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Influence of oxygen mass transport on bioreactors for Tissue Engineering
(Influencia del transporte de materia de oxígeno en biorreactores aplicados a Ingeniería Tisular)

Para acceder al Título de

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Me gustaría mostrar mi agradecimiento, en primer lugar, a mi directora de proyecto Nazely Diban, por su tiempo, dedicación, ayuda y, sobre todo, su cercanía; así como por darme la oportunidad de desarrollar este trabajo.

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Mª del Mar Hervella Baturone

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1. INTRODUCTION

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1.1. Tissue Engineering

The loss or failure of an organ or tissue is one of the most harmful and costly problems in human health care. Also, there is scarcity of compatible donors and rejections are often limiting the success transplantation. For these reasons a new field, tissue engineering (TE), applies the principles of biology and engineering to the development of functional substitutes for damaged tissue [1]. Therefore, constructing *in vitro* a replacement of engineered tissue is considered an excellent alternative to overcome the problems associated to direct transplantation of donor organs and, consequently, a large number of research groups focus on TE [2, 3].

The replacement of organs since long has been the subject of debate, however, the field of engineering tissue *in vitro* to repair damaged tissue *in vivo* originated only about two decades ago [2, 3]. The term TE was used for the first time at a meeting of the National Science Foundation (NSF) at Granlibakken, Lake Tahoe, California, in 1987. However, it was widely accepted in 1993, with the publication of an article in the journal Science [1].

TE is defined as the interdisciplinary field applying the principles and methods of engineering and life sciences to fundamentally understand and develop biological substitutes to restore, maintain or improve tissue functions [1]. Basically, TE attempts to create artificial tissue that mimic the function of natural tissue. To optimize the evolution of functional biological substitutes, the natural circumstances of the specific tissue have to be fundamentally understood [2].

Biological tissues basically consist of cells, signaling systems and extracellular matrix (ECM). The cells are the core of the tissue, nevertheless, cannot function in the absence of signaling systems and/or of the ECM [2].

Accordingly, TE approaches can generally be sub-divided based on these two procedures, either studied single or combined [2]:

- Cell-based therapies
- Biocompatible support (scaffold)

In this study only the last procedure, a biocompatible support or scaffold, is used.

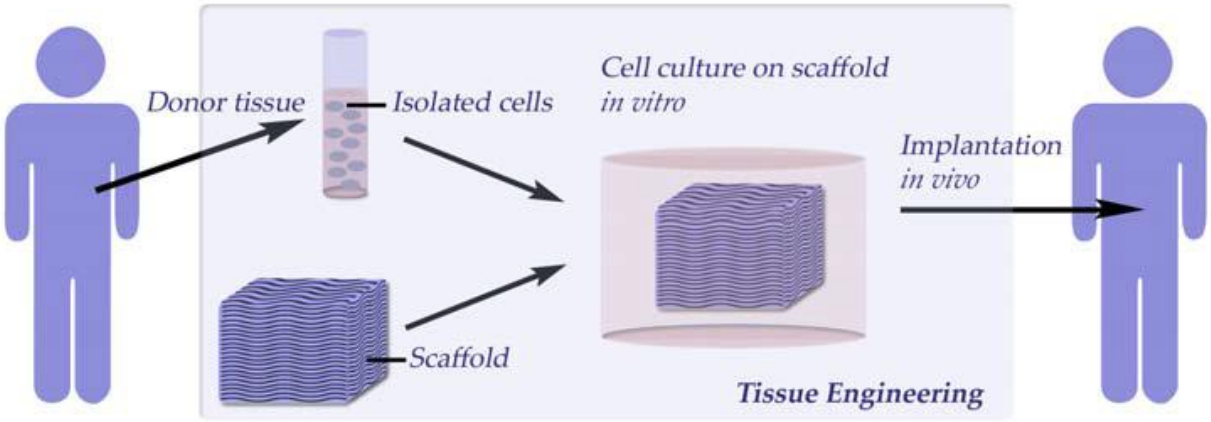


Figure 1 Schematic illustration of the tissue engineering principle [2]

Figure 1 illustrates the basic principle of the general TE approach based on the introduction of a scaffold. Cells are isolated from either the patient (autogenic) or from a donor (allogenic). After isolation, the cells are introduced to a scaffold and cultured *in vitro* in a bioreactor. Finally, the cell-cultured scaffold is implanted *in vivo* into the patient [2].

Many aspects in this process contribute to the final suitability and functionality of the tissue engineered construct. Isolation of the cells and *in vitro* culturing requires optimal processing and environmental conditions, e.g. pH, temperature, medium composition. Each type of tissue requires distinct conditions and therefore, demands the understanding of the specific natural biological environment *in vivo* to allow optimization of culturing *in vitro* [2]. The control and stablishment of the required conditions *in vitro* is achieved by means of bioreactors for cell culture.

1.2. Tissue Engineering Bioreactors

The term “bioreactor” refers to a system in which conditions are closely controlled to permit or induce certain behaviour in living cells or tissues. The concept of bioreactors is neither new nor restricted to TE. Microbiologists use bioreactors to grow cultures of microorganisms under defined conditions. Bioreactors are also used in the brewing, food, pharmaceutical and biotechnology industries. The fact that physical stimuli can modulate cell function and tissue development has motivated the development of biomechanically active simulation systems to recellularise tissues *in vitro* by exposing them to physiologically relevant mechanical and/or hydrodynamic stimulation. Bioreactor technologies intended for TE can be used to grow functional cells and tissues for transplantation, and for controlled *in vitro* studies on the regulation effect of biochemical and biomechanical factors on cell and tissue development [4].

The requirements for a TE bioreactor will vary depending on the dimensions, complexity, and physiological environment of the tissue to be engineered [4]. However, its design should be as simple as possible.

The overall goal is to have systems that reliably and reproducibly form, store, and deliver functional tissues that can sustain function *in vivo*. In essence, the bioreactor needs to provide the appropriate physical stimulation to cells, continuous supply of nutrients (e.g. glucose, amino acids), biochemical factors and oxygen, diffusion of chemical species to the inner construct, as well as continuous removal of by-products of cellular metabolism (e.g. lactic acid). Moreover, such a bioreactor has to be able to operate over long periods of time under aseptic conditions since maturation of a functional tissue may take up to 3-4 months [4].

In general, these biological reactors are designed to perform the following five functions: 1) providing uniform cell distribution, 2) maintaining the desired concentration of gases and nutrients in the medium, 3) providing mass transport to the tissue, 4) exposing tissue to physical stimuli, or 5) providing information about the formation of three-dimensional (3D) artificial tissue [5].

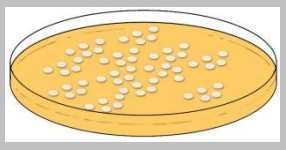
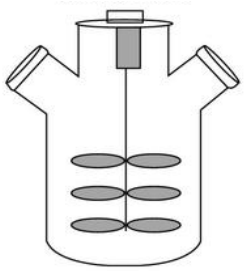
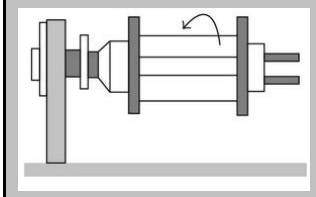
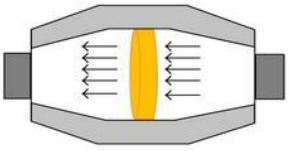
Currently, the most widely used bioreactors in TE are static and mixed flasks, rotating wall and perfusion bioreactors (Table 1). These bioreactors offer three distinct flow conditions (static, turbulent, and laminar), and hence a different rate of nutrient supply to the surface of tissue construct. They also differ in mass transfer and shear stress rates experienced by the cultured cells. Table 1 compares engineering parameters of different TE bioreactors [6].

