University of Strathclyde
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Studies on Collagen Stability and Mechanical Properties in Tissue Engineering Applications

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Copyright Statement

Date: Signed:

Abstract

Objective: This project aims to measure the stiffness of collagen based gel matrix. This work provides mechanical study of 0.3 % collagen hydrogel monolayer properties under compression. In order to understand how the collagen gel properties change in relation with time and cell concentrations, the mechanical analysis was performed with different cell densities and time rates. They have been analysed before the culture (to report the stiffness characteristics of non-altered collagen gel) and during the culture, with the influence of the cells.

Background: Cells are profoundly affected by the physical properties of the environment, including the stiffness of the matrix. The stiffness of cell adhesion substrates is increasingly appreciated as an important mediator of cell behaviour; it can regulate cell signalling broadly, with effects on growth, survival, differentiation and motility.[22] The stiffness of ECM-based collagen gels can be manipulated, and so varied to be suitable for growing cells derived from soft tissues. However, the cells are capable of remodelling the gel, altering its stiffness during culture; this could damage the final culture characteristics, leading to altered cell properties.

Methodology: The 0.3 % collagen hydrogel was prepared from collagen type I, obtained from rat tail tendons; it was then placed in the wells of a standard 24-well plate. The collagen gel monolayer was seeded with human hepatoma cells (HepG2) at different densities: 10^5, $5*10^4$, $2.5*10^4$, 10^4 and $0.5*10^4$. Before testing the wells, the viability of the cells was ensured with the MTT assay. The normal sample was represented by a single well of collagen hydrogel which was not seeded with cells, but left for the same period of the culture filled with medium in the same incubator

as the samples. The confined compression strength was measured through *BOSE ElectroForce* machine together with the WinTest software. The data acquired were analysed with *Matlab* software in order to evaluate the Aggregate Modulus (H_A), Hydraulic Permeability (k), Coefficient of Permeability and Correlation Coefficient *(r).*

Results and Conclusions: The data showed that the Aggregate Modulus has a general trend of increasing with culture time, except for the collagen gel without cells; the differences between culture durations are statistically significant just for the cases of $0.5*10^4$ and 10^5 cells per cm^2 cultures. Cells grow and increase in number during culture time, indeed cells in gels cultured for one day present a smaller cell concentration than the ones cultured for 5 days. The increase in number can contribute to increase in the Aggregate Modulus, therefore in the stiffness, since, as showed in the literature, the stiffness tends to increase with cells because they add resistance to the gel (Saddiq *et al.,* 2008). It is generally true that cells act to weaken the collagen gel, but the rate in which they make it soft (by causing the secretion of degradative enzymes and by exerting mechanical forces) could be probably lower than the rate of cells growth, in the specific case of HepG2 cells. For this reason, the cell growth effect outweighs the effect of weakness. Seeding cells on collagen gels will usually lead to one of two possible effects on the Aggregate Modulus: the Aggregate Modulus can reduce which will be due to cells weakening the gels or the Aggregate Modulus can increase which can be due to physical presence of cells contributing to higher stiffness measurements. However, for HepG2 cell line, the increase in stiffness seems to be the predominant action.

On the other hand, regarding the changes in stiffness during the different cell densities, the data acquired do not show a trend which can be interpreted with logical deductions.

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Introduction

1.1 Overview

Tissue engineering is a multidisciplinary science the primary purpose of which is to design and develop new tissues in order to create biological substitutes which can improve, repair or treat parts of the body. It is characterised by *in vitro* production of tissue, through growing cells on scaffolds (matrices).

The ultimate goal of tissue engineering is to revolutionize medicine allowing tissues and organs to regenerate instead of just repairing them. This could lead to new treatment possibilities, with a consequent increase in the quality of patients lives. Moreover, it should be possible to gradually overcome the problem of the chronic shortage of organs for transplant, and, at the same time, to avoid the risk of rejection related to tissue donations.

The main elements of tissue engineering are the cells of a specific tissue, the matrix (scaffold) on which cells grow and the environmental factors, essential to simulate the *in vivo* conditions in which the tissue arises.

The cell sources, the way to culture and the scaffold (material and shape) range between a huge variety, depending on the type of culture and the different results desired. Thanks to the culture techniques, few cells are needed from a donor to create cultures. However, isolated cells cannot form tissues, as they require specific environments for a proper growth; for this reason specific scaffolds are necessary to

play a central role allowing the tissue growth. [1]

1.2 The scaffold : collagen hydrogel

Before beginning culturing cells, it is necessary to chose an appropriate scaffold with mechanical and chemical properties desired in relation with the kind of culture goals wished. The scaffold is a structure the purpose of which is to support the neo-tissue formation, and provide specific signals in order to drive the cells during the regeneration process. It has to guarantee adhesion of cells to the substrate, a necessary condition for cell migration, proliferation, differentiation and biosynthesis.[2] Therefore, cells are profoundly affected by the physical properties of the scaffold.
The scaffold acts as extracellular matrix (ECM), organizing cells into a three-dimensional architecture and releasing stimulants, which direct the growth and formation of the desired tissue. It has to provide support and it has to be able to allow the growth and diversify of the cells; it needs specific mechanical characteristics, which are different from tissue to tissue. The scaffold guarantees appropriate mechanical properties to the cells, particularly the mechanical stress. Additional factors to consider are the porosity and permeability; these allow the release of nutrients and other compounds, which are able to provide appropriate stimuli to cells. The membrane transport allows the transition of nutrients and molecules; this takes place through passive diffusion or active transport (against gradient, so requiring expenditure of energy from the cell).

One of the most important factors in scaffold development is the material used to produce them; according to the type of cell culture for which it is destined and the specific applications, different materials can be used. The main characteristics which

the material has to satisfy are biocompatibility and biomechanical performance. Indeed, the main goal is to avoid, as much as possible, undesired response of the organism to the implant, and to create an environment which is simulating the *in vivo* conditions as closely as possible, for both physiological and mechanical characteristics. Moreover, it could be necessary to make a resorbable scaffold, which is capable of being absorbed simultaneously with the cell growth and then be gradually replaced by the new tissue, with a reasonable degradation time, without releasing toxic compounds into the organism.

There are several alternatives for scaffold materials: metals (stainless steel, cobalt alloys, titanium alloys), ceramic (alumina, zirconia, calcium phosphate, bioglass), natural polymers, synthetic polymers, composites and hydrogels.

Examples of different scaffolds used for different approaches are shown in the figure below *[Fig1.1].*

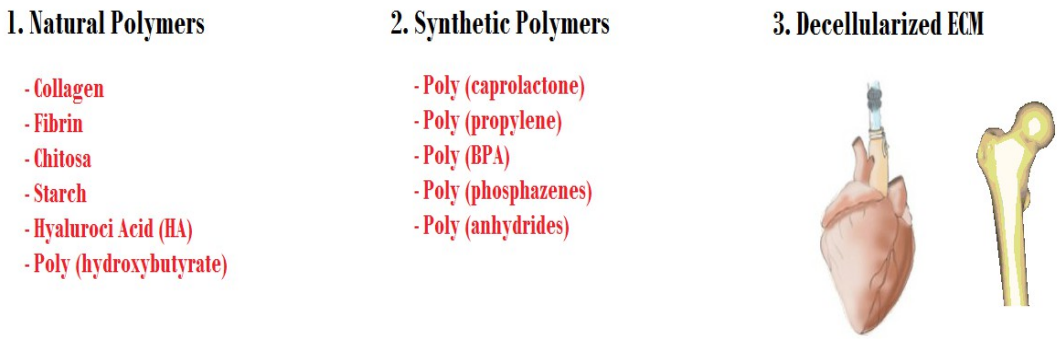


Fig 1.1 : Schematic diagram showing different scaffolding approaches in tissue engineering [2]

Among the hydrogels, collagen gel is the most commonly used, because of its physical and chemical properties. [48]

Collagen represents the main structural protein in the human body, and it is the major composition of the extracellular matrix.[3]

There are at least 33 distinct peptide chains (amino acid sequence) identified in collagens until now, and the different collagen types characterized, formed by assembling there peptide chains, are 19. In its most common form (type I collagen),

collagen protein has a triple helix structure.

This protein is generated in the endoplasmic reticulum of cells; the typical dimensions of type I collagen are 300 nm long, 1.5 nm in diameter and 2.9 nm of propeller pitch. The Elastic Modulus of type I collagen ranges from 2 to 4 GPa.

The collagen molecule is composed of three helices that wrap around each other *[Fig1.2]*; each helix is called α chain. Type I collagen is generally in eterotrimera form (in 95% of cases), and its structure is $[\alpha1(I)]_2$ α2(I) ; eterotrimera means that the subunits (the α chains) are different among each other (in this specific case, there are two different subunits, α1 and α2). The remaining 5% is omotrimera form (composed by just one type of subunit) and it is often associated with pathological conditions.[21]

The collagen triple helices are linked within each other by hydrogen bonds (intramolecular bonds), while the adjacent triple helix are linked together by covalent bonds between the globular triple helices extremes (telopeptides); the triple helices are assembled forming substructures of higher hierarchical level until reaching the collagen fibrils and fibres, characterized by a diameter of approximately 0.01-0.8 μM.

Collagen fibres are chemically very stable. Regarding the mechanical behaviour, in the absence of a load, the fibres are arranged without order in space; under a load, they change their geometry until reaching a linear arrangement. When the protein chains are stretched, the mechanical properties increase becoming dependent on intra- and inter- molecular bonds.

Collagen hydrogel as a scaffold is made most commonly from type I collagen; it has already been used successfully in tissue engineering because of its excellent biocompatibility, low antigenicity and high biodegradability (Jimenez and Jimenez 2004; Jones et al. 2002; Auger et al. 2004; Lee et al. 2001; Vaissiere et al. 2000).

It has the intrinsic capacity to modulate cell behaviour and to reduce immunogenicity of the seeded cells.[4]

The chemical structure of a collagen hydrogel is characterised by crosslinked networks, which have the property to be hydrophilic; moreover, collagen hydrogel does not dissolve in water at physiological temperature (approximately 37 ^{0}C). Generally, hydrogels have the characteristic to swell considerably in an aqueous solutions, increasing in volume; this behaviour is due to the presence of hydrogen

atoms, which tend to bond with the hydroxyl group of the water molecules. However, this does not happen with collagen hydrogel, since the collagen chains are crosslinked through hydrogen bonds, and this characteristic make impossible to the hydrogens to bind with water molecules.[5]

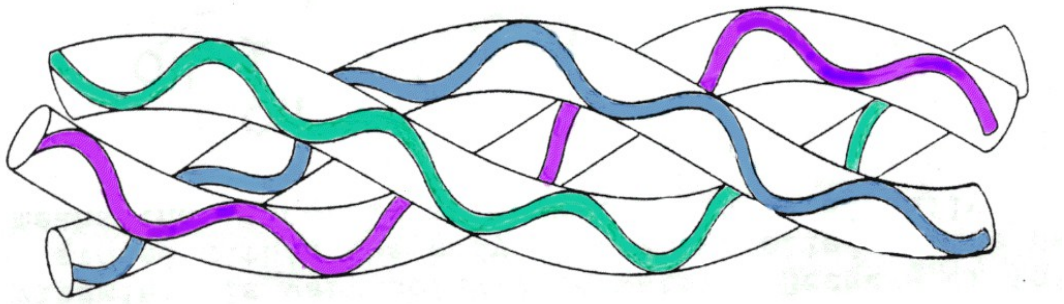


Fig1.2 : Collagen protein structure, in evidence the three helices [21]

Cells seeded on a collagen hydrogel tend to aggregate into clusters; particularly, studies have observed that hepatocytes can preserve mitochondria functionality. Moreover, collagen gel allows hepatocytes attachment, spheroid formation and functionality.[8]

However, the number of cells seeded per unit volume is limited to small orders of magnitude (approximately 10^4 cells per cm^2) and the adaptation to large scale is complex. [6, 7]

Moreover, collagen hydrogel is easily susceptible to degradation by enzymes; the rate which the enzyme action takes place is related with the multitude of cleavage sites on the collagen and with the number of enzymes in the culture. [48, 49, 50, 51, 52, 53]

1.3 The cells : HepG2

The second important characteristic to take into consideration is the choice of the kind of cells to use. The goal of tissue engineering is to reproduce viable cells which can as best as possible reproduce the natural characteristics; for this reason, the cell selection has to be accurate and related with the desirable result.[9, 10]

The ideal source is represented by the human cells. In the specific case of the liver, the cells can be isolated by perfusion; this technique was developer by Seglen in 1976. However, this kind of source brings several disadvantages, since the liver cells are particularly complex and delicate to treat without damage to their functions.

The first difficulty is related with the source; indeed, liver tissue from which to extract the cells is barely available, because it is not possible to use cells from cadavers (the liver cells lose their physical and functional characteristics very quickly after the body death).

Secondly, the senescene effect can occur (losing of liver function in vitro). Hepatocytes do not grow after isolation, and this impedes the creation of cell lines.[12]

Finally, another important problem is represented by the dedifferentiation phenomenon; it occurs mainly using primary human cells then cell lines (such as HepG2), which dedifferentiate over the course of approximately 1 week in ECM-sandwich and in suspension cultures.[61, 62] However, it was reported that cells in contact with collagen hydrogel or Matrigel™ (a natural hydrogel used as three-dimensional scaffold) show a decrease in the dedifferentiation rate and this is strongly related with the ECM molecules, (Money *et al.*, 1992). [60, 62, 63, 88]

For the reasons mentioned above, hepatocytes cell line was preferred to primary human cells; particularly, HepG2 cells are one of the most widely lines selected in tissue engineering. HepG2 (hepatoma-derived) is a human liver carcinoma cell line, which was isolated for the first time by Aden *et al.* in 1972 from a primary hepatoblastoma of an 11-year-old Argentinian boy. [65]

These cells are widely used in culture because their ability to maintain some of the specialized functions, such as plasma protein secretion, which are normally lost by primary hepatocytes; moreover, they can easily grow and differentiate without the problems related with human primary cells mentioned above.

Examples of their successful applications in tissue engineering are largely shown in the literature. Bokhari *et al.* (2007) have demonstrated that HepG2 grows maintaining their abilities and viability.[46] A study of 2013 of Erro *et al.* has explained the successful use of HepG2 encapsulated in alginate to develop an artificial liver machine, in order to overcome the problem related with the shortage of liver donors.[54]

However, as shown in Wilkening *et al.* (2003), HepG2 cells gene expressions for the enzyme metabolism strongly differ from that in normal human liver.[47] Moreover, Want *et al.* (2005) have shown that the use of HepG2 to treat Liver Acute Failure is generally successfully (the study was performed with a dog affected by hepatic failure), but HepG2 cell line presents limitations when comparing their ability to clear the ammonia with the normal hepatocytes.[55]

Comparing the advantages and disadvantages mentioned above, the choice of HepG2 for the present study appeared the most indicated; indeed, firstly of all, there was the necessity to grow a high number of hepatocytes on collagen hydrogel to carry out the tests, and the hypothetical use of primary cells appears to be limiting due to their issue of poor growth. HepG2 were obtained from liquid nitrogen, stored in the Department of Biomedical Engineering.

It is very important to choose the right culture model, and the literature shows several ways to culture hepatocytes with their respective results; indeed, the final goal during a culture is to maintain the hepatocytes phenotype and functions and, in order to reach it, several approaches have been investigated. An appropriate *in vitro* model has several advantages, such as the reduction of animal assays, saving time when evaluating new drug candidates or the development of safer drugs. [80]

Firstly, it is important to choose the cell culture material and the system. The most commonly used systems are as following: monolayer cultures, sandwich cultures,

microcarriers, the Unisyn-Hollow fibre bioreactor and 3D-membrane bioreactor. The matrices can be adherent or non-adherent; non-adherent matrices tend to alter hepatocytes chemical properties. [12]

The monolayer culture model is characterised by a single layer of scaffold (such as collagen gel), which can be placed in Petri dishes or in well plates.

The sandwich culture provides the use of two collagen gel layers with hepatocytes seeded in the middle of them. Nussler *et al.* (2001) have shown that the so called "Sandwich" culture is the best method to maintain hepatocyte functions over an extended period of time, since it simulates the *in vivo* microenvironment of hepatocytes. Moreover, this system is able to maintain the hepatocytes phenotype, morphology, metabolic capacity, and stable albumin secretion.[80]

The microcarriers have been proved to be a good model particularly for stem cells; Demetriou *et al.* (1986) have proved that Cytodex 3 is a good microcarrier for primary hepatocytes, since it led to prolonged viability and function *in vivo*. [82, 83]

Additional factors have been studied in order to improve *in vitro* culture conditions, allowing cells to provide sufficient metabolic capacity in order to be studied properly. Examples are given by supplying the culture with medium, oxygen, metabolite removal or using hollow fibre bioreactor systems which allow cells to attach to the surface of the fibres and reorganise themselves into three-dimensional structures close to the *in vivo* conditions. [80]

It is also important the medium choice, since different media are destined to different cultures. Media can be divided in two categories: short-term and long-term culture medium. Short-term medium is usually used for culture of only few days (<7); the most common is the Williams' E medium. [81]

1.4 Hepatocytes culture applications

Having a look into the literature, it is possible to observe how hepatocyte cultures have a lot of attention in tissue engineering studies and research in the recent years, keeping the attention of the international scientific community. The reason of such a great interest can be found in their very large number of possible applications. The most common are as follows.

- Temporary liver support (bridge) for patients waiting for a liver transplant due to Acute Liver Failure
- Models for studies of metabolism, enzyme induction or inhibition
- Research for new alternative methods to animal use, for the chemical evaluation of chemicals
- Pharmacological and toxicological *in vitro* screening, used in the early stages of the process of development of new molecules
- Study of toxic effects of pharmaceutical compounds and potential environmental pollutants
- Bio-transformation studies: analysing the changes which a chemical undergoes in the body and the formation of metabolites

Among the applications mentioned above, the evaluation of the hepatotoxicity for pharmaceuticals, cosmetics and food is a necessary step before they can be launched on the market. The pharmaceutical field is characterised by long lead times and high costs incurred by the industries for the development of new drugs, required to obtain the best result. For this reason, studies in this area appear more urgent in order to develop *in vitro* systems which can recreate conditions closed to the *in vivo* situation, and which are able to predict the toxicity of new molecules at an early development stage. In addition, this great interest is also linked to the recent new EU legislation which regulates chemicals and their safe use: REACH (Registration, Evaluation,

Authorisation of Chemical), EC Regulation no. 1907/2006. [58, 59]

The test for the carcinogenesis evaluation involves the use of hepatocytes, since it analyses how these cells repair the organism damage in relation to the severity of the intoxication caused by a certain substance. This methodology is particularly important because most of the chemical transformations occur in the liver.

This technique represents an alternative to experimental animals in toxicity tests; however, its major limitation consists in the short duration of hepatocytes functional life time. [56, 57]

Another important application of *in vitro* (cultured) cells is the preparation of hepatocytes for transplantation and bioartificial liver applications.

A damaged liver of a patient who is not considered suitable for transplantation can be treated with primary human hepatocytes; it could be provided for the treatment of fulminating hepatitis, employing an extracorporeal bioartificial liver. The much broader potential indications are Acute Liver Failure (primitive or secondary) in the course of chronic liver disease or other debilitating and metabolic diseases of the liver, treated with hepatocyte transplantation. [56, 57]

Acute Liver Failure (ALF) is a clinical syndrome, often fatal, due to massive liver cell necrosis with sudden and severe hepatic impairment. The most common causes are viruses B, C and delta, drugs (halothane, acetaminophen, isoniazid) and poisoning (amanita phalloides, chloroform, carbon tetrachloride, trichlorethylene). It is potentially reversible if diagnosed within 8 weeks from the presence of the symptoms.

All the four degrees of the syndrome present hepatic encephalopathy, mental confusion, behavioural changes, difficulty in speaking, modification of the sleep-wake rhythm, slowness of thought, inappropriate behaviour and stupor. In addition, the fourth ALF grade can lead to coma, with limb spasticity and no response to painful stimuli, headache, vomiting, right upper quadrant pain, fetor hepaticus and hyperpyrexia.

The biochemical consequences to the body include abnormalities in blood pH, elevated bilirubin and ammonia concentration, lower glucose level and defective

blood clotting. It can lead to Central Nervous System and kidney problems.

ALF requires an intensive care with continuous monitoring of vital functions, administration of intravenous fluids to restore water balance, glucose solutions (tendency to hypoglycemia), branched chain amino acids intravenously with lactulose enemas, antibiotics, plasma to correct the deficiency of coagulation, and mannitol to prevent cerebral edema. For extreme cases in which the liver function is irreversibly impaired, a liver transplantation is necessary.[12, 15]

In the UK, the majority of the patients admitted to Liver Failure Units in hospitals have taken a paracetamol overdose. The annual incidence of acute liver failure in the Scottish population is approximately 0.62 per 100,000.[16]

For the reasons mentioned above, it is clear why research centres are focusing their studies on liver cells; in particular, hepatocytes *in vitro* behaviour, how they grow, differentiate and their functions in culture are carefully under study.

The present study observes in this context, trying to overcompensate for a lack in the literature regarding HepG2 cells mechanical behaviour. In particular, mechanical tests will be performed in order to understand the physical characteristics of cultured cells in collagen gels. The research question and aim will be exactly explained in the second chapter.

1.5 Confined compression test and the biphasic theory

Among the mechanical properties which can be tested in a sample (scaffold and cells), the stiffness of a culture is one of the most representative, since it gives information about how cells act on the scaffold and how the scaffold (in this case, the collagen hydrogel) reacts to the cell influence. The background regarding this topic,

how it was treated and studied in the literature, and how it can be improved, will be debated in the next chapter.

The stiffness of a given sample, such as a collagen gel scaffold seeded with cells, can be tested with two different techniques:

- Compression test
- Tensile test

The tensile test is performed by stretching the sample in order to see its resistance, so its stiffness. It can be carried out until fracture occurs or just up to a certain established threshold.

One of the most innovative tensile tests for ensuring the stiffness of cells on scaffold was developed by *Lam et al.* in 2012. This test is characterised by a stretchable micropost array membrane (mPAM) on which cells can adhere. The mPAM exerts a tensile force on the cell, stretching it, as shown in the figure below *[Fig 1.3]*. [64]

The tensile test is very precise and accurate, but it presents the disadvantage that it is complex to perform, then expensive, since it requires sophisticated test machines.[20 ,64]

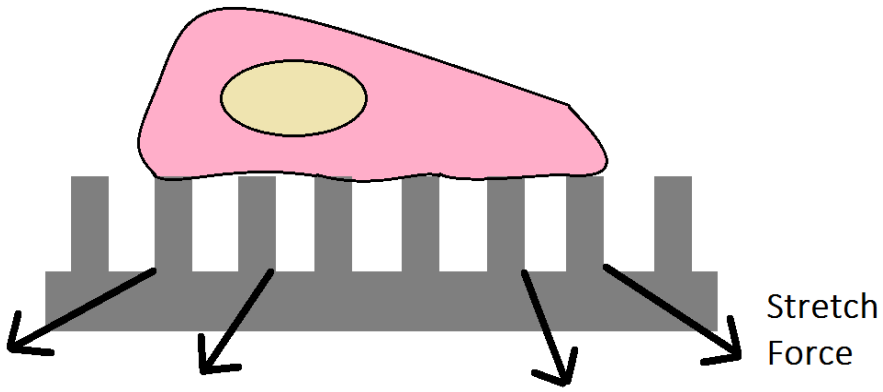


Fig 1.3 : Tensile forces (black arrows) applied to the cell by mPAM causing cell stretching. [64]

The compression test is most simply carried out by applying a compressing force to the sample, and then measuring the related changes in length or strain, in order to evaluate the stiffness of the specimen. [67] This test can be divided into two groups, according to the physical conditions in which the sample is tested: unconfined compression test and confined compression test.

During an unconfined compression test, the specimen is loaded with an uniaxial force; since there are no containment walls, the sample is subjected to both a radial and axial displacement, as shown in the figure below *[Fig 1.4].* However, this test requires an initial hypothesis, such as zero friction and elasticity, in order to make the evaluation of the parameters not excessively complex; these hypotheses make this kind of test not suitable for specimens characterised by a biphasic nature, as solid and liquid phases, such as the collagen hydrogel. In this case, the confined compression test is more to be recommended.[20]

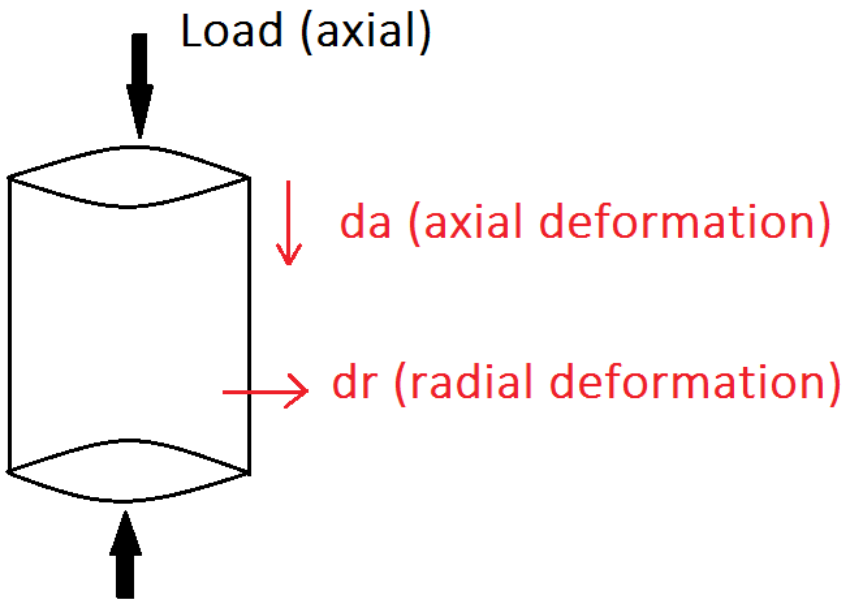


Fig 1.4 : Unconfined compression (axial load) which causes both an axial deformation (da) and a radial deformation (dr). [20]

The confined compression test has the advantage to give the possibility to measure both the solid and the liquid phase of a sample. Indeed, as mentioned above, the collagen hydrogel is characterised by a liquid phase (the interstitial fluid, which represents approximately 99.5% or more of the total volume of the gel) and a solid phase (the collagen fibrils and cells); this characteristic makes it a biphasic material. The confined compression can evaluate the stiffness of the solid phase and the resistance opposed by the fluid to flow (liquid phase). The chemical bonds between collagen fibrils provide tensile strength and elasticity to the collagen hydrogel, and the water of the gel is responsible for the resistance to compression.

