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The role of perfusion bioreactors in bone tissue engineering

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Tissue engineering has emerged as a possible alternative to current treatments for bone injuries and defects. However, the common tissue engineering approach presents some obstacles to the development of functional tissues, such as insufficient nutrient and metabolite transport and non-homogenous cell distribution. Culture of bone cells in three-dimensional constructs in bioreactor systems is a solution for those problems as it improves mass transport in the culture system. For bone tissue engineering spinner flasks, rotating wall vessels and perfusion systems have been investigated, and based on these, variations that support cell seeding and mechanical stimulation have also been researched. This review aims at providing an overview of the concepts, advantages and future applications of bioreactor systems for bone tissue engineering with emphasis on the design of different perfusion systems and parameters that can be optimized.

Introduction

Current medical treatments for large bone defects or injuries caused by trauma, tumor, tissue degeneration or congenital deformities are based on bone-grafting surgeries.[1,2] The bone grafts consist of autografts, allografts, xenografts, synthetic materials or de-mineralized bone matrix, depending on the bone source. The most common treatment is the autograft transplant. However, it is restricted by the limited availability of bone, which is harvested from the iliac crest, and by medical complications following the harvesting such as infection, nerve and arterial injury and chronic pain.[2-4] Allografts and xenografts are also limited due to the risk of rejection and disease.[5,6] The synthetic materials that can be used as bone substitutes include mainly hydroxyapatite, coralline hydroxyapatite, tricalcium phosphate, biphasic calcium phosphates and bioglasses. De-mineralized bone matrix, produced through the decalcification of cortical bone, can also be used to try and reduce the potential for immunogenic host response.[7,8] All of the synthetic materials commonly used are hard, porous and osteoconductive, yet medical applications are limited due to possible resorption after long-term implantation and brittleness.[9,10] Tissue engineering has emerged as a possible alternative for the described treatments as it would provide functional substitutes for the native tissues and models for studies on tissue formation and development. A tissue engineering approach involves the implantation in the site of injury of a 3D porous biodegradable structure seeded with osteoblastic or mesenchymal stem cells, which are one valuable cell source for orthopedic applications and its growth rate and osteogenic differentiation have been studied for years.[11] The construct would provide a template for tissue development and should be biodegradable in order to eliminate the need for surgical removal.[12,13] Biodegradable polymers used for bone tissue engineering include silk, chitosan, collagen and polyglycolic acids.[14] For this strategy to lead to a functional substitute it would be important that certain parameters such as cell seeding density, culture period, scaffold architecture, scaffold composition, cell source, matrix deposition and mineralization are optimized.[15-17] Although the described approach allows cell growth and proliferation, when dealing with 3D structures in static culture (where there is not any mixing or circulation of the culture medium), nutrient transport only occurs by diffusion which leads to higher concentrations of nutrients and metabolites at the scaffold's surface which in turn may constrain cell migration to the interior of the construct and tissue formation.[6,18] Limitations of cell culturing on three-dimensional scaffolds under static conditions include insufficient nutrient and oxygen transport and waste removal which will cause decreased proliferation and differentiation and non-uniform cell distribution. This also limits the dimensions of the scaffolds that can be used on static culture.[19] In traditional two-dimensional culture techniques diffusion is enough to provide nutrients and oxygen to all the cells and waste removal, but when using three-dimensional constructs, diffusion is not sufficient.[20] Hence more complex bioreactor systems can be used to improve culture media circulation and convective transport of nutrients to the cells, allowing the development of a more uniform tissue. Bioreactors use materials that are widely used for bone tissue engineering. These materials are seeded with cells capable of proliferating and differentiating into osteoblasts and are maintained under dynamic culture. Bioreactors bring several advantages into the culture of functional tissues. They not only increase mass transport inside three-dimensional structures, but also reduce the number of handling steps, hence reducing contamination potential. They

