this novel bioreactor system in terms of ability to commit naive cells towards an osteogenic phenotype, perfusion and compression were transferred, onto 3D CHT/G scaffolds (Figure 4.12, [A],[B]) hosting onboard human mesenchymal stem cells, (hMSCs) as biomechanical stimuli aiming to improve cell proliferation and differentiation respectively. Platelet lysate was used as soluble growth factor source, in a way to supply biochemical stimuli to both static and dynamic cultures.

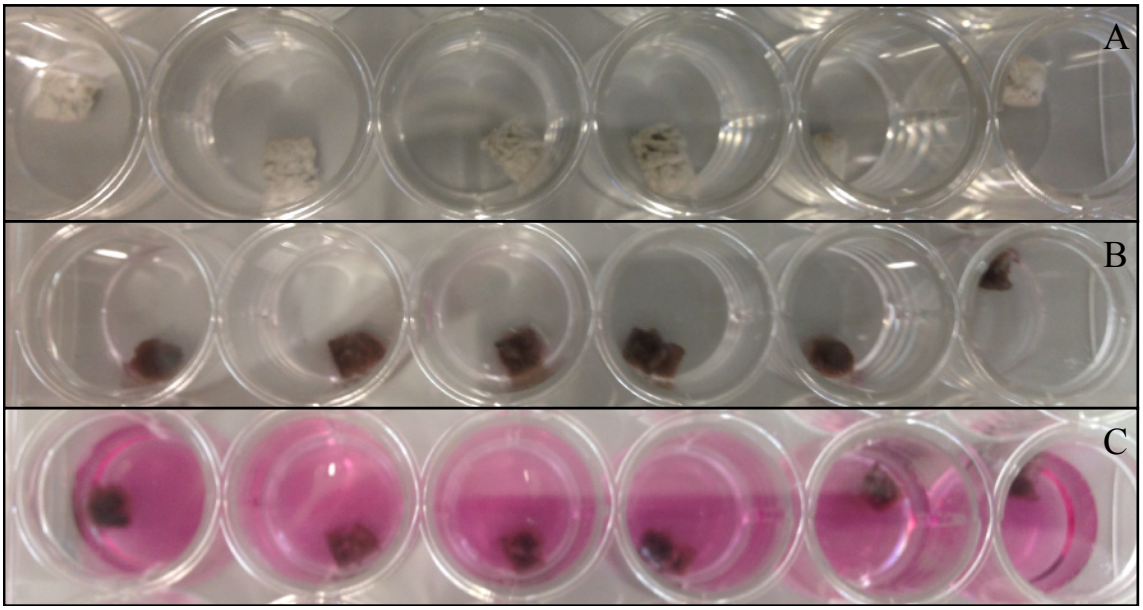

Figure 4.12 - CHT/G scaffolds before bioreactor cell culture. A, empty scaffolds after their making and sterilization; B, scaffolds during hMSCs seeding; C, scaffolds maintained in a traditional static culture.

Both groups were initially maintained in a traditional static culture condition (Figure 4.12, [C]), to promote cell adhesion and proliferation over/within the scaffold. Subsequently, perfusion - aiming at improving nutrient exchange and waste removal - was applied to a group (n = 6) of cell-bearing scaffolds for 7 additional days together with compression aiming at the transfer to the cells of a mechanical stress intended as the stimulus for phenotypic cell commitment. To evaluate cell viability, 3 days after the beginning of dynamic culture (i.e. total culture day 10th), one scaffold from the bioreactor system and one maintained in static condition were evaluated by the live/dead® assay.

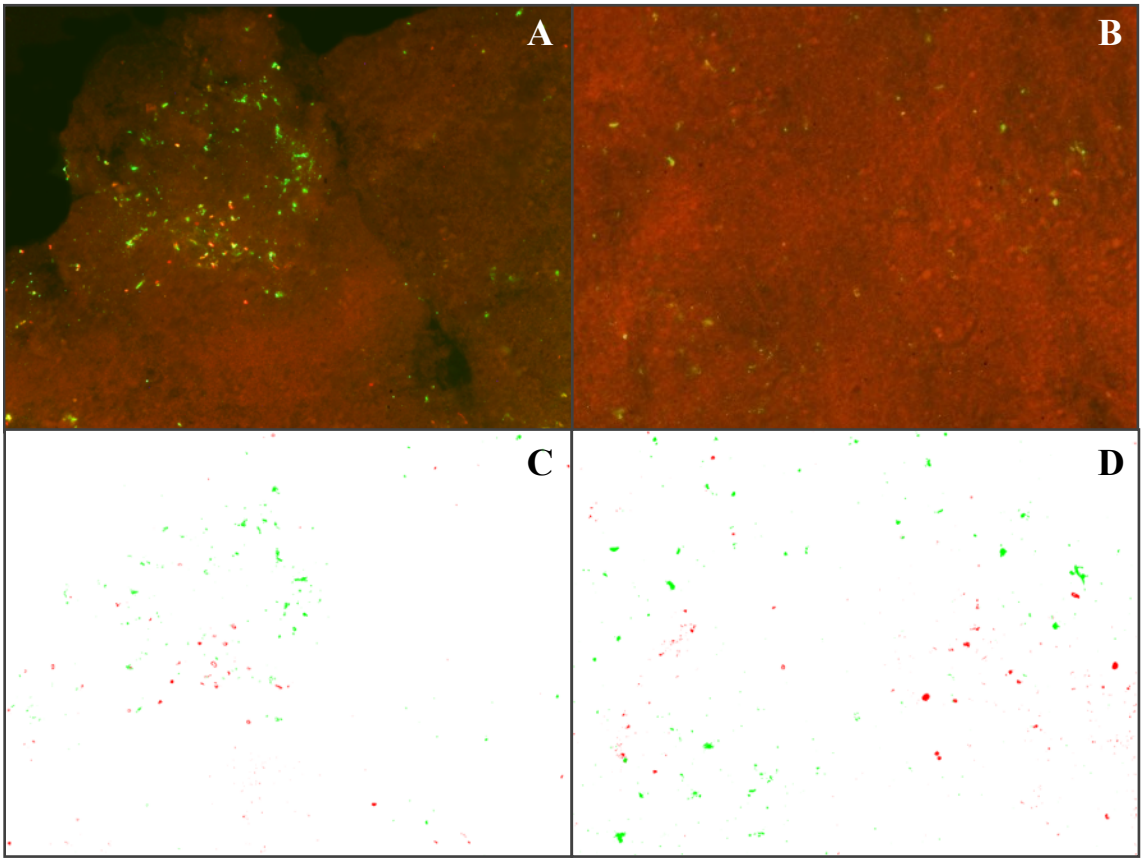

Figure 4.13 - Cell viability/proliferation (live/dead assay) within the 3D CHT/G scaffold under static (A / C = outer layer; B / D inner layer).