This test can be performed in two different ways: a creep test or a relaxation test. The creep test provides the application of a constant load, whereas the relaxation test is performed applying an established deformation, as shown in the figure below *[Fig 1.5]*.

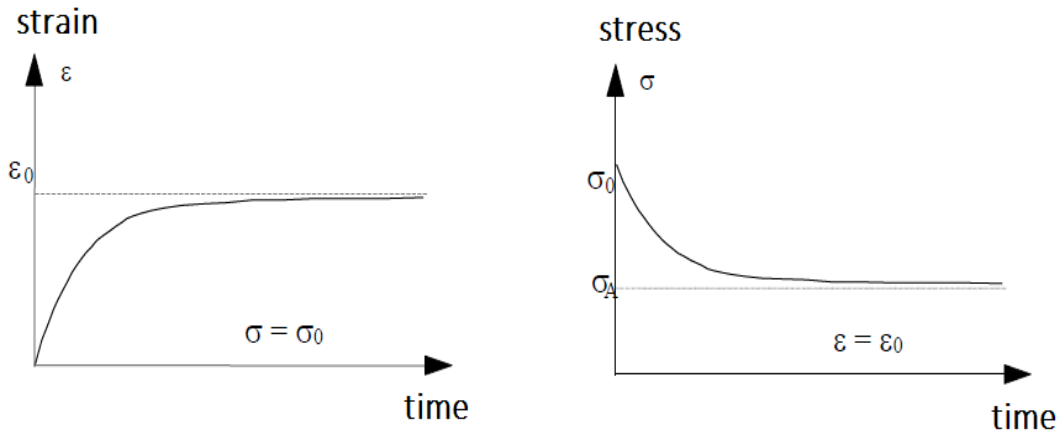


Fig 1.5 : Creep tests (on the left) and relaxation test (on the right). The creep test is characterised by the application of a stress, maintained constant during the time, analysing the occurred deformation. The relaxation test provides the application of a constant defined deformation, analysing the stress necessary to perform it. [21]

During a creep test, an axial load is applied to the top of the sample *[Fig 1.6]*. The compression proceeds to an established threshold, after which the relaxation phase can occur; at the end of the compression, the specimen reaches equilibrium, which is the condition with internal and external forces balanced.

Fluid is exuded from the gel through a porous platen until there is no more fluid loss, which is the equilibrium condition.

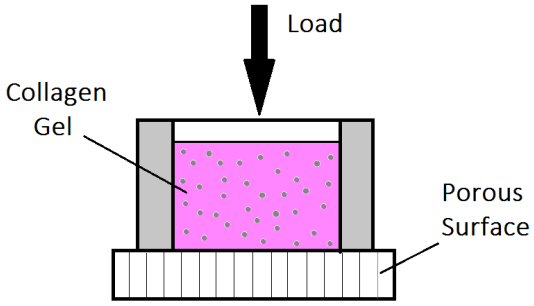


Fig 1.6 : Confined compression test : schematic structure [20]

After performing the confined compression tests, it is necessary to analyse the data. Several models and theories can be chosen, in relation to the kind of material analysed and the parameters to evaluate. Among these, one of the most commonly used to analyse collagen hydrogel is the poroviscoelastic biphasic model; however, the recently studied linear biphasic poroelastic theory (Busby *et al.*, 2013) appears to be a good substitute to the poroviscoelastic model because it is not sensitive to the small changes in mechanical properties due to the behaviour of collagen, which is susceptible to degradation and variations in the amount during the synthesis process. This biphasic poroelastic theory was firstly developed by civil engineers for soil mechanical purposes; its biomedical application hails from Mow *et al.* (1980), who developed an out-and-out mathematical model.

The theory is based on three initial assumptions:

- the material is homogeneous
- the sold phase is elastic, whereas the liquid phase is an incompressible viscous fluid
- the pores of the sample are connected to each other, so that the fluid is able to flow through the solid phase

The theory is based on the premise that by applying a gradual load to a certain porous biphasic sample, the stress is just supported by the fluid phase; the liquid is then compressed and, since the pores are interconnected to each other (as for assumption), it can gradually flow out of the specimen, until reaching the equilibrium (when all the liquid is completely thrown out). At this point, the collagen fibrils and cells (the solid phase) are responsible for supporting the load. The fluid leakage is gradual, with different rate of speeds (initially faster, and then constantly decreasing).
This theory allows the evaluation of four important parameters: the Aggregate Modulus (H_A), the Hydraulic Permeability (k_0), the Stretch Ratio (λ) and the Stress Response.
The Aggregate Modulus is estimated with the following equation:

$$H_A = E(1-\gamma) / [(1+\gamma)(1-2\gamma)]$$

It represents the stiffness of the specimen, since it is related to the Young's Modulus (E) and the Poisson's ratio (γ), as it is possible to observe from the formula above. In particular, it is directly proportional to the Young's Modulus, and with the degree of stiffness of the sample, which represents the transversal expansion and constriction of a certain material. The Aggregate Modulus is measured in Pascal units.
The Hydraulic Permeability represents the resistance to fluid flow; it is connected with the Poisson's Ratio as to the Aggregate Modulus. In particular, their relation is regulated by Darcy's equation:

$$\gamma = k_0 \nabla p$$

Where ∇p is the pressure gradient and k_0 (the permeability, constant of proportionality) is a function of time and displacement: $k_0 = k_0(\lambda(z,t))$.

The Stretch Ratio is a measure of the extensional or normal strain; it can reach values of 3 or 4, if the material is elastomer.

The linear biphasic theory is defined by the following equation:

$$\delta^2 u/\delta z^2 = 1/(H_A k_0)(\delta u/\delta t)$$

Where *u* represents the displacement along the z-axis.

The formula above gives parameters physically and structurally significant; however, it requires a complex numerical fitting procedure which make the solution particularly complicated to estimate. [19, 20]

In conclusion, the confined compression test associated with linear biphasic analysis appears to be the best combination in order to analyse the collagen hydrogel seeded with hepatocytes.

The next chapter will show the background reasons and the resulting research question in order to compensate for lack of literature about the mechanical relation between HepG2 cells and collagen gel scaffolds.

The State of the Art

2.1 The mechanics of cells and tissues

Human tissues have the property to be viscoelastic, so they are characterised by both elastic and viscous behaviour.

The cells of a tissue can be anchorage-dependent or -independent.

The majority of them are anchorage-dependent, and they are termed adherent cells; it means that they are attached both to their neighbouring cells and to the Extracellular Matrix (ECM) through specific anchorage points, termed focal adhesions *[Fig2.1]*. For these kinds of cells, adhesion is essential for their survival.

Anchorage-independent cells do not need to attach to ECM or other cells; examples are give by blood cells, which are suspended in plasma, and cancer cells, which are able to proliferate separately from their neighbours. [66]

Discher *et al.* (2005) have analysed the behaviour of anchorage-dependent cells cultured on different kinds of materials; in particular, the paper focuses on gel substrates, since their elasticity can be varied to match the soft tissue *in vivo*. The study shows that different cells have different ways to relate with other cells and with their substrate. However, in general, adherent cells on a stiffer substrate have better cytoskeleton organization and more stable focal adhesion points, so they are more strongly anchored to the matrix than it could prove to be on softer matrix; cytoskeleton assembly is evident since its structure is more organised and this leads

to a better cell support, with a larger and more stable adhesion *in vitro*. This characteristic is particularly evident for cells which tend to be mechanical in their actions since they generally model or remodel the structure of the tissues, such as fibroblasts. [67]

The resistance to deformation provided by a stiff matrix is higher than that given by a soft matrix. Resistance to deformation is represented by the Young's modulus (*E*), measured in Pascal (Pa), and it represents the stiffness of the matrix as discussed in the previous chapter. [22 , 67]

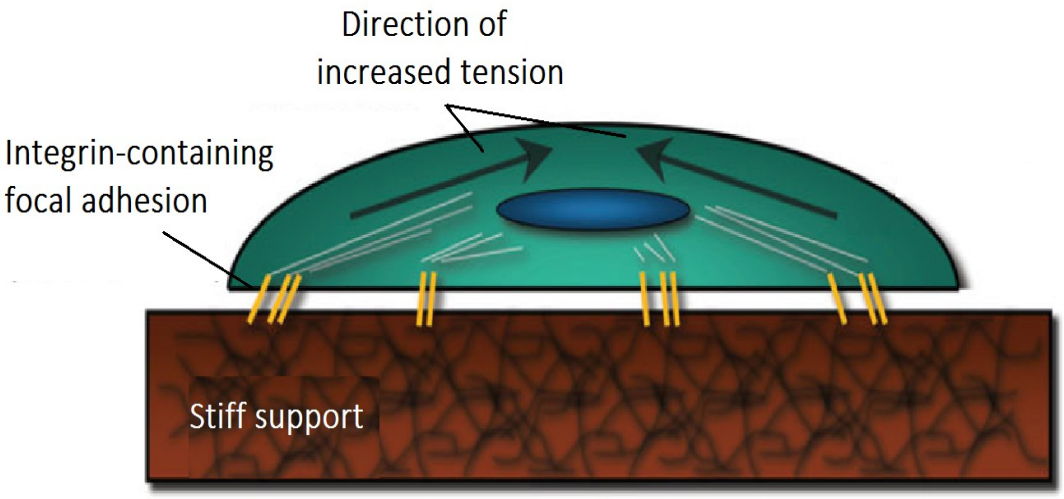


Fig2.1 : Static mechanical forces sensed by cells : schematic of a cell on a stiff support, with attachment to the matrix via integrin containing focal adhesions. [22]

As explained above, anchorage-dependent cells are attached both to their neighbour and to the scaffold they grow on and they are able to sense the mechanical stiffness of ECM and other cells. These kind of cells are termed mechanosensing.

Mechanosensing cells are influenced by the stiffness of the substrate and, reciprocally, they influence the scaffold mechanical properties; indeed, cells act modifying the scaffold through mechanical forces generated by cross-bridging interactions of actin and myosin filaments, and by secreting degradative enzymes,

such as collagenase (Saddiq *et al.,* 2008); these forces and secretions influence the substrate, causing strains.[26, 44, 67] Particularly, Young's modulus of cells is determined by their actin-myosin-based cytoskeleton. Especially, mechanosensing cells on the top of a culture adopt the stiffness of the substratum layer.

Cell-cell mechanosensing plays an important role in tissue organization and development; and indeed, cells can change significantly the ECM properties, among which is its stiffness. In particular, the stiffness is regulated by cell-directed organization, fibronectin and post transitional modifications of ECM, such as non-enzymatic glycation and cross-linking enzymes (tissue transglutaminase, the lysyl oxidases, and the lysyl hydroxylases), ECM components such as proteoglycan expression can increase liver stiffness (Wakatsuki *et al.*, 2000).[22]

How significant these mechanical changes are, and whether they are sensed by cells, is still unknown.

In 2006, Bershadsky *et al.* have reviewed all the experiments carried out to uncover the major signaling pathways involved in the response of adhesion sites to force. The experiments have shown that integrins, the heterodimeric receptors for ECM, are the primary cellular mechanosensors for adhesion-dependent mechanical forces. Indeed, integrin regulates downstream signalling in response to matrix stiffness. This signalling is as defined bidirectional, since there is a reciprocal action between the matrix (which directs the forces through integrin proteins) and cellular cytoskeleton (which in turn resists these forces). The central role is performed by the focal adhesion integrins, as shown in the figure above *[Fig2.1]*. [22, 70]

2.2 Stiffness and hepatocyte behaviour

The Young's Elastic modulus (*E*) of human liver is approximately 640 Pa analysed with compression testing method,[84] and it can increase fourfold if the liver is affected by

diseases, such as fibrosis and cirrhosis.[27, 30]

The goal of tissue engineering is to try to recreate the physiological conditions in culture. To achieve this, collagen gels have been used *in vitro* and their ability to provide culture conditions of physiological stiffness have been proved. [27]

As shown in the figure below *[Fig2.2]*, cells are profoundly affected by substrate stiffness.

Basically, the stiffness regulates cell growth and viability, and it can cause or prevent apoptosis. The differentiation status, the motility and the degree and size of cell-matrix adhesion are strongly influenced by matrix mechanical properties.[22]

In general, cells tend to migrate from softer to stiffer environments, and this phenomenon is termed "durotaxis". In the specific case of primary rat hepatocytes, Georges *et al.* (2005) reported that they adhere more strongly on scaffolds whose elastic modules have been shown ranging between 22 kPa and 64 kPa. [23] Moreover, Semler *et al.* (2004) have analysed that hepatocyte isolated from rats have growth factors and fibronectin density which increase with substrate stiffness; the study shows that these kind of cells are more proliferative and fibrogenic when cultured on a stiff substrate. This is actually a non-desired effect because it is far from the *in vivo* minimally proliferative characteristic. of On the other hand, primary hepatocytes cultured on stiff matrix tend to assume flattened morphology, which is far from the physiological shape. *[23, 68, 69]*

Other evidences show that cells in general tend to be less adherent, minimally adhesive and minimally proliferative (or even growth arrested) when cultured on soft surfaces, such as 5% hydrogels, close to the *in vivo* situation; moreover, they are prone to apoptosis even if they assume a rounded morphology, which reflects the *in vivo* morphology, *[Fig 2.2]*.[22, 68, 69]

However, there is an optimal stiffness value, over which cells do not adhere more; infact, cells tend to respond better to substrates that resemble most of the tissue of origin in terms of stiffness. The optimal level of stiffness and the way in which it influences the behaviour of cells changes between different kind of cells and the

mechanism of these effects is still unknown.[22]

Despite that, the cells proliferate and differentiate better in substrates which can reproduce as well as possible the *in vivo* conditions.[22]

A study by Hansen *et al.* (2006) has proved that primary rat hepatocytes grow on a thin film of collagen gel, they spread and enter in S phase, whereas they remain growth arrested if cultured on soft collagen gel. Moreover, hepatocytes on collagen gel quickly form clumps (spheroidal aggregates) and reorganize the matrix, while, in contrast, they become polygonal and do not aggregate effectively on stiffer matrix *[Fig2.3]*.[28]

It is necessary that hepatocytes aggregate to maintain differentiated function, especially in the context of functional tissue-replacement design.[23]

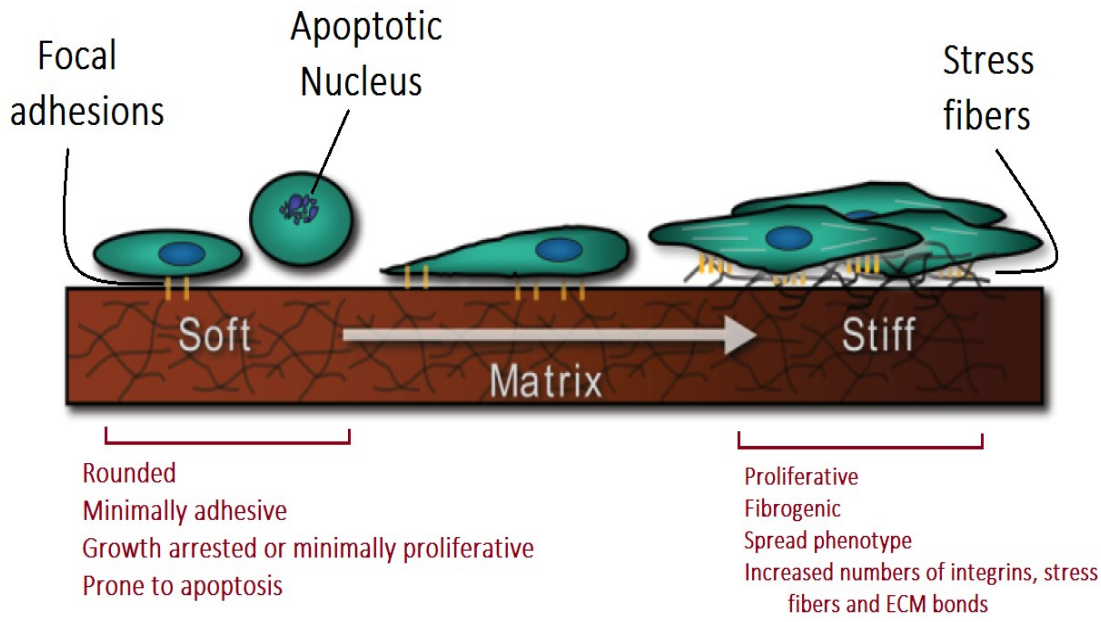


Fig2.2 : General effects of matrix mechanics on anchorage-dependent cell behaviour: schematic of the general changes in cell behaviour observed as matrix stiffness increases. [22]

Semler *et al.* (2001) have investigated the optimal collagen gel scaffold Young's modulus optimal for a primary hepatocytes culture; it was reported as being 34 Pa

compared with the slightly crosslinked modulus of 180 Pa, both below the physiological modulus of liver tissue and both relatively soft. The 34 Pa gel, however, may be too soft for hepatocytes to anchor to the substrate and send signals via force transduction to neighbouring cells.[23, 71]

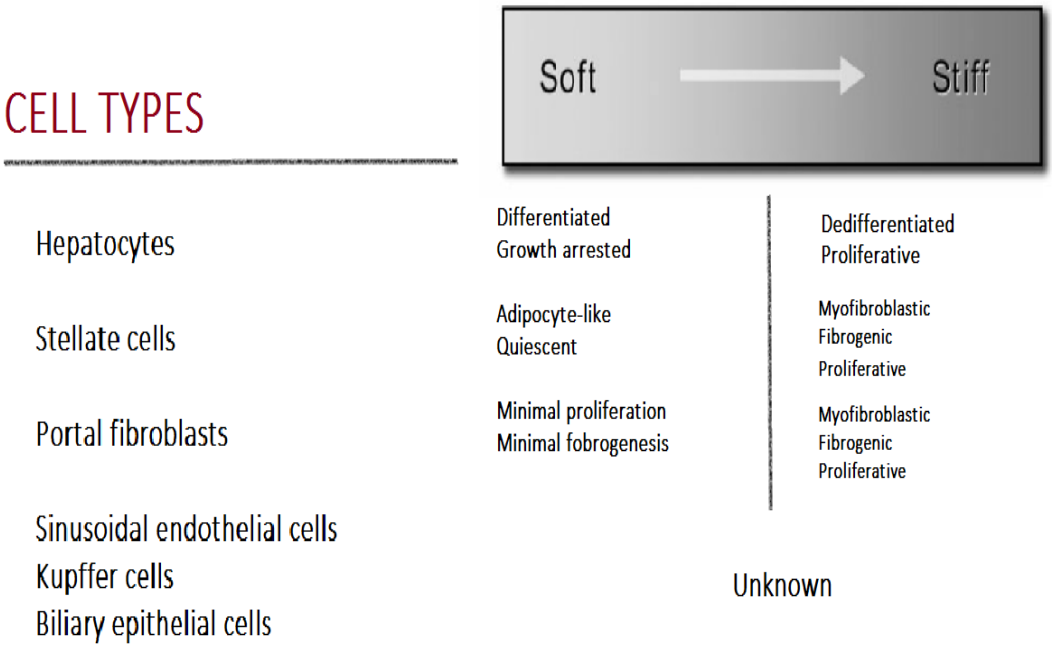


Fig2.3 : The effect of matrix stiffness on liver cells: the general effects of changes in matrix stiffness on parenchymal and non-parenchymal cells of the liver. [22]

Regarding the effects produced by stiffness to the actual function of hepatocytes, there are several studies which have investigated this topic.

As analysed above, stiffness influences the degree of cells spreading; however, cell spreading is connected with changes in important cell functions, such as DNA synthesis, motility, cytoskeleton organization and differentiation. [78]

In conclusion, mechanosensitive cells change their behaviour in relation to the substrate stiffness when cultured *in vitro [Fig2.3]*. However, the absolute stiffness values for each response have not been defined, yet.

For this reason, the matrix stiffness is an important parameter to take into consideration during culture, since it has significant implications for hepatocyte functions.

2.3 Liver stiffness measurement

The liver stiffness (LS) is a parameter widely used in diagnostic medicine, in order to reveal possible abnormalities in liver functions. Indeed, there are several factors which affect the stiffness value, such as age, sex, ascites, narrow intercostal space, obesity, necroinflammation of the liver, cholestasis and congestive heart failure; some of these factors are physiological (like age and gender) and others are related to pathologies.[30] For the reasons mentioned above, liver stiffness measurement has been used to diagnose abnormalities, such as in the case of significant fibrosis.

Nowadays, many techniques are used to investigate liver stiffness, both invasive and non-invasive. The most common ones are ultrasound, magnetic resonance imaging (MRI) and computer tomography (CT), which are non-invasive approaches, or the invasive biopsy and laparoscopy. [86]

The FibroScan device is a non-invasive elastography technique, which is characterised of markers capable of measuring the liver stiffness through ultrasonic waves; the ultrasonic signal is emitted by a multifrequency probe (3-5 MHz) and the return signal is detected by sensors. The data are integrated by a computer, which is able to evaluate the stiffness. [31, 72, 73]

It is possible to observe from the literature how different measurement approaches lead to different stiffness values; for example, magnetic resonance technique, acoustic radiation force impulse and vibration-controlled transient elastography (FibroScan), show the normal liver stiffness values which ranging between 3.7 kPa

and 7 kPa in men and from 3.3 kPa to 6.8 kPa in women; on the other hand, analyses conducted with compression tests performed on a specimen extracted by biopsy show stiffness values from 350 Pa to 700 Pa. [30, 86, 87]

The shift of values is approximately one order of magnitude; this significant difference is due to the limitations related with both the techniques. Firstly, the ultrasound technique is limited by the presence of different organs and sheaths with different inferences values and this can influence the return signal reading; moreover, the tissue in *in vivo* conditions is characterised by hydrostatic pressure due the the blood pressure, which can lead to a non-reliable measurement. On the other hand, confined compression has limitations related to the tissue behaviour which can be compromised after the biopsy; the sample can lose its mechanical *in vivo* characteristics.

In the case of fibrosis, the liver stiffness is higher than the normal value, ranging between 7 kPa and 8 kPa in women and between 7.9 kPa and 10 kPa for men, if tested with ultrasound machine. In the case of tests with cyclic compression-relaxation method, the fibrotic liver values from 586 Pa to 1733 Pa. [88]

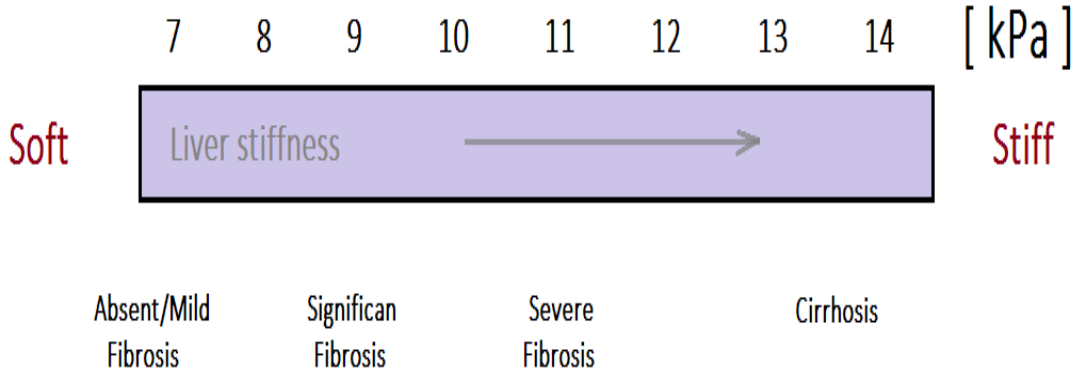


Fig2.4 : Young's modulus values with the related levels of fibrosis which can be associated with the liver stiffness; increasing in pathology seriousness lead to an increase in liver stiffness. [31]

The figure above shows the relationship between increasing liver stiffness values and the degeneration of fibrosis in hepatic cirrhosis *[Fig2.4],* for the data evaluated through FibroScan approach.[31] For example, fatty liver stiffness values are between 5.3 kPa and 5.8 kPa.[31]

2.4 Research question and aims

The work of Busby *et al.* (2013) investigated the mechanical behaviour of collagen hydrogels in confined compression using biphasic theory. Particularly, the authors analysed whether the confined compression technique was sensitive enough to determine differences in collagen stiffnesses between collagen hydrogels at different percentages of collagen content (0.2%, 0.3% and 0.4%). The results confirmed that the confined compression test is evidently able to define the differences in stiffnesses and, together with biphasic theory, to evaluate the mechanical properties of collagen hydrogels. [29]

According to the study mentioned above, confined compression technique and biphasic model can now be applied in order to determine quantitatively how cells actually influence the collagen gel stability and mechanical properties. This method was applied to human liver derived hepatoma cells in culture. It was not possible to obtain human hepatocytes, so in their place human hepatoma cells were used.

As analysed, the previous literature shows a lack of data regarding the stiffness characteristics and its effects during cell culture. In particular, how it influences hepatocytes behaviour and, reciprocally, how the cells influence them.

This present study aims to measure the stiffness of collagen based gel matrices in

order to understand how the hepatoma cells influence the matrix, in relation with their densities and culture time. Therefore, the relationships between hepatoma cells and the collagen hydrogel scaffold on which they are cultured will be investigated; in particular, how the gel stiffness is affected by the cells over the time in culture will be evaluated, trying to show a logical explanation of cause-and-effect.

The collagen hydrogel stiffness will be evaluated in relation with the culture time and with different cell density combinations, in order to provide a general picture of the dynamics established between them. Collagen hydrogel seeded with hepatoma cells in five different cell densities will be analysed, retained the most significant combinations, as better explained in the next chapter (Nakajima *et al.* (1996), Dai *et al.* (2009)). Moreover, samples of collagen gels without cells seeded on it (just incubated in medium) will be tested in order to have control value with which compare the values obtained with hepatoma cell cultures.

Particularly, the combinations of culture duration and cell densities will be set up as shown in the table below *[Tab 2.1].* The selection of the cell densities and culture durations have been inspired from the Department experience and from the literature analysed. [77]

CULTURE DURATION / CELL DENSITIES	1 - Day	3 - Days	5 - Days
10^5	4	4	4
$5 * 10^4$	4	4	4
$2.5 * 10^4$	4	4	4
10^4	4	4	4
$0.5 * 10^4$	4	4	4
Collagen gel (NC)	2	2	2

Table 2.1 : Combination between cell densities (plus collagen gel without cells) and culture duration which will be performed and analysed in the next chapters

The cells selected will be the HepG2 cells; HepG2 cell line is preferred than human normal hepatocytes since, as explained in the chapter before, primary cells cannot be readily obtained and HepG2 cell line appears to be a good substitute (Wilkening *et al.* (2003), Wang *et al.* (2005), Bokhari *et al.* (2007), Erro *et al.* (2013)).