Although static culture is simply designed and operated, there are nutrient diffusion limitations with large constructs since both external and internal mass transfers are undertaken by diffusion. This situation can be improved by the use of stirred flask bioreactors. However, in order to reduce the levels of shear stress experienced by cells on this system the tissue engineered can be cultivated in rotating wall bioreactors, but the limitations of the diffusional transfer of oxygen to the construct interior still remain [6].

Perfusion bioreactors, in which the culture medium is forced through the pores of solid porous 3D scaffolds, is superior compared to other nonperfusion or static bioreactors [7], thereby enhancing nutrient transport and providing mechanical stimuli to the cells.

In such systems, oxygen and nutrients are supplied inside the construct by both diffusion and convection. The flow rate can be optimized with respect to the limiting nutrient, which is mostly oxygen due to its low solubility in the culture medium. Perfusion bioreactors can offer greater control of mass transfer than other conventional systems but the potential for flow to follow a preferential path through the construct and the possible shear stress of the cells still remain common problems. The phenomenon of preferential path, particularly happens for scaffolds with a wide pore size distribution and nonuniformly developing tissues, leaving some regions poorly nourished, while others are perfused strongly [6].

Table 1 Comparison of engineering parameters in different TE bioreactors [6]

Bioreactor type	General descriptions	Mass transfer mechanism	Shear stress	Special usage	Considerations
Static culture 	Batch culture with no flow of nutrient	Diffusion (high)	Very low	Cell proliferation	Homogeneous structure of cell constructs and nutrient diffusion limitations
Stirred flasks 	Magnetically stirring of medium	Convection (high)	High	Dynamic seeding of scaffolds	Appropriate scaffold and balance between increasing mass transfer and modulating shear stresses
Rotating wall 	Rotating at a speed so the constructs in the reactor are maintained "stationary" in a state of continuous free fall	Convection (high)	Low	Tissue constructs which need dynamic laminar flow	Operating conditions (e.g., speed of rotating) especially for growing large tissue mass
Perfusion 	Flow of medium over or through a cell population or bed of cells	Convection (moderate) and diffusion (high)	Moderate	Tissues physico-chemical and environmentally relevant to human tissues	Seeding and attachment of human cells especially within the scaffold body

1.2.1 Scaffolds

As the membrane or scaffold is the most important part of a TE bioreactor, one of the main research lines in the field of TE focuses on scaffold design. A scaffold is a 3D construct which serves as temporary support for isolated cells to grow into a new tissue before transplantation back to the host [2].

As high nutrients and oxygen delivery through the scaffolds is necessary as a consequence of the requirements of nutrients by the cells in the bioreactor, the design of the scaffold becomes very important (i.e., improving the scaffold design will aid efficient mass transfer). Its design determines the functionality of the construct, that is, on the one hand, the structure and surface morphology on which the culture is performed have special influence on cell growth and tissue formation; on the other hand the porosity and pore interconnectivity affects the transport properties through the membrane and ultimately the nutrients accessibility to the cells. Although the final requirements are dependent on the specific purpose of the scaffold, several general characteristics need to be considered for all designs [3]. The scaffold should be biocompatible, biodegradable, bioactive and easy to connect to the vascular system of the host, and should have suitable mechanical strength, surface morphology and topography and, finally, good transport properties [2, 8]. This last requirement is important to ensure sufficient nutrient transport towards the cells and removal of waste products. In order to fulfill these properties the scaffold should be highly porous with good pore connectivity, however, it should maintain sufficient mechanical strength implying that the optimization of porosity is necessary.

The most used materials in scaffolds for TE are polymers, ceramics, bioactive glass (bioglass) and some composite materials and metals [8]. Among those, biodegradable synthetic polymers are the most used, because they show certain advantages over other materials, such as their ability to tailor mechanical properties and degradation kinetics to suit various applications [9]. Some of these are Poly(esters) as for instance Poly(lactic acid) (PLA) and Poly(Ɛ-caprolactones) [2].

1.2.2 Oxygen limitation and supply

Generally, the major mission of a bioreactor is to provide biomechanical stability and biochemical environment that facilitates the nutrient and oxygen transfer to the cells and removes the metabolic products from the cells. The size of most engineered tissues is limited as they do not have their own blood system and the cells are only nourished by diffusion. Since tissue constructs should have dimensions larger than 5 mm to become functional, mass transfer limitation can be considered as one of the greatest engineering challenges [6, 10].

After distributing cells throughout porous scaffolds, the key is the maintenance of cell viability, especially within the interior of the construct during prolonged culture. Nutrients, oxygen, and regulatory molecules have to be efficiently transferred from the bulk culture medium to the tissue surfaces (i.e., external mass transfer) as well as to the interior cells of the tissue construct (i.e., internal mass transfer). In addition, metabolites and CO_2 are to be removed from the cells within the tissue to the bulk medium [6].

Nevertheless, many studies conclude that oxygen supply appears to be one of the most important factors limiting tissue growth, due to poor solubility of oxygen in culture medium, compared to the high concentration of other nutrients, such as glucose [6, 11].

Adequate oxygen transport is vital to the success of a TE construct developed *in vitro*. If sufficient oxygen concentration is not maintained, cells will not survive and the device will fail.

Oxygen supply to cells depends on a variety of factors, including [11]:

1) The oxygen permeability of the membrane.
2) The oxygen consumption rate of the tissue.
3) The geometric characteristics of the implant device.
4) The tissue density and spatial arrangement of the cells or tissues.

The diffusive penetration depth of oxygen within tissues *in vitro* is in the range of only 100 to 200 µm, this means that diffusion alone is capable of providing enough oxygen for a ≈100 µm thick outer layer of a tissue construct, whereas the interior remains relatively acellular or becomes necrotic due to hypoxia [6, 10]. Thus, maintaining the balance between oxygen delivery to cells and their oxygen consumption is critical, considering this diffusive distance. Therefore, the oxygen adjustment is a critical matter in the design process of any bioreactor [6].

In applications related to TE, the oxygen demand will fluctuate each time. During the initial expansion stage, cell density increases with time, and consequently, the overall demand of oxygen also increases. Cells may change from a proliferative state to differentiation during the later stages of the culture. This change has implications for oxygen transfer, since proliferating cells typically have a higher oxygen demand per cell than differentiating cells. Therefore, during the differentiation stage, the oxygen demand is likely to decline gradually [6].

In order to improve the oxygen supply to the cells, the medium can be reoxygenated via a gas exchanger, by aeration (bubble oxygenator) or by membrane exchange (membrane oxygenator) [10, 12]. The oxygenation element may be integrated within the reactor or situated in series with the bioreactor in the circuit [13]. However, by applying oxygenation, the oxygen concentration present in the culture medium is still limited to its soluble limit (0,2 mmol/L) [14].

In order to enhance the oxygen concentration in the culture medium to achieve values more similar to those present in *in vivo* systems, some researchers have incorporated oxygen carriers (e.g. hemoglobin-based oxygen carrier, HBOCs), since several studies [15][16] showed that supplementation of red blood cells in the circulating cell culture media improved O_2 transport to cultured cell. O_2, CO_2 and H^+ are transported by the hemoglobin. The hemoglobin is present in the blood in a concentration of 150 g/L, each molecule of hemoglobin links 1 molecule of O_2 and each gram of hemoglobin dissolved 1,34 mL of oxygen. So, in total 200 mL of oxygen are transported in one liter of blood [17] increasing the concentration of oxygen up to 16 mmol/L.

