# Engineering of Cartilage Tissue Constructs in a 3-Dimensional Perfusion Bioreactor Culture System under Controlled Oxygen Tension

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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Basel (Schweiz), 2007

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Basel, den 22. Mai 2007

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# TABLE OF CONTENTS

# INTRODUCTION

# CHAPTER 1

# CARTILAGE BIOLOGY

## 1.1 Articular Cartilage: structure and function

*Types of cartilage in the human body*

Cartilage is a specialized avascular connective tissue comprising of only one single type of cell called chondrocyte which is sparsely populated in a collagen and proteoglycan rich hydrated extracellular matrix (ECM). Based on the biochemical composition and structure of the ECM, the mechanical properties and structural characteristics of the tissue, three major types of cartilage (elastic cartilage, fibrous cartilage and hyaline cartilage) can be distinguished.

Elastic cartilage is found in the pinna of the ear, in the walls of the auditory and eustachian canals and tubes, as well as in the larynx and in the epiglottis. This type of cartilage with a more elastic property maintains tubes-like structures permanently open and provide intermediate mechanical stability. Elastic cartilage mostly consists of type II collagen matrix elements and elastic fiber bundles (elastin) which manifest in aligned fiber structures. This structural composition provides a tissue which is stiff yet elastic.

Fibrocartilage is most prominently found in areas which require greater tensile strength and support such as between intervertebral discs and at sites of tendons or ligaments connected to bone tissue. Typically, fibrocartilage is found at locations which are under considerable mechanical

stress (i.e. tendon and ligaments) but still provides properties which allow flexible body movement. Accordingly, fibrocartilage mainly consists of type I collagen fibers which are aligned in thick fiber bundles and chondrocytes arranged in parallel rows between these fibers. The fibrous type of cartilage is usually associated with a dense connective tissue, namely the hyaline type cartilage which defines the third type of cartilage (Buckwalter and Mankin, 1998).

The hyaline type cartilage is the most abundant type of cartilage and is found in the nose, Larynx, trachea, bronchi, in the ventral ends of the ribs, and at the articular ends of the long bones. Characterized by the arrangement of the chondrocytes in multicellular stacks which prominently produce a type II collagen and a proteoglycan rich matrix, the hyaline type of cartilage provides the flexible support in nose and ribs but can also sustain mechanical load during body motion as shown at the surface of articular joints. This hyaline type of cartilage is lining as a thin layer of deformable, load bearing tissue at the bony ends of diarthrodial joints and is more specifically called articular cartilage (Buckwalter and Mankin, 1998).

*Articular cartilage function, structure, and mechanical environment*

The primary function of articular cartilage is the absorption and distribution of forces, generated during joint loading and to provide a lubricating tissue surface which prevents the abrading and degradation of the joint and the subchondral bone structure during joint motion. Indeed, the articular type of hyaline cartilage has to bear and tolerate enormous physical stress and load during its entire lifetime.

Despite the rather primitive composition of articular cartilage, characterized by chondrocytes entrapped in hydrated extracellular matrix molecules such as collagen type II, IV and VI, and proteoglycan aggregates, the tissue shows unique, highly defined structural organization to maintain its mechanical and functional integration.

Articular cartilage has two different structural characteristics: (i) the matrix micro-environmental structure surrounding the single chondrocyte and (ii) the structural segmentation of the entire tissue.

The extracellular matrix which directly surrounds the chondrocytes is a highly ordered structure and can be divided in three compartments, such as the pericellular region adjacent to the cell body, the territorial region enveloping the pericellular matrix, and the interterritorial compartment which defines the space between these cellular regions (Figure 1) (Buckwalter and Mankin, 1998).

The pericellular region which is rich in proteoglycan, decorin, aggrecan, collagen type VI, and cell membrane associated molecules like anchorin and decorin (Hagiwara et al, 1993;Keene et al, 1988;Poole et al, 1982) defines a narrow rim of a filamentous matrix network which fulfills the functions of the interlink between the chondrocyte cell body and the territorial matrix structure.

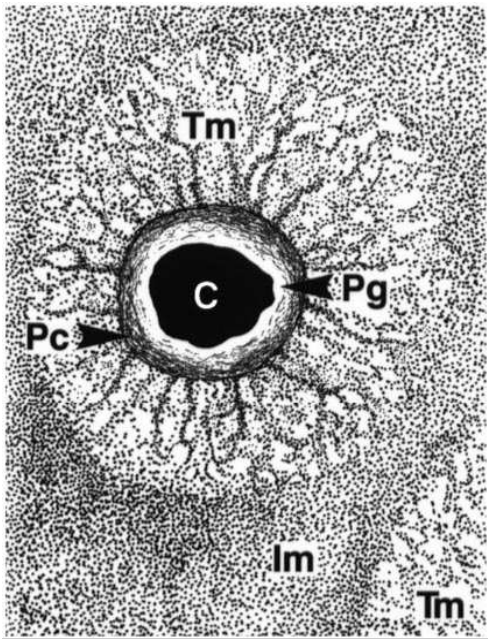


**Figure 1.** Horizontal view of circumferential collagen organisation in the deep layer showing chondrocyte (C), pericellular matrix (Pg, Pc), territorial matrix (Tm) and interterritorial matrix (Im). (Reproduced from Poole CA (Poole, 1997); Articular cartilage chondrons: form, function and failure).

The territorial region describes an envelop surrounding the cells or cluster of cells with their pericellular matrix. Thin collagen fibrils (most prominently collagen type II) bind to the

pericellular matrix and form a basket like structure which protects the cell from damage during loading and deformation of the cartilage tissue. Moreover these structures may also contribute to transmit mechanical signals to the chondrocytes during joint-loading (Poole et al, 1984;STOCKWELL, 1975).

The interterritorial region confines the most volume of the articular cartilage tissue and contains intermolecular cross linked collagen fibrils (collagen type II), non collagen proteins and aggregates of glycoproteins (Poole et al, 1982). This extracellular matrix composition provides the tissue with its functional characteristic to absorb mechanical load.

The structure and composition of the entire articular cartilage tissue varies according to the distance from the tissue surface. Four different zones arranged as layers horizontally to the tissue surface can be distinguished and are characterized according to the extracellular matrix composition and cellular morphology (Figure 2).

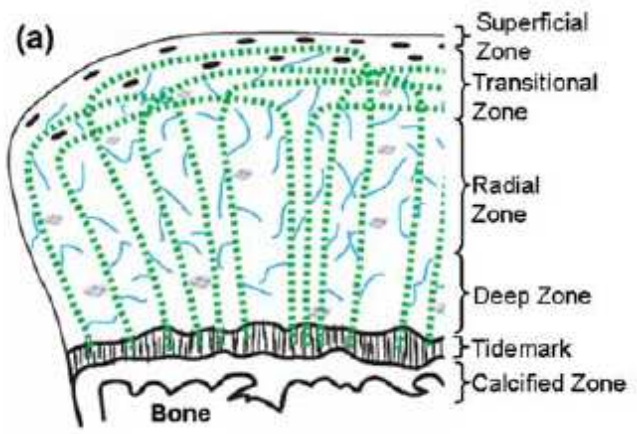


**Figure 2.** Schematic drawing of articular cartilage demonstrates the zonal arrangement and macromolecular organization by the illustration of PGs (blue) and collagen fibrils (green). (Reproduced from Schulz RM. and Bader A. (Schulz and Bader, 2007); Cartilage tissue engineering and bioreactor systems for the cultivation and stimulation of chondrocytes)

In the superficial zone the layer of tissue is composed of flattened ellipsoid-shaped chondrocytes and a high concentration of thin collagen fibers arranged in parallel to the articular surface (Bayliss et al, 1983). In this layer the pericellular matrix structure mentioned above can not be found. The thin layer of cells is covered with an acellular sheet of collagen fibers (lamina splendes) which functions as a protective barrier between the synovial fluid and the cartilage tissue

and controls the in- and egress of larger size molecules (Takada et al, 1999). Its rather low permeability regulates the diffusion transport of nutrients and oxygen to the underlying cartilage structures. Only within this zone chondrocytes synthesize and secret the superficial zone protein lubricin (Flannery et al, 1999;Schumacher et al, 1999) responsible to reduce surface friction during joint motion. The specific arrangement of the collagen fibrils which lay in parallel to the joint surface, provides a high mechanical stability of the tissue layer and mainly contributes to the tensile stiffness and strength of articular cartilage (Akizuki et al, 1986;Kempson et al, 1973;Roth and Mow, 1980).

In the transitional zone the chondrocytes appear in a more spherical shape and produce higher amount of proteoglycan compared to the superficial layer. The collagen fibers are synthesized at a lower quantity but show larger diameter fibrils which are aligned obliquely or randomly to the articular surface and describe an intermediate structure between the superficial zone and the adjacent radial zone.

In the radial and deep zone, the chondrocytes have a round morphology and are arranged in cell columns perpendicular to the cartilage surface. The extracellular matrix contains a high content of glycosaminoglycans and large diameter collagen fibers which form arcades perpendicular to the joint surface (Dallek et al, 1983).

The partially calcified cartilage zone defines the boundary of cartilage tissue to the subchondral bone. This rather thin layer of calcified cartilage with intermediate mechanical properties functions as a buffer between the cartilage and bone tissue. The cells have a smaller volume and are partially surrounded by calcified cartilage matrix. The chondrocyte in this zone usually persist in a hypertrophic cell stage which correlates with the expression of collagen type X. Finally this boundary provides an optimal integration to the subchondral bone tissue and prevents vascular invasion.

*Mechanical environment in mature cartilage*

Chondrocytes and cartilage tissue during joint motion are exposed to body weight load which creates a rigorous mechanical environment for articular cartilage tissue such as direct compression,

shear, and hydrostatic pressure. The function of articular cartilage to undergo tissue deformation is dependent on the specific arrangement of macromolecules in the extracellular matrix. Especially the organization of collagen fibers into a three dimensional arranged collagen network can balance the swelling pressure of the proteoglycan-water "gel" (de Bont et al, 1986;Jeffery et al, 1991). Cartilage is considered as a viscoelastic material composed of three principal phases: a solid phase composed of a dense, collagen fibrillar network and charged proteoglycan aggregates, a fluid phase of water and an ion phase with ionic species for neutralizing the charged matrix components (Lai et al, 1991;Mow et al, 1999). Under physiological condition these three phases define an equilibrium where the extension of the proteoglycan-water gel volume is restricted by the firm collagen frame (Maroudas, 1976). The bound water in the cartilage tissue and finally the mechanical properties of the cartilage tissue are influenced by the interaction of water with the large, negatively charged proteoglycan aggregates (LINN and SOKOLOFF, 1965). The negatively charged proteoglycans mostly driven by chondroitin sulphate residues are balanced by a high concentration of cations dissolved in the cartilage tissue (Yoshikawa et al, 1997).

In summary, the mechanical function of articular cartilage tissue bases on the matrix structure surrounding each single cell, the arrangement of the extracellular matrix fibres within the single zonal compartment and the proportional composition of the different extracellular matrix components.

## 1.2 From cartilage tissue development to tissue aging

*The multistep cell differentiation process in cartilage development*

Articular cartilage as a part of the limb skeleton develops in a well defined and controlled multistep differentiation process of cells from the mesenchymal origin (Cancedda et al, 1995;Cancedda et al, 2000;Olsen et al, 2000).

The establishment of the cartilage structure follows precise and distinct patterns of cell differentiation and cell rearrangement driven by environmental factors such as cell-cell and cell matrix interaction, growth factor and morphogen mediated signaling (Ganan et al, 1996;Vogel et al, 1996) as well as defined biomechanical conditions (Heegaard et al, 1999).

The steps of development are divided in 3 phases. In the first phase mesenchymal precursor cells migrate from the lateral mesoderm towards the presumptive skeletogenic site and determine the cartilage anlagen (Hall and Miyake, 2000). In the second phase, the epithelial-mesenchymal interactions results in the mesenchymal condensation. The pre-chondrogenic condensation is a prerequisite for the future establishment of the limb skeleton (Thorogood and Hinchliffe, 1975) and is associated with an increased cell to cell contact which facilitate the intercellular communication and the transfer of small molecules between the cells (Coelho and Kosher, 1991). It has been shown that such a high cell density is required to allow chondrogenic development (Ahrens et al, 1977) and that the level of cell condensation correlates with the stage of chondrogenic development (DeLise et al, 2000;San Antonio and Tuan, 1986). Additionally, cell-matrix interactions appeared to play an important role in mesenchymal condensation (Dessau et al, 1980). For example the integrin mediated binding of chondrocytes to collagen, has been shown to be essential for chondrocyte survival (Cao et al, 1999;Lee et al, 2004). Finally, the overt differentiation of immature pre-chondrocytes into fully committed chondrocytes is manifested by an increased cell proliferation and by the up-regulation of cartilage specific matrix components like collagen type IIα1, IX and XI and aggrecan. In the final commitment of the chonodrogenic phenotype the cells reduce their proliferative activity and maintain the functional integrity of the mature cartilage tissue (Cancedda et al, 1995;Cancedda et al, 2000;Olsen et al, 2000).

Within these developmental processes growth promoting factors act on the cell and contribute to establish a mature cartilage tissue.

*Soluble growth factors in the cartilage development*

Within the multi step cell differentiation process a number of growth factors and morphogenes are involved and essential during chondrocyte maturation and cartilage tissue

formation. The most prominent growth factors belong to the transforming growth factor (TGF-β) superfamily which are responsible for chondrocyte proliferation (TGF-β1), terminal differentiation (TGF-β3; bone morphogenic protein; BMP) (Thorp et al, 1992) or to promote cell-cell interaction in the early stage of chondrogenesis (BMP) (Chen et al, 2004). The insulin like growth factor 1 (IGF-1) which belongs to the IGF family of peptide hormones (including insulin) regulates many cellular functions during cartilage maturation such as induction of chondrocyte differentiation (Oh and Chun, 2003) and proliferation (Phornphutkul et al, 2004). In mature cartilage IGF-1 promotes and maintains the anabolic synthesis of proteoglycan and type II collagen (Martel-Pelletier et al, 1998) and inhibits the nitric oxide-induced de-differentiation of articular chondrocytes (Oh and Chun, 2003). Furthermore members of the fibroblast growth factor (FGF) family of morphogenes influence processes correlated with cell division and chondrocyte proliferation and have been shown to promote chondrocyte proliferation in a human growth plate ex vivo culture system (Olney et al, 2004).

Finally, only the combinatorial action of these growth and morphogenic factors specifically expressed in selective tissue areas in different developmental phases and at defined concentrations establishes the precise structure of the articular cartilage tissue.

*Oxygen tension in cartilage development*

Due to the avascularity of the cartilage tissue in the adult body but also during the developmental phase it has been speculated that chondrocytes are exposed to a low oxygen environment (Brighton and Heppenstall, 1971). Indeed, recent findings demonstrated hypoxic regions within the fetal growth plate and that hypoxia and hypoxia associated signals have a central function during the process of chondrocyte differentiation and cartilage development (Schipani et al, 2001). Hypoxia regulates the expression and activity of a wide range of proteins. In particular the hypoxia inducible factor 1α (HIF1α), a member of the basic helix-loop-helix transcription factor family has been shown to be stably expressed in the central region of the growth plate and to have a major role in the adaptive response to hypoxia (Schipani et al, 2001). Hif-1α can thus form an active transcriptional complex and up-regulate target genes such as, glucose transporter

(Mobasheri et al, 2005), angiogenic factors (Cramer et al, 2004) or protein involved in cell cycle regulation (Schipani et al, 2001). The Hif-1 α -mediated up-regulation of type II collagen as well as a group of procollagen hydroxylases which are involved in the collagen fiber formation was reported in in vitro culture of chondrocytes under physiological oxygen conditions (Pfander et al, 2003;Takahashi et al, 2000). Therefore, besides conventional growth factor mediated signals, the hypoxic environment has been demonstrated to be a critical factor in the regulation of chondrocyte differentiation and to increase cartilage specific matrix deposition during fetal development (Pfander et al, 2003).

*Maintenance and aging of articular cartilage*

Once the articular cartilage tissue structure is established, chondrocytes reduce their metabolic activity and persist in a anabolic and catabolic equilibrium of the matrix components. Although the two major extracellular matrix proteins, collagen type II and aggrecan, have a relatively long turnover time span (Maroudas et al, 1998), they have to be maintained in a balanced state of production and degradation. The key factors to maintain the equilibrium of tissue metabolism are found in the physicochemical environment of cartilage tissue such as: (i) mechanical load during joint motion; (ii) growth factor responsiveness of chondrocytes; (iii) the balanced molecular composition of the matrix (proportions of the matrix components). These factors contribute to the preservation of the functional properties of the mature articular cartilage surface.

After the third decade in human the properties of the weight bearing articular cartilage tissue significantly change with progressive age (Kempson et al, 1973;Kempson, 1982). The process of cartilage aging has been shown to cause changes in the mechanical properties of articular cartilage (Kempson, 1991), in the molecular composition, structure and organization of the extracellular matrix (Koepp et al, 1999;Thonar et al, 1986;Verzijl et al, 2001) and in the synthetic and metabolic activity of chondrocytes (Bolton et al, 1999;Dozin et al, 2002). In advanced age individual the number of cells, the size of the cartilage tissue and the content of bound water diminish (STOCKWELL and BARNETT, 1964). The anabolic activity of chondrocytes required for the

balance of cartilage tissue matrix homeostasis declines and thus the imbalance of matrix turn-over causes the loss of tissue matrix structure. Furthermore, in line with the decreased ability of chondrocytes to respond to a variety of extrinsic stimuli (e.g. growth factors) the sensitivity to catabolic regulative cytokines is enhanced in age. Moreover the imbalance of the tissue homeostasis can be moreover manifested by the increased expression of catabolic mediators such as matrix metalloproteinases (Forsyth et al, 2005). Finally, these change in the molecular structure of extracellular matrix components leads a softening of the cartilage tissue which increase the risk of synovial joint degeneration, often provoking the clinical syndrome of osteoarthritis (Buckwalter et al, 2005).

However, not only the reduced tissue function in elderly individuals but also the generally low metabolic activity of cartilage tissue in combination might explain the limitation in the self-repair function of cartilage with increasing age (Paulsen et al, 1999;Verbruggen et al, 2000).

## 1.3 Cartilage healing and defect treatment

*Natural healing capacity of articular cartilage*

According to the size of cartilage tissue damage in the cartilage surface, several grades of tissue injury can be distinguished which lead to different healing response (Bauer and Jackson, 1988;Outerbridge, 2001). In the case of and distinct chondral or partial thickness fractures, the classical self-repair of injured cartilage tissue goes through conserved mechanisms of cell and tissue necrosis followed by the proliferation of surviving chondrocytes adjacent to the site of the lesion. Although these cells aggregate in clusters and demonstrate a temporary increased type II collagen synthesis, in long term the formed tissue shows a lost of hyaline like cartilage characteristics. Thus these chondral lesions remain almost unchanged and can proceed towards osteoarthritic diseases (Hunziker, 1999).

Another mechanism of cartilage tissue regeneration occurs in osteochondral or full thickness defects where the lesion penetrates to the subchondral bone part. In this more sever case of tissue damage towards the underlying bony tissue the access to the vascular system provoke bleeding into the lesion and the formation of a fibrin clot which is filling the defect site. Thereafter a population of marrow derived mesenchymal progenitor or stem cells (MSC) can invade into the fibrin clot and start to re-model the previously formed tissue (Coutts et al, 1997). These progenitor cells then differentiate into chondrocyte like cells characterized by the up-regulation of collagen and proteoglycan synthesis which finally leads to the complete re-filling of the former defect site with a tissue similar to hyaline type cartilage. Anyhow, the decreased deposition of extracellular matrix components and the formed tissue with fibro-cartilage structures lack the strength, the mechanical properties and duration of the original articular cartilage tissue as it has been demonstrated in longer time follow-up studies (Caplan et al, 1997;Shapiro et al, 1993).

The two mechanisms of the spontaneous self healing show limitations in the quality and mechanical duration as compared to the native cartilage tissue and can increase the risk of tissue and joint degeneration (Buckwalter et al, 2005). Therefore, procedures to regenerate the functional properties of the cartilage surface are crucial to avoid the progression of secondary joint disease.

*Cartilage defect treatment and its limitation*

The different approaches to treat cartilage defects vary from more conservative approaches, like physiotherapeutic measures or application of pharmaceuticals (i.e. corticosteroids, hyaluoronic acid and growth factors) towards more invasive (i.e. surgical) procedures (O'Driscoll, 1998;Temenoff and Mikos, 2000). Such invasive procedures to substitute damaged cartilage tissue aim to more immediately re-establish a functional cartilage surface.

The substitution of the defect area by small autologous cartilage plugs from adjacent sites or from a different cartilage tissue source can be performed by the press-fitting of these grafts into lesion site. This procedure provides the re-establishing of a functional cartilage surface which can absorb body weight load but has limitation in terms of poor tissue integration within the adjacent

native cartilage tissue. Furthermore, the surgical intervention damages intact host tissue and might enhance the donor site morbidity.

Similarly to the natural process of fracture healing in osteochondral defects the drilling or abrasion of the cartilage tissue towards the subchondral bone provides an alternative method to the procedure described above. According to the process of fibrin clot formation and mesenchymal progenitor cell invasion a hyaline cartilage like tissue can develop. However, the outcome of tissue quality varies from hyaline cartilage, to fibro cartilage to no cartilage, and dependent on the patient the tissue does not accomplish the mechanical properties and durability of the original tissue.

Even though such invasive procedures hold promise and showed acceptable results in some cases the outcome of these procedures shows generally limitations in terms of quality and reproducibility (Temenoff and Mikos, 2000).

An alternative approach proposed by Brittberg and colleagues utilizes extracted chondrocytes from a small biopsy of the cartilage tissue, expand the cells in in vitro culture dishes and subsequently re-infused them directly into the defect site. Although this method is a promising approach to re-store the tissue structure (less invasive), the outcome in tissue quality from expanded chondrocytes is limited (Brittberg et al, 1994;Temenoff and Mikos, 2000).

In conclusion, the available surgical procedures to re-establish the cartilage surface currently show limitations such as strong donor site morbidity and the generation of insufficient hyaline tissue characteristics and quality as compared to the native tissue. Therefore alternative approaches like fully in vitro engineered tissue substitutes are proposed to overcome the current limitations in cartilage tissue resurfacing (Langer and Vacanti, 1993). The requirements and limitations of such in vitro tissue engineering approaches are discussed in chapter 2.

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# CHAPTER 2

# CARTILAGE TISSUE ENGINEEING

## 2.1 Requirements in cartilage tissue engineering

Already in 1913 Carrel and colleagues initiated the ex vivo culture of cells derived from human connective tissue. They proposed the prerequisite of appropriate culture conditions to establish these cells in in vitro culture dishes and stated that “certain modification of the milieu interior” can lead to the acceleration of cell growth in vitro and that it would become possible to artificially activate the process of tissue repair. Starting from his rather rough description to grow cells and tissues under optimal culture condition, recent approaches consider the combination of different cell culture techniques and the integration of advanced cell culture systems for the improvement of engineering functional grafts towards tissue regeneration.

The term “tissue engineering” was first defined by Langer and Vacanti (Langer and Vacanti, 1993) as “an interdisciplinary field of science that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ". In line with the improved investigations, the activities in the field of tissue engineering broaded and the term “tissue engineering” required an extended definition which moreover emphasizes the "understanding of the principles in tissue growth, which then applied, leads to production of functional tissue replacements for clinical use" (MacArthur and Oreffo,

2005). The additional interest not only to engineer functional tissue but to understand biological aspects of tissue development and growth in vitro may allow greater success in developing therapeutic concepts aiming towards the replacement, repair, maintenance, or enhancement of tissue function (Wendt et al, 2005;Wendt et al, 2006).

Successful engineering of cartilage grafts which follows a cell-scaffold based approach requires optimized in vitro culture condition. The successes is dependent on three key elements: i) the selection of a cell source, able to produce a new tissue with hyaline like cartilage characteristics; ii) the choice of an appropriate scaffold material and design which allow cell seeding and promote the chondrogenic differentiation process; iii) the application of bio-inductive molecules supplemented in the culture growth media (i.e. growth factors, cytokines, hormones, vitamins, glucose and oxygen) and an optimal conditioning of the physical environment (e.g. shear or compression) which enable the cells to differentiate and to re-organize a cartilage like matrix structure.

The three key elements per se but also approaches combining these parameters are currently under investigation and open a broad field of research where only an interdisciplinary approach might be able to overcome the current limitations of in vitro chondrocyte differentiation and cartilage tissue re-formation (Temenoff and Mikos, 2000). In the following sections these requirements will be discussed regarding their potential and limitations to successfully engineer functional cartilage tissue grafts.

## 2.2 Cell sources to engineer cartilage tissue

Among the different parameters which influence the outcome of in vitro tissue engineering procedures the selection and definition of a convenient cell type or cell source is the first issue to

deal with. The indispensable demands on cells for cartilage tissue engineering are: (i) not to provoke hostile immune reaction (ii) not to induce tumorigenic development and (iii) to integrate within the site of insertion in a controlled way.

The requirements on these cells to moreover improve the quality of in vitro engineered cartilage tissue are: (i) to provide sufficient number of cells from the biopsy site which enables the culture of cells at a high cellular density to improve the induction of cartilage development in vitro; (ii) to harvest a population of cells which is able to properly recover a chondrogenic phenotype and (iii) to harvest the cells from body sites with low donor site morbidity caused by additional surgical interventions.

The use of xenogenic (animals derived) or allogeneic (human derived) cells and tissues could provide a source of cells with an almost unlimited availability and with a high accessibility to different populations of cells to most simply engineer tissue constructs in vitro. Anyhow, the use of an allogeneic or xenogenic cell source is usually correlated with possible adverse immunogenic effects (Platt, 1996).

The most evident choice for a non-immunogenic cell source is the use of autologous cells harvested from the patient's own tissue. These cells provide an optimal source which does not induce an immunogenic respond. For the implementation in cartilage tissue engineering the most promising attempts have been made by the isolation of bone marrow derived mesenchymal progenitor cells (progenitor from mesenchymal origin) or by the use of adult chondrocytes from cartilage tissue itself.

The use of undifferentiated, multipotent mesenchymal progenitor cells (MPCs) which characterize a population of cells multipotent for the mesoderm cell line (Caplan, 1991), can be isolated from the bone marrow and adipose tissue (Guilak et al, 2004), expanded in vitro and kept in their undifferentiated properties when maintained in appropriate culture condition (Pittenger et al, 1999;Reyes et al, 2001). Although, subsequent culture of MPCs in the presence of specific growth factors was shown to induce chondrogenic differentiation in three-dimensional micromass

culture (Awad et al, 2003;Barry et al, 2001;Johnstone et al, 1998), or on polymeric cell carrier scaffolds (Lee et al, 2004), bone marrow derived MPC differentiated towards the chondrogenic lineage were shown to express markers specific of hypertrophic chondrocytes (Mackay et al, 1998a;Winter et al, 2003) thus indicating a potential instability of the acquired chondrocytic phenotype. Despite a series of recent studies reporting the use of MPC for osteochondral defect repair in different animal models (Gao et al, 2001;Oshima et al, 2004;Uematsu et al, 2005), the long-term efficacy of bone marrow derived MPC and their contribution to the regeneration of hyaline cartilage which does not remodel into bone in the long term, still has to be demonstrated.

Anyhow, the harvesting of MSC from bone marrow or adipose tissue usually requires a second invasive procedure which correlates with the risk to induce additional morbidic effects to the patient.