For the reasons explained in the chapter before as well, collagen hydrogel reveals to be a good choice as scaffold since it provides good performance for hepatocytes culture, such as structural support and biocompatibility (Lee *et al.* (1995), Varum *et al.* (1996), Tomihata *et al.* (1997), West *et al.* (1999), Mann *et al.* (2001), Risbud *et al.* (2003), Drury *et al.* (2003)).

The present work was conducted in parallel with another project, the aim of which is to measure how HepG2 cell morphology, functions and viability change in relation with the environment changes. The combination between these two works will provide an overall percentage about how hepatoma HepG2 cells can be influenced during culture, increasing the knowledge over this matter, which can turn out to be useful for future studies.

Materials and Methods

3.1 Overview

This work provides mechanical study of 0.3% collagen hydrogel monolayer properties under compression. In order to understand how the collagen gel properties change in relation with time and cell concentration, the mechanical analysis was performed with different cell densities and time rates.

The 0.3% collagen hydrogel was prepared from collagen type I, obtained from rat tail tendons; it was then placed in the wells of a standard 24-well plate. The structure analysed is a monolayer of collagen gel.

The collagen gel monolayer was seeded with human hepatoma cells (HepG2) at different densities: 10^5, $5*10^4$, $2.5*10^4$, 10^4 and $0.5*10^4$.

Before testing the wells, the viability of the cells was ensured with the MTT assay.

The normal sample was represented by a single well of collagen hydrogel which was not seeded with cells, but left for the same period of the culture filled with medium in the same incubator as the samples.

The confined compression strength was measured through BOSE ElectroForce machine. The data acquired were managed by Microsoft Excel and then analysed with Matlab in order to evaluate the Aggregate Modulus (H_A), Hydraulic Permeability (k), Coefficient of Permeability (M) and Correlation Coefficient *(r)*.

The Coefficient of Permeability allows us to evaluate the relation occurred between

the initial value Hydraulic Permeability (k_0) and the final one (k). The equation which regulates this relation is as follow:

$$k = k_0 \exp(-M\varepsilon)$$

However, since the values of Hydraulic Permeability have small orders, the difference between k and k_0 is negligible; so, it is possible to affirm that

$$k = k_0$$

For this reason k and k_0 will be used as the same parameter.

3.2 Collagen gel setup

The 0.3% collagen hydrogel setup is a procedure performed in order to create the scaffold used during the hepatoma cells during culture.
Before starting, it is important to sterilize a beaker in which the reaction between chemicals will take place.
In order to perform this, the beaker is wrapped in clingfilm and then positioned in a microwave. The microwave is actioned for 5 minutes at 750 W. This technique is practical and cheap; the principle of sterilization is related to thermal and energy effects.[36]

5.8 ml of *Collagen (5.1 mg/ml)* was placed into the beaker with a pipette. Then, 1000 µl of *DMEM/0.4 M NaOH 2:1* was added to the collagen and it was gently shaken.

3.1 ml of *Acetic acid 1/1000* was added and then shaken again.
In order to regulate the pH of the collagen gel obtained, it was necessary to add gradually drops of *Sterile NaOH 1 M* with a syringe till the solution became red (it means that the pH is regulated at 8.8). The solution was continuously blended.
Using a pipette, the collagen gel obtained was dispensed into 24 well plates. Particularly, each well contained 0.4 ml of collagen gel.

In order to decrease the rate of gelling of the collagen gel, it was important to work with the beaker in a box filled with ice. The low temperature decreases the rate of gel formation allowing more accurate despensinging into the wells.
The collagen gel had to stand for a couple of hours before being seeded with cells; the storage was at room temperature; it was washed with full medium for one single washes per well. The DMEM (Dubecco's Modified Eagle's Medium) culture medium was composed of 4.5 g/L glucose with L-glutamine 2 mmol/L, penicillin 100 U/mL and streptomycin 100 g/mL. For the wells cultured for three and five days, the culture medium was changed every 48 hours.

3.3 Seeding cells

Two hours after collagen hydrogel set up, it was possible to proceed with the cell seeding.
The HepG2 cells were collected from a culture in a flask.

The figures below *[Fig 3.1]* show pictures of HepG2 cells growing routinely in a flask. The pictures represent the same culture, the first taken with a lens of X10, the second X20.

The two figures represent identical fields except for the lens zoom. The cells appear alive and with “rounded” shape; morphology of HepG2 is said to be epithelial-cell like: they are polygonal in shape. Round cells are actually not a typical shape for HepG2 cells. It may also be noticed that many cells are aggregated in clumps, creating cell clusters: this is a peculiarity of HepG2 cells at early time points (such as 24 hours), which are used to form small clusters of cells and this is clearly seen in the pictures.

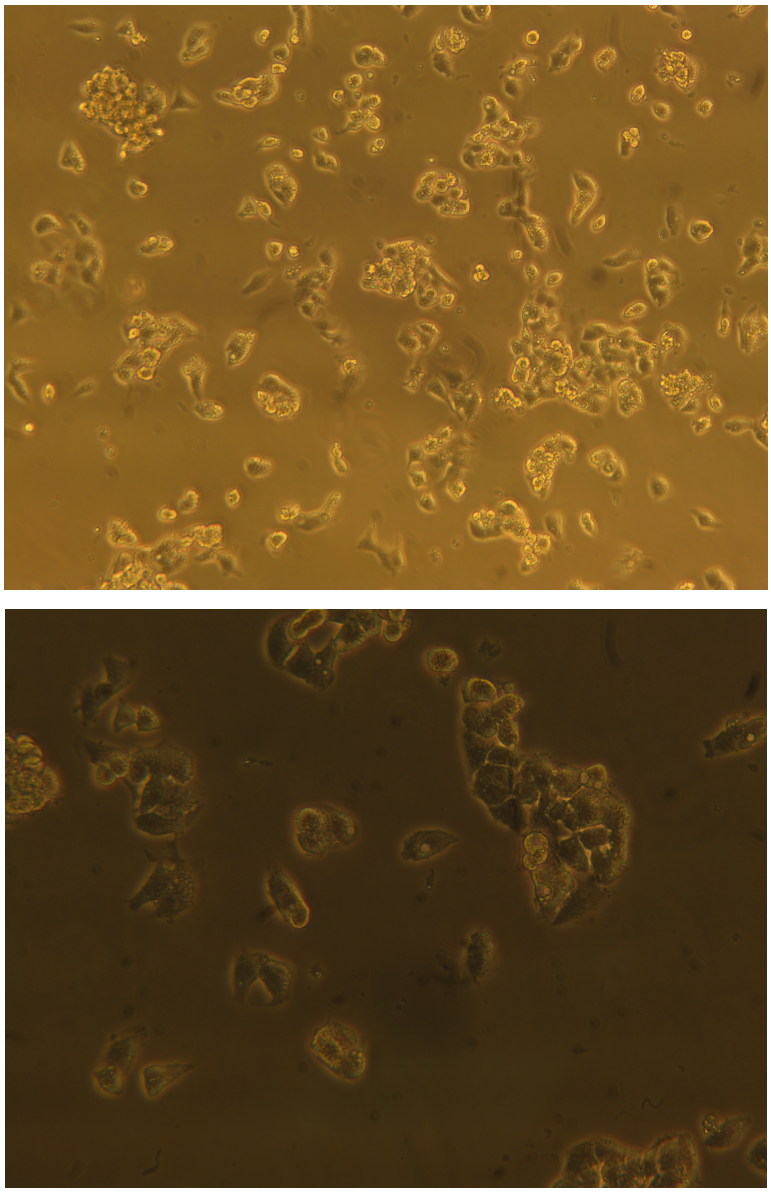


Fig3.1 : The figure shows HepG2 cells on the flask , with respectively X10 and X20 lens, one day after culture

During the cell passaging procedure the medium was poured off from the flask and discarded into a beaker. 5 ml of *Versene* was added into the flask; the fluid was gently swirled over the cell monolayer, in order to wash it. *Versene* removes the residual medium which contains a trypsin inhibitor, and EGTA, a calcium chelator which is in the *Versene* solution. *Versene* was then removed and discarded.

These two actions were repeated with less of *Versene,* to be sure that it had worked efficiently.

Approximately 1 ml (for a small flask of 25 cm^2) of trypsin, *Trypsin in versene,* was added to the flask. *Trypsin* is a serine protease enzyme capable of removing the cells which are adhered onto the flask surface.

The flask was left for a few minutes and then gently tapped, in order to create a stress which helped to remove the cells.

A check was performed under the microscope in order to guarantee that the cells were not still adhering to the flask surface: it was observed that they freely floated in the *Trypsin.*

Approximately 5 ml of medium was added into the flask and gently pipetted up and down to get an even suspension of cells.

The cells can be taken with the pipette and positioned in a universal tube.

Through a pipette it was possible to seed the collagen gel. It was important to constantly shake the universal tube during the seeding, in order to avoid sedimentation of the cells at the lowest part of the tube and in order to guarantee as much as possible that the amount of cells positioned in each well was homogeneous.

In order to evaluate the concentration of cells in the tube, and ensure seeding the right density on the wells, they were counted using a haemocytometer. Haemocytometer is a traditional method which consists in a modified and calibrated microscope slide designed to allow quick estimation of the concentration of cells in a sample *[Fig 3.2]*.[34] It is composed of two grids, positioned in the counting chamber as shown in the figure*;* a glass coverslip was positioned over the grids and stuck through moistening. After positioning the glass coverslip, a thin space is created due the geometry of the countring chamber. 10 µL of the solution containing the cells was

drawn up and delivered into the gap between the coverslip and the chamber. Helped by the grid, it was possible to count through a microscope the average number of cells contained in each 1 mm^2 square of the grid; from this value, knowing that the volume of the liquid in each square is 0.1 µL, it was easy to estimate approximately the number of cells in the solution.

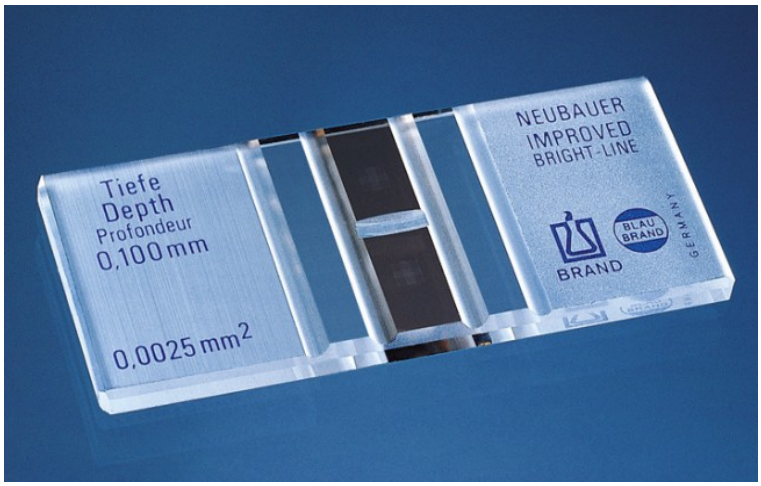


Fig3.2 : Haemocytometer from Brand company [37]

The seeding was performed at five different cell concentrations per cm^2:

- 10^5
- $5 * 10^4$
- $2.5 * 10^4$
- 10^4
- $0.5 * 10^4$

Indeed, previous experiments conducted in the Biomedical Engineering laboratory of University of Strathclyde and previous papers found in the literature established the optimum cell density of HepG2 cells in collagen gel to be $5*10^4$ per cm^2 for immediate treatment and $2.5*10^4$ per cm^2 for prolonged treatment.[40, 41]

For this reason, a combination between the cell densities and the lifetimes of the culture mentioned above seemed a good compromise in order to understand the

dynamics and the influences on the mechanical properties of the collagen gel in relation with time in culture and cell concentration.

In order to obtain 1 ml of solution per well (2 cm^2), after adding the right concentration of cells, the residual volume was filled with medium; 1 ml is the volume of solution contained in every single well.
The well plates seeded were left in the 37 ^{0}C incubator for three different lifetimes: one day, three days and five days.
The 4 wells intended to be tested were seeded and other two wells were left with just a layer of collagen gel, covered with 1 ml of medium; these two non-seeded wells represented the control sample. Other two wells well were seeded with cells and destined to the MTT viability control, which was carried out 4 hours before performing the mechanical test, in order to be sure that the cells in the culture were not died or contaminated; the choice of 4 hours was in order to guarantee that MTT test to have the enough time to show the viability of the cells.

The figures below show the HepG2 cells after being seeded on the wells. The figure [*Fig 3.3-A*] shows the well with a concentration of $2.5*10^4$ cells, the figure [*Fig 3.3-B]* the $5*10^4$ concentration.
The pictures were been taken from the microscope with a X10 lens.

At 1 day on a single layer of collagen gel, HepG2 cells increased in number and they exhibit polygonal morphology typical of these kind of cells. As in the case of the HepG2 cells grown in the flask, *[Fig 3.1],* they aggregated into clusters on 24 well plates as well.

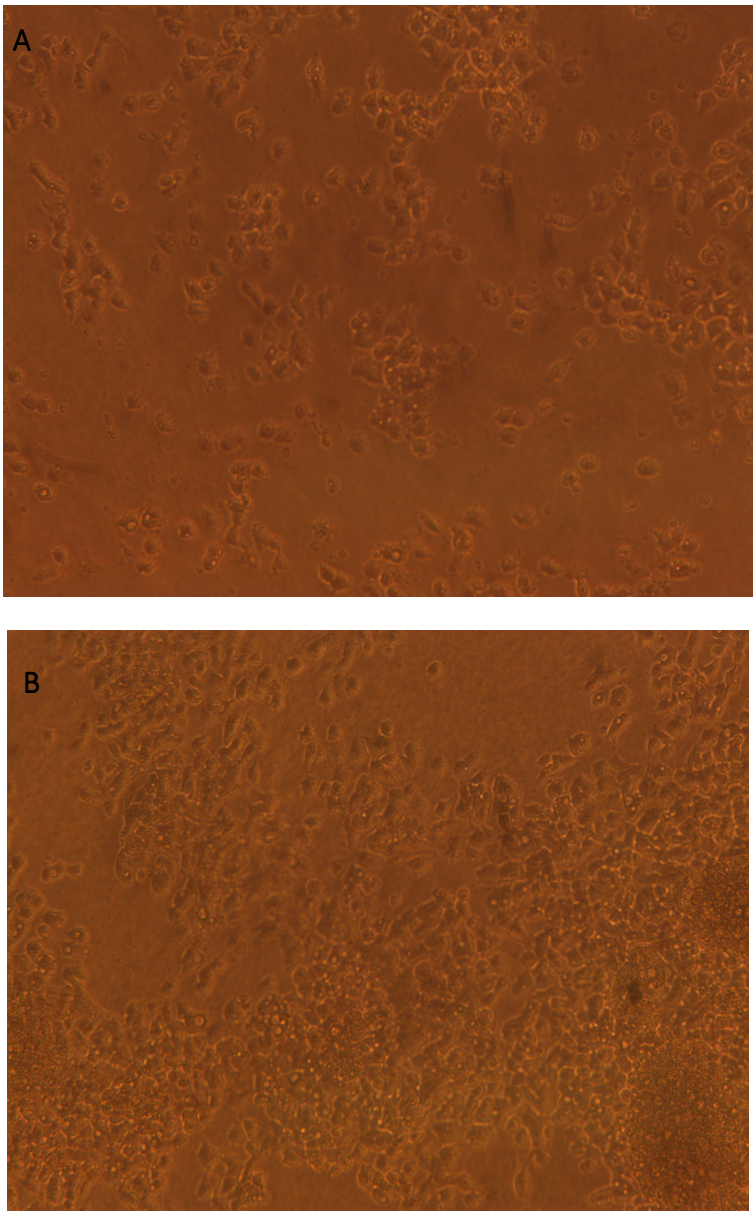


Fig 3.3 : Fig 3.3-A shows the hepatocytes with a concentration of $2.5*10^4$, Fig3.3-B hepatocytes at $5*10^4$ density; both the pictures were taken one day after culture. Magnification X10 lens.

3.4 MTT microplate assay

The MTT assay is a widely used quantitative *in vitro* test. It was performed in order to evaluate if the hepatocytes on the 24 well plate were alive before proceeding with the mechanical tests.

In order to set up the MTT assay used for the viability test, the first step was to make up 10 mM solution of *MTT in PBS* (0.4143 g/100 mL) at pH 6.75 . It was essential to make it sterile, for this reason the solution was filtered through a 0.2 µm filter.

The medium was removed from the cell seeded well destined to the MTT test, and 50 µL of MTT solution was added to it.

The yellow tetrazolium salt *((3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide)* is reduced inside viable cells to form a blue product which is a formazan salt. The reduction takes place in the cytosol and mitochondria by reductase enzymes.

When using this method it is important to remember that the amount of MTT reduced inside the cells depends on the availability of NADH/NADPH so it is strongly related with the redox state of the cells.

Although it is widely used as a measure of cell number, it is in reality measuring the activity of reductase enzymes in the cells.[35]

The microscope was used to confirm that the cells were alive.

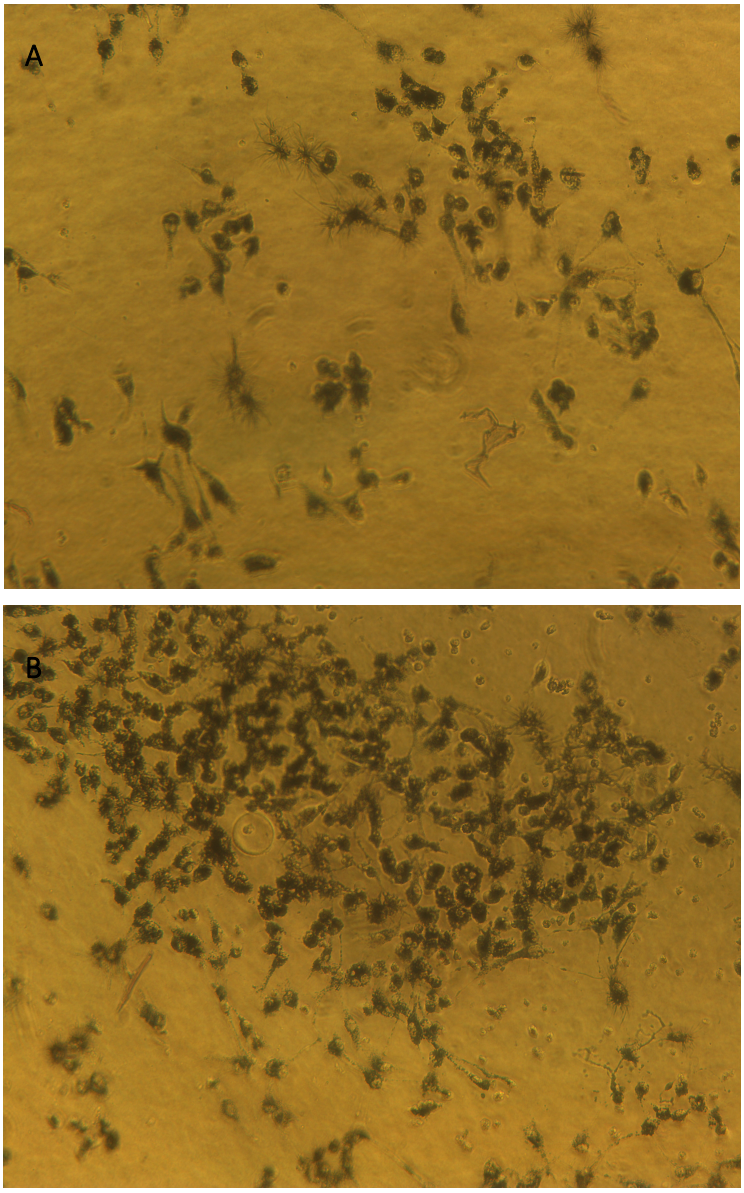


Fig 3.4 : Fig 3.4-A shows the hepatocytes with a concentration of $2.5*10^4$, Fig 3.4-B hepatocytes at $5*10^4$ density, after being treated with MTT .

The well treated with MTT was left to incubate at 37 ^{0}C for approximately 4 hours. After this time, it was possible to observe at the microscope the cells: the ones which appeared blue represented the living ones.

The figure *[Fig3.4 A-B]* show the cells as they appeared in the microscope after MTT assay. The first picture represents a 3-days culture seeded with $2.5*10^4$ of cells per cm^2, the second picture is 1-day culture seeded with $5*10^4$ cells per cm^2.

3.5 Mechanical tests

After proving the cells were alive, mechanical parameters of the collagen gels were determined using a confined compression experiment. Previous work in department had successfully validated the technique of confined compression and biphasic theory as suitable for the determination of mechanical properties of collagen hydrogels (Busby et al., 2013). The set up and test parameters used in this project and outlined below were the same as previously used and reported by Busby et al. (2013).

The compression platen was specifically designed and constructed to fit into the well of a 24-well plate [*Fig. 3.5*]. The platen consisted of a cylindrical shaft with a circular porous indenter at its end. The platen was clamped and attached to a load cell which was displacement controlled using a BOSE ElectroForce® Load Frame System 3200 (BOSE, UK) and accompanying WinTest® software used also for data acquisition. The BOSE ElectroForce 3200 is a test instrument capable of measuring relatively small loads with the maximum load cell available having a capacity of 450 N maximum force. In this case, the mechanical compressive characteristics of each collagen gel, unseeded or cell seeded, were estimated using a load cell of 22 N (BOSE, UK) in a dry state.[39]

The bathing fluid was removed from every well immediately prior to testing. The gel was then gently detached from the sides of the well using a micropipette tip. The surface of each well was found by lowering the platen until a preload of 0.01 N (0.05 kPa) was achieved, and then resting the system until the stress response equilibrated. Samples were compressed by 250 µm (~ 11% strain) at 25 µm/s (~ 1.1% strain/s) with a hold phase of 300 seconds immediately following the ramp. Since the amount of collagen gel placed on each well was of 0.4 mL and the wells have a diameter of 2 cm2, it was possible to estimate that the gels have a diameter of 16 mm and

thickness of 2.2 mm.

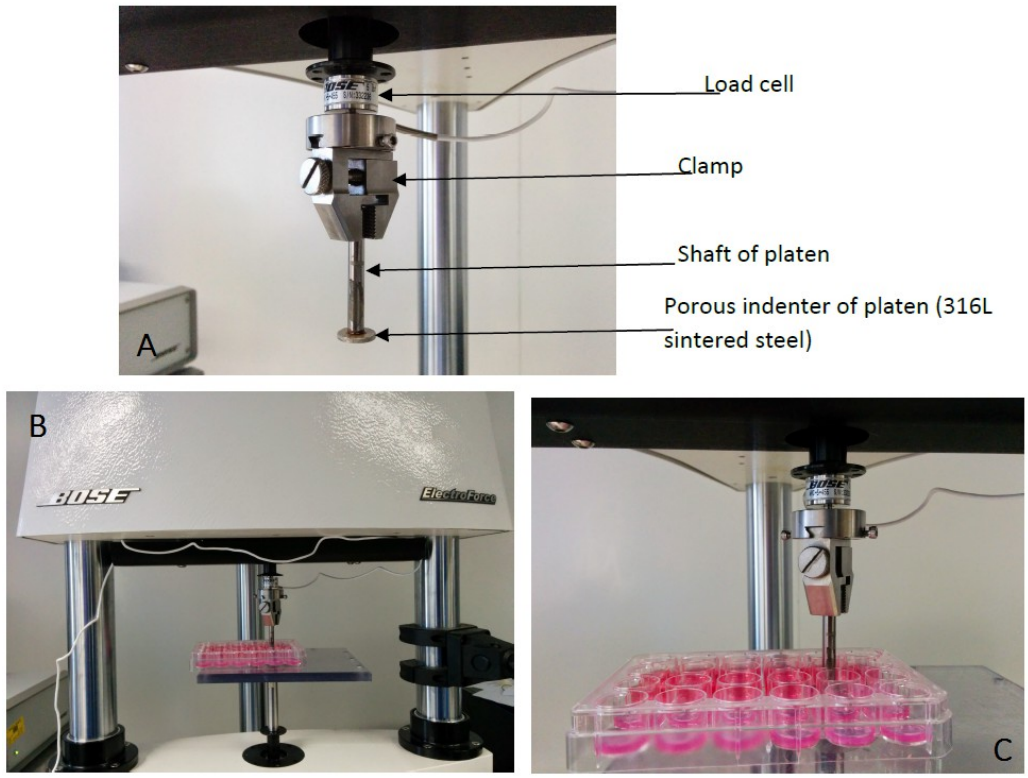


Fig 3.5 : a) Photograph of the compression platen showing it clamped, attached to the load celland fitted to the BOSE Electroforce 3200 Test Instrument. b) Photograph showing setup forcompression of collagen gels. c) Photograph of close-up of compression setup.

The data were extracted into Microsoft Excel and analysed with MATLAB® using biphasic theory as previously reported (Busby et al., 2013). The Matlab code was written and obtained from Dr. Phil Riches. It was possible to estimate aggregate modulus (H_A), hydraulic permeability (k_0), coefficient of permeability (M) and correlation coefficient (r^2) with this biphasic theory model following the Matlab analyses of the data.

3.6 Statistical analyses

The statistical analysis was performed with the data obtained.

The data has been divided in groups, according to the three culture durations and the five cells densities plus collagen gel without cells; so, as better showed in the paragraph 2.4 of "The State of the Art" chapter, there were a total of 18 groups. For each of the groups of gel seeded, 4 different wells were tested, since it was considered the best compromise between statistical reliability and time availability to conduct the tests. For the three groups of collagen gel without cells on it, 2 samples have been set up an tested.

The data were analysed with *Minitab17* software.

Statistical analyses were carried out using analysis of variance (ANOVA) followed by Tukey test for two group and multiple group comparisons respectively.

The test selected was the ANOVA test since it is a statistical inference technique which allows to compare two or more groups, comparing the internal variability of the groups with the variability between the groups. The null hypothesis is that the data have the same origin. [75]

In this work, the three groups representing the 3 culture durations (1 day, 3 days and 5 days) were compared among each other for the same cells concentration and then the 6 groups representing the five cell concentrations and the collagen gel without cells were compared for the same culture period.

The confidence level was set up at 95 % of confidence, assuming equal variances, with two-sided type of confidence interval. The comparison procedure was the Tukey, with an error rate of comparison of 5. This method allows to find means that are significantly different from each other.