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also permit control and monitoring of environmental conditions such as pH, temperature, oxygen and carbon dioxide concentrations and nutrient supply.[1,21] For bone tissue engineering it is also worth considering the fluid shear stress caused by mixing or perfusion of culture medium that will expose the cells to mechanical stimulation. In vivo, mechanical loading driven fluid can increase production of prostaglandins, alkaline phosphatase (ALP), collagen type I, along with osteoblast proliferation and mineralization.[22] It is thought that interstitial fluid flow through lacunar and canalicular spaces in bones is caused by mechanical loading of the skeleton. The cells lining these spaces are then influenced by the mechanostimulation provided by the fluid flow, differentiating or proliferating accordingly.[23,24] Thus, this becomes a clear advantage when using fluid flow in in vitro culture. Bioreactor design should account for the following basic requirements: controlled and fast cell expansion, efficient exchange of nutrients, oxygen and metabolites in all parts of the scaffold, enhanced cell seeding and provision of physical or biochemical stimuli.[25] The most common bioreactor types are spinner flasks,[26] rotating wall vessels[7,27] and perfusion systems.[28-30] Spinner flask and rotating wall vessel are two alternatives to static culture that try to minimize gradients in nutrient and metabolite concentrations. Both use convection to ensure that culture media around the scaffolds is well mixed thus leading to enhanced nutrient transport into the porous structure.[27] Perfusion systems, on the other hand, are the most complex as they can perfuse fluid directly through the structures, ensuring good mass transport inside the constructs, and they have been shown to upregulate expression of osteoblastic markers.[27,31,32]

Spinner Flasks

To address the issues of low seeding efficiency and other limitations present in static culture, spinner flask bioreactors were introduced in order to create a convective flow and produce hydrodynamic forces that will help increase mass transport.[28] Spinner flasks are simple bioreactor systems composed by a cylindrical container with side arms for removal of cells and medium and a stirring element at the bottom ensuring circulation and mixing of culture medium. The scaffolds used in these systems are in fixed positions, threaded in needles attached to the cap of the container.[14,33,34] Spinner flask bioreactors can mimic some aspects of native bone environment which allows them to be used for several studies regarding bone tissue formation and cell function.[26] The use of spinner flask culture systems has shown a positive effect in accelerating human mesenchymal stem cells (hMSCs) differentiation into osteoblasts cultured in silk fibroin scaffolds. These were maintained in both static and dynamic culture for 84 d and the peak for ALP activity was, respectively, on days 28 and 56. Also, constructs cultured on spinner flasks showed connective tissue and mineralized nodule formation after 14 d of culture whereas in static culture this was only seen on day 56.[22] ALP activity is used as a marker for early osteoblast differentiation because it peaks during the matrix maturation phase when the cells are committing to the differentiated phenotype and decreases at a later stage when the cells are starting to mature and form mineralized ECM.[35] hMSCs cultured in PLGA [poly(lactic-co-glycolic acid)] foams in this system also had higher ALP activity and calcium deposition, compared with those cultures in static conditions and gene expression of COLA1A (collagen type I α 1), Runx2 (runt-related transcription factor-2), OSX (osterix), ON (osteonectin), BMP2 (bone morphogenetic protein protein-2) genes was upregulated at days 7 and 21 of culture.[19] In collagen scaffolds these cells also exhibit higher ALP activity and its peak was followed by mineralization but mineralized matrix only formed in the outer rim of the scaffold, not penetrating more than 0.5 mm to 1 mm of the 11 mm diameter scaffolds, which corresponds to the penetration depth of the fluid.[17] Culture of immortalized hMSCs on coralline hydroxyapatite scaffolds showed that the pore size affects in-growth and differentiation of hMSCs.[36,37] ALP expression and rate of differentiation was higher on 200 μm pore size scaffolds compared with 500 μm pore size scaffolds. Although spinner flasks present advantages when compared with static culture and, in some cases, even with rotating wall vessels, they only permit extracellular matrix production at the scaffolds surface. Mixing the media is associated with turbulent shear at the surfaces which can also be detrimental to cell growth and tissue formation.[17,30] It is then necessary to look at alternatives that allow for a better penetration of cells and culture medium in the scaffolds.

Rotating Wall Vessels

The rotating wall vessel was first developed to create a microgravity environment, but when it was first tested using cell suspensions it was observed that the cells aggregated and formed tissue-like spheroids which opened the possibility of using this type of reactor for co-cultures of different cell types or for differentiation in early steps of tissue formation.[38] Rotating bioreactors provide controlled supply of oxygen and have low shear stress and turbulence, which are major advantages when compared with the stirred flasks.[39] The most common rotating wall bioreactor is composed by two concentric cylinders: the outer cylinder consists of the culture chamber filled with culture medium and where the scaffolds or microcarriers are placed and the inner cylinder is static and permits gas exchange.[6] The culture chamber rotates horizontally around its axis, randomizing gravitational forces that act on the cell surface and as it rotates the culture medium inside is accelerated until the entire fluid mass is rotating at the same angular rate as the wall.[38] Comparing the use of a rotating wall bioreactor and a spinner flask using PLGA scaffolds seeded with rat MSCs, it was observed that the ALP activity for cells in scaffolds cultured in the rotating wall vessel was lower than for samples cultured in the spinner flask and in static culture.[27] Osteocalcin secretion was also observed to be lower than in samples cultured in stirred flasks. In both systems cell growth and mineralization were limited to the outside region of the scaffolds.[6] hMSCs cultured in gelatin-hyaluronic acid scaffolds also presented similar behavior.[26] Particle dynamics studies show that the shear stress endured by a microcarrier increases with the density difference between the culture medium and the microcarrier. Most of the scaffolds used in these systems are

