Introducing monitoring and automation in cartilage tissue engineering, toward controlled clinical translation

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
Vorgelegt der
Medizinischen Fakultät
der Universität Basel
von

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Mailand , Italien

Basel, 2011

Genehmigt von der Medizinischen Fakultät
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Basel den …….

……
(Dekan)

Basè l[*] after *basèl* I am close to reaching the PhD landing; looking back at the steps I have been walking on, I reflect upon all the illuminated guides and the good friends I have met during these years. I sincerely wish to thank them in the next few lines.

Prof. Martin who gave me the possibility to join the Tissue Engineering group and supported me during my work

Prof. Müller and Dr. Moretti who accepted to be members of my committee

Dr. Wendt, for being a patient mentor. Thanks for supporting my aptitudes and for teaching me the power of both word and silence

All the members of Tissue Engineering, Oncology, Cell and Gene Therapy I met and I worked with, both for the scientific support and for the nice intercultural environment
Uta, my best German friend,
Anna, for the nice runs and yoga,
Chitra, for the nice discussions on the balcony eating mangos,
Francine, for making of me a "spinning addict",
Sinan, Karo, Ben, Sandra and Andrea who made of my hours in the "403 cage" always a surprise. In particular to Andrea, the wiser member of the cage, for the great scientific support
Waldemar for all the German translations
Mr Strittmarten and Antonio, from the Biozentrum mechanical workshop, for your precious time and your flawless (*tip top*) work

Manuel, Susi, Anselmo, Carmelina, Miri, Angela, Gabri and all the other members of my big "Swiss- Italian family in Basel"

Arcangelo and William who made me experience the truth of Bauman's message "*fluidity* is the leading metaphor for the present stage of the modern era. Fluids travel easily. They *flow, spill, run out, splash, pour over, leak, flood, spray, drip, seep, ooze*; […]. From the meeting with solids they emerge unscathed, while the solids they have met, if they stay solid, are changed, get moist or drenched"

Ilaria, for more than 400 e-mails that filled up my account

Antonio, for sharing deep thoughts and good time, words and music

My parents, Gigio, Roby, for their care, for their fate in me, for sharing the steps forward and the setbacks, in one word for being a grate family

Teo, "*darei le stelle questa barca questo mare, per poterti solo sfiorare" (S. Vergani)*

Thanks!

[*] *in Milan dialect the word "basèl" means "step"*

Magna eloquentia, sicut flamma, materia alitur et motibus excitatur et urendo clarescit.

Tacito, Dialogus de oratoribus

Abstract

The clinical application of tissue engineered products requires to be tightly connected with the possibility to control the process, assess graft quality and define suitable release criteria for implantation. The aim of this work is to establish techniques to standardize and control the *in vitro* engineering of cartilage grafts. The work is organized in three sub-projects: first a method to predict cell proliferation capacity was studied, then an in line technique to monitor the draft during in vitro culture was developed and, finally, a culture system for the reproducible production of engineered cartilage was designed and validated.

Real-time measurements of human chondrocyte heat production during in vitro proliferation

Isothermal microcalorimetry (IMC) is an on-line, non-destructive and high resolution technique. In this project we aimed to verify the possibility to apply IMC to monitor the metabolic activity of primary human articular chondrocytes (HAC) during their in vitro proliferation. Indeed, currently, many clinically available cell therapy products for the repair of cartilage lesions involve a process of in vitro cell expansion. Establishing a model system able to predict the efficiency of this lengthy, labor-intensive, and challenging to standardize step could have a great potential impact on the manufacturing process. In this study an optimized experimental set up was first established, to reproducible acquire heat flow data; then it was demonstrated that the HAC proliferation within the IMC-based model was similar to proliferation under standard culture conditions, verifying its relevance for simulating the typical cell culture application. Finally, based on the results from 12 independent donors, the possible predictive potential of this technique was assessed.

Online monitoring of oxygen as a non-destructive method to quantify cells in engineered 3D tissue constructs.

This project aimed at assessing a technique to monitor graft quality during production and/or at release. A quantitative method to monitor the cells number in a 3D construct, based on the on-line measurement of the oxygen consumption in a perfusion based bioreactor system was developed. Oxygen levels dissolved in the medium were monitored on line, by two chemo-optic flow-through micro-oxygen sensors connected at the inlet and the outlet of the bioreactor scaffold chamber. A destructive DNA assay served to quantify the number of cells at the end of the culture. Thus the oxygen consumption per cell could be calculated as the oxygen drop across the perfused constructs at the end of the culture period and the number

of cells quantified by DNA. The method developed would allow to non-invasively monitoring in real time the number of chondrocytes on the scaffold.

Bioreactor based engineering of large-scale human cartilage grafts for joint resurfacing

The aim of this project was to upscale the size of engineered human cartilage grafts. The main aim of this project consisted in the design and prototyping of a direct perfusion bioreactor system, based on fluidodynamic models (realized in collaboration with the *Institute for Bioengineering of Catalonia*, Spain), able to guarantee homogeneous seeding and culture conditions trough the entire scaffold surface. The system was then validated and the capability to reproducibly support the process of tissue development was tested by histological, biochemical and biomechanical assays. Within the same project the automation of the designed scaled up bioreactor system, thought as a stand alone system, was proposed. A prototype was realized in collaboration with *Applikon Biotechnology BV, The Netherlands*. The developed system allows to achieve within a closed environment both cell seeding and culture, controlling some important environmental parameters (e.g. temperature, CO2 and O2 tension), coordinating the medium flow and tracking culture development. The system represents a relevant step toward process automation in tissue engineering and, as previously discussed, enhancing the automation level is an important requirement in order to move towards standardized protocols of graft generation for the clinical practice.

