Frontiers of Biomechanics 3

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Multiscale Mechanobiology in Tissue Engineering

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Frontiers of Biomechanics

Volume 3

Aims and Scope:

Biomechanics is a rapidly expanding field that focuses on exploring the mechanics of structure–function relationships underlying the functionality of living systems with the aim of gaining insights for medical applications. The field cuts across diverse disciplines: physics, biology, medicine, mechanical engineering, and biomedical engineering, and incorporates principles and concepts from these fields in the study of living systems and their dynamics.

A major objective of this series is to pioneer new frontiers of biomechanics by including concepts and ideas from new research frontiers yet to be addressed within the existing paradigm of biomechanics. The series highlights the role of mechanics in the realization of multicomponent and hierarchical interactions that characterize living systems, based on the context of open systems. The series also presents in-depth coverage of cutting-edge research in frontiers of biomechanics that explores fundamental concepts of what constitutes life, based on mechanics as an entity of energy principles and information science. It is hoped that this series will develop as an open system (analogous to living systems) that is admissible of future research dynamics and expansion in the field of biomechanics.

Distinctive features of the series:

- Each volume will cover core topics in the frontiers of biomechanics.
- The series will be multidisciplinary in that it will consist of volumes written by authors who are at the forefront of research in diverse fields including physics, mechanical engineering, biology, and medicine.
- Each volume will place emphasis on not only providing readers with new knowledge and techniques in biomechanics, but also presenting the underlying concepts and principles concisely and precisely so that the readers are able to discern unresolved and challenging problems in biomechanics.
- The series aims to contribute to further expansion and development of the field of biomechanics by including concepts and ideas from new research frontiers yet to be addressed within the existing paradigm of biomechanics.

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Preface

This book focuses on the mechanobiological principles in tissue engineering with a particular emphasis on the multiscale aspects of the translation of mechanical forces from bioreactors down to the cellular level. It aims to contribute to a better understanding on the design and use of bioreactors for tissue engineering and the use of mechanical loading to optimise in vitro cell culture conditions.

It covers experimental and computational approaches and the combination of both to show the benefits that computational modelling can bring to experimentalists when studying in vitro cell culture within a scaffold. With topics from multidisciplinary fields of life sciences, medicine and engineering, the book provides a novel approach to the use of engineering tools for the optimisation of biological processes and its application to regenerative medicine. The research described in this book was based on a European Research Council grant (258321) entitled 'Finite element simulations of mechanobiology in tissue engineering' partially funded by the European Commission.

A review of current state-of-the-art bioreactors is presented in Chap. 1. Spinner flasks, rotating and perfusing bioreactors are extensively reviewed as systems for control over shear stress applied forces and seeding capabilities. Then, bioreactor systems used to apply tension and compression on seeded constructs and the effect of these stimuli on cell differentiation are presented. Finally, the effect of electromagnetic fields on osteogenic differentiation are also presented.

Cell seeding in biomaterial scaffolds is often achieved through a perfusion bioreactor. However, not all scaffolds and perfusion bioreactors are optimised to each other. The scaffold and the bioreactor used to generate the engineered tissue have usually been developed empirically. As the research area is going further and the computational possibilities as well, a virtual physiological human cell tool to improve and optimise this process is presented in Chap. 2. A workflow from a single cell modelling to the bioreactor modelling and the biomaterial scaffold is presented

to create a patient specific implant. Combining experimental measurements with fluid, structure and fluid/structure analysis, we are able to calculate the mechanical stimuli at the cell, scaffold and bioreactor levels in a patient or sample specific manner.

The mechanical properties of a polycaprolactone rapid-prototyping scaffold are examined in Chap. 3 with particular focus on strain/stress curve, relaxation behaviour, apparent elastic modulus and dynamic mechanical analysis. The effect of different scaffold architectures and boundary conditions on mechanical properties are examined and the total error quantified. Then, the necessity for the development of collagen-polycaprolactone scaffolds to transmit mechanical stress to cells are explained and the organisation of the collagen inside the scaffold is shown. The effect of mechanical compression on the polycaprolactone scaffold cell proliferation and differentiation is presented. Results on seeding efficiency, proliferation and differentiation are further discussed and compared with other studies found in literature.

