The evolution of simulation techniques for dynamic bone tissue engineering in bioreactors

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Abstract

Bone tissue engineering aims to overcome the drawbacks of current bone regeneration techniques in orthopaedics. Bioreactors are widely used in the field of bone tissue engineering, as they help support efficient nutrition of cultured cells with the possible combination of applying mechanical stimuli. Beneficial influencing parameters of in vitro cultures are difficult to find and are mostly determined by trial and error, which is associated with significant time and money spent. Mathematical simulations can support the finding of optimal parameters. Simulations have evolved over the last 20 years from simple analytical models to complex and detailed computational models. They allow researchers to simulate the mechanical as well as the biological environment experienced by cells seeded on scaffolds in a bioreactor. Based on the simulation results, it is possible to give recommendations about specific parameters for bone bioreactor cultures, such as scaffold geometries, scaffold mechanical properties, the level of applied mechanical loading or nutrient concentrations. This article reviews the evolution in simulating various aspects of dynamic bone culture in bioreactors and reveals future research directions. Copyright © 2013 John Wiley & Sons, Ltd.

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Keywords dynamic tissue engineering; bone; bioreactor; simulation; mechanical stimuli; scaffold

1. Introduction

Bone tissue engineering combines the principles of engineering and life sciences to overcome drawbacks of traditional bone regeneration techniques used in orthopaedics (Sittichokechaiwut et al., 2010). The structural and mechanical characteristics of a tissue-engineered construct are intended to mimic the natural tissue as closely as possible and determine its success in clinical applications. The first strategies in three-dimensional (3D) bone tissue engineering consisted of static cultures, in which bone cells were seeded on a scaffold and placed in a well-plate for a defined period of time. This culture strategy led to different drawbacks: the cells tended to concentrate at the periphery of the scaffold, causing poor nutrient and waste exchange in the middle of the scaffold (Goldstein et al., 2001). This further led to cell necrosis in the centre of the scaffold. Dynamic bioreactors could help prevent such problems in cell culture.

Bioreactors are defined as devices that enable a closely monitored and tightly controlled environment to allow biological and biochemical processes to develop. Bioreactors provide a high degree of reproducibility, control and automation, which is favourable for specific experimental processes (Martin et al., 2004). Additionally, some bioreactors are able to apply physical stimuli, such as compression or shear stress, to the constructs. It was shown in vitro that mechanical stimulation improved cell behaviour and structure of engineered bone tissue using osteoblasts and stem cells (Botchwey et al., 2001; David et al., 2008; Sikavitsas et al., 2003).

Despite the numerous bioreactor designs, device parameters leading to improved reproducibility in bone tissue cultures have not yet been determined systematically, possibly because, until now, the parameters have been chosen by trial-and-error-approaches. A possible strategy to determine the effect of parameters influencing tissue-engineered outcomes is to simulate tissue-engineering systems. Simulations are able to compute stress and strain distributions, fluid shear stresses and velocities, bone ingrowth and several other aspects of bone tissue-engineering cultures, depending on the scaffolds’ properties. These simulations can then be compared with the
obtained in vitro results and parametric studies can indicate which factors have a significant effect on the engineered output. The first simulations of bone tissue engineering in bioreactors were performed in the early 1990s. Since then, the field of simulations in bone tissue engineering has evolved vastly. In the last decade the method of finite element (FE) modelling became more and more important, replacing earlier, simpler models. Micro-computed tomography (μCT) is a commonly applied imaging technique to obtain the 3D geometry of the simulated bioreactor–scaffold system, which then can be directly implemented into an FE model.

In this review we focus only on bone tissue engineering and the four most commonly used bioreactors in dynamic bone tissue engineering: (a) rotating wall vessel bioreactor; (b) spinner flask bioreactor; (c) compression bioreactor; and (d) perfusion bioreactor (Table 1). Hydrodynamic bioreactors will not be discussed, due to the lack of simulation studies in these bioreactors. For the same reason, some spinner flask studies cited in this publication were primarily intended for cartilage tissue engineering, but their results are potentially applicable to bone tissue engineering. Given the volume of work in the field of mathematical modelling in dynamic bone tissue engineering, it is not possible to be fully comprehensive. This review therefore aims to summarize past and current work and reveal future research directions that may be most relevant to optimizing bone tissue engineering. Optimizing cell-seeding strategies will not be covered in this review.

2. Bioreactors for dynamic bone tissue engineering

The first dynamic bioreactors were developed in the early 1990s (Dixit, 1994; Freed et al., 1993; Halberstadt et al., 1994; Wang and Wu, 1992), about 5 years after the evolution of the field of tissue engineering (Nerem, 1992). Dynamic bioreactors have been designed to overcome the drawbacks of static cultures, such as poor nutrient and waste exchange (Cartmell et al., 2003; El Haj and Cartmell, 2010). The four most prevalent bioreactor designs for bone tissue engineering are described in the following sections.

2.1. Rotating wall vessel bioreactor

The rotating wall vessel bioreactor is composed of two concentric cylinders (Figure 1A). The outer cylinder is capable of rotating and the space between the two cylinders is filled with culture medium (Schwarz et al., 1992). Scaffolds are freely suspended in the culture medium and are subjected to dynamic laminar flow (Vunjak-Novakovic et al., 1999), leading to low shear stresses and high mass-transfer rates (Singh and Hutmacher, 2009). Rotating wall vessel bioreactors have been primarily used for culturing cartilage tissue in vitro (Vunjak-Novakovic et al., 1999) and only a few studies on bone tissue engineering exist. It has been shown that rotating wall vessel bioreactors can improve the osteogenic differentiation of cells, increase mineralized extracellular matrix (ECM) production and enhance the distribution of cells throughout the 3D scaffolds. However, cell growth and mineralization were still limited to the outer surfaces of the 3D scaffolds, because internal diffusion limitations were not eliminated or cell sheets encapsulated the scaffold. The transport of nutrients to the centre of the scaffold was still limited because the convective forces could not extend to the interior of the scaffold (Goldstein et al., 2001; Sikavitsas et al., 2002).