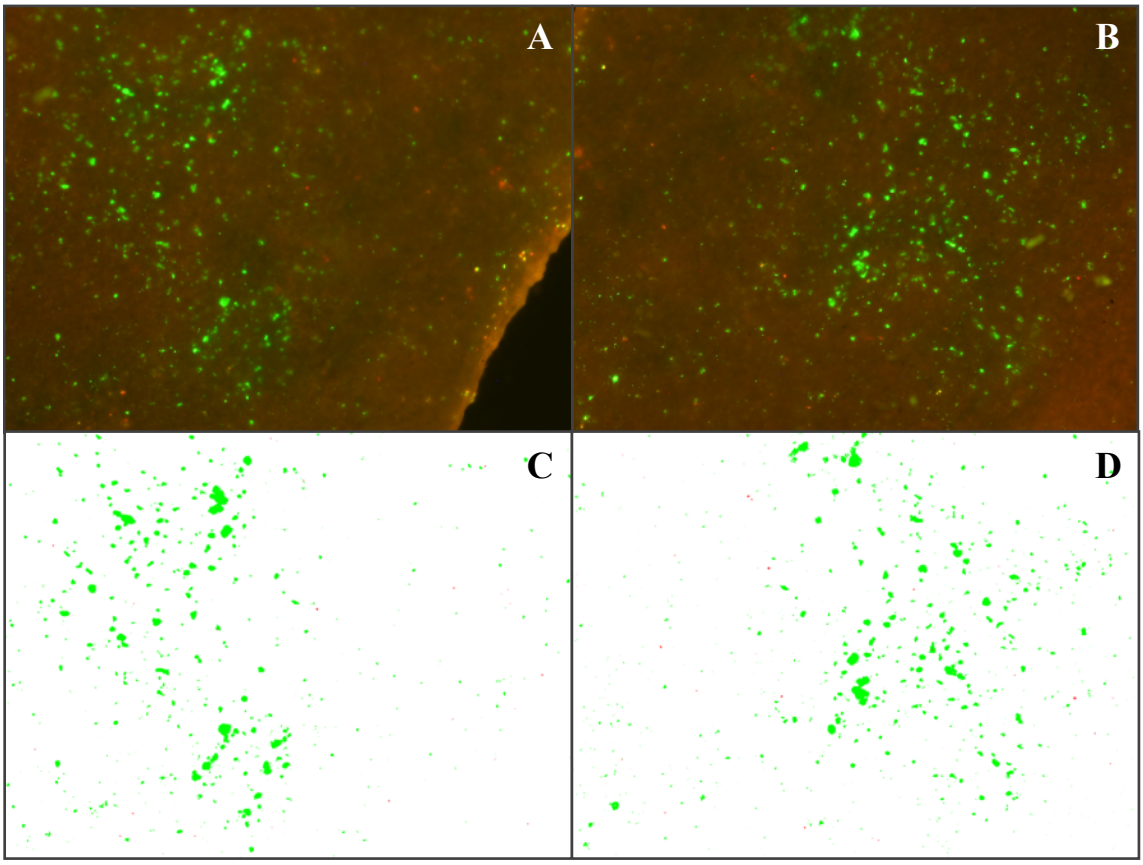

Figure 4.14 - Cell viability/proliferation (live/dead assay) within the 3D CHT/G scaffold under dynamic (perfusion and compression) (A / C = outer layer; B / D inner layer).

Figures 4.13 and 4.14 show cell distribution and viability inside (outer and inner layers) the CHT/G scaffolds comparing the static (Figure 4.13 [A], [B]) vs. the dynamic (Figure 4.14 [A],[B]) culture. Comparing [B] panels from both Figures 4.13 and 4.14 show the increase of alive cells (green spots) in the core region of the dynamic vs. static condition. The expected perfusion contribution in improving nutrient exchange and waste removal determined a larger amount of viable cells under dynamic culture conditions, together with a consistently reduced number of necrotic cells

when compared to a traditional static culture protocol. A quantitative analysis was performed to confirm this initial evaluation. Image segmentation was used as tool to highlight the amount of scaffold area covered by either live or dead cells in static (Figure 4.13 [C],[D]) vs. dynamic (Figure 4.14 [C],[D]) condition, detailing the outcome measured within the outer and the inner layers of the scaffold, respectively.

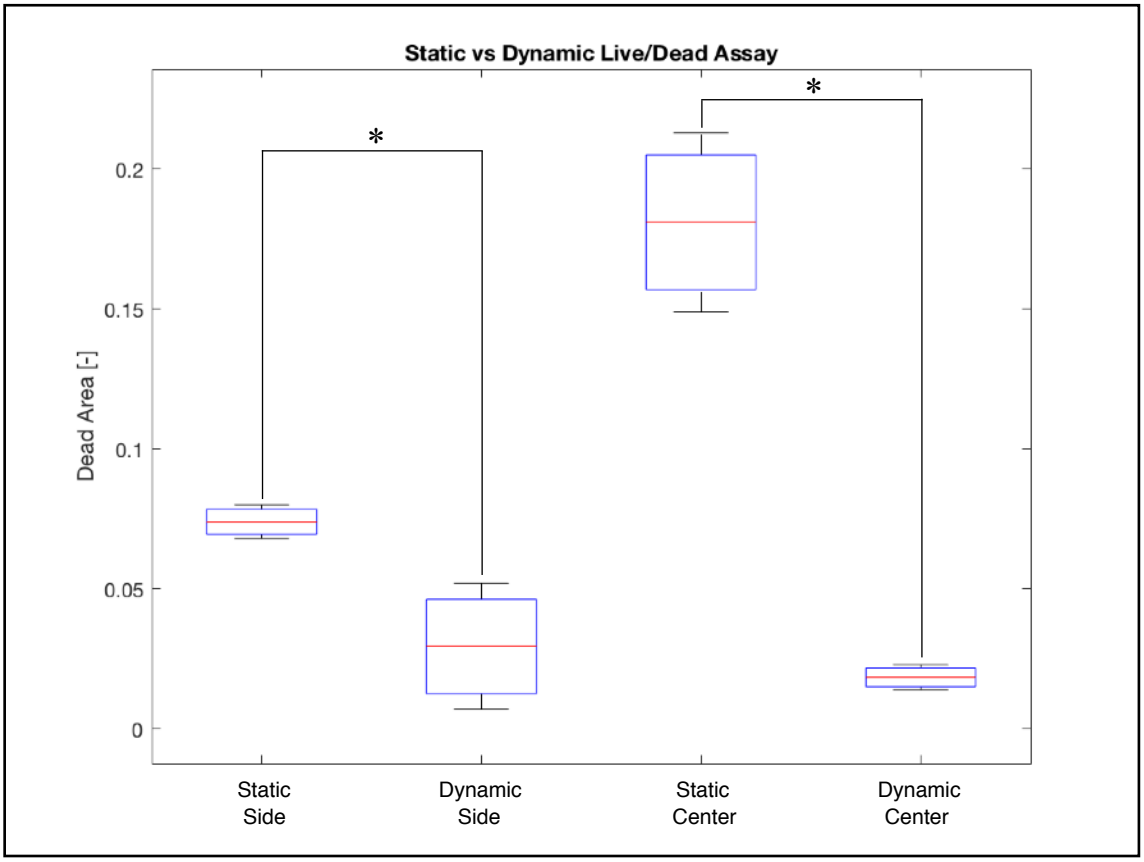

Figure 4.15 - Dead area evaluated by a cell viability/proliferation (live/dead) assay on CHT/G scaffolds. *Statistically significant difference (Student's t-test; $P<0.05$).