Despite the ability of rapid-prototyping techniques to fabricate regular structures, the consistency with which these regular structures are produced throughout the scaffold and from one scaffold to another needs to be quantified. Small variations at the pore level can affect the local mechanical stimuli sensed by the cells thereby affecting the final tissue properties. Most studies assume rapid prototyping scaffolds as regular structures without quantifying the local mechanical stimuli at the cell level. In Chap. 4, a computational method using a micro-computed tomography-based scaffold geometry is presented to characterise the mechanical stimuli within a real scaffold at the pore level. Five samples from a commercial polycaprolactone scaffold are analysed and computational fluid dynamics analyses are created to compare local velocity and shear stress values at the same scaffold location. The high variability amongst samples is shown. This chapter shows that regular scaffolds need to be thoroughly analysed in order to quantify real cell mechanical stimuli so inspection methods should be included as part of the fabrication process.

Chapter 5 presents a Computational Fluid Dynamics (CFD) simulations combined with micro Particle Image Velocimetry (μPIV) experiments to predict seeding efficiency and optimise experimental parameters. The chapter shows that cells reach all pores inside the scaffold mainly following streamlines with a higher number of cells passing by the centre of the pores where fluid velocities are higher. Since cells do not intercept with scaffold substrate, low cell seeding efficiency was observed and compares well with the CFD model. In this chapter, an experimental approach was developed to investigate cell seeding inside a 3D scaffold, and a computational model was able to predict local fluid dynamics and cell seeding efficiency.

Mechanical forces and 3D topological environment can be used to control differentiation of mesenchymal stem cells. However, the effects of physical and mechanical cues of the microenvironment on cell fate determination have not yet been fully understood. In Chaps. 6 and 7, an investigation and comparison of the

effect of mechanical stimulations on soft cellular microspheres are presented when subjected to dynamic fluid compression in three different in vitro systems: microfluidic chamber, compression bioreactor and orbital shaker. Mechanical forces can stimulate the differentiation of mesenchymal progenitors in microenvironment. Results showed that despite similar cell viability, on average the level of ALP activity in 5 days dynamic compression regime was nearly two times higher than 10 days compression. Also, free floating samples presented the highest cell number and ALP activity compared to other conditions. Application of compression cycles on mesenchymal stem cells could be used as a model to study the effect of mechano-stimulation on osteogenesis.

In Chap. 8, the formulation of poro-viscoelastic behaviour of collagen hydrogels presented in previous chapters is described using different finite element solvers. The computational approach enables to understand better the contribution of collagen on the mechanical stimuli that affects cell behaviour, by modelling a complex system including a scaffold, the collagen medium and cells.

Cell cytoskeleton provides a bridge to transmit information between the extracellular and the intracellular environments. It has been suggested that the cytoskeleton components may have distinct mechanical roles in the cell and that they might form the structure that defines cell rigidity. One approach to studying the mechanosensing processes is to understand the mechanical properties of cells' constitutive components individually. The development of a multi-structural 3D finite element model of a single-adherent cell is described in Chap. 9 to investigate the biophysical differences of the mechanical role of each cytoskeleton component. The multi-structural model not only illustrates that a combination of cytoskeletal structures with their own properties is necessary for a complete description of cellular mechanics, but also clarifies the effects of cytoskeletal heterogeneity on the interpretation of force-deformation measurements.

The ability to predict the mechanical responses of different adherent cell types presents many opportunities to mechanobiology research to further identify changes from cell physiological conditions to disease. Using the multi-structural cell model presented in Chap. 9, the effect of the variation of the material properties of the intracellular components on the cell response after compression and shearing is shown in Chap. 10. A parametric study was performed to understand the key mechanical features from different cell types, focussing on variation of the mechanical properties of specific cytoskeleton components and prestress. The time dependent responses observed were remarkably similar to those reported for a variety of measurements with atomic force microscopy, suggesting this model is a consensus description of the fundamental principles defining cell mechanics.