2.2. Spinner flask bioreactors

Like the rotating wall vessel bioreactor, the spinner flask bioreactor uses convection to ensure that the culture medium surrounding the scaffold is well mixed (Goldstein et al., 2001). Spinner flask systems consist of a dual-side arm cylindrical flask with a rubber stopper serving as a cover. 3D scaffolds are attached to needles that pierce the rubber stopper, fixing them in place within the stirring medium. The distance from the scaffolds to the stir bar can be controlled by the position of the scaffolds on the needles. Scaffolds are completely covered with culture medium and a magnetic stirrer is placed at the bottom of the flask (Figure 1B), stirring the culture medium (Goldstein et al., 2001; Lee et al., 2011; Sikavitsas et al., 2002). A specialized spinner flask design is the wavy-walled bioreactor. This modified spinner flask bioreactor is fabricated by altering the radius of conventional spinner flasks by introducing grooves in the wall (Figure 1B). This design enhances the mixing of the culture medium while minimizing fluid shear forces (Bilgen et al., 2005; Bueno et al., 2005). Spinner flask cultures showed improved osteogenic differentiation compared to static or rotating wall vessel bioreactor cultures and increased calcium deposition at the scaffold’s surface (Goldstein et al., 2001; Meinel et al., 2004; Sikavitsas et al., 2002). Despite these advantages, spinner flask cultures showed sparse distribution of cells and mineralized ECM was only located at the outer surfaces of the 3D scaffolds (Meinel et al., 2004; Sikavitsas et al., 2002; Uebersax et al., 2006).

2.3. Compression bioreactors

Compression bioreactors are intended to mimic the macroscopic mechanical stimulus of bone in vivo (Figure 2). They are made up of a compression chamber with a piston, which applies compressive loads directly to the scaffold (Figure 1C) (Baas and Kuiper, 2008; Hagenmüller et al., 2010). Compression loading closely represents the in vivo mechanical stimulation of bone cells (Milan et al., 2009). Compression studies have been shown to improve cell ingrowth, ECM synthesis and alkaline

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<table>
<thead>
<tr>
<th>Scaffold properties</th>
<th>Scaffold material</th>
<th>Simulation/method</th>
<th>Experimentally validated</th>
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phosphatase (ALP) activity (Bolgen et al., 2008; David et al., 2008; Sitichockechaivut et al., 2009). The upregulation of ALP in vitro has been generally associated with the onset of osteogenic differentiation (Hutmacher, 2000). However, there are still large differences between different papers about the ideal level, duration and frequency of the applied loading condition.

2.4. Perfusion bioreactors

The perfusion bioreactor aims to mimic the microscopic mechanical loading of bone in vivo (Figure 2) (Allori et al., 2008). Perfusion bioreactor systems pump culture medium through the scaffold’s interconnected pores and the scaffold is press-fitted into a culture chamber (Figure 1D) (Bancroft et al., 2003; Gomes et al., 2003; Sailon et al., 2009). Flow perfusion enhances the mass transfer at the interior of the 3D scaffold and exerts shear forces on the cultured cells. Several studies have shown that the increased mass transport led to improved distribution of ECM throughout the 3D scaffold, increased cell number, enhanced expression of the osteogenic phenotype and improved mineralized ECM deposition, compared to other bioreactor systems (Bancroft et al., 2002, 2003; Gomes et al., 2003; Sikavitsas et al., 2002, 2003).

Although dynamic bioreactors could overcome diffusion limitations at the surface of a scaffold, only the perfusion bioreactor was able to eliminate diffusion limitations inside a scaffold (Bancroft et al., 2003; Meinel et al., 2004). Consequently, the perfusion bioreactor seems to be a very useful dynamic culture technique for bone tissue engineering (Goldstein et al., 2001) and is the most commonly used dynamic bioreactor nowadays (Lacroix et al., 2009).
3. Simulation techniques

Simulations are widely performed in bone tissue engineering today (Byrne et al., 2007; Cioffi et al., 2006; Maes et al., 2009; Sandino et al., 2001). Generally, simulations in bone tissue engineering can be divided into simulations of the mechanical environment (Milan et al., 2009; Qiu et al., 1999; Sucosky et al., 2004), simulations of the biological environment (Galbusera et al., 2007) or simulations of both environments (Baas et al., 2010; Lappa, 2003; Milan et al., 2010).

The mechanical environment is the sum of all physical forces acting in a system. Early simulation strategies of mechanical environments in bioreactors were based on simple numerical models. These models solve a problem and give a solution in numbers. Numerical models were most prevalently used to simulate the motion of scaffolds in rotating wall vessel bioreactors (Botchwey et al., 2001; Boyd, 1990; Pollack et al., 2000). The advantages of numerical models are that they are easy to solve, can be calculated in a short time and give a rough estimate of the solution. The latter, however, is also a major disadvantage of numerical models. Usually, numerical models apply a lot of rough assumptions, e.g. rigorous geometrical simplifications. This leads to inaccurate results that are very far from the reality. Experimental approaches analysing the motion of a fluid or scaffolds in a bioreactor evolved in the late 1990s and were used until the early twenty-first century. Particle image velocimetry (PIV) is one of these approaches. PIV is an optical method to determine the velocity field of moving fluids. The speed of scaffolds can be calculated from the temporal description of the scaffold motion tracked by PIV. Compared to numerical models, experimental approaches are measurement-based and resemble reality more closely. Major limitations arise due to measurement devices. In the case of PIV, the image sensor limits the resolution and when using one sensor the method is limited to two dimensions (2D) (Pollack et al., 2000).

The evolving bioreactors, spinner flask, compression and perfusion, hold the scaffolds fixed in place and the motion of the scaffolds is no longer an issue. In 2001 a simple mechanical model, the cylindrical pore model, was introduced for the estimation of shear stresses in perfused porous structures (Goldstein et al., 2001). It is based on the assumptions that flow is uniformly distributed across a structure surface of a given diameter and the flow of the culture medium is parabolic, and the pores are represented as a bundle of parallel cylindrical pores with a diameter equal to the average pore diameter. Wall shear stresses can then be calculated according to:

$$\tau_{\text{wall}} = 8\mu V_m / d$$

where $\mu$ is the viscosity of the fluid, $V_m$ is the mean velocity of the fluid in the pores and $d$ is the mean diameter of the pores. The cylindrical pore model is still frequently used today (Grayson et al., 2011; Jungreuthmayer et al., 2009). It gives researchers a quick indication of approximate values of shear stresses in porous scaffolds under perfusion. The geometrical simplifications, however, do not resemble the mostly complicated, porous and interconnected geometry of 3D scaffolds. Additionally, shear stresses in highly irregular scaffolds must be described by a shear stress distribution, as shear stresses in small pores are lower than in large pores (Boschetti et al., 2006).