Thus, differentiated mature chondrocyte harvested from the cartilage tissue itself provide a more convenient source for cartilage tissue engineering. Similar to the previously mentioned procedure of the autologous chondrocyte implantation (ACI) the chondrocytes can be isolated by an invasive procedure from the adjacent site of the tissue lesion.

Primary articular chondrocytes isolated from cartilage tissue can be successfully maintained in in vitro culture (Guerne et al, 1995;Quarto et al, 1997). The application of different growth factors during the 2D culture phase enables the cells to proliferate and while exposed to growth factor chondrocyte progressively lose their typical differentiated phenotype and appear fibroblastic. The exposure of chondrocytes to a variety of growth factors (i.e. bFGF-2; TGFβ-1) not only enhance the de-differentiation of chondrocytes but can additionally improve the capacity to re-gain a differentiated phenotype during subsequent culture in a permissive chondrogenic environment (Barbero et al, 2003). Again, such a permissive environment can consist of soluble growth factors like TGFβ-3; insulin or ascorbic acid. Beside the treatment of the cells with soluble chondrogenic inducer the maintenance of the cells in a 3-dimensional environment at a high cellular density

during the phase of chondrogenic re-differentiation can additionally promote the differentiation process (Tacchetti et al, 1992).

The advantage to use these cells which are considered to have high a chondrogenic potential and to be obtained in a high cell number after growth factor mediated expansion, allows the culture of these cells at a high cellular density to establish and increase cell to cell contacts and the induction of the chondrogenic differentiation process (Tacchetti et al, 1992). Finally these rationales support the use of adult chondrocytes as a source for the implementation in cartilage tissue engineering approaches.

A critical issue associated with the use of autologous articular chondrocytes is the acquirement of the biopsy from the individual. The harvesting of a cartilage biopsy in the joint represents an additional injury to the cartilage surface, and might be detrimental to the surrounding healthy articular cartilage (Lee et al, 2000). To circumvent this problem an alternative approach would be based on the use of chondrocytes obtained from non-articular cartilage tissues. For instance, biopsies of nasal or rib cartilage can be harvested by a less invasive procedure than excising tissue from distinct areas of the joint. The potency of morbidity is also reduced by the fact that the donor site (ear and nose) is not subjected to high levels of physical forces, as in the joint. Various studies have been shown that chondrocytes derived from human nasal septum or ear cartilage proliferate and generate cartilaginous tissue after monolayer expansion with similar or superior capacity to those derived from articular cartilage (Tay et al, 2004;Van Osch et al, 2004;Kafienah et al, 2002). However, to demonstrate whether the tissue generated by non-articular chondrocytes is adequate for articular cartilage tissue repair, extensive data from in vivo orthotopic experimental studies and from in vitro loaded models will be needed.

Considering the implementation of chondrocytes from the articular surface harvested from adult individuals in cartilage engineering approaches the outcome of the tissue quality shows limitations in terms of donor variability which might be influenced by the clinical background, the disease history of the patient or on the age of the individual. In particular the age of the individual

significantly reduce the capacity of the ex vivo cultured chondrocytes to respond to growth stimulation and thus the quality produced cartilage tissue from cells of elderly donors are limited (Verbruggen et al, 2000).

Based on these considerations for each single study in this work articular chondrocytes, harvested from the articular surface of knee joints, from individuals of the same age range were used.

## 2.3 Scaffolds: demands on material and design

The scaffold materials implemented within tissue engineering approaches provide a preliminary template for the cells to attach but additionally provide the mechanical stability for a potential engraftment into the tissue defect site. A large number of scaffold designs and concepts were tested experimentally, in animal models and received the approval in clinical applications (Bonzani et al, 2006a).

An ideal scaffold material or architecture must provide the following characteristics: (i) biocompatibility and not provoke a hostile immune response; (ii) bio-absorbability with a controlled degradation and absorption rate which allows tissue in-growth; (iii) a three-dimensional frame with a highly interconnected structure which enables cell invasion, tissue growth and transport of nutrients and metabolic waste; (iv) mechanical stability for in vitro handling and subsequent implantation within surgical procedures; (v) and provide a suitable surface chemistry or the ability to absorb proteins to improve chondrocyte attachment, proliferation, or differentiation and thus to promote and support tissue specific development (Bonzani et al, 2006b).

The two most commonly used solid scaffold architectures reported in the literature are porous sponges and non-woven fiber meshes (Putnam and Mooney, 1996). They implicate

properties which enable the modulation of the mesh fiber diameter and density, or the scaffold porosity and the pore size and interconnectivity, according the requirement for the invasion, homing and the nourishing of hosted cells. The scaffold matrices used in tissue engineering approaches are mostly natural or synthetic polymer materials (Woodfield et al, 2002).

Various synthetic polymer scaffold materials have been validated in cartilage tissue engineering such as polylactic- or polyglycolic acids (Chu et al, 1995;Freed et al, 1998;Vunjak-Novakovic et al, 1998), polycaprolactones, polycarbonates or co-polymer containing ethylene-terephthalate (Radder et al, 1994;Sakkers et al, 1998). In contrast to the advantage to provide initial mechanical stability, non-immunogenicity and bio-resorbability these scaffold polymers have been shown to potentially provoke adverse cytotoxic effects due to the release of acidic products (Sung et al, 2004). Moreover synthetic polymers per se would not have biological properties to induce cartilage tissue regeneration.

Scaffolds based on natural biopolymeric compounds (i.e. hyaluronan or collagen based scaffolds) mimic and resemble the natural cartilage environment. The presentation of bioactive surface structures can induce signals to the entrapped chondrocytes and potentially stimulate the chondrogenic differentiation process which leads to the cartilage tissue neogenesis (Raghunath et al, 2007).

Furthermore the possibility to design specific scaffold characteristics (i.e. porosity, pore size or pore interconnectivity) could provide the basics to establish a model system to study the influence of physical means on the chondrocyte differentiation and the tissue development (Wendt et al, 2005).

In our system we used a synthetic PEGT/PBT (poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) co-polymer (IsoTis, Netherlands) type scaffold with a highly porous, interconnected pore structure (Malda et al, 2004;van Dorp et al, 1999). It has been shown that this type of scaffold material can be instructive for expanded human chondrocytes to generate 3D cartilaginous tissues (Miot et al, 2005) which, incorporated in our direct perfusion

bioreactor system, allows to investigate the influence of defined and controlled culture environment (such media perfusion flow rate and oxygen levels) on chondrocyte differentiation and the effect of enhanced mass transport on the uniform cartilage matrix deposition.

## 2.4 Media supplements and culture environment

As described earlier (Chapter 1.2), soluble mediators are mostly involved during the event of cartilage growth, metabolism and development, such as in the mesodermal differentiation of the cartilaginous skeleton in the embryo, the process of endochondral bone formation and the onset of articular cartilage "repair" (Cancedda et al, 2000). As a common basis of various approaches considered for cell-based engineering of cartilage tissue, there is a general accepted concept that during the in vitro culture of chondrogenic cells, it is suitable to apply specific growth factors, cytokines, hormones or enzymatic co-factors (e.g. vitamins) in order to enhance cell proliferation, migration or cell differentiation, and in consequence obtaining sufficient cells with the potency to re-induce cartilaginous tissue structures.

In general, growth factors and cytokines are cell secreted molecules and when bound to cell membrane receptors can induce intracellular signaling pathways which lead to cell adhesion, proliferation or promote cell differentiation, by the up- or down regulation of target genes.

As compared to the mophogenic action in vivo, several growth factors and mitogens are applied in in vitro tissue engineering approaches. Basic Fibroblast growth factor (bFGF) is a known mitogen that stimulates RNA and DNA synthesis in chondrocytes (Kato et al, 1983). Many in vitro studies have shown that FGF plays a key role in chondrocytes proliferation (Kato et al, 1983), prevents chondrocytes from terminal differentiation (Kato and Iwamoto, 1990) and promotes the de-differentiation process of primary chondrocyte in monolayer culture (Martin et al, 2001). Growth media supplementation with transforming growth factor β (TGFβ) induces chondrogenic differentiation as shown in a pre-chondrogenic cell line (Han et al, 2005) or in MSC micromass

pellet culture (Mackay et al, 1998b), and has been reported to up-regulate aggrecan and type II collagen when applied synergistically with insulin or insulin like growth factor (IGF) in chondrocyte alginate culture (Yaeger et al, 1997). Indeed, there are evidences that the combination of several specific growth factors during the phases of chondrocyte expansion and subsequent 3D micro-mass culture can have additive effects on the cell proliferation or chondrocyte differentiation process (Jakob et al, 2001).

To re-establish a proper matrix structure during the re-differentiation process in 3-dimensional chondrocyte culture, enzymatic co-factors can additionally be supplemented. For instance, ascorbic acid known as a co-factor for proline and lysine hydroxylase is required for the assembly and stabilization of collagen fibrils (Meier and Solursh, 1978).

Moreover oxygen molecules foremost included in the cell energy production, is additionally recognized as a key signalling mediator in the oxygen sensing pathway of chondrocyte and critical in the establishment of the chondrocyte phenotype (Schipani et al, 2001). The absence or low level of oxygen has been shown to inactivate the degradation of the hypoxia inducible factor 1α (Hif-1α) protein which is considered as a molecular inducer for chondrocyte differentiation and cartilage growth in vivo (Carmeliet et al, 1998;Schipani et al, 2001;Semenza, 1999). In vitro culture of germinal chick cells exposed to low (physiological) amount of oxygen induced chondrogenesis (Hall, 1969). Hansen and colleagues (Hansen et al, 2001) observed higher proliferation and collagen type II production in chondrocytes cultured under physiological $O_2$. Therefore the non-physiological oxygen environment (20% $O_2$) traditionally applied in in vitro cell culture systems might negatively influence the maintenance of chondrocytes in vitro (Henrotin et al, 2005).

In this work we assessed the effect of low oxygen tension on the proliferation capacity of primary human articular chondrocytes, exposed to a specific growth factor “cocktail” (TGFβ-1/ FGF-2/ PDGF) during the phase of expansion (de-differentiation). Moreover we assessed the effect of low oxygen tension on the ability of expanded articular chondrocytes to re-gain a chondrogenic cell phenotype during the phase of re-differentiation (exposed to TGFβ-3/ insulin/ ascorbic acid;

(Jakob et al, 2001) in 3 dimensional cell culture systems (i.e. micro mass pellet and polyactive foam scaffold).

## 2.5 Bioreactors to culture cell-scaffold constructs

Cell-scaffold based approaches with the ultimate goal to in vitro engineer functional cartilage substitutes require the recruitment of an optimal cell source (chapter 2.2), scaffold material and design (chapter 2.3) and optimal cell culture supplements (chapter 2.4). Furthermore, the success in the production of a functional cartilage tissue grafts which follows a cell-scaffold approach is dependent on the environmental culture condition. These environmental condition should provide a milieu where chondrocytes remain viable and functional entrapped in scaffold constructs, and additionally provide an environment which favors the differentiation of chondrocytes in 3-dimension. The possibilities and requirements to establish a 3 dimensional cell culture in vitro either with or without scaffolds are discussed in the following section.

The culture of chondrocytes in a three dimensional environment can maintain, induce or re-induce the differentiated phenotype of these cells (Schulze et al, 2000). Various in vitro chondrocytes culture techniques to re-establish a 3-dimensional tissue like structure have been developed either by the implementation of different biomaterials like agarose gel (Quinn et al, 2002;Buschmann et al, 1992), alginate (Domm et al, 2000;Ehlers and Vogel, 1998); and fibrin (Perka et al, 2000) or by a polymer-free system where a chondrocyte cell suspension is centrifuged to form cell aggregates. In fact such spherical aggregates with a high cellular density provide improved cell to cell contacts and serve as an auspicious in vitro model system to study chondrocyte differentiation (Barbero et al, 2003;Schulze-Tanzil et al, 2002) and cartilage tissue formation (Barbero et al, 2003;Stewart et al, 2000).

However, the engineering of cell-scaffold based cartilage constructs at clinically relevant size (4mm thickness) still show limitation in terms of: (i) the differentiation capacity of chondrocytes on 3D polymeric scaffolds, (ii) the homogenous deposition of cartilage specific extracellular matrix (iii) and the understanding of the influence of physiochemical culture parameter.

The most critical step in establishing a 3-dimensional cell-scaffold construct is the seeding of chondrocytes within a scaffold. The dissemination of cells within polymeric matrices determines the development of tissue formed during the subsequent culture phase(Vunjak-Novakovic et al, 1998). Therefore the initial allocation of chondrocytes within the scaffold after the seeding phase correlates with the distribution of tissue formed during the culture phase, assuming seeding uniformity as the a key attribute for homogenous tissue development (Holy et al, 2000;Ishaug-Riley et al, 1998;Kim et al, 1998).

The static seeding method is the most commonly used procedure to load cell onto scaffolds but shows limitations in cell seeding efficiency and uniformity which is associated with a non-uniform tissue generation (Wendt et al, 2003;Wendt et al, 2005). The manual seeding technique is highly operator dependent. The seeding outcome shows high variation and limited reproducibility and therefore might be insufficient for the manufacture of implantable grafts. To overcome the operator-dependency in the process of construct manufacturing, bioreactor systems can be implemented to generate a reproducible environment in the tissue culture stimulation (Wendt et al, 2005)

The seeding of chondrocyte into porous interconnected scaffolds or meshes can be performed in a stirred-flask bioreactor. The dynamic loading of cells onto matrices leads to a more uniform cell distribution and seeding efficiency as compared to the conventional seeding techniques (Carrier et al, 1999;Vunjak-Novakovic et al, 1998). However, such dynamic seeding protocols are sufficient to accommodate cells in the most types of scaffold but this again has limitations when applied to larger scale matrices whereas the cells preferentially allocate in the periphery of the scaffold matrix and avoid the core regions (Wendt et al, 2003).

The possibility to directly perfuse porous interconnected foam scaffolds and fiber meshes with a cell suspension has been successfully translated into a labor practice and showed to increase the seeding efficiency and the distribution uniformity of chondrocytes within the scaffolds as compared to other seeding processes (Wendt et al, 2003).

Although a uniform cell distribution is provided initially, the subsequent static culture of cell seeded constructs in a culture dish, a sufficient supply of nutrients and oxygen can only be provided within a short distance from the construct surface (Malda et al, 2003) and thus the constructs consists of a layer of cells and matrix at the periphery and an essentially a void interior region. Indeed enhanced mass transport of nutrients and oxygen by dynamic culture condition was proposed and showed to eliminate cell necrosis towards the core region of the construct and to improve the uniformity of extracellular matrix deposition (Wendt et al, 2006).

The possibility not only to seed chondrocytes into polymer scaffolds but grow cartilage tissues in 3-dimension has been considered by implementation of bioreactor systems to first and foremost enhance mass transport by the application of media perfusion but additionally direct biological and biochemical processes under highly defined environmental operating conditions (i.e. pH, temperature, pressure, nutrient supply, waster removal, and biomechanical stimuli) (Wendt et al, 2005). The variety of bioreactor model systems display different methods to nourish cells within the constructs and can provide different physiochemical environments to the cells within the construct.

Firstly, the external mass transfer can be enhanced by exposing immobilized cell-scaffold constructs to convective flow and shear forces in spinner-flask (Falsafi and Koch, 2000), or to a dynamic laminar flow on microgravity floating constructs in rotating wall vessels (Klement et al, 2004;Unsworth and Lelkes, 1998). Secondly, dynamic compression enhances the in- and out-flux of media and waste components and additionally mimics the physiological loading condition in the human joint and thus can mechanically stimulate chondrocytes to produce cartilage specific matrix components (Demarteau et al, 2003a;Demarteau et al, 2003b;Mauck et al, 2000). Lastly,

bioreactors based on direct perfusion of fluid throughout porous scaffolds or meshes aim to most efficiently nourish chondrocytes allocated in scaffolds by providing nutrients and oxygen towards the core of the constructs(Wendt et al, 2006).

Moreover the combination of a direct perfusion bioreactor system with the use of scaffolds with an appropriate architecture and design (regularly assembled pore size and structure) could additionally represent a model system to specifically characterize the chondrogenic differentiation and the development of cartilage tissues in a defined and controllable culture environment (i.e. fluid-dynamic microenvironment and nutrient supply) (Carver and Heath, 1999;Davisson et al, 2002;Mauck et al, 2000;Wendt et al, 2006).

In our study we used a direct perfusion bioreactor system to uniformly seed cells onto porous Polyactive (Polyactive™, IsoTis, Netherlands) foam scaffolds and subsequently culture these cell-seeded constructs under prolonged perfusion media flow to maintain cells uniformly distributed and results in uniform tissue development.

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# CHAPTER 3

# APPLICATION OF PHYSIOLOGICAL OXYGEN TENSION ON HUMAN ARTICULAR CHONDROCYTES IN 3D TISSUE CULTURE SYSTEMS

## 3.1 Rationale of the study

One of the major challenges within the approach of in vitro engineering cartilage tissue constructs of clinically relevant size is to determine the needs of cells to be grown in 3D and to provide a favorable culture environment to reproducibly fabricate a suitable cartilaginous graft to substitute damaged host tissue.

Up to date, the quality of ex vivo engineered cartilaginous constructs is limited due to restricted understanding of the regulatory role of physical and biochemical culture parameters on cartilage development.

In particular, oxygen in cell function, clearly responsible for cell survival and energy metabolism is considered as an important factor in several cell regulatory aspects during development and maintenance of the cartilage tissue (Wilson, 1986).

The effect of oxygen in the in vitro culture of chondrocytes in general and the influence on the in vitro engineering of 3D cartilaginous grafts in particular is still under controversial discussion and remains to be investigated (Malda et al, 2003). In the context of reproducibly

engineering 3-dimensional cartilage substitutes, the application of sufficient oxygen and nutrients might be essential to (i) avoid cell necrosis towards the core of the construct and (ii) promote the homogenous deposition of extracellular matrix (ECM) components throughout the cell-scaffold construct. Moreover the application of oxygen at more physiological levels could provide a favoring environment which enhances chondrogenic differentiation and cartilage tissue formation. The application of enhanced mass transport under a defined culture environment (i.e. oxygen tension), together with the monitoring of important culture parameters to control the tissue growth progression can be possible within specialized bioreactor systems.

The integration of such bioreactor systems not only provides the possibility to engineering uniform cartilaginous tissue constructs, but also the technological basis to reveal profound of chondrocyte function in a three dimensional environment. Finally, such systems could support the establishment of automated and standardized manufacturing protocols to improve the current quality of engineered cartilage tissue.

## 3.2 Aims of the thesis

*Ultimate goal*

The ultimate goal of this study is the development of concepts to engineer functional cartilaginous tissue constructs at clinical relevant size. The proposed concepts might lead towards establishing protocols to reproducibly and automated manufacture uniform cartilage tissue implants, possibly using cells of elder donors.

*Specific aims*

The specific aims of this thesis were: (*i*) to determine the influence of physiological oxygen levels on the in vitro differentiation capacity and development of cartilaginous tissue applied on

adult human articular chondrocytes from elderly individuals (Chapter 4), (*ii*) to develop a bioreactor system which directly perfuse culture media throughout chondrocyte seeded porous foam scaffolds with the possibility to monitor, control and maintain defined levels of oxygen applied to the chondrocytes to generate a homogenous graft with a uniform distribution of cells and ECM (Chapter 5) and (*iii*) to use the developed perfusion bioreactor system to selectively apply and modulate culture parameters (i.e. flow rate, oxygen levels) to control and improve the process of chondrocyte differentiation and cartilage tissue formation in a 3D environment (Chapter 6).

*Aim 1 (i)*

For the first aim we tested whether chondrocytes from advanced aged individuals, known to have low metabolic activity in vivo (Paulsen et al, 1999;Verbruggen et al, 2000) and currently showed to be limited in their ability re-differentiate in vitro (Barbero et al, 2004), can improve their capacity to regain a chondrogenic cell phenotype under a physiological oxygen environment and if so, whether the environmental oxygen level influence the regulation of cartilage specific genes involved in the ECM formation process .

The chondrocytes were extracted from biopsies of elderly donors and monolayer (2D) expanded in cell culture media with or without supplementation of a growth factor cocktail (TGFβ-1; FGF-2; PDGF) (Jakob et al, 2001) at conventional (20% $O_2$) or under reduced oxygen (5% $O_2$) tension. Subsequently the expanded chondrocytes were micro-mass cultured, at either normoxic (20% $O_2$) or at a more physiological (5% $O_2$) oxygen tension. The effect of oxygen was assessed on the differential proliferation activity during the expansion phase as well as on the capacity of these chondrocytes to re-induce a chondrogenic phenotype in micromass pellet culture. The chondrogenic phenotype of the cells and the maturation of the developed tissue was monitored histologically (Safranin-O; immunohistochemically, IHC), biochemically by the quantification of extracellular matrix components (glycosaminoglycans), DNA and by the mRNA expression levels of specific cartilage related anabolic and catabolic genes (RT-PCR).

*Aim 2 (ii)*

The second aim was to develop a bioreactor system which applies direct perfusion media flow throughout cell seeded porous Polyactive scaffolds to reduce mass transfer limitations and to overcome diffusion limitations currently obtained in cartilage tissue construct cultures at a clinically relevant size (up to 4mm) (Malda et al, 2004).

Therefore we used a previously developed direct perfusion bioreactor system to uniformly seed cells onto porous scaffolds (Wendt et al, 2003) and extended the system to a second direct perfusion fluid cycle to prolong culture the cell seeded constructs under direct perfusion. Within this extended perfusion bioreactor system we furthermore integrated flow through oxygen sensors to monitor and apply defined culture parameters such as oxygen levels and media flow rates (extensively described in chapter 5). As a cell carrier model system, we used the highly porous and interconnected PolyActive® scaffold (compression molded PEGT/PBT 45/55; Isotis, The Netherlands) which was press-fit integrated into a culture chamber. The oxygen levels in the perfusion system were monitored non-invasively by the integrated flow-through micro-oxygen sensors at the inlet and outlet of the constructs (Presens GmbH, Regensburg, Germany; Appendix B). All components incorporated into the bioreactor system were tested on adverse cytotoxic effects according to an ISO standardized protocol (ISO/EN 10993 part 5, see Appendix A). The cell-scaffold constructs were analyzed histologically, on the maintenance of cell distribution uniformity and cell viability as well as on the homogeneity of matrix deposition.

*Aim 3 (iii)*

In the third aim we used the developed perfusion bioreactor system (Wendt et al, 2006) to assess whether physiological oxygen levels, known to promote the chondrogenic differentiation on expanded human chondrocytes (Murphy and Polak, 2004) can improve cartilaginous tissue formation at defined media flow rates.

The quality of the developed tissue was assessed histologically (Safranin-O), biochemically (quantification of glycosaminoglycans and DNA) and by the mRNA expression profile of specific cartilage related marker genes (RT-PCR).

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# METHODS AND RESULTS

# CHAPTER 4

# ANABOLIC AND CATABOLIC RESPONSES OF HUMAN ARTICULAR CHONDROCYTE FROM ELDER INDIVIDUALS TO CULTURE UNDER LOW OXYGEN TENSION

Enclosed is the Paper currently in preparation.

# ANABOLIC AND CATABOLIC RESPONSES OF HUMAN ARTICULAR CHONDROCYTES FROM ELDER INDIVIDUALS TO CULTURE UNDER LOW OXYGEN TENSION

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**Keywords**: cartilage, chondrocyte, micromass culture, differentiation, age, oxygen tension, metabolic activity

**Abstract**

Aging is known to negatively modulate the metabolism of chondrocytes. We investigated whether culturing human articular chondrocytes (HAC) from elderly donors at low oxygen tension during monolayer-expansion or 3D-redifferentiation would improve their chondrogenic capacity and reduce the expression of specific catabolic mediators. We additionally investigated whether expansion with TGFβ1/FGF-2/PDGFbb (TFP), previously shown to enhance HAC de-differentiation, modulates responsiveness to low $O_2$.

HAC isolated from cartilage biopsies of four patients (mean age 65years) were expanded either at 20% or 5%$O_2$ in 10%FBS medium without (CTR) or with TFP. Post-expanded cells where then cultured as pellets in medium promoting chondrogenesis under the two different oxygen tensions. The generated cartilaginous tissues were assessed histologically, biochemically, immunohistochemically and by RT-PCR.

HAC expanded in CTR medium produced pellets with low quality at all the oxygen conditions tested. HAC expanded in TFP medium under the two oxygen tensions produced tissues with similar low quality and GAG/DNA contents following differentiation under 20%$O_2$. Instead, differentiation at 5%$O_2$ (vs 20%$O_2$) of HAC expanded at 20%$O_2$ improved the intensity of staining for GAG and collagen-II, the GAG/DNA content (2.8-fold) and the expression of aggrecan (8.5-fold) and collagen-II (86.6-fold) mRNA. Moreover, pellets cultured under lower oxygen tension expressed lower MMP-1 (7.7-fold) and MMP-13 (3.5-fold) and higher TIMP-1 (3.6-fold) mRNA.

The use of TFP during monolayer-expansion in combination with the application of low oxygen tension during re-differentiation in 3D-culture not only enhances matrix production but also reduces the expression of catabolic mediators by HAC. This culture condition might be useful to enhance the outcome of cartilage engineering techniques in elderly individuals.

## Introduction

Articular cartilage has limited capacity in self repair (Buckwalter and Mankin, 1998b). Therefore, a defects in cartilage tissue is often associated with the onset of osteoarthritic degenerative changes(Buckwalter and Mankin, 1998a). In vitro tissue engineering using autologous chondrocytes is considered as a promising approach in the re-establishing of cartilage surfaces after a tissue trauma. However, in correlation with the loss of cartilage tissue quality during life span (Leutert, 1980), tissue engineering approaches might have limited outcome when using cells of advance aged patients (Giannoni et al, 2005).

It has been shown that with progressive age the properties of articular chondrocytes to respond on extrinsic stimulus are reduced and the biosynthetic and metabolic activity decline after skeletal maturity (Bayliss et al, 1983). Such age related differences could be explained by a progressive loss of physiological function due to an increased imbalance of in vivo tissue homeostasis by enhanced expression of degenerative mediators (Aurich et al, 2002).

The use of specific growth factors during the monolayer expansion have been shown to enhance the proliferation capacity of human articular chondrocytes reducing age-related differences and to enhance the chondrogenic capacity of chondrocytes from donors younger then 40 years but not of cells from elder individuals. (Barbero et al, 2004).

The application of low oxygen tension in vitro to levels similar to that in vivo has been shown to enhance the biosynthetic activity of articular chondrocytes of *animal* origin under certain culture conditions (Murphy and Sambanis, 2001;Domm et al, 2002). Two studies, in which the responsiveness of *human* chondrocytes was investigated, showed that dedifferentiated chondrocytes derived from nose (Malda et al, 2003a) or hip (Murphy and Polak, 2004) cartilage had higher re-differentiation capacity when cultured in three-dimensional structure under low oxygen tension.

These studies suggested that the application of low oxygen maybe a possible solution to enhance the utilization of chondrocyte from elderly individuals for articular cartilage repair.

With this ultimate goal, we investigated the effect of lower (more physiological) oxygen during the expansion or the re-differentiation in 3D pellet culture of human articular chondrocytes (HAC) from elderly donors. Moreover, since the growth factor combination TGFβ1/FGF-2/PDGFb (TFP) have been shown to accelerate the process of de-differentiation allowing chondrocytes to reach a more 'plastic' state, we investigated whether expansion with TFP can influence the chondrocyte responsiveness to low oxygen. The quality of the chondrocytes was assessed both in terms of anabolic and catabolic activities.