The results appeared showed the analysis of variance, the model summary and the means, as it will appear in the appendix for the data statistically significant. Data are expressed as mean ± S.E.M. and a p value of ≤ 0.05 considered significant.

Results

4.1 Data analyses

Before any of the cell–seeded collagen samples were tested for mechanical properties the MTT test was carried out and the distribution of viable blue cells throughout the culture were confirmed. Some representative pictures of the cultured Hep G2 cells showing crystals of formazan are shown in the appendix.

The data were acquired with a BOSE *ElectroForce 3200* machine together with the WinTest software. The stress responses obtained had well defined ramp, peak and hold phases, as shown in the figures below *[Fig4.1], [Fig4.2]* and *[Fig4.3].*

The displacement of the figure *[Fig4.1]* was applied by the machine, in order to obtain a compressive strain with a rate of 10 % of displacement per second. It is possible to observe the characteristic behaviour of a sample processed with a creep confined compression test (as explained in the first chapter): the highest value of load corresponds to 0.064 N, and it is reached at approximately 10 seconds; after this, relaxation occurs since load reduced until the equilibrium is reached approximately at 0.01 N, with a global test duration of 310 seconds.

The strain applied increased linearly for approximately 10 seconds, which corresponds with the load peak; the rate of increase is 10 % of strain every second. Then, it is maintained constant for the entire duration of the test (300 seconds), at a value of 0.25 millimetres in order to obtain the equilibrium value, as shown in the

figure *[Fig4.2]*.

The stress is then easily evaluated with the following formula:

$$\text{Stress [Pa]} = \text{Load [N]} / \text{Area(well) [m}^2\text{]}$$

The stress function has the same shape as the load, since there is a peak value reached at approximately 10 seconds and then the relaxation with the final equilibrium measure. In the specific case of the figure below *[Fig4.3],* the stress peak is approximately at 0.35 kPa and the equilibrium at 0.05 kPa.

The three figures were obtained through the data acquired from a well of collagen hydrogel seeded with a concentration of 10^5 cells per cm^2 for a culture duration of 5 days.

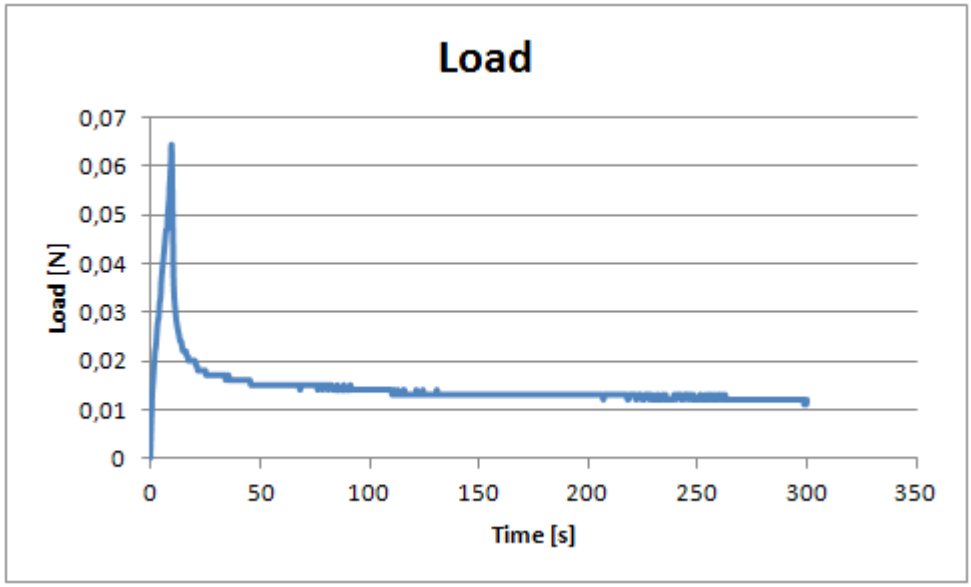


Fig 4.1 : Compressive load in response to ramp-hold compressive strain (10% per second) for 0.3% collagen hydrogel for 5-days culture, 10^5 cells per cm^2

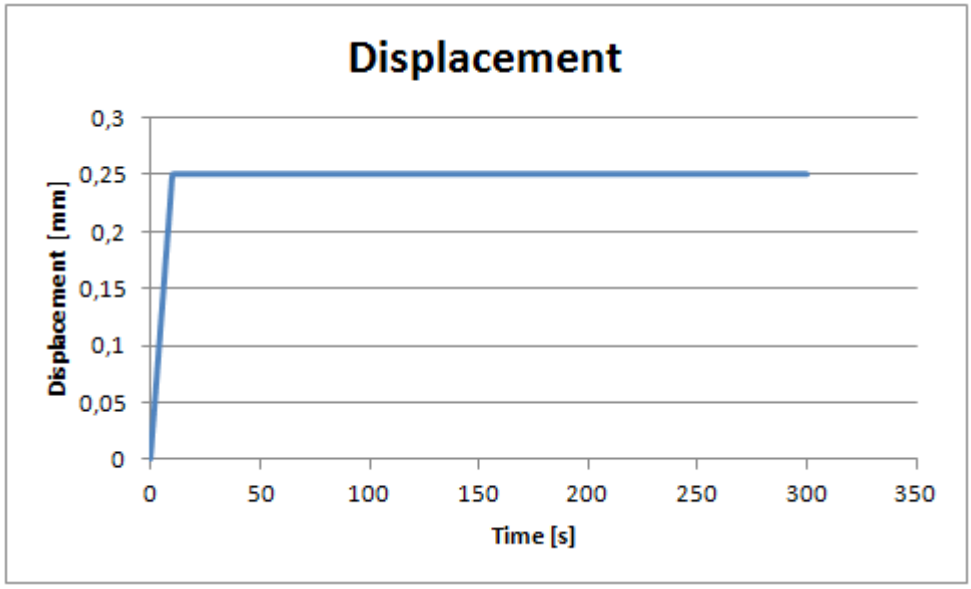


Fig 4.2 : Compressive strain with a rate of 0.5% of compression per second for 0.3% collagen hydrogel of 5-days culture, 10^5 cells per cm^2

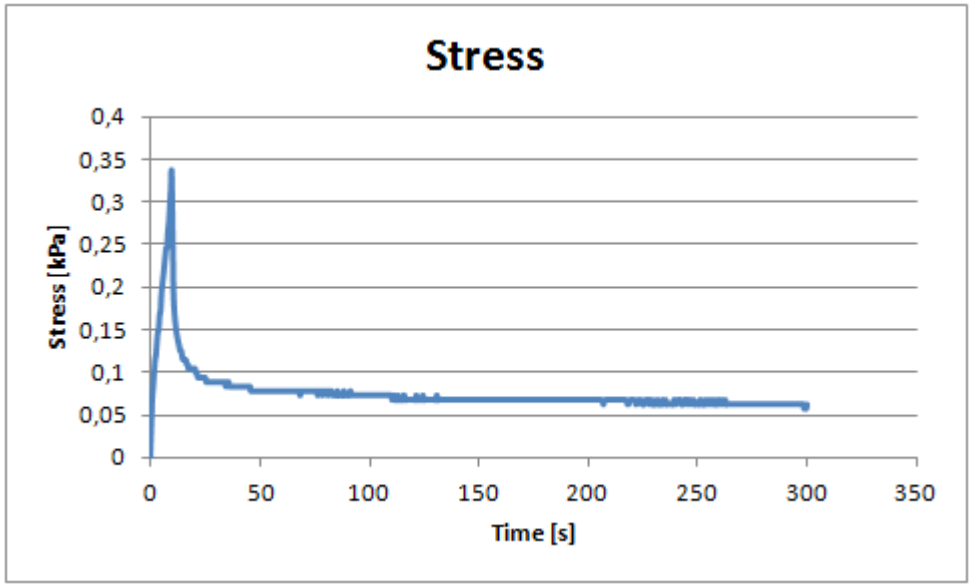


Fig 4.3 : Compressive stress in response to ramp-hold compressive strain (10 % per second) for 0.3 % collagen hydrogel of 5-days culture, 10^5 cells per cm^2

The data acquired have been manipulated with MATLAB R2013a; the software could evaluate the Hydraulic Permeability (k_0), the Aggregate Modulus (H_A), the non-linear Permeability Coefficient (M), the Peak Stress and the Equilibrium Stress, as shown in the figure below *[Fig 4.4].*

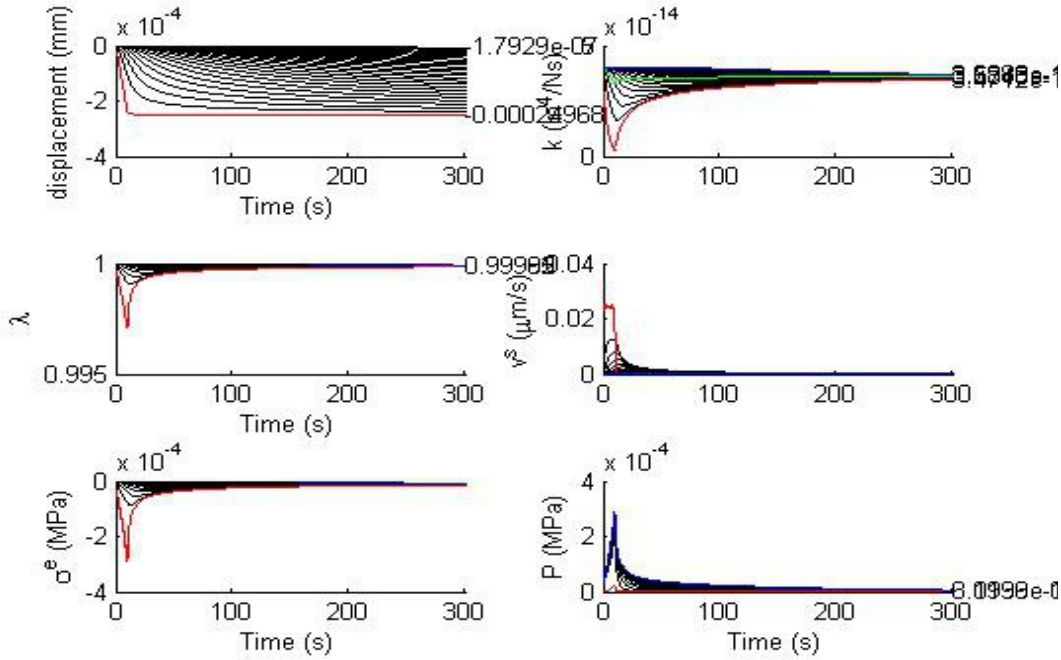


Fig 4.4 : Four graphs which represent from the top left to right respectively displacement, Hydraulic Permeability, Stretch Ratio, velocity, stress and pressure, as plotted from MATLAB software. The data shown are examples and they are acquired from one generic well, as sample.

All the samples have been compared; the comparison has been performed between wells seeded with the same concentration of cells, but left for different culture durations before being tested, and between wells with different cell densities on it, grown for the same period.

The statistical analysis has been carried out with *Minitab17* software; particularly, the test used was the one-way Analysis Of Variance (*ANOVA*), which can determine whether there are any significant differences between the means of three or more independent groups.

In this case, the comparison have been performed between three groups of data representing the three culture durations (1 day, 3 days and 5 days) with the same cell density and between six groups of data for six different cell densities (10^5, $5*10^4$, $2.5*10^4$, 10^4, $0.5*10^4$ and collagen gel without cells on it) cultured for the same

period. Both cases are characterised by three or more independent groups, for this reason *ANOVA* test is recommended.

Shown below, the results of the statistical analyses are reported on the graphs. The report of the statistical analyses conducted by *Minitab17* will be illustrated in the appendix chapter; in particular, the appendix will show the histograms which report the asterisks symbolizing the statistical significance of a comparison ($p < 0.05$).

4.2 The Aggregate Modulus

As observed in the figure below *[Fig 4.5]*, the data acquired and compared show that the Aggregate Modulus (H_A) of collagen hydrogel increases with the duration of the culture in the presence of cells. Specifically, gels seeded with $0.5*10^4$ and 10^5 cells per cm^2 show a statistically significant increase ($p < 0.05$) in H_A , whereas H_A of wells with $2.5*10^4$ cells per cm^2 have a trend of increasing although it is not statistically significant.

As explained above, the in-depth statistical analysis was performed trough *Minitab17* for the data reported and the relation between the average Aggregate Modulus of $0.5*10^4$ cells per cm^2 culture and 10^5 cells per cm^2, are shown in the appendix chapter.

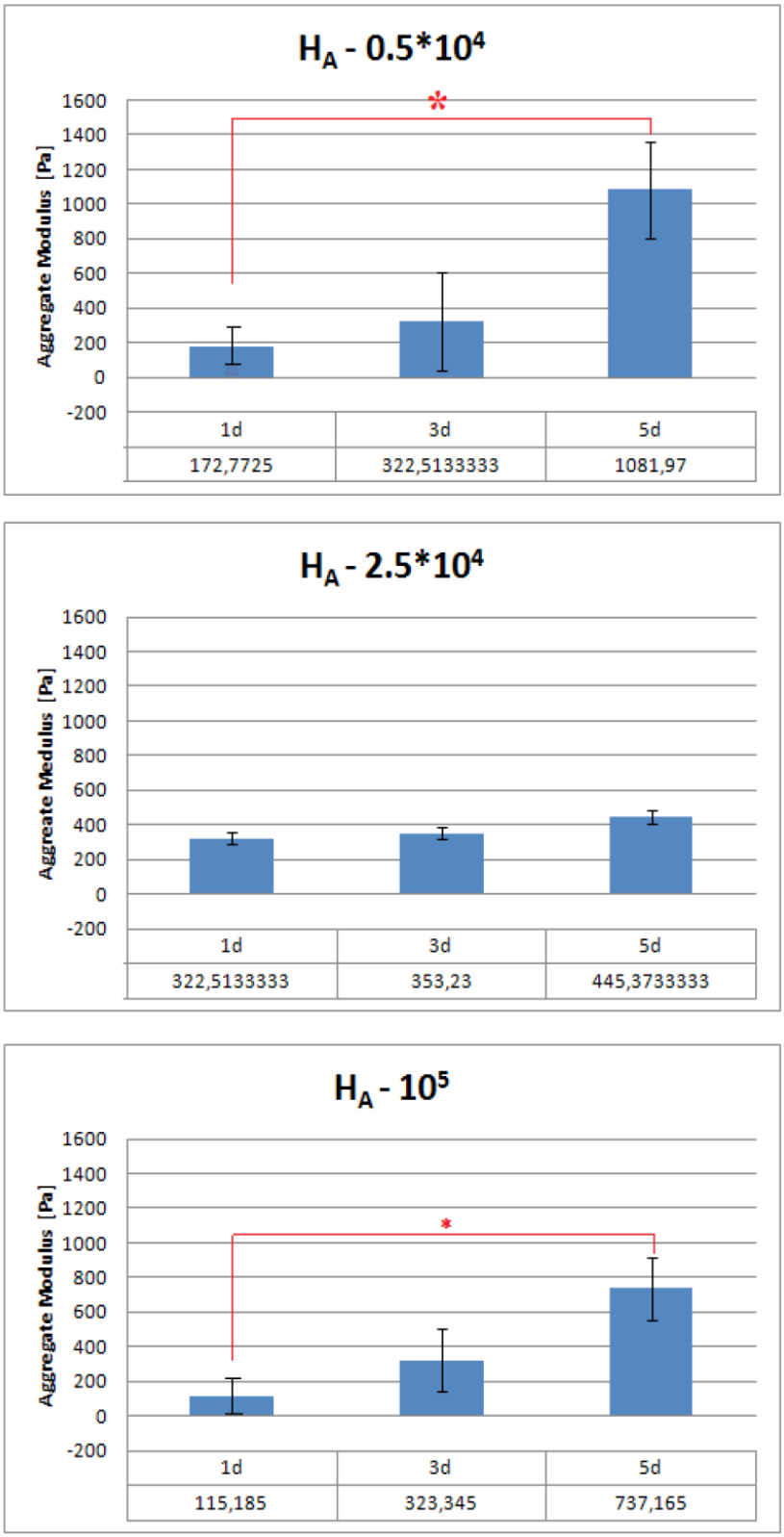


*Fig 4.5 : Variation in the average Aggregate Modulus (H_A) with duration of the culture, for three different cell densities (from top, $0.5*10^4$, $2.5*10^4$ and 10^5). Error bars indicate +/- one standard error of the mean with * indicates $p < 0.05$ by ANOVA comparing the three means among each other.*

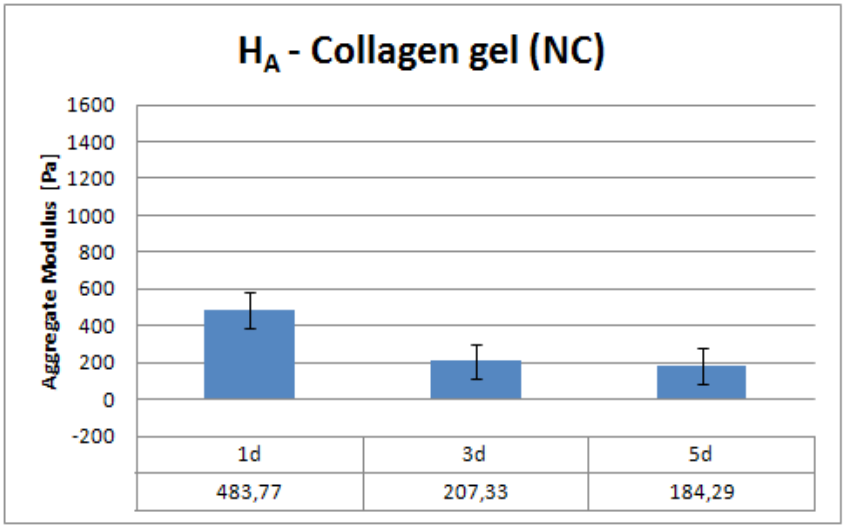


Fig 4.6 : Variation in the average Aggregate Modulus (H_A) with duration of the culture for collagen gel without cells. Error bars indicate +/- one standard error of the mean.

4.3 The Hydraulic Permeability

The Hydraulic Permeability (k_0) represents the ability of a liquid to flow through the matrix. As expected, there is a trend of decreasing k_0 during time, for the same cell culture, as shown in the figure below *[Fig 4.7],* even if it does not represent a statistical significant variation*;* in particular, this trend can be observed for the wells seeded with $2.5*10^4$ cells per cm^2 and for the wells characterised by collagen gel without cells. The small number of measurements (four measurements per each group) do not allow to have statistical significance, even if the difference between 1-day and 3-day is considerable. It is possible to observe that after 1 day there is a drastic decrease in k_0, more than after 3 days.

Indeed, cells add resistance to fluid flow and create a “barrier” which makes difficult

for the fluid to permeate. [29]

Moreover, it is possible to observe that the hydraulic permeability of the gels seeded with HepG2 is considerably lower than the one of collagen gel without cells. This evidence is in agreement with what explained above: cells add resistance to the fluid, so k_0 of wells seeded results much lower than the one of collagen gel without cells.
The graph *[Fig 4.8]* compares all the cell densities means with culture durations. In this case, the software have highlighted statistically differences between 1-day culture wells between different concentrations; these evidences agree with what explained above, since Hydraulic Permeability appears to increase while cells densities decrease.

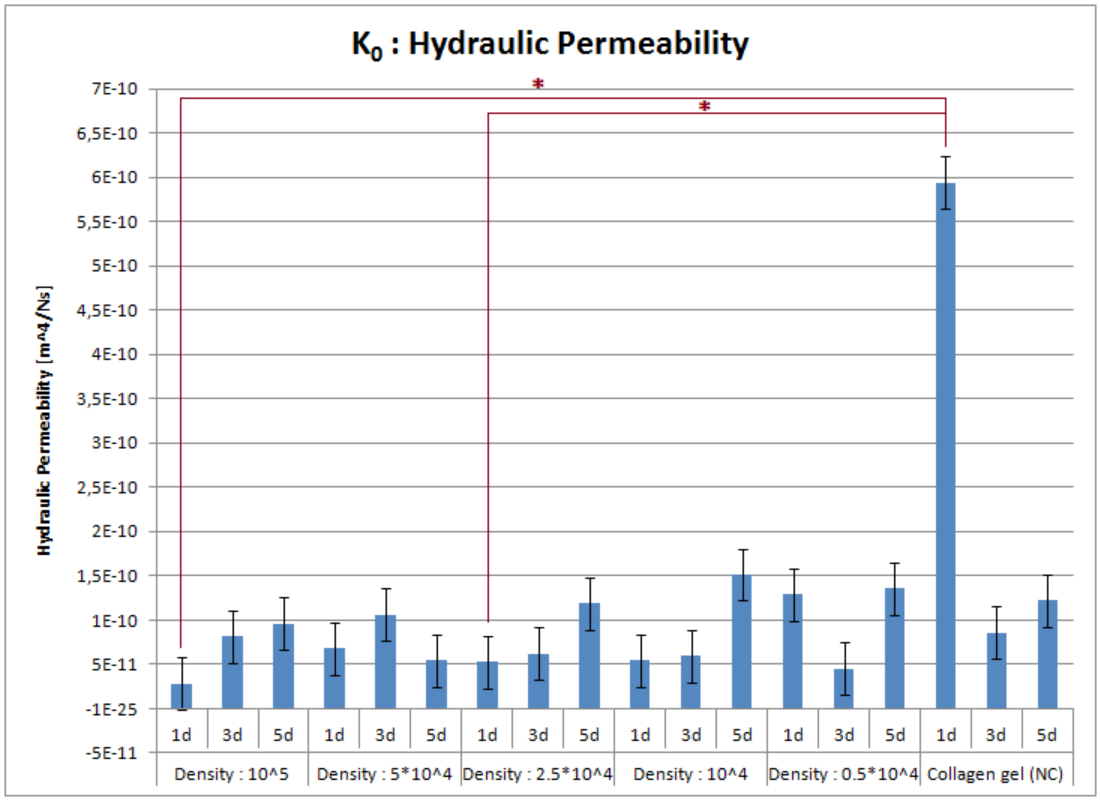
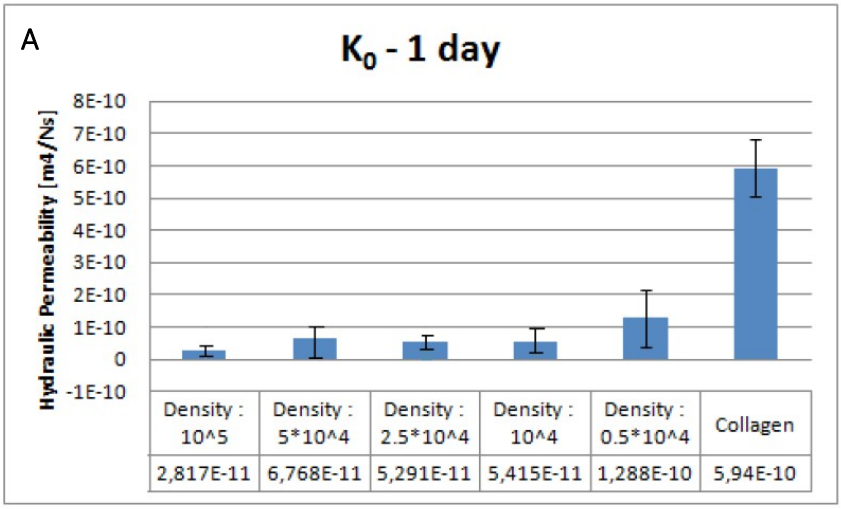
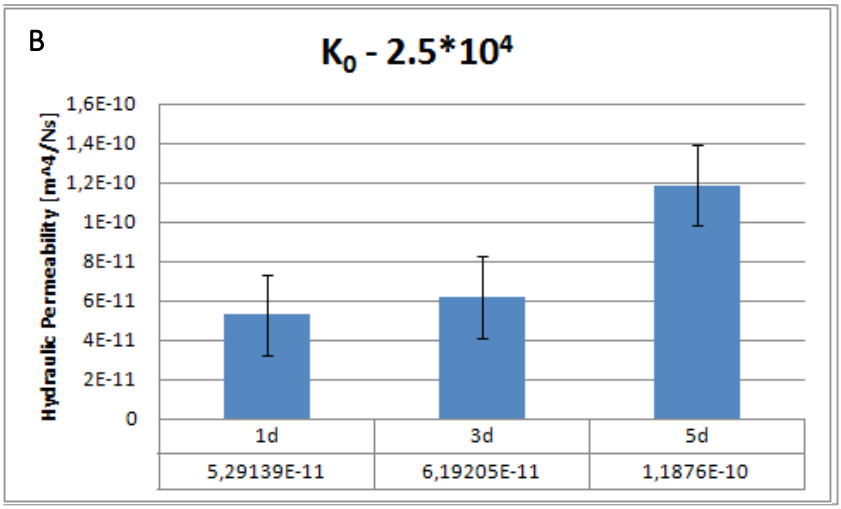


*Fig 4.8 : Variation in the average Hydraulic Permeability (K_0) with duration of the culture and cell densities. Error bars indicate +/- one standard error of the mean with * implying $p < 0.05$ by ANOVA comparing every mean value with the corresponding for a different cell density or culture duration.*





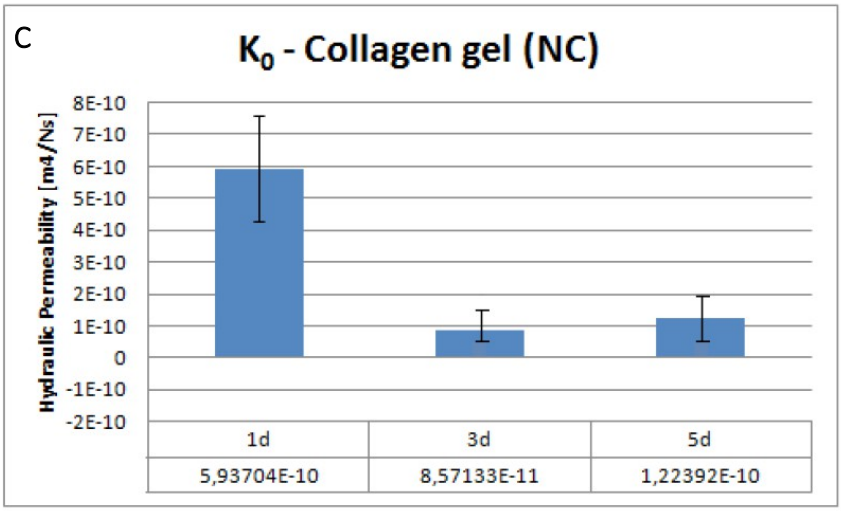


Fig 4.7 : Variation in the average Hydraulic Permeability (K_0) with duration of the culture in both unseeded collagen gels and in gels seeded with 2.5 x 10^4 cells (Figure B), and cell densities in cultures at 1 day since seeding (Figure A), and finally non-cell seeded collagen gels (Figure C). Error bars indicate +/- one standard error of the mean.