Over time, mechanical models became more complex. FE modelling is a numerical technique for finding the solutions to differential and integral equations. For FE modelling, a geometrical shape is subdivided into a finite number of small elements. The displacement of the elements under loading is then modelled for each element and stresses and strains are computed. A subgroup of FE modelling is computational fluid dynamics (CFD). In CFD a fluid flow through the void space of a shape is simulated, and fluid shear stresses are then calculated from the fluid velocities. For all FE models the underlying geometries are obtained either directly by imaging or with the help of computer-aided design (Figure 3). FE models have the advantage that they are much more accurate than simple analytical and numerical models. The accuracy of the geometry is highly increased because it is based on a real geometry. FE modelling is a powerful tool and is able to predict local mechanical effects, even at cellular level (Boccaccio et al., 2011). Nevertheless, calculating mechanical environments through complete scaffolds is far from evident because of the lack of computational power, especially in CFD models (Maes et al., 2009). Today, models often no longer reflect a

Figure 3. Meshed scaffold structures. (A) Silk fibroin scaffold: the scaffold structure was obtained by microcomputed tomography imaging at a resolution of 6 μm; the scaffold was coarsened for the meshing procedure. Reprinted with permission from Simpleware Ltd (Exeter, UK). (B) Gyroid shape and (C) hexagonal prism: these two structures were built artificially and meshed with a triangle surface mesh. Reprinted from Olivares et al. (2009), with permission from Elsevier
whole system because they have been reduced in size or accuracy.

In addition to the mechanical environment, the biological environment in bioreactors can be simulated. The biological environment includes concentrations of nutrients and waste products, tissue growth and cells. Simulations of the biological environment in bioreactors started in the late 1990s with numerical simulations of bone growth, based on mechanobiological models (Galbusera et al., 2007; Grayson et al., 2011; Lappa, 2003; Sanz-Herrera et al., 2008). A mechanoregulatory algorithm was first introduced by Prendergast et al. (1997). Briefly, mechanical stimuli are calculated with FE modelling in each element of a material. The tissue phenotype is then determined according to predetermined tissue thresholds. Material properties are updated according to the determined tissue phenotype, because each tissue phenotype has different material properties. The differentiation thresholds can be adapted to match other tissue phenotypes or even to under- or overloading (Milan et al., 2010). Originally, the mechanoregulation theory was strain-based, but was further adapted to take shear stresses into account (Olivares et al., 2009; Sandino and Lacroix, 2011). The downside of the mechanoregulation theory is the choice of threshold levels and material properties. The growing tissue is highly irregular in shape and composition. Additionally, the stiffness of one tissue type changes depending on its phenotype (e.g. cortical/cancellous bone). Hence, it is not valid to set only one threshold for an entire structure. Despite these limitations, very good results have been observed for simulations of osteogenesis in scaffolds using the mechanoregulatory model (Milan et al., 2009). Several other models have been adapted and individualized to simulate specific attributes of bone cell cultures in bioreactors. An organic crystal growth model was used to model tissue growth over time (Lappa, 2003), mass conservation, diffusion–convection, and enzymatic kinetics were used to model oxygen concentration as well as oxygen transport in scaffolds (Pierre and Oddou, 2007; Truscello et al., 2011) and a random walk algorithm simulating cell migration (Galbusera et al., 2007), just to name a few.

The application of these mathematical models will now be described for the four major types of bioreactors used in bone tissue engineering, with respect to mechanical, biological or combined mechanical and biological environments. For some bioreactor types, literature is lacking for one or more of those environments. In consequence, the three environments are not discussed for all bioreactor types.

3.1. Simulation of rotating wall vessel bioreactors

3.1.1. Simulation of mechanical environment

Boyd (1990) was one of the first to model the flow field of circulating culture medium in a rotating wall vessel bioreactor. He showed that the circulation in the middle of the rotating wall vessel was poor and that maximum shear stresses occurring at the scaffold’s surface were in the range 0.0002–0.0013 Pa. Nevertheless, the study was purely mathematical and did not contain comparisons to, or implementation of, experimental observations. About 10 years later Qiu et al. (1999) calculated maximum shear stresses on scaffolds, based on experimentally recorded trajectories (Figure 4). A camera recorded the locations of the scaffolds while the rotating wall vessel was turning. Based on trajectory recording, the maximum shear stresses were calculated to be around 0.06 Pa, which were about 50 times higher than the maximum values observed by Boyd. A bone cell line culture did not show any detrimental effects to the cells, and cells were able to attach to the scaffolds and form ECM and mineral nodules (Qiu et al., 1999). A similar study developed a numerical model to describe the trajectory of a scaffold in a rotating wall vessel bioreactor, which was validated with PIV; the authors could show that the predicted results from the numerical model were in excellent agreement with experimental measurements (Pollack et al., 2000). The results were compared to an experimental cell study performed under similar culture conditions. A bone cell line was cultured for 7 days in a rotating wall vessel bioreactor on poly(β,γ-lactic-co-glycolide acid) (PLGA) scaffolds. It was shown that cells penetrated as deep as 800 μm into the scaffold (Botchwey et al., 2001). This is four times higher than a penetration depth of 200 μm under static conditions in a scaffold with a similar pore size distribution (Ishaug et al., 1997). ALP activity was higher for cells cultured in the rotating wall vessel bioreactor than in static culture after 7 days. This confirmed the results predicted by the simulation: (a) the motion of the scaffold in the bioreactor created a convective flow at the scaffold’s surface, which could have led to the increased penetration of the cells; and (b) maximum shear stresses were determined between...
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0.27 to 0.47 Pa, which could have led to the increased ALP expression (Botchwey et al., 2001). The combination of simulations with experimental observations in rotating wall vessel bioreactors built the basis for further simulations on tissue-engineering cultures in other bioreactors. The simulations of rotating wall vessel bioreactors were able to reveal distinct influences on bone cell cultures; however, the results are of a qualitative nature and indicate only a rough estimation of culture properties.