## Materials and Methods

### Cartilage samples collection

Macroscopically normal human articular cartilage samples was obtained *post mortem* (within 24 hours after death) from the knee joints of a total of 3 patients with no clinical history of joint disorders (age 65 years, males), after informed consent by relatives and in accordance with the local ethics committee (University Hospital Basel, Switzerland).

### Chondrocyte isolation and expansion

Chondrocytes were isolated using 0.15% type II collagenase for 22 hours and resuspended in Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco) containing 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.29 mg/ml L-glutamine (complete medium; CM). The isolated chondrocytes were plated in tissue culture flasks at a density of $10^4$ cells /$cm^2$ and cultured in complete medium, either without growth factors (control medium, CTR) or with the addition of 1 ng/ml of Transforming Growth Factor-β1 (TGF-β1; R&D Systems Inc., Minneapolis, USA), 5 ng/ml of Fibroblast Growth Factor-2 (FGF-2; R&D Systems Inc., Minneapolis, USA) and 10 ng/ml of Platelet-Derived Growth Factor-BB (PDGF-BB; R&D Systems Inc., Minneapolis, USA) (growth factor medium, TFP) in a humidified incubators (37°C/5% $CO_2$) at either normoxic condition (20%

$O_2$; 75% $N_2$) or more physiological oxygen tension (5% $O_2$; 90% $N_2$). The chondrocytes were expanded as previously described (Barbero et al, 2003). In brief, sub-confluent chondrocytes at passage 1 were trypsinized (0.05% trypsin/0.53mM EDTA) and re-seeded at 5 x $10^3$ cells/$cm^2$. Subsequently second passage (for TFP expansion, P2) or third passage (for CTR expansion; P3) chondrocyte at sub-confluent stage were detached and induced to re-differentiate in pellet cultures as described below.

**3D pellet cultures**

The chondrogenic capacity of expanded chondrocytes was investigated in pellet cultures under the two oxygen conditions used for the expansion. HAC were resospended in DMEM complete media (CM) supplemented with 10 μg/ml insulin (ACTRAPID HM), 0.1 mM ascorbic acid 2-phosphate (SIGMA), 10 ng/mL TGF-β3 (Novartis, Schweiz) (chondrogenic medium; CHM). Aliquots of $5x10^5$ cells/0.5 ml were centrifuged at 250 g for 3 minutes in 1.5 ml polypropylene conical tubes (Sarstedt, Germany) to form spherical pellets and cultured for 2 weeks, with medium changes twice a week.

**Analysis**

*Proliferation rate*

Cell proliferation rate was defined as the total number of doublings during the expansion phase (to P2 or P3) divided by the time required for the expansion, and was expressed as doublings/day.

*Histological and immuhistochemical analyes*

Cultured cell pellets were fixed in 4% formalin, embedded in paraffin and cross-sectioned at thickness of 5 μm. The sections were stained with Safranin O for sulfated glycosaminoglycans (GAG) and processed for immunohistochemistry to visualize collagen type II (II-II6B3, Hybridoma Bank, University of Iowa, USA), as described in (Grogan et al, 2003).

*Biochemical analyses*

To measure total GAG amounts of chondrocyte pellet cultures, each sample was digested with protease K (0.5 ml of 1 mg/ml protease K) for 15 hours at 56°C) (Hollander et al, 1994). DNA amounts were measured spectrofluorometrically using the CyQUANT Kit (Molecular Probes, Eugene,

OR) and with calf thymus DNA as a standard. GAG amounts were assessed spectrophotometrically using dimethylmethylene blue with chondroitin sulfate as a standard (Farndale et al, 1986), normalized to the amount of DNA and reported as μg GAG / μg DNA.

*Total RNA extraction and cDNA synthesis*.

For total RNA from cell pellet cultures the Trizol extraction method (Life Technologies, Basel, Switzerland) and the standard single-step acid-phenol guanidinium method was used (Chomczynski and Sacchi, 1987). In brief, pellet cultures were firstly mechanically destroyed and sonicated for 1 minute while in Trizol. The isolated RNA was treated with DNAseI using the DNA-free Kit (Ambion, Austin, TX), quantified spectrophotometrically and re-transcribed in cDNA from 3 μg of RNA by using 500 μg/ml random hexamers (Promega, Madison, WI) and 1 μl of 50 units/ml Stratascript reverse transcriptase (Stratagene, Cambridge, MA), in the presence of dNTPs. following a standard protocol (Martin et al, 2001).

*Quantitative Real-time reverse transcriptase-polymerase chain reaction (RT-PCR).*

PCR reactions were performed and monitored using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, Switzerland). Cycle temperatures and times as well as primers and probes used for the reference gene (18-S rRNA) and the genes of interest (type II collagen and aggrecan) were as previously described (Jakob et al, 2001). To assess the expression of MMPs and TIMP-1 the Assays on-Demand (Applied Biosystem) was used: MMP-1 (Hs00233958_m1), MMP-2 (Hs00234422_m1), MMP-9 (Hs00234579_m1), MMP-13 (Hs00233992_m1), TIMP-1 (Hs00171558_m1). For each cDNA sample, the threshold cycle (Ct) value of each target sequence was subtracted to the Ct value of 18-S, to derive ΔCt. The level of gene expression was calculated as $2^{\Delta Ct}$. Each sample was assessed at least in duplicate for each gene of interest.

*Statistical Analysis*

Statistical evaluation was performed using SPSS software version 7.5 software (SPSS, Sigma Stat). Values are presented as mean ± standard deviation (SD). Differences between groups were assessed Mann Whitney test and considered statistically significant when P values were less then 0.05.

## Results

### Proliferation rates of HAC under different oxygen tensions

HAC cultured at 20% oxygen proliferated at higher rate (5.6-fold) in TFP medium as compared to CTR medium (0.48 ± 0.14 and 0.09 ± 0.03 doubling/days respectively). Expansion under lower oxygen tension (5%) did not influence proliferation rates of HAC neither in CTR nor in TFP medium (0.49± 0.12 and 0.08 ± 0.02 doubling/days respectively) (Fig. 1). To obtain sufficient number of HAC for subsequent cell pellet culture, chondrocytes were expanded to passage 2 in TFP medium (corresponding to 7.5 ± 1.3 doublings) and to passage 3 in CTR medium (corresponding to 3.4 ±0.9 doublings).

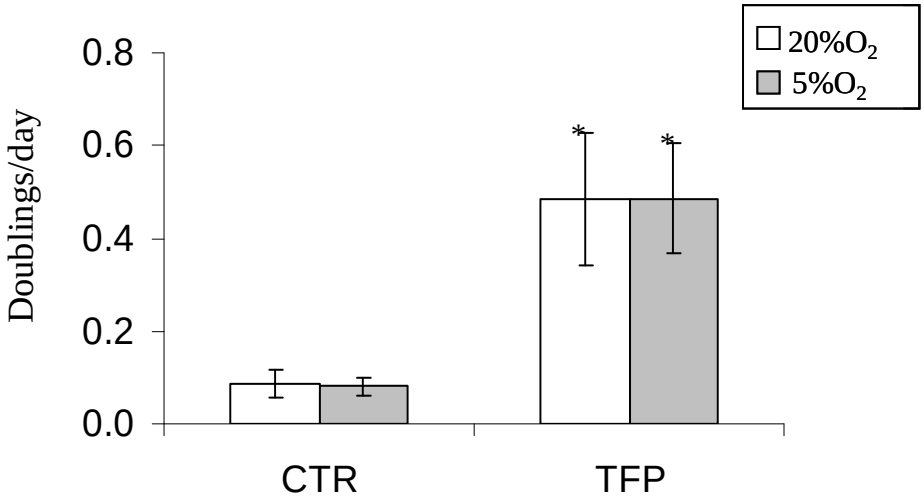



**Figure 1**. **Proliferation rates of human articular chondrocytes (HAC).** HAC were isolated from cartilage biopsies of four elderly individual (mean age: 65 years) and expanded in medium without (CTR) and with the growth factors TGFb-1, FGF-2 and PDFG-BB (TFP) under 5% and 20 % oxygen. Values are reported as mean ± SD.

**Chondrogenic differentiation of HAC expanded and cultured under different oxygen tensions**

HAC expanded under 5% or 20% oxygen were that cultured in three-dimensional pellets at the two different oxygen tensions. Therefore the four oxygen culture conditions considered in this study were: *expansion* 20%$O_2$/*differentiation* 20%$O_2$; *expansion* 20%$O_2$/*differentiation* 5%$O_2$; *expansion* 5%$O_2$/d*ifferentiation* 20%$O_2$; *expansion* 5%$O_2$/*differentiation* 5%$O_2$.

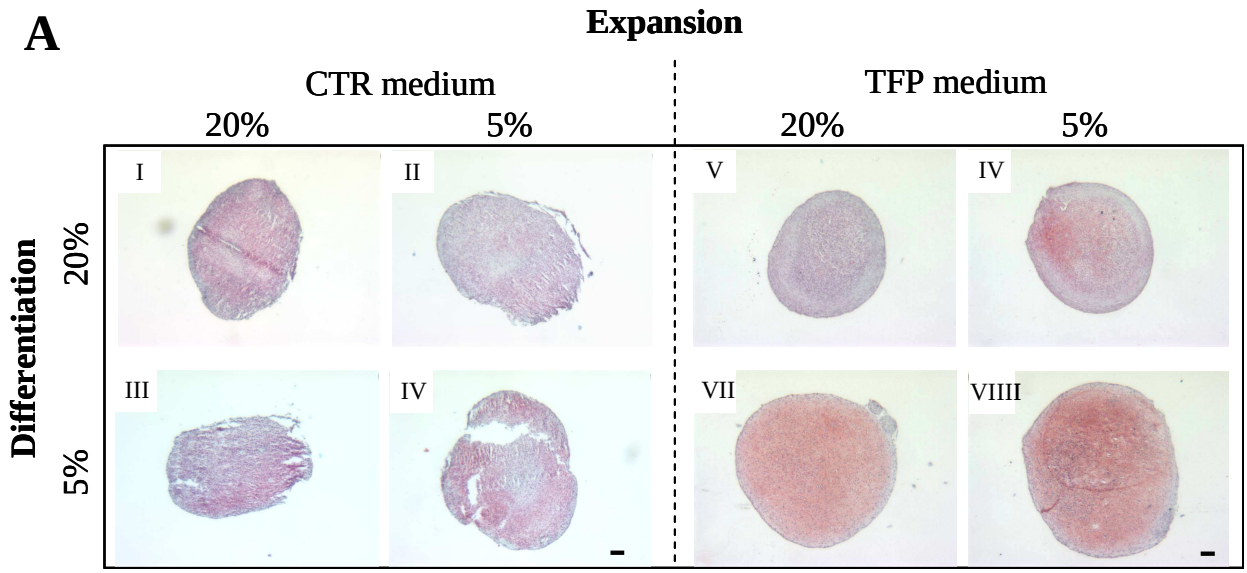
When CTR medium was used during the expansion, HAC differentiated producing pellets small in size and of poor histological quality that did not varied among the different oxygen condition used neither during the expansion nor during the differentiation. Biochemical analysis of the pellets confirmed no statistically differences in the GAG and DNA contents among pellets generated under the culture conditions considered (Fig. 2).

HAC expanded in TFP medium under the two oxygen tensions, also produced tissues with similar and poor histological quality when subsequently differentiated under 20%$O_2$ so that no difference among tissue generated by CTR- or TFP-expanded HAC were observed under such conditions. Instead, differentiation at 5%$O_2$ of HAC expanded in TFP under both oxygen condition, enhanced the volume of the generated tissue and improved the intensity of Safranin-O staining. Biochemical analysis confirmed statistically higher amount of GAG and GAG/DNA contents in pellets generated under the condition *expansion* 20%$O_2$/*differentiation* 5%$O_2$ and *expansion* 5%$O_2$/*differentiation* 5% versus the conditions *expansion* 20%$O_2$/*differentiation* 20%$O_2$ and *expansion* 5%$O_2$/*differentiation* 20%$O_2$ (2.6- and 2.1-fold respectively) (Fig. 2). The DNA contents of pellets did not largely differ among cells expanded with or without TFP and under lower oxygen condition, thus the increase in pellets size were mainly due to a higher GAG accumulation (up to 3.8- fold) (Fig. 2B). Due to a lack of responses of CTR expanded HAC to the low oxygen, further assessments were performed in pellets generated by cells expanded in TFP.

In agreement with higher amount of GAG, immunohistochemical analysis of pellets generated by TFP-expanded HAC showed that higher amount of collagen type II was accumulated in tissues produced during 3D culture under lower oxygen conditions (Fig.3).

RT-PCR analysis confirmed higher expression of the cartilage specific genes aggrecan and collagen type II by TFP-expanded HAC under low oxygen tension during the 3D culture. The largest up-regulation in aggrecan (8.5-fold) and type II collagen (86.6-fold) mRNA were measured among the culture conditions *expansion* 20%$O_2$/*differentiation* 5%$O_2$ and *expansion* 5%$O_2$/*differentiation* 5% (Fig. 4).

In summary our results indicated that under expansion with TFP (but not with CTR) medium, HAC responded to low oxygen tension during the 3D (but not 2D) culture expressing/accumulating larger amount of cartilage-specific extracellular matrix proteins.



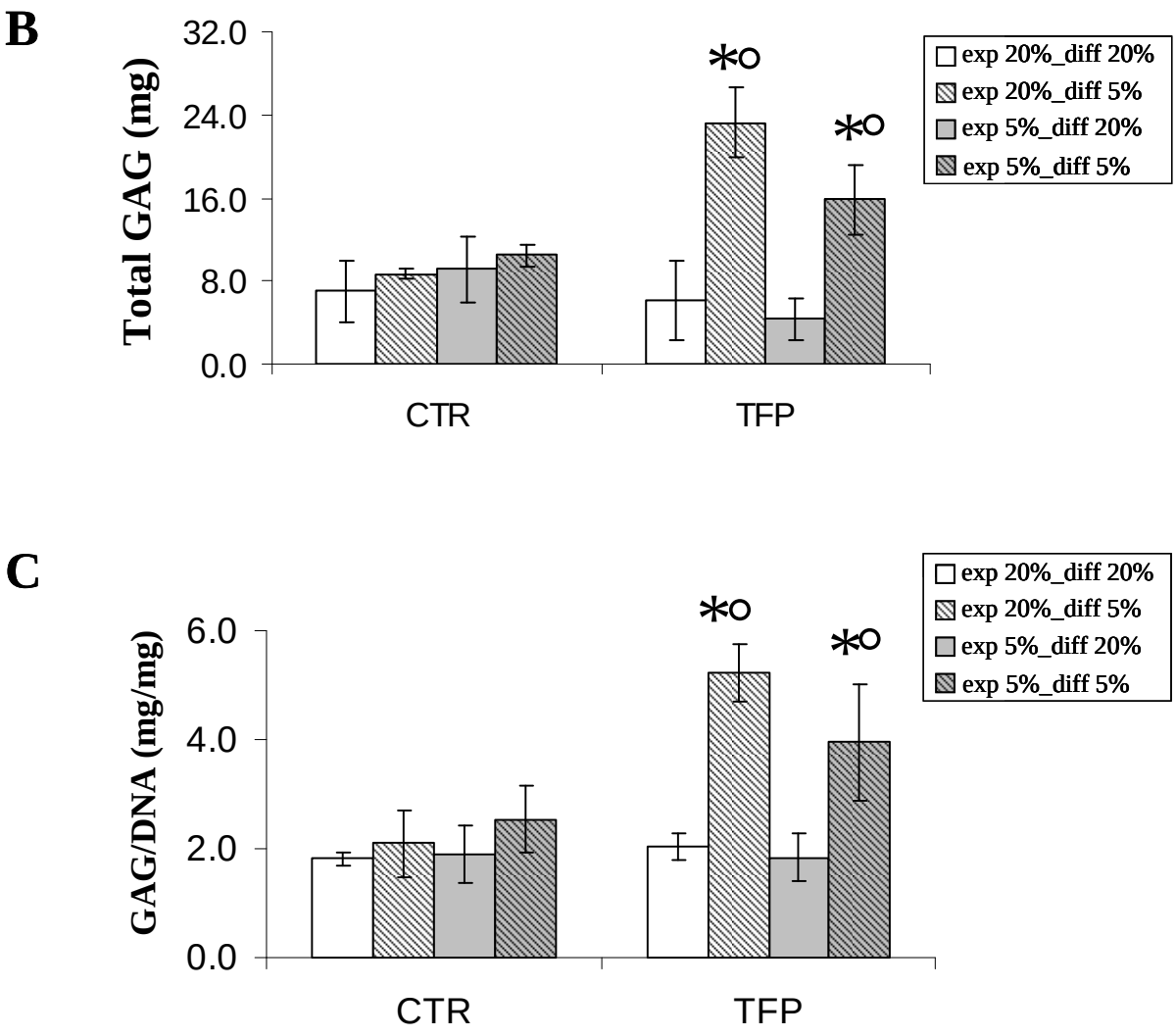



**Figure 2. Chondrogenic capacity of human articular chondrocytes (HAC).** HAC were expanded (exp) at 5 or 20% oxygen and re-differentiated (diff) at the both oxygen tension. **(A)** Representative Safranin-O stained histologies of pellets generated by HAC expanded in medium without growth factors (CTR) (**I** – **IV**) or with the growth factors TGFβ-1, FGF-2 and PDFG-BB (TFP) (**V** – **VII**I) under 20% oxygen (**I**, **III**, **V**, **VII**) or 5% oxygen (**II**, **IV**, **VI**, **VIII**). Bar = 100 μm. (**B**) Sulfate glycosaminoglycan (GAG) content and (**C**) GAG normalized to the amount of DNA (GAG/DNA) in the pellets. Values are reported as mean ± SD. ° = $p < 0.05$ from 20% oxygen during 3D culture; * = $p < 0.05$ from expansion in CTR medium.

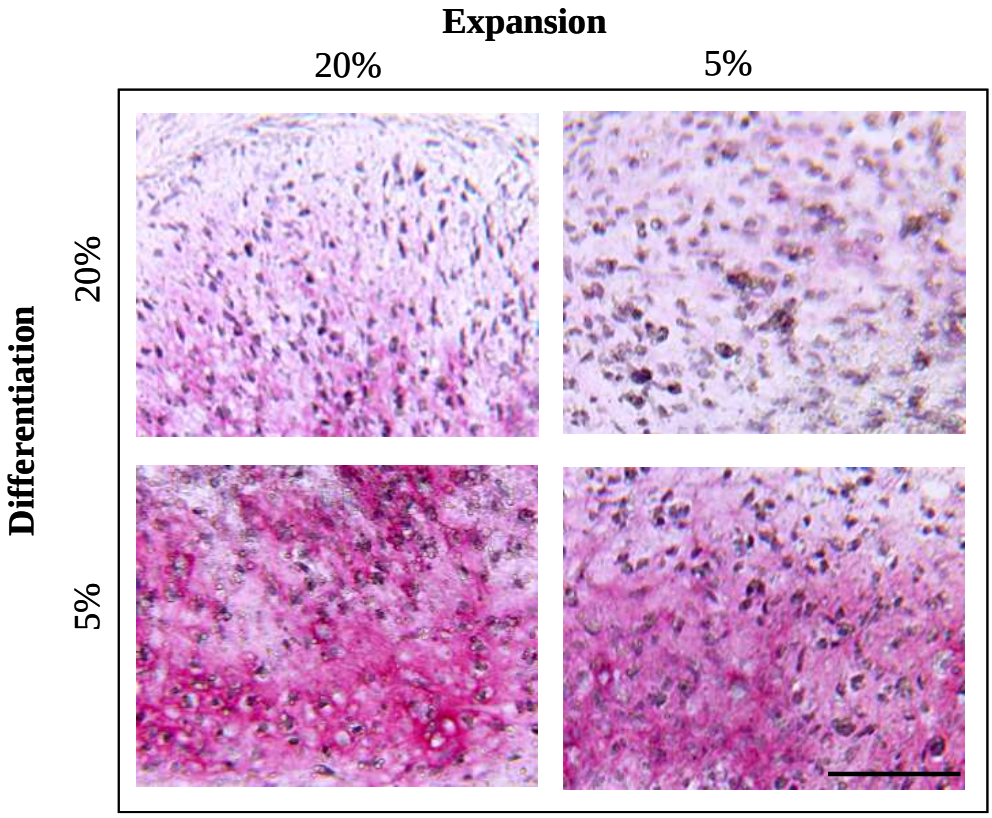



**Figure 3**. **Collagen type II immunohistochemistry staining** of representative pellets generated by human articular chondrocytes expanded in medium containing the growth factors TGFβ-1, FGF-2 and PDFG-BB at 20% oxygen (**I** and **III**) or 5% oxygen (**II** and **IV**) following culture in chondrogenic medium under 20% oxygen (**I** and **II**) and 5% oxygen (**III** and **IV**). Bar = 100 μm.

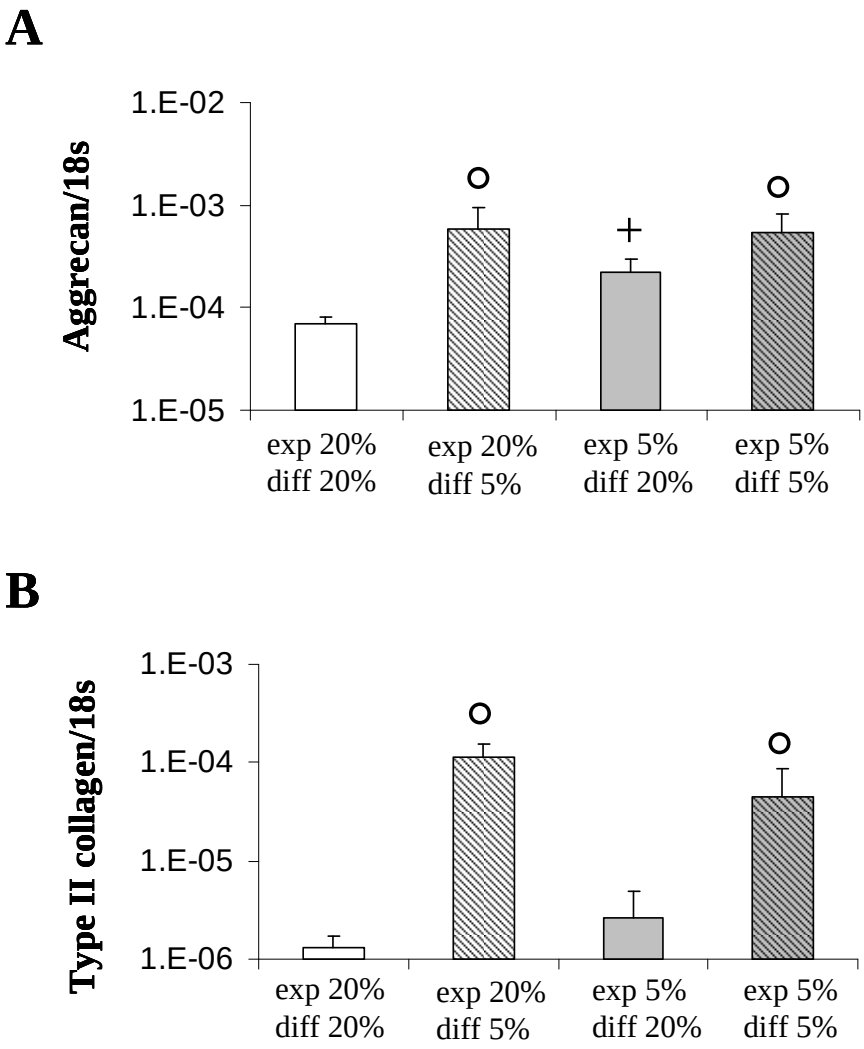



**Figure 4. Real time RT-PCR analysis** of the mRNA expression of aggrecan (**A**) and type II collagen (**B**) normalized to the reference gene 18S in the pellets generated by human articular chondrocytes expanded in medium containing the growth factors TGFβ-1, FGF-2 and PDFG-BB at 20% and 5% oxygen (exp) following culture in chondrogenic medium (diff) under the two oxygen conditions. Values are reported as mean ± SD. ° = $p < 0.05$ from 20% oxygen during 3D culture; $^{+}$ = $p < 0.05$ from 20% oxygen during expansion.

**Expression of catabolic mediators**

To investigate the possible role of low oxygen tension in modulating the expression of catabolic mediators, the two culture conditions among them the largest differences in the expression of cartilage

specific protein were measured (namely *expansion* 20%$O_2$/*differentiation* 5%$O_2$ and *expansion* 5%$O_2$/*differentiation* 5%) have been used. RT-PCR analysis of specific metalloproteinases (i.e.: MMP-1, MMP-2, MMP-9, MMP-13) and metalloproteinases inhibithors (TIMP-1) indicated that low oxygen tension applied during the 3D culture induced downregulation of MMP-1 and MMP-13 mRNA (7.7- and 3.5-fold respectively) and upregulation of TIMP-1 mRNA (3.6-fold). MMP-2 gene was not modulated by oxygen and the expression of MMP-9 gene was below the limit of detection at both oxygen tension culture conditions (Fig. 5).

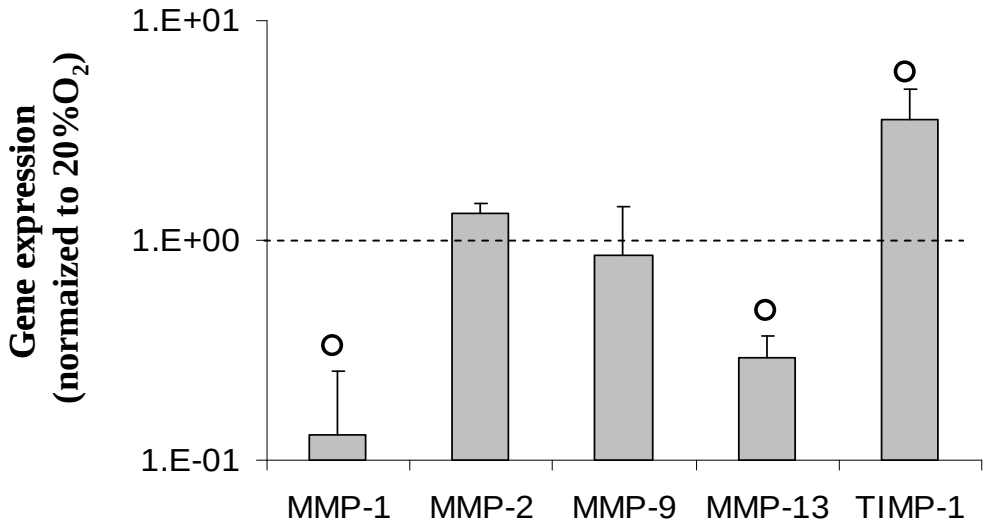



**Figure 5. Real time RT-PCR analysis** of the mRNA expression of the matrix metalloproteinases (MMP) -1, -2, -9, -13 and the tissue inhibitor of MMP (TIMP)-1 in the pellets generated by human articular chondrocytes (HAC) expanded in medium containing the growth factors TGFβ-1, FGF-2 and PDFG-BB at 20% oxygen following culture in chondrogenic medium under 5% oxygen. Levels are expressed as fold of difference from those measured in pelletes cultured at 20% oxygen. Values are reported as mean ± SD. ° = $p < 0.05$ from 20% oxygen during 3D culture

**Discussion**

In the present study, we investigated the effect of low (more physiological; 5%) oxygen tension on the proliferation rates and *in vitro* cartilage forming capacity of human articular chondrocytes (HAC) from elderly individuals. Proliferation rates of HAC expanded in medium without (CTR) or with TGFβ-1/FGF-2/PDGF-BB (TFP) were not modulated by physiological oxygen tension. The application of physiological oxygen tension during the three-dimensional (but not the monolayer) culture enhanced the cartilage tissue-forming capacity of TFP- (but not of CTR-) expanded HAC. Furthermore, low oxygen tension applied during pellet culture of HAC previously expanded with TFP, reduced the expression of specific matrix metalloproteinase (i.e.: MMP-1, -13) and enhanced the expression of tissue matrix metalloproteinase inhibitor TIMP-1.