denser than the surrounding medium in rotating vessels, thus they impart higher shear stress and centrifugal forces cause them to collide with the walls of the culture chamber during rotation.[7] This leads to cell damage and interferes with cell attachment and deposition of mineralized matrix.[2] This could be the explanation behind the disappointing results obtained for the comparison of rotating wall reactors with spinner flasks. Bearing this problem in mind, several groups developed lighter-than-water scaffolds or microcarriers that exhibit migration toward the center and avoid collisions with the walls.[40] Using lighter-than-water microcarriers of PLGA seeded with human osteoblastic cells (Saos-2), ALP staining was positive on day 7 and was significantly higher for samples cultured in the rotating wall bioreactor compared with non-rotating 3D controls. Substantial amount of calcified matrix was also detected by alizarin histochemical staining.[41] These cells, however, appear to migrate toward the inner region of the microcarriers as cell in-growth covered the entire depth of the 2.5 mm PLGA microcarriers.[42] When rat calvarial osteoblastic cells were used, similar results were found for ALP expression and matrix mineralization and also the expression levels of osteopontin and osteocalcin significantly increased under rotating conditions.[2] Values of fluid shear were estimated and were in the range of 0.16–32 N/m^2 (1.6–320 dyn/cm^2), which are similar to the estimates of physiological level of shear stress on osteocytes under flow.[43] hMSCs were cultured in silk scaffolds in rotating bioreactors for 36 d and the presence of mineralized matrix and collagen type I were visible by staining and microCT. Also the mineralized structures were distributed throughout the entire volume of the constructs.[44] Hollow ceramic microspheres were also used as microcarriers for rotating bioreactors with rat MSCs and rat osteosarcoma cells (ROS 17/2.8). The formation of an aggregate was seen after 10 d of culture. Most of the beads were entirely covered with cells and extensive production of extracellular matrix was also visible. Early stages of mineralization were identified by the presence of nodules in the matrix.[39] Similar results were obtained using rat MSCs.[45] A variation of the common rotating bioreactor was developed and it consists in a new rotational oxygen-permeable bioreactor system (ROBS). The objective of this system is to supply optimal oxygen levels and continuous hydrostatic pressure to biodegradable polymer scaffolds. It consists of a polypropylene centrifuge tube modified with a silicon elastomer to allow gas exchange. The tube is then placed in a roller device and maintained in an incubator.[46] PLGA foams were seeded with rat MSCs and cultured for 5 weeks in this system and histological analysis showed that the density of the extracellular matrix produced increased and became partially calcified after 2 weeks. After 3 weeks extensive calcification was observed. The same system was also used with polycaprolactone (PCL) scaffolds and after 4 weeks there was abundant presence of calcified matrix and collagen type I and cell migration had occurred inside the scaffold.[47] One other variation of the rotating wall bioreactor system that consists on attaching the scaffolds to the vessels wall was used to culture rat osteoblasts on bio-derived bone scaffolds and showed higher proliferation rates, more ECM production and mineralization when compared with both spinner flasks and static culture.[48]

Rotating wall bioreactors managed to solve some of the problems associated with static cultures and partially improved culture conditions with regards to stirred flasks, but when it comes to expression of osteoblastic markers the results are not satisfactory probably due to the low shear stresses or collisions with the vessel wall. Still, the advantages over the stirred flasks are not clear which is why it is important to try and develop better alternatives.[6,26,27,38,48]