Conclusions and final remarks

These techniques will be critical towards a controlled and standardized procedure for clinical implementation of tissue engineering products and will provide the basis for controlled *in vitro* studies on cartilage development. Indeed the resulting methods have already been integrated in a streamlined, controlled, bioreactor based protocol for the *in vitro* production of up scaled engineered cartilage drafts. Moreover the techniques described will serve as the foundation for a recently approved Collaborative Project funded by the European Union, having the goal to produce cartilage tissue grafts. In order to reach this goal the research based technologies and processes described in this dissertation will be adapted for GMP compliance and conformance to regulatory guidelines for the production of engineered tissues for clinical use, which will be tested in a clinical trial.

Contents

General introduction

Cartilage: structure and properties

Cartilage is a connective tissue of mesoderm origin, found in many areas in the human body; there are three types of cartilage: elastic cartilage, fibrocartilage and hyaline cartilage. The elastic cartilage is the constituent of the epiglottis and the eustachian tube and it is characterized by high content of elastin, providing high flexibility to this tissue. The fibrocartilage often exists temporarily at bones fracture sites, but it is also permanently present in the intervertebral disks of the spine, as a covering of the mandibular condyle in the temporo-mandibular joint, and in the meniscus of the knee; compared with elastic cartilage it contains less elastin, but more collagen. The third type of cartilage, hyaline cartilage during childhood forms the growth plate by which long bones grow, and in adults is found in diarthroidal joints covering long bones. The presence of healthy hyaline cartilage on the joints surface is fundamental for the tribology, by providing lubrication, homogenizing the loads applied on the joint surfaces and absorbing the shock generated by impulsive loads.

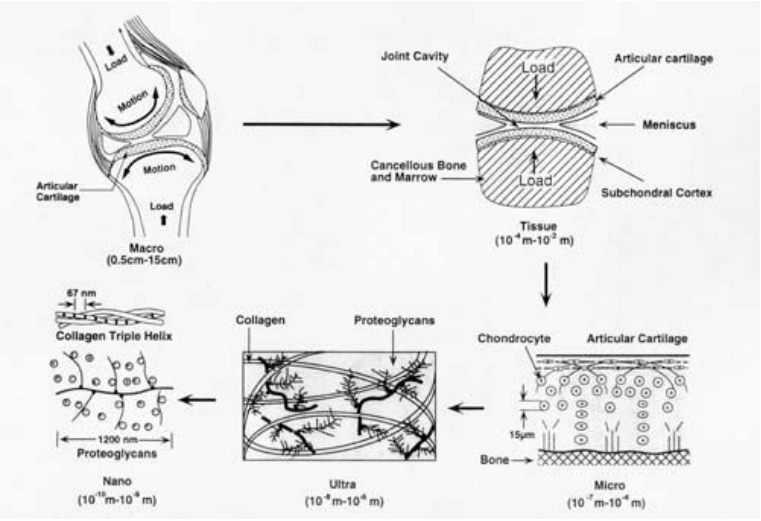

Figure 1 Cartilage components can be divided in relation with the observation scale. Looking at the microstructure (100μm-100nm) it is possible to observe the chondrocytes, the collagen matrix and the high molecular weight proteoglycans (PG) (such as aggrecans and hialuronans). At the ultrastructure (1μm-10nm) there are the biochemical constituents of the tissue (single fiber of collagen and of PG). At all the different levels, the molecules of collagen and PG, which constitute the solid component of ECM, actively electro-statically interact with water and other electrolytes. This balanced interaction between the solid and the liquid phase is crucial to the good mechanical response of cartilage to the load in vivo. (Mow and Ratcliffe, 1997)

Articular cartilage is a well-characterized tissue. It has the lowest volumetric cellular density of any tissue in the human body: chondrocytes are the exclusive cell type and in humans they contribute only about 1% of the tissue volume, and the remaining 99% is made up of a complex ECM (Buckwalter and Mankin, 1998; Heath and Magari, 1996; Stockwell, 1979). The chondrocytes are situated in small cavities (lacunae) within the cartilage tissue. The spherical cells are found as single, isolated cells, or in a chondron, an aggregate of several chondrocytes. The cells sense the structure and composition of the ECM and carry out their primary function that is to maintain it. The chondrocytes themselves synthesize all necessary ECM components (Buckwalter and Mankin, 1998; Cohen et al., 1998). The unique viscoelastic properties of articular cartilage are a consequence of the molecular nanoarchitecture and zone-specific organization of the matrix components in this tissue, which are synthesized by the chondrocytes (Figure 1). The ECM of articular cartilage consists (as wet mass) of about 60–85% water and dissolved electrolytes. The complex solid framework is composed of collagens (mainly type II) (10–30%), proteoglygans (3–10%) and non collagenous proteins and glycoproteins (Buckwalter and Mankin, 1998; Hardingham and Muir, 1973; Mankin and Thrasher, 1975). The tensile behavior of cartilage is believed to be mainly due to the collagen fibers, while proteoglycans affects swelling pressure as well as fluid motion under compression. Moreover about 30% of the total water exists within the intrafibrillar space of collagen. The collagen fibril diameter and the amount of water within the collagen are determined by the swelling pressure due to the strong negative electric charges of the proteogylcans. The proteoglycans are constrained within the collagen matrix. Because the proteogylcans are bound closely, the closeness of the negative charges creates a repulsion force that must be neutralized by positive ions in the surrounding fluid. The higher concentration of ions in the tissue compared to outside the tissue leads to swelling pressures. The exclusion of water raises the density of fixed charge, which in turn raises the swelling pressure and charge-charge repulsion. The amount of water present in cartilage depends on the concentration of proteoglycans, the organization of the collagen network, and the stiffness and strength of the collagen network. The collagen network resists the swelling and if the collagen network is degraded, the amount of water in the cartilage increases, because more negative ions are exposed to draw in fluid, changing the mechanical response of the tissue to the load. In addition, with a pressure gradient or compression, fluid is squeezed out of the cartilage. When the fluid is being squeezed out, there are drag forces between the fluid and the solid matrix that increase with increasing compression and make it more difficult to exude water. This behavior increases the stiffness of the cartilage as the rate of loading is increased.