Influence of low oxygen during expansion

According to our previous study, the supplementation of TFP during the expansion phase at normoxic (20% $O_2$) condition drastically enhanced the proliferation rate of HAC (Barbero et al, 2004). Expansion at low oxygen tension (5%$O_2$) did not modulate the proliferation rate of HAC in TFP or in CTR medium. In agreement with our finding, Malda and co-workers showed that bovine articular chondrocytes cultured on microcarrier beads at different low oxygen levels (4%, 10.5%, 21%), proliferated at similar doubling rate (Malda et al, 2004b).

Additionally to the proliferation ability, we investigated whether the application of physiological oxygen tension during the monolayer expansion would modulate the chondrogenic capacity of HAC. Again, chondrocytes expanded at 5% oxygen with or without TFP exhibited chondrogenic capacity, similar to that observed in normoxic condition. Overall our results indicate that physiological oxygen does not play an evident role during 2D proliferation. One possible interpretation could be that the proliferation in a 2D environment does not represent a physiological condition and cells do not respond as in the native environment.

Influence of physiological oxygen during re-differentiation

In our study the general poor tissue forming-capacity of CTR- and TFP-expanded HAC from elderly individual under normoxic condition is in agreement with our previous finding (Barbero et al, 2004). The low responsiveness of aged chondrocytes to a chondrogenic stimulus is in line with the finding of Loeser and co-workers who observed that oxidative damage caused by the overproduction of reactive oxygen species in aging cartilage contributed to the resistance of chondrocytes to IGF-1 stimulation (Loeser et al, 2002).

The application of physiological oxygen tension during the three-dimensional culture of chondrocytes has been investigated in several studies. However, the outcome is largely inconsistent and often controversial (Malda et al, 2003b), which might arise by the use of different cell types (different animal, anatomical origin, stage of differentiation), culture conditions (alginate, pellets, micro carrier) and the oxygen tensions applied (0.1% - 20%). We have shown that the application of 5%$O_2$ oxygen tension during the three-dimensional culture of *human* articular chondrocytes from elderly donors previously expanded in medium without growth factors did not enhance the production of GAG and type II collagen. At difference from our finding, Murphy and Polak (Murphy and Polak, 2004) showed that post-expanded chondrocytes better re-differentiated (fully re-acquiring the original phenotype) in alginate beads cultured at 5% as compared to 20% oxygen levels. The discrepancy between our results and those of this study could be explained by the different cell types used (cells from *knee* vs. *hip* cartilage) or the age of the patient (mean age of 49 years vs. 65years). Using human *nasal* chondrocytes, interestingly, Malda and co-workers(Malda et al, 2004a), observed that low oxygen tension positively modulated the re-differentiation of post-expanded chondrocytes derived from a young donor (age 28 years) but not from an old individual (age 71 years). In this study, the author suggested that the stimulatory effects of physiological oxygen tension on the redifferentiation of dedifferentiated human chondrocytes may be age-related and diminish in elderly donors.

An important finding of our study was that only when chondrocytes from elderly donors were expanded with TFP, the process of re-differentiation was largely enhanced under reduced oxygen tension. TFP-expanded HAC cultured in pellets under 5%$O_2$ condition expressed higher level of type II collagen (up to 86.6-fold) and aggrecan mRNA and produced large amount of GAG (up to 2.6-fold) than under normoxic conditions. The strong chondrogenic response to physiological oxygen tension by TFP-expanded chondrocytes could be due to the undifferentiated stage acquired by exposure to these growth factors along by an induction of progenitor cell features (Barbero et al, 2003). Indeed, mesenchymal progenitor cells isolated from fat tissue underwent enhanced chondrogenic differentiation when exposed to physiological oxygen environment (Wang et al, 2005).

Alternatively, the extrinsic application of TFP might directly modulate the expression of genes involved in the responsiveness to hypoxia. In various cell lines it has been shown that TGF-β can down-regulate CITED2 (Chou and Yang, 2006) a transactivator nuclear protein which is involved in the hypoxia-mediated signaling. In particular, CITED2 has been shown to negatively regulate hypoxia mediated signaling by competing with Hif-1α CBP/p300 transcriptional complex formation (Bhattacharya and Ratcliffe, 2003;Freedman et al, 2003). Thus, the application of TGF during the cell expansion phase might inhibit CITED2 expression and could provide a rational for the enhanced Hif-1α mediated cartilage specific matrix production under hypoxic condition.

Influence of physiological oxygen in the catabolic activity of HAC

In our study we also investigated whether increased tissue forming capacity of TFP-expanded HAC under culture at 5% oxygen tension would be related to modulation of specific metalloproteinases involved in the degradation of different extracellular matrix protein (i.e.; MMP-1, -2, -9, -13) and the MMP inhibitor TIMP-1. We observed that cells cultured at 5% oxygen the expression of MMP-1 and MMP-13 were down-regulated and TIMP-1 was enhanced as compared to conventional $O_2$ culture condition. Interestingly MMP-1 (or collagenase-1) and MMP-13 (or collagenase-3) are the metalloproteinases involved in the initial phase of the breakdown of type II

collagen (Dahlberg et al, 2000;Wu et al, 2002) and MMP-13 is the collagenase whose affinity for collagen type II is the greatest (Dahlberg et al, 2000;Reboul et al, 1996). Additionally, the up-regulation of TIMP-1 under low oxygen tension has been correlated with the regulatory role of the Hif-1 α signaling pathway (Yang et al, 2006). Therefore the larger content of collagen type II in the tissues generated under low oxygen tension seems to be derived both by an increased synthesis (in agreement with higher expression of the collagen type II mRNA) and by a reduced MMP-mediated degradation. Controversially, higher expression of such catabolic mediators, and possible other ones not investigated in this study, in tissues generated at 20% oxygen may partially explain their lower quality. However differences in the mRNA expression of MMPs mRNA in response to lowered oxygen measured in our study are not large (less then one order or magnitude) and require confirmation at the protein level.

There are strong evidences that MMP mediated tissue degenerative processes can be correlated with the presence of reactive oxidative species (ROS). ROS and the decline in the specific antioxidative defense of HAC from elderly individuals (Jallali et al, 2005) could provide additional rationales for the decrease of the cartilage tissue forming capacity under oxygen culture condition conventionally used in vitro.

## Conclusion

In conclusion, we have demonstrated that more physiologic oxygen tension applied during the monolayer expansion does not enhance the proliferation activity and re-differentiation capacity of human articular chondrocytes from elderly individuals. In contrary, the application of low oxygen tension during the phase of re-differentiation in 3D-culture can enhance matrix production and reduce the expression of catabolic mediators in HAC, but only when cells were previously expanded in medium containing de-differentiating factors (namely TGFβ-1/FGF-2/PDGF-BB, TFP). Thus the use of TFP during the monolayer expansion of chondrocytes and the further application of low oxygen

tension during the three-dimensional culture might be useful tools to enhance the outcome of cartilage engineering techniques in using cells from elderly individuals.

**Acknowledgements**

We would like to acknowledge the European Union for financial support ("STEPS"; FP6- #NMP3-CT-2005-500465, www.stepsproject.com).We are grateful to Francine Wolf for her assistance with immunohistological processing.

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# CHAPTER 5

# UNIFORM TISSUES ENGINEERED BY SEEDING AND CULTURING CELLS IN 3D SCAFFOLDS UNDER PERFUSION AT DEFINED OXYGEN TENSIONS

Enclosed is the paper published in Biorheology 2006;43:481–488.

Biorheology 43 (2006) 481–488
IOS Press


# Uniform tissues engineered by seeding and culturing cells in 3D scaffolds under perfusion at defined oxygen tensions

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**Abstract.** In this work, we assessed whether culture of uniformly seeded chondrocytes under direct perfusion, which supplies the cells with normoxic oxygen levels, can maintain a uniform distribution of viable cells throughout porous scaffolds several milimeters in thickness, and support the development of uniform tissue grafts. An integrated bioreactor system was first developed to streamline the steps of perfusion cell seeding of porous scaffolds and perfusion culture of the cell-seeded scaffolds. Oxygen tensions in perfused constructs were monitored by in-line oxygen sensors incorporated at the construct inlet and outlet. Adult human articular chondrocytes were perfusion-seeded into 4.5 mm thick foam scaffolds at a rate of 1 mm/s. Cell-seeded foams were then either cultured statically in dishes or further cultured under perfusion at a rate of 100 $\mu$m/s for 2 weeks. Following perfusion seeding, viable cells were uniformly distributed throughout the foams. Constructs subsequently cultured statically were highly heterogeneous, with cells and matrix concentrated at the construct periphery. In contrast, constructs cultured under perfusion were highly homogeneous, with uniform distributions of cells and matrix. Oxygen tensions of the perfused medium were maintained near normoxic levels (inlet $\cong$ 20%, outlet $>$ 15%) at all times of culture. We have demonstrated that perfusion culture of cells seeded uniformly within porous scaffolds, at a flow rate maintaining a homogeneous oxygen supply, supports the development of uniform engineering tissue grafts of clinically relevant thicknesses.

Keywords: Bioreactor, fluid flow, chondrocyte, mass transport, functional tissue engineering

## 1. Introduction

Tissues engineered *in vitro* by seeding and culturing cells into 3D porous scaffolds are frequently reported to have an inhomogeneous structure, consisting of a dense layer of cells and extracellular matrix (ECM) concentrated along the periphery, and a necrotic interior region. Such an inhomogeneous structure may limit the initial mechanical functionality and subsequent *in vivo* development of grafts of clinically relevant size.

In order to generate homogeneous tissue grafts, cells may have to be initially seeded into porous 3D scaffolds with a uniform distribution [7,8], thereby establishing a template for spatially uniform ECM deposition. The cells seeded within the interior regions must then be supplied with sufficient nutrients during prolonged 3D culture in order to maintain viability and support the production of ECM; possibly

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requiring the application of convective fluid flow. Since oxygen has a low solubility in culture medium, and is likely to be supplied to cells within the scaffold can help to determine whether the flow rate applied is sufficient.

We previously described a bioreactor for the perfusion of cell suspensions through the pores of 3D scaffolds in alternate directions, resulting in high efficiency and high spatial uniformity of cell seeding [8]. In this work, we first developed an integrated bioreactor system to allow prolonged perfusion of culture medium through 3D constructs following cell seeding by perfusion, within a single device. To continuously monitor the range of oxygen levels in the perfused constructs, in-line oxygen sensors were incorporated within the culturing pathway near the inlet and outlet of the constructs. Using the developed bioreactor system, we then tested the hypothesis that cells seeded uniformly throughout a 3D scaffold, when cultured under direct perfusion that supplies a normoxic range of oxygen, will generate a homogeneous graft with a uniform distribution of cells and ECM. The hypothesis was tested using a *human* chondrocyte/foam scaffold model system.

## 2. Methods

### *2.1. Integrated perfusion bioreactor system*

The bioreactor system is designed to perfuse first a cell suspension (Fig. 1a), and subsequently culture media (Fig. 1b), through the pores of a 3D scaffold within a single closed system. To avoid any risk of mechanically induced cell damage from a pump, we aimed to design a seeding flow path which did not recirculate the cell suspension through a peristaltic pumphead. Therefore, based on our previous seeding bioreactor, the seeding pathway is designed to instead pump the headspace above the cell suspension back and forth from one Teflon column to the other, thereby generating an alternating flow of the cell suspension through the scaffold. Cell settling and cell attachment to bioreactor components are minimized by its vertical orientation, component material properties, and by minimizing the surface area of horizontal surfaces where cells tend to accumulate. Scaffolds are lightly press-fit and clamped within a scaffold chamber (Fig. 1c), such that fluid flow cannot deviate around the scaffold but must flow through its pores. The chamber is manufactured from polycarbonate and polished until translucent, thus permitting the detection of possible air bubbles. Teflon FEP tubes (6 mm i.d.; Cole Parmer) are connected to disposable three-way stopcocks (Hi-Flow™; Medex GmbH) via polypropylene luer adaptors (EM-Technik GmbH), and stopcocks are then connected to the scaffold chamber via its luer connections.

Following the cell seeding phase, stopcocks can simply be rotated to divert flow through a separate perfusion loop for prolonged culture. Although the seeding path minimizes cell attachment to the bioreactor components, the separate pathway for prolonged culture eliminates the inadvertent culture of any attached cells which would nonetheless proliferate on the bioreactor components and consume vital nutrients. Gas exchange for medium oxygenation and pH buffering is achieved through platinum-cured silicon tubing (1/32″ i.d., 1/16″ o.d., 2 m length; Cole Parmer). Media is exchanged through Interlink® injection sites (Becton Dickinson) connected to the reservoir bottle and is performed without removing the system from the incubator.

To ensure accurate, controlled, and reproducible perfusion through each construct (i.e., avoiding either preferential channelling or negligible flow through one particular construct), each construct can be seeded and cultured independently from the others using independent flow pathways and a multi-channel peristaltic pump. Up to eight integrated bioreactor units can be placed into a standard-sized incubator.

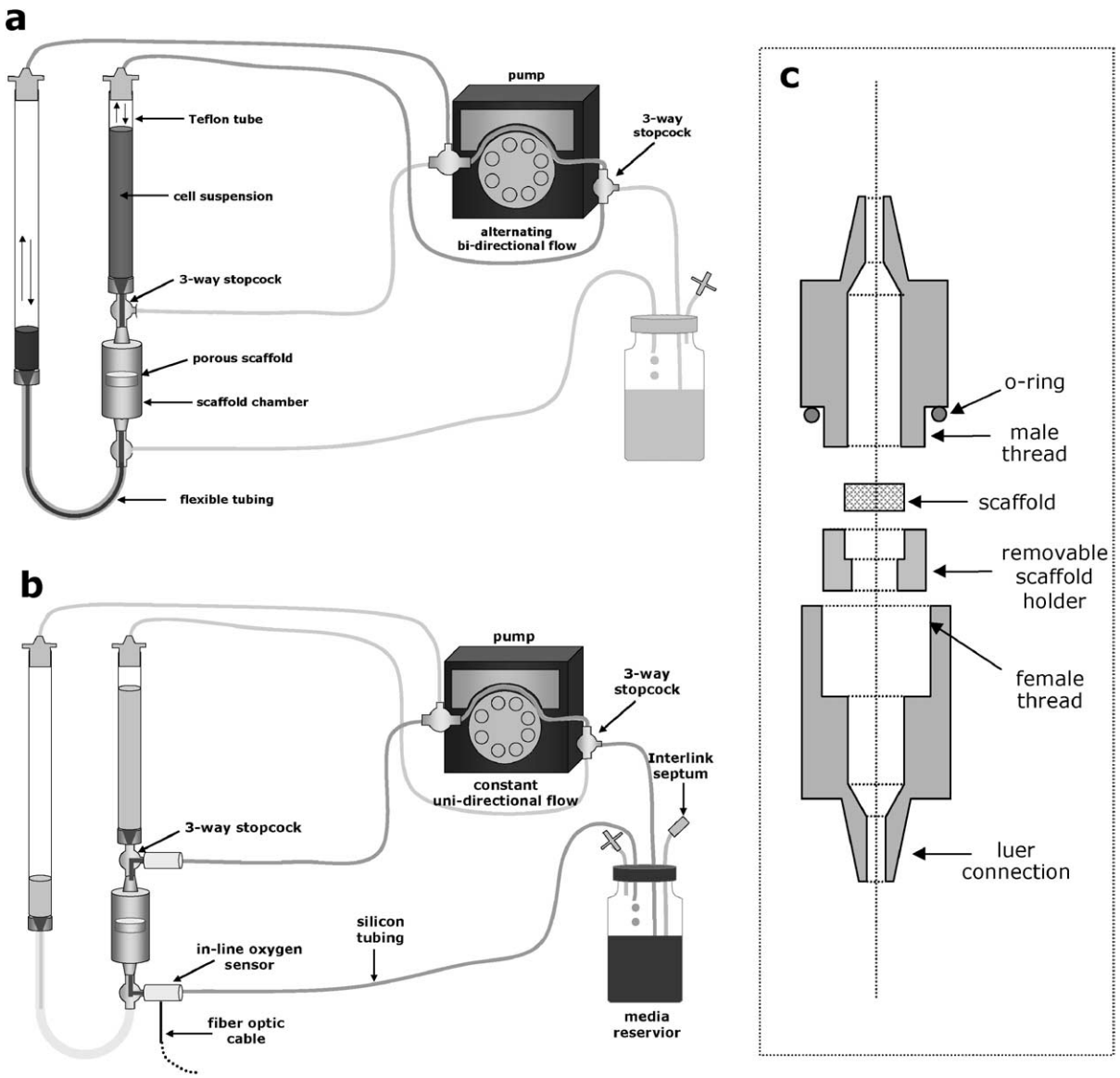


Fig. 1. Integrated perfusion bioreactor system. The bioreactor system streamlines the phases of cell seeding and prolonged culture of the cell-seeded scaffolds within a single device. (a) Cell seeding pathway. Alternating bi-directional flow of the cell suspension through the scaffold is generated by pumping the headspace above the cell suspension back and forth from one Teflon column to the other. In this way, cells are not recirculated through the pump, eliminating potential mechanically induced cell damage from the pumphead. (b) Prolonged culturing pathway. Following the cell seeding phase, the bioreactor system remains within the incubator, cell-seeded scaffolds are not handled or transferred to a separate bioreactor, but instead, stopcocks simply divert flow to a separate perfusion loop for prolonged culture. (c) Scaffold chamber. The scaffold is inserted into a removable holder, which simplifies sterile assembly and construct harvesting. The top of the chamber clamps the scaffold in place by its outer 1 mm periphery. The flow path contains a gradual expansion and contraction to reduce flow separation, and has a straight cylindrical region to fully develop the flow before reaching the construct.

Excluding the scaffold chamber, all components were selected for disposable use, eliminating risks of contamination and endotoxin build up associated with reuse. All bioreactor components were assessed for cytotoxicity in accordance with ISO10993-5.

### *2.2. Oxygen sensors*

In order to eliminate artifacts (i.e., inadvertent media re-oxygenation) associated with sampling and off-line analysis, *in-line* oxygen sensors were required. However, a perfusion system for the current tissue engineering application imposes unique requirements for the sensors (e.g., small size, accurate at low flow rates, long-term stability), necessitating non-traditional sensor technologies. Therefore, chemo-optic flow-through micro-oxygen sensors (FTC-PSt-3; PreSens GmbH, Germany), based on the quenching of luminescence by oxygen, which do not consume oxygen, are independent of flow rate, and maintain long-term stability, are incorporated into the culturing perfusion loop (Fig. 1b). A fiber optic cable transmits the optical signals between the sensor and oxygen meter (Fibox-3; PreSens GmbH).

Sensors are connected directly to the stopcocks at the inlet and outlet of the scaffold chamber, in order to prevent oxygen ingress into the system through tubing and other components, as seen in preliminarily experiments if sensors were placed further upstream/downstream. Nitrogen-sparged water (i.e., 0% oxygen) was perfused through the system to demonstrate that negligible oxygen from the incubator was diffusing into the bioreactor between the inlet and outlet sensor.

### *2.3. Experimental model system*

In two independent experiments, culture expanded adult human articular chondrocytes (AHAC) (1.3E+07 AHAC suspended in 8 ml media) were perfusion seeded onto foam disks made of poly(ethylene glycol terephthalate)/poly(butylene terephthalate) (PolyActive, 300/55/45 composition, compression molded, 8 mm diameter, 4.5 mm thick; IsoTis OrthoBiologics, The Netherlands) ($n = 12$ per experiment) at a superficial velocity of 1 mm/s overnight using an oscillating bi-directional flow regime (0.008 Hz). Following perfusion seeding, four constructs were examined by confocal microscopy to determine the cell viability (staining with LIVE/DEAD® Viability/Cytotoxicity Kit; Molecular Probes) as previously described [8], and were assessed histologically for the uniformity of the cell distribution (hematoxylin and eosin (H&E) stained cross-sections). Four seeded foams were transferred to agarose-coated dishes for static culture, and four remained within the bioreactor system for perfusion culture with continuous uni-directional flow at a superficial velocity of 100 $\mu$m/s. Constructs were cultured in 25 ml of DMEM (4.5 g/l glucose with nonessential amino acids) supplemented with 10% FBS, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 0.1 mM ascorbic acid 2-phosphate, 1 U/ml insulin, and 10 ng/ml TGF$\beta$-3 for two weeks in a 20% oxygen/5% $CO_2$ incubator, with media exchanged three times per week. Cultured constructs were examined with confocal microscopy for the distribution of viable cells and histologically for the uniformity of the cell and matrix distribution (H&E staining).

## 3. Results

### *3.1. Cell seeding into porous scaffolds*

Following cell seeding, essentially all AHAC detected in the foams were viable, likely due to efficient nutrient transport and the flushing of dead cells out from the scaffold pores resulting from perfused

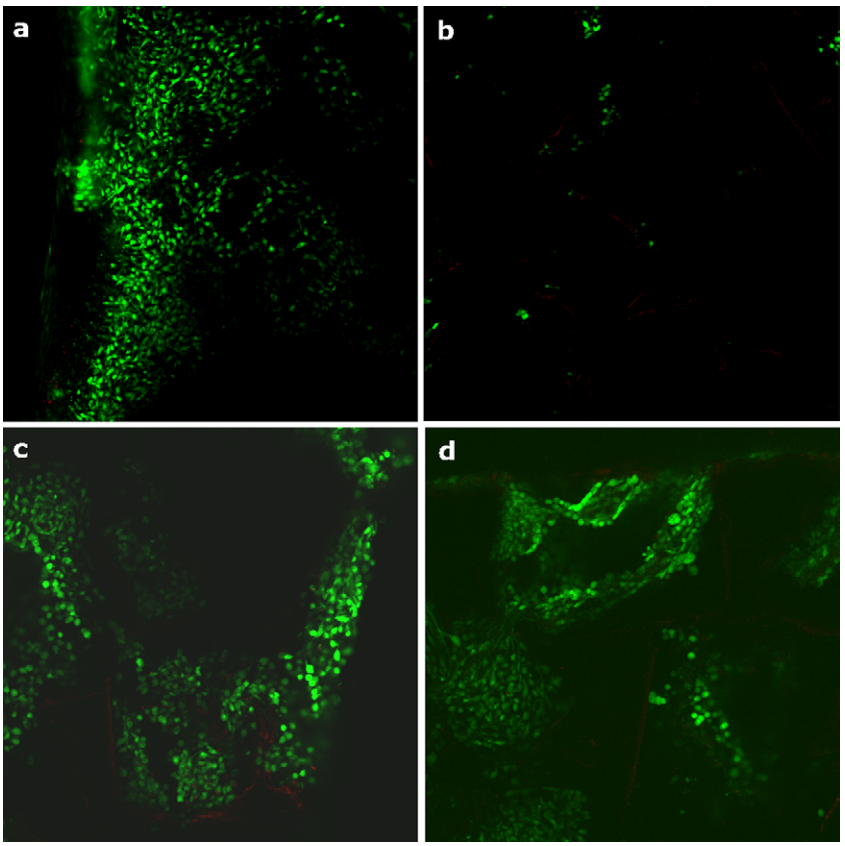


Fig. 2. Cell viability after two weeks of culture. Presented confocal microscopy images show only the channel for viable cell staining. Statically cultured constructs contained (a) a high density of viable cells concentrated at the periphery, and (b) an essentially non-viable internal region. In contrast, perfusion cultured constructs contained high densities of cells both (c) at their periphery, and (d) throughout their internal region.

flow. Furthermore, cells were seeded with a highly uniform distribution throughout the cross-sections, consistent with results obtained using a previously described perfusion bioreactor [8].

### *3.2. Static culture of cell-scaffold constructs*

Perfusion seeded foams cultured statically for 2 weeks contained a dense layer of viable cells along the outer 0.5–1.0 mm periphery of the construct which encapsulated an internal region with predominantly non-viable cells (Fig. 2a). Histologically, constructs cultured statically were highly heterogeneous, containing cells and ECM concentrated only along the outer 1 mm periphery (Fig. 3a).

### *3.3. Perfusion culture of cell-scaffold constructs*

At all times throughout the culture period, oxygen tensions measured at the inlet remained near saturation levels (i.e., 20%), and those measured at the outlet remained above 15% (Fig. 4). These data confirmed that at the flow rate used, the bioreactor maintained an efficient and rather homogeneous oxygen supply to the cells within the perfused constructs, close to normoxic levels.

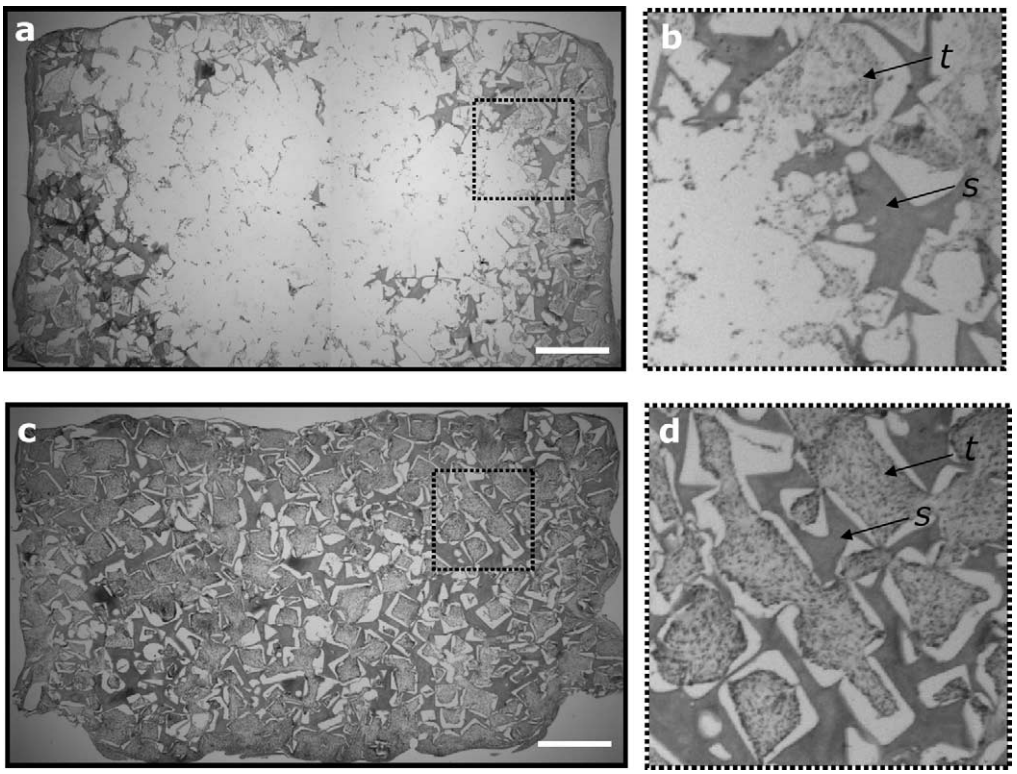


Fig. 3. Representative H&E stained cross-sections following two weeks of culture. (a,b) Statically cultured constructs; (c,d) perfusion cultured constructs; (a,c) low magnification images show the tissue distribution throughout the entire cross-section (scale bar = 1 mm); (b,d) higher magnification images identify the tissue "$t$" and scaffold "$s$" within the cross-sections. Statically cultured constructs contained cells and matrix only at the construct surface, reaching a depth of approximately 1 mm into the scaffold. In contrast, perfusion cultured constructs were highly homogeneous, containing a uniform distribution of cells and matrix throughout the cross-section.