1.2.3 Mathematical oxygen transport model

In order to gain a better understanding on how physical factors modulate tissue development, it is necessary to integrate bioreactor studies with quantitative analyses and computational modeling of changes in mass transfer and physical forces experienced by cells [6].

The amount of delivered oxygen is a significant factor in designing the cell culture bioreactors. One major obstacle preventing proper understanding of oxygen in TE constructs is a lack of mathematical models that can predict which parameters are beneficial for avoiding oxygen limitation and increasing oxygen diffusion across serial resistances [6].

Generally, distribution of oxygen in a bioreactor could occur due to two different phenomena depending on the flow characteristics: convection and diffusion. In flow dominated systems, the oxygen mass transport can typically be modeled using the convective-diffusion equation (Eq. 1), which links the flow field to the concentration gradients.

$$\nabla \cdot \left(-D\nabla C_{O_2}\right) + u \cdot \nabla C_{O_2} = r_A \qquad (Eq.1)$$

where, C_{O_2} is the concentration of oxygen (mol/m^3), D is the diffusivity of the species (m^2/s) and u is velocity (m/s). The rate expression (r_A) is representative of the rate of oxygen consumption in the system (mol/m^{3}s) and typically, the Michaelis-Menten equation is used (Eq.2) [18]:

$$-r_A = \frac{V_m C_{O_2}}{K_m + C_{O_2}} \qquad (Eq.2)$$

where V_m is the maximum reaction rate and K_m is the Michaelis constant.

The cellular oxygen consumption (r_A) can also be calculated using the cell specific Oxygen Uptake Rate (OUR), which is the rate of oxygen consumption per cell unit and depends on the type of cell. Its units are mol/(cell s) [19].

1.3. Level of implementation

The use of bioreactors, understood as any device used to carry out any kind of bioprocess, is a well-know and implemented process in some applications, like waste water treatment [20]. Nevertheless, the use of bioreactors in TE is still under research, because mammalian cells are more sensitive to culture conditions *in vitro* than the cells of plants or the enzymes or microorganisms [21].

The use of these apparatus has brought us a step closer to engineering numerous tissue types in the laboratory. At present, most bioreactors are specialized devices with low volume output. Their assembly is often time consuming and labour intensive. While scaled-up versions of some devices may be useful for developing larger amounts of tissue, problems with process consistency and process contamination may persist. A better understanding of the different effects of mechanical stimulation on cell signalling and mechanotransduction is also needed. This can be achieved through the use of existing simple bioreactors in conjunction with numerical simulation of culture conditions to minimise the number of experiments needed. In the future, ways of minimising the time and effort needed in order to form tissue must be found if costs are to be minimised and the use of engineered tissue is to become a clinical routine [5].

Similarly, the use of membranes as scaffolds to support host cell proliferation and differentiation for direct *in vivo* applications such as dental problems [22], burn injury [23] or even cardiovascular is already implemented and, currently, surgical procedures for the treatment of these damages in human health have been carried out. One example is the work performed by Matsumura et al. who reported application of tissue-engineered in cardiovascular surgery on children with various complex heart diseases. They applied a tissue engineering technique where patients own cells were isolated, cultured and subsequently seeded on a biodegradable polymer scaffold. The first operation was performed in May 1999, and over 40 patients have been treated by then. During post-operative analysis no complications related to the TE scaffold were observed [3].

The direct implantation *in vivo* of scaffolds has lower problems related to nutrients and oxygen delivery. However, scaffolds for bioreactors in TE are under research because the conditions to be hold by the cells *in vitro* are much more challenging.

Even though TE is a recent field in the regenerative medicine, there are some companies (i.e. Synthecon, Incorporated and Instron TERM) or research groups (i.e. Fraunhofer Institute for Interfacial Engineering and Biotechnology) that are investigating, innovating and improving cell culture systems; thereby contributing to a healthier world [24-26].

Figure 2 Companies or research groups in TE [24-26]

1.4. Objectives

Oxygen supply in TE bioreactors is one of the most important limiting factors for tissue growth, due to the low solubility of oxygen in culture medium. Several studies, including research groups and companies, are working to improve this situation, by enhancing the design of the bioreactors to maximize the oxygen mass transfer to the cell layers, introducing oxygen carriers and studying the mathematical models that simulate the oxygen mass transport to the cells in order to achieve tissue sizes of clinical relevance. This achievement for TE would represent a major advance in medicine.

The aim of this project is the study of the theoretical influence of oxygen mass transport on the size achieved in an engineered tissue developed *in vitro*. For this, a mathematical model considering the mass transport of oxygen through a cell cultured scaffold on a perfusion bioreactor was developed. The theoretical evaluation was made considering a laboratory experimental set-up using a polymeric membrane scaffold previously developed in the Research group Advanced Separation Processes (ASP) of the Chemical and Biomolecular Department made of poly(Ɛ-caprolactone) (PCL) by the phase inversion method [8]. Using this scaffold, the concentration profile of oxygen across the thickness of the cell side at steady-state of two kind of common cells (normal human lung fibroblasts and chondrocyte cells) was analysed.

The influence of the oxygen concentration on the culture medium on the potential cell tissue size achieved was evaluated. Initially, with the maximum oxygen concentration allowed by its limit of solubility in the culture medium, the maximum cell thickness could be determined, in order to know if, with this scaffold, cells can exist; taken into account that the clinically relevant thickness for engineered tissue is approximately 5 mm [10]. Secondly, an oxygen carrier was introduced (HBOCs) to increase the concentration of oxygen in the culture medium and to achieve a higher thickness. The results of both systems have been studied and evaluated.

2. DEVELOPMENT

2. DEVELOPMENT

2.1 Experimental model

Since different types of bioreactors have been used in various TE applications in the literature, in this project a dynamic flow perfusion bioreactor has been proposed due to, as indicated earlier, is superior compared to other nonperfusion or static bioreactors, thereby enhancing nutrient transport and providing mechanical stimuli to the cells. Figure 3a shows the diagram of the different elements conforming the experimental bioreactor set-up considered for the present work. The knowledge of the experimental set-up used is important to identify the performance of the system and to describe the subsequent mathematical model.