In Chap. 11, current perspectives are presented to indicate that more efforts need to be put into the development of such advanced studies presented earlier, and a new workflow including the use of computer modelling for the development of new tissue engineering product is proposed.

This unique book provides a valuable resource for researchers and graduate students studying mechanobiology and tissue engineering. It also offers a deep insight of tissue engineering and its use in the design of bioreactors for undergraduate students. This book has been supplemented with extensive references for each chapter to enable the reader to progress through the study of each chapter.

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Contents

Chapter 1
A Review of Bioreactors and Mechanical Stimuli

The increased need to accelerate the healing process of critical size defects in the bone led to the study of optimal combination of cells, materials and external stimuli to obtain fully differentiated tissue to the injured site. Bioreactors play a crucial role in the control over the development of functional tissue allowing control over the surrounding chemical and mechanical environment. This chapter aims to review bioreactor systems currently available for monitoring mesenchymal stem cells (MSCs) behaviour under mechanical stimuli and to give an insight of their effect on cellular commitment. Shear stress, mechanical strain and pulsed electromagnetic field bioreactors are presented, and the effect of multiple conditions under varying parameters such as amplitude, frequency or duration of the stimuli on bone progenitor cells differentiation is considered and extensively discussed with particular focus on osteogenic and chondrogenic commitment.

1.1 Introduction to the Tissue Engineering Approach

1.1.1 Mesenchymal Stem Cells and External Environment

Long bone fracture gaps can be repaired through the use of natural and synthetic grafts seeded with cells to enhance tissue formation. For this purpose, the tissue engineering (TE) approach aims to use cells directly harvested from the donor and then expand them in cultures to reach the desired number. Osteoblasts are the most obvious choice for bone TE purposes as they are the main precursors of the bone. Despite this, their low proliferation rate and their fully differentiated state present issues. Moreover, there are problems related to the lack of tissue source and morbidity (Finkemeier 2002). As a consequence, mesenchymal stem cells (MSCs) currently are the next cellular target (Salgado et al. 2004) to satisfy the demand for an increased proliferation rate and a reduced amount of surgical intervention. Indeed, MSCs present high proliferation rates and can also be obtained from several sources such as bone marrow, adipose tissue or cord blood. Their undifferentiated state allows them to differentiate towards diverse lineages such as osteoblasts, chondrocytes, adipocytes or myocytes (Caplan 2007). After expansion and seeding

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onto the scaffolds, cells usually are stimulated through bioreactors to drive their differentiation towards a defined pathway and to obtain fully differentiated tissue to implant. Applying external stimuli, cells activate biochemical pathways defining the functional properties of the resulting engineered tissue (Hoffman et al. 2011). For example, chemical stimulation was found to be particularly promising. On this regard, fibroblast growth factors (FGFs) showed to increase self-renewal and to maintain cell multi-lineage differentiation potential, transforming growth factors (TGFs) and serum-free medium-induced chondrogenesis; bone morphogenic proteins (BMPs) and dexamethasone were instead the most relevant chemical factors inducing osteogenesis and have already been employed for clinical treatments such as spinal fusion and long bone fractures (Wilson et al. 2005).

Another stimuli having an impact on cells differentiation is the mechanical load. As a matter of fact, the bone is constantly under loading condition arising from the daily activities. Vigorous exercise induces up to 1000 microstrain in human bone, where 1000 microstrain equal to 0.01% change in length compared to the initial length, and are associated with bone mass increase (Klein-Nulend et al. 2012). As many evidences have shown the possibility to influence cell behaviour through mechanical stimulation (Ehrlich and Lanyon 2002; Kelly and Jacobs 2010), the use of external mechanical stimuli on cell differentiation has become an increasingly common practice nowadays.