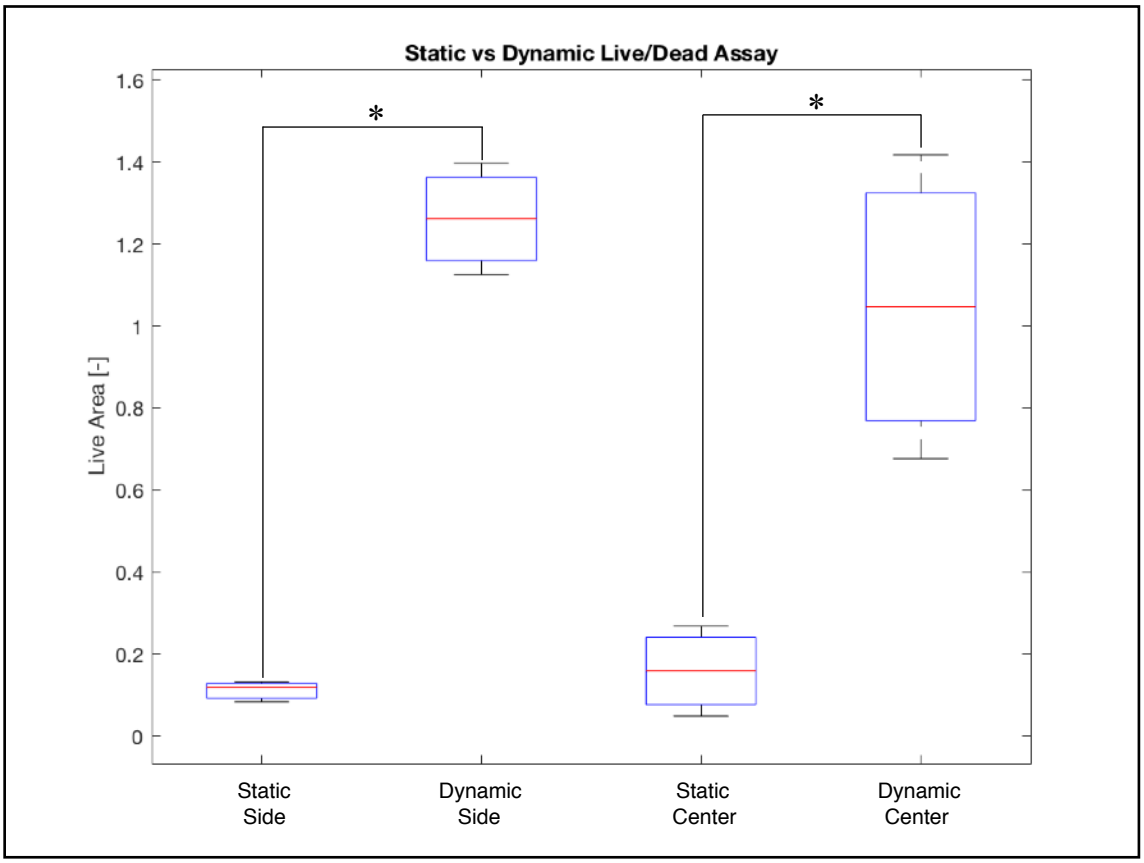

Figure 4.16 - Live area evaluated by a cell viability/proliferation (live/dead) assay on CHT/G scaffolds. *Statistically significant difference (Student's t-test; $P<0.05$).

Figure 4.15 and 4.16 show the size of the scaffold area that can be attributed to viable/necrotic cells. A sizable effect of perfusion exchange enhancement under dynamic culture condition is particularly evident within the inner scaffold core, where more viable and less necrotic cells are present, when compared to traditional culture condition. On the other hand, apparently the absence of significant differences is observed when the outer scaffold layers are compared in static vs. dynamic conditions. This suggests that cell growth and proliferation are adequately sustained within the limit

of diffusion from surrounding medium in standard culture conditions. On the other hand, nutrient diffusion into the core of 3D tissue engineered tissue constructs was always considered to be hampered by the traditional static culture. The results obtained with our bioreactor system strengthen the strategy of supplying an appropriate perfusion flow to support nutrient influx and waste removal into/from the core of the scaffold.

In addition to improving cell viability, our bioreactor system is expected to prime cell phenotype towards the osteogenic phenotype. Extracellular matrix (ECM) mineralization is among the hallmarks of the expected commitment, thus we evaluated our scaffolds upon Von Kossa staining of the CHT/G texture, after 7 days of dynamic vs. static culture.

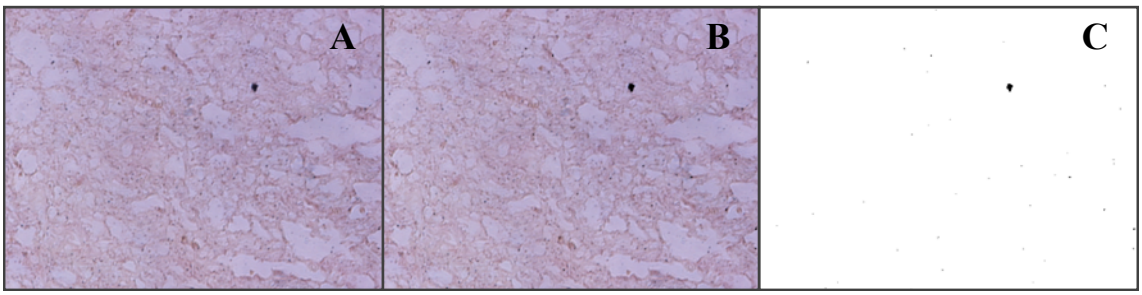

Figure 4.17 - ECM mineralization (Von Kossa staining) within the core of the 3D CHT/G scaffold under static.

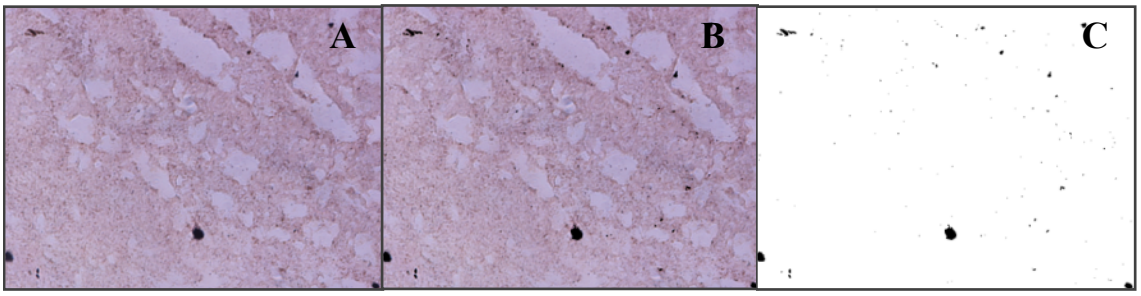

Figure 4.18 - ECM mineralization (Von Kossa staining) within the core of the 3D CHT/G scaffold under dynamic (perfusion and compression).