4.4 The Peak and Equilibrium Stress

The histograms below, *[Fig 4.9],* show how the peak stress changes during culture duration, for four different cell densities ($0.5*10^4$, $2.5*10^4$, $5*10^4$ and 10^5) and for the pure collagen gel.

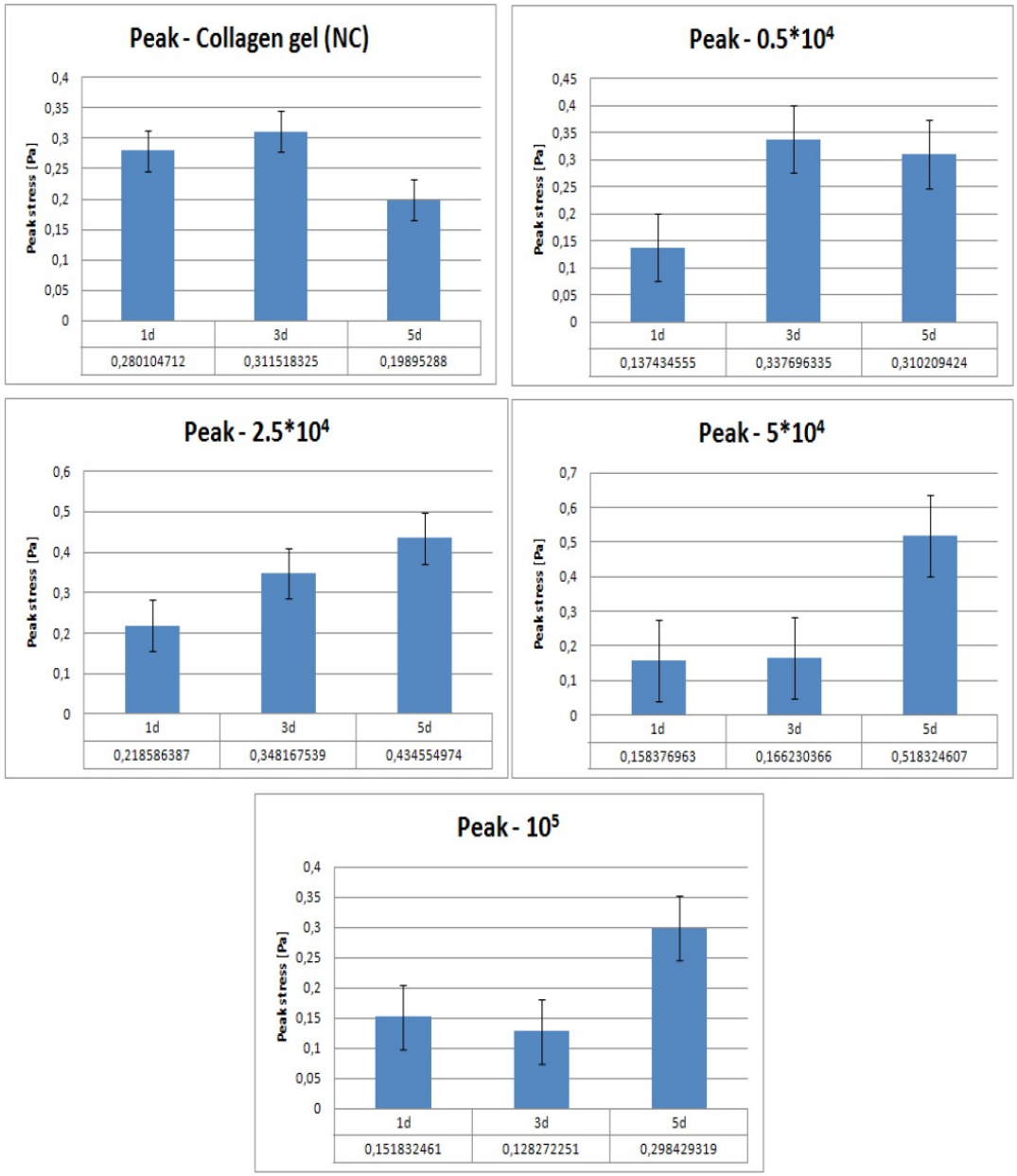


Fig 4.9 : Variation in the average Peak Stress in relation with duration of the culture in both unseeded collagen gels and in gels seeded with 0.5 x 10^4 cells (Figure B), 2.5 x 10^4 cells (Figure C), 5 x 10^4 cells (Figure D) and 10^5 cells (Figure E). Error bars indicate +/- one standard error of the mean

It is possible to observe that there is a trend of increasing peak stress during time for all the cell concentrations, albeit non-significantly; on the other hand, non-seeded collagen gel stress peak appears to remain stable till the third day and then it decreases. As shown above, the stiffness of gels seeded seems to increase during time; this means that a stiffer sample needs higher levels of stress in order to reach a certain deformation (0.25 millimetres in this case). This is the reason why peaks increase during time, in relation with the increase in stiffness.

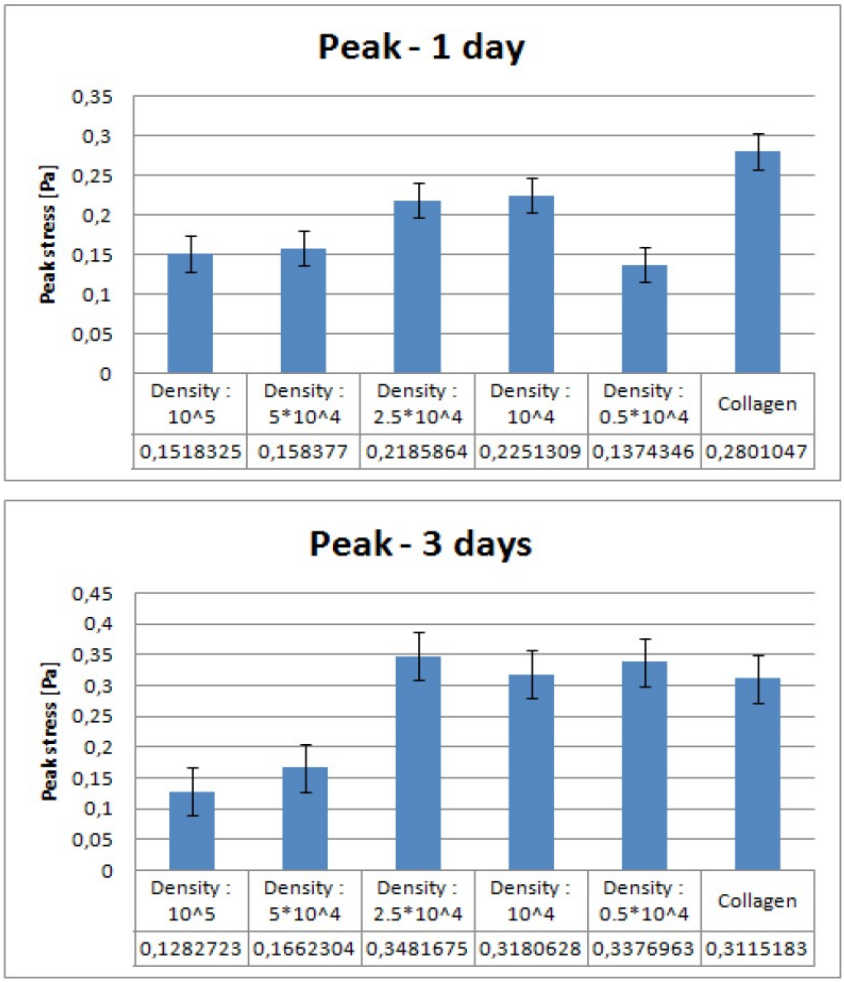


Fig 4.10 : Variation in the average Peak Stress in relation with different with cell densities. Error bars indicate one standard error of the mean.

In confirmation of what has just been asserted, the collagen gel graph shows a small decrease in stress peak; and indeed, the Aggregate Modulus analysis has shown that the gel stiffness tends to decrease during time and, for the same reasons as just mentioned, it can lead to a decrease in stress value necessary to produce the deformation.

The two graphs of *[Fig 4.10]* draw attention to the fact that the peak stress increases not only in relation to the culture duration, but also with time, even if it is not statistically significant. The explanation can be the same of the case discussed above.

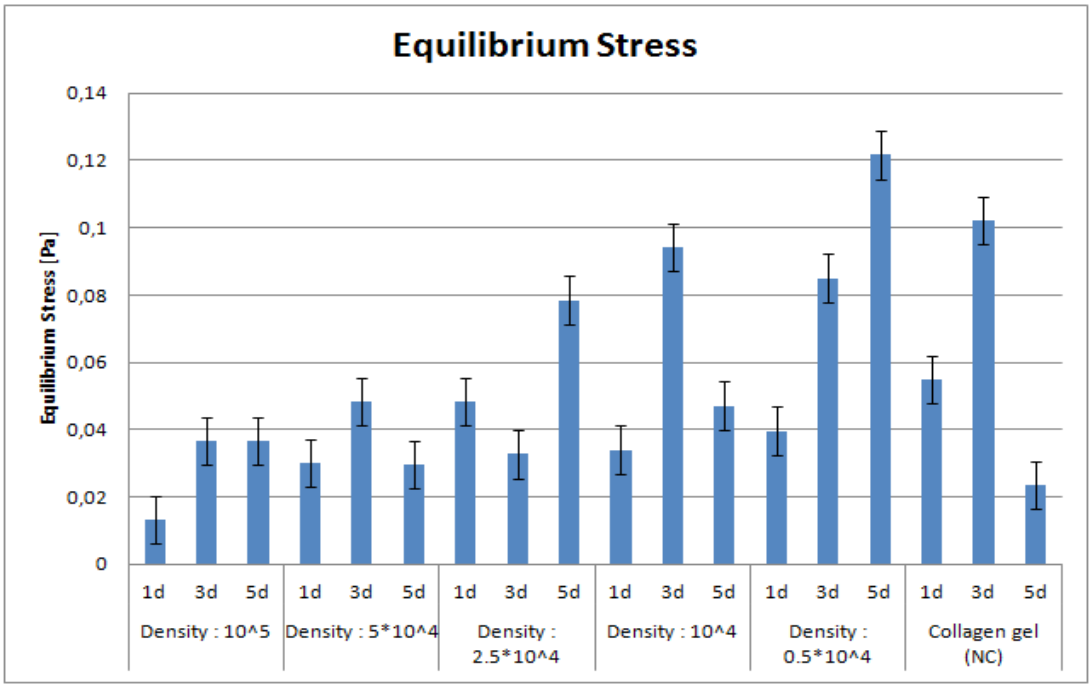


*Fig 4.11 : Variation in the average Equilibrium Stress with duration of the culture and cell densities. Error bars indicate +/- one standard error of the mean with * implying p < 0.05 by ANOVA comparing every mean value with the corresponding for a different cell density or culture duration.*

Finally, the comparison between all the Equilibrium Stress average data shown in the figure above, *[Fig 4.11],* displays a trend of increasing values during culture duration in the same cell density group, and a trend of decreasing when densities become higher.

Effectively, as for the case of the peak Stress, it is possible to affirm that if the sample

is stiffer, the equilibrium will reach a higher value compared with softer samples. The only exception is represented by the collagen gel without cells behaviour: after an initial increase in value there is a strong reduction of the equilibrium stress.

All of the other histograms, which have been not reported or analysed here because they were not considered significant, have been arranged in the appendix chapter.

Discussions

5.1 Overview

The stiffness of a tissue engineering scaffold is an important parameter to take into consideration during a culture, since it was proved that it influences cells viability, proliferation and functions (Wells (2008), Li *et al.* (2008), Semler (2004)) . [22, 68, 69]

Considering hepatocyte cultures, the literature shows lack of information regarding how scaffold stiffness changes during the culture; given that, no studies have been carried out yet in order to understand the dynamics behind this. Comprehending the behaviour of hepatocytes during a culture is an important point because of their wide applications in pharmacology, toxicology and tissue engineering research (Paine (1990), Hawksworth (1994), Lasser *et al.* (2002)). [56 - 58]

For these reasons, this study has analysed the mechanical properties of collagen hydrogel as a scaffold on which to grow hepatoma HepG2 cells; in particular, the stiffness property is the focus of interest. Changes in the stiffness of the gel (increasing or decreasing) were analysed in relation to the culture duration and with cells concentration. Effectively, the goal was to understand how HepG2 cells influence the stiffness of collagen gel in order to compensate for the lack of information in the literature regarding the their behaviour during culture. Three culture durations (1 day, 3 days and 5 days) and six cell densities ($0.5*10^4$, 10^4, $2.5*10^4$, $5*10^4$, 105 cells per cm^2 and collagen gel without cells on it) have been selected, considered the most

significant and representative combinations, according to the literature and to the previous department experiences (Torok *et al.* (2001), Dvir-Ginzberg *et al.* (2003)).[76, 77] The choice of collagen hydrogel was related to its many applications in tissue engineering because of its properties as a scaffold, so that this work can result in useful future developments (Risbud *et al.* (2003), Drury *et al.* (2003), Lee *et al.* (1995), Varum *et al.* (1996), Tomihata *et al.* (1997), West *et al.* (1999), Mann *et al.* (2001)). [8, 48 - 53] HepG2 cell line was preferred to primary human hepatocytes because of their ability to proliferate in a culture, without being limited in growth such as the primary cell line (Bokhari *et al.* (2007), Wilkening *et al.* (2003), Erro *et al.* (2013), Wang *et al.* (2005)). [46, 47, 54, 55]

The stiffness of the gels has been tested with confined compression tests, which are able to provide several useful parameters to investigate scaffold mechanical properties; particularly, the parameters analysed were the Aggregate Modulus (H_A), the Hydraulic Permeability (k_0) and the Peak and Equilibrium Stress (Discher *et al.* (2005)). [20, 67]

The monolayer model is characterised by two structures: the collagen gel (with a thickness of 2.5 mm, easily evaluating knowing the volume of collagen gel per well, 0.4 mL, and the size of the well) and the HepG2 cells on it; according to the average size of HepG2 cells (which is of approximately 18 µm of diameter[91]) and modelling them with a round regular shape, it is possible to estimate that one layer of cells has a thickness of approximately 18 µm. Considering a single layer of HepG2 cells on the gel, the ratio of depths is 2500µm : 20µm ; this means that the cells layer depth represents approximately 0.7 % of the entire thickness. Therefore, it is possible to affirm that the thickness of the cells layer is negligible compared with the collagen gel one. This assertion takes important during confined compression test, because it is important to make sure of testing the actual stiffness of collagen gel with the minimum effect of other parameters, such as cells stiffness.

The tests have been performed with *BOSE ElectroForce* machine together with the WinTest software, and the data have been analysed with *Matlab R2013a* and *Minitab17* software packages.

5.2 Relevant literary background

The mechanical properties of collagen gels cultured without cells have been established in the work of Busby *et al.* (2013) who incubated collagen gels in culture medium at 37 ^{0}C in an incubator. It was proved that the stiffness increases with the percentage of collagen concentration (0.2 % of collagen gel appears softer than the 0.4 % one), due to the further polymerisation of the collagen; the average Aggregate Modulus of 0.3% collagen gel without cells estimated is approximately 1000 Pa.[29]

The mechanical properties of collagen hydrogel with cells cultured on it, have been tested in 2008 by Saddiq *et al. ;* the cells seeded were the 3T3 mouse and human fibroblasts. It was shown how the presence of fibroblast cells clearly results in an overall reduction in scaffold stiffness. The stiffness of anchored collagen hydrogel remains relatively stable in growth medium, but it is altered by cell ingrowth. Indeed, as explained better in the third chapter, the cells act to weaken the scaffold through microscopic mechanical forces exerted by cells and by secreting degradative enzymes, such as collagenase.[44]

For the Hydraulic Permeability, Saddiq *et al.* (2008) have shown that after 6 days in culture it is possible to observe large differences between the calculated permeabilities of the cell-seeded and cell-free gels. In addition to decreasing the stiffness of the scaffolds, cell ingrowth also acts to decrease the permeability; usually, tissues characterised by lower stiffness have higher permeabilities. The cells themselves contribute to the reduction of the Hydraulic Permeability by adding resistance to the fluid flow, impeding the passage of fluid through the scaffold.[29]

In summary, the proliferation of cells in collagen hydrogels results in a reduction in overall stiffness and a decrease in the Hydraulic Permeability.[29]

Sharma *et al.* (2009) have analysed the Young's Modulus for HepG2 cells cultured on Matrigel^{TM} (several microns of thickness), Laminin and Collagen I scaffolds. The modulus was evaluated 24 hours after seeding with the Hertz model (Touhami *et al.*, 2003) that the average Young's Modulus was 0.72 kPa for Matrigel^{TM} scaffold, 0.06

MPa for Laminin and 0.05 MPa for Collagen I. This study showed that HepG2 cells stiffening for Matrigel™ and its elasticity values are closer to hepatocytes cell elasticity than for Laminin and Collagen I scaffold. [89, 90]

The data acquired with BOSE *ElectroForce 3200* have been statistically analysed and compared with the literature knowledge mentioned above.

5.3 Discussions

According to the data obtained, it is possible to affirm that the Aggregate Modulus has a general trend of increasing with culture time, except for the collagen gel without cells; the differences are particularly evident for low seeding densities between 3-days and 5-days cultures. However, the differences between culture durations are statistically significant just for the cases of $0.5*10^4$ and 10^5 cells per cm^2 cultures, and so the above interpretation can still hold.

These results disagree with the work of Saddiq *et al.* (2008) conducted with fibroblasts, since this previous study showed that stiffness modulus decreases along the culture duration.

However, a possible way to interpreter this phenomenon can be as follows. Cells grow and increase in number during culture time, indeed cells in gels cultured for one day present a smaller cell concentration than the ones cultured for 5 days. The increase in number can contribute to increase in the Aggregate Modulus, therefore in the stiffness, since, as showed in the literature, the stiffness tends to increase with cells because they add resistance to the gel (Saddiq *et al.,* 2008).

It is generally true that cells act to weaken the collagen gel, but the rate in with they make it soft (by causing the secretion of degradative enzymes and by exerting

mechanical forces) could be probably lower than the rate of cells growth, in the specific case of HepG2 cells. For this reason, the cell growth effect outweighs the effect of weakness.

The collagen gel weakening in those instances (where the Aggregate Modulus at 3-days culture is higher than the one of 5-days culture) outweighs the physical presence of cells causing a rise in stiffness. Indeed HepG2 cells are principally chemical processors rather than structure altering cells; for this reason, differently from cells such as fibroblasts analysed in the paper of Saddiq *et al.* (2008), HepG2 cells tend to weaken collagen gels much less.

Another interesting result is represented by the collagen gel behaviour without HepG2 cells seeded on it (expressed with the abbreviation NC, *no-cells*). It is possible to observe from the graph *[Fig 4.6]* that collagen hydrogels without cells have a trend of decreasing H_A, even though this is not statistically relevant.

The collagen gel is characterised by a constant polymerisation activity, which lead to an increase of cross-linkings during time; this might lead to thought that stiffness increases during time, till reaching an equilibrium value, approximately at the third day after set up, after which the stiffness should remain stable, cause polymerisation process stops to act.

However, a possible interpretation is given analysing the paper of Nakagawa *et al.* (1989); in this work is explained that in some cases collagen hydrogels have a trend of decreasing during time because a fraction of collagen cannot be properly incorporated leading to a non-specific loss of collagen from gels over time and decreasing in stiffness.

In summary, seeding cells on collagen gels will usually lead to one of two possible effects on the Aggregate Modulus:

- The Aggregate Modulus can reduce which will be due to cells weakening the gels
- The Aggregate Modulus can increase which can be due to physical presence of cells contributing to higher stiffness measurements

However, for HepG2 cell line, the increase in stiffness seems to be the predominant action.

The Hydraulic Permeability seems to decrease during culture durations, and this can be interpreted as if the increase in cells proliferation leads to an increase in the obstruction exercised by them to the fluid flow.
This effect is most visible after 1 day because of the characteristic of cells to proliferate faster the first hours of culture. [85]
Moreover, it is evident from the histogram *[Fig 4.7 (a)]* that collagen gel without cells has higher k_0 average value than the ones of wells seeded with cells, even if the shortage of data do not allow a statistical relevant conclusion; according to the previous explanation, collagen gels without cells do not present the same resistance to what as the ones with cells on them.

On the other hand, regarding the changes in stiffness during the different cell densities, the data acquired do not show a trend which can be interpreted with logical deductions.

Comparing the data with the *in vivo* situation analysed in the second chapter (*The State of the Art*), it is possible to observe that the values of the Aggregate Modulus estimated have the same order of magnitude of the *in vivo* measurements. In particular, the values for the 1-day culture ranges from 0.115 kPa to 0.322 kPa, with an average of 0.2535 kPa, whereas the range for the 5-days culture is between 0.261 kPa and 1.081 kPa, with 0.588 kPa as average. The normal value for a healthy liver tested with compression-relaxation test has been estimated from 0.35 kPa to 0.7 kPa (*[Tab 5.1]*).

	Healthy liver [kPa]	Fibrotic liver [kPa]
Compression test	0.35 – 0.7	0.586 – 1.733
Ultrasound technique	3.3 – 7.0	7.0 – 10.0

Tab 5.1 : Comparison between values of liver stiffness for health liver condition and fibrotic pathological condition, tested with two different techniques. [30, 86, 87]

Therefore, the 1-day and 3-days cultures appear to agree with the normal *in vivo* condition, whereas the 5-days culture seems to come close to values which represent pathological situations in human body, such as fibrosis (*[Tab 5.2]*). A possible interpretation is that after 3 days the HepG2 cells start to assume a non-physiological behaviour, characterised by mechanical properties which do not respect the *in vivo* conditions.

	1-day [kPa]	3-days [kPa]	5-days [kPa]
$0.5*10^4$	0.172	0.322	1.081
10^4	0.299	0.522	0.414
$2.5*10^4$	0.322	0.353	0.445
$5*10^4$	0.266	0.428	0.261
10^5	0.115	0.323	0.737
Collagen gel (NC)	0.483	0.207	0.184
AVERAGE VALUES (no collagen gel)	0.235	0.390	0.588

Tab 5.2 : Stiffnesses comparison between cells densities plus collagen gel without cells vs. their culture durations; it is reported the average values of stiffnesses for every culture duration, collagen gel (NC) excluded.

Comparing the values obtained with the same parameter found in the literature, estimated for other studies, such as the paper of Sharma *et al.* (2009) whit HepG2 cells on Matrigel™ scaffold, it is possible to observe that collagen hydrogel has an elastic modulus close to the *in vivo* conditions, such as Matrigel™ scaffold. Indeed, the work of Sharma *et al.* shows that Matrigel™ has an average Young's Modulus of

0.72 kPa after 24, whereas collagen hydrogel's average value is 0.235 kPa (*[Tab 5.2]*); both these two values are slightly out of the stiffness range of healthy liver sample, which is 0.35 kPa – 0.7 kPa (*[Tab 5.1]*), but the differences between the scaffold stiffnesses and the healthy liver sample stiffness (delta values Δ, respectively 0.29 for collagen hydrogel and 0.195 for Matrigel™) are strongly lower than the ones found for the Laminin and Collagen I scaffold, which are one order of magnitude bigger.

The data obtained are scientifically considerable, because they show a trend of transformation in collagen hydrogel stiffness which could have significant implications. The reliability of the data measured is guaranteed by the Correlation Coefficient (*r*), as shown in the *Appendix D.* Indeed, the integrity of the measurements is proved if this parameter ranges between 0.5 and 0.9, and their vicinity.

5.4 Possible future developments

The results presented in this study suggest that collagen hydrogel stiffness tends to increase during time in culture when seeded with cells; this effect is more evident when analysing the culture with a low starting cell densities, such as 0.5*10. However, the collagen gel stiffness tends to decrease if not seeded, and this is clear shown by the Collagen Gel (NC) histogram.

It must be noted that the shortage of the data replicate numbers has made it difficult to obtain results which could be considered statistically significant. Indeed, 4 wells have been analysed for every group with the exception of collagen gel without cell groups, which were composed of two well samples. This issue was mainly connected

with the shortage of time available to make tests. For this reason, having the same kind of experiments with an higher number of sample may be more suited for a most reliable stiffness analysis. Particularly, the number of collagen gel samples should be equal to the number of the other groups tested.

Moreover, future developments could be directed to the study of different culture designs, which can outdo design limitations related with the monolayer culture with cells on top of it. The mainly limitation is due to the fact that cells do not really affect homogeneously the layer of collagen, because they are located on top of it; moreover, as explained above, the ratio of depths between the collagen gel and the cell layer thickness has to be appropriate (the thickness of the cell layer has to be negligible compared with the gel one) in order to be sure that the compression test performed is actually measuring the stiffness of the collagen hydrogel, without being influenced by the cell ones. However, no studies have been carried out yet in order to quantitative establish the proper rate. For this reason, keep to the monolayer structure, a possible alternative could be found setting a monolayer of gel with cells seeded inside of it; this could lead to a more homogeneous distribution of cells, therefore a uniform effect of them on the collagen gel, and the certainty to test the actual stiffness of the hydrogel without influence of the cell layer, as in the present model.

Another designer improvement could be found in the set up of collagen gel sandwich. This is structure can overcome the problem of the test reliability mentioned above; however, the limitations of this model are related with the cells action: as explained above, the cells could not act homogeneously on the two layers of collagen, leading to non-reliable stiffness results.

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Appendix

Appendix A : The statistical analyses

Appendix A reports the prove of the statistical analyses conducted with *Minitab17* software. Shown below the graphs and the tables which represent the data appeared statistically significant and analysed in the fourth chapter.

- The Aggregate Modulus (H_A) for the wells seeded with $0.5*10^4$ cell density per cm^2 compared during the three culture durations (1 day, 3 days and 5 days). The analysis reported significant difference between the 1-day and 5-days wells.

  The tables showing the method of comparison (Turkey Method, which is a multiple comparison procedure[75]), the basic statistical calculations (such as mean, confidence, standard deviation, etc.) and the results with the model summary. Then, the graphs show the difference of means and the confidence intervals.

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
5d	4	1082	A
3d	3	322,5	A B
1d	4	172,8	B

Means that do not share a letter are significantly different.