In conclusion, articular cartilage functionality is tightly related with the balance between the different components of its ECM.

Cartilage lesions treatments.

Progressive reduction of extracellular matrix in articular cartilage, due to traumatic lesions or degenerative diseases like osteoarthritis (OA), inducing loss of joint function and excessive morbidity is an open clinical challenge; in particular damages of the knee joint are quite common. The appearance of lesions is frequently associated with pain, disturbed function and disability, and, if not successfully treated, often results in total replacement of the joint. The replacement of the entire joint or of part of it with metallic prosthesis is a high invasive technique and due to the limited durability of the devices, the procedure is especially critical in case of young patients. Due to the absence in cartilage of blood vessels and nerves pharmaceutical treatments are ineffective and the normal mechanisms of tissue repair, involving the recruitment of cells to the site of damage, do not occur. Some of the most popular treatment options, referred to as marrow-stimulating techniques (e.g., microfracture or subchondral drilling of the bone) are based on the principle of inducing invasion of mesenchymal progenitor cells from the underlying subchondral bone to the lesion site, in order to initiate cartilage repair (Mitchell and Shepard, 1976). In the absence of a material which appropriately "instructs" the mesenchymal progenitors to differentiate into articular chondrocytes in stable fashion, the outcome of these procedures is highly variable and often results in repair tissue composed of fibrocartilage, with limitations in quality and duration as compared to native hyaline tissue (Kreuz et al., 2006).

A breakthrough in the field, for localised injuries, was the introduction of cell-based repair techniques, such as autologous chondrocyte implantation (ACI) (Brittberg, 2008). In this procedure, *in vitro* expanded autologous articular chondrocytes are introduced into the defect site as a cell suspension or in association with a supportive matrix (matrix-assisted ACI, MACI) (Marlovits et al., 2005), where they are expected to synthesize new cartilaginous matrix. The clinical outcome of these chondrocyte-based techniques is generally good, as they lead to lessening of symptoms for the patient (Bentley et al., 2003; Peterson et al., 2000), but in many cases results in the formation of fibrous repair tissue with inferior mechanical properties and limited durability (Brittberg, 2008; Grigolo et al., 2005; Roberts et al., 2003). A recent study proposed a correlation between the symptomatology of patients treated with ACI and the quality of the repair tissue, suggesting that the persistence of symptoms after surgery reflected the presence of non-hyaline cartilage repair tissue (Brun et al., 2008). These

observations underline the importance of improving the quality of the generated repair tissue following treatment of cartilage defects. A way to reach this goal is the implant of a more mechanically functional graft; in fact it has been demonstrated that in vivo, in ectopic mouse models, the implant of more developed construct leads to the generation of a more hyline like tissue (Demarteau et al., 2003; Moretti et al., 2005). This is the aim of "functional cartilage tissue engineering".

Cartilage Tissue Engineering.

Tissue engineering has been defined as the application of principles and methods of engineering and life sciences for the development of biological substitutes, to restore, maintain or improve tissue function.

The challenge for cartilage tissue engineering is to produce cartilage tissue with suitable structure and properties *ex vivo*, which can be implanted into joints to provide a natural repair that, with time, will become integrated with the patient's tissues. Regeneration of a hyaline-like tissue could be facilitated by the implantation of a pre-engineered, functional cartilage tissue, as opposed to the delivery of a chondrocyte suspension, as described before. In the most typical approach (Figure 2) the ex vivo generation of a functional graft starts with the isolation of human cells and their monolayer expansion on plastic; when a sufficient number of cells is reached the cells are seeded on a 3D structure (scaffold) which provides an initial frame for the development of the final functional graft. Biochemical and biomechanical stimulus can be needed in order to induce the cell differentiation and organization (Demarteau et al., 2003; Jakob et al., 2001), thus generating a graft approaching the properties of native cartilage. Indeed, the presence of extracellular matrix (ECM) around cells was reported to enhance donor cell retention at the repair site (Ball et al., 2004) and possibly protect the cells from environmental factors such as inflammatory molecules (Francioli et al., 2011). Furthermore, precultivation under conditions inducing cell differentiation was shown to support enhanced *in vivo* development of engineered cartilage at ectopic sites in mice (Moretti et al., 2005) and improved cellular response to a compressive deformation conditioning resembling a mild rehabilitation regime (Demarteau et al., 2003). Importantly, from the clinical point of view the improved mechanical stability of the more mature and stable engineered graft would also allow easier surgical handling, application specially in critically sized defects (Farhadi et al., 2006) and possibly earlier postoperative loading.