3.1.2. Combined simulation of biological and mechanical environment

Lappa introduced a 2D mathematical model in 2003 including not only mechanical properties but also bioreactor-specific features for growth, nutrient transport, nutrient transformation into organic tissue, mass variation of the specimen, and growing tissue. A combined model simulated mass transfer at the scaffold surface to determine the concentration field of glucose in the liquid phase. The model was further adapted by including biological tissue growth rate to take the three main aspects of growth behaviour into account: (a) availability of nutrients; (b) slow surface kinetics; and (c) the effect of surface shear stress. The simulations were compared to an experimental study, using the same bioreactor (Obradovic et al., 2000).

It was shown that especially the corners and edges of the scaffold were supplied with a higher amount of glucose. The growth simulation was able to reproduce the morphological evolution observed by Obradovic et al. (2000) (Figure 5). Fluid shear stress distribution was the major factor influencing oxygen absorption kinetics and tissue growth. Shear stresses at the scaffold’s surface were non-uniformly distributed and changed over time due to tissue growth. In regions with lower shear stresses, tissue growth was increased, likely because fluid close to stagnation allows for better surface absorption kinetics of glucose. However, these results are in disagreement with other studies showing increased growth with increased shear stresses. This study was one of the first concerning the biological environment and the influence of growing tissue as a moving boundary condition in rotating wall vessel bioreactors. The results, however, have thus far only been observed in 2D cartilage constructs (Lappa, 2003).

3.2. Simulation of spinner flask bioreactors

3.2.1. Simulation of mechanical environment

Sucosky and colleagues (2004) introduced one of the first simulations describing the flow field in a spinner flask bioreactor. A CFD approach and experimental PIV were compared to assess the validity of the results. Both the simulation and the experiment were conducted under the same culture conditions. CFD simulations showed that the velocity components differed approximately 20% from the results of the PIV, but both the trends and amplitudes of the velocities were similar. The simulated maximum shear stress of the CFD was 0.21 Pa and was located near the lower surface of the scaffold as well as along the vertical wall. Computational simulation of the shear stresses was in agreement with the experimental results. The maximum shear stress of the PIV was 0.25 Pa and occurred at the lower surface of the scaffold, which was expected for a spinner flask with a magnetic stir bar placed at the bottom. The maximum shear stress level differed by about 16% from the simulation, because the vertical position of the scaffold in the experimental model was lower, and therefore the construct experienced higher fluid velocities because of the vicinity to the stir bar. This study showed that differences between experimental and computational simulations can be up to 20%. The outcomes were very encouraging, but they only constituted an initial step in the design of a reliable tool to investigate effects of culture medium convection (Sucosky et al., 2004) and independent experimental verification is missing.

Bilgen et al. (2005) and Bueno et al. (2005) performed simulations using a wavy-walled bioreactor. They characterized the effect of the wavy walls on flow patterns by CFD and compared the results to the simulation of a regular spinner flask. The wavy-walled bioreactor showed a more uniform distribution of flow patterns in the bioreactor. The velocity in the middle, where the scaffolds were placed, was doubled compared to a regular spinner flask, indicating
a beneficial effect of the wavy walls on the mixing of culture medium. Compared to the regular spinner flask, the resulting average shear stresses increased by 6%. The authors predicted the larger shear stresses to enhance aggregation of cartilaginous cells and increase nutrient and gas transport at the scaffold’s surface. Bueno et al. (2005) verified the CFD results by performing a cell study using calf chondrocytes. After 4 weeks of culture, chondrocyte proliferation and matrix deposition were enhanced, confirming the positive effects of the wavy-walled design. This study revealed how important the design of the bioreactor itself is. Not only should scaffold geometry be incorporated in simulations, but also the geometry of the bioreactor itself.

In general, spinner flask bioreactors were used primarily for cartilage tissue engineering. Initially, the mechanical environment in spinner flask bioreactors seemed to be beneficial for bone tissue engineering cultures as well, although compression and perfusion bioreactors have evolved, leading to better results in bone tissue cultures than with spinner flask bioreactors.

3.3. Simulation of compression bioreactors

Compression bioreactors are often combined with the application of perfusion (Baas et al., 2010; Sandino et al., 2008). This combination closely resembles the in vivo loading of bone: macroscopic compression load and microscopic perfusion flow (Figure 2). Therefore, combined compression–perfusion studies will be mentioned in the following sections, along with conventional compression studies.

3.3.1. Simulation of mechanical environment

Lacroix et al. (2006) investigated the effect of the load transfer from three differently prepared calcium phosphate (CaP)-based scaffolds to the cells. Scaffolds were scanned using μCT and scans were divided into smaller cylindrical volumes of interest (VOIs), due to computational limitations. A compressive axial strain of 0.5% was applied on the upper part of the scaffolds. The obtained results showed a variation in strain values by a factor of 3–4 between the different materials, but the preparation of the CaP-based scaffolds did not influence subsequent mechanical behaviour.

Sandino et al. (2008) showed that compressive loads applied to irregular scaffolds led to different strain levels throughout the scaffold, according to its morphology and geometry. Under 0.5% global strain, local compressive strains were in the range 0.2–0.6%. High changes in fluid velocity were observed at 1, 10 and 100 μm/s, with regions of almost no flow and regions with high-velocity fluid flow. The majority of shear stress values were around $5 \times 10^{-3}$ Pa. Maximum shear stress values were about 800 times higher than mean stress values (0.0004 Pa). The distribution of stresses and strains was highly heterogeneous throughout the scaffold structure (Baas et al., 2010; Sandino et al., 2008). Irregular scaffolds are mostly used in bone tissue engineering today, due to the variety in manufacturing methods and because they mimic the geometry of cancellous bone. However, this irregularity leads to a high variation of the mechanical loads acting on cells in vitro, which makes it hard to control the mechanical stimulation of cells. Such studies should be combined in the future with in vitro studies to improve the understanding of tissue differentiation in a scaffold.