Means

Factor	N	Mean	StDev	95% CI
1d	4	172,8	115,2	(-297,0; 642,6)
3d	3	322,5	92,1	(-220,0; 865,0)
5d	4	1082	651	(612; 1552)

Pooled StDev = 407,469

Method

Null hypothesis	All means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0,05$

Equal variances were assumed for the analysis.

Factor Information

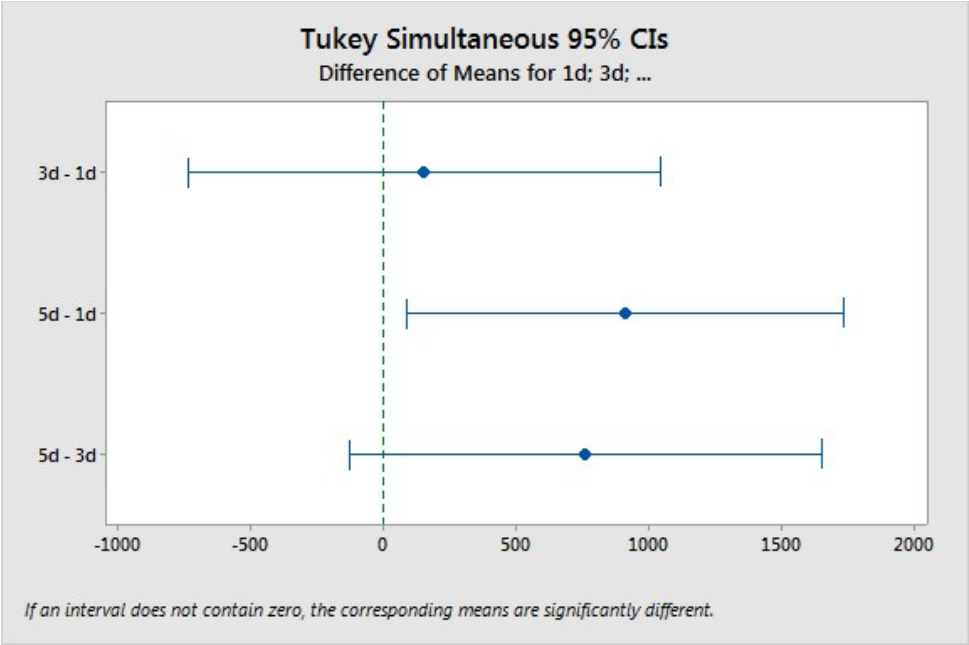
Factor	Levels	Values
Factor	3	1d; 3d; 5d

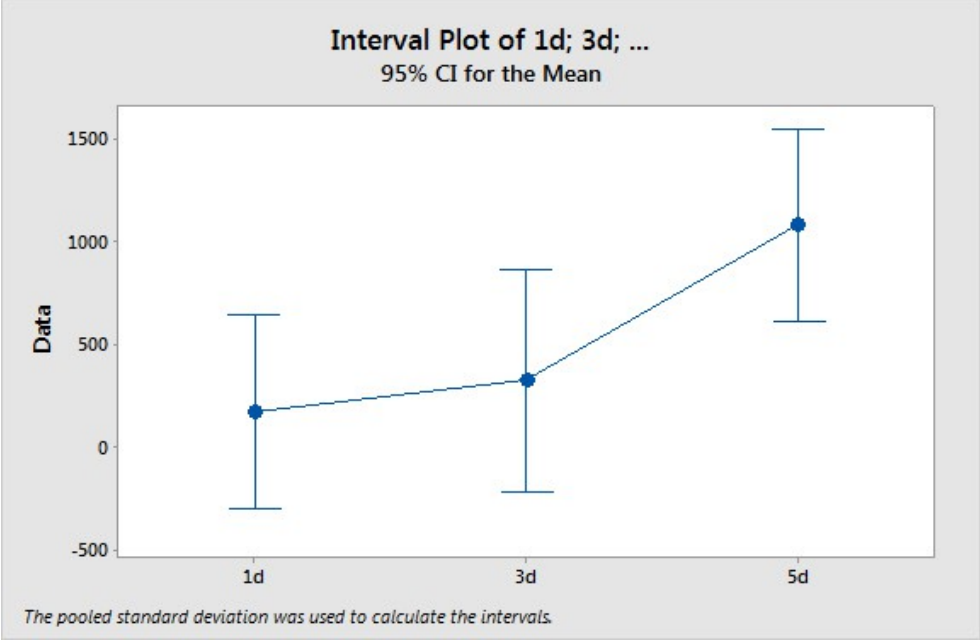
Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	1856055	928027	5,59	0,030
Error	8	1328249	166031		
Total	10	3184304			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
407,469	58,29%	47,86%	25,59%





- The Aggregate Modulus (H_A) for the wells seeded with 10^5 cell density per cm^2 compared during the three culture durations (1 day, 3 days and 5 days). The analysis reported significant difference between the 1-day and 5-days wells.

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
5d	4	737	A
3d	2	323,3	A B
1d	2	115,2	B

Means that do not share a letter are significantly different.

Means

Factor	N	Mean	StDev	95% CI
1d	2	115,2	32,6	(-204,0; 434,4)
3d	2	323,3	131,5	(4,1; 642,6)
5d	4	737	213	(511; 963)

Pooled StDev = 175,618

Method

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0,05$

Equal variances were assumed for the analysis.

Factor Information

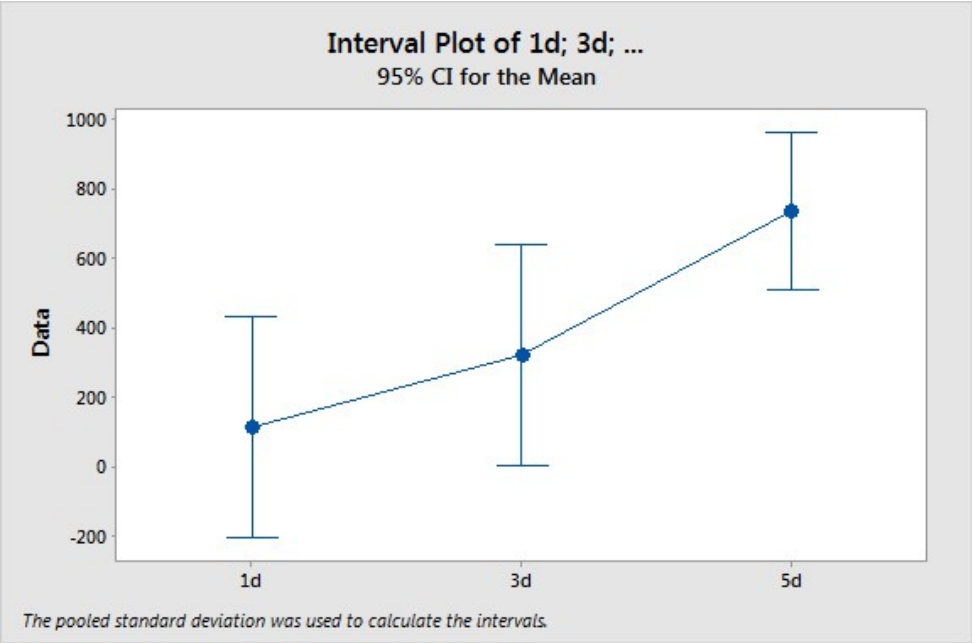
Factor	Levels	Values
Factor	3	1d; 3d; 5d

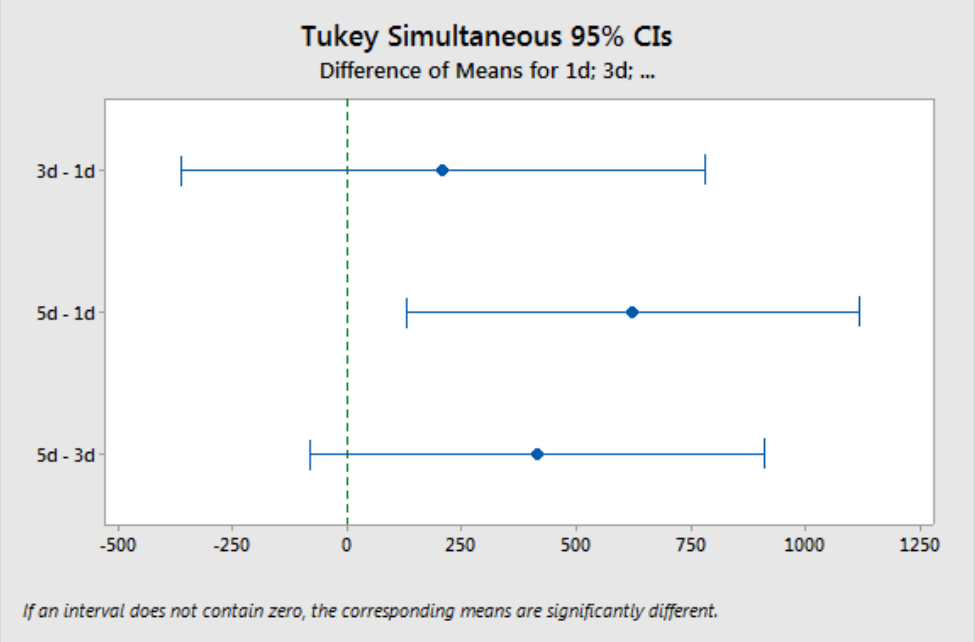
Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	579771	289886	9,40	0,020
Error	5	154209	30842		
Total	7	733980			

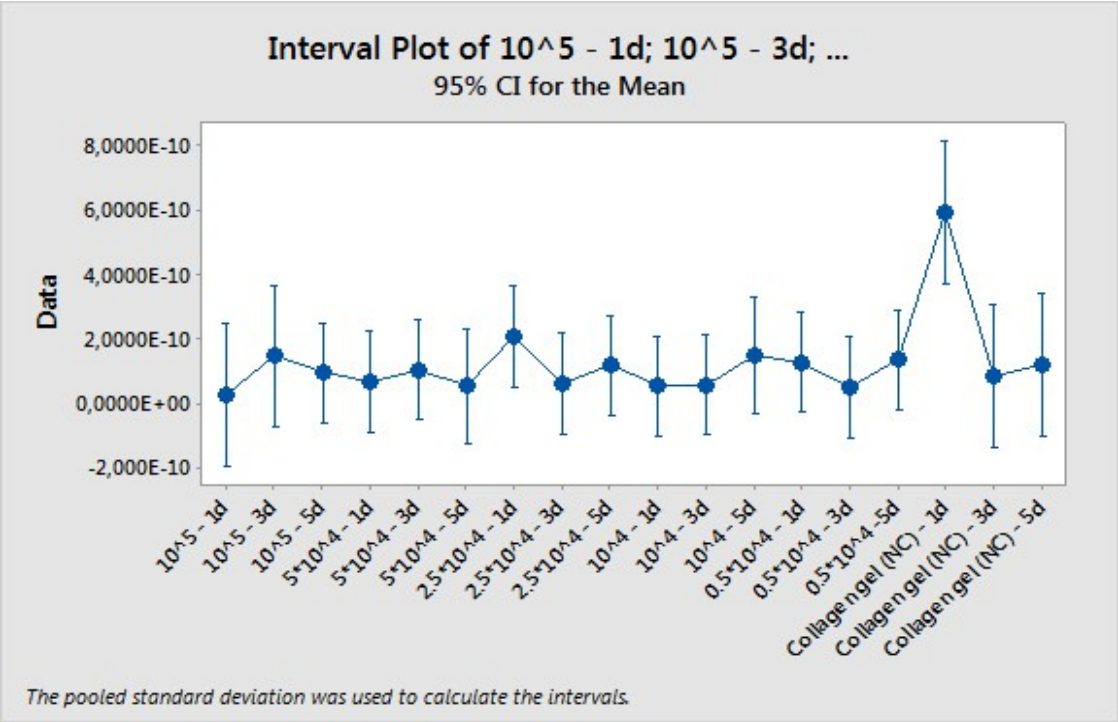
Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
175,618	78,99%	70,59%	57,09%





- Hydraulic Permeability (K_0) comparing all the cell densities and culture durations. The analysis reported significant difference between the 1-day culture of collagen gel without cells on it and 1-day culture of wells seeded with 10^5 and $2.5*10^4$ cells per cm^2.



Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Collagen gel (NC) - 1d	2	0,000000	A
2.5*10^4 - 1d	4	0,000000	A B
10^4 - 5d	3	0,000000	A B
10^5 - 3d	2	0,000000	A B
0.5*10^4 -5d	4	0,000000	A B
0.5*10^4 - 1d	4	0,000000	A B
Collagen gel (NC) - 5d	2	0,000000	A B
2.5*10^4 - 5d	4	0,000000	A B
5*10^4 - 3d	4	0,000000	A B
10^5 - 5d	4	0,000000	B
Collagen gel (NC) - 3d	2	0,000000	A B
5*10^4 - 1d	4	0,000000	B
2.5*10^4 - 3d	4	0,000000	B
10^4 - 3d	4	0,000000	B
10^4 - 1d	4	0,000000	B
5*10^4 - 5d	3	0,000000	B
0.5*10^4 - 3d	4	0,000000	B

Appendix B : Hepatocytes pictures and viability

The following pictures show the hepatocytes culture and viability reported as seen on the collagen gel after seeded.

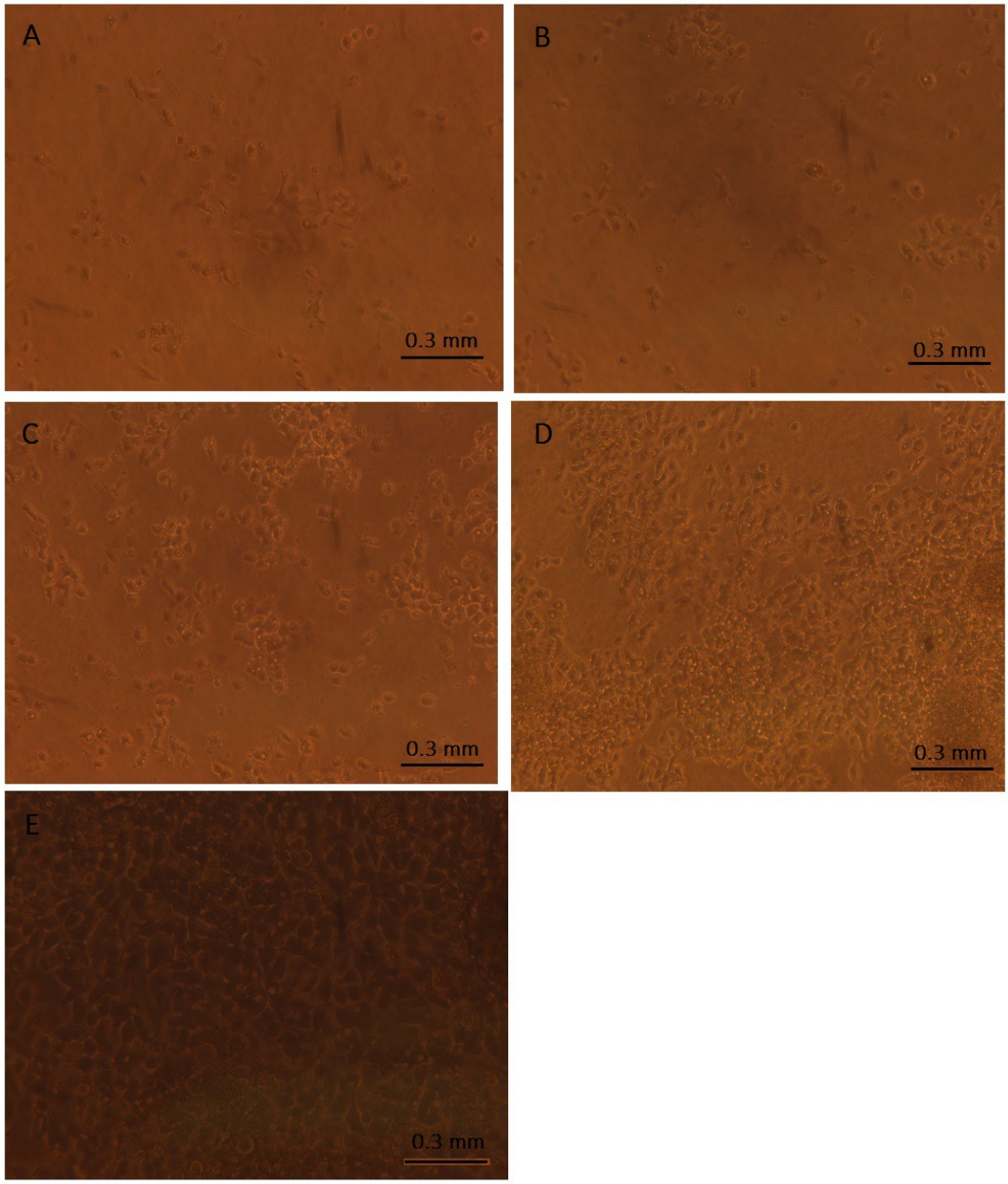


Fig 6.1 : Hepatocytes on collagen gel. 10X lens

The picture *[Fig 6.1]* shows the six different hepatocytes densities analysed with a

X10 lens: from A to E the concentrations are respectively $0.5*10^4$, 10^4, $2.5*10^4$, $5*10^4$ and 10^5 cells per cm^2. The pictures have been taken one day after seeded the wells.

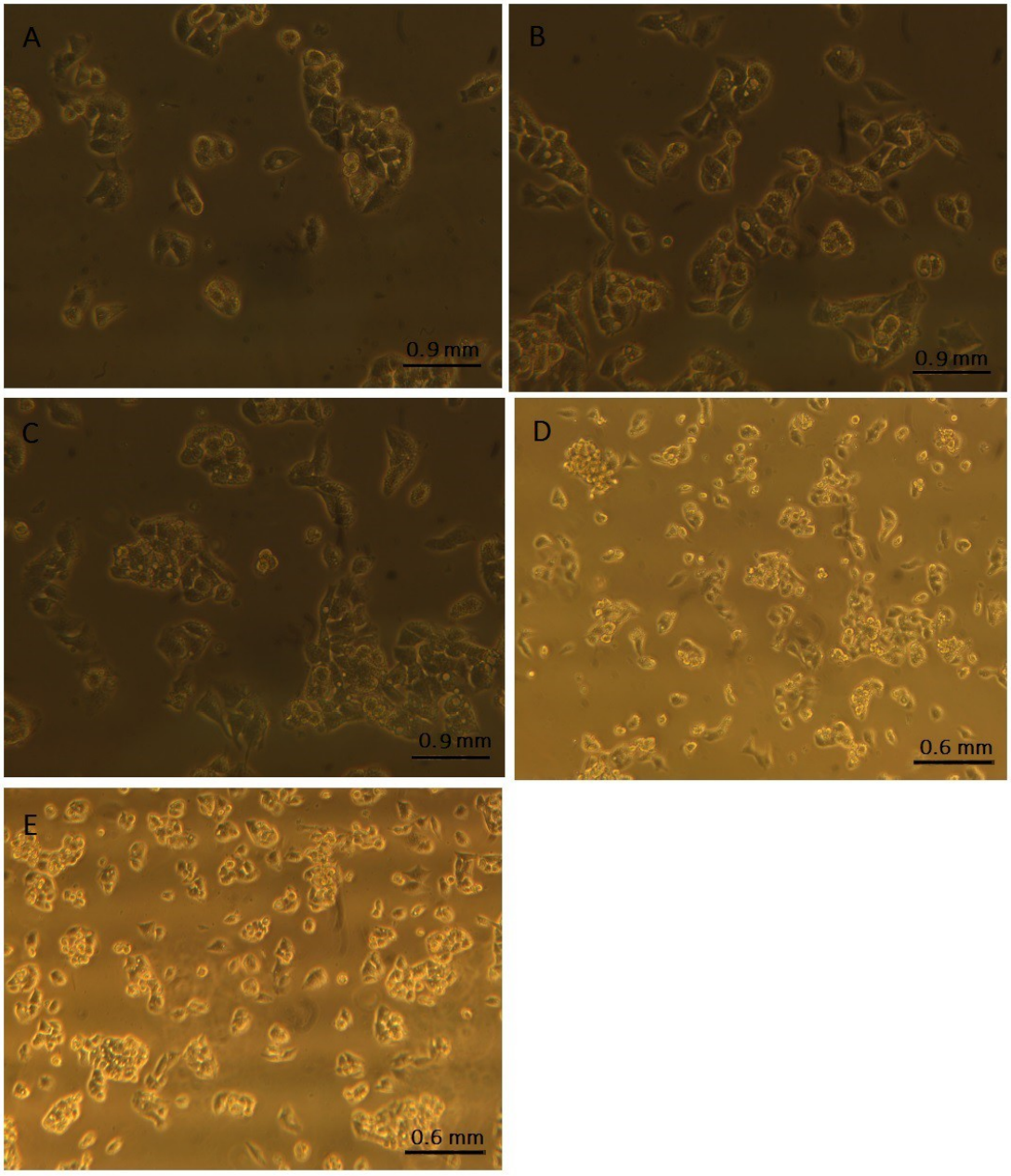


Fig 6.2 : Hepatocytes on collagen gel. 20X and 30X lenses

The figure *[Fig 6.2]* shows the six different hepatocytes densities analysed with a X20 and X30 lenses: from A, B and C correspond to $0.5*10^4$, 10^4 and $2.5*10^4$ cells per cm^2

with the X30 lens, whereas D and E show the wells with $5*10^4$ and 10^5 cells per cm^2 seeded on it, taken with X20 lens. The pictures have been taken one day after seeded the wells.

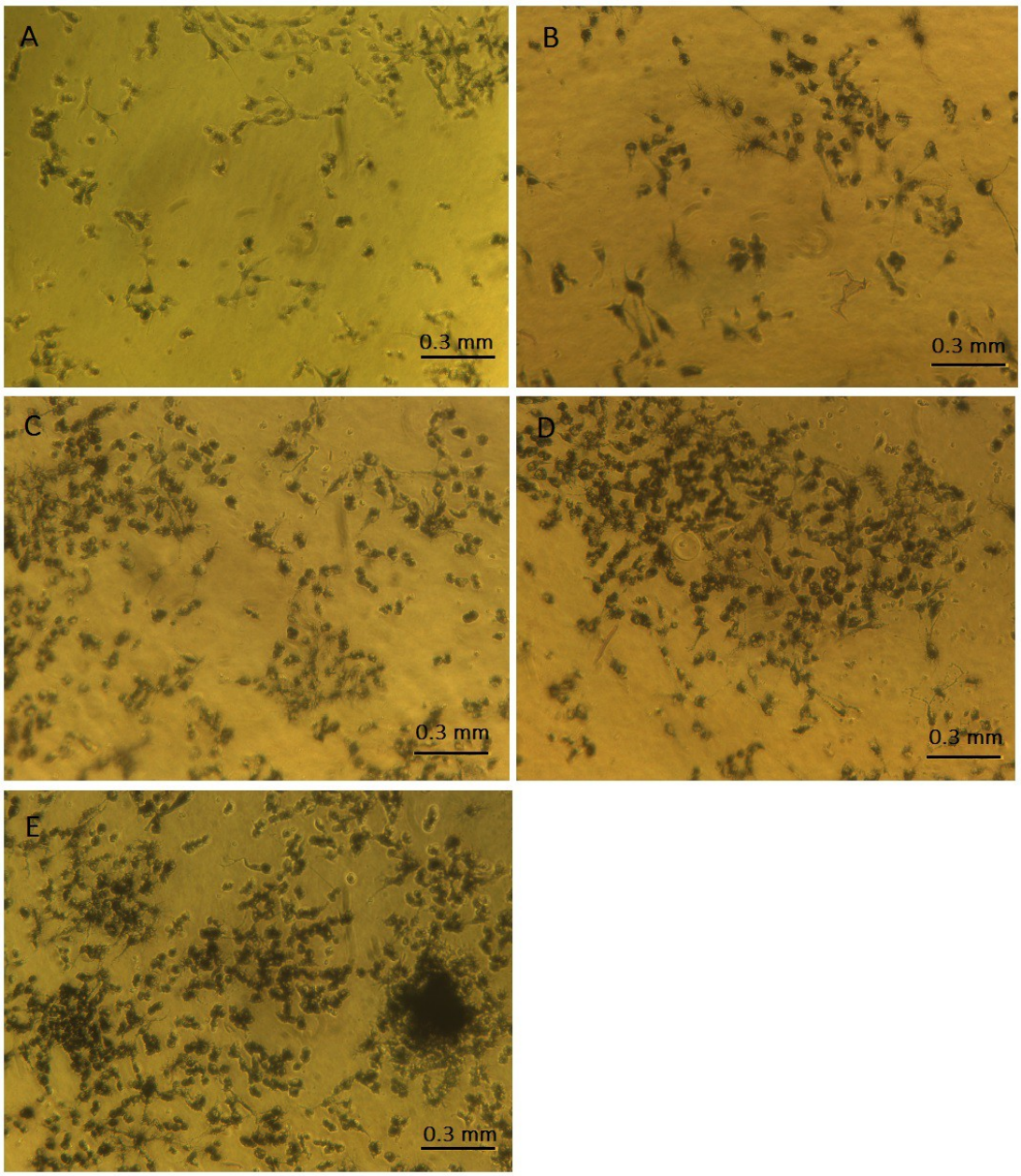


Fig 6.3 : Hepatocytes on collagen gel. Viability tested with MTT assay. 10X lens

The figure *[Fig 6.3]* shows the six different hepatocytes densities analysed with a X10

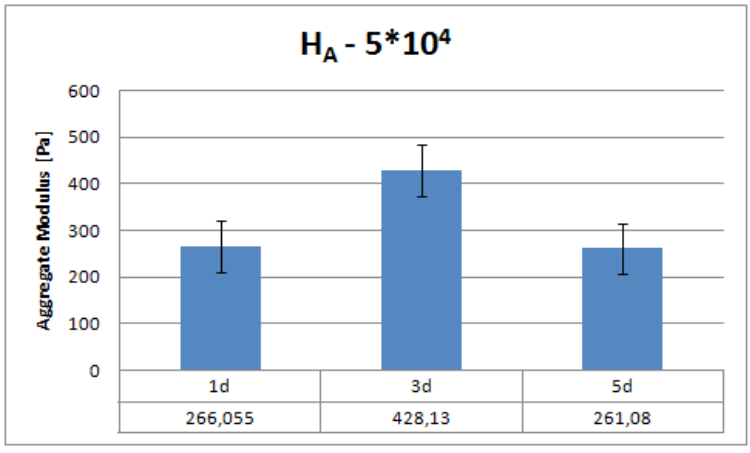
lens, as they appear when their viability is tested with MTT assay. The have been conducted few hours before the cells were tested with BOSE *ElectroForce* machine; the culture durations of the wells were respectively 1 day for the pictures A, B and C and 3 days for the pictures D and E.