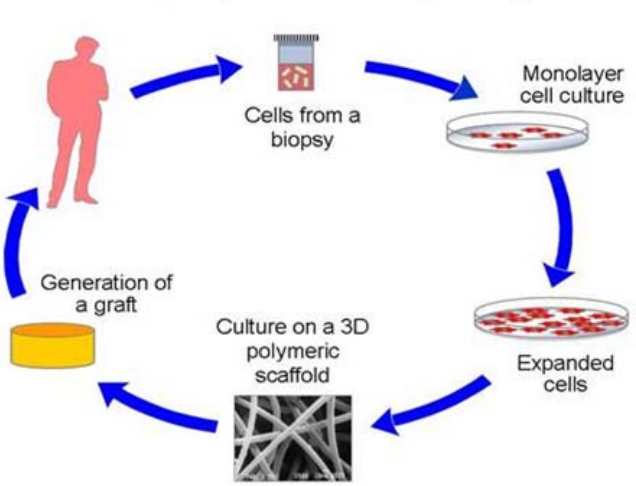

Figure 2 In the most typical approach, tissue engineering is a cycle, starting with the harvesting of a cell biopsy. The cells are, then, isolated and expanded *in vitro*, aiming to reach a number of cell sufficient to perform a high density seeding of a 3D scaffold. The hybrid cell-scaffold construct in further cultured *in vitro*, where is exposed to biochemical and biomechanical stimuli, until the level of maturation required for the *in vivo* implant is reached.

Some products, such as Carticel® and Hyalograft-C®, have been well established in the clinic for the treatment of traumatic focal cartilage defects, and follow up show good results (Manfredini et al., 2007). However no tissue engineered product is currently available to treat large defects associated with wide traumatic lesions or advanced diseases such as osteoarthritis. Beyond the biological challenges that must be addressed to treat chronic joint disorders, it remains a significant engineering challenge to generate cartilage grafts with dimensions sufficient for the repair of large, advanced, and deep (4 mm) defects. Indeed the homogeneity of the final graft, is directly dependent upon the homogeneity of the culture condition overall the graft volume. Moreover one non unimportant obstacle in delivering cartilage tissue engineering products to routine clinical use are the costly, labour-intensive and time-consuming manual processes which are difficult to control and standardise. To be attractive for clinical applications, engineered cartilage will need to demonstrate cost-effectiveness and cost-benefits over existing therapies, absolute safety for patients, manufacturers and environment, and compliance with the evolving regulatory framework in terms of quality control and good manufacturing practice (GMP). To meet these targets and translate research-scale production into clinically compatible manufacture, quality control

methods, automation and streamlining of the process and up-scaling of the constructs are required.

Proliferation capacity as a marker of cell quality.

As autologous chondrocytes can only be harvested from a small biopsy (280 mg, (Brittberg, 2008)) of articular cartilage in relatively low numbers, currently, many clinically available cell therapy products for the repair of cartilage lesions involve a process of *in vitro* cell expansion (Marcacci et al., 2007). This step, consisting in an extensive proliferation on plastic dishes, is lengthy, labor-intensive, and challenging to standardize. Indeed the capacity of isolated chondrocytes to proliferate and to regenerate a tissue is not only dependent on the health state of the joint (Tallheden et al., 2005) and on the age of the donor (Loeser et al., 2000), but is also extremely variable between individuals in the same age range and with no history of joint disease (Barbero et al., 2004). Therefore establishing physics-based model systems aimed at better understanding and predicting chondrocyte function during proliferation could help in the development of protocols to reduce the time of expansion and improve its reproducibility, ultimately having a great potential impact on the manufacturing process. The evaluation of the metabolic rate of freshly isolated chondrocytes could be used to estimate their response to the *in vitro* environment and their proliferation capacity. Being cell metabolism tightly related with heat generation, this could be an eligible parameter to be monitored.

Isothermal microcalorimetry (IMC)

Isothermal microcalorimetry is a technique by which the heat flow generated by an arbitrary chemical, physical or biological process is continuously monitored while the sample is kept at constant temperature. Isothermal microcalorimeters (Figure 3) are defined as instruments measuring heat flow in the microwatt range (μW) and operating at nearly constant temperature (Wadsö, 2002). In this type of calorimeter the heat produced or consumed in the calorimetric ampoule is allowed to flow between the ampoule and a heat sink (usually an aluminum block) thus keeping the calorimetric ampoule and its contents within a few millidegrees of the temperature at which the heat sink is maintained by the thermostatic system in which the calorimeter operates. The real sensing elements are the thermoelectric modules (i.e., Seebeck or Peltier modules) placed between the sample and the heat-sink. These thermoelectric modules allow any slight temperature difference to be converted into an electrical signal, which can be easily recorded.

The use of a label-free, passive, dynamic measurement opens a wide range of potential use in biomedical sciences. In terms of biomedical and clinical applications it allows investigating the metabolism and growth of human cell cultures and also potentially infectious, contaminated or genetically modified organisms in an environment of choice. Of equal importance, the measurements can be run in closed ampoules, under safe and controlled conditions. In addition, many processes relevant to biomaterial degradation or material stability can be studied using IMC. Beyond the more traditional thermodynamic applications within the physical sciences, a sufficiently sensitive calorimeter can be used in the study of living systems, for instance to quantify cell metabolic activities. Calorimetry-based techniques have been employed in a broad range of biological applications including fermentation (Wadsö and Gomez Galindo, 2009), pharmaceuticals (Buckton, 1995), and environmental studies (Rong et al., 2007). The use of IMC as a fast and inexpensive alternative to traditional diagnostic and prognostic tools has also been investigated for clinical applications (Monti, 1990), for example in the rapid detection of bacterial infections (von Ah et al., 2009; Xi et al., 2002; Yang et al., 2008) and to monitor the metabolic activity of tumor cells (Bäckman, 1990). Finally, calorimetry has the potential to represent an innovative tool to define and optimize specific cell culture parameters, as well as to provide non-invasive and non-destructive methods in quality control assessments (Kemp and Guan, 2000).