Milan et al. (2009) analysed the mechanical environment induced by dynamic compression loading and perfusion flow on the basis of a μCT scan of a polyactic acid (PLA)–glass composite scaffold. A steady fluid flow of 100 μm/s and a dynamic compression of 5% at a strain rate of 1/s were applied to the PLA–glass scaffold within a cylindrical bioreactor. The highest fluid-flow velocities were found in the centres of the scaffold pores, whereas the lowest velocities were allocated near the pore walls. Mean stress occurring in the glass part was four times higher than the mean stress calculated for the PLA part, because the glass part was 20 times more stiff than the PLA part. Large standard deviations showed a heterogeneous distribution of stresses and strains within the scaffold. The authors could show that the architecture of the scaffolds could have a big influence on mechanical stimulation of seeded cells, but they did not perform a cell study to confirm this statement. The mechanical environment analysis performed is of great interest for bone tissue engineering, because it closely resembles the in vivo macroscopic and microscopic mechanical stimulation of bone. However, the authors did not simulate both loading conditions simultaneously. It would be interesting to analyse the combined effects of simultaneous compression and perfusion loading. Apart from that, a heterogeneous, composite scaffold design could introduce interesting effects on cultured cells, such as increased bone density at stiffer sites, or decreased bone density at softer sites.

3.3.2. Combined simulation of biological and mechanical environment

Milan et al. (2010) simulated cell differentiation under dynamic compression. The geometry of a PLA–glass scaffold was reconstructed from μCT scans and a biological material containing fluid, cells and matrix was simulated to fill the scaffold pores. A mechanoregulation algorithm was applied to determine the differentiation of the biological material. The algorithm predicted formation of immature and mature bone for compressive strains of 0.5–1% at strain rates of 0.0025–0.005/s in the middle of the pores. Cartilage and fibrous tissue formation was predicted at higher strain levels and close to the pore walls (Milan et al., 2010). This simulation study was a further development of their study in 2009, where the authors simulated compression and perfusion loading separately. The combination of compression and perfusion loading closely resembles the in vivo loading conditions (Figure 2). Concerning tissue-engineering applications, the simulation
of biological tissue completely filling the scaffold pores is not applicable for early time points, because cells and matrix would not yet be filling the pores completely. Therefore, this model should only be used for simulating late stages of tissue-engineering applications where the pore volume is already completely filled with tissue. The results of Milan et al. (2010) were in agreement with the study of Olivares et al. (2009). Olivares and colleagues studied the interactions between scaffold morphology and applied culture conditions on two regular scaffold structures: gyroid and hexagonal (Figure 3B, C). The resulting strains and fluid shear stresses were calculated at an axial strain of 5% and an inlet velocity of 1 mm/s, respectively. The mechanoregulation theory of Prendergast et al. (1997) was adapted to take the combined effects of strains and shear stresses into account. Strain values of 0.5–2.5% showed a prevalence of osteogenic differentiation, whereas chondrogenic differentiation appeared at 2.5% strain. An inlet velocity of 0.001 mm/s was favourable for bone stimulation in both geometries. Hexagonal scaffolds showed fewer changes in fluid path, leading to a better distribution of strains for bone phenotype. Beside the fact that artificial scaffolds do not represent physiological bone geometry, they are often advantageous to simulating the effects of mechanical stimulation in a controlled manner. The study presented is one of the first combining simultaneous compression and perfusion stimulation. However, the results were again presented concerning only compression or perfusion effects. Under physiological conditions, a bone is always affected by both loading regimes and this should therefore be considered in the future.

Sandino and Lacroix (2011) confirmed the results of their earlier study from 2008, in which they simulated the mechanical environment of compression bioreactors. Additionally, they determined tissue differentiation under 0.5% strain load and 10 μm/s perfusion flow, using the mechanoregulation theory. Stimulus distribution was highly heterogeneous. Some stimuli increased up to the load range of cell death in regions with very high shear stresses. This effect occurred because the predicted tissue formation decreased the porosity until the pores were completely filled with tissue (Sandino and Lacroix, 2011). This effect can also be observed in vitro. Growing tissue within a scaffold leads to smaller or obstructed pores, which results in increased shear stresses acting on the cells in these pores, assuming the cells to be sitting on top of the growing ECM. The global mechanical stimulation should therefore be adapted to the growing tissue. This study demonstrated one possible solution to simulating in vitro tissue growth in porous scaffolds and, according to these results, the in vitro culture conditions could be adapted.

Baas et al. (2010) combined an FE model with an experimental bone cell study (Baas and Kuiper, 2008). The same experimental conditions for macroscopic compression were simulated as previously described (Baas and Kuiper, 2008). Scaffolds were scanned with μCT before the start of the cell study. The seeded scaffolds were maintained in static culture for 2–3 weeks and were then dynamically loaded in a compression–perfusion bioreactor at 1.5% strain and 1 Hz for 1 h daily, for 1 week. In addition to the compression loading, the scaffolds were perfused continuously at a rate of 0.1 ml/min. After a total of 4 weeks of culture time, all the scaffolds were scanned again. Both datasets from the pre- and post-scans were then compared for each scaffold separately to correlate local principal strain at the start of the culture with local mineralization at the end of the culture (Figure 6). The average value of principal strain before the culture was significantly higher at sites where mineralized ECM had formed compared to sites where no mineralized ECM had formed. The results showed that bone cells in a 3D environment are sensitive to surface strain, leading to mineralized ECM formation in locations with higher local strains. This study is one of the few studies showing a direct connection between local mechanical stimuli and mineralized tissue formation in a 3D environment (Baas et al., 2010).

An important future step in simulating the behaviour of compression bioreactors is the combination with perfusion loading, because until now compression and perfusion have been modelled separately (Olivares et al., 2009; Sandino and Lacroix 2011; Sandino et al., 2008).