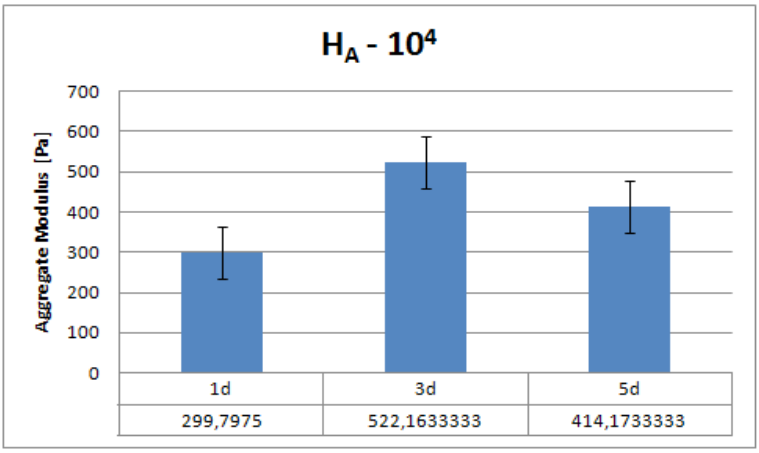
Appendix C : Data histograms

As follows, all the histograms obtained from the comparisons between the means of all the samples.
Missing the graphs reported in the fourth chapter.

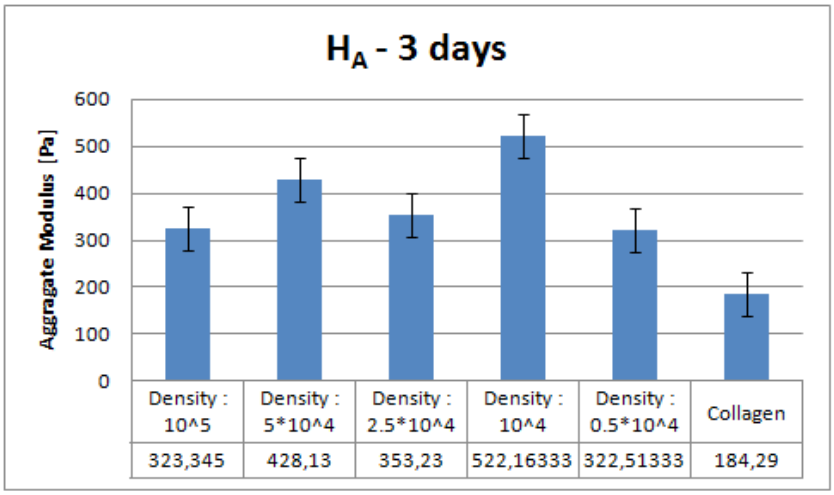
THE AGGREGATE MODULUS

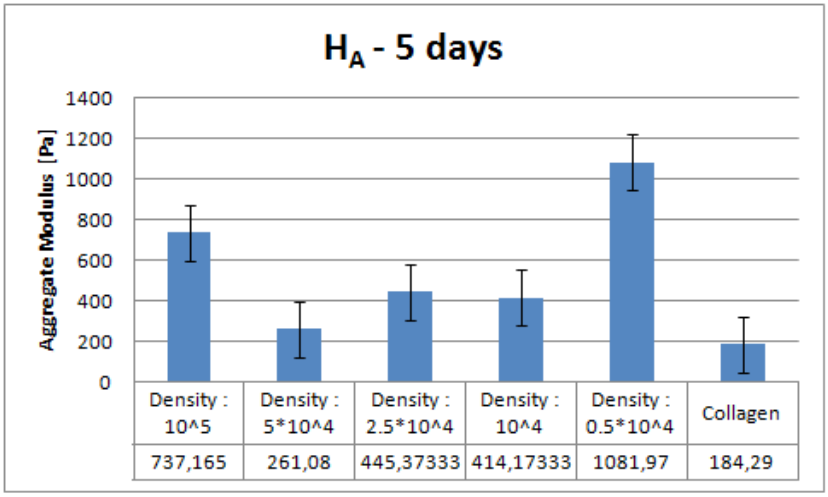
- Culture duration comparison for the same day concentration



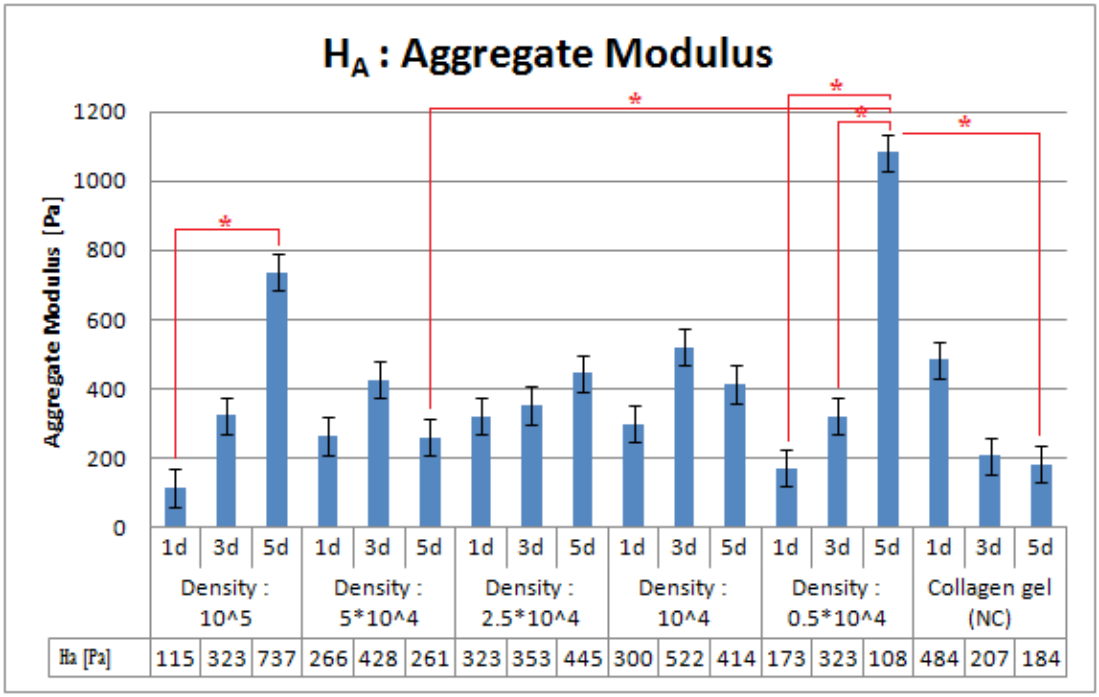


- Comparison between cell densities for the same culture duration



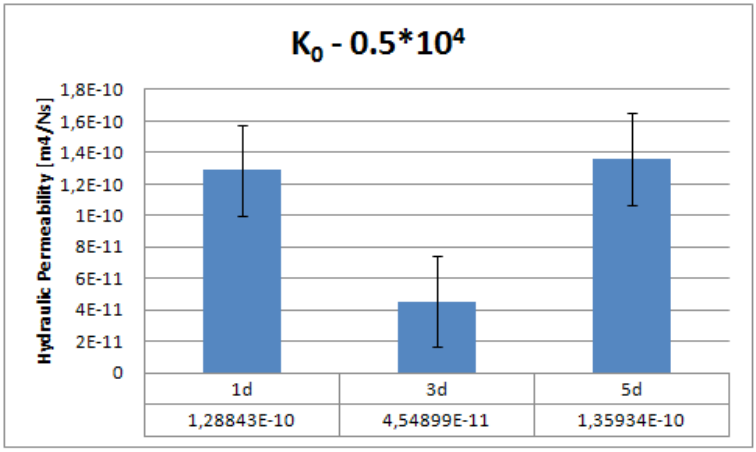


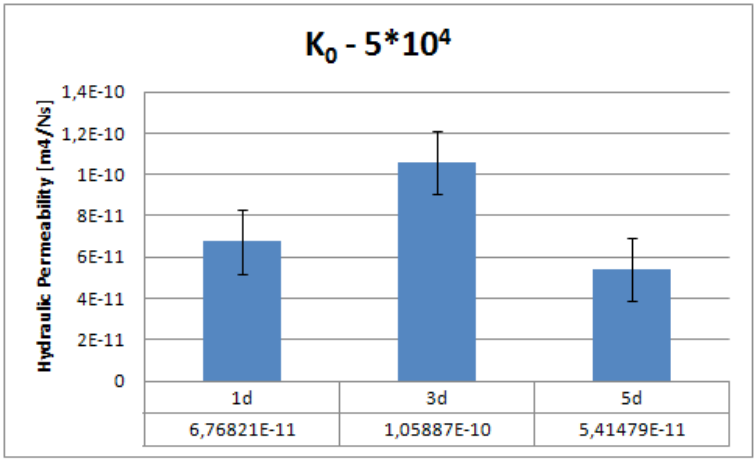
- Overview of all the comparisons

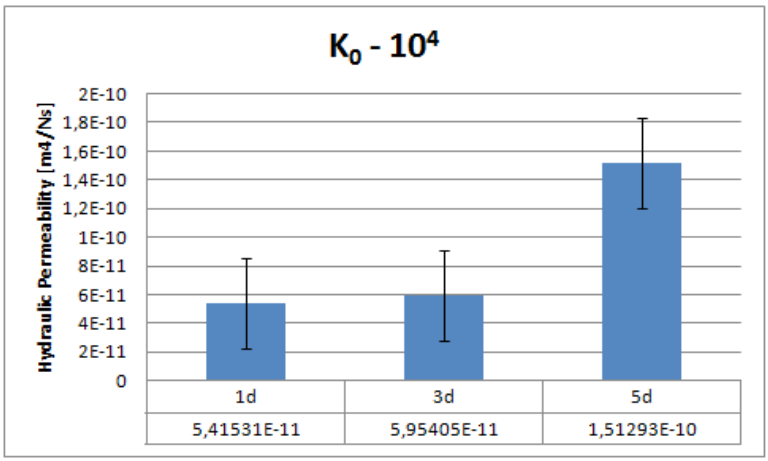


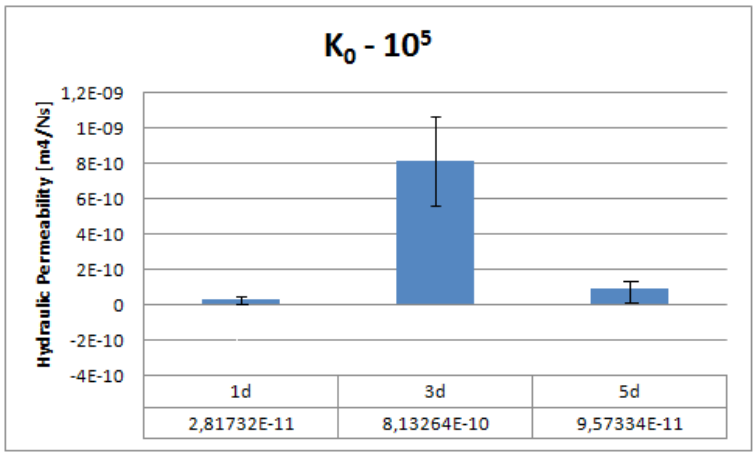
THE HYDRAULIC PERMEABILITY

- Culture duration comparison for the same day concentration

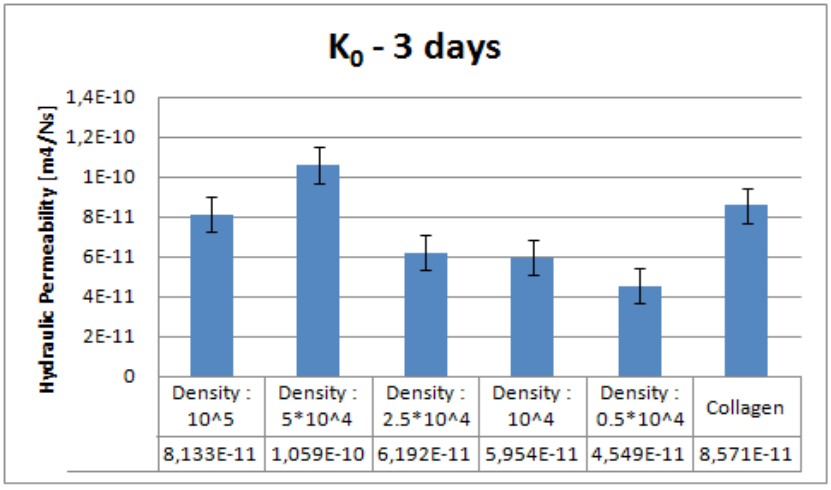


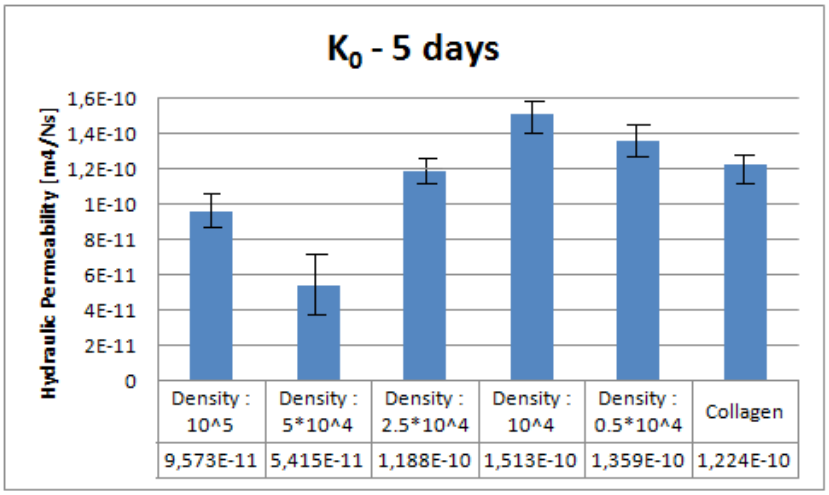






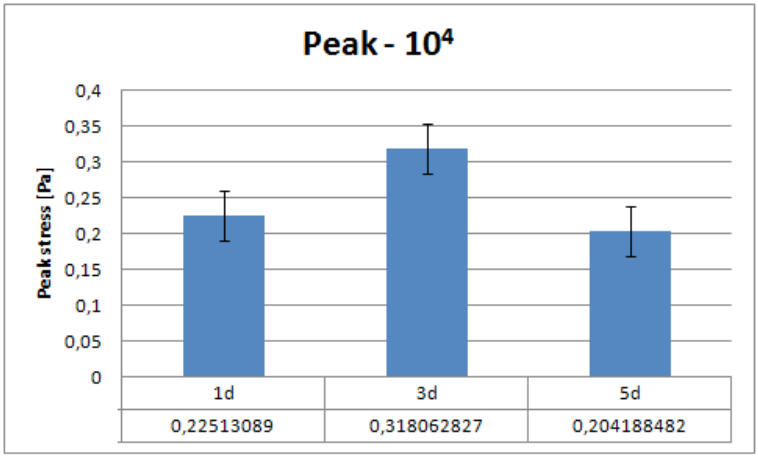
- Comparison between cell densities for the same culture duration



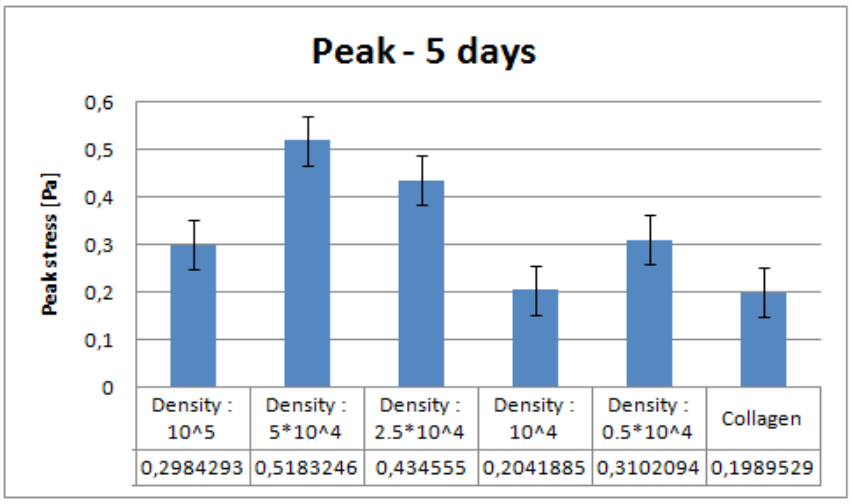


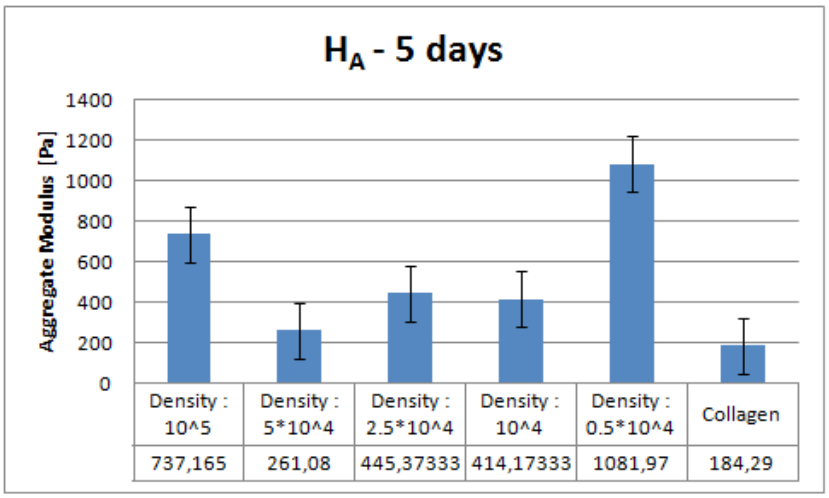
THE PEAK STRESS

- Culture duration comparison for the same day concentration

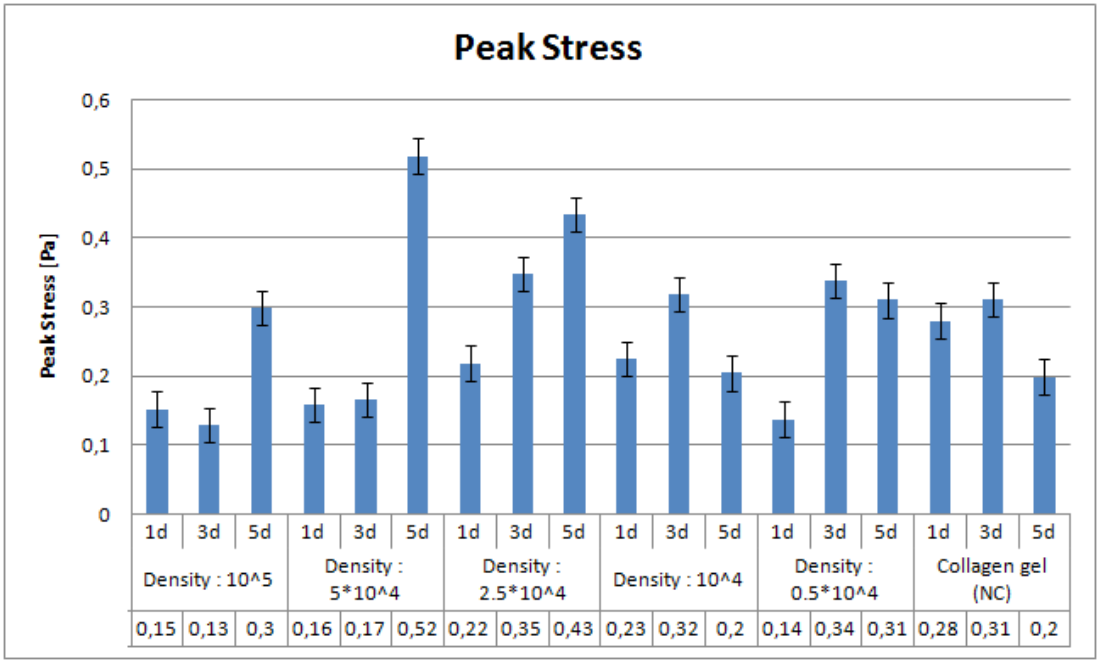


- Comparison between cell densities for the same culture duration



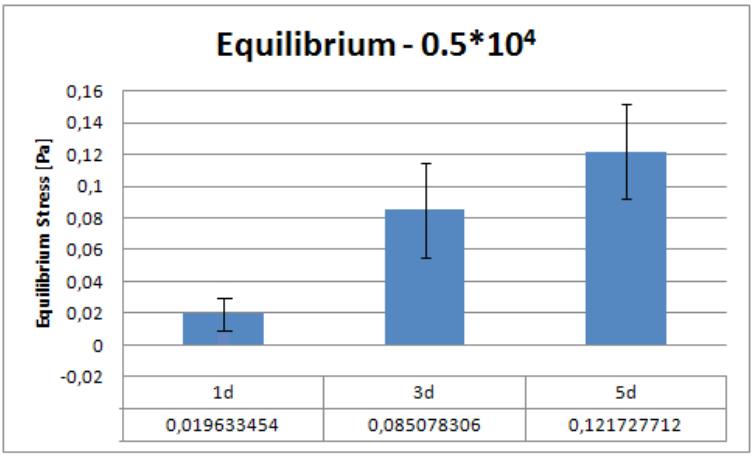


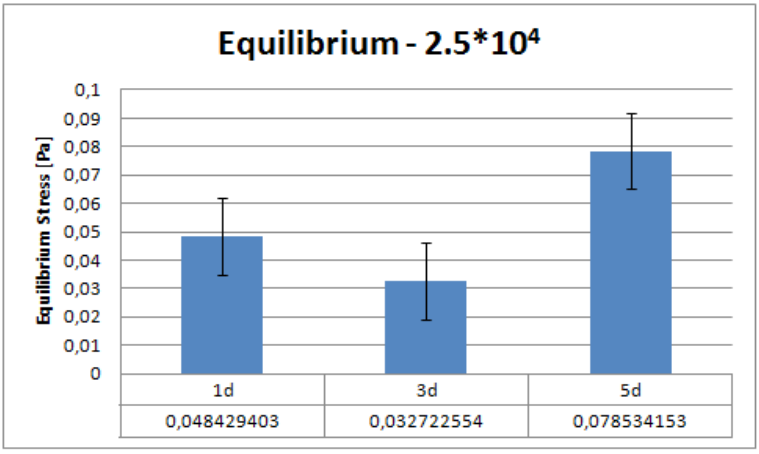
- Overview of all the comparisons

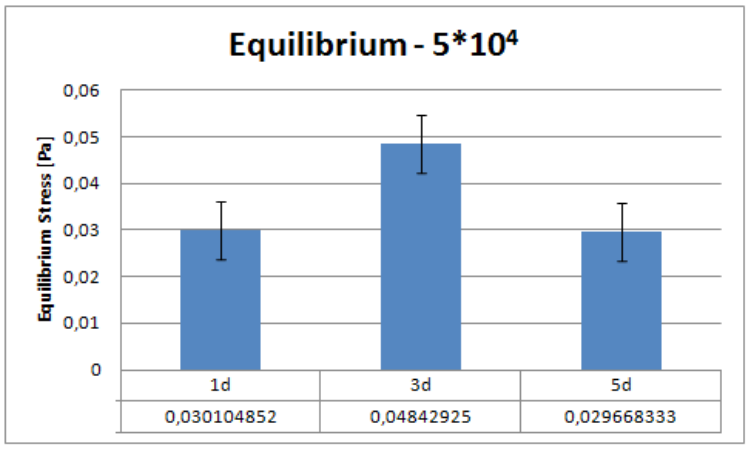


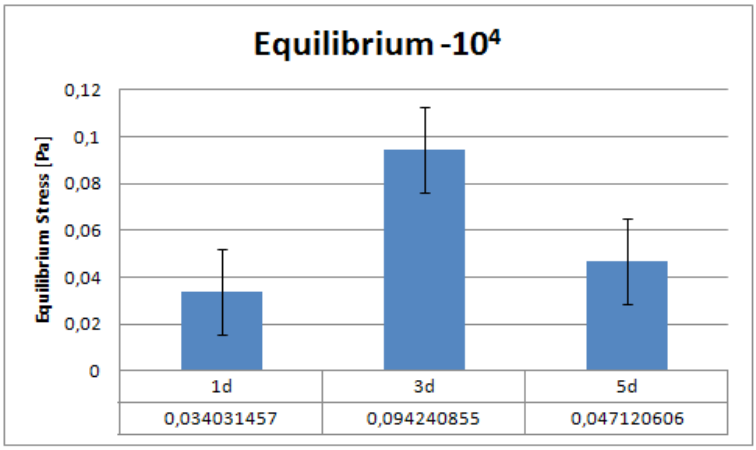
THE EQUILIBRIUM STRESS

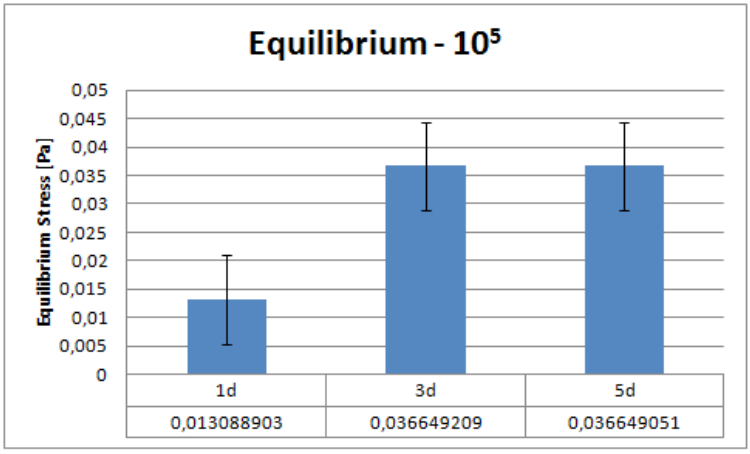
- Culture duration comparison for the same day concentration

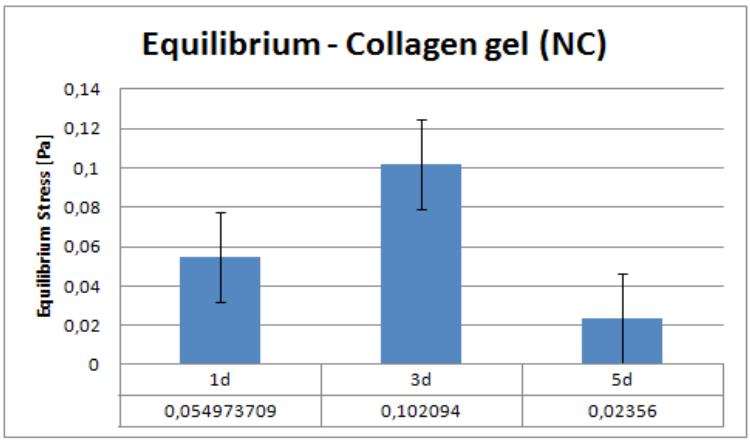



Equilibrium - 2.5*10⁴
Equilibrium Stress [Pa]
0,1
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
1d
3d
5d
0,048429403
0,032722554
0,078534153