1.1.2 Mechanical Stimuli and Cell Behaviour

Cell behaviour can be triggered by hydrostatic pressure, fluid shear stress, mechanical strain and electrical fields generated by interstitial flow passing on charged bone crystals. For example, continuous hydrostatic pressure decreases collagen production by osteoblasts, while intermittent compressive forces enhance osteoblast activity and decreased osteoclast resorption (Rubin et al. 2006). Hydrostatic pressure has also shown to play a role on chondrocyte behaviour as a constant stimulus was proved to lead to chondrogenesis, while intermittent strain led to hypertrophy (Rubin et al. 2006). When bone is loaded in tension, compression or torsion, the interstitial fluid is moved towards regions of low pressure to come back when the load is removed, inducing an oscillatory fluid flow of 0.8 Pa up to 3 Pa in vivo. This regime results in a dramatic amplification of local strains in proximity of the osteocyte processes (Klein-Nulend et al. 2012; Klein-Nulend et al. 2005). Osteocytes are able to sense this variation in the interstitial fluid as demonstrated by multiple studies where shear stress was found to trigger mechano-activated biochemical pathways regulating NO production in osteocytes (Vezeridis et al. 2006; Rubin et al. 2006). Osteocytes were found to be more responsive to mechanical stimuli than other cell types and are believed to play a role in regulating the activity of osteoblasts and osteoclasts (Klein-Nulend et al. 1995). Furthermore, mechanical stimuli were shown to regulate calcium deposition with osteoblast cells increasing mineralization as a result of cyclic loading (Sittichockechaiwut et al. 2009; Damaraju et al. 2014).

1.1.3 Cell Mechanotransduction

The effect of mechanical forces on bone cells is currently under investigation aiming to define a relationship between stimuli and differentiation. The key cues to better understand the effect of mechanical stimuli on cell commitment are (1) the forces applied by the cytoskeleton and the contractile components of cells on the surrounding environment, (2) how the stiffness of the surrounding environment influences cells through durotaxis and (3) how external mechanical stimuli generated by gravitational action, muscles and other cells are translated into biochemical processes. In skeletogenesis the differentiation of stem cells towards the osteogenic or chondrogenic pathway is regulated by many external factors (Kelly and Jacobs 2010; Mauck 2003) influencing cytoskeletal organization, shape, motility (Lim et al. 2010; McBeath et al. 2004) as well as the expression of transcriptor factors (Salazar and Ohneda 2012). For example, the Wnt/ß-catenin or Rho/ROCK signalling pathways are known to play a crucial role in controlling cell commitment towards the osteogenic or chondrogenic pathway through the expression of Sox9 and Runx2 at early stage of differentiation (Kelly and Jacobs 2010). Sox9 is put alongside with expression of collagen II, TGFβ and glycosaminoglycan (GAG) genes and identifies differentiation towards the chondrogenic lineage, while Runx2 identifies osteogenic differentiation and induces expression of collagen I and non-collagenous proteins such as alkaline phosphatase (ALP), osteocalcin (OC) and osteopontin (OP). OC and OP are markers for bone mineralization and help in regulating the size of mineral crystals deposited by mature osteoblasts (Clarke 2008).

1.1.4 Bioreactors for Tissue Engineering

To find a correlation between mechanical forces and cell differentiation, complex bioreactors providing a controlled micromechanical environment were developed combining advanced scaffold designs and mechanical conditioning systems (Zhang et al. 2010; Tanaka 1999; Thorpe et al. 2013). Bioreactors facilitate the monitoring and control of biological or biochemical processes undergoing within the scaffold during the bone-forming process. Bioreactors are generally adapted to fit within an incubator that controls the external environment guaranteeing physiological conditions: 37 °C temperature, 5% CO_2 concentration and 99% humidity. A requirement for cell culture bioreactors is inertia to the harsh chemistry of the biological environment preventing corrosion and toxic reactions. Moreover, the diffusion limit and uniform distribution of cells in the scaffolds are key factors to consider in the development of functional tissue. With this purpose, bioreactors aim to maximize the supply of nutrients and oxygen to cells seeded in internal areas exceeding the diffusion limit distance of 100–200 μm (Ratcliffe and Niklason 2002) in order to maintain their viability. Exchange of substances within the scaffold during the seeding can be also used to help increasing seeding efficiency and uniform