Figures 4.17 and 4.18 show the Von Kossa stain within the core of a CHT/G scaffold in static (Figure 4.17, [A],[B],[C]) vs. dynamic (Figure 4.18, [A],[B],[C]) culture. Panels 4.17, [A] and 4.18, [A] represent qualitative optical microscopy images where black spots from the Von Kossa stain appear supernumerary in the dynamic condition (Figure 4.18 [A]).

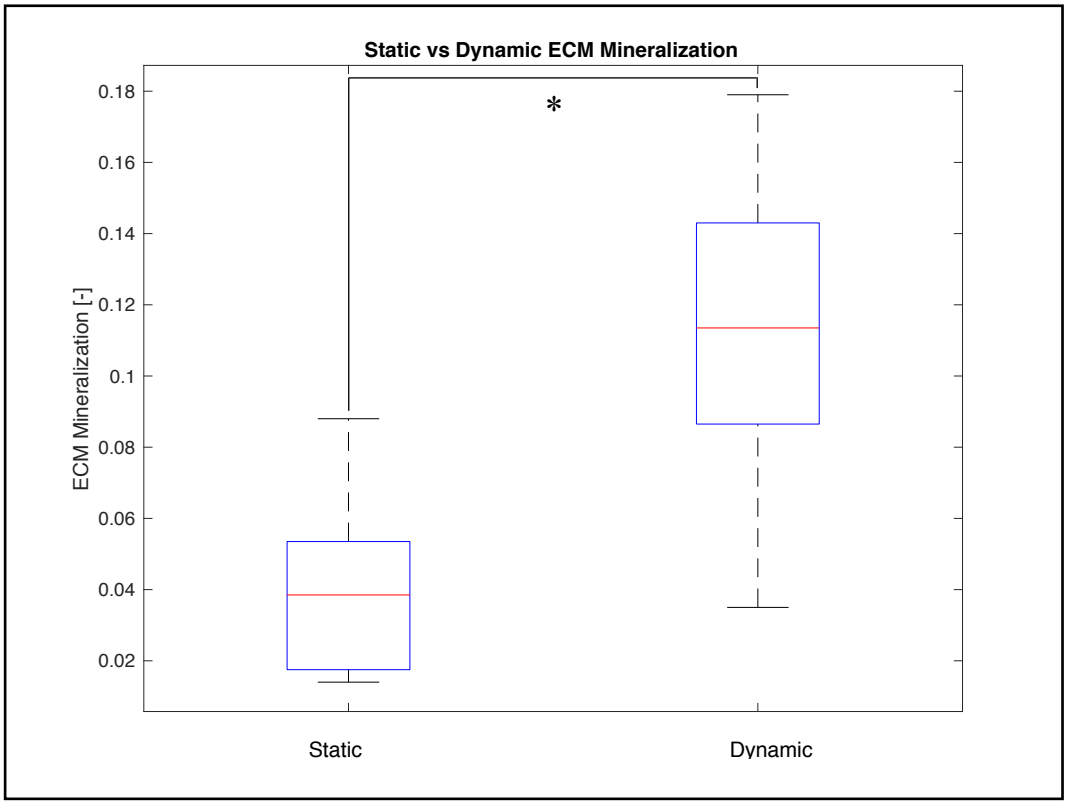

Figure 4.19 - ECM Mineralization evaluated by Von Kossa staining within the core of the 3D CHT/G scaffolds under static (left) and dynamic (perfusion and compression) (right). *Statistically significant difference (Student's t-test; $P<0.05$).

This was confirmed performing a quantitative analysis where image segmentation, obtained by using the max entropy method, highlights the amount of scaffold area covered by the Von Kossa elicited black spots in static (Figure 4.17 [B],[C]) vs. dynamic (Figure 4.18 [B],[C]) conditions.
The ECM Mineralization obtained under static (Figure 4.19, [left]) and dynamic (perfusion and compression) (Figure 4.19, [right]) conditions was evaluated. A statistical significance, related to the effect of the compression stimulus, is particularly evident within the core of the 3D CHT/G scaffolds maintained under dynamic culture condition, where a sizable ECM mineralization is present, when compared to traditional culture condition.

4.5 Preliminary evaluation of the real-time/in-line monitor

Each single mobile unit described above (Figure 4.6, [A]) might undergo real-time/in-line analysis (e.g. spectro(fluoro)scopy, X-ray μCT 3D imaging) to monitor the evolution of phenotype commitment before going back to the differentiation protocol within the device. This allows to keep track of a very same tissue construct during different days of dynamic cell culture.