Figure 6. Micro-computed tomography images of a scaffold before and after culture: (A) empty scaffold before culture; (B) strain distribution in the empty scaffold; (C) scaffold after culture with mineralized nodules formed (orange). Mineralized nodules have formed at locations of higher strains. Reprinted from Baas et al. (2010), with permission from Elsevier.
3.4. Simulation of perfusion bioreactor

3.4.1. Simulation of mechanical environment

Goldstein et al. (2001) predicted shear stresses with the cylindrical pore model for a porous PLGA scaffold. The applied flow of 0.03 ml/s/scaffold led to a shear stress of 0.034 Pa. Experimental results showed that this fluid flow applied to the cultured cells improved cell distribution in the scaffold and led to increased osteogenic differentiation (Goldstein et al., 2001). Similar studies were performed by others (Grayson et al., 2011; Vance et al., 2005). Vance et al. exposed bone cells seeded on CaP scaffolds to high-rate oscillatory flow, low-rate perfusion flow and static culture conditions. With the use of the cylindrical pore model, the shear stresses in continuous perfusion at a flow rate of 0.025 ml/min and under oscillatory flow at 1 Hz, with a peak flow rate of 40 ml/min, resulted in 0.0007 and 1.2 Pa, respectively. At both shear-stress levels, the release of prostaglandin E2, which is thought to have an anabolic effect on bone but is also an inflammatory marker, was significantly increased. DNA content was not affected, despite the high fluid flow velocity (Vance et al., 2005). Grayson et al. (2011) investigated the influence of perfusion flow velocities in the range 80–1800 μm/s on human mesenchymal stem cells (hMSCs) seeded on bone scaffolds, calculating shear stresses using the cylindrical pore model. Shear stresses increased with increasing fluid flow velocity from 0.0006 to 0.02 Pa. An optimal range of flow velocities resulting in the highest ECM deposition for hMSCs seeded on bone scaffolds was determined to be between 400-800 μm/s. The range of shear stresses shown to be beneficial for bone cell cultures is very broad, with the highest value being 2000 times higher than the lowest value (0.0006–1.2 Pa). This shows the low specificity of the cylindrical pore model, due to numerous assumptions. Jungreuthmayer et al. (2009) compared the results of the cylindrical pore model to a μCT-based CFD model. Analytical results of the wall shear stress using a velocity of 235 μm/s showed values of 0.022 and 0.903 Pa for collagen–glycosaminoglycan (CG) and CaP scaffolds, respectively. Mean wall shear stresses acting on CG scaffolds were 0.019 Pa, and CaP scaffolds experienced a mean wall shear stress of 0.745 Pa, as determined by CFD. These results suggested that the analytical model of Goldstein et al. (2001) overestimates the wall shear stresses, especially at higher fluid flow velocities. This could lead to a suboptimal stimulation of cells when using the analytical model to determine culture parameters. At high flow velocities the difference between the two models is > 20%, confirming the assumption of the low specificity of the cylindrical pore model.

Scaffold properties such as connectivity, porosity and pore size play an important role in perfusion cultures. Simulations of regularly shaped scaffolds are a straightforward method to investigate different scaffold properties. Boschetti et al. (2006) simulated the influence of porosity and pore size on mechanical environment with a simple 2D scaffold model. The scaffold was modelled by subtracting a solid sphere from a concentric solid cube. The dimensions of the sphere and the solid cube were varied to obtain different pore sizes and porosities. The velocity map looked the same when simulating with constant porosity, independent of pore size. The local velocity gradient was bigger for smaller pores. As expected, the wall shear stresses were higher with a smaller pore size. Wall shear stresses appeared to be independent of the porosity at constant pore size, except for a small area around the inlet and outlet, where high shear stresses were observed (Figure 7). The values of shear stresses were roughly constant with increasing porosities but increased with decreasing pore size, which shows that the pore size is a parameter strongly

![Figure 7](image-url)
influencing the predicted wall shear stress. This study had the advantage that the observed results were qualitative and could be easily transferred into 3D and more complex scaffolds, where the same rules apply, as Yao et al. (2010) showed; they modelled an entire scaffold and compared the results against those of Boschetti and colleagues, who modelled only a microdomain of the scaffold containing 27 pores. Yao et al. (2010) confirmed that the velocity map showed the same trend with constant porosity: shear stress decreased with increasing porosity and marginal regions showed higher shear stresses than the rest of the scaffold.

CFD simulations are widely used in combination with μCT (Cioffi et al., 2006; Maes et al., 2009; Porter et al., 2005; Voronov et al., 2010). CFD models are especially suitable for predicting fluid velocities, fluid pressure and the fluid shear stresses acting on cells (Boschetti et al., 2006; Olivares et al., 2009; Yao et al., 2010). Porter et al. (2005) performed one of the first studies combining a μCT scan of human trabecular bone, defining the physical boundary conditions for the CFD model. The highest flow velocities were observed at the centre of small orifices and the lowest flow velocities were observed at the scaffold surface and bioreactor chamber walls. These results confirmed the basic conceptual simulations of Boschetti et al. (2006) on a physiological sample. Local shear stresses experienced by cells at a constant flow rate can be vastly different, ranging from 0 to 0.0002 Pa, because of the irregular scaffold geometry. A similar phenomenon was that with a smaller VOI scaffold size and porosity led to a more constricted oxygen distribution and transport within perfusion bioreactors. The authors concluded that the minimal model size should be at least twice the pore size, but as large as possible to cover the heterogeneity and the actual micro-architecture of the scaffold completely. Compared to other simulations in the context of porous material properties, the minimum size of a representative VOI for a continuum domain was determined to be at least 3.5 times the pore diameter (Petrasch et al., 2007; Zermatten et al., 2011). Defining the smallest valid VOI representing the whole scaffold is of significant importance for reducing the computational power of very large models.

Unfortunately, experimental data to validate perfusion bioreactors is mostly missing. One paper quantitatively evaluating the effects of shear stresses on hMSCs, both mathematically and experimentally, is the study of Zhao et al. (2007). At a flow rate of 1.5 ml/min the velocity in the scaffold decreased from 2.0 μm/s at the surface to 0.1 μm/s at a depth of 70 μm. The corresponding shear stresses decreased approximately one order of magnitude. For a fluid flow velocity of 0.1 ml/min, the velocity and shear stresses at the surface were all lower than 0.1 μm/s and 10⁻⁵ Pa, respectively. A cell study was performed with identical settings. Poly(ethylene terephthalate) scaffolds were seeded with hMSCs and were cultured at a flow rate of either 0.1 or 1.5 ml/min for 20 days. The results of the cell study showed that perfusion flow, even at very low flow velocities, had a significant effect on the cultured hMSCs. Low flow velocity led to higher cell numbers, whereas higher flow led to statistically increased ALP activity and calcium deposition (Zhao et al., 2007). These results were in agreement with previous studies showing the same effects of fluid flow in osteogenic cultures (Cartmell et al., 2003; Datta et al., 2006; Van den Dolder et al., 2003).