distribution of cells (Sobral et al. 2011). For this purpose, current techniques employ convection of medium by perfusion, centrifugation and spinner flasks (Zhang et al. 2010). Moreover, bioreactors can be designed to apply shear strain forces, mechanical strain or pulsed electromagnetic fields with a high control over the stimulation in order to reproduce the biological environment and clarify the relationship between mechanical stimulation and tissue formation.

1.2 Bioreactors for Fluid Flow-Induced Cell Differentiation

A homogeneous cellular distribution and a good exchange of nutrients and oxygen within the scaffolding material are the first step in the development of functional engineered tissue. Due to the three dimensional architecture of novel scaffolds, static seeding is no longer an optimal method as it leads to a low seeding efficiency, cellular inhomogeneous distribution and low diffusion of fluids or gases in the internal regions causing cell apoptosis. In order to overcome these limitations, different systems were considered which are spinner flask (SF), rotating wall vessel (RWV), biaxial rotating (BXR) and perfusion bioreactors. These systems are more efficient compared to the static methods where molecule exchange occurs by diffusion because those novel systems induce a convective flow, enhancing cell attachment, proliferation and differentiation.

1.2.1 Rotating Bioreactors

SF bioreactors consist in a vessel provided with side arms for gas exchange and a stirring mechanism able to create a flow though the culture media (Fig. 1.1a). In order to avoid scaffolds fluctuation, pins are connected to the top lid for allocating samples. SF bioreactors were shown to increase the seeding efficiency compared to static methods (Mauney et al. 2004) and to induce osteogenic differentiation though the expression of ALP and OC and increased calcium deposition (Meinel et al. 2005).

RWV bioreactors consist in a hollow cylinder provided with an external chamber for scaffolds allocation and working as medium reservoir, rotating along the radial axis (Fig. 1.1b). The laminar flow generated by the rotating motion results in low shear stress preventing cell detachment and partially overcome the diffusional limitations characteristic of static and SF seeding methods. Despite this, lower cell number and matrix production were observed compared to SF methods because scaffolds are free to float inside the chamber hitting against the walls of the rotating vessel. Solutions include (1) fixing scaffolds to the cylindrical structure as in rotating