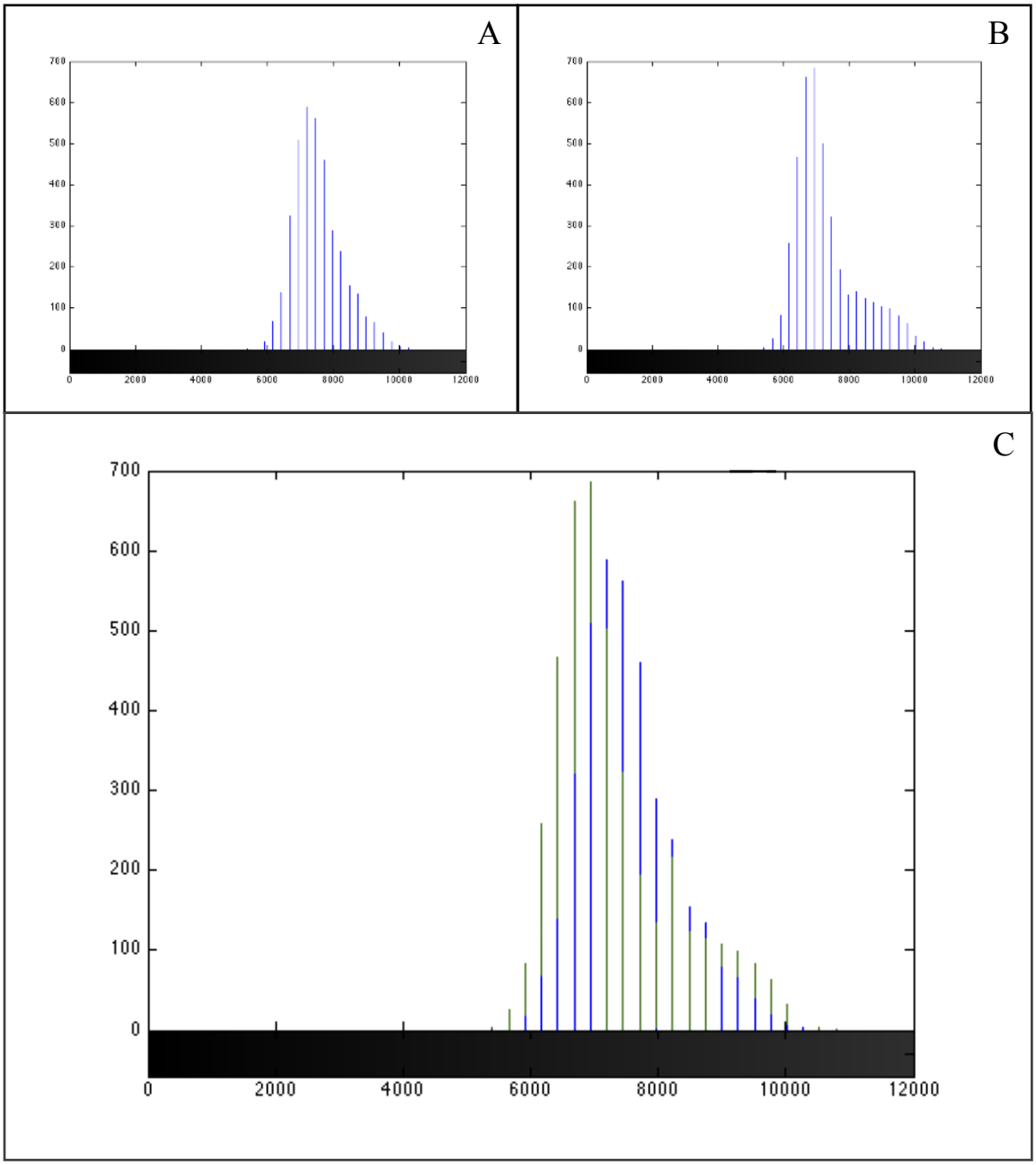

Figure 4.20 - Histograms of one scaffold processed by X-ray μCT. A, sample-pre; B, sample-post; C, pre (blu) and post (green) samples overlapped.

Figure 4.20 shows very preliminary results about one scaffold hosted within a mobile unit and cultured for 10 days in static condition followed by 4 days into the bioreactor system. Histograms of images obtained by X-ray μCT acquisition at day 11th (sample-pre, Figure 4.20, [A]) and day 14th (sample-post, Figure 4.20, [A]) were extracted. Figure 4.20 shows the overlap between pre- (blue) and post- (green) samples histograms. An increase in the gradient distribution of the post-sample is present. The relationship with a biological effect will be evaluated in future experiments. On the other hand, is possible to confirm that real-time/in-line analysis can be performed without to interfere with the cell culture.

5. Discussion

Although engineered bone tissue is been viewed as a potential alternative to the conventional use of natural bone grafts, due to its virtually limitless supply and the "clean" manufacturing procedure, however, bone tissue engineering practices have not proceeded to clinical practice yet.

Likely responsible for this delayed progress are the poorly standardized applicative protocols, being the *ex vivo* fabrication of tissue engineered bone substitutes a complex process whose aspects need to be more carefully determined. In other words, there is still a need to recognize, assess, and arrange in order of importance the criteria to proceed towards tissue substitutes of high quality.

Over the past decade, the field of tissue engineering (TE) has witnessed a significant progress, as a result of our improved understanding of biology, materials science, chemistry and engineering strategies, and the convergence of these disciplines [96]. As a consequence, present biofabrication technologies have enabled the manufacturing of complex 3D artificial tissues heralding better strategies for tissue/organ repair, with respect to the current traditional options. Thus, the TE approach has gained considerable attention as a promising strategy to heal bone defects, which are a significant health problem (resulting in billion annual healthcare costs) currently treated with grafts, decellularized bone, or synthetic bone grafts, with variably successful results. Looking towards future treatments for such defects, modern medicine was looking to bone grafts based on tissue-engineered substitutes grown in vitro and able to integrate with the

host [97]. To this aim, various biomaterials - both biomimetic synthetic polymers and biological molecules - manufactured via several fabrication techniques, were used as extracellular matrix substitutes with adequate biological and mechanical properties able to provide support for cell attachment, proliferation, and differentiation [98]. Autologous human Mesenchymal Stem Cells (MSCs), more specifically bone marrow-derived MSCs (hBM-MSCs) are suitable candidates to populate these scaffolds. In fact, given their proliferation potential, biomolecular production, cell-to-cell signaling, and formation of appropriate extracellular matrix (ECM), they efficiently differentiate down the osteogenic path, and also secrete paracrine factors that may aid survival and vascularization of engineered bone [99]. Mechanostimulation also acts as a significant input to maintain/ induce bone phenotype [100], while increasing culture diffusive regimes [101]. In particular, compressive load was shown to significantly increase osteogenic markers in MSCs subjected to mechanical reconditioning [102]. Several bioreactors were used to impact mechanical stimuli to cells in culture. Some of them can also act as a stand-alone cell culture incubator able to transfer a controlled, recordable, and adjustable (cyclic) deformation to a 3D scaffold [70].

In summary, a combination of appropriate cells, biomaterials/scaffolds, and physical stimulation seems a successful approach to bone TE, but a standard protocol to optimally stimulate a 3D scaffold with MSC cells onboard, using a controlled mechanical deformation, to induce MSC fast osteogenic lineage commitment has to be released yet.

Current scientific literature about using bioreactor system for bone tissue engineering is converging towards this objective.

Ding et al. 2016, shown as the combination of a mineral scaffold (i.e.: high Young's modulus) with a low flow rate is enough to promote bone formation; on the other hand, Nguyen et al. 2016 and Sinlapabodin et al. 2016 applied higher flow rates onto soft scaffolds (i.e.: low Young's modulus) to obtain bone differentiation. From those, there is an evidence on how perfusion flow rate and scaffolds mechanical properties are straightly related. Different limits can be highlighted: (i) considering bone physiological stimuli, perfusion and compression are present; particularly, compression plays a fundamental role during differentiation, thus, perfusion alone is not enough to reproduce correctly the bone physiological behavior. (ii) To apply compression stimulus, hard scaffold materials (i.e. PLA, PLGA, CaP, etc.) might be not appropriate, because an high load value might be applied to obtain a proper strain. (iii) soft scaffold materials (i.e.: alginate, chitosan, etc.) are useful to apply compression stimulus, but they might require high flow rates which might interfere with cells viability during dynamic culture.

Brunelli et al. 2017, Maeda et al. 2017 and Revichandran et al. 2016 described compression as a stimulus promoting mineralization but, at the same time, suppressing proliferation. As mentioned above, for a proper bone tissue differentiation, both perfusion and compression are necessary. Thus, compression alone is not enough to reproduce bone physiological behavior. Furthermore, compression effect on suppressing proliferation can be related with the perfusion absence, that is, a not appropriate nutrient diffusion and waste removal.

To satisfy the presence of both perfusion and compression, suitable bioreactors were designed by Teng et al. 2016, Baumgartner et al. 2015, Li

et al. 2014, Petersen et al. 2012. Their results shown as: (i) perfusion is useful to enhance/increase cell proliferation; (ii) compression is a trigger for the osteogenic differentiation; particularly, comparing low and high frequencies cyclic compression, low frequencies are more effective; (iii) scaffold stiffness has only a temporary effect on cell behavior, while the impact of mechanical loading is preserved over time.
Furthermore, Li et al. 2014 designed a stand-alone bioreactor, that is, a device that not require a dedicated cell culture incubator.

The proposed current scientific literature shows how mechanical actuation devices play a fundamental role in obtaining an engineered bone tissue construct. Different kind of cells and 3D supports can be used without significative differences; on the other side it is clear how perfusion and compression fulfill each a specific role during tissue proliferation/ differentiation. Perfusion flow rate in a range of 1-3 mL/min leads to shear stresses promoting cell proliferation; on the other hand a flow rate higher than 3 mL/min causes shear stresses that lead to a decrease in the number of cells. Compression frequency in a range of 1-5 Hz is useful for a proper differentiation; compression at frequency lower than 1 Hz inhibits both cell proliferation and differentiation.
Finally, only two of the proposed devices might be defined stand-alone. Having a standalone device can be considered an important feature: in fact, most of the bioreactor systems needs of a committed incubator to guarantee the maintenance of the standard cell culture parameters, requiring las space and resources that can be spared in the stand-alone configuration.