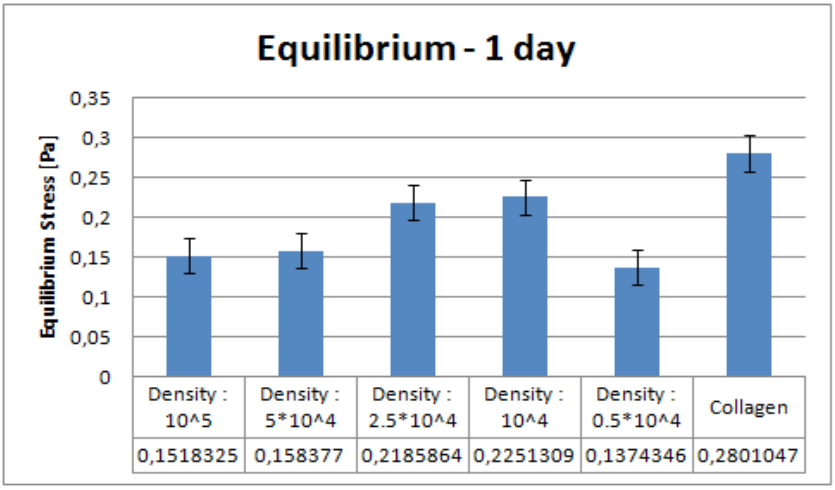

Equilibrium - 5*10⁴
Equilibrium Stress [Pa]
0,06
0,05
0,04
0,03
0,02
0,01
0
1d
3d
5d
0,030104852
0,04842925
0,029668333

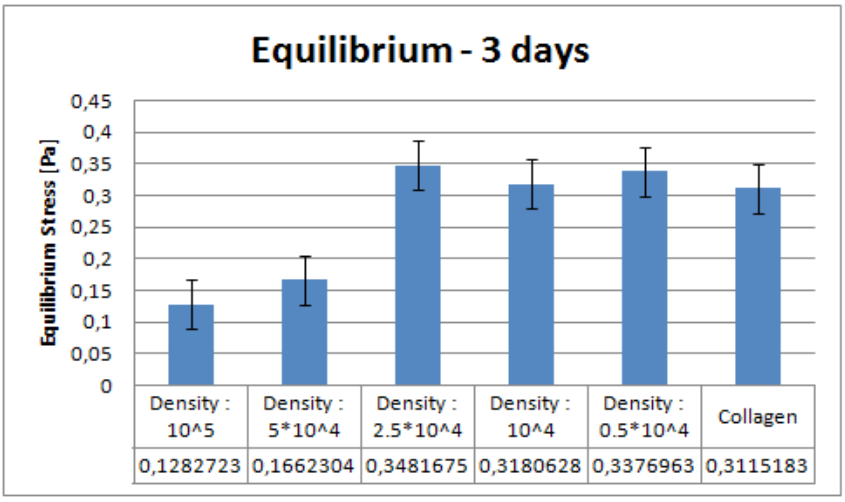

Equilibrium -10⁴
Equilibrium Stress [Pa]
0,12
0,1
0,08
0,06
0,04
0,02
0
1d
3d
5d
0,034031457
0,094240855
0,047120606

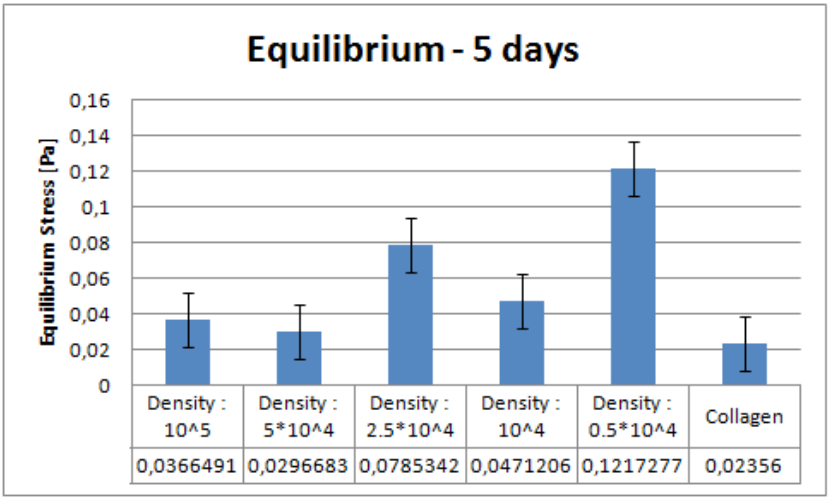




- Comparison between cell densities for the same culture duration

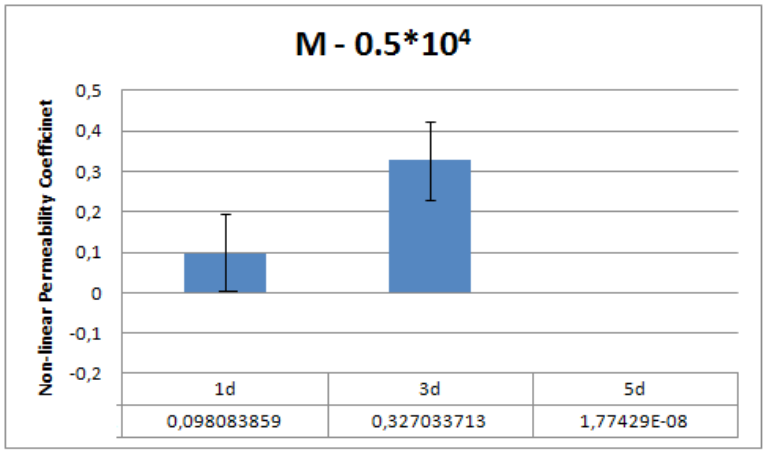


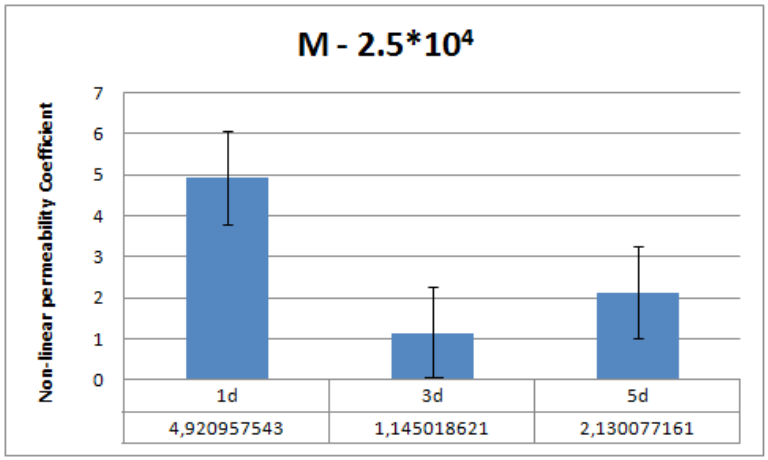


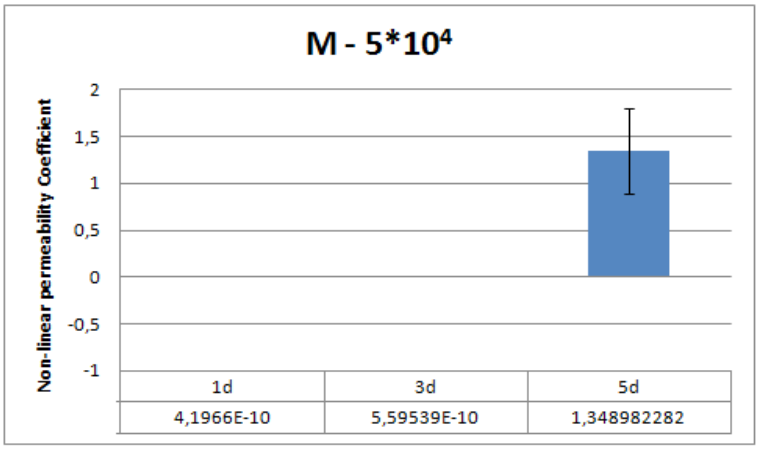


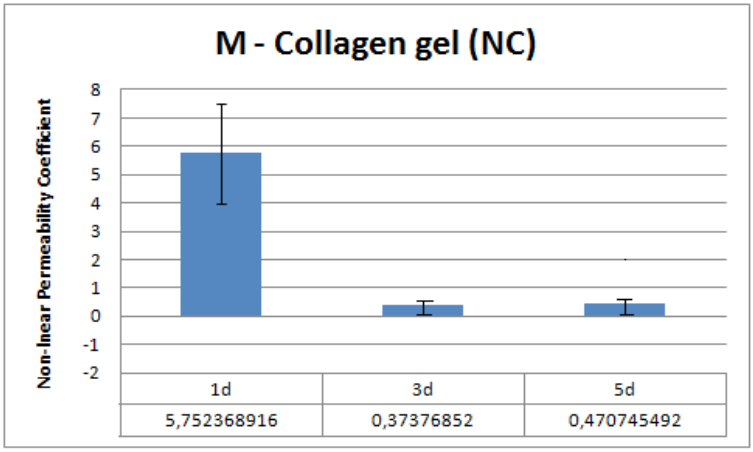
THE NON-LINEAR PERMEABILITY COEFFICIENT

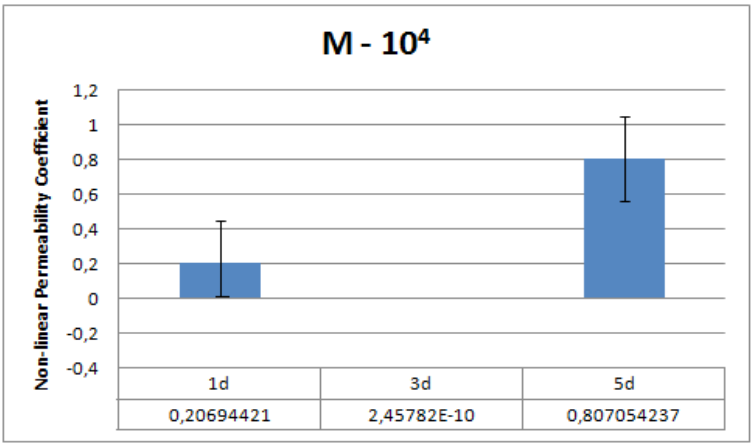
- Culture duration comparison for the same day concentration

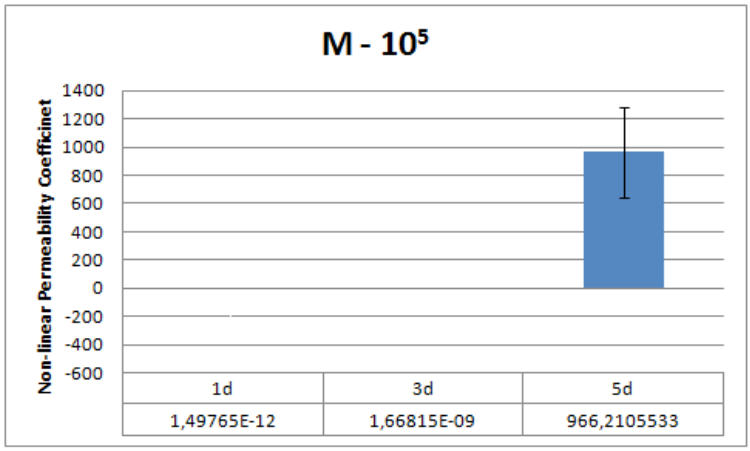




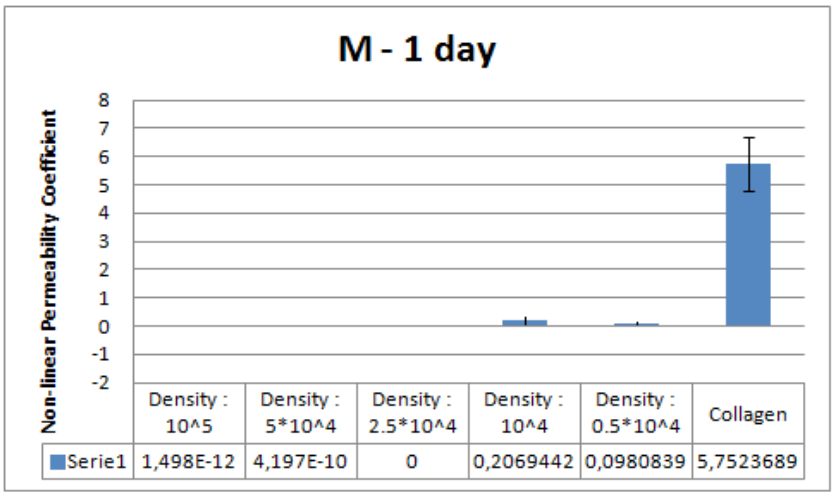


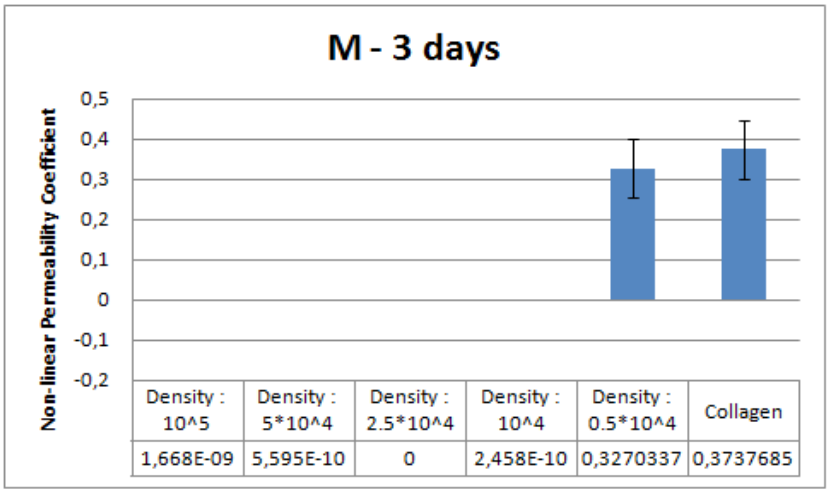

M - Collagen gel (NC)
Non-linear Permeability Coefficient
8
7
6
5
4
3
2
1
0
-1
-2
1d
3d
5d
5,752368916
0,37376852
0,470745492

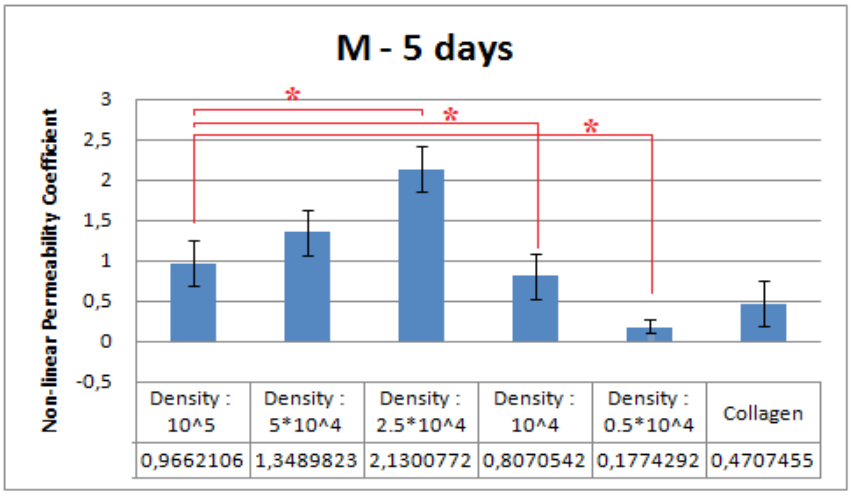

M - 10⁴
Non-linear Permeability Coefficient
1,2
1
0,8
0,6
0,4
0,2
0
-0,2
-0,4
1d
3d
5d
0,20694421
2,45782E-10
0,807054237


M - 10⁵
Non-linear Permeability Coefficinet
1400
1200
1000
800
600
400
200
0
-200
-400
-600
1d
3d
5d
1,49765E-12
1,66815E-09
966,2105533

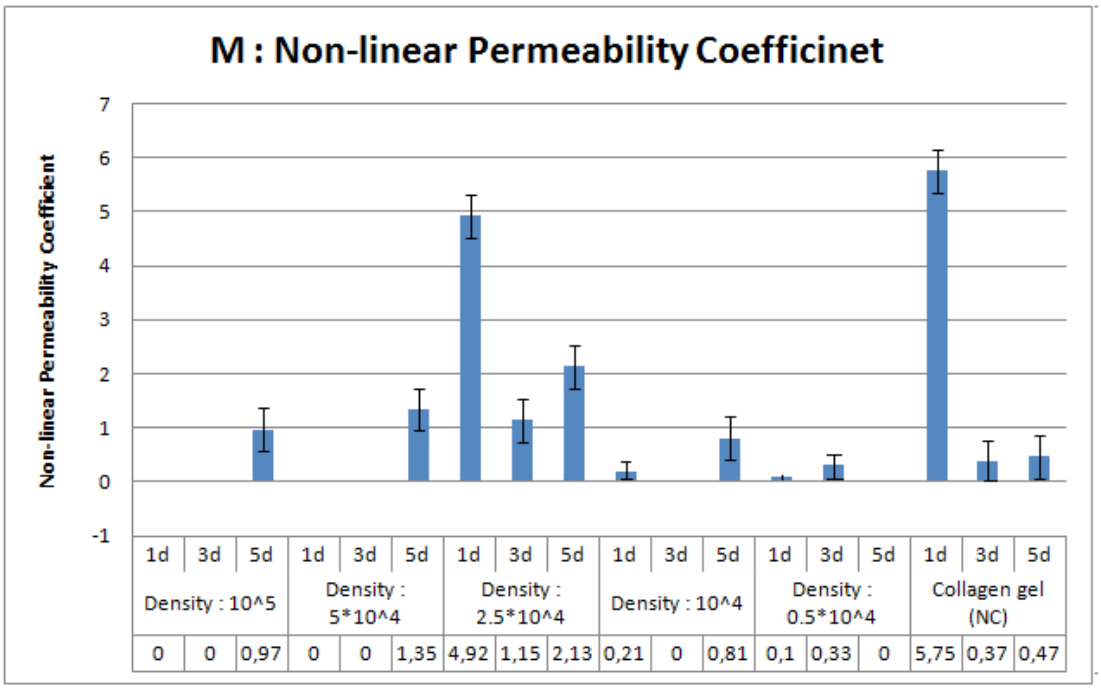
- Comparison between cell densities for the same culture duration







- Overview of all the comparisons



Appendix D : The Biphasic Model data

DATA OBTAINED AND ANALYSED WITH MATLAB SOFTWARE:

		Ha	k	M	r^2	peak	equilibrium
10^5	1d						
		92.15	2.48E-011	2.99E-012	0.68050698	0.125654	0.010471
		138.22	3.15E-011	1.23E-015	0.74140362	0.17801	0.015706806
	3d						
		230.37	1.49E-010	1.13E-009		0.104712	0.026178
		416.32	1.48E-009	2.21E-009	0.58634966	0.151832	0.047120419
	5d	552.87	8.79E-011	1.82E+003	0.9063578	0.335079	0.062827
		921.46	3.17E-011	8.30E+002	0.94946628	0.319372	0.010471204
		921.46	4.84E-011	1.21E+003	0.965515	0.387435	0.010471
		552.87	2.15E-010	4.33E+000	0.7503636	0.151832	0.062827
5*10^4	1d	276.44	4.99E-011	8.47E-013	6.86E-001	0.204188	0.031414
		230.37	5.43E-011	2.22E-011	0.76515884	0.17801	0.026178
		188.82	6.88E-011	8.68E-012		0.109948	0.020942408
		368.59	9.76E-011	1.65E-009	0.54617165	0.141361	0.041885
	3d	230.37	7.07E-011	1.34E-010	0.7428113	0.204188	0.026178
		506.81	1.20E-010	2.04E-009	0.62979444	0.162304	0.057592
		650.23	1.35E-010	3.34E-012	0.55412546	0.162304	0.073298
		325.11	9.84E-011	5.65E-011	0.54803673	0.136126	0.036649
	5d	322.51	1.09E-010	2.23E+000	0.95175229	0.251309	0.036649
		276.44	3.57E-011	7.62E-001	0.94410774	0.366492	0.031414
		184.29	1.73E-011	1.06E+000	0.93205129	0.937173	0.020942
2.5*10^4	1d	230.37	7.79E-011	2.18E+000	0.94086114	0.361257	0.026178
		460.73	1.42E-010	1.19E+001	0.62778597	0.115183	0.052356
		276.44	5.56E-010	2.87E-001	0.91693292	0.26178	0.031413613
		737.17	5.59E-010	5.32E+000	0.8899084	0.136126	0.08377
	3d	230.37	7.74E-011	2.32E+000	0.90606747	0.439791	0.02617801
		322.51	7.90E-011	2.52E-010	0.88811261	0.188482	0.036649
			4.00E-011	9.69E-001	0.9864686	0.308901	0.010471204
		506.81	5.12E-011	1.29E+000	0.84533736	0.455497	0.057592
	5d	598.95	3.60E-010	7.26E+000		0.256545	0.068063
		276.44	2.32E-011	2.37E-001	0.92953618	0.408377	0.031413613
		460.73	1.24E-011	1.02E+000	0.85643512	0.712042	0.052356
			7.93E-011	2.04E-010		0.361257	0.162304
10^4	1d	138.77	4.47E-011	8.28E-001	0.97280518	0.251309	0.015707
		185.03	7.59E-011	9.47E-012	0.61292725	0.125654	0.020942
		276.44	3.42E-011	1.01E-012	0.91371379	0.282723	0.031414
		598.95	6.19E-011	6.75E-011	0.68520723	0.240838	0.068062827
	3d	184.29	6.17E-011	2.45E-012	0.75949397	0.151832	0.020942
		414.66	9.37E-011	4.58E-013	0.84125363	0.204188	0.047120419
			4.37E-011	9.67E-010		0.502618	0.198953
		967.54	3.91E-011	1.36E-011	0.67029478	0.413613	0.109948
	5d						
		367.12	9.27E-011	2.42E+000	0.93568284	0.329843	0.041884817
		506.81	1.79E-010	7.01E-010	0.68835653	0.151832	0.057592
		368.59	1.82E-010	2.04E-008	0.82596999	0.13089	0.041885
0.5*10^4	1d	138.22	2.85E-011	3.92E-001	0.97279819	0.246073	0.015707
		322.51	6.62E-011	5.16E-013	0.74865313	0.193717	0.036649215
		46.07	1.69E-010	2.84E-013	0.86584437	0.041885	0.005235602
		184.29	2.51E-010	5.82E-010	0.74140362	0.068063	0.020942
	3d	414.66	5.25E-011	3.72E-012	0.73587932	0.267016	0.04712
		322.51	4.43E-011	1.31E+000	0.98508187	0.371728	0.036649215
			6.88E-011	7.83E-010	0.6266437	0.429319	0.230366
		230.37	3.97E-011	5.66E-012	0.92142497	0.282723	0.02617801
	5d	1474.35	7.91E-011	9.49E-010	0.62872274	0.34555	0.167539
		1793.84	3.94E-011	5.37E-008	0.63873843	0.628272	0.198953
		552.88	2.32E-010	2.49E-009	0.64777231	0.125654	0.062827225
		506.81	1.93E-010	1.39E-008	0.58252217	0.141361	0.057591623
Collagen	1d	414.66	2.60E-011	6.62E-002	0.90924961	0.429319	0.047120419
		552.88	1.16E-009	1.14E+001		0.13089	0.062827
	3d	184.29	5.54E-011	7.48E-001	0.9424906	0.251309	0.020942
			1.16E-010	3.59E-010		0.371728	0.183246
	5d	184.29	3.29E-011	2.54E-003	0.92443846	0.293194	0.020942
		230.37	2.12E-010	9.39E-001	0.96766517	0.104712	0.026178

MEANS ON THE DATA OBTAINED:

MEANS		Ha	k	M	r^2	peak	equilibrium
Density : 10^5	1d	115.185	2.8173155E-011	1.4976479E-012	0.7109553	0.15183246	0.0130889031
	3d	323.345	8.1326406E-010	1.6681520E-009	0.58634966	0.12827225	0.0366492094
	5d	737.165	9.5733388E-011	0.9662105533	0.89292567	0.29842932	0.036649051
Density : 5*10^4	1d	266.055	6.7682111E-011	4.1966033E-010	0.6657094133	0.15837696	0.0301048521
	3d	428.13	1.0588665E-010	5.5953898E-010	0.6186919825	0.16623037	0.04842925
	5d	261.08	5.4147856E-011	1.3489822823	0.9426371067	0.51832461	0.0296683333
Density : 2.5*10^4	1d	322.5133333	5.29E-11	4.9209575425	0.8438721075	0.21858639	0.0484294031
	3d	353.23	6.1920457E-011	1.1450186211	0.90649651	0.34816754	0.0327225537
	5d	445.3733333	1.1876040E-010	2.1300771606	0.89298565	0.43455497	0.0785341531
Density : 10^4	1d	299.7975	5.4153092E-011	0.2069442098	0.7961633625	0.22513089	0.0340314568
	3d	522.1633333	5.9540543E-011	2.4578176E-010	0.7570141267	0.31806283	0.0942408547
	5d	414.1733333	1.5129252E-010	0.8070542371	0.8166697867	0.20418848	0.0471206056
Density : 0.5*10^4	1d	172.7725	1.2884348E-010	0.0980838591	0.8321748275	0.13743455	0.0196334542
	3d	322.5133333	4.5489856E-011	0.3270337127	0.817257465	0.33769634	0.0850783063
	5d	1081.97	1.3593425E-010	1.7742918E-008	0.6244389125	0.31020942	0.121727712
Collagen gel (NC)	1d	483.77	5.94E-10	5.7523689157	0.90924961	0.280104712	0.0549737094
	3d	207.33	8.5713345E-011	0.3737685202	0.9424906	0.31151832	0.102094
	5d	184.29	1.2239248E-010	0.4707454921	0.946051815	0.19895288	0.02356