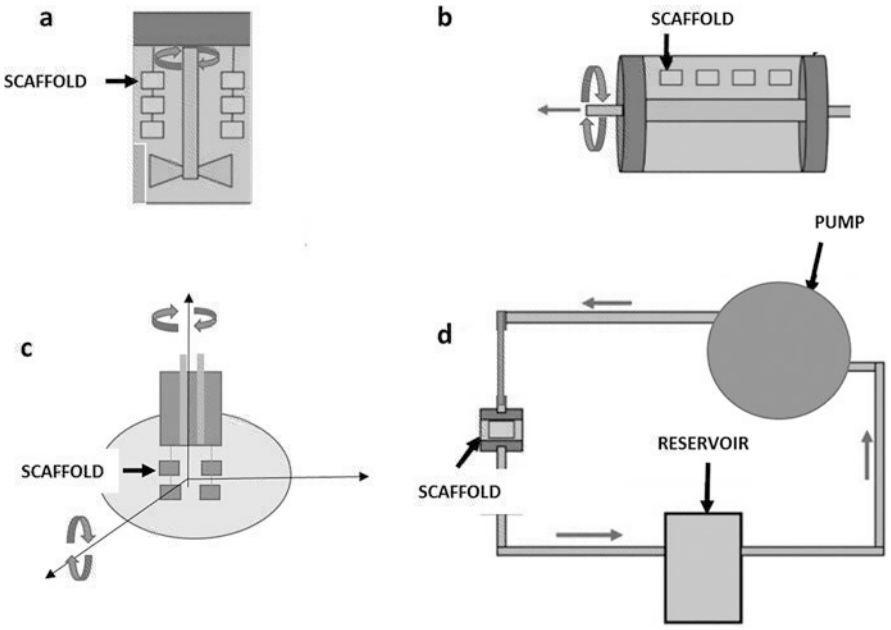

Fig. 1.1 Bioreactors for seeding and differentiation of MSCs due to effect of fluid flow. Spinner flask (**a**) and rotating wall vessel (**b**) bioreactors provide rotation towards an axis, while the biaxial rotating wall vessel (**c**) systems allow rotation in two directions providing homogeneous shear stress distribution in the culture chamber. Closed loop perfusion bioreactor (**d**) scheme employing a serial multichamber configuration. (Figures adjusted from Zhang et al. 2010)

bed bioreactors (Rauh et al. 2011), (2) employing scaffolds with lower density than water (Yeatts and Fisher 2011) or (3) preventing contact with the walls by optimization of the rotation rate (Zhang et al. 2010).

According to a study by Zhang (Zhang et al. 2010), the gold standard seeding performances are given by biaxial rotating bioreactor (BXR). It consists in a spherical chamber equipped with pins for scaffolds allocation, a reservoir for culture media and a perfusion system (Fig. 1.1c). The spherical chamber is able to rotate simultaneously in two perpendicular axes overcoming diffusion problems observed with SF. Moreover, it prevents cell detachment phenomena observed in RWV, thanks to the spaces for scaffold allocation. In summary, BXRs provide all the advantages of the perfusion systems while overcoming the "cell washout" phenomena observed in perfusion bioreactors. Indeed by not allocating the scaffold directly in the flow stream, cell detachment from the side of the scaffold facing the oncoming flow is prevented, resulting in higher homogeneous distribution of cells. BXRs increase considerably cell attachment, proliferation, molecule diffusion and osteogenic differentiation compared to SF, RWV and even perfusion bioreactors working in optimal conditions (Zhang et al. 2010).

1.2.2 *Perfusion Bioreactors*

In the last decade, the attention turned towards perfusion bioreactors (Fig. 1.1d) composed by a chamber fitting the geometry of the scaffold, a medium reservoir for supply of nutrients and a waste reservoir. Some perfusion bioreactors are closed loop and do not use a waste reservoir but nutrients are continuously pumped into the system (Kausar and Kishore 2013). Perfusion bioreactors force the fluid through the entire scaffold allowing cells to reach the interior of the structure and enhancing homogeneous distribution and optimal supply of gases and nutrients. The first challenge developing perfusion systems is related to prevent air bubbles formation as the presence of air is the main cause of local stress variation as it blocks the passage of fluid increasing the local flow rate and inhomogeneous condition inside the culture chamber, which might compromise the seeding process. A similar effect is observed when scaffolds are not completely anchored to the walls of the bioreactor chamber. In this case, void areas arise and become the preferred pathway for fluid to flow. The shear stresses generated by the fluid flowing through the scaffold are not only dependent on the inlet flow rate but also on the scaffold pore size and interconnectivity (Melchels et al. 2011; Porter et al. 2005; Chen et al. 2011). Despite the difficulties in developing efficient perfusion systems, a number of studies have studied the effect of perfusion flow on cell attachment, proliferation, matrix production and differentiation. While turbulent flow caused mainly cell detachment or programmed cell death due to the high shear stress (Cherry 1993), laminar regimes such as continuous, oscillating and pulsating flow led to satisfactory results and increased performances compared to static conditions. The effect of velocity and number of cycles on cell attachment was elucidated by Koch et al. who applied an oscillatory perfusion flow showing that velocities up to 5 mm/s were necessary in order to obtain uniform cell distribution in the interior of the scaffold (Fig. 1.2). He also demonstrated that the main effects on seeding efficiency were elicited by the number of cycles applied rather than the velocity used. Indeed, a lower number of cycles led to higher seeding efficiency. This suggests a dual role of shear stress which promotes cell attachment at the early stages of the seeding process but causes cell detachment if applied for long periods of time. The velocity of fluid flow was also found to significantly affect the viability of cells on the exterior of the scaffold as increased cell apoptosis was found associated to increasing shear stress regime (Fig. 1.3). These outcomes underline the need to define the optimal conditions enhancing uniform cell distribution, high seeding efficiency and cell viability.