Starting to this paradigm, the aim of this study was to develop a novel perfusion/compression bioreactor system as efficient tool for tissue engineering approach. Based on this idea, the bioreactor was designed as a compact unit including a mix among: proved features as perfusion/ compression and stand-alone apparatuses; innovative features as automatic media replacement system and real-time/in-line monitor.

Perfusion and compression apparatus was included to apply proper physical stimuli during bone tissue commitment. As mentioned above, perfusion is aimed at improving diffusive exchanges, i.e. nutrient uptake and waste product removal; on the other hand, compression is applied to exert a mechanical strain over the cell onboard the scaffold, with the aim of addressing their phenotype commitment.

A stand-alone apparatus was introduced in a way to avoid the use of a dedicated cell culture incubator, that is, to locate the device over a standard lab benchtop.

A system of automatic replacement of the media was incorporated in order to: facilitate the replacements of the media avoiding to administer to the cell culture the stress that is typically occurring with sheer media changes within traditional subculture procedures; prevent contamination due to manual operation.

A real-time/in-line monitor was designed to easily perform at different time point on the same tissue construct specific analysis (e.g.:

spectrofluorometric analysis, fluorescence analysis; X-ray μCT for 3D imaging analysis).

Before to use the bioreactor device a series of test were performed: (i) culture parameters (i.e.: temperature of 37°C and ph of 7.4) were monitored for a week aiming to verify the stability of the system: no fluctuation were detected; (ii) each bioreactor component was sterilized, placed in the bioreactor and the absence of possible contamination was monitored for a week: no contamination was detected; an *in silico* evaluation was carried out to estimate the stress affecting the scaffolds, therefore the cells onboard, when perfusion and/or compression are applied: no apparent turbulences were detected and stress values in a range of $10^{-5} \div 10^{-1}$ [Pa] were measured

During the dynamic culture, a perfusion flow rate of 1 [ml/min] was used to guarantee appropriate diffusive exchange without, at the same, interfere with the cell viability. A dynamic compression 2 times/day for 30 min each time, with a 1 % strain at 1 Hz was applied to address specific differentiation stimuli. The effect of perfusion and compression stimuli on cell behavior (proliferation/differentiation respectively) was evaluated at different time point.

Proliferation was investigated, at day 3 of the dynamic culture, through a cell viability assay: qualitative/quantitative data shown as the expected perfusion contribution in improving nutrient exchange and waste removal determined a larger amount of viable cells under dynamic culture

conditions, together with a consistently reduced number of necrotic cells when compared to a traditional static culture protocol. This suggests that cell growth and proliferation are adequately sustained within the limit of diffusion from surrounding medium in standard culture conditions. On the other hand, nutrient diffusion into the core of 3D tissue engineered tissue constructs was always considered to be hampered by the traditional static culture.

Osteogenic differentiation, this is, ECM matrix mineralization was evaluated through Von Kossa staining: qualitative/quantitative data confirmed that our bioreactor system is apt to transfer to the scaffold a relevant mechanical cue which is translated into a sizable biological effect.

A specific test for the assessment of the real-time/in-line monitor was performed. Very preliminary results shown that the same sample, thus, the same construct can be processed by X-ray μCT, at different time point, without to interfere with the cell culture. The relationship among the obtained data and the biological effect will be evaluated in future experiments.

Comparing this with the previously manufactured bioreactors, it is possible to conclude how the developed device: (i) is able to reproduce the perfusion/compression regimens useful for a proper proliferative/ differentiative response; (ii) is a stand-alone device, which means that different experiments can be performed onto a standard lab bench top,

without the need of a committed incubator; (iii) allows an automatic cell media replacement preventing the risk of contamination; (iv) includes an innovative feature which allows to perform a real time/inline analysis, at different time points on the same scaffold, avoiding the necessity to apply destructive technique to asses the tissue construct during the whole culture.

6. Conclusion

The main focus of this work is the development of an innovative bioreactor system for human bon tissue engineering. This device is useful to reproduce physiologic mechanical solicitations, recognized as fundamental cues actives over natural tissues. Especially, human bone tissue is normally subjected to two forms of biomechanical stimuli: compression/tension loading generating fluid movement through lacunae and physical deformation generating structural change. In order to address the bone tissue phenotype commitment the perfusion aimed at improving diffusive exchanges was coupled with compression intended to exert a mechanical strain over the cell onboard the scaffold.

The developed bioreactor was designed integrating both proved and innovative features aiming to work easily and safety improving the processes involved during osteogenic lineage commitment.

This is realized through the use of a series of sensors, actuators and suitable biocompatible materials used for the cell interfacing environment, while polycarbonate, silicon, aluminum, or stainless steel were used for the other components of the device.

The complete description of the device and of its realization is reported in detail together with a validation obtained comparing the results of the tissue construct cultured in dynamic condition vs. static condition (control). Furthermore, a really preliminary result about real-time/in-line monitor is shown, to evaluate the possibility of performing the same analysis on the same tissue construct at different time point.

The obtained results confirming that this bioreactor system is apt to transfer to the scaffold a relevant mechanical cue which is translated into a sizable biological effect.

A series of future developments are required: (i) a set of generic experiments to produce robust biological data; (ii) a set of specific experiments to evaluate how the compression stimulus may promote the phenotype commitment without the need of soluble growth factors added to the culture; (iii) a revision of the device structure aiming to shift from a prototype version to a final version of the device; (iv) a standard protocol to optimally stimulate a 3D scaffold with MSC cells onboard, using a controlled mechanical deformation, to induce MSC fast osteogenic lineage commitment.

Acknowledgments

When I decided to begin this journey I was certain that it would be an awfully big adventure. It was characterized by several events and each difficulty that I have encountered was engaged head-on. A lot of things have changed in this time, me included. I have enriched my self, I have met new people and unfortunately, along the way, I have lost a part of my life.

Despite this, I reached the end of the journey and it would not be possibile without the support of all the people that are part of me. I would like to thank and to dedicate this work to them. First and foremost to my mother, she is always by my side, ready to support and to encourage me. To my father, who has always believed in me. To Alessandra, I would never have gotten here without you. To my brother, that I hope will follow my path as an example for his life.

Then I would like to thank all the people that shared this journey with me.

Emanuele, Paolo, Óli, my supervisors, always present and ready to guide me during this adventure. Giovanna, Magnús and Þórður the others traveling companions. All of you are more than just colleagues.

Giulia, Valeria, Alan, Alessandro, Claudio (in alphabetical order, women first), my colleagues, my friends, the Cavalcavia's gang, thank you to shared all the events of this journey with me.

Alice and Marilisa, the colleagues and friends of the ICMLab's gang, thank you for the support, the long talk and the help.

Alex, Andrea, Bob, Danilo, Paola, the Apice's gang, thank you for all the "interesting" lunches and funny talk we have shared.