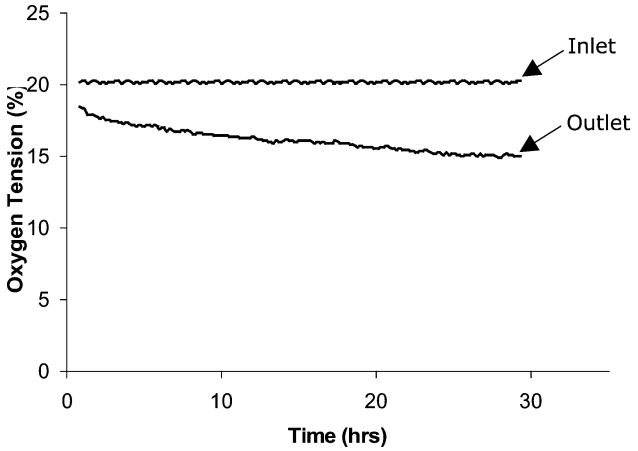


Fig. 4. Profiles of the oxygen tensions measured at the inlet and outlet of the scaffold chamber. Inlet oxygen concentrations remained near saturation levels throughout the two week culture period. Oxygen levels measured at the outlet were less than 5% lower than the inlet, indicating that cells within the construct were exposed to relatively homogeneous oxygen tensions.

Perfusion seeded foams cultured under perfusion contained viable AHAC distributed throughout both the exterior and interior regions of the foams, with few non-viable cells detected (Fig. 2b). In contrast to static cultures, constructs cultured under perfusion were remarkably homogeneous, containing a uniform distribution of both cells and ECM throughout the entire cross-section (Fig. 3b). However, despite the remarkable tissue uniformity in perfused constructs, only in small localized areas did AHAC have a rounded cell morphology, characteristic of differentiated chondrocytes.

## 4. Discussion

In this paper, we described the design of an integrated bioreactor system, which streamlines within a single device the phases of perfusion cell seeding and prolonged perfusion culture of the cell-seeded scaffolds. Using the developed bioreactor we then demonstrated that culture of uniformly seeded AHAC under direct perfusion, maintaining cells in a normoxic range of oxygen levels, can maintain a uniform distribution of viable cells throughout thick porous scaffolds, and support the development of uniform tissue grafts is necessary to generate a homogeneous tissue with a uniform distribution of both cells and ECM.

Previous studies have used perfusion systems to culture chondrocytes into porous scaffolds, but either did not address the uniformity of the resulting tissues [1,5,6] or reported the formation of heterogeneous tissues, despite using scaffolds that were rather thin [2]. The discrepancy between the latter study and our results could be explained by the fact that in our experiments perfusion culture was introduced following the uniform seeding of cells by perfusion, and/or by the higher flow rate (approximately 10-fold higher) used in our system, which may have provided a more efficient and homogeneous oxygen supply to the chondrocytes within the 3D constructs.

Despite initially having a uniform distribution of cells, statically cultured foams contained only a thin layer of cells and matrix concentrated at the construct periphery. Although oxygen tensions within statically cultured constructs were not assessed in this study, the heterogeneity could be explained by the steep oxygen gradients (from 20% at the surface to 2% at a depth of 1 mm from the surface) previously predicted [3] and measured [4] using the same polyactive foams cultured in the absence of perfusion. The dramatically improved tissue uniformity generated under perfusion as compared to static culture may be attributed to the maintenance of a normoxic range of oxygen levels across the constructs, as well as to the increase of mass transport of other nutrients (e.g., glucose) and metabolic waste products. In this context, studies are ongoing, using the developed bioreactor system, to understand the specific influence of oxygen on chondrocyte metabolism and function within a 3D construct.

In conclusion, we have developed an integrated perfusion bioreactor system for the engineering of uniform 3D grafts based on AHAC. The described device could be used in conjunction with other cell types, not only for generating uniform tissue grafts, but also as a controlled model system to investigate fundamental mechanisms of cell function in a 3D environment.

## Acknowledgements

This work was supported by the Swiss Federal Office for Education and Science (B.B.W.) under the Fifth European Framework Growth Program (project SCAFCART, grant No. G5RD-CT-1999-00050). We are grateful to Jeroen Pieper from IsoTis OrthoBiologics for kindly providing the Polyactive foams.

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# CHAPTER 6

# INFLUENCE OF PHYSIOLOGICAL OXYGEN LEVELS ON ADULT HUMAN CHONDROCYTES CULTURED IN 3D SCAFFOLDS UNDER PERFUSION

Enclosed is the Paper currently in preparation.

# Influence of physiological oxygen levels on adult human articular chondrocytes cultured in 3D scaffolds under perfusion

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**Keywords**: oxygen, cartilage, bioreactor, redifferentiation, fluid flow, mass transport, functional tissue engineering

**ABSTRACT**

In smaller-scale model systems (i.e., pellet and alginate beads), chondrocyte redifferentiation may be enhanced when cultured under physiological oxygen (≈5%) vs normoxic (≈19%) levels. Under normoxic conditions, culturing expanded adult human articular chondrocytes (AHAC) in 3D scaffolds under direct perfusion can enhance cell viability and matrix deposition as compared to static culture conditions. In this study, we tested the hypothesis that supplying physiological ranges of oxygen to AHAC cultured in 3D scaffolds under perfusion would enhance the chondrogenic redifferentiation and glycosaminoglycan (GAG) deposition as compared to supplying normoxic ranges. We then assessed whether the response of AHAC to low oxygen is dependent upon the applied perfusion rate (100µm/s or 10µm/s) or to the developmental stage of the construct. AHAC cultured in a 3D pellet model system served as controls.

Compared to 3D pellets cultured at 19%$O_2$, those cultured at 5%$O_2$ stained more intensely for GAG, contained significantly more GAG/DNA (2.1-fold higher), and had 600-fold higher collagen type II mRNA expression. At 100µm/s, compared to 19%$O_2$ constructs, 5%$O_2$ cultured constructs stained slightly more for GAG, contained more GAG/DNA (25% higher), and upregulated expression of collagen type II mRNA 6-fold. At 10µm/s, constructs cultured under 5%$O_2$ stained more intensely for GAG and contained significantly more GAG/DNA (31% higher) than those under 19%$O_2$, but the effects of oxygen was not more pronounced than at the higher flow rate. Applying low and high oxygen tensions at different stages of construct development also did not affect AHAC redifferentiation and matrix production in longer-term cultures.

In this work we have used a controlled bioreactor model system to demonstrate that a physiological range of oxygen supplied to dedifferentiated AHAC in a PolyActive foam enhanced the redifferentiation and GAG deposition as compared to supplying normoxic oxygen levels. Nevertheless, these effects were significantly less pronounced than in the 3D pellet model system, and the extent to which the resulting engineered cartilage was actually improved under low oxygen would not likely have a significant clinical impact on the functionality of the graft when implanted

in vivo. Considering the marked effects observed in the 3D pellet system, future efforts should aim to understand how physiological oxygen could be used as a tool to improve the functionality of engineered cartilage grafts for clinical applications.

## INTRODUCTION

Injuries to articular cartilage are known to have limited self-healing capacity, and if left untreated have the tendency to develop into early degenerative joint changes. Conventional surgical treatments for joint lesions are not fully satisfactory, because they often result in a repair tissue that is fibrocartilaginous and exhibit poor mechanical properties. Autologous cartilage grafts engineered *in vitro*, which resemble the composition and function or native cartilage, have the potential to support the regeneration of full-thickness cartilage defects, allowing early postoperative rehabilitation and function. To engineer articular cartilage grafts of clinically relevant size, the few number of chondrocytes that can be obtained from a typical tissue biopsy must be extensively expanded in monolayer culture prior to seeding and culturing within a three-dimensional porous biomaterial. However, a significant challenge lies in this process given that adult human articular chondrocytes ("AHAC") have a rather limited capacity to redifferentiate and synthesize cartilaginous extracellular matrix (ECM) following monolayer expansion.

While growth factors are recognized to be potent regulators of chondrocyte redifferentiation and biosynthetic activity (Cancedda et al, 1995), basic environmental parameters such as oxygen levels may also have a strong influence on the biology of chondrocytes during *in vitro* culture. As mature articular cartilage is an avascular tissue, AHAC *in vivo* rely on the transport of oxygen from the synovial surface via diffusion and forced convection during joint loading, and thus are exposed to particularly low oxygen concentrations (2-7% $O_2$) (Brighton and Heppenstall, 1971). The unusual milieu of the chondrocyte has driven much speculation that the application of physiological levels of oxygen during *in vitro* cell/tissue culture could drive or maintain chondrocytes in a more chondrogenic state as compared to nominal "normoxic" levels within typical cell culture incubators (19-21% $O_2$).

In small scale model systems it was recently shown that physiological oxygen tensions can enhance the redifferentiation capacity and/or the biosynthetic activity of primary human chondrocytes cultured on collagen membranes (Kurz et al, 2004;Wang et al, 2005), expanded

human nasal chondrocytes in micromass culture (Malda et al, 2003a), and expanded human articular chondrocytes in alginate beads (Murphy and Polak, 2004). However, when larger scale model systems have been employed, nonetheless with animal derived cells, the application of different oxygen tensions have provided seemingly conflicting conclusions. Whereas Obradovic et al. found primary bovine chondrocytes to synthesize more glycosaminoglycans (GAG) when cultured in polyglycolic acid meshes under aerobic conditions (Obradovic et al, 1999), Saini and Wick reported higher GAG synthesis if cultured in poly-L-lactide acid meshes under a low oxygen tension (Saini and Wick, 2004). On the other hand, physiological or normoxic oxygen tensions were found to have little influence on redifferentiated porcine chondrocytes cultured on the surface of ceramic scaffolds (Nagel-Heyer et al, 2006).

The aforementioned studies illustrate the controversy currently ongoing in the literature (Malda et al, 2003b), and highlight that no conclusion can be drawn as to the effects of physiological oxygen levels on human chondrocytes, and moreover, as to whether applying such oxygen tensions would be advantageous or unfavorable to the engineering of clinically relevant engineered grafts. The lack of a clear consensus is likely due in part to the wide variety of model systems that have been employed, including different species (human or various animals), differentiation stages (primary or expanded cells), and culturing systems (monolayer culture, engineered 3D tissues, or explants). To further confound interpretation of the existing data, studies have typically only controlled and reported the oxygen level in the incubator, which however, due to mass transport limitations in culture media and within the 3D tissues, does not allow to accurately control or precisely define the actual oxygen concentration applied to the *cell*.

We previously developed a bioreactor system that perfused culture media directly through the pores of 3D scaffolds, thereby reducing diffusion controlled oxygen transport and oxygen gradients within the construct, and facilitating control over the oxygen applied to the cells (Wendt et al, 2006). Inline micro-oxygen sensors were integrated into the system to monitor the range of oxygen in the culture medium supplied to cells within the 3D constructs throughout the culture

period. Using this bioreactor system, we demonstrated that when AHAC where supplied with a normoxic range of oxygen (i.e., 15-19%), a graft of clinically relevant size could be generated, which contained a uniform distribution of viable cells and ECM. In this previous work, AHAC were cultured in a specific composition of PolyActive foam, which has an equilibrium modulus and dynamic stiffness (Miot et al, 2005) comparable to native cartilage in the human knee (Treppo et al, 2000), and would thus be particularly appealing for the engineering of articular cartilage grafts for weight bearing joints. Despite the advantage of having favorable mechanical properties, human chondrocytes have been shown to have a rather limited capacity to redifferentiate and generate cartilage specific ECM when cultured in this scaffold under conventional normoxic culture conditions (Miot et al, 2005). Therefore, in this work, we used our controlled perfusion bioreactor model system to test the hypothesis that supplying a physiological range of oxygen (i.e., 1-5%) to dedifferentiated AHAC cultured in a PolyActive foam would enhance the redifferentiation and GAG deposition as compared to supplying normoxic oxygen levels. We then assessed whether the response of AHAC to low oxygen could be dependent upon the applied flow rate or to the developmental stage of the construct.

## MATERIAL AND METHODS

### Chondrocyte isolation and expansion

Human articular cartilage was collected from the femoral condyle of cadavers (18-43 years of age), with no known clinical history of joint disorders, within 24 hours after death, after informed consent of the relatives and approval by the local ethical committee. Chondrocytes were isolated using 0.15% type II collagenase (Worthington Biochemical Corporation, Lakewood, NJ) for 22 hours and resuspended in Dulbecco's Modified Eagle's Medium (DMEM; 10938-025) containing 10% fetal bovine serum, 1mM sodium pyruvate, 100mM HEPES buffer, 100U/ml penicillin, 100µg/ml streptomycin, and 0.29mg/ml L-glutamine ("complete medium"). Human articular

chondrocytes were expanded for two passages in complete medium supplemented with 1ng/mL Transforming growth factor-β1 (TGF-β1), 5ng/mL Fibroblast growth factor-2 (FGF-2) and 10ng/mL Platelet-derived growth factor-bb (PDGF-bb), a cocktail of factors previously shown to increase human chondrocyte proliferation rate and post-expansion chondrogenic capacity (Barbero et al, 2003).

Chondrocyte redifferentiation

*Scaffold-free controls:* Culture expanded AHAC were transferred into 1.5ml polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 250 x g to form spherical 3D pellets (5E+05 cells per pellet) to serve as scaffold-free controls. 3D pellets were cultured in 0.5mL complete medium supplemented with 10µg/mL Insulin, 0.1mM ascorbic acid 2-phosphate, and 10ng/mL Transforming Growth Factor-β3 ("chondrogenic medium") for two weeks within incubators (37°C, 5%$CO_2$) with either normoxic (19%$O_2$) or physiological (5%$O_2$) oxygen levels.

*Engineered constructs:* Engineered constructs were generated in a bioreactor system previously shown to support development of thick tissue grafts with a uniform distribution of viable cells and extracellular matrix (Wendt et al, 2006). The bioreactor system perfused first a cell suspension, and subsequently culture media, through the pores of a 3D scaffold within a single closed system. Culture expanded AHAC were perfusion seeded (1.3E+07 AHAC suspended in 8ml complete media) onto foam disks made of poly(ethylene glycol terephthalate)/poly(butylene terephthalate) (PolyActive, 300/55/45 composition, compression molded, 8mm diameter, 4.5mm thick; IsoTis OrthoBiologics, The Netherlands) as previously described (Wendt et al, 2003). Following perfusion cell seeding, constructs remained within the bioreactor system and were cultured under perfusion at superficial velocities of 100µm/s (4 independent experiments) or 10µm/s (2 independent experiments) for two weeks. Alternatively, perfusion seeded constructs were removed from the bioreactor and transferred to agarose coated flasks to serve as static culture controls. Flasks and

bioreactors were placed within incubators (37°C, 5%$CO_2$) with either normoxic (19%$O_2$) or physiological (5%$O_2$) oxygen levels. Static and perfused constructs were cultured in 25mL of chondrogenic medium per construct, which was replaced twice per week.

To assess the influence of oxygen at different stages of construct development, in one experiment perfusion seeded foams were perfusion cultured for up to four weeks in one of three conditions: i.) 19%$O_2$ at 100µm/s, ii.) 5%$O_2$ and adjusting the flow rate in order to maintain the outlet oxygen measurements at 1.5%$O_2$, or iii.) two weeks in 19%$O_2$ at 100µm/s followed by two weeks in 5%$O_2$ with the variable flow rate condition (group: "19%/5%"). Variable flow rates were adjusted once per day, and increased from 18µm/s to 85µm/s during the four week culture under 5%, and ranged from 33µm/s to 90µm/s during the last two weeks under 5% for group 19%/5%.

Analytical methods

*Oxygen measurements:* To monitor the range of oxygen applied to AHAC within the perfused constructs, in-line oxygen sensors were incorporated into the perfusion flow path (Wendt et al, 2006). Chemo-optic flow-through micro-oxygen sensors (FTC-PSt-3; PreSens GmbH, Germany) were connected to the bioreactor scaffold chambers and the oxygen concentration of the media near the outlet of the construct was measured every 10 minutes throughout the culture period. Prior to reinfusion at the inlet of the construct, medium was fully reoxygenated to saturation levels (i.e., either 5% or 19%) by recirculation through highly gas permeable silicon tubing.

*Quantitative real-time RT-PCR:* Total RNA of cells cultured in pellets and constructs was extracted using Trizol (Life Technologies, Basel, Switzerland) and the standard single-step acid-phenol guanidinium method. RNA was treated with DNAseI using the DNA-free™ Kit (Ambion, USA) and quantified spectrometrically. cDNA was generated by using 500µg/ml random hexamers (Catalys AG, CH) and 1µl of 50 U/ml Stratascript™ reverse transcriptase (Stratagene, NL), in the presence of dNTPs. Real-time RT-PCR reactions were performed and monitored using the ABI

Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, Switzerland). Primers and probes used for the reference gene (18-S rRNA) and the genes of interest (collagen type II and collagen type I), as well as the reactions setting, were previously described (Barbero et al, 2003). Each sample was assessed at least in duplicate.

*Biochemical quantification:* Engineered constructs and 3D pellets were digested with 0.5 ml protease K solution (1mg/ml protease K in 50mM Tris with 1mM EDTA, 1mM iodoacetamide, and 10µg/ml pepstatin-A for 15 hours at 56°C) as previously described (Hollander et al, 1994). DNA was quantified with the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR), with calf thymus DNA as a standard. GAG was quantified with the dimethylmethylene blue colorimetric assay, with chondroitin sulfate as a standard (Farndale et al, 1986).

*Histological analysis:* Engineered constructs and 3D pellets were rinsed in phosphate buffered saline, fixed in 4% formalin, embedded in paraffin, cross-sectioned (10µm thick) and stained with Safranin-O for GAG.

*Statistical analysis:* Differences between the experimental groups of two week cultures were evaluated by ANOVA with donor, and oxygen tension or flow rate as factors, and considered to be statistically significant with $p \leq 0.05$. Differences between the experimental groups of four week cultures were evaluated by non-parametric Mann Whitney U tests and considered to be statistically significant with $p \leq 0.05$.

**RESULTS**

Scaffold-free controls

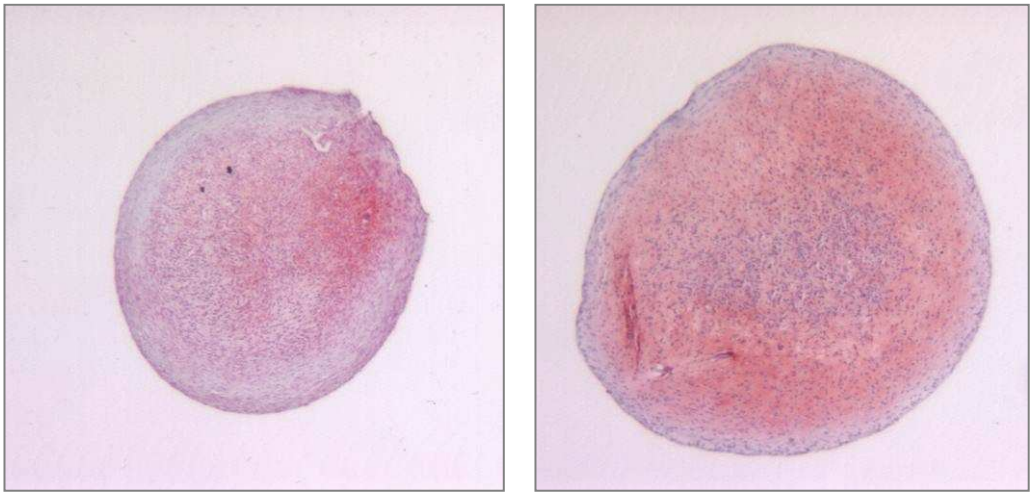

**Figure 1.** Safranin-O stained cross-sections of 3D pellets cultured in incubators with (A) normoxic (19%) or (B) physiological (5%) oxygen levels for 2 weeks.

AHAC 3D pellets cultured in 5%$O_2$ were significantly larger and more intensely stained for GAG than pellets cultured in 19%$O_2$ (Figure 1). Chondrocytes within the 5%$O_2$ cultured pellets had a round morphology and were embedded within lacunae. Biochemical analyses were consistent with histological observations, indicating that pellets cultured in 5%$O_2$ contained significantly higher ratios of GAG/DNA than pellets cultured in 19%$O_2$ (2.1-fold higher, p=0.05) (Figure 2). Moreover, expression of collagen type II mRNA was nearly 600-fold higher in pellets cultured under 5%$O_2$ than 19%$O_2$ (p=8.8E-04), whereas expression of collagen type I was similar between the two groups.

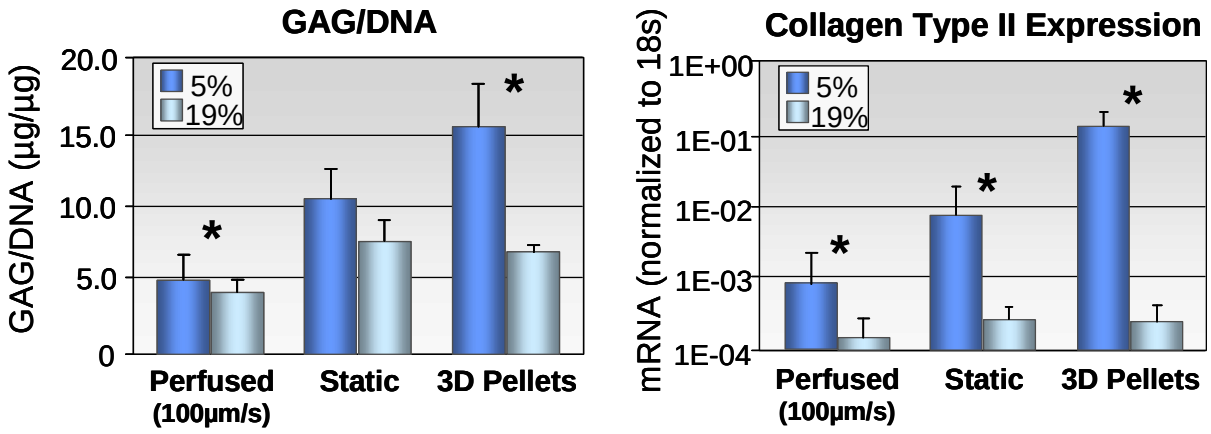


**Figure 2.** GAG/DNA ratios and collagen type II mRNA expression of AHAC foam constructs and 3D pellets cultured for two weeks. * indicates a statistically significant difference between the two oxygen tensions, determined by ANOVA and controlling for donor variability.

Static culture controls

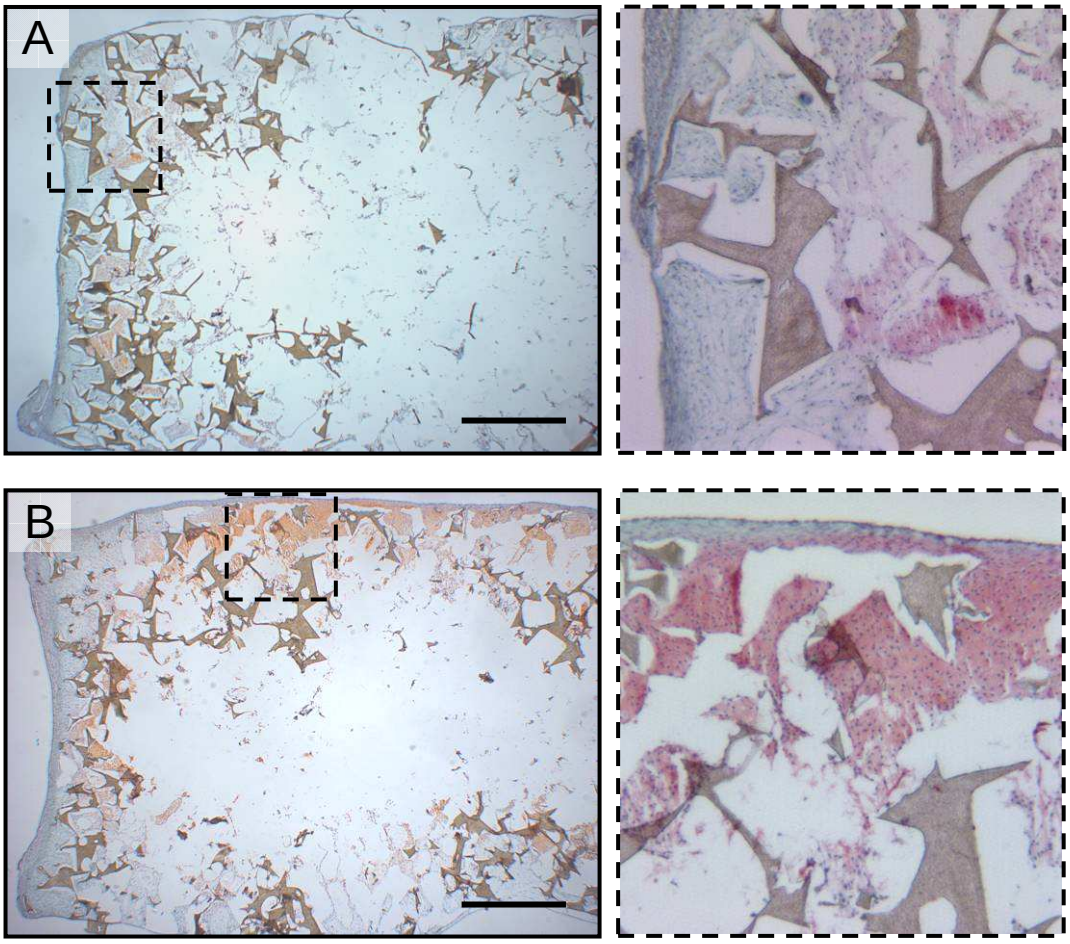


**Figure 3**. Safranin-O stained cross-sections (transversally) of constructs cultured statically in incubators with (A) normoxic (19%) or (B) physiological (5%) oxygen levels for 2 weeks. Scale bar = 1mm

Perfusion seeded foams cultured statically for 2 weeks in either 5% or 19% $O_2$ incubators contained a dense layer of cells along the outer 0.5-1.0mm periphery that encapsulated an internal region essentially void of cells and matrix (Figure 3). The matrix at the periphery of 5%$O_2$ static constructs was more intensely stained for GAG than that of the 19%$O_2$ static constructs. However, both the total amount of DNA and the ratios of GAG/DNA were similar in constructs cultured statically at either oxygen tension (Figure 2). Analogous to trends observed in 3D pellet controls, expression of collagen type II mRNA was 28-fold higher in 5% vs. 19% static constructs, and expression of collagen type I was similar between the two groups.

Perfusion cultured constructs

*Two week cultures:* Representative profiles of the oxygen levels in the culture media at the outlet of the perfused constructs throughout the two week culture period are shown in Figure 4.