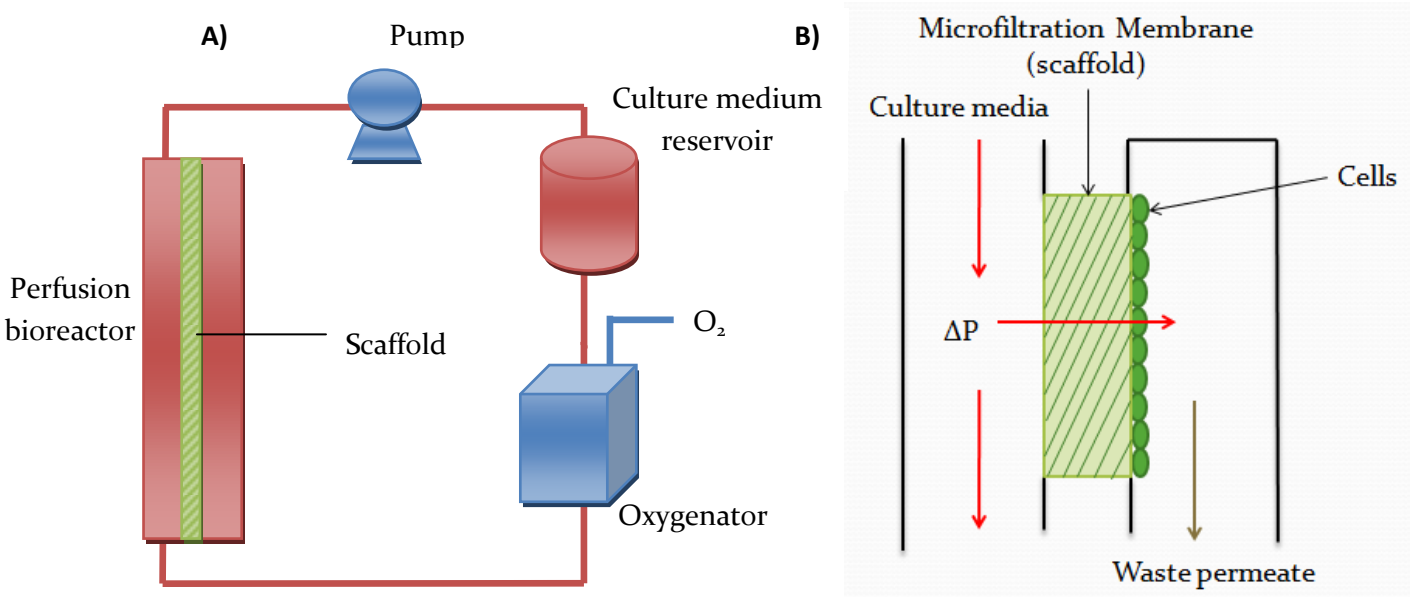


Figure 3 Diagram of the experimental flow perfusion bioreactor set- up (A) and detail of the cell seeded scaffold of the bioreactor and the circulation conditions of the feed and waste streams (B)

In this case, the flow perfusion bioreactor consists of culture medium reservoir, a pump, an oxygenator and a scaffold chamber. The culture medium flows tangentially to the membrane scaffold and is forcely perfused through the scaffold

by applying pressure, thus enhancing fluid transport [5] (see Figure 3b for more details). An oxygenator is required to force the solubility of the oxygen in the exhausted culture medium leaving the perfusion bioreactor. This oxygenation of the culture medium provides a constant concentration of oxygen in the bioreactor along experimental time. It is supposed that the efficiency of the oxygenation is maximum, and thus, the oxygen concentration reaches always its solubility in the culture medium.

The scaffold consists of a microfiltration membrane in which the components of the culture medium (proteins (i.e. BSA), oxygen, other nutrients as glucose…) passed through it, applying constant pressure as driving force. It is assumed that the cells (fibroblasts and chondrocytes) grow uniformly on the right hand of the scaffold and not inside it, so it is not possible to clog the pores of the membrane. The exhausted waste permeate can exit the cell side compartment of the bioreactor.

The dimensions of the scaffold to be fitted in the bioreactor are shown in Figure 4A. The thickness is around 100 µm, the length is 5 cm, the width is 2 cm. Besides the distance between the top of the scaffold and the shell depth is 0,3 cm (Figure 4B).

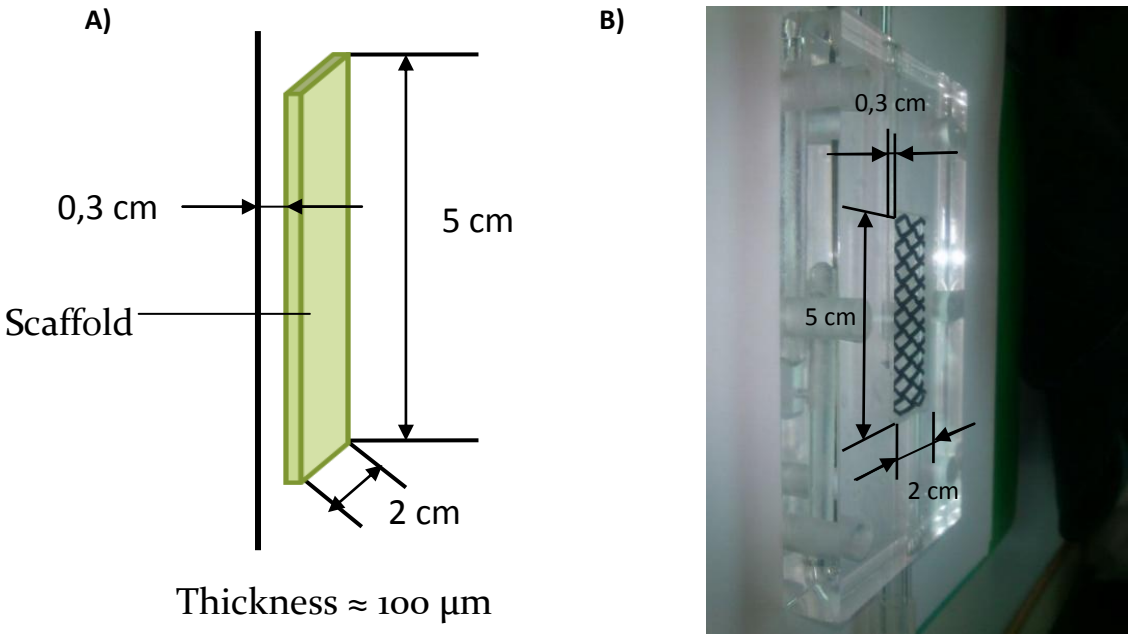


Figure 4 Scaffold dimensions (A) and bioreactor culture compartment dimensions (B)

2.2 Mathematical model

Once the experimental set-up has been described, the mathematical model to describe the oxygen mass transport through the membrane perfusion bioreactor developed is explained below.

2.2.1 General hypotheses of the model

Firstly, some general comments or assumptions have to be done, in order to define and simplify the overall mass transport resistances considered in the process:

- Steady-state conditions are considered both in the membrane bioreactor and in the culture medium reservoir. This could be assumed because we considered always complete replenishment of the oxygen concentration in the culture medium reservoir due to the presence of the oxygenator and the retention time of the culture medium inside the bioreactor was negligible in comparison to that in the culture medium reservoir.
- The concentration of oxygen is assumed to be constant across the length and the width of the scaffold as it is assumed that there is not oxygen rejection by the membrane nor back flux of the permeated culture medium to the feed side. This is a rough approximation to simplify the modeling. However, in future works, the convection of oxygen within the bioreactor length position should be incorporated.
- There is no polarization concentration phenomenon in the proximities of the membrane scaffold; it only varies across the thickness of the cells. This is a simplification of the mass transport resistances in series assuming the mass transport resistance of oxygen due to its diffusion across the cell layer represents the highest one in this system, and the rest of mass transport resistances (concentration polarization and membrane resistance) are negligible.

- Cells grow on layers on top of the right hand (in Figure 3) of the membrane scaffold (it is considered that cell do not penetrate inside the membrane).
- In the cell layer, the diffusion term and the consumption term of the oxygen are taken into account. This diffusivity is supposed to be constant as cells do not proliferate, but they are in the differentiation stage and therefore, they remain at the same cell density and thus, the cell layer porosity is also constant (cellular proliferation and death are not considered.)
- The rest of nutrients (glucose, proteins, etc) are considered to be present in excess in the culture medium.

2.2.2 Mathematical model

In this section, the mathematical model proposed for the experimental set-up is presented.

Figure 5 shows the oxygen mass transport resistances in series, according to the previous general hypotheses of the model.