Appendices

Appendix A

Equipment

Reagent	Manifacturer
DMEM F12 + Glutamax medium	Life Technologies, Carlsbad, CA, USA
Penicillin-Streptomycin	Life Technologies, Carlsbad, CA, USA
Fetal Bovine Serum (heat inactivated or MSC screened)	Life Technologies, Carlsbad, CA, USA
PBS	Life Technologies, Carlsbad, CA, USA
Confluent hBM-MSCs cells in monolayer	Lonza inc, Allendale, NJ, USA
0.1% Gelatin	Sigma Aldrich, St. Louis, MO, USA
0.25% Trypsin	Life Technologies, Carlsbad, CA, USA

Table 1. Reagents used for harvesting stems cells

Component	Manifacturer
75cm cell culture flasks	Nunc, Penfield, NY, USA
Tubes (15 and 50 ml)	Falcon, Franklin Lakes, NJ, USA
Pipettes and tips	Gilson, Middleton, WI, USA

Table 2. Components used for harvesting stems cells

Device	Manifacturer
Centrifuge	Thermo Scientific, Vantaa, Finland
Incubator (5% CO_2, 95% H_2O)	N/A

Table 3. Devices used for harvesting stems cells

Protective equipment should be worn while handling the components of this assay. Wear lab coat, protective gloves and safety googles at all times. hES-MP002.5 are cells of human origin. Same procedures that apply to other tissue of human origin such as blood should be followed.

Component	Storage	Hazards and Precautions
DMEM F12 +glutamax	4-8°C	Not dangerous
Penicillin Streptomycin	-20°C	**Danger** Skin corrosion, serious eyes damage, respiratory and skin sensitization, reproductive toxicity
Fetal bovine serum	-20°C	Not dangerous
Platelet lysate	-20°C	Not dangerous
Sterile Gelatin	4-8°C	Not dangerous
Sterile PBS	RT	Not dangerous
Trypsin	-20°C	Not dangerous

Table 4. Precautions for the components used during harvesting stems cells

Procedure

Preparation of solutions

For the preparation of cell culture media, the following protocols was applied:

- Preparation of Culture media with 10% FBS in DMEM F12 +glutamax.

Preparation of 0.1% gelatin solution

- Bringing 2% gelatin stock solution to room temperature.
- Working under sterile conditions in a clean hood with the fan on.
- In a 50 ml tube mixing 47,5 ml sterile H2O with 2,5 ml 2% gelatin.
- Storing at 4-8°C.

Coating cell culture flasks and preparation

- Blending media or obtaining already prepared media
  - Making sure that media has 0.1% Penicillin streptomycin and 10% supplement (either bbPL or FBS)
  - If using platelet lysates (PL) making sure that media has 4IU/ml of heparin
- Bringing the media and the trypsin to 37°C in the shaking plate incubator

- If preparing cells to be reseeded, working sterile and add 5 ml of 0.1% gelatin solution to a sterile 75cm2 cell culture flask (67µl/cm2) or other culture vessel of your choice.
- Close the flask, allowing the gelatin to completely float over the culture surface and keeping at 4-8°C for at least 30 min.
- Making sure that your flask is carefully labeled with your name, date, cell type, media type and passage number.

Harvesting cells

- Preparing the laminar flow hood with clean o Pipettes and tips
  - o Waste container with chlorinated water o Empty lab tubes
  - o Reagents to be used
- Obtaining the cell culture from the incubator and placing inside the hood.
- Loosening the cap and pouring the media into the waste container.
- Transfering 5 ml sterile PBS (67µl/cm2) into the cell culture flask. Close the flask and allowing the PBS to float over the culture surface by rocking the flask gently.
  - o This will wash excess media from the culture flask and ensure better effectiveness of the trypsin in next steps.
- Pouring the PBS off and then putting 5 ml of warmed Trypsin into the flask.
- Close the flask and putting it into the incubator for 5 min
  - o The trypsin, as a digestive enzyme, will release the cells from the

plastic surface.

- When the cells are released they will appear small, round and bright in the microscope and float around creating a “snow storm” impression.
- In this step the cells should be monitored carefully since prolonged trypsin digestion can harm the cells. When the cells are released proceed immediately to next step.

- Returning the cell culture flask into the hood and adding 5 ml of pre-warmed media into the flask.
  - The supplemented media will help neutralize the trypsin and stop the digestion.
- With the aid of a pipettboy, using the cell solution inside the flask to wash the culture surface and then transferring the solution to a 15 ml tube.
- Centrifugating for 5 min at 1750 rpm.
- Pouring of the supernatant into the waste container and resuspending the cells in 1 ml culture media.
  - If the cell solution is dense, like when combining cells from many culture flasks into a single tube, resuspend the cells in as many ml as the number of culture flasks. 1 culture flask = 1 ml.
- Proceed to cell counting. Seeing the following protocol:
  - Counting cells with hemocytometer
- When the number of cells was obtained they can be reseeded, frozen or used for experimentation.

- If reseeding, using gelatin coated culture flask and using 5500 cells/cm2 seeding density.

Precautions

Use sterile working techniques without exceptions when handling cells for culture.

This includes cleaning everything with ethanol prior to entering the hood, including gloved hands.

Always use a clean water-bath. Mark the medium bottle as supplemented and write your name and date (as for all solutions you work with). Always take the volume you expect to need from the a stock bottle for media or PBS and place in a different sterile container (like 50 ml plastic tubes) before continuing work. This is to prevent contaminating your medium bottle. Do not spray ethanol on culture flasks because the cells are sensitive to ethanol.

Appendix B

Equipment

Reagent	Manifacturer
Trypan Blue	Gibco, Grand Island, NY, USA
PBS	Gibco
Cell solution of unknown concentration	-

Table 1. Reagents used for counting cells

Component	Manifacturer
Microtubes	Sarstedt, Nümbrech, Germany
Pipettes and tips	Gilson, Middleton, WI, USA

Table 2. Components used for counting cells

Device	Type	Producer
Hemacytometer	Neubauer	Assistant, Munich, Germany
Cell counter	-	N/A
Microscope	Leica DM IRB	Lumenera, Capella court, Ottawa, ON, Canada
Calculator	-	N/A

Table 3. Devices used for counting cells

Protective equipment should be worn while handling the components of this assay. Wear lab coat, protective gloves and safety googles at all times.

Component	Storage	Hazards and Precautions
Trypan Blue	RT	**Danger** May cause cancer Suspected of causing genetic defects Suspected of damaging fertility or the unborn child
PBS	RT	Not dangerous

Table 4. Precautions for the components used during counting cells

Procedure

Preparation of stained cell solution

- To a microtube adding
  - 50µl Trypan Blue
  - 30 µl PBS
  - 20µl Cell solution.
- Mixing well
- **Note! Never bring your cell solution outside of the cell hood.**
  - You want your cell solution to remain sterile. Simply transfer a sample

of the cell solution into the staining solution inside the hood under sterile operating procedures.

- Afterwards, the staining solution containing the cell sample can be brought outside the hood.