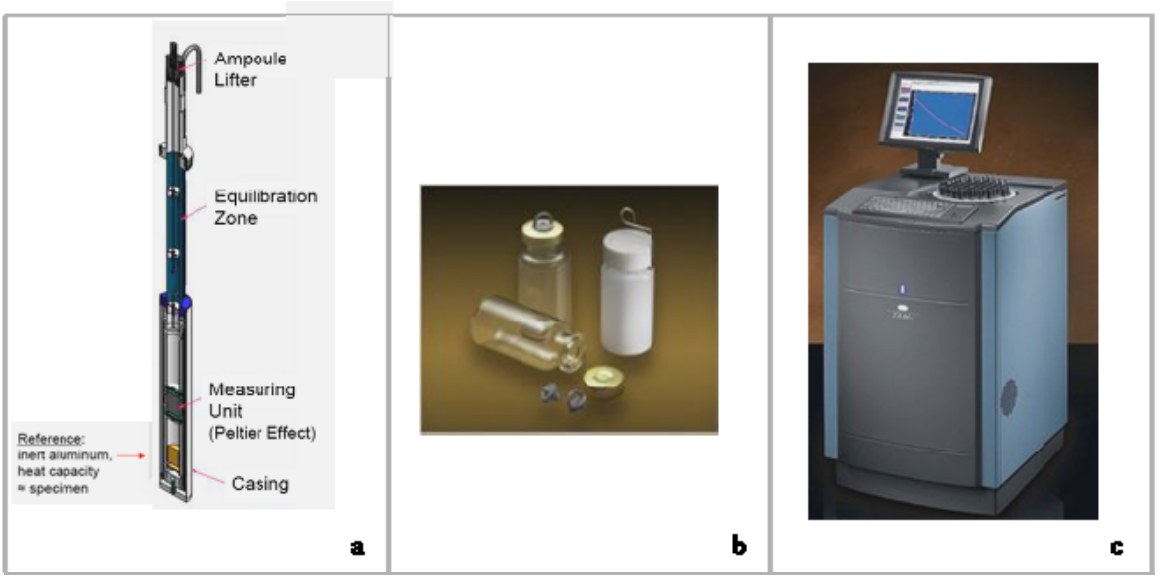

Figure 3 Panel (a) shows the internal structure of an isothermal microcalorimeter: the core is the measuring unit, which measures the heat flow between the sample, placed in a sealed ampoule (b), and a reference. Modern machines, as the one shown in panel (c) (TAM48), can monitor several samples with high sensitivity (e.g. 5E-2 $mW \times L^{-1}$ in this model)

Bioreactors for cartilage tissue engineering: towards process automation and quality control.

The term *bioreactor* refers to a system in which conditions are closely controlled to permit or induce certain processes in living cells or tissues. The concept of bioreactor is not restricted to tissue engineering; indeed bioreactors are classically used in industrial fermentation processing, wastewater treatment, food processing and production of pharmaceuticals and recombinant proteins. Bioreactor technologies intended for tissue engineering can be used to grow functional cells and tissues for transplantation, and for controlled in vitro studies on the regulation effect of biochemical and biomechanical factors on cell and tissue development. In essence, the aim of a bioreactor is to provide the appropriate physical stimulation to cells, continuous supply of nutrients (e.g. glucose, amino acids), chemical species, biochemical factors and oxygen to the construct interior, as well as continuous removal of products of cellular metabolism (e.g. lactic acid), aiming to reliably and reproducibly form, store, and deliver functional tissues. Moreover, a bioreactor has to be able to operate over long periods of time under aseptic conditions (S.A.Korossis et al., 2005).

Seeding

One of the applications of a bioreactor system is the support at the seeding phase. Cell seeding of scaffolds is the first step in establishing a 3D culture, and might play a crucial role in determining the progression of tissue formation (Vunjak-Novakovic et al., 1998). Engineering autologous grafts for clinical applications limiting the biopsy size and/or the extent of cell expansion requires the cells to be seeded with the highest possible efficiency. Furthermore, the initial distribution of cells within the scaffold after seeding has been related to the distribution of tissue subsequently formed within engineered constructs (Freed et al., 1998; Holy et al., 2000; Kim et al., 1998), suggesting that uniform cell-seeding could establish the basis for uniform tissue generation. However it can be a significant challenge to distribute cells efficiently and uniformly throughout the scaffold volume. Although static loading of cells onto a scaffold is by far the most commonly used seeding method, several studies reported low seeding efficiencies and non-uniform cell distributions within scaffolds (Kim et al., 1998; Xiao et al., 1999; Li et al., 2001), owing, in part, to the manual- and operator-dependent nature of the process. Significantly higher efficiencies and uniformities can be obtained by forcing the flow of a cell suspension directly through the pores of 3D scaffolds using a multi-pass filtration seeding technique(Wendt et al., 2003; Li et al., 2001), performed in a bioreactor, under controlled and reproducible flow conditions.