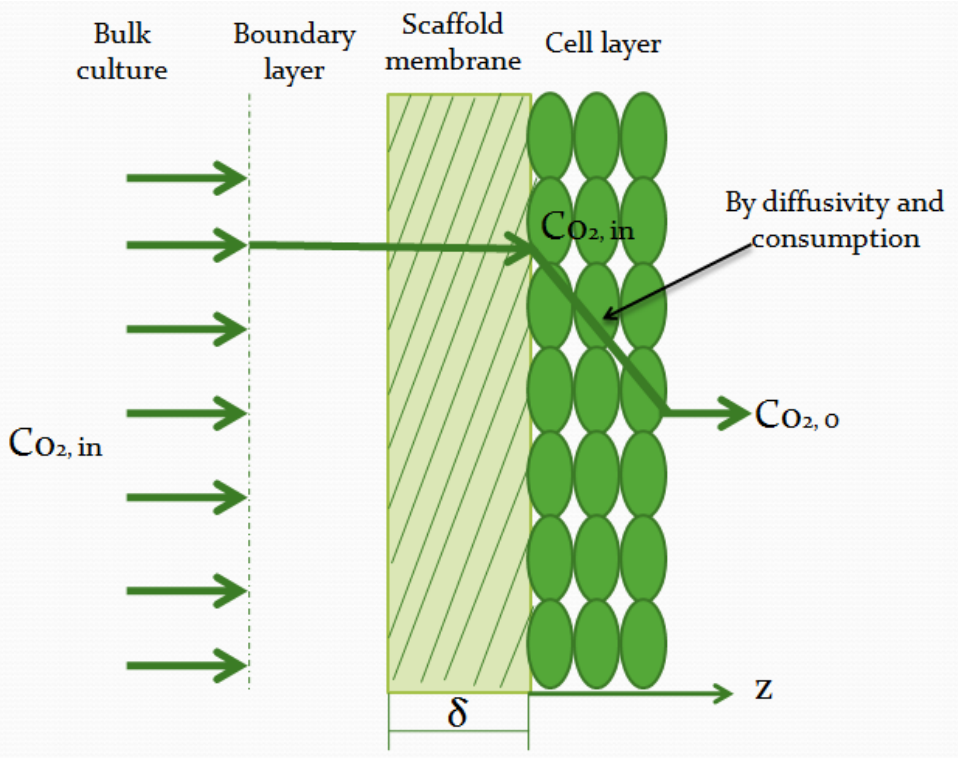


Figure 5 Oxygen the mass transport resistances in series at the microscale

δ represents the scaffold membrane thickness, z is the thickness of the cell layer, $C_{O_2,in}$ is the oxygen concentration at the inlet (in the culture medium) and $C_{O_2,0}$ is the oxygen concentration after the cell culture

Once the hypotheses are clear, the mathematical model developed is presented below. Several articles in literature [27-29] suggest a mathematical model for the cell side of similar systems with this structure:

$$\frac{\partial C_{O_2}(z,y,x,t)}{\partial t} = -D\left[\frac{\partial^2 C_{O_2}}{\partial z^2} + \frac{\partial^2 C_{O_2}}{\partial y^2} + \frac{\partial^2 C_{O_2}}{\partial x^2}\right] - r_A(z,y,x,t) \quad (Eq.3)$$

However, in this project the concentration of oxygen does not vary with time (steady state) and also does not change with the length and the width of the bioreactor in the feed side. It only varies with the thickness of the cell layer, which depends on the number of monolayers developed. Hence the oxygen transport occurs only via diffusion and consumption and the expression can be simplified as:

$$0 = -D\left[\frac{\partial C_{O_2}}{\partial z}\right] - r_A(z) \quad (Eq.4)$$

C_{O_2} is the oxygen concentration in mol/m^3, z the thickness of the cell culture, D is the effective diffusivity of the oxygen through the cell layer expressed in m^2/s (assumed constant in the general hypotheses) and $r_A$is the cellular oxygen consumption rate per unit of area of the tissue (mol/m^{2}s).

The oxygen profile on the cell thickness z is evaluated in the range between 0 to 5 mm, which is the clinically relevant thickness for engineered tissue [10]. In this model different concentration of oxygen are calculated for different cell thickness as it is shown in the Figure 5.

The boundary condition at z=0 is:

$$J_{o_2} = -D\left.\frac{\partial C_{O_2}}{\partial z}\right|_{z=0} - r_A(0) \quad (Eq.5)$$

Where J_{O2} is the oxygen flux.

The cellular oxygen consumption is modeled using the cell specific OUR [19], where r_A depends on the available number of cells:

$$r_A(z) = \frac{q_{O_2} \cdot N_{cell} \cdot z}{A_m \cdot d} \quad (Eq. 6)$$

q_{O_2} is the cell specific OUR expressed in mol/(cell s), A_m is the membrane area (10^{-3} m^2), d is the diameter of the cells in m and N_{cell} is the number of cells per layer, calculated dividing the membrane area by the cell area.

Table 2 shows the value of the parameters required in the model which depend on the type of cell (fibroblasts or chondrocytes):

Table 2 Model parameters [19, 28, 29, 30, 31]

Parameter	Type of cell	
	Fibroblasts	**Chondrocytes**
Effective diffusivity (D)	$1{,}7 \cdot 10^{-9}$ m^2/s [28]	$1{,}5 \cdot 10^{-9}$ m^2/s [29]
Oxygen uptake rate (q_{O_2})	$1{,}78 \cdot 10^{-17}$ mol/cell s [30]	$2{,}78 \cdot 10^{-17}$ mol/cell s [19]
Cell diameter (d)	$12{,}5 \cdot 10^{-6}$ m [31]	$20 \cdot 10^{-6}$ m [31]
Number of cells/monolayer (N_{cell})	$8{,}15 \cdot 10^{6}$ cells/monolayer	$3{,}14 \cdot 10^{6}$ cells/monolayer

Lastly, the influence of the oxygen concentration in the culture medium has been evaluated for both systems: first considering the maximum oxygen concentration allowed to its limit of solubility in the culture medium (0,2 mmol O_2/L [14]) and then considering the addition of HBOCs to increase the concentration of oxygen above its soluble limitation. Hence, the new concentration of oxygen in the model would be 16 mmol/L [15-17]; considering that hemoglobin is totally saturated of oxygen. Both values of oxygen concentration remain constant in the culture medium thanks to the oxygenator.

Once all the parameters are described, eq. 3 and 4 were implemented and solved using the mathematical program Aspen Custom Modeler (see Appendix section).

2.3 Results and discussion

In this part, the results of the study are shown and analysed.