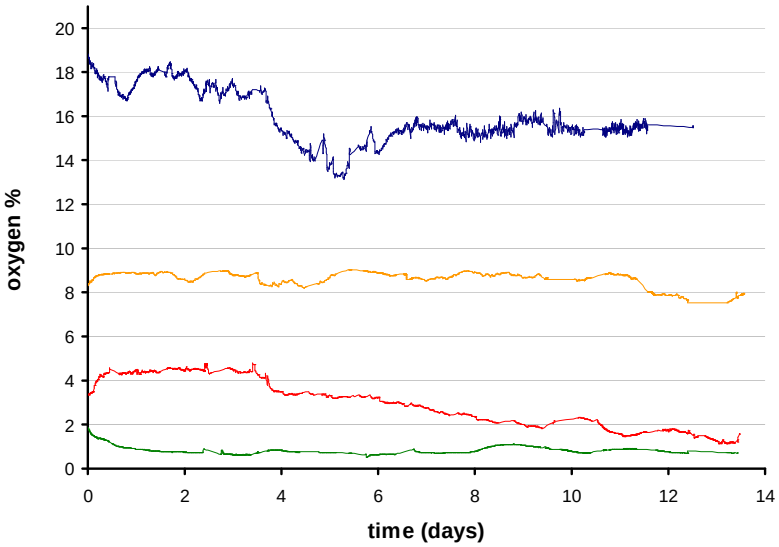


**Figure 4**. In line oxygen measurements at the outlet of uni-directionally perfused constructs at constant inlet oxygen levels (19%O2 or 5%O2) and different perfusion flow rates (100µm/s or 10µm/s) over 2 weeks culture period.

When perfused at 100µm/s, oxygen levels at the construct outlet remained above 14% and 1.5%$O_2$ when constructs were supplied with 19% or 5%$O_2$ at the inlet, respectively. When perfused at

10µm/s, oxygen levels at the construct outlet remained above 8%$O_2$ when supplied with 19%$O_2$ at the inlet, but dropped to less than 0.5%$O_2$ when 5%$O_2$ was supplied to the inlet.

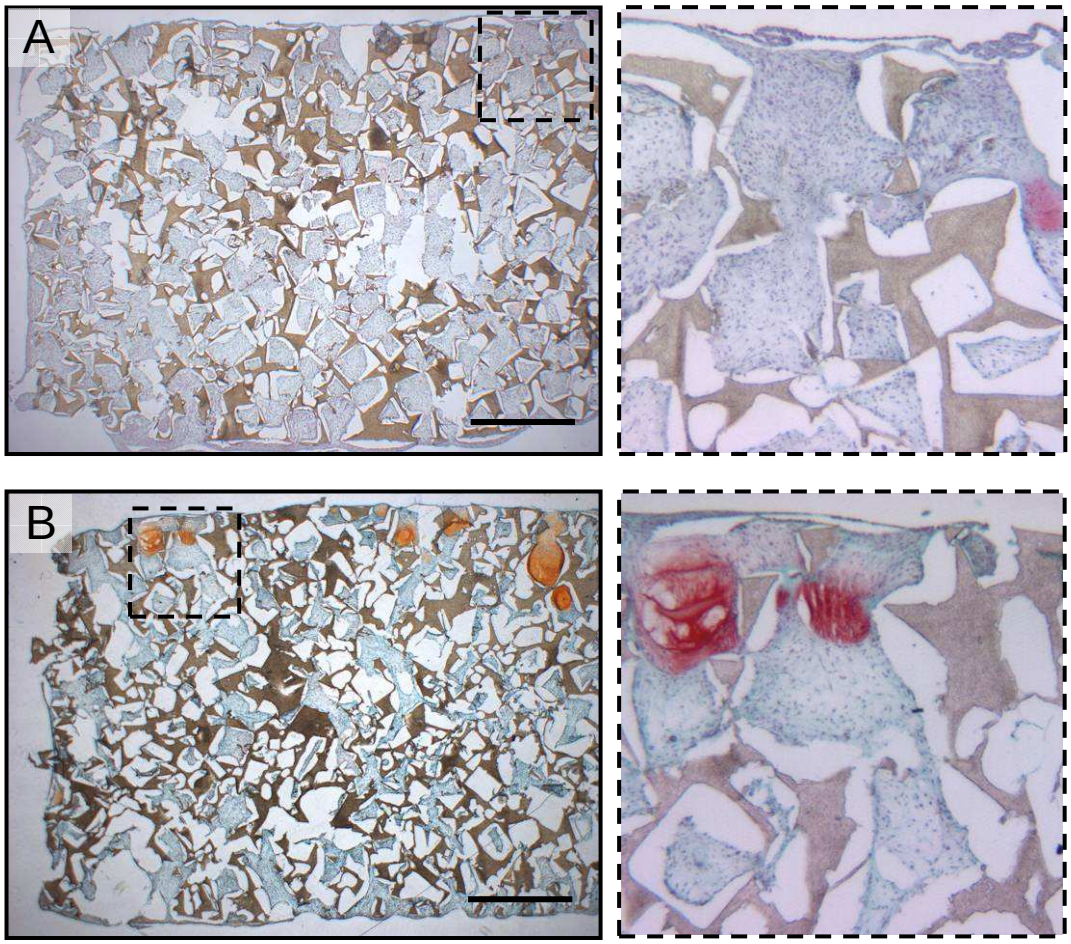


**Figure 5**. Safranin-O stained cross-sections (transversally) of constructs cultured under perfusion at the higher perfusion rate (100µm/s) under a (A) normoxic (14-19%$O_2$) or (B) physiological (1.5-5%$O_2$) range of oxygen for 2 weeks. Scale bar = 1mm.

In contrast to static cultures, constructs supplied with 5% or 19% inlet oxygen levels at the higher perfusion rate (100µm/s) contained a highly uniform distribution of both viable cells and matrix (Figure 5). Despite the homogeneous tissues obtained, constructs perfused under 19%$O_2$ stained rather faintly for GAG and generally contained chondrocytes with a fibroblastic-like morphology. Constructs perfused under 5%$O_2$ generally stained more intensely for GAG, and along with regions with fibroblastic-like chondrocytes, displayed areas with round chondrocytes

embedded within lacunae. Biochemical analyses were consistent with histological observations. Constructs perfused at 100μm/s under 5%$O_2$ contained significantly higher ratios of GAG/DNA (25% higher, p=0.037), and significantly less DNA (29% less, p=0.031) than the corresponding 19% perfused constructs (Figure 2). Due to the high donor to donor variability associated with the redifferentiation capacity of human chondrocytes (Barbero et al, 2004), significant differences were identified when ANOVA with donor as a factor was performed, but not by simple T-tests in which all constructs for a given oxygen tension where simply pooled together. The fraction of GAG released into the media, relative to the total GAG synthesized, was similar using the two oxygen tensions (84.4±3.7% at 5%$O_2$, 81.1±6.0% at 19%$O_2$). Consistent with the general trends observed for 3D pellet and static controls, expression of collagen type II mRNA was 6-fold higher in 5% than 19% constructs, and expression of collagen type I was similar between the two groups. Ratios of glucose to lactate conversion indicated a predominant anaerobic metabolism, but were not significantly affected by the oxygen tension (data not shown), consistent with the redifferentiation of human articular chondrocytes in alginate beads (Murphy and Polak, 2004).

Since fluid-induced shear stresses could adversely affect chondrocyte redifferentiation (Lee et al, 2002), and potentially influence the response to different oxygen tensions, constructs were also perfused at one-tenth the flow rate. AHAC-foams perfused at the lower perfusion rate (10μm/s) under 19%$O_2$ contained a highly uniform distribution of both viable cells and matrix (Figure 6). However, chondrocytes were fibroblastic-like in morphology, and the ECM stained faintly for GAG. In contrast, chondrocytes within constructs perfused at 10μm/s under 5%$O_2$ were round, embedded within lacunae, and surrounded by ECM rather intensely stained for GAG. Nevertheless, these constructs only contained cells and matrix within the top 1.5-2mm of the scaffold, consistent with the low oxygen levels measured in the media at the construct outlet. Constructs perfused under low flow and low oxygen contained significantly higher ratios of GAG/DNA (31% higher, p=0.017), had similar amounts of DNA (p=0.21), and expressed similar levels of both collagen II and collagen I than the corresponding constructs perfused under 19%.

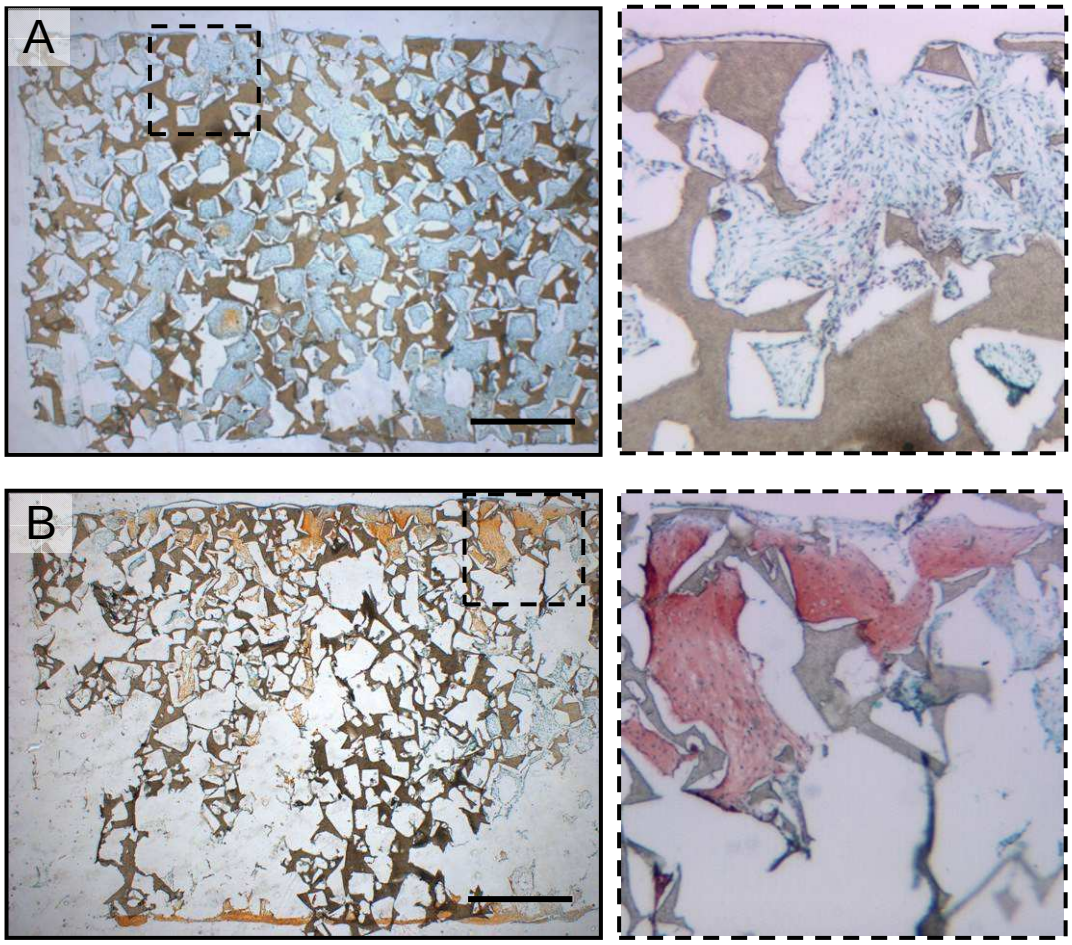


**Figure 6.** Safranin-O stained cross-sections (transversally) of constructs cultured under perfusion at the lower perfusion rate (10µm/s) under a (A) normoxic (8-19%$O_2$) or (B) low (0.5-5%$O_2$) range of oxygen for 2 weeks. Scale bar = 1mm.

Constructs cultured at either 5% or 19% contained significantly more DNA when perfused at the higher vs lower flow rate (87% more under 5%$O_2$, p=5.7E-07; 54% more under 19%$O_2$, p=5.2E-05). The ratios of GAG/DNA were not affected by flow rate under either oxygen tension. Expression of collagen type II was significantly higher at the lower flow rate under both oxygen tensions (800-fold higher under 5%, 1400-fold higher under 19%). The fractions of accumulated GAG, relative to total synthesized GAG, were similar in constructs perfused at 10µm/s as constructs cultured under the same oxygen tension at 100µm/s.

*Four week cultures*: Since the response of AHAC to different oxygen tensions could be regulated by the stage of construct maturation, constructs were perfused with high or low ranges of oxygen during two subsequent two week culture periods. All constructs perfusion cultured for four weeks contained a highly homogeneous distribution of cells and matrix throughout the volume of the scaffold (Figure 7). In general, four week constructs appeared to have lower densities of cells but more dense matrix than two week constructs. No differences were observed between any of the four week groups in terms of cell morphology, cell density and distribution, or GAG staining. Biochemical analyses were consistent with histological observations, indicating no significant differences in the total amount of DNA or the ratios of GAG/DNA between any of the four week experimental groups. However, expression of collagen type II mRNA was respectively 10-fold and 30-fold higher in the 19%/5% and 5% cultured constructs as compared to those cultured at 19% for the entire four weeks.

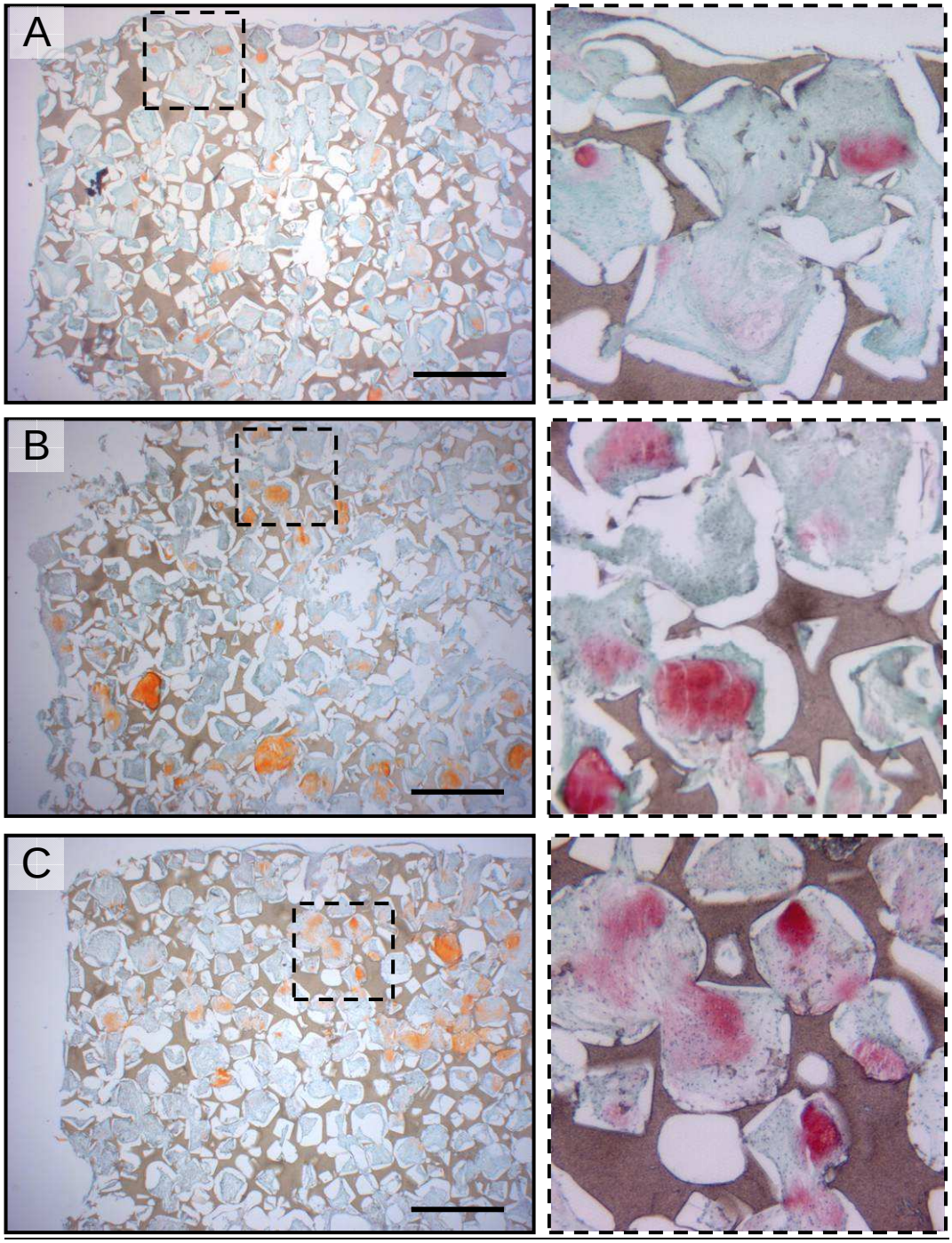


**Figure 7**. Safranin-O stained cross-sections (transversally) of constructs cultured under perfusion (A) for four weeks under normoxic oxygen and 100µm/s, (B) for four weeks under physiologic oxygen at flow rates maintaining the outlet oxygen measurements above 1.5%$O_2$, or (C) for two weeks under normoxic oxygen and 100µm/s followed by two additional weeks under normoxic physiologic oxygen at flow rates maintaining the outlet oxygen measurements above 1.5%$O_2$. Scale bar = 1mm.

## DISCUSSION

In this work, we used a previously developed perfusion bioreactor as a controlled model system to study the effects of defined oxygen tensions applied to expanded human chondrocytes in 3D porous scaffolds. Using this system we have shown that homogeneous and viable grafts, with clinically relevant thicknesses, can be generated by supplying AHAC not only with a normoxic range of oxygen (Wendt et al, 2006), but also with a defined physiological range of oxygen as well (i.e., between 1.5-5%). Moreover, we have shown that AHAC cultured within a porous scaffold that were supplied with physiological oxygen levels showed enhanced chondrogenic redifferentiation and deposited more GAG than those supplied with normoxic oxygen levels. However, from a clinical perspective, given the absolute magnitude of the observed effects, it is unlikely that these differences would in fact lead to improved functionality following implantation in a cartilage defect.

Expanded AHAC cultured in the 3D pellet model system (i.e., scaffold-free controls) showed a remarkably higher redifferentiation capacity under the low oxygen tension than under normoxic conditions. Although more pronounced effects were observed in the current study, our 3D pellet results are consistent with other studies employing small-scale model systems to investigate the effects of low oxygen tensions on expanded *human* chondrocytes. Expanded AHAC cultured in small alginate beads for four weeks were shown to have 3-fold higher collagen type II expression, similar collagen type I expression, and 1.6-fold higher ratios of GAG/DNA under 5% and 20%$O_2$ (Murphy and Polak, 2004). Interestingly, expanded human *nasal* chondrocytes cultured in 3D pellets have shown rather similar responses to low oxygen, resulting in more intense GAG and collagen type II staining than under normoxic conditions (Malda et al, 2004a).

AHAC seeded by perfusion and then cultured statically in the PolyActive foam scaffolds better resembled native chondrocytes when constructs were cultured at 5%$O_2$ than those at 19%. However, under both oxygen tensions, severe cell necrosis was observed at a depth of less than 1mm from the periphery of the scaffold, likely the result of mass transport limitations within the

constructs. Although which limiting nutrient(s) and/or excess metabolic waste product(s) contributed to these results cannot be determined decisively, the pattern of cell necrosis and cell proliferation would be consistent with oxygen transport limitations and steep oxygen gradients, which have been predicted (Lewis et al, 2005) and measured (Malda et al, 2004b) in 3D scaffold model systems. These steep oxygen gradients do not allow clear interpretation of results pertaining to defined oxygen tensions since cells within these constructs would be subjected to a highly heterogeneous oxygen environment, which may encompass both normoxic and physiological levels (e.g., from 20% at the surface to 2% in the interior (Malda et al, 2004c). In fact, it has been suggested that under severe oxygen diffusional limitations, constructs cultivated under *nominal* low and high oxygen tensions may in effect experience only slight differences in oxygen tensions (Nagel-Heyer et al, 2006). Therefore, despite local histological differences observed at the periphery of our statically cultured constructs, it may not be surprising that significant differences were not detected in the more global-scale quantitative measurements.

Our perfusion bioreactor system, developed to minimize oxygen gradients within engineered constructs, not only dramatically improved cell viability throughout the thick constructs, but also, provided a well-defined model system to assess the effects of defined oxygen tensions applied to AHAC within our 3D scaffold. Using our model system, we found that AHAC cultured in PolyActive foams for two weeks under a physiological range of oxygen (i.e., between 1.5% and 5% at 100µm/s) were indeed both qualitatively and quantitatively more chondrogenic than AHAC cultured under a normoxic range of oxygen (i.e., between 14% and 19%). Nevertheless, AHAC within constructs perfused under low oxygen tended to be more fibroblastic in morphology and embedded in less positively stained GAG matrix than AHAC within 5%$O_2$ 3D pellets or even AHAC at the outer periphery of 5%$O_2$ static constructs. Additionally, although expression of collagen type II was in fact upregulated 6-fold at low oxygen tensions under perfusion, expression was upregulated nearly 600-fold under 5% in the 3D pellet system. We therefore speculated that fluid induced shear stresses, clearly absent in the 3D pellets and static constructs, may have

contributed to the fibroblastic morphology observed in perfused constructs and inhibited the redifferentiation of AHAC at physiological oxygen. While it still remains unknown how particular magnitudes of shear stress affect chondrocytes in prolonged 3D cultures, regions of engineered cartilage exposed to shear have previously been associated with the formation of fibrous tissue comprised of multiple layers of elongated chondrocytes and little GAG matrix (Vunjak-Novakovic et al, 1999). It has also been reported that chondrocytes cultured in monolayer and exposed to shear can undergo cell alignment and elongation (Smith et al, 1995), and reduce the expression of both collagen type II and aggrecan mRNA (Lee et al, 2002).

To reduce potential adverse effects of fluid-induced shear stress, AHAC-foam constructs were perfused for two weeks at 10µm/s (one-tenth the initial rate), a speed approximately in the range estimated for interstitial fluid flow through articular cartilage (1µm/s) during 1MPa of loading (Mow et al, 1999). Based on an approximate linear relationship between flow rate and the median wall shear stress in 3D scaffolds (Cioffi et al, 2006b;Raimondi et al, 2002), the lower perfusion rate would reduce the wall shear stresses by approximately 10-fold. Constructs perfused at 10µm/s were in fact histologically and biochemically more cartilaginous when cultured under the low vs higher range of oxygen, however, the effects of low oxygen were not markedly more pronounced at the lower flow rate as compared to the initial high flow rate. The flow rate however had induced significant effects: for both low and high oxygen ranges, cell proliferation was significantly enhanced at the high flow rate, whereas collagen type II expression was markedly upregulated at the low flow rate, suggesting that the perfusion rate could be manipulated to direct AHAC towards proliferation or differentiation pathways.

Since the authors are undoubtedly aware that mass transport and shear are closely coupled in this model system, and simply lowering the flow rate reduces both mass transport and shear stresses, efforts are currently ongoing to quantify both hydrodynamic and mass transfer phenomena within our 3D constructs, to better elucidate their relative contributions. For this purpose, a novel computational fluid dynamics model has been developed, based on a µCT reconstruction of the

porous PolyActive microarchitecture, and combined with experimentally determined chondrocyte oxygen consumption data, to ultimately predict local velocity, shear, and oxygen levels within the actual pore microarchitecture of the PolyActive foam (Cioffi, 2006a). Such models could inevitably be used to also predict transport phenomena of other key biochemical species, such as glucose (Heywood et al, 2006), which could be vital for chondrocyte survival and matrix production in 3D constructs.

In addition to the absence of applied mechanical stresses, AHAC within the 3D pellet system are immediately exposed to a three-dimensional microenvironment with initial high density cell-cell contacts. In contrast, cells seeded into PolyActive foams under perfusion were previously shown to be uniformly distributed throughout the scaffold pores, virtually outlining the surface area of the scaffold. (Wendt et al, 2003). This particular cell distribution initially results, in effect, in a three dimensional "monolayer" culture on the PolyActive walls, which remodels over time into a three dimensional tissue that fills the volume of the pore (Figures 5-7). It is well known that chondrocytes cultured in monolayer do not show the same differentiation capacity in response to chondrogenic stimuli (i.e., growth factors) as chondrocytes cultured in 3D (Albrecht et al, 2006). Interestingly, it has also been shown that expanded bovine chondrocytes cultured under 5% oxygen deposited collagen type II protein only when cultured in 3D systems, but not when cultured in monolayer (Kurz et al, 2004). We therefore postulated that within the two week duration of our experiments, the initially uniformly seeded AHAC had not sufficiently remodeled to form a high density 3D microenvironment within the scaffold pores to be responsive to the differentiating signal of physiological oxygen.

To address this question, we used our bioreactor system to assess whether we could modulate AHAC redifferentiation and matrix production in longer-term cultures by applying low and high oxygen tensions at different stages of construct development. This question is in line with the hypothesis recently proposed by Wang et al., suggesting that high and low oxygen tensions could be used to differentially induce proliferation and differentiation of progenitor cells (Wang et al,

2005). We have previously shown that chondrocyte-PolyActive constructs, cultured under normoxic conditions, contained similar numbers of cells at either two or four weeks, but showed increasing matrix development with significantly higher ratios of GAG/DNA at four weeks versus two (Miot et al, 2005). Therefore, AHAC-foam constructs were first cultured for two weeks in order to generate a high density three-dimensional environment within the pores of the foam, applying the conditions found to support cell proliferation, namely the high perfusion rate and normoxic oxygen levels. We subsequently adjusted the bioreactor parameters and cultured the constructs for an additional two weeks, minimizing the flow rate and supplying the AHAC with a physiological range of oxygen, to support chondrogenic redifferentiation and GAG production. Nevertheless, no significant differences were observed between constructs cultured under the sequential proliferation/differentiation regime and those cultured for four weeks under either only normoxic or physiological oxygen tensions. It is possible that even after two weeks of culture, the cell densities in the foam pores, which were still significantly lower than in 3D pellets, remained too low to establish sufficient cell-cell contact for cells to enter a complete program of redifferentiation in response to low oxygen.

While cell density could be one plausible explanation for markedly greater effects observed in the 3D pellet system than in the perfused constructs, cell-matrix interactions, which are known to affect chondrocyte proliferation and/or differentiation (Li et al, 2003;Mahmood et al, 2006), may also play a role in the response of cells to different oxygen tensions (Wang et al, 2005). Redifferentiation of expanded bovine chondrocytes under low oxygen was shown to be significantly more pronounced using alginate beads as compared to collagen scaffolds (Kurz et al, 2004). While the study once again raises the question as to influence of the high cell density in the alginate system, interactions with the collagen scaffold, which was shown to be less supportive of chondrogenic redifferentiation than alginate, may have also inhibited the chondrocyte response to low oxygen. Although the foam scaffold used in our current study possesses highly attractive mechanical properties for engineering cartilage grafts for weight bearing joints, this composition

was previously shown to have a limited capacity to support chondrogenic redifferentiation under normoxic culture conditions (Mahmood et al, 2006;Miot et al, 2005). One of the main objectives of this study was thus to determine whether the application of physiological oxygen tensions could be used to enhance the redifferentiation and GAG production of AHAC cultured within this scaffold. However, the surface chemistry of this material may have such a strong regulation on the phenotype of the human chondrocyte (Mahmood et al, 2006) that the chondrogenic signal of physiological oxygen could not induce complete redifferentiation.

## CONCLUSIONS

In this study, we have used a controlled bioreactor system to minimize diffusional limitations associated with previous model systems, which allowed us to study the effects of defined levels of oxygen to cells within three-dimensional porous scaffolds. Although physiological oxygen levels significantly affected AHAC in the PolyActive foams, the extent to which the resulting engineered cartilage was actually improved would likely not have a significant clinical impact on the functionality of the graft when implanted in vivo. Based on the marked effects of low oxygen on the redifferentiation of AHAC in the 3D pellet system, future efforts should aim to understand how low oxygen could be used as a tool to improve the functionality of engineered cartilage grafts for clinical applications. Future studies should further investigate the influence of cell-cell contacts and specific cell-biomaterial interactions in the response of chondrocytes to different oxygen tensions. By providing well-defined and controlled model systems, bioreactor systems that control and monitor physicochemical culture parameters in 3D cultures can play a crucial role in advancing our understanding in the proposed areas.