Continuous unidirectional flow of cell suspension was also demonstrated to increase cell attachment and distribution (Vunjak-Novakovic et al. 1999; Wendt et al. 2003), ECM production and osteogenic differentiation (Scaglione et al. 2006; Bjerre et al. 2011; Papadimitropoulos et al. 2013; Koch et al. 2010; Sikavitsas et al. 2005). Moreover, a laminar flow oscillating in nature mimics the in vivo conditions applied to bone cells and stimulates calcium production in osteoblast-like cells (Koch et al. 2010) and human bone marrow stromal cells (Li et al. 2004). However, pulsating flow was found to be the most efficient in enhancing mineralization

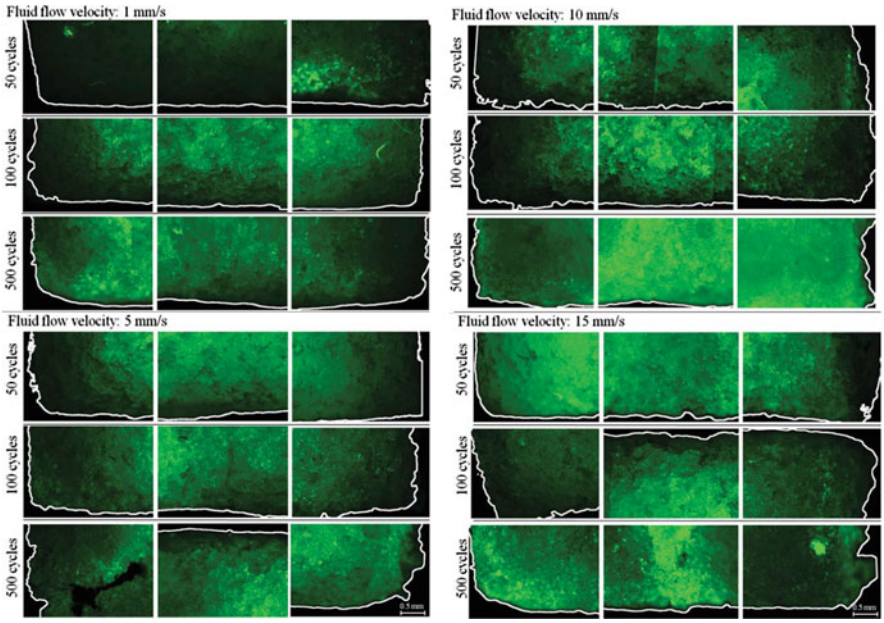

Fig. 1.2 Effect of velocity and number of cycles on cell attachment in the interior of the scaffold. (Koch et al. 2010)

(Jacobs et al. 1998; Bancroft et al. 2002), inhibiting cell apoptosis (Tan et al. 2008) and regulating matrix deposition (Vezeridis et al. 2006; Tan et al. 2007). The main drawback of perfusion bioreactors is the high amount of reagents needed, which has led to the development of perfusion microfluidic systems.

Microfluidic systems are easy to develop, require a low amount of reagents and above all allow to perform parallel experiments (Beebe et al. 2002). The new generation "lab on a chip" microfluidic devices permit repeatability of experimental conditions, testing simultaneously multiple samples. Due to their high versatility, they have already found application in the development of in vitro vascular implants (Khan et al. 2012). Polydimethylsiloxane (PDMS) is the most commonly used material for microfluidic perfusion culture systems since it is non-cytotoxic, autoclavable, gas permeable, flexible and easy to mold. Moreover, PDMS has low autofluorescence, and it is light transparent finding application for fluorescence and optical imaging (Kim et al. 2007). For cellular culture purposes, a glass-PDMS configuration is the preferred choice (Plecis and Chen 2007) as PDMS can be easily covalently bonded to glass substrates by surface activation through gas plasma treatments (Bhattacharya et al. 2005; Millare et al. 2008). Microfluidics systems made of glass-PDMS are currently used as support for 2D and 3D culture studies on the differentiation towards muscular tissue (Tourovskaia et al. 2005); the effect of different flow rates on cell morphology and proliferation (Kim et al. 2006), liver toxicology (Kane et al. 2006), cell seeding and monitoring (Toh et al. 2007); and