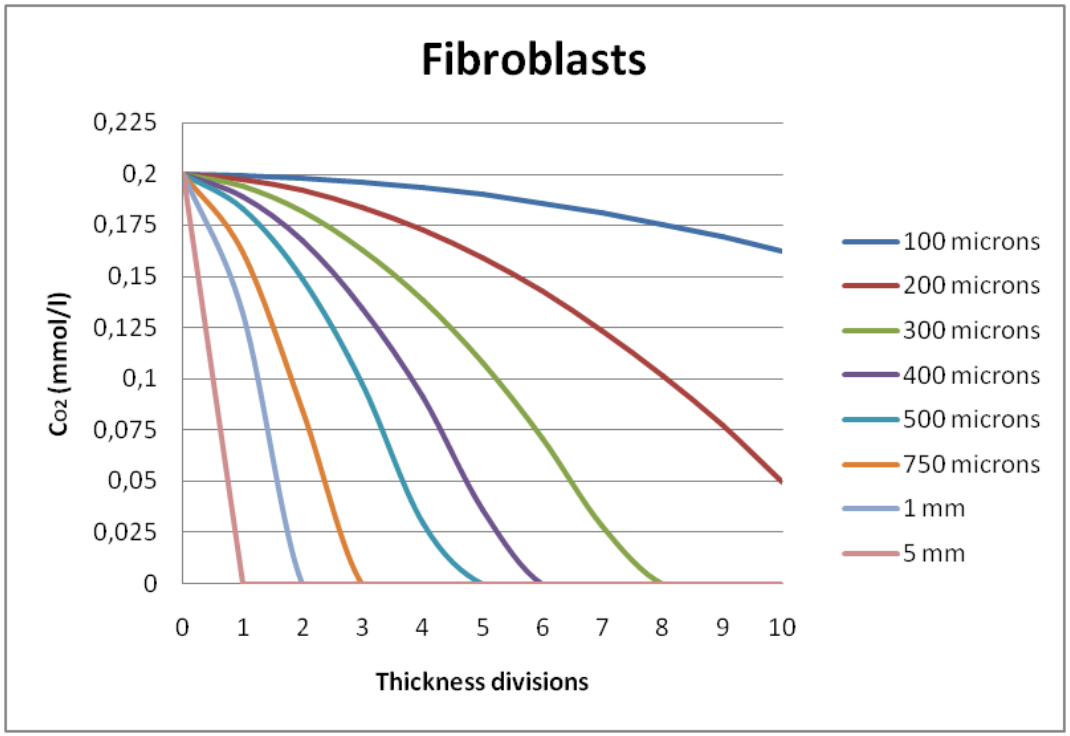
2.3.1 Results

The results of this project are expressed as the concentration profile of oxygen across the cell thickness layer of the two types of cells with and without HBOCs supplementation.

In Figure 6, the oxygen concentration profile of fibroblasts and chondrocytes with the position of the different cell thickness without HBOCs supplementation is shown.

Figure 7 presents the oxygen concentration profile of fibroblasts and chondrocytes with the position of the different cell thickness with the addition of HBOCs.

Finally, a comparison between the maximum cell thickness achieved by the two types of cells (fibroblasts and chondrocytes) distinguish a normal culture medium a culture medium supplemented with HBOCs is shown in Figure 8.



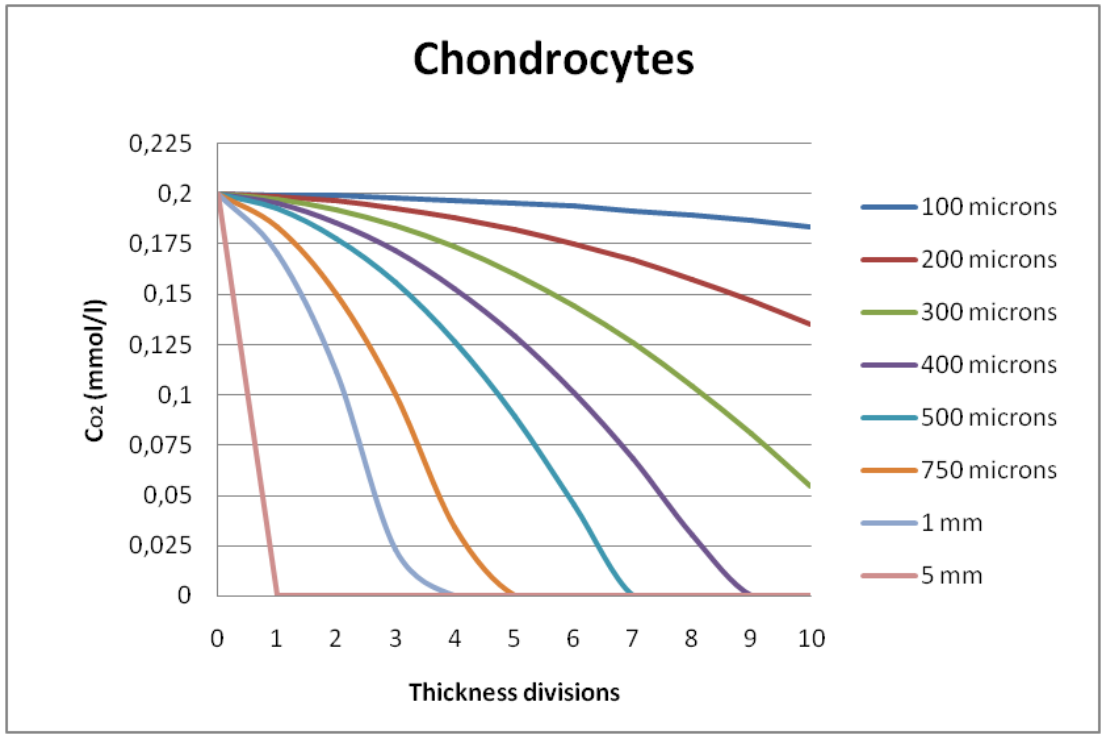


Figure 6 Oxygen concentration profile with the position of the different cell thickness without HBOCs supplementation