## ACKNOWLEDGEMENTS

We would like to acknowledge the European Union for financial support ("STEPS"; FP6- #NMP3-CT-2005-500465, www.stepsproject.com).

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# SUMMARY AND CONCLUSION

# CHAPTER 7

# SUMMARY AND CONCLUSIONS

## 7.1 Summary: aims and results of this work

The specific aims of this thesis were: (i) to determine the influence of physiological oxygen levels on the in vitro differentiation capacity and development of cartilaginous tissue applied on adult human articular chondrocytes from elderly individuals (Chapter 4), (ii) to develop a bioreactor system which directly perfuses culture media throughout chondrocyte seeded porous foam scaffolds with the possibility to monitor, control and maintain defined levels of oxygen applied to the chondrocytes (Chapter 5) and (iii) to use the developed perfusion bioreactor system to selectively apply and modulate culture parameters (i.e. flow rate, oxygen levels) to control and improve the process of chondrocyte differentiation and cartilage tissue formation in a 3D environment (Chapter 6).

Growth factors during chondrocyte expansion not only influence cell proliferation and differentiation, but also the cell potential to redifferentiate and respond to regulatory molecules upon transfer into a 3D environment (Jakob et al, 2001). These growth factor mediated effects of enhancing the in vitro chondrogenic capacity of chondrocytes are reduced in the cultivation of chondrocytes from elderly individuals (Barbero et al, 2004). To improve the re-differentiation

ability of chondrocytes from patients with a low capacity to re-gain a chondrogenic phenotype, we investigated whether the exposure to lower levels of oxygen during the expansion and/or subsequent 3D micromass pellet culture of chondrocytes influences their capacity to re-gain a chondrogenic cell phenotype and the potency to form a cartilaginous tissue. We have found that the oxygen levels which were tested and applied *during cell expansion* did not influence the cell proliferation and the capacity of the cells to re-gain a more chondrogenic phenotype. Instead, physiological oxygen tension applied *during subsequent pellet culture* was able to enhance the chondrogenic differentiation, but only if cells were previously expanded in medium supplemented with a specific growth factor combination (TGFb-1, FGF-2 and PDGF). Additionally we have shown a potential correlation of low oxygen culture environment with the down-regulation of extracellular matrix associated proteases during the 3D culture phase.

Considering the potential application of physiological oxygen tension on cell-scaffold constructs at clinical relevant size, we first developed an integrated bioreactor system which allows the application and monitoring of defined oxygen levels on prolonged perfusion of culture media through 3D cell seeded constructs to generate homogenous tissue grafts. We could demonstrate that culture of uniformly chondrocyte seeded scaffolds under perfusion, maintaining cells in a normoxic range of oxygen levels, could maintain a uniform distribution of viable cells throughout thick porous scaffolds, and support the development of uniform tissue grafts.

To improve the chondrogenic ability of the human chondrocytes cultivated in these constructs, we further tested the concept of cultivation at physiological oxygen ranges. Using our perfusion bioreactor as a controlled model system we have demonstrated, that constructs cultivated under perfusion at more physiological oxygen levels were both qualitatively and quantitatively more chondrogenic than those cultured under a normoxic range of oxygen. However, these effects of low oxygen tension on chondrocyte constructs under perfusion were less pronounced as compared to cells in statically cultured constructs or in 3D micromass pellets. Under a 10-fold lower flow rate applied to reduce potentially adverse flow induced shear stress, the constructs

supplied with low ranges of oxygen were histologically and biochemically more cartilaginous as compared to higher range of oxygen but again the effects of low oxygen were not as marked as in the pellet culture system. Interestingly, at both oxygen ranges, cell proliferation was significantly enhanced at the high flow rate whereas collagen type II expression was markedly up-regulated under low flow rate. The extension of the perfusion culture phase of chondrocytes seeded on scaffolds to 4 weeks resulted in a more mature cartilage tissue formation as compared to 2 weeks culture. However, the sequential application of high medium flow and high $O_2$ during a first phase of culture, to allow the cells to re-establish a high density 3-dimensional environment, and the subsequent exposure to physiological oxygen tension, to enhance the chondrogenic differentiation, did not improve the cartilage tissue forming capacity.

## 7.2 Relevance of the achieved results and future perspectives

*Relevance of the results and potential applications*

Relevance of the results: This work has significant implications in the establishing of chondrocyte culture conditions and the in vitro engineer cartilage tissue constructs of clinically relevant size which can be further implemented in a streamlined and automated manufacturing process of cartilage tissue substitutes for clinical application (Wendt et al, 2005).

Our results indicate relevant culture conditions which influence the differentiation and matrix production of human chondrocytes in 3D tissue culture: (i) a low oxygen environment can enhance the chondrogenic differentiation ability of chondrocytes with usually limited chondrogenic capacity (e.g. from elderly individuals) (ii) a minimal direct perfusion flow applied to cell seeded constructs

can maintain normoxic or physiological oxygen ranges required to provide sufficient mass transport to maintain chondrocytes viable.

The developed system also represents a promising tool towards the establishing of a 3D in vitro model to investigate the fundamental mechanism of chondrocytes in a 3D environment under defined culture conditions.

In particular, it was used as a tool to investigate and understand the complexity of chondrocyte response to oxygen levels by studying interactions with other culture parameters (e.g. flow rate, cellular density, etc.)

One of the most obvious relevance of the present work is represented by the possibility to enhance the chondrogenic capacity of expanded de-differentiated chondrocytes (especially from elderly donors) by the application of more physiological oxygen tension during the in vitro culture in a 3D environment.

Alternative possible applications of the system: The perfusion bioreactor, as a system to study chondrocyte cell function under given physicochemical conditions, could also provide a tool to test computational fluid dynamic (CFD) models simulations (media flow velocity; shear profiles; oxygen concentration within the scaffold pores) (Cioffi et al, 2006;Raimondi et al, 2002) and prove whether the biological outcomes in agreement with the modelled prediction.

Moreover, the developed bioreactor system could provide the basics towards the extended application in the engineering of osteochondral grafts which would consider the use of different cell sources and specific, more complex scaffold materials to finally produce functional osteochondral substitutes (Martin et al, 2007).

The model could also be extended to other types of cells to study the biological mechanisms in a 3D environment, which may be more physiological than classical culture in monolayer.

*Possible improvements of the system to engineer functional cartilage grafts*

The approach used in this work to ultimately engineer cartilage tissue grafts was based on (i) primary adult human articular chondrocytes (AHAC) as a cell source (ii) compression moulded porous poly (ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) co-polymers scaffolds as a 3D environment for the cells (iii) 3D perfusion flow and micromass pellet culture as a culture system (iv) the supplementation of the media with growth stimulating factors during the phase of cell expansion (TGFβ-1; FGF-2; PDGF) or during the phase of cell differentiation (TGFβ-3; insulin, ascorbic acid), and (v) the exposure of AHAC to physiological oxygen tension as a culture environment to promote chondrogenic differentiation. The combination of these elements resulted in the chondrogenic differentiation of cells in a 3D micromass pellet culture system and in the generation of uniform cartilage like tissue constructs using a cell-scaffold approach. The results obtained in this work were promising and encouraging but alternatives for the mentioned elements of the system could further improve the proposed culture methods.

Cell source: The ideal source of autologous cell should provide a large number of cells harvested from the individual with a minimal invasive procedure to minimize patient morbidity. Alternatively to a cartilage biopsy in the joint which represents an additional injury to the cartilage surface and reported to be detrimental to the surrounding healthy articular tissue (Lee et al, 2000), biopsies from the nasal or rib cartilage can be harvested by a less invasive procedure. Several studies have shown that chondrocytes derived from human nasal septum or ear cartilage proliferate and generate cartilaginous tissue after monolayer expansion with similar or superior capacity to those derived from articular cartilage (Kafienah et al, 2002;Tay et al, 2004;Van Osch et al, 2004). Indeed, a recent in vitro study provide evidences that as compared to chondrocytes from the articular surface chondrocytes harvested from the nasal septum regained a higher chondrogenic phenotype and this in particular when exposed to a low oxygen culture condition (unpublished data). However, it remains to be demonstrated whether the tissue generated by non-articular chondrocytes fulfil the biomechanical requirements for the cartilage repair at articular sites.

Scaffold material: The ideal 3D-scaffold for engineering cartilage tissue should provide the initial template for the seeded chondrocytes to deposit extracellular matrix component. A high porosity and a high degree of interconnectivity among the pores are required to allow the cells to invade and to support exchange of nutrients and waste products. The polyactive scaffold (compression moulded) used in this study has a high porosity (~75%) and highly interconnected pore structure. This architecture allowed to seed chondrocytes within the pores and to apply direct perfusion of media throughout the scaffold structures. Anyhow, the poor ability to control the manufacturing process of a precise size and geometry of the pores on the micrometer level represents the limiting structural feature of the polyactive scaffold. These design characteristics restrict the defined computable modelling of media flow pattern within the construct and thus show limitations in the prediction of fluid flow and shear forces development at the cellular level. New scaffold manufacturing protocols (e.g. 3D printing)(Limpanuphap and Derby, 2002) would enable the precise design of the scaffold architecture, allowing a more predictive process/procedure of cell distribution and seeding densities within the single pore and the modelling of the fluid flow during the deposition and development of tissue under direct media perfusion. Finally, the implementation of such "customized scaffolds" would improve the perfusion bioreactor system as a tool to investigate fundamental mechanisms (e.g. impact of shear) on chondrocytes in a 3D environment.

Monitoring and control of culture parameter: The improvement of the perfusion bioreactor system as a model to more systematically approach the key regulatory parameters in chondrocyte function and cartilage tissue generation would require the integration of additional monitoring and control features. Such features would enable (i) the on-line monitoring and active control of essential media ingredients (e.g. glucose, amino acids, and buffer components) and pH (ii) the active regulation of these culture parameters to maintain an optimal culture environment.

Monitoring chondrocyte differentiation and tissue formation: The detection of secreted cartilage specific marker proteins (Bosserhoff and Buettner, 2003) could provide the monitoring of the chondrogenic differentiation processes within the culturing phase. Furthermore, the on-line

monitoring of matrix deposition provides the quality control element to evaluate the anabolic performance of the cells and the tissue growth progression. Such monitoring tools could be adapted from nuclear magnetic resonance (Bashir et al, 1996) or ultrasound techniques (Martino et al, 1993). An additional way to monitor the tissue quality could be provided by assessing the reduced tissue construct permeability, which would correlate with an increased fluid pressure force to maintain the constant direct perfusion flow. Thus the monitoring could be done by integrated fluid pressure sensors. These additional features can improve the system as a tool to understand profound of chondrocytes in a 3D environment and the quality of produced cartilage tissues.

Extension of the system towards a clinical application: The process of engineering functional cartilage substitutes in vitro still considers the 2D expansion of chondrocytes and the subsequent culture within 3d scaffold matrices. Thus to bypass the process of 2D-expansion prior to seed and culture the cells within porous scaffolds under perfusion would give the possibility to directly seed and expand freshly isolated chondrocytes within the scaffolds and would facilitate a more streamlined and automated process of engineering cartilage tissue towards the establishment of tissue manufacturing unit.

Furthermore the developed perfusion bioreactor system to engineer grafts of clinically relevant size could provide the basic for a cartilage tissue production unit allocated and used in the clinic. Further improvements regarding (i) the control of key culture parameters by extended monitoring tools (ii) the reproducible application of these parameters and finally (iii) process automation (e.g. media change; modulation of optimal culture conditions) in order to avoid operator dependencies would required to potentially lead to the allocation of such tissue manufacturing units in hospitals.

## 7.3 Schematic summary

The most relevant results generated in this thesis can be summarized as follow:

- Adult human articular chondrocytes (AHAC) from elderly individuals expanded in culture medium supplemented with the growth factors TGFβ-1, FGF-2 and PDGF and subsequently cultured in 3-d pellets had an enhanced chondrogenic capacity when exposed to more physiological (i.e. 5%) oxygen levels.
- In correlation with the enhanced tissue forming capacity of AHAC from elderly donors under low oxygen tension, the mRNA expression levels of selective matrix degrading enzymes were reduced as compared to conventional in vitro oxygen culture condition.
- We developed an integrated bioreactor system, which streamlines within a single device the phases of perfusion cell seeding and prolonged perfusion culture of cell seeded scaffolds in vitro.
- The culturing of uniformly seeded adult human articular chondrocytes under direct perfusion, where cells are continuously exposed to a normoxic range of oxygen levels, can maintain a uniform distribution of viable cells throughout thick porous scaffolds as compared to statically cultured constructs.
- The culturing of constructs uniformly seeded with adult human articular chondrocytes under a more physiological range of oxygen resulted in a higher chondrogenic differentiation as compared to culture under normoxic levels. Anyhow, this effect was less pronounced as compared to statically cultured cell constructs or micromass cell pellets, possibly due to the flow induced shear forces.
- Reduced perfusion flow rates applied to chondrocytes on porous scaffolds significantly induced more cartilaginous tissue in the presents of low vs. high oxygen levels. However the effects of low oxygen were not as marked as in pellet culture.

- The final cell number in scaffolds perfused at a higher flow rate (at both high and low oxygen ranges) was significantly higher as compared to perfusion at a lower flow rate.
- The re-establishing of cells in a high density 3-dimensional environment in a first phase of perfusion culture did not enhance the cartilage tissue forming capacity under the subsequent application of physiological oxygen tension.

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# APPENDIX

# Appendix: Perfusion bioreactor system validation

## A) Cytotoxicity-Test:

To assess the cytotoxicity of the components integrated in the developed perfusion bioreactor (pBR) system (Table 1) the ISO/EN 10993 part 5 protocols was used. Components with cytotoxic effects were excluded from the system and substituted by non-toxic materials. In brief, the cytotoxicity was assessed as follows.

| Component | Material |
|---|---|
| Control media (CTR) | DMEM |
| Tubing PharMed® | BPT |
| Silicon Tubing (platinium cured) | Silicone |
| Tubing (Chemfluor®) | FEP |
| Valves | Polycarbonat ( PC) |
| Scaffold Chamber | Polycarbonat ( PC) |
| O-ring (Viton®) | Fluorelastomer |
| O-ring (Buna-N®) | Nitrile-polymer |
| Tube connector (PVDF) | Polyvenyldiene fluoride |
| Tube connector (PP) | Polypropylene |

Table 1. Components and material polymers integrated in the perfusion bioreactor system.

**Material and cell preparation:**

*Material extraction:*

In brief, DMEMs (Doulbeccos ME media, supplemented with Peniciline (100 U/ml), Streptovadine (100 µg/ml), HEPES (1mM), Na-pyrovate (1mM) was applied at a media to surface ratio of 1:2 ($ml/cm^2$) for 48hrs at 37°C and 5%$CO_2$ to extract substance from heat-sterilized pBR components. DMEM extraction solution was sterile filtered with 22um sterile filters (Millex GH; Millipore, Switzerland) and supplemented with FBS at a final concentration of 10%.

*Cell culture:*

Primary bovine chondrocytes (BCh) from a 6 month calf was harvested from the femur condyle and digested with 0.3% collagenase solution. Cells were plated at a density of 10'000cells/cm2 in TCP cell culture treated dishes using TFP- DMEM-CM. Dishes were incubated at incubator environment (37°C and 5%$CO_2$) for 48hrs. BCh were processed according to cell culture protocols described elsewhere (Barbero et al, 2003) and re-plated at 20'000cells/cm2 in 24 well plates (TPP, Switzerland) in DMEM-CM for additional 48hrs. Cells were washed twice in 1xPBS and reinoculated with DMEM extraction solution (1ml) at incubator environment (37°C and 5%$CO_2$) for 72hrs.

**Analysis:**

Cytotoxicity of extracted material was assessed by cell counting and metabolic activity of the cells.

*Cell count:*

BCh were harvested according to standard cell culture protocols and counted with a Coulter Counter model Z2 (Beckman Coulter; Z2 Counter®, Germany) in triplicates at each condition.

*MTT (Thiazolyl blue) activity test:*

MTT cell activity assay was assessed according to manufactures instruction (Sigma Product No. M 5655). In brief, Isopropanol/HCl solution was transferred to flat bottom 96-well plate and transmission wave length was measured at 575nm with a photo spectrometer (SpectraMax® 250, Mediacl Devive, CA). The values were normalized to untreated controls (CTR).

*Data analysis:*

Data were analysed for significance differences using Denova test after normalizing to control media (CTR).

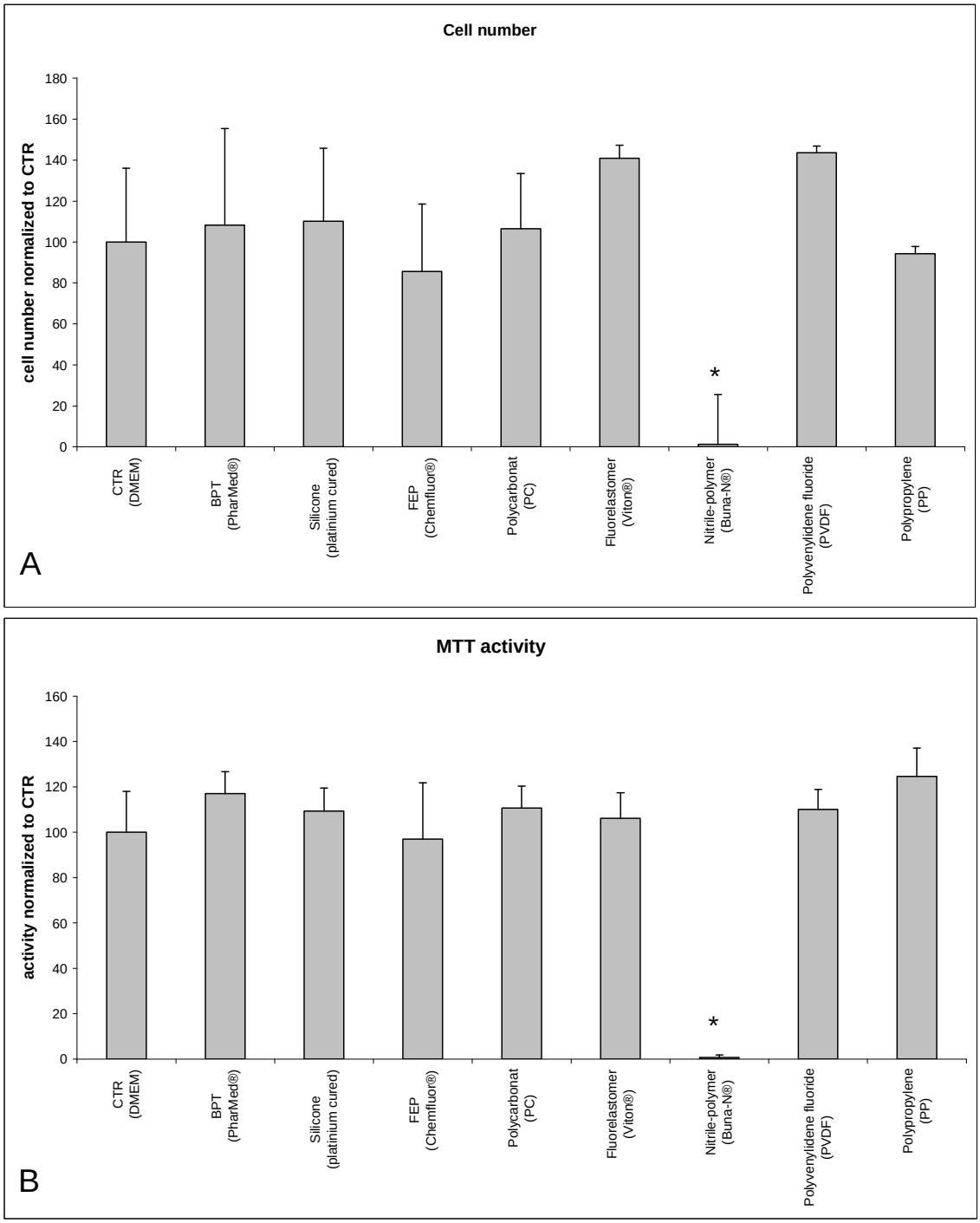


**Figure 1.** Cytotoxicity test (according. to ISO/EN 10993 part 5 ). Chondrocyte cell number and MTT activity were normalized to cells treated with DMEM-complete media (indicated as 100). Significant differences were indicated by (*) ($p<0.05$).

*References*

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## B) Flow through oxygen sensors

The flow though oxygen sensors (Presens GmbH, Regensburg, Germany) were integrated and validated according to the manufacture instructions (Presens GmbH, file name: FB3-PSt3-Info1.pdf).

With the official permission of Presens GmbH the instruction manual is enclosed as a PDF-file.

PreSens
Precision Sensing GmbH

PreSens GmbH Josef-Engert-Str. 9 D – 93053 Regensburg Germany

# Fibox 3
# Single Channel Fiber-Optic Oxygen Meter for Minisensors

## General:

The **Fibox 3** is a precise single channel, **temperature compensated oxygen meter** with fiber-optic oxygen minisensors based on a 2 mm polymer optical fiber (POF). The Fibox 3 system detects oxygen (the oxygen partial pressure) in both solutions (dissolved oxygen) as well as in the gaseous phase.

The small outer dimensions, low power consumption and a robust box make it ready for indoor and **outdoor** applications.

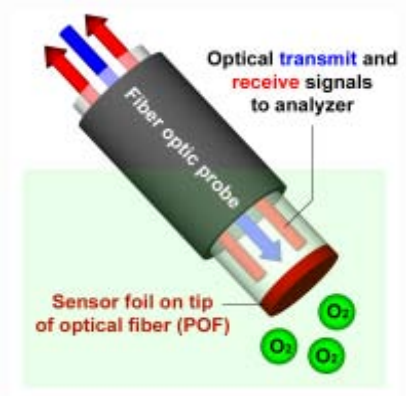
For operation, a PC/Notebook is required. The Fibox is controlled using a comfortable software, which also saves and visualizes the measured values.

The **Fibox 3** has 2 analog outputs (0-5 V) and one trigger input (TTL) to be connected to a data logger. Analog connectors are BNC connectors.


The analog outputs are programmable to deliver oxygen, temperature, or the raw values (phase shift or amplitude), and the data are called via computer and RS232 (digital) or using the external trigger input (analog).

## Principle

The principle of the sensor operation is based on the quenching of luminescence caused by collision between molecular oxygen and luminescent dye molecules in the excited state. The detection of the luminescence lifetime as oxygen dependent parameter makes the PreSens oxygen meter superior to other optical oxygen meter which are based on luminescence intensity measurements, since oxygen measurements are not affected by light source or detector stability. Intensity fluctuations caused by fiber bending or changes of the optical properties of the sample (coloration, turbidity, refractive index, etc.) do not interfere with the oxygen measurement. The technology is immune to interference caused from pH, ions (e.g. sulfide) or salinity change or from changes in viscosity.




## Features

- **Non-invasive** oxygen measurements
- Excellent long-term stability
- Wide variety of sensor housings available
- Measurement in-vitro in sealed glass chambers
- No oxygen consumption during experiment
- Oxygen sensing based on luminescence lifetime detection (luminescent indicator dye)
- Measures oxygen in both liquids as well as in the gas phase
- Oxygen measurement range 0 - 500% air saturation
- Immune to electrical and magnetic interference
- The system is controlled by a comfortable user-friendly software
- RS232 interface allows to connect several Fibox 3 instruments to create multi-instrument set-up
- 2 x 12bit programmable analog outputs, with galvanic isolation and 1 x external trigger input, with galvanic isolation
- Small size robust housing and 12VDC power makes it ideal for portable and field use

## Extension to a multi-channel multi-analyte system

Using a computer port extender providing multiple RS232 ports, up to eight single PreSens devices (Fibox, Microx pH-1 mini, …) can be connected to one single computer. This multi-instrument set-up offers a highly flexible method to create multi-channel, multi-analyte measuring systems including additional temperature-compensation of each channel.

## Specifications

| | | | |
|---|---|---|---|
| **INSTRUMENT** | | | |
| **Channels** | 1 x optical channel (SMA connector) , designed for mini-sensors<br>1 x PT1000 connector | | |
| | **Dissolved Oxygen** | | **Gaseous & Dissolved Oxygen** |
| **Measurement range** | 0 - 45 mg/L (ppm)<br>0 - 1.4 mmol | | 0 - 500 % air-sat.<br>0 - 100 % oxygen-sat.<br>0 - 760 Torr<br>0 - 1013 hPa |
| **Limit of Detection (LOD)** | 0.15 % air-saturation,<br>15 ppb dissolved oxygen | | 0.31 hPa,<br>0.23 Torr |
| **Resolution at 20 °C and 1013 hPa** | 0.09 ± 0.005 mg/L (ppm)<br>2.72 ± 0.01 mg/L (ppm)<br>9.06 ± 0.05 mg/L (ppm)<br>22.65 ± 0.15 mg/L (ppm) | | 1 ± 0.05 % air-sat.<br>30 ± 0.1 % air-sat.<br>100 ± 0.5 % air-sat.<br>250 ± 1.7 % air-sat. |
| | | | 0.21 ± 0.01 % oxygen<br>6.3 ± 0.02 % oxygen<br>20.9 ± 0.1 % oxygen<br>52.4 ± 0.35 % oxygen |
| | 2.83 ± 0.14 µmol<br>85.0 ± 0.28 µmol<br>283.1 ± 1.4 µmol<br>798.0 ± 4.7 µmol | | 1.55 ± 0.08 Torr<br>46.7 ± 0.2 Torr<br>155.5 ± 0.75 Torr<br>388.8 ± 2.6 Torr |
| | | | 2 ± 0.1 hPa<br>60 ± 0.3 hPa<br>200 ± 1 hPa<br>500 ± 0.3 hPa |
| **Accuracy** (20 °C) | ± 1% at 100 % air-saturation; ± 0.15% at 1 % air-saturation | | |
| **Temperature (PT1000)** | **Range** | **Resolution** | **Accuracy** |
| | 0-50°C | ±0.2°C | ±1°C |
| **Digital interface** | RS 232 interface with galvanic isolation (19200 Baud, Databits 8),<br>RJ connector (RJ 6/4 - SUB-D 9 cable for RS232 PC port included) | | |
| **Analog outputs** | Dual 12bit programmable outputs with galvanic isolation<br>(BNC connectors) | | |
| **Specification** | **Range** | **Resolution** | **Accuracy** |
| **Analog output (Oxygen)** | 0-400% air-sat. | ±0.2% air-sat. | ±0.5% air-sat. |
| **Analog output (Temperature, PT1000)** | 0-50°C | ±0.5°C | ±1.5°C |
| **External Trigger** | TTL compatible with galvanic isolation (BNC connector) | | |
| **Acquisition time** | Programmable (default 1 second, min: 250 ms) | | |
| **Power supply** | 12 VDC/max.550 mA (110-240 VAC, 50/60 Hz adapter) | | |
| **SOFTWARE OxyView** | | | |
| **Oxygen units** | User selectable from:<br>air-saturation, oxygen-saturation, hPa, Torr, mg/L (=ppm), µmol | | |
| **Compatibility** | Windows 95/98/2000/Millenium/NT4.0/2000/XP | | |
| **Calibration** | Conventional two-point calibration with oxygen-free environment<br>(nitrogen, sodium sulfite) and air-saturated environment | | |
| **ENVIRONMENTAL CONDITIONS** | | | |
| **Operating Temperature (°C)** | 0 to +50 | | |
| **Storage Temperature (°C)** | -10 to +65 | | |
| **Relative humidity (%)** | up to 95 | | |
| **Dimensions, DxWxH (mm)** | 185 x 110 x 45 | | |
| **Weight (kg)** | 0,63 | | |

## *Cross sensitivity:*

There exists **no** cross sensitivity for ***carbon dioxide*** ($CO_2$), ***hydrogen sulfide*** ($H_2S$), ***ammonia*** ($NH_3$), ***pH***, any ionic species like ***sulfide*** ($S_2^-$), ***sulfate*** ($SO_4^{2-}$), ***chloride*** ($Cl^-$) or ***salinity***. Turbidity and changes in the stirring rate have no influence on the measurement.