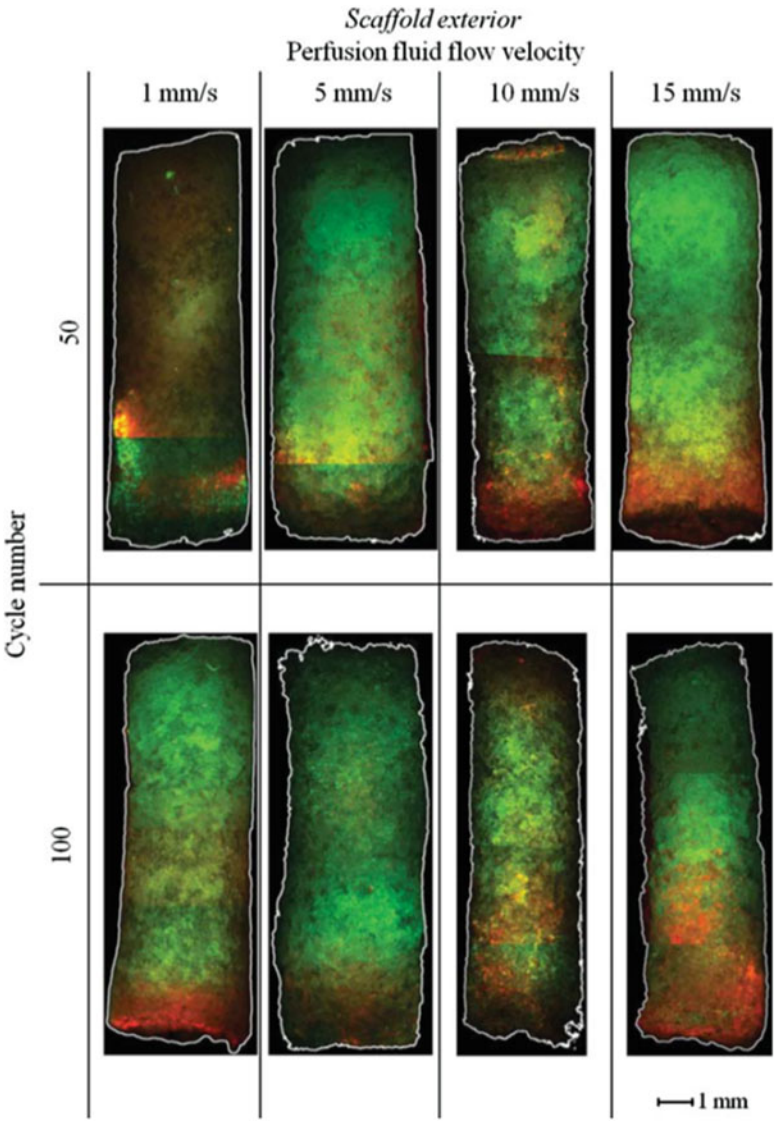

Fig. 1.3 Cell distribution on the exterior of the scaffold employing different velocities and number of cycles. Alive cells are shown in green, while apoptotic/dead cells are shown in red. (Koch et al. 2010)

comparison between cell lineages response to hydrostatic pressure (Park et al. 2012). Creating a robust sealed channel and avoiding bubble formation (Kim et al. 2007) are among the main challenges to currently face in the design of an efficient microfluidic system. In general, the fluid flow in a microfluidic perfusion system defines cell seeding efficiency and nutrients and gases delivery and can be used to transport