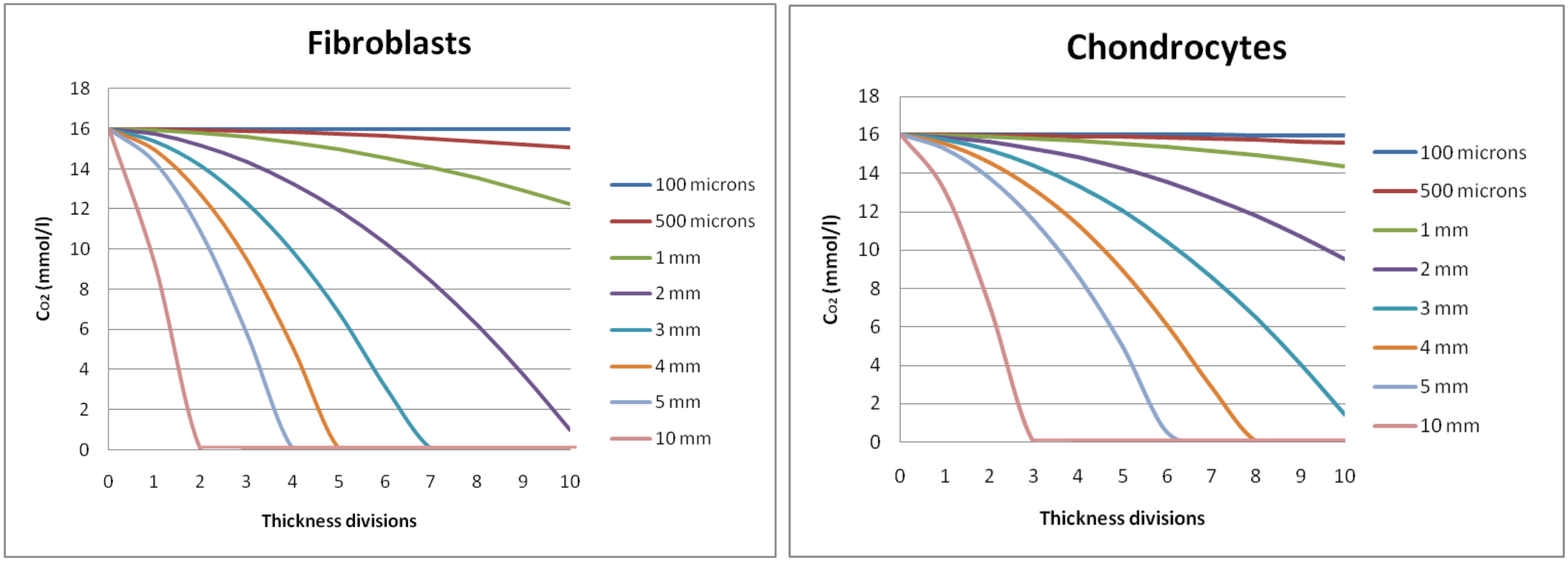
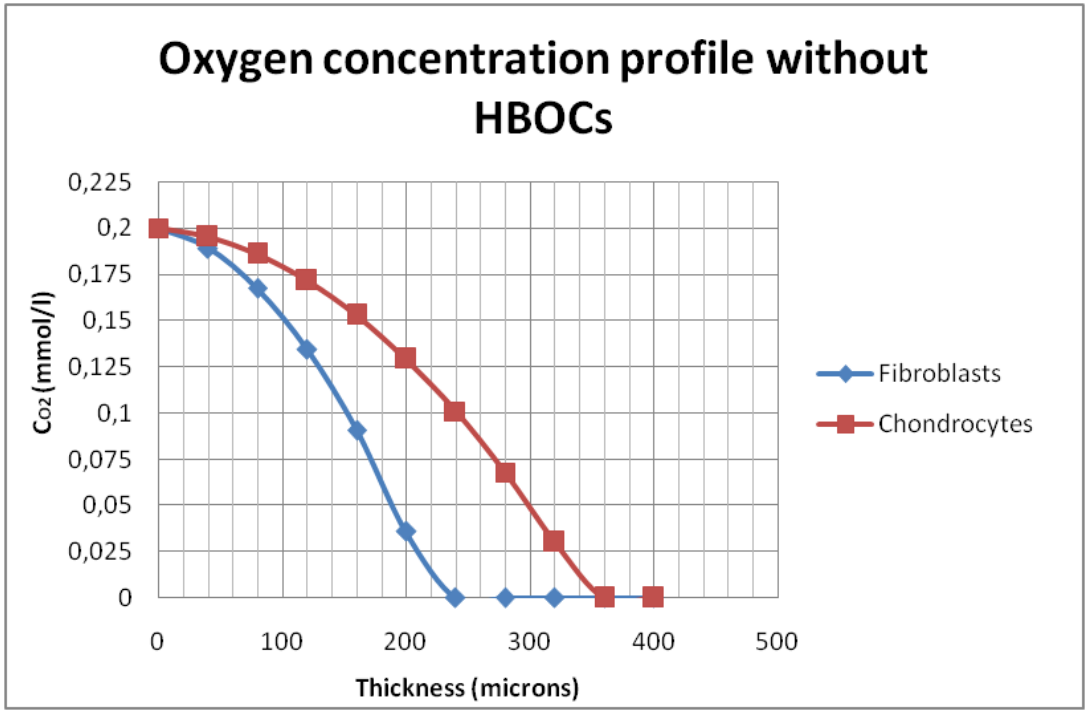


Figure 7 Oxygen concentration profile with the position of the different cell thickness with addition of HBOCs



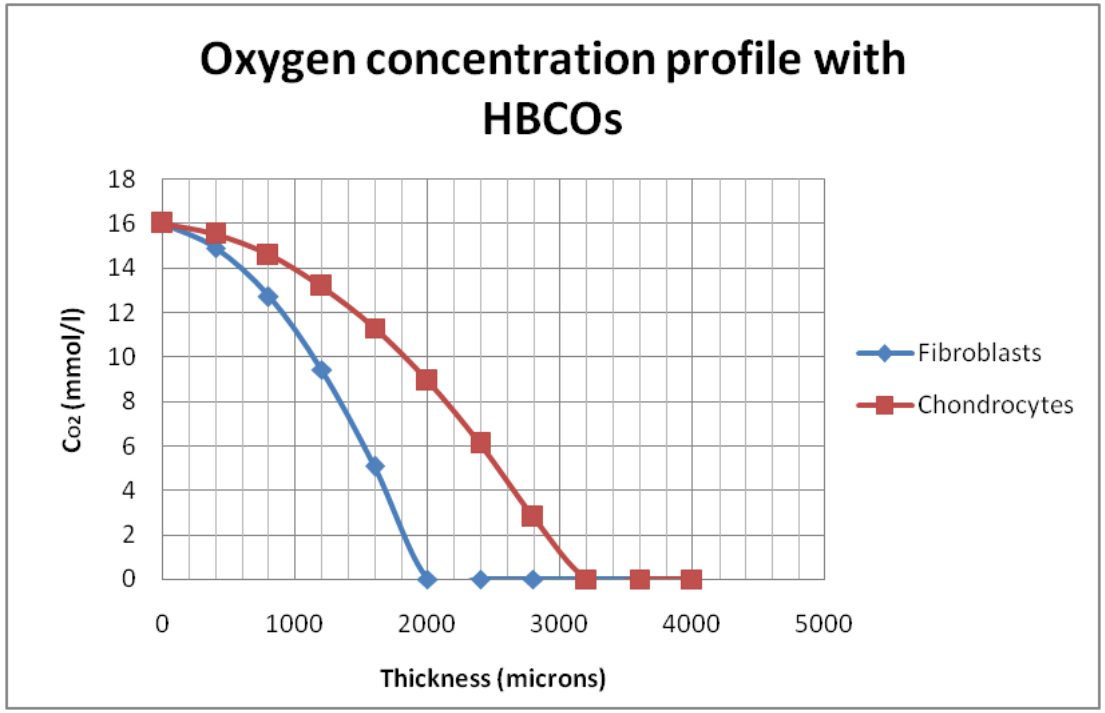


Figure 8 Comparison of maximum cell thickness achieved with and without addition of HBOCs

2.3.2 Discussion

Figure 6 and Figure 7 shows that, the concentration of oxygen is reduced when the cell thickness is increased, as expected, due to the diffusion term and the consumption of oxygen by cells [27-29].

Also, these oxygen concentration profiles reveal the difference between both systems: with and without HBOCs. As it is possible to see in Figure 6 (with an initial oxygen concentration of 0,2 mmol/L) the cell thickness studied is in the range of 100-5000 microns and there is no concentration of oxygen beyond 400 microns. In Figure 7, adding the HBOCs to increase the concentration of oxygen up to the value of 16 mmol/L, the studied cell thickness range increases; having oxygen, even when it is reached 2 mm of thickness.

Assuming that cells can grow with an oxygen concentration higher than 0 mmol/L, Figure 8 shows the maximum thickness achieved for both systems with more detail than the others figures.

Fibroblasts and chondrocytes reach, approximately, a maximum cell thickness of 250 and 375 microns, respectively, without HBOCs addition. These values are in the range of cell thicknesses developed *in vitro* reported in previous works (100-200 μm) [6,10]. The slight deviation from the reported cell thicknesses may be explained by the model simplifications assumed. However, the clinically relevant thickness for engineered tissue (approximately 5 mm) [10] cannot be achieved with this type of bioreactor.

In the case of supplementing the culture medium with HBOCs, fibroblast and chondrocyte still receive oxygen supply up to 2250 and 3250 microns of cell tissue thickness, respectively. These higher values of cell tissue thickness demonstrated that adding HBOCs to the culture medium improves the potential of cell growth *in vitro* up to tissue sizes in the range of clinical relevance [10, 15, 16]. As the available recent literature concludes [32], it is very difficult that cells grow beyond a 4 mm diameter size, apparently because the lack of a blood supply limits access to nutrients; therefore, this situation agrees with the results obtained. Similarly

than in the non supplemented culture medium results, this is a first approximation of the real mass transport phenomena that actually takes place, so the results might be different. Although there is still a long way up to predict real cell growth, it is possible to consider that the strategy of implementing HBOCs in the culture medium for cell regeneration *in vitro* could become the key to overcome the limitation to reach clinical relevant cell thickness in TE applications.