3.4.2. Simulation of biological environment

Truscello et al. (2011) and Pierre and Oddou (2007) investigated the effect of perfusion flow on oxygen distribution and transport within perfusion bioreactors. Truscello et al. (2011) simulated oxygen distribution in a perfusion bioreactor and determined the critical length of the scaffold to guarantee a given target range of oxygen tension. Cells were simulated either as an attached cell layer on the scaffold walls or suspended in the culture medium. The critical scaffold length was determined to be proportional to the inlet velocity and inversely proportional to the cellular consumption rate and cell density, using a 1D model (Truscello et al., 2011). Pierre and Oddou (2007) investigated oxygen concentration subject to the inlet velocity for a large bone implant (d = 10 mm, l = 25 mm). Cells were modelled as a monolayer attached to the scaffold surface. The simulation showed that having a local oxygen concentration sufficient for cell metabolism, 64% of the scaffold surface was considered to be loaded under detrimental mechanical conditions (shear stresses > 10⁻³ Pa). Considering a lower inlet velocity, scaffold surface is loaded under adequate mechanical conditions but oxygen concentration on 32% of the scaffold surface is under hypoxic conditions. Compared to the study of Truscello and colleagues, this study shows the importance of not looking only at one factor (e.g. mechanical loads) of cell culture study. The study presented is very promising for a transformation into a 3D model, because it simulates the multiple biological properties of a cell culture: (a) cells; (b) oxygen concentration; and (c) oxygen consumption (Pierre and Oddou, 2007). Galbusera and colleagues (2007) performed an even more detailed study, including cell population dynamics. They determined oxygen transport and
consumption, hydrodynamic environment and cell movements for the prediction of in vitro tissue growth. All culture conditions but the hydrodynamic environment were simulated by a cellular automaton. A cellular automaton usually consists of a regular grid of cells. Briefly, the scaffold was divided into equally spaced points defined as scaffold or fluid. Each of these points could host one cell and was able to be in one of three states: (a) moving at a migration speed of 1 μm/s; (b) stationary, due to a collision with another cell; and (c) stationary due to a collision with the scaffold wall. If the cell is in state (a), it keeps moving. Cell divisions were also taken into account (Figure 8). A homogeneous oxygen concentration of 0.2 nM/ml was initialized in the whole fluid. The simulations showed a first stage of exponential cell growth, followed by a deceleration when the volume fraction of occupied nodes was > 0.2 and a final decrease of growth rate due to the filling of available spaces. A higher cell number had a significant effect on oxygen concentration, as cell consumption led to decreased oxygen concentration. Perfusion flow reduced the drop of oxygen concentration between the inlet and outlet, as compared to statically cultured scaffolds. The study focused on oxygen concentration only while nutrient diffusion and waste removal were not taken into account. Another important limitation was the absence of an ECM in the simulation, which modifies the cellular microenvironment, especially the oxygen concentration. Additionally, the influence on fluid velocity due to filling of the available pore space was not investigated (Galbusera et al., 2007).

The increasing number of papers published on bone tissue engineering with perfusion bioreactors reflects the importance of this bioreactor type. Experimental studies were able to show promising effects of perfusion on cell number (Cartmell et al., 2004; Zhao et al., 2007) or mineralized ECM formation (Sikavitsas et al., 2003, 2005). Simulations strongly support the need to determine the best influencing parameters for bone tissue cultures.

4. Discussion and future directions

The application of simulations in dynamic bone tissue engineering using bioreactors has evolved over the last two decades as a crucial research field, building bridges between biology and engineering. Since the early 1990s simulations have played an important role in bone tissue engineering, advancing from simple numerical models (Boyd, 1990; Goldstein et al., 2001) to very complex, but more accurate, computational simulations (Baas et al., 2010; Sandino and Lacroix, 2011; Voronov et al., 2010). Most of the studies presented in this review are still in their infancy concerning their expected use in predicting the development of bone-like tissue cultures over time. The future of simulations of bone tissue engineering in bioreactors is promising. Technological improvements such as imaging techniques with higher resolution and increased computational power will enable simulations of whole scaffolds and an increased number of parameters for more accurate and physiologically relevant simulations in the future.

The understanding of tissue construct development and growth must be enhanced to catch the relationship between the bioreactor’s environment and cellular responses. The environment provided by bioreactors needs to be precisely controlled to produce: (a) more reproducible methods; (b) more realistic in vitro studies, imitating environmental cues acting on cells in vivo; and (c) precise and controlled application of environmental cues to improve and optimize cell response (Lacroix et al., 2009).

Most simulations modelling biomechanical laws remain poorly developed (Singh and Hutmacher, 2009). This is mainly attributable to moving boundary conditions and the assumptions made in computational models. Simulations performed in 2D do not reflect the geometry of bone in vivo. It was shown in vitro that the dimension has a major influence on the effect of mechanical stimulation (Yu et al., 2004). Despite the fact that the true geometry of the scaffolds can be obtained by various imaging techniques, regularly shaped scaffolds were often used to perform simplified simulations (Bilgen et al., 2005; Boscetti et al., 2006; Olivares et al., 2009). These scaffolds do not resemble the architecture present in native bone or the scaffolds used in current in vitro experiments.

Cells were mostly neglected in the models (Milan et al., 2009; Sandino and Lacroix, 2011) or, if cells were modelled, no comparisons were made to a no-cell situation.
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(Galbusera et al., 2007). Cells have been modelled as attached to the surface of the scaffold in a regular manner or evenly suspended in the culture medium. In *vitro* it is almost impossible to seed cells uniformly on a scaffold, and assuming evenly suspended cells in simulation models does not resemble reality. It was shown that the distribution of cells does have an important effect on oxygen concentration (Truscello et al., 2011), but the effect of cells, and especially the ECM produced by them, on the mechanical environment has not yet been investigated.