Perfusion Bioreactors

To overcome the limitations of the system already described, systems that use flow perfusion have started being used for bone regeneration. These reactors present an advantage when compared with stirred flasks and rotating wall vessels because they provide more uniform mixing of the media hence allowing for a better environmental control and physical stimulation of the cells in large constructs.[49] These bioreactors use a pump system that can perfuse media through the scaffolds in a continuous or non-continuous way. Several types of perfusion bioreactors have been tested, but most of them have a similar basic design which consists of a pump, a culture media reservoir, a tubing circuit and cartridges, chambers or columns that hold the scaffolds.[1,16,50] The scaffolds need interconnective pores and should have between 70% to 99% porosity in order to facilitate direct perfusion. In most cases the major difference between the systems is the design of the perfusion chamber because it is the key element to ensure thorough perfusion of the center of the structures. One of the most common systems that uses perfusion is described in detail by Bancroft et al.[29] This system consists of six flow chambers and two media reservoirs. Each flow chamber contains a cassette where the scaffold is held between two neoprene o-rings that are held tightly against the cassette screw on top. The media flows from top to bottom through the scaffold to prevent the trapping of air bubbles.[29,51] A schematic representation as well as a detail of the perfusion chamber can be seen in **Figure 1**.

This perfusion system has been used with several types of materials and scaffolds. Perhaps one of the most used is the titanium fiber mesh scaffold.[23,35,51,52] When cultured with rat MSCs, these scaffolds in the flow perfusion system have been shown to accelerate differentiation, as shown by ALP activity. Several studies showed that flow perfusion, when compared with static culture, leads to significantly higher values of ALP activity.[27,53,54] Late osteoblast differentiation is evaluated by osteopontin expression. Osteopontin is a glycoprotein synthesized by osteoblasts that is produced during the stages of differentiation that precede mineralization.[51] It has also been shown that flow perfusion culture facilitates an increase in the osteopontin secretion, which leads to believe that osteoblast differentiation is enhanced by flow perfusion culture.[52] Similar results have been obtained using porous calcium phosphate ceramics.[55] Real-time RT-PCR performed on hMSCs cultured on silicate-substituted tricalcium phosphate on the same perfusion system also corroborated the increase of ALP activity and osteopontin expression, showing significantly higher expression than in static culture.[31] The endpoint of osteoblastic differentiation is the

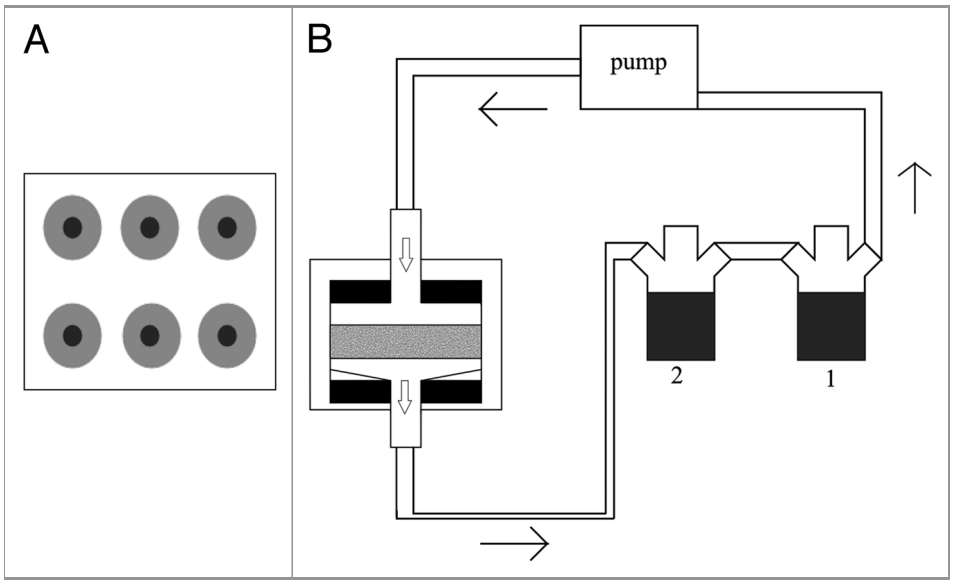

Figure 1. Schematic of the perfusion system described by Bancroft et al. (**A**) Top view of the perfusion chamber with six scaffold holders. (**B**) Representation of the complete system with the scaffold represented in gray, press-fit between the two O-rings, in black. The two medium reservoirs, 1 and 2, allow for complete medium change when the connection between the two is closed. Arrows represent medium flow.

production of a mineralized matrix and should be seen in long-term culture if the cells are differentiating.[23] A mineralized calcium matrix has been obtained in a number of studies that aim at differentiating marrow stromal cells under flow perfusion,[55-57] but most importantly a more uniform ECM distribution has been obtained in flow perfusion systems, as shown by micro CT with PLLA non-woven scaffolds, while the matrix produced under static conditions was denser in the scaffold peripheral regions.[23,57]