The sensors can also be used in ***methanol***- and ***ethanol-water*** mixtures as well as in ***pure methanol*** and ***ethanol***.

We recommend to avoid other organic solvents, such as acetone, chloroform or methylene chloride, which may swell the sensor matrix.

Interferences were found for gaseous sulfur dioxide ($SO_2$) and gaseous chlorine ($Cl_2$). Both of them mimic higher oxygen concentrations.

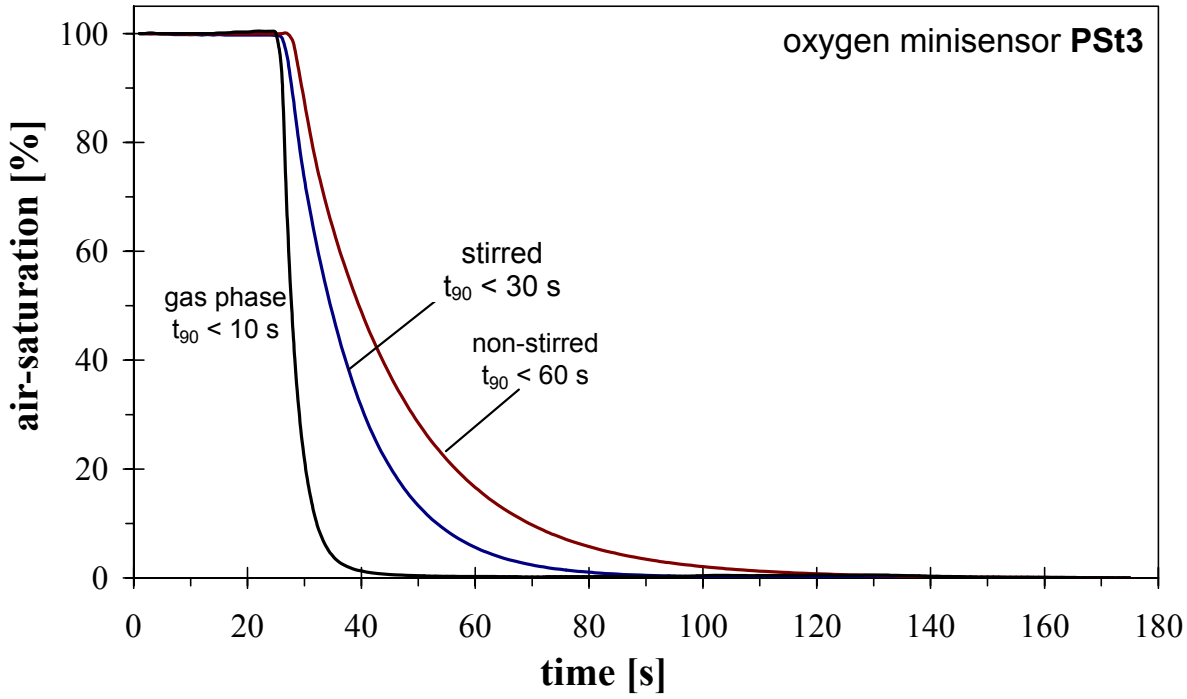
## *Response time*

The response time (t90, 90 % of the signal change has occurred) of the PSt3 oxygen sensor is less than 30 s in solution (non-stirred) and even less than 8 s in the gas phase.

The response time ($t_{90}$) of the oxygen sensor is dependent from the diffusion rate of oxygen through the sensor layer, hence, the response time is dependent from the thickness of the sensor layer and the stirring rate. A typical oxygen response curve of sensor membrane **PSt3** in a non-stirred and stirred sample solution is shown in Figure below. The response times ($t_{90}$) of sensor membrane **PSt3** is listed in Table below.

Unlike electrodes, optical sensors do **not** consume oxygen and the signal is independent of changes in flow velocity which means that stirring decreases the response time, but has no effect on the measured value.

Optical isolation of the oxygen-sensitive layer which is applied to exclude ambient light and improve chemical resistance will slow down the sensor response.




Response characteristic of an optical isolated oxygen sensor **PSt3** in a stirred, a non-stirred sample solution and in the gas phase.

Response time ($t_{90}$) of the PSt3 oxygen sensors.

| | dissolved oxygen | | gaseous oxygen |
|---|---|---|---|
| | stirred | non-stirred | |
| ***oxygen sensor PSt3*** | | | |
| $t_{90}$ without optical isolation | < 20 s | < 40 s | < 6 s |
| $t_{90}$ with optical isolation | < 30 s | < 60 s | < 10 s |

### *Optical isolation*

Optical isolated sensor tips are required, if your sample shows intrinsic fluorescence between 540 - 700 nm. Consequently, an optical isolation is recommended measuring in whole blood, urine or chlorophyll containing samples. Using optical isolated sensors exclude the impact of colored samples and ambient light on measurements. Furthermore, the optical isolation layer is applied to exclude strong ambient light, to improve chemical resistance especially against oily samples as well as to reduce bio-fouling on the sensor membrane.

Optical isolated sensor tips of oxygen sensors enable measurement in photosynthetically active samples, since stimulation of photosynthesis, due to emission of blue-green light from the fiber tip, is avoided.

PreSens offers additional optical isolation for all types of oxygen sensors.

### *Sensor Stability*

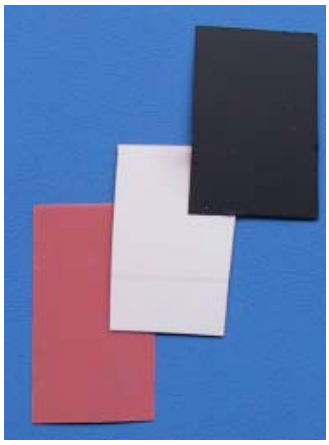
The oxygen-sensitive membrane stands gamma-sterilization, sterilization by ethylene oxide, steam autoclavation (140 °C, 1.5 atm), CIP conditions (cleaning-in-place, 5% NaOH, 90°C), as well as a 3% $H_2O_2$ solution.
The oxygen-sensitive material may be subject to photo-decomposition resulting in a signal drift. Photo-decomposition takes place only during illumination of the sensor tip and depends on the intensity of the excitation light.

*Drift in % air-saturation at 100% air-saturation when illuminating the oxygen sensor **PSt3** for 1, 12 and 24 hours in the continuous mode (1 sec. mode).*

| | Drift per hour | Drift per 12 hours | Drift per 24 hours |
|---|---|---|---|
| ***PSt3*** | --- | --- | < 0.4 % air-saturation |

## Housings of Oxygen-Sensitive Minisensors

PreSens fiber-optic oxygen sensors are based on 2 mm polymer optical fibers (POF). Depending on the respective application, PreSens offers a set of different standard designs.



planar oxygen sensitive sensor foils

Flow through cell design connected to a 2 mm POF

2 mm POF with coated sensor foil

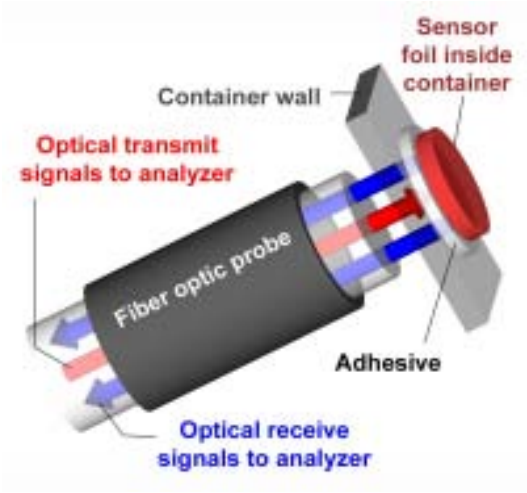
Of course, it is possible to build ***customer-specific*** designs. Please feel free to contact our service team to find the best solution for your application.

# Planar Oxygen-Sensitive Foils Designed for the Fibox 3 Oxygen Meter

Planar oxygen sensors immobilized onto different supports (polyester, glass) are available for customer specific applications. Sensors based on a polyester support can be easily cut into small pieces using a razor blade. Round spots (sensorspots) of 3 mm in diameter can be punched.

The **sensor spots** can be glued, for example, inside glass vials such as cell culture flasks, bags, and disposables. The oxygen concentration can be measured **non-invasive** and **non-destructive** from outside through the wall.

***Only prerequisite***: The wall has to be transparent and non fluorescent




## *Features*

- non-invasive and non-destructive measurement from outside through the wall of the flask
- excellent mechanical stability and long-term stability (more than 100000 data points without drift)
- online monitoring
- response time ($t_{90}$) in the order of 30 s

*Oxygen sensor immobilized onto a glass support*

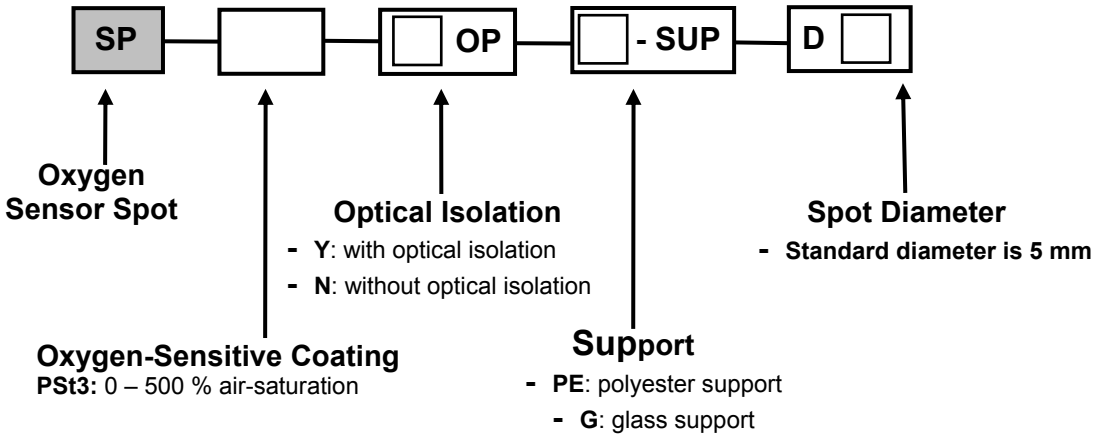
- stands CIP (Cleaning In Place) conditions
- sterilizable (autoclave (130 °C, 1.5 atm), ethanol, ethylene oxide, $H_2O_2$)

*Oxygen sensor immobilized onto a polyester support*

- stands CIP (Cleaning In Place) conditions
- sterilizable (ethanol, ethylene oxide, gamma irradiation, $H_2O_2$)
- not autoclavable
- flexible

A polymer optical fiber is used as a light guide between the Fibox 3 oxygenmeter and a sensor foil (SF-xx) which was glued inside a glass vial to read out the analyte concentration non-invasive and non-destructive **from outside through the transparent wall of the flask**.

## Ordering information




## Example


With this code you will order a planar oxygen sensor spots, type ***PSt3*** (0 - 500 % air-saturation) with optical isolation (***YOP***) immobilized onto a glass support (***G-SUP***). The spot diameter is 5 mm.

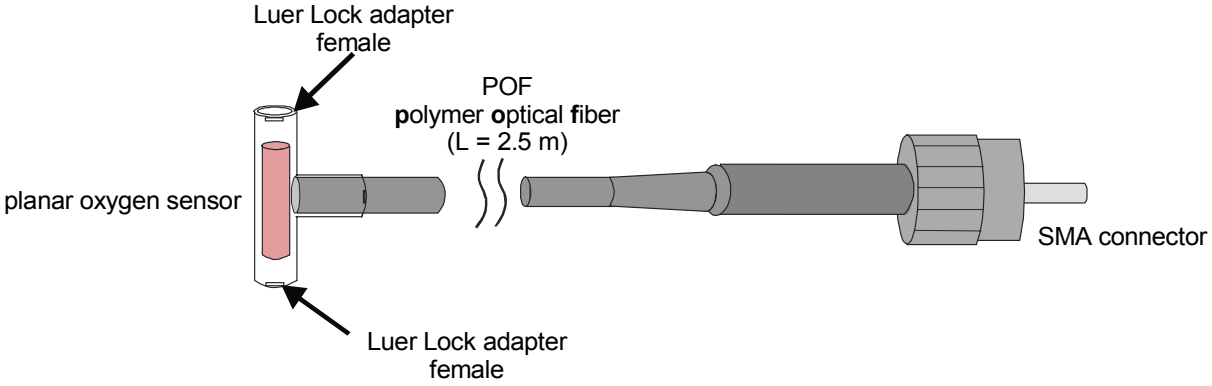
# Flow-Through Cell with Integrated Planar Oxygen Sensor Designed for the Fibox 3 Oxygen Meter

Flow-through oxygen minisensor (FTC-PSt3) is a miniaturized fiber optic chemical sensor integrated in a T-shape flow through cell.

The flow-through cell is connected to the PreSens oxygen meter Fibox by a polymer optical fiber (POF) with 2 mm diameter as a light guide. A glass tube with 2mm inner diameter (4mm outer diameter) is coated with oxygen sensitive dye at its inner wall. The volume for liquid inside the FTC cell is about 100 (+- 10) microliter.

The standard T-shape flow cell can be easily connected via Luer-Lock adapters to external tubings. Liquids (like water, blood, etc.) can be pumped through the cell.

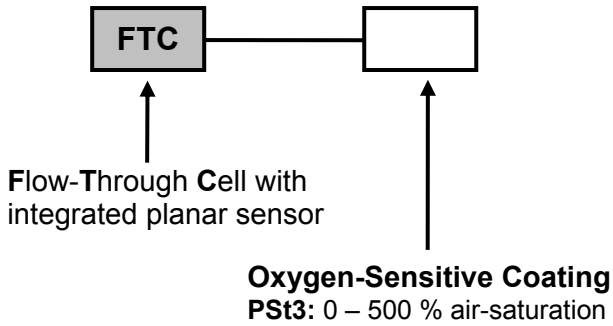
## *Schematic drawing of flow-through cell oxygen sensors*




## *Features*

- very robust sensor with an excellent long-term stability (more than 100000 data points without drift)
- online monitoring
- sterilizable (autoclave (130 °C, 1.5 atm), ethanol, ethylene oxide)
- response time ($t_{90}$) in the order of 1 minute
- stand CIP conditions (cleaning-in-place, 5 % NaOH,90°C)

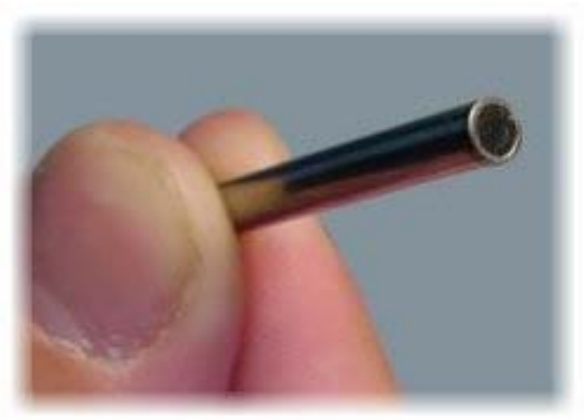
## *Ordering Information*




## Example

With **FTC—PSt3** you will order flow-through cell with integrated **PSt3**, an oxygen sensor which can measure up to 500 % air-saturation (100 % pure oxygen). The standard cable length is 2.5 m, the maximum length is 25 m.

# Oxygen Dipping Probe Designed for the Fibox 3 Oxygen Meter

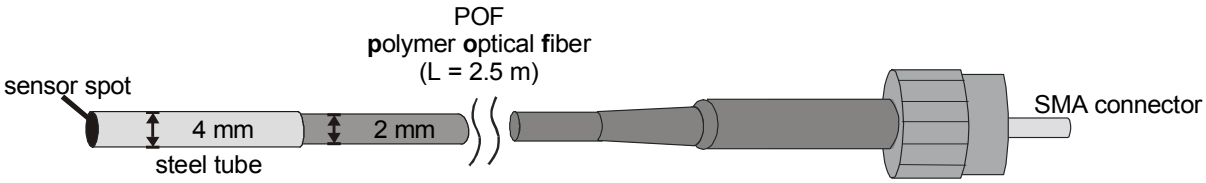


This oxygen sensor consists of a polymer optical fiber (POF) with a polished distal tip which is coated with a planar oxygen-sensitive foil.

The end of the polymer optical fiber is covered with a high-grade steel tube, to protect both the sensor material and the POF.

Usually, the fiber is coated with an optical isolated sensor material in order to exclude ambient light from the fiber tip.

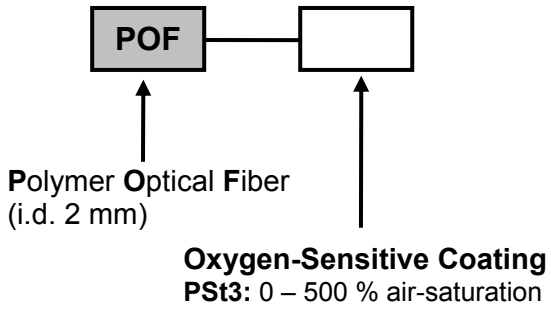
## *Schematic drawing*




## *Features*

- usable for process application
- very robust sensor with an excellent long-term stability (more than 100000 data points without drift)
- sterilizable ($H_2O_2$, ethanol, ethylene oxide)
- not autoclavable (POF does not stand autoclaving conditions (130 °C, 1.5 atm))

## *Ordering information*

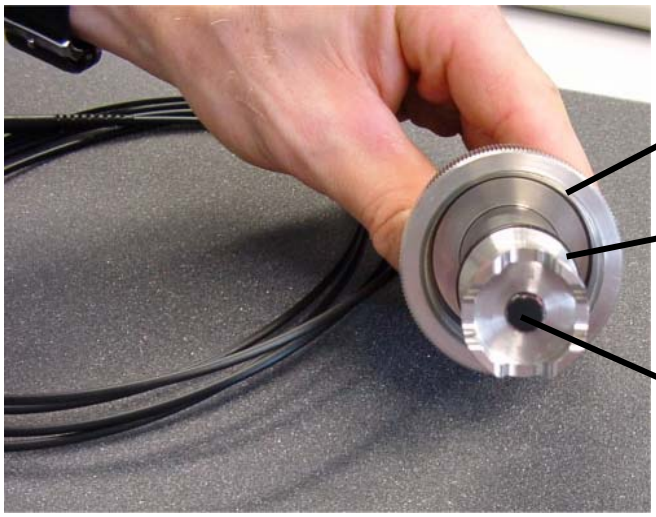



### Example

With **POF—PSt3** you will order a polymer optical fiber (POF) coated with **PSt3**, an oxygen sensor which can measure up to 500 % air-saturation (100 % pure oxygen). The standard cable length is 2.5 m, the maximum length is 25 m.

## OxyFerm Chemo-Optical DO Probes for Fermenters and Wastewater Treatment (OFM)

OxyFerm consists of a fitting made from stainless steel. The oxygen sensor is integrated in the top of the metal fitting (as shown on the right). The metal fitting is connected to the instrument via a polymer optical fiber (POF).The standard fiber cable length is 2.5 m. OxyFerm is available in different sizes (12 mm, 25 mm) and standard OxyFerm fits to B. Braun Biostat B and B.Braun Biostat C fermenters.

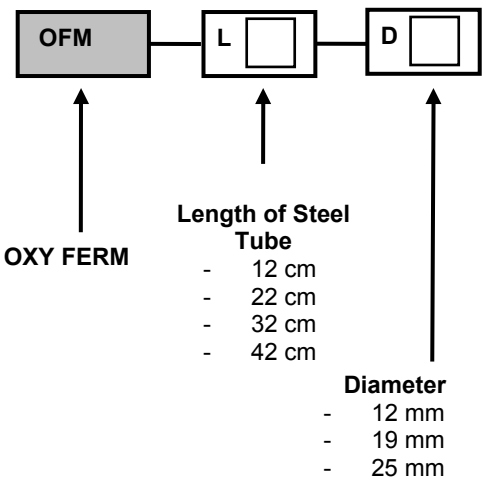



**This chemo-optical DO probe has outstanding properties:**

- The system can be used after autoclavation without recalibration
- The system is fully autoclavable up to 100 times
- In contrast to classical oxygen electrodes, membrane cleaning and replacement is not necessary because there are no membranes
- There are no electrolyte solutions to poison or replenish
- No time for polarization needed
- Long shelf-life

| Response time | Stirred | Not stirred | Gaseous Oxygen |
|---|---|---|---|
| $t_{90}$ without optical isolation | < 50 s | < 100 s | < 6 s |
| $t_{90}$ with optical isolation | < 60 s | < 120 | < 20 s |

## Ordering information



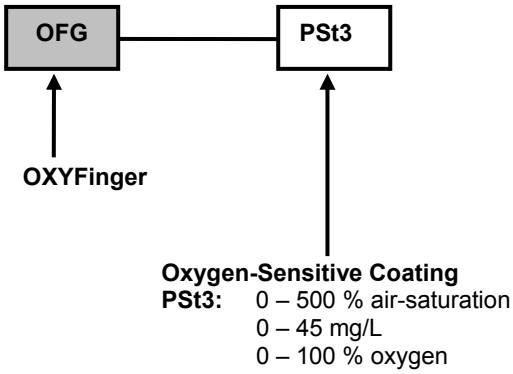

# OxyFinger Chemo-Optical DO Probe for Mini-Fermenters

OxyFinger consists of a glass test tube (glass finger) which is coated with an oxygen-sensitive foil. The sensor signal is transmitted to the instrument (Fibox 3, OXY-4 , OXY-10, Fibox 3-Trace, OXY-4 trace, OXY-10trace) via a polymer optical fiber.
Cable lengths between 2 and 15 meters are available.
OxyFinger will be manufactured following your specifications. Please specify both length and diameter of the glass finger and the size of the vessel closure.
Please contact our service team directly at 'info@presens.de'.

### The OxyFinger Chemo-Optical DO Probe for Mini-Fermenters has outstanding properties:

- The system can be used after autoclavation without recalibration
- The system is fully autoclavable up to 100 times
- In contrast to classical oxygen electrodes, membrane cleaning and replacement is not necessary because there are no membranes
- There are no electrolyte solutions to poison or replenish
- No time for polarization needed
- Long shelf-life

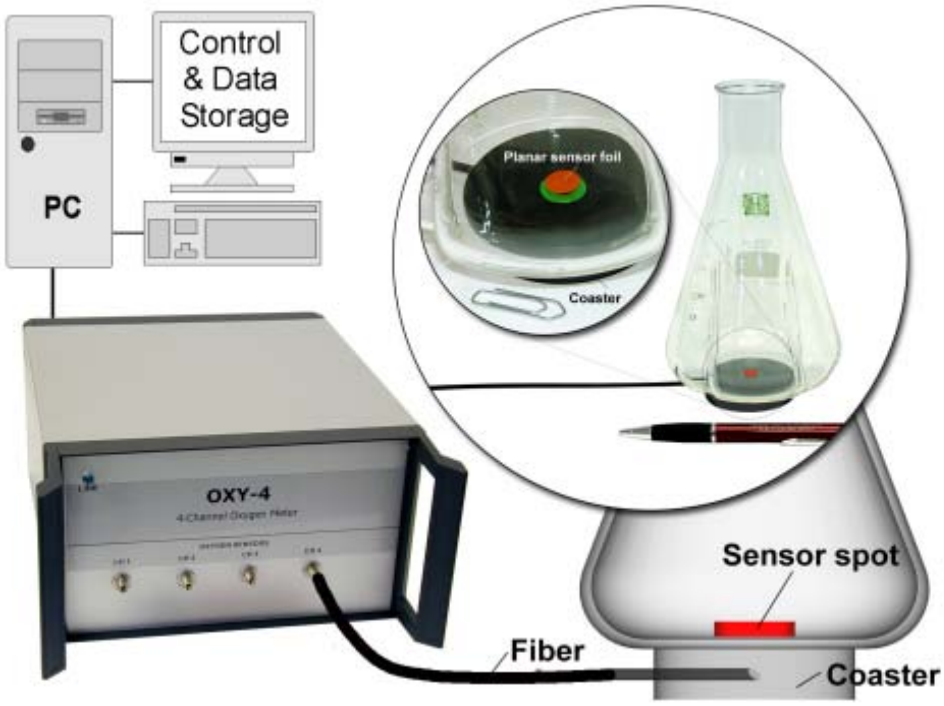
## Ordering information



# Coaster for Shaking Flasks and Spinner Flasks (CSF)

## Application:

Online control of oxygen in shaking flasks and spinner flasks.




System set-up for online DO measurement in shaking flasks

## Specifications:

The coaster for shaking flasks and Spinner flasks is a tool for online monitoring of dissolved oxygen concentration in shaking flasks. The coaster for shaking flasks does not contain a sensor. It redirects the light so that oxygen sensors in shaking flasks can be read out.
The coaster has a colored circle which makes it easy to position it right under the sensor.
The standard cable length is 2.5 m. The cable has an outer diameter of 2.6 mm.

**Please note:**
The coaster for shaking flasks and spinner flasks can only be used in combination with a Fibox 3, OXY-4 , OXY-10or Fibox 3 Trace, OXY-4 trace or OXY-10trace and does not contain a sensing layer. It is designed to read out sensor foils which are attached to the inner side of a shaking flask, spinner flask or a similar vessel (e.g. beaker).

## Ordering Information:

**Order code for the Coaster for Shaking Flasks: CSF**

# AKNOWLEDGEMENT

I would like to thank Michael Heberer who offered me the opportunity to enter the Tissue Engineering group, thus giving me the chance to experiencing these years in such an interesting field of research.

I would like to express my gratitude to Ivan Martin for his support, patience, and availability, his precious advices, and his contagious enthusiasm throughout these years.

A special thank to David Wendt who first introduced me to the fascinating world of bioreactors and tissue engineering, Andrea Barbero who supported me with his knowledge and expertise, and finally all the members of the Tissue Engineering, Oncology and gene therapy groups for their friendship, help, and collaboration.

I would like to specially acknowledge Karl and Marcel Jakob for their considerable help in the manufacturing all bioreactor components used in this work.

I like to thank Daniel Vonwil for all the inspiring scientific and non-scientific discussions.

I sincerely thank Ueli Aebi and Dan Daniels who accepted to be members of my PhD committee.

This work was financially supported by the European Union “STEPS” project (FP6- #NMP3-CT-2005-500465, www.stepsproject.com).

# CURRICULUM VITAE

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[redacted]

**Education:**

[redacted]

**Research education:**

[redacted]

[redacted]

**Lectures: teachers' list**

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Prof. M. Chiquet
Prof. R. Chiquet-Ehrisman
Prof. M. Hall
Prof. R. Landmann
Prof. U. A. Meyer
PD Dr. B. Fahrenkrog
PD Dr. C. Schönenberger

To my family