Simulations of bone tissue cultures are often stationary and the formation of new ECM and neo-tissue over time is neglected. Growing tissue is a major issue, especially in perfusion cultures. As tissue mass increases, the interconnected pores of the scaffold become more and more obstructed. This process gives rise to an increase in mechanical shear stress acting on the cells. If the mechanical stimulation is not adapted to the growing tissue, it may lead to detrimental effects on the cells. Growing tissue can also lead to a change in mechanical properties of the whole construct. Especially under compression, growing tissue would have an influence on the mechanical stimuli a cell experiences. Another effect is the deformation of the scaffold in perfusion studies, an effect that is thought to be small and negligible (Milan et al., 2009). In turn, hydrodynamic stimulation caused by moving fluid due to compression of the fluid or movement of the load-applying piston is most often neglected in compression studies. Nevertheless, if the understanding of a bone tissue culture is to include as many of the influencing aspects affecting the culture as possible, these parameters will need detailed investigation in the future. Moving boundary issues remain a challenging task to be solved in future studies.

The implementation of quantitative imaging techniques contributes vastly to computational simulations, as only factors that can be determined quantitatively can serve as parameters affecting the simulation. For example, scaffold geometry at the beginning of an *in vitro* culture can be determined and reconstructed as a 3D image; simulations can then be based on these images. Additionally, scaffold parameters such as porosity, pore size, surface area/unit volume and interconnectivity can be calculated from reconstructed data. Image-based models have investigated the effect of scaffold structural properties on bone regeneration (Tuan and Hutmacher, 2005), their influence on mechanical stimuli distribution (Lacroix et al., 2006), fluid flow through the interconnected pores of the scaffold (Porter et al., 2005) and fluid shear stress within a scaffold (Cioffi et al., 2006). Image-based models led to more accurate and more realistic models, making the method essential for future studies. A particular aim should be to look for a possible strategy to determine the exact location of cells in a tissue-engineered construct to include actual position, size and occupied volume of cells in the simulations. An advantage of imaging techniques is the possibility of obtaining patient- or site-specific data. Prior to implanting a tissue-engineered construct into a specific anatomical site of a patient, its mechanical and structural properties can be evaluated by combining computational simulations and imaging techniques. Nonetheless, scanning of patients can be difficult at this point, because high-resolution scans (around 10 m) of large volumes are time consuming and the radiation dose for the patient would be too high. For simulations investigating processes and scaffold properties at the cellular scale, the resolution must be in the sub-μm range; however, the resolution is still limited by scanner settings, scanning time, radiation dose and a lack of computational power to solve high-resolution simulations. Image-based models have one critical disadvantage: large computational power is required to model the already small volumes of irregular scaffolds. Simulating the fluid flow through a complete scaffold at high resolution (around 10 m) with a volume of about 20 mm is currently far from realizable in terms of computational power (Maes et al., 2009). In recent studies, a VOI was chosen which was considered to be representative of the whole scaffold (Maes et al., 2009; Porter et al., 2005; Sandino et al., 2008; Voronov et al., 2010). Another technique that was applied to reduce computational power is structure coarsening. Nevertheless, coarsening leads to a loss of information concerning the scaffold’s architecture, due to decreased resolution, and was found to be accountable for underestimations in wall shear stresses (Maes et al., 2009). However, a resolution of 1 m or less is required to understand all influencing parameters on a cellular scale, such as scaffold microporosity or surface characteristics. Future work needs to include optimized algorithms to reduce computational power, or must be run on supercomputers. This will lead to more complex and bigger computational simulations at higher accuracy and potentially lower cost.

In simulation studies the connection between biology and mechanics must be emphasized. Most of the studies so far have focused on mechanics only and neglected the biology. The effects of nutrient and waste distribution, culture medium concentration, oxygen concentration and consumption, physical and chemical surface properties and so forth have rarely been included in modelling studies. It is necessary to include these effects to effectively model a complete bone tissue-engineering system.

In many cases, experimental data or experimental validation of the simulations is missing. It is very important to show the significance of a simulation by comparison with an experimental model. Only experimental models allow the effects of certain parameters on the cellular environment to be verified. More experimental data has to be produced in the future to validate and test the numerical models.

A general issue in tissue engineering and also in simulating tissue engineering cultures is the variability between different studies, which makes any comparison difficult. As influential parameters and boundary conditions are still not determined and the magnitude of their effect is still unknown, even a small change could have a significant effect in *vitro*. Selecting boundary conditions for simulations has led to additional disparity between the different studies. This complexity should be reduced
to make comparisons easier and reduce the number of studies performed.

In the future it will hopefully be possible to determine cellular volume and cellular spread within a scaffold. A prediction of the mechanical load a cell feels and its response in terms of gene expression and ECM production should be possible, and a temporal forecast about the development of the tissue culture will be feasible. The ultimate goal of simulations in tissue engineering is to develop a predictive model. If a model can simulate all essential factors acting on a tissue-engineered system, it will be capable of simulating tissue growth and differentiation. Like this, influential parameters can be chosen in advance and can be determined continuously and adapted to the actual situation. It may even be possible to automate the whole process, using feedback-controlled mechanisms. If such knowledge can be translated from the in vitro to the in vivo situation, even prediction of patient-specific in vivo performance might be possible in the future.

5. Conclusion

The field of simulations of dynamic bone tissue engineering in bioreactors has evolved rapidly over the last two decades. The simulation approach is multidisciplinary and builds a bridge between biology and engineering. However, this bridge is still narrow and a lot of effort must be expended to improve the dialogue between experts in experiments and experts in simulations. It is crucial to understand the construct development in response to mechanical loading for the improvement and continuation of current tissue-engineering strategies. With the help of simulations, more realistic in vitro studies mimicking the in vivo environment will be possible. Only with such studies can we improve our understanding of the biological processes in tissue-engineering cultures. Bioreactors play a central role in bone tissue engineering because they provide a high degree of reproducibility, control and a possibility of automation, and therefore have the capability to improve the quality of engineered bone tissue. Simulations will play a major part in advancing the field of bone tissue engineering by enabling the understanding of causal relations between environmental cues and the final construct.

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Conflict of interest

The authors declare that there is no conflicts of interest.

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