To increase biological relevance biodegradable scaffolds were also used with the same perfusion system.[53,58,59] One study was performed using starch-based biodegradable scaffold and again, ALP activity was used as a marker for early differentiation of marrow stromal cells and calcium deposition for late differentiation. Although there was an increase in ALP activity for both dynamic and static cultures, it was significantly higher for the scaffolds culture under flow perfusion. Also calcium deposition increased dramatically after two weeks of culture under flow perfusion suggesting that it enhanced mineralization and differentiation due to fluid shear induced mechanical stimulation and improvement of possible nutrient transport limitations in static culture.[53] PLLA non-woven resorbable scaffolds were also cultured with rat MSCs, and an increase was also seen in calcified matrix deposition in dynamic culture.[59] A modification of the system was used to allow the use of oscillating flow and study its influence on cell seeding, showing that oscillating perfusion yields higher seeding densities, more homogenous cell distributions and stronger cell-matrix interactions.[28,60] Looking only at the results it seems that perfusion systems present clear advantages when compared with spinner flasks and rotating wall vessels. Mass transport limitations are not an issue with most perfusion reactors and better results have been obtained with respect to differentiation, proliferation and expression of osteoblastic phenotype markers. Yet, further research is still required to improve these systems to allow standardization of the procedures necessary to the development of a functional bone substitute in a bioreactor. Parameters that can help toward the development of more efficient systems are described in following sections.

The Use of Different Perfusion Chambers

The design of the perfusion chamber is of the outmost importance. Bancroft et al. have described a system that holds six samples in individual chambers. The culture medium enters though a single hole on the top of the chamber and exits through another one in the bottom.[29] The culture medium is forced through the chamber and goes around and through the porous scaffold (**Fig. 1**). Another system (**Fig. 2**) incorporates a chamber for a single scaffold with perforated lid and bottom.[30,61] In this case this perforated basket is placed in the medium flow path and the medium is forced through the scaffolds. Because this chamber presents a perforated top there seems to be a better distribution of medium on the surface of the scaffolds which may allow for a more uniform penetration throughout the structure than with the previous one. The system described by Cartmell et al. is similar to these two with the exception that it holds eight samples in individual chambers (**Fig. 3B**), but the scaffolds are held in a very similar manner.[62] Finally another example is the one described by Grayson et al.[32] This one consists on a circular chamber with the dimensions of a Petri dish where six samples are held in individual spaces (**Fig. 3A**). The medium goes in through one end and it is distributed equally between the six scaffolds.

All of these examples have shown interesting results and there is not one that seems superior to the other, but it is important to consider that they are other parameters to consider and that depending on the material being used and on its dimensions there may be need to optimize the design of the chamber. Most