Mass transport

It has long been known that the supply of oxygen and soluble nutrients becomes critically limiting for the in vitro culture of 3D tissues. The consequence of such a limitation is exemplified by early studies showing that cellular spheroids larger than 1 mm in diameter generally contain a hypoxic, necrotic center, surrounded by a rim of viable cells (Sutherland et al., 1986). Similar observations were reported for different cell types cultured on 3D scaffolds under static conditions. Because cartilage engineered constructs should be at least 2-4 mm thickness in size to serve as grafts for tissue replacement, mass-transfer limitations represent one of the greatest challenges to be addressed. Aiming to optimize the mass transfer, during the last years, several culture systems have been tested, like stirred flask or rotating vessels. Indeed these models can enhance external mass transfer, but the resulting grafts still show the effects of a limited mass transfer in the more internal zone. Trying to overcome this limitation, bioreactors were designed based on the perfusion of the culture medium directly through the pores of the cell-seeded 3D scaffold (Figure 4). These bioreactors provided the advantage of reducing the mass transfer limitations both at the construct periphery and within its internal pores, enhancing cell survival, growth and function, as GAG synthesis and accumulation (Davisson et al., 2002b; Pazzano et al., 2000). Being the effect of direct perfusion so relevant for the effective result of the culture, prove a homogeneous distribution of the flow is important. For this reason a bioreactor system should be designed on careful estimation of the flow patterns, based on computational fluid dynamic models in conjunction with further experimental validation.

Additionally, perfusion seeding can be readily integrated into a perfusion bioreactor system capable of forming both seeding of the scaffold and subsequent culturing of the construct (Davisson et al., 2002b). These systems not only streamline the engineering process but also reduce the safety risks associated with the handling and transferring of constructs between separate bioreactors.

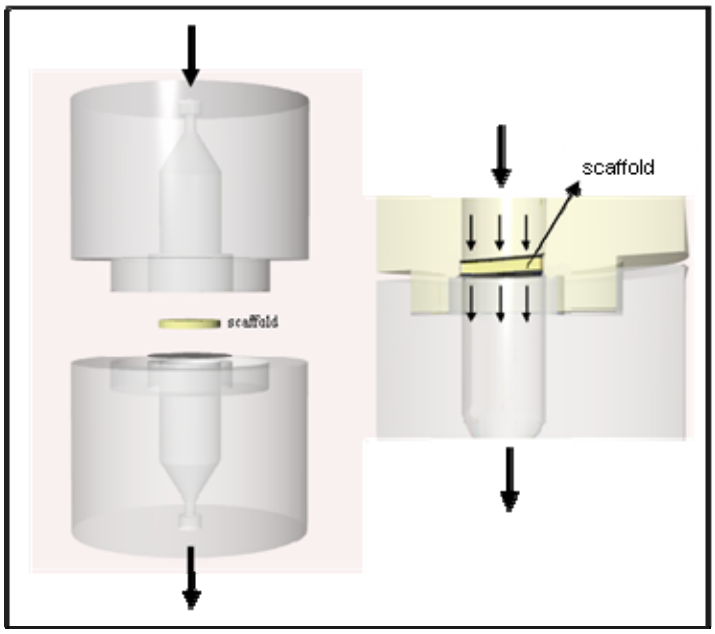

Figure 4. The image on the left shows the exploded view of a representative direct perfusion bioreactor. The chamber is composed by two sub-unites, that create a place for the scaffold. The detail in the assembled view on the right underlines how critical is the design in ensuring the absence of lateral flows around the scaffold. Black arrows underline the flow distribution.

Physical stimuli

Another role of bioreactor systems is to provide physical stimulus to the culture. Increasing evidence suggests that mechanical forces, which are known to be important modulators of cell physiology, might increase the biosynthetic activity of cells in bioartificial matrices and, thus, possibly improve or accelerate tissue regeneration in vitro (Butler et al., 2000). Regarding cartilage, dynamic deformational loading and shear of chondrocytes embedded in a 3D environment stimulated GAG synthesis (Davisson et al., 2002a; Mauck et al., 2000; Waldman et al., 2003) and increased the mechanical properties of the resulting tissues (Mauck et al., 2000; Waldman et al., 2003); a role in the matrix development is played also by dynamic tension (Eschenhagen et al., 1997; Matthews et al., 2001; Vandenburgh, 1992; Young et al., 1998), compression (Buschmann et al., 1995) or hydrodynamic pressure (Carver and Heath, 1999). Nowadays still little is known about the specific mechanical forces or regimes of application (i.e. magnitude, frequency, continuous or intermittent, duty cycle) that are stimulatory for a particular tissue, specially if it is taken into account that different stages of development might require different regimes of mechanical conditioning owing to the increasing accumulation of extracellular matrix and developing structural organization. In this

context, bioreactors can have an important role, providing controller environments for reproducible and accurate application of specific regimes of mechanical forces to 3D constructs (Demarteau et al., 2003).

Control

One of the major challenges to translate research products into clinically applicable manufacturing is to establish a production process allowing obtaining products that are reproducible, clinically effective and economically acceptable, while complying with Good Manufacturing Practice (GMP) requirements (Ratcliffe and Niklason, 2002). Advanced bioreactor controlled closed systems would facilitate streamlining and automation of the numerous labor-intensive steps involved in the in vitro engineering of 3D tissues. Typical environmental factors (e.g. temperature, pH and oxygen) will have to be maintained at defined levels to ensure reproducibility and standardization, as is routinely achieved in classical bioreactors for the production of recombinant proteins. Moreover, because the development of engineered tissues might progress at varying rates for different cell batches, additional parameters to be monitored would be cell number, phenotype and metabolism, or specific tissue mechanical properties. Development of the tissue could be further monitored through the incorporation of advanced technical tools for online micro or macroscopic observation of the structural properties of the tissue (e.g. video microscopy, magnetic resonance imaging and microcomputerized tomography). All collected inputs could be analyzed by a microprocessor unit and fed back to the bioreactor system to optimize the control of culture parameters at pre-defined levels (Figure 5).