It is also interesting to note that, depending on the type of cells, the oxygen demand is very different as it was observed in this work. For instance, chondrocyte cells [29] present a lower demand than human lung fibroblasts [28]. Therefore, the strategies applied for each type of cells should be different.

3. CONCLUSIONS

3. CONCLUSIONS AND FUTURE CHALLENGES

In this project, the theoretical influence of oxygen mass transport on the size achieved in an engineered tissue developed *in vitro* was studied and it has been demonstrated that, as the size of the cell layer (number of cells) increases, the oxygen concentration is reduced until complete consumption, so cells would stop to grow as they need oxygen to live. To perform this evaluation, a simplified mathematical model has been developed able to make a first approximation to the study of the mass transport of oxygen through different cell type layers, as it is considered the most critical part of the TE process.

One of the most important conclusions achieved in the present work was that by adding HBOCs to the culture medium it is possible to increase the concentration of oxygen from 0,2 mmol/L to 16 mmol/L, enhancing the thickness of the cell layer from 300 microns to 3000 microns approximately (one order of magnitude) meaning that the clinically relevant thickness for engineered tissue could be reached. Finally, human cartilage chondrocytes reveal a better behavior (higher cell thickness achieved) than normal human lung fibroblasts for the same oxygen concentration. Therefore, depending on the tissue type to be regenerated, the oxygen demands should be determined experimentally to be implemented in the mathematical model.

In order to improve this work and continue this line of research some future challenges should be overcome. First, the mathematical model has to be more realistic by introducing changes in time, considering all the mass transport resistances, incorporating the convection of oxygen within the bioreactor length or taken into account that cells can grow inside the scaffold. Secondly, it is necessary to demonstrate that this mathematical model is correct (model validation), following the same procedure experimentally and if it works properly, the model could be used to predict the behaviour at different working conditions. Finally, in order to really achieve clinically relevant tissue sizes (and even organs) the bioreactors should facilitate the vascularisation of the tissue engineered construct; this could be done by enhancing the design of perfusion hollow fiber bioreactors.

4. REFERENCES

5. REFERENCES

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6. ANNEXES

7. ANNEXES

5.1 Aspen Custom Model

a) Mathematical model command for fibroblasts without HBOCs supplementation

```
Model cell_layer

// Not dependent variables of x
Deef as notype; // Efecctive diffusivity = 1.7*10^-9 m2/s
qo2 as notype; // Cell specific oxygen uptake rate = 1.78*10^-17 mol/cell s
Am as notype; // Membrane area = 10-3 m2
Ncell as notype; // Number of cell per layer = 8.15*10^6 cell/layer
D as notype; // Diameter of cell = 1.25*10^-5 m


// Domain of the spatial dimension. Thickness= 250 microns
x as lengthdomain (discretizationmethod: "BFD1", highestorderderivative:1, length:0.00025,
spacingpreference:0.000025);


// Derived variables from x
Co2 as distribution1d (XDomain is x,highestorderxderivative:1) of conc_mole;
// Oxygen concentration = 0.2 mmmol/l= 0.2 mol/m3


// Distributed variables (not derived)
ro2([0:x.endnode])as notype; // mol/m2 s


// Boundary conditions
Co2(0)=0.2;


// Equations
For i in (x.interior+x.endnode) do
0=-Deef*(Co2(i).ddx)-ro2(i);
ro2(i)=qo2/Am*Ncell/D*x(i);
endfor


End
```

b) Mathematical model command for chondrocytes without HBOCs addition

```
Model cell_layer

// Not dependent variables of x
Deef as notype; // Efecctive diffusivity = 1.5*10^-9 m2/s
qo2 as notype; // Cell specific oxygen uptake rate = 2.78*10^-17 mol/cell s
Am as notype; // Membrane area = 10-3 m2
Ncell as notype; // Number of cell per layer = 3.18*10^6 cell/layer
D as notype; // Diameter of cell = 2*10^-5 m

// Domain of the spatial dimension. Thickness=375 microns
x as lengthdomain (discretizationmethod: "BFD1", highestorderderivative:1, length:0.000375,
spacingpreference:0.0000375);


// Derived variables from x
Co2 as distribution1d (XDomain is x,highestorderxderivative:1) of conc_mole;
// Oxygen concentration = 0.2 mmmol/l= 0.2 mol/m3


// Distributed variables (not derived)
ro2([0:x.endnode])as notype; // mol/m2 s


// Boundary conditions
Co2(0)=0.2;


// Equations
For i in (x.interior+x.endnode) do
0=-Deef*(Co2(i).ddx)-ro2(i);
ro2(i)=qo2/Am*Ncell/D*x(i);
endfor


End
```

c) Mathematical model command for fibroblasts with HBOCs supplementation

```
Model cell_layer

// Not dependent variables of x
Deef as notype; // Efecctive diffusivity = 1.7*10^-9 m2/s
qo2 as notype; // Cell specific oxygen uptake rate = 1.78*10^-17 mol/cell s
Am as notype; // Membrane area = 10-3 m2
Ncell as notype; // Number of cell per layer = 8.15*10^6 cell/layer
D as notype; // Diameter of cell = 1.25*10^-5 m

// Domain of the spatial dimension. Thickness= 2,25 mm
x as lengthdomain (discretizationmethod: "BFD1", highestorderderivative:1, length:0.00225,
spacingpreference:0.000225);


// Derived variables from x
Co2 as distribution1d (XDomain is x,highestorderxderivative:1) of conc_mole;
// Oxygen concentration = 16 mmmol/l= 16 mol/m3


// Distributed variables (not derived)
ro2([0:x.endnode])as notype; // mol/m2 s


// Boundary conditions
Co2(0)=16;


// Equations
For i in (x.interior+x.endnode) do
0=-Deef*(Co2(i).ddx)-ro2(i);
ro2(i)=qo2/Am*Ncell/D*x(i);
endfor


End
```

d) Mathematical model command for chondrocytes with HBOCs addition

```
Model cell_layer

// Not dependent variables of x
Deef as notype; // Efecctive diffusivity = 1.5*10^-9 m2/s
qo2 as notype; // Cell specific oxygen uptake rate = 2.78*10^-17 mol/cell s
Am as notype; // Membrane area = 10-3 m2
Ncell as notype; // Number of cell per layer = 3.18*10^6 cell/layer
D as notype; // Diameter of cell = 2*10^-5 m

// Domain of the spatial dimension. Thickness= 3,25 mm
x as lengthdomain (discretizationmethod: "BFD1", highestorderderivative:1, length:0.00325,
spacingpreference:0.000325);


// Derived variables from x
Co2 as distribution1d (XDomain is x,highestorderxderivative:1) of conc_mole;
// Oxygen concentration = 16 mmmol/l= 16 mol/m3


// Distributed variables (not derived)
ro2([0:x.endnode])as notype; // mol/m2 s


// Boundary conditions
Co2(0)=16;


// Equations
For i in (x.interior+x.endnode) do
0=-Deef*(Co2(i).ddx)-ro2(i);
ro2(i)=qo2/Am*Ncell/D*x(i);
endfor


End
```