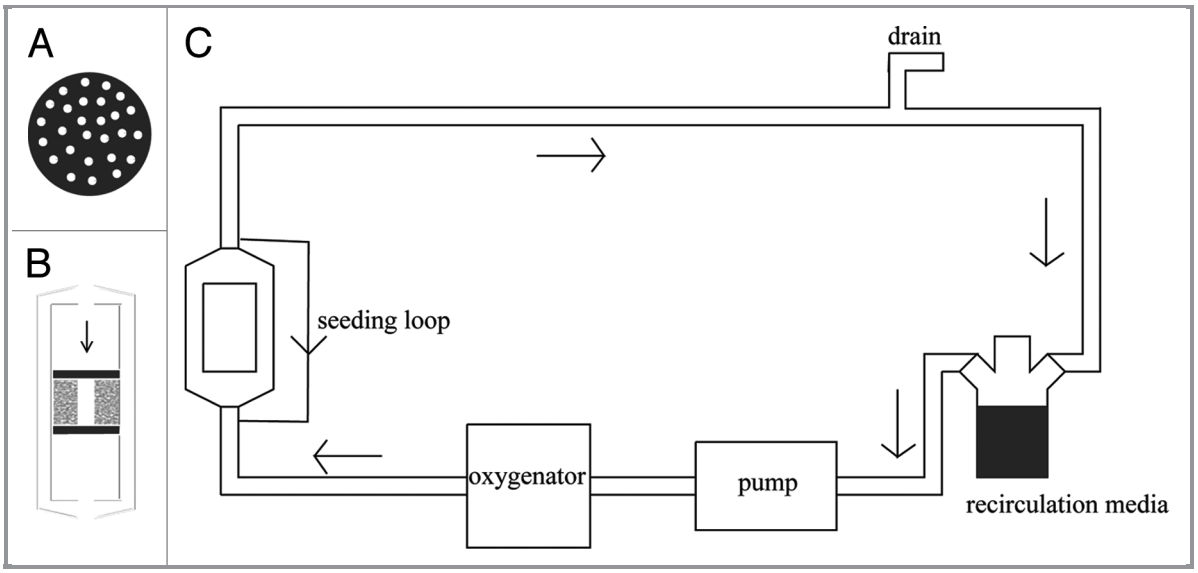

Figure 2. Schematic of the perfusion system described by Janssen et al. (**A**) Top view of the perforated lid and bottom, (**B**) detail of the perfusion chamber (scaffolds in gray and O-rings in black) and (**C**) representation of the complete system. Oxygen sensors are placed before and after the perfusion chamber.

importantly to actually obtain clinically relevant amounts of regenerated bone the perfusion chambers described might not be suitable as it is more complicated to force medium through a thicker or larger structure and ensure a uniform distribution of media and oxygen throughout the scaffold's depth and surface.

The Importance of Flow Rate and Shear Stress

One of the most important parameters when optimizing a bioreactor for this application is the flow rate and it needs to be considered that its value will also be affected by the design of the reactor and by the architecture of the three dimensional structure used in the system. Bearing this in mind, different flow rates have been experimented on some of the different perfusion systems investigated. In **Table 1** the values of flow rate for some of the reactors available can be seen. Still, there is a big variation of values and there are not many studies that compare a significant range of flow rates. However, in some cases, it is possible to detect a tendency. It appears that, up to a point, the increase in flow rate leads to an increase in the deposition of mineralized matrix.[51] Very low flow rates such as 0.01 ml/min have been reported to lead to higher cell viability than higher values, but this does not seem an optimal flow rate for bone tissue engineering applications as it might be too low to actually accomplish an adequate distribution of nutrients, oxygen and removal of waste products.[51,62] It is also necessary to bear in mind that lower values of flow rate will provide lower values of shear stress which might facilitate cell attachment and spreading, hence leading to higher values of cell viability. Despite the wide variation of flow rates tested, it seems that the optimal values would range from 0.2 to 1 ml/min, depending obviously on the system being used. This is the range of values that seems to have a more positive effect on osteoblastic differentiation, ECM deposition and distribution. Higher values such as 4 ml/min have been tested in comparison with static culture and after 19 d of culture there was not a significant difference in the amount of newly formed bone between the two.[30] Although this is not of the most disappointing results, very high flow rates can have a negative effect on cell attachment and disrupt formation and deposition of ECM, therefore obliterating the benefits associated with perfusion systems. Considering all of this, it seems of the outmost importance to optimize this parameter, but most studies in the field only look at distribution of seeded cells, micro-architecture and cell differentiation without actually optimizing the design and its flow characteristics. One example that shows the importance of this is described by Jaasma et al.[63] and in this work a new reactor design is validated by evaluating its performance within a range of flow rates of interest

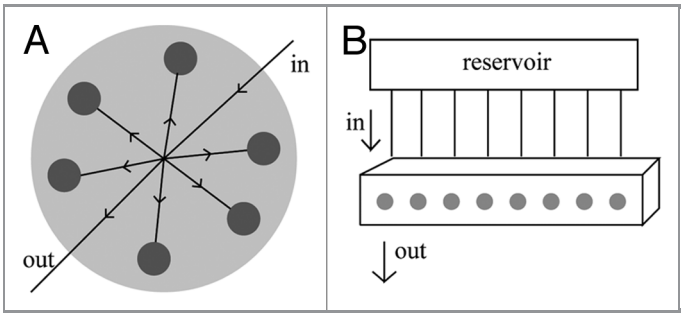

Figure 3. (**A**) Representation of the perfusion chamber described by Grayson et al.[32] The media goes in through one end and it is distributed equally by the six individual chambers (each holding one scaffold shown in gray) and finally goes out through the opposite end. (**B**) Representation of the system described by Cartmell et al.[62] The perfusion block is composed by eight individual chambers (each holding one scaffold, entrance of the chamber shown in gray). Each chamber is fed individually by a tube that comes from the reservoir.