An advantage in the area would be the development and employment of computational or experimentally validated models which could test the quality of the tissue non-invasively and on-line. This would allow for a higher efficiency in the tissue engineering process, as well as a high degree of certainty in harvesting tissues within the pre-determined manufacturing specifications. At the state of the art, both metabolites (e.g. oxygen, glucose) and catabolites (e.g. CO2, lactate) dissolved in the medium culture were proposed as parameters eligible to describe the state of the culture. Of the various sensors traditionally used to monitor milieu parameters, oxygen sensors have been successfully adapted to the tissue engineering field. Indeed, despite the technological success of producing small, sterile and high sensitive probes, the application of oxygen measurements to monitor and control the bioreactor based culture has not been achieved yet. In fact the most relevant studies in the field use oxygen

measurements either to provide local data for the boundary conditions of computational models (Malda et al., 2004) or describe qualitative relations (Janssen et al., 2006).

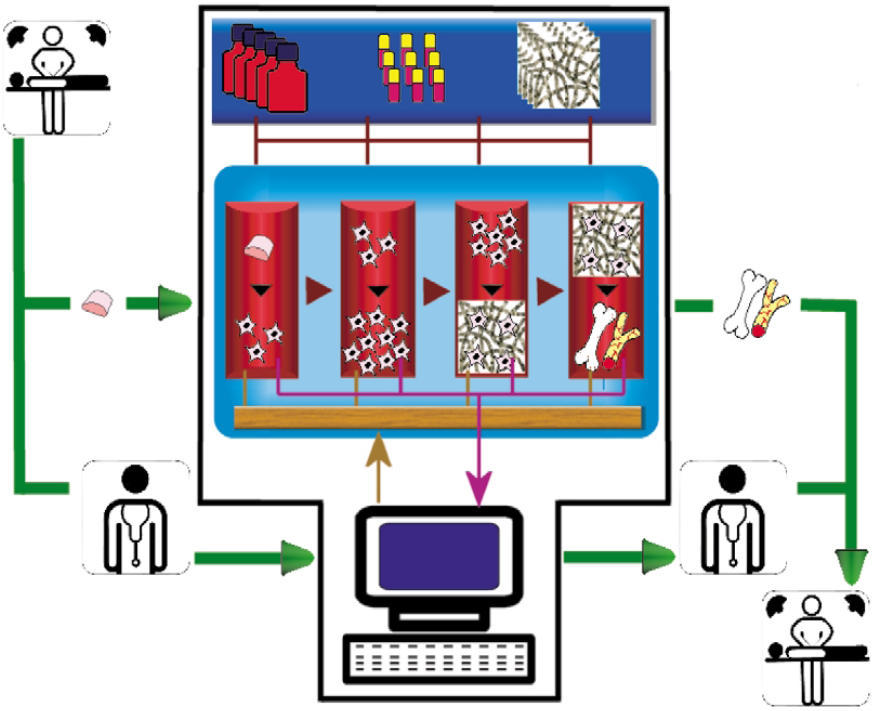

Figure 5. Vision for a closed-system bioreactor for the automated production of tissue-engineered grafts. Within the same bioreactor system the cells would be isolated from the biopsy, seeded, expanded and cultured. Environmental culture parameters and tissue development would be monitored and inputs fed into a microprocessor unit for analysis. In conjunction with data derived from clinical records of the patient, the inputs would be used to control culture parameters at pre-defined optimum levels automatically and provide the surgical team with data on the development of the tissue, enabling timely planning of the implantation. Reproduced from (Martin et al., 2004).

Thesis Outline

General Aim

The clinical application of tissue engineered products is tightly connected with the possibility to control the process, assess graft quality and define suitable release criteria for implantation. The aim of this work is to establish techniques to standardize and control the in vitro engineering of cartilage grafts. Attention will focus first on development of non destructive techniques for the estimation of the proliferation capacity of cells isolated from the biopsy. Moreover a method for the on-line quantification of the cell proliferation in the cell-scaffold construct will be validated. Finally a controlled bioreactor for the engineering of clinically relevant sized constructs will be designed and validated. These techniques will be critical towards a controlled and standardized procedure for clinical implementation, and will provide the basis for controlled in vitro studies on cartilage development.

Chapter 1 *Real-time measurements of human chondrocyte heat production during in vitro proliferation*

Isothermal microcalorimetry (IMC) is an on-line, non-destructive and high resolution technique. In this project we aimed to verify the possibility to apply IMC to monitor the metabolic activity of human articular chondrocytes (HAC) during their in vitro proliferation. Indeed, currently, many clinically available cell therapy products for the repair of cartilage lesions involve a process of in vitro cell expansion. Establishing a model system able to predict the efficiency of this lengthy, labor-intensive, and challenging to standardize step could have a great potential impact on the manufacturing process. In this study an optimized experimental set up was first established, to reproducible acquire heat flow data; then it was demonstrated that the HAC proliferation within the IMC-based model was similar to proliferation under standard culture conditions, verifying its relevance for simulating the typical cell culture application. Finally, based on the results from 12 independent donors, the possible predictive potential of this technique was assessed.

Chapter 2 *Online monitoring of oxygen as a non-destructive method to quantify cells in engineered 3D tissue constructs.*

This project aimed at assessing a technique to monitor graft quality during production and/or at release. A quantitative method to monitor the cells number in a 3D construct, based on the on-line measurement of the oxygen consumption in a perfusion based bioreactor system was developed. Oxygen levels dissolved in the medium were monitored on line, by two chemo-optic flow-through micro-oxygen sensors connected at the inlet and the outlet of the bioreactor scaffold chamber. A destructive DNA assay served to quantify the number of cells at the end of the culture. Thus the oxygen consumption per cell could be calculated as the oxygen drop across the perfused constructs at the end of the culture period and the number of cells quantified by DNA. The method developed would allow to non-invasively monitor the number of chondrocytes on the scaffold.