Cell Counting

- Preparing the counting chamber
  - Making sure the counting surface of the Neubauer hemacytometer is clean
  - Placing the cover slide on top of the counting surface
    - The cover slide will stay in place on the counting chamber via capillary force. Trying to breathe gently on the glass and then placing it immediately on the counting chamber.
- With a pipette transferring a small amount of the cell solution into the hemocytometer by placing the tip at the end of the cover slide and gently allowing the solution to be drawn under cover slide until the area is covered. Do not overfill.
- Counting all the cells in the four big corner squares. Excluding cells that stain blue.
- If more than 200 cells are counted per big square, diluting the solution and repeating.

Calculation

- The volume of each square is 1 mm^3 or 10^{-3} cm^3. 1 cm^3 equals 1 ml so the count can be found with the following formula:

Cells/ml = Average count per square * dilution factor * 10^4

- If the protocol above is followed the total cell count is divided by 4 and the dilution factor is 5

$$\frac{cells}{ml} = \frac{Total\ cell\ count}{4} \cdot 5 \cdot 10^4$$

- To obtain the total cell count of the original solution, the outcome is simply multiplied by the original volume of the solution
- It's recommended to count each stained solution few times (2-4 times) and mix thoroughly between counts to obtain more accurate results.

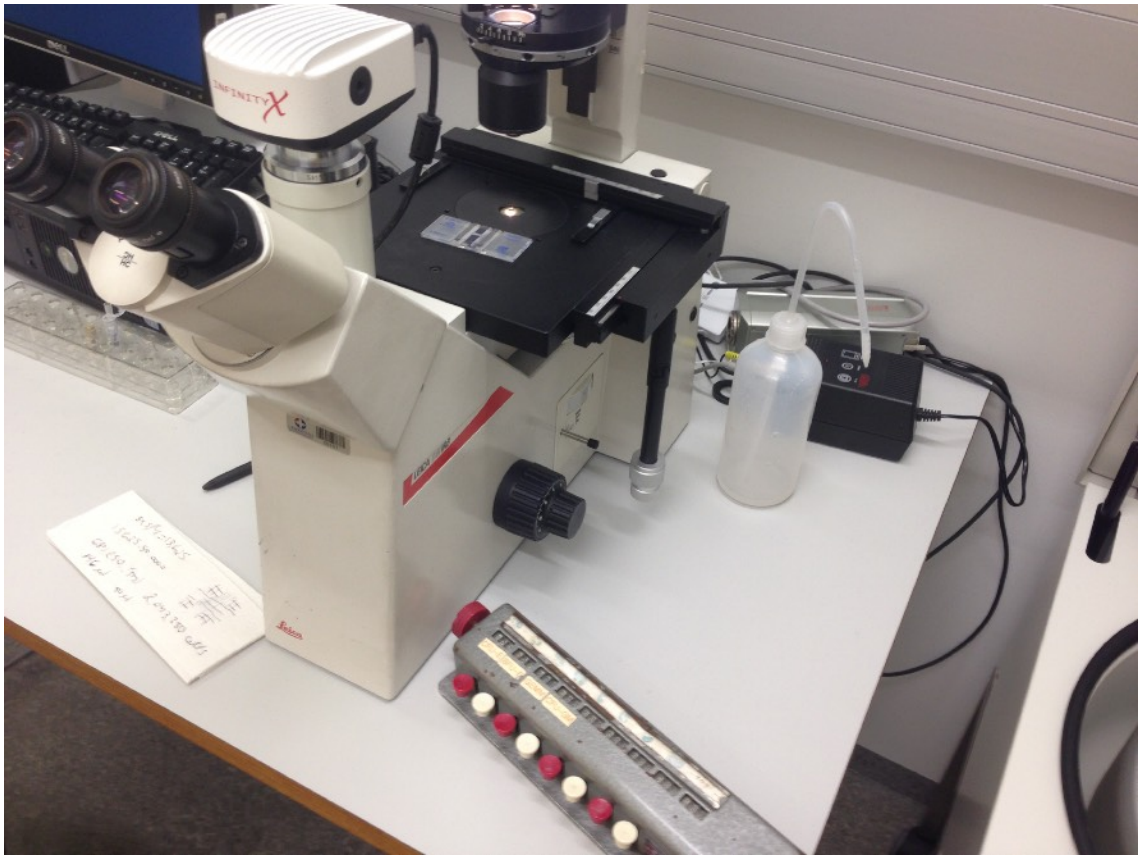

Figure 1. Microscope and cell counter.

Precautions

Use sterile working techniques without exceptions when handling materials intended for cell culture.

This includes cleaning everything with ethanol prior to entering the hood, including gloved hands.

Tryphan blue is a histological dye to stain tissues. Wearing protective gloves and safety googles is recommended since both fingers and eyes are covered with tissue that you don't want to stain!

Wear lab coat, the stain will not wash out of clothes.

Appendix C

Equipment

Reagent	Manifacturer
Cell culture media (e.g. a-MEM, DMEM)	Life Technologies, Carlsbad, CA, USA
Cell culture supplement (e.g. FBS, hPL)	-
Cell solution (e.g. MC3TE, MSC or hES-MP)	-

Table 1. Reagents used for cell culture

Component	Manifacturer
24 well plate – non-tissue treated	BD Falcon, Franklin Lakes, NJ, USA
Sterile chitosan/graphene scaffolds	Bloodbank, Reykjavik, Iceland
Pipettes and tips	Gilson, Middleton, WI, USA

Table 2. Components used for cell culture

Device	Producer
Centrifuge	Thermo Scientific, Vantaa, Finland
Incubator (5% CO_2, 95% H_2O)	N/A

Table 3. Devices used for cell culture

Protective equipment should be worn while handling the components of this assay. Wear lab coat, protective gloves and safety googles at all times.

Procedures

- Obtaining the plate with 6 scaffolds in cell culture medium from the overnight incubation.
- Removing the scaffolds from the medium and placing in a new 24-well plate.
- Allowing the scaffolds to dry for 1 hour at 37°C in the bench top incubator
- Adjusting your cell solution to 4000 cells/µl (100.000 cells/ 25µl)
- Obtaining the correct amount of cells needed in a test tube.
  - You need 100.000 cells per scaffold
- Centrifuging for 5 min at 1750 rpm (545 x g)
- Discarding the supernatant
- Resuspending the cells in 25µl of cell culture media for each scaffold.

- Seeding 25 µl (100.000 cells) drop by drop on each scaffold and incubating in the CO_2 incubator for 15 min.
  - **Mixing the cell solution well prior to seeding each scaffold.**
- After 15 min, removing the scaffold-plate from the incubator and aspirating and reseeding any medium that has leaked from the scaffolds.
- Returning the plate to the incubator for another 15 min.
- Repeating for a total of 4 times.
- At the end of the 4th seeding
  - Getting 3 of 6 scaffolds and add it into the 3 wells of 24-well plate;

after adding 1 ml media for each well.

- Getting the remaining 3 scaffolds and adding it into the bioreactor; after adding the necessary media.

- Returning to the incubator for culture.

Precautions

Use sterile working techniques without exceptions when handling cells for culture.

This includes cleaning everything with ethanol prior to entering the hood, including gloved hands.

Mixing the cell solution prior to seeding is important. No blending will result in inaccurate seeding density and negatively affect the experiment.

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