Table 1. Selected perfusion systems corresponding flow rates and scaffolds used with them and respective pore sizes

First described by	Type of scaffold	Pore size	Flow rate
Bancroft et al.[29]	PLA nonwoven scaffolds	17 μm	0.6 ml/min[59]
	Titanium nonwoven fibers	Not reported	1 ml/min[52]
		250 μm	0.3, 1 and 3 ml/min[51]
		250 μm	0.3 ml/min[23]
		29.8 and 65.3 μm	1 ml/min[35]
	Skelite™	200 to 500 μm	0.1 ml/min[31]
	Coralline hydroxyapatite	200 to 500 μm	0.1 ml/min[36]
Cartmell et al.[62]	Trabecular bone	645 μm	1, 0.2, 0.1 and 0.01 ml/min
Grayson et al.[32]	Trabecular bone	600 to 1,000 μm	0.85 ml/min
Janssen et al.[30]	Biphasic calcium phosphate	> 100 μm	4 ml/min
Sailon et al.[20]	Polyurethane	200 μm	1 ml/min

and assessing flow profiles for steady, oscillatory and pulsatile flow. However, it is obvious that testing so many different parameters is exhausting and time consuming. One possible alternative is the use of computational fluid dynamics,[64,65] but these simulations are computationally intensive and can end up being as time consuming as the actual testing. To avoid these problems it has been suggested that approximations to model the fluid flow can be done based on the Darcy law (Eqn. 1, where u represents the volume-averaged velocity and it is proportional to *K*, the permeability tensor divided by the viscosity, μ, and to the pressure gradient, *p*) that describes fluid flow through a porous medium and this can be a simpler approach to understanding what actually goes on inside the constructs and the perfusion chamber.[66]

$$\bar{u} = \frac{K}{\mu}\nabla p$$ Equation 1: general form of the Darcy law.

Another very important parameter that is closely related with the flow rate (τ) is the shear stress. The shear stress on a point *y* at a distance ∂y from the surface is given by Equation 2, where μ is the viscosity and the velocity of the fluid on the surface.[67]

$$\tau(y) = \mu\left(\frac{\partial v}{\partial y}\right)$$ Equation 2: shear stress in fluids.

In vivo, bone cells are subjected to shear stresses that range from 8 to 30 dyn/cm^2 and in vitro has been shown that values from 2 to 10 dyn/cm^2 are sufficient to stimulate osteoblasts.[59,68] In the three dimensional constructs used under flow perfusion the values of shear stress to which cells are subjected are represented on **Table 2**. It can be seen that in these cases, the shear stress is very low, barely reaching 1 dyn/cm^2, which is lower than the values that have been shown to stimulate osteoblasts. As seen in **Equation 2**, the viscosity also influences the shear stress. This has also been studied by the supplementation of culture medium with different concentrations of dextran (0%, 3% and 6%). The increase of the concentration of dextran leads to an increase in viscosity. It was seen that the increasing concentration led to an increase in shear stress from 0.1 to 0.3 dyn/cm^2 and that it also improved distribution and amount of mineralized matrix.[23] Varying viscosity might be another alternative to study the effect of shear stress without altering scaffold architecture.

Table 2. Selected perfusion systems and respective shear stresses

Described by	Shear stress
Sikavitsas et al.[59]	0.05 dyn/cm^2
Bancroft et al.[51]	1 dyn/cm^2
Sikavitsas et al.[23]	0.1 to 0.3 dyn/cm^2
Sailon et al.[20]	0.02 dyn/cm^2
Goldstein et al.[27]	0.34 dyn/cm^2

Bearing in mind the importance of this parameter, it is necessary to try and optimize it as it might have a great influence on the osteoblastic behavior. Still, it is a difficult parameter to alter as it is influenced by characteristics such as pore size and flow rate and, although it can be easily estimated in some cases, there are situations where it is not possible to obtain accurate values and this is mainly due to the scaffold's architecture. The commonly used fluid flow model assumes that the scaffolds present a cylindrical pore geometry which is not precise in cases where fibrous meshes are used, for example, but the approximation can be made nonetheless.[52]

As seen in **Table 1**, the pore size of the constructs used varies greatly and there are only a few studies comparing different pore sizes. One study that used two titanium meshes with different diameters (20 and 40 μm) showed that the large diameter had a positive influence on early osteoblast differentiation and the smaller diameter had a more significative influence on the later differentiation and matrix deposition. The authors explain this by stating that the decrease in mesh size increases the shear stress and it seems to prove that the importance of shear stress on cell differentiation may vary depending on its stage.[52] A different group also tested different pore sizes (from 200 to 500 μm) in coralline hydroxyapatite but the results were not very conclusive regarding the effect of the varying pore size.[36] These studies clearly show that there is still a lot of work to be done regarding pore size and scaffold architecture and these have to be investigated alongside flow