Chapter 3 *Bioreactor based engineering of large-scale human cartilage grafts for joint resurfacing*

The aim of this project was to upscale the size of engineered human cartilage grafts. The main aim of this project consisted in the design and prototyping of a direct perfusion bioreactor system, based on fluidodynamic models (realized in collaboration with the *Institute for Bioengineering of Catalonia*, Spain), able to guarantee homogeneous seeding and culture conditions trough the entire scaffold surface. The system was then validated and the capability to reproducibly support the process of tissue development was tested by histological, biochemical and biomechanical assays. Within the same project the automation of the designed scaled up bioreactor system, thought as a stand alone system, was proposed. A prototype was realized in collaboration with *Applikon Biotechnology BV, The Netherlands*. The developed system allows to achieve within a closed environment both cell seeding and culture, controlling some important environmental parameters (e.g. temperature, CO2 and O2 tension), coordinating the medium flow and tracking culture development. The system represents a relevant step toward process automation in tissue engineering and, as previously discussed, enhancing the automation level is an important requirement in order to move towards standardized protocols of graft generation for the clinical practice.

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Real-Time Measurements of Human Chondrocyte Heat Production During In Vitro Proliferation

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Received 19 April 2011; revision received 31 May 2011; accepted 8 July 2011

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23268

ABSTRACT: Isothermal microcalorimeters (IMC) are highly sensitive instruments that allow the measurement of heat flow in the microwatt range. Due to their detection of minute thermal heat, IMC techniques have been used in numerous biological applications, including the study of fermentation processes, pharmaceutical development, and cell metabolism. In this work, with the ultimate goal of establishing a rapid and real-time method to predict the proliferative capacity of human articular chondrocytes (HAC), we explored to use of IMC to characterize one of the crucial steps within the process of cartilage tissue engineering, namely the in vitro expansion of HAC. We first established an IMC-based model for the real-time monitoring of heat flow generated by HAC during proliferation. Profiles of the heat and heat flow curves obtained were consistent with those previously shown for other cell types. The average heat flow per HAC was determined to be 22.0 ± 5.3 pW. We next demonstrated that HAC proliferation within the IMC-based model was similar to proliferation under standard culture conditions, verifying its relevance for simulating the typical cell culture application. HAC growth and HAC heat over time appeared correlated for cells derived from particular donors. However, based on the results from 12 independent donors, no predictive correlation could be established between the growth rate and the heat increase rate of HAC. This was likely due to variability in the biological function of HAC derived from different donors, combined with the complexity of tightly couple metabolic processes beyond proliferation. In conclusion, IMC appears to be a promising technique to characterize cell proliferation. However, studies with more reproducible cell sources (e.g., cell lines) could be used before adding the complexity associated with primary human cells.

Biotechnol. Bioeng. 2011;xxx: xxx–xxx.

KEYWORDS: calorimetry; tissue engineering; cartilage; metabolism

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Introduction

Isothermal microcalorimeters (IMC), which have the capacity to measure heat flow in the range of microwatts, have long been used as analytical tools for thermodynamic and kinetic studies (Wadsö, 1996, 2002). In addition to the high thermal sensitivity, the power of IMC techniques is also related to the ability to perform rapid, passive, non-invasive, and real-time measurements (Braissant et al., 2010; Lewis and Daniels, 2003). Moreover, during heat flow data acquisition, samples can be kept in sealed ampoules, intact for further investigations, thus allowing IMC to be used as a non-destructive technique.

Beyond the more traditional thermodynamic applications within the physical sciences, a sufficiently sensitive calorimeter can be used in the study of living systems, for instance to quantify cell metabolic activities. Calorimetry-based techniques have been employed in a broad range of biological applications including fermentation (Wadsö and Gomez Galindo, 2009), pharmaceuticals (Buckton, 1995; Tan and Lu, 1999), and environmental studies (Rong et al., 2007). The use of IMC as a fast and inexpensive alternative to traditional diagnostic and prognostic tools has also been investigated for clinical applications (Monti, 1990), for example, in the rapid detection of bacterial infections (von Ah et al., 2009; Xi et al., 2002; Yang et al., 2008) and to monitor the metabolic activity of tumor cells (Bäckman, 1990).

Despite the power and sensitivity of calorimetric techniques for biological applications, such methods have

yet to be implemented within the rapidly growing field of cellular therapy and tissue engineering. The generation of cellular tissue grafts in vitro provides an attractive alternative to traditional treatments aimed at repairing or replacing damaged tissues to establish normal function. Calorimetry has the potential to represent an innovative tool to define and optimize specific cell culture parameters, as well as to provide non-invasive and non-destructive methods in quality control assessments (Kemp and Guan, 2000).

Currently, many clinically available cell therapy products for the repair of cartilage lesions involve a process of in vitro cell expansion. In this process, a limited number of chondrocytes, which can be obtained from the digestion of a small cartilage biopsy (only 100–250 mg of tissue), must be extensively proliferated on plastic dishes in order to obtain a sufficiently high number of cells to be re-implanted back into the patient. This step of the manufacturing process is lengthy, labor-intensive, and challenging to standardize. Establishing physics-based model systems aimed at better understanding and predicting chondrocyte function during proliferation could help in the development of protocols to reduce the time of expansion and improve its reproducibility, ultimately having a great potential impact on the manufacturing process.

Therefore, in this study, we