Table 3. Selected perfusion systems that support oscillatory or pulsatile flow and some relevant parameters

Described by	Flow rate	Shear stress	Frequency
Du et al.[69,70]	0.5 ml/min	0.04 dyn/cm^2	1/60 Hz
Jagodzinski et al.[71]	10 ml/min	Not reported	0.1 to 20 Hz (Cyclic compression of 10%)
Sharp et al.[73]	3 to 6 ml/min	2.3 to 4.3 dyn/cm^2	0.015, 0.044 and 0.074 Hz
Kavlock et al.[72]	3.1 to 6.1 ml/min	0.21 to 0.42 dyn/cm^2	0.083, 0.05 and 0.017 Hz

rate and shear stress to better understand the how the fluid behaves in the perfusion systems.

The Effect of Dynamic Flow

Most of the systems described in previous sections support steady perfusion, meaning that there is a continuous flow through the scaffolds, but there are several systems that incorporate oscillatory or pulsatile flow (see **Table 3** for details). Overall oscillatory flow seems to have a beneficial effect over static culture. Du et al.[69] showed that it leads to increases in the amount of DNA and in ALP activity, with the last one being justified by the mechanical stimulation due to the oscillatory shear flow. This work only compares the oscillatory flow with static culture and it could be of interest to also compare with steady flow. The authors also suggest that the use of a non-continuous flow might be an advantage in a way that allows cells that detach to reseed again on the scaffold. Other studies have compared the use of continuous and non-continuous flow. The same author has performed this comparison and also showed an increase in ALP activity over the unidirectional continuous flow. The system described by that work also supports seeding and it also showed good results: it was seen a uniform distribution and proliferation of cells throughout the scaffolds.[70] Another work reporting cyclic compression also showed improvements when compared with steady perfusion, as it led to an increase in OCN and Runx2 expression after 21 d of culture.[71] Although there are a few studies showing that non-continuous flow has several advantages when compared with continuous flow, there are also a few studies that show that, although there is a slight increase in osteogenic markers, there doesn't seem to be a significant difference between the two and that it is not possible to detect a trend in the varying frequencies, but this might only be due to the use of different perfusion systems, different cell types, scaffold and range of frequencies.[72] The use of pulsatile flow also appears to have an effect on the expression of BMPs 2, 4 and 7, TGF-β and prostaglandin E_2 expression.[73,74]

Although it has not been shown that these systems are clearly superior to the ones that only support steady flow, there seems to an advantage when looking at cell seeding. Most of the dynamic flow systems support seeding and it seems to lead to more homogenous cell distributions and more uniform proliferation. Also, in most perfusion systems, the seeding is performed statically which will definitely have an effect on the final cell distribution and might not be an ideal alternative when studying dynamic culture.[69,70] Nevertheless there are a few systems that have been developed to support cell seeding and have shown promising results.[30,61]

Concluding Remarks

Bioreactor systems allow monitoring of environmental factors that provide the means to a better understanding of biological, chemical and physical factors involved in the formation of a specific tissue. Spinner flasks and rotating wall vessels have shown improvements over static culture, but they still are unable to promote adequate cell migration to the inner regions of the three-dimensional constructs and uniform formation of extracellular matrix. Although they still present some disadvantages, these systems are quite easy to implement and might be optimized. Although perfusion systems present a more complex alternative, they have shown better results regarding the induction of osteogenic differentiation and de novo bone formation in vivo. Still, there is a lot of progress to be made with these systems. Flow rates need to be optimized to try and supply the cells with a shear stress that approaches the in vivo values. It is also relevant to look at the cell seeding. Most systems don't support dynamic seeding and they should as this would reduce handling steps and it would make the reactors more clinically relevant. Also the reactors that support oscillatory or pulsatile flow seem to be a promising approach to solving this problem. More progress needs to be made to allow the implementation of these systems in a clinical environment. To achieve that they should support cell extraction from the patient, cell proliferation in order to obtain the necessary cell number, seeding in a biodegradable construct and automatic culture medium change to decrease the number of handling steps and supply adequate biochemical and physical differentiation cues, thus producing a ready-to-use functional substitute. For a generalized use of a tissue engineering approach based on bioreactors it is also necessary to make the up-scaling step from the laboratory to an industrial-scale. Only then tissue engineering based treatments will be economically viable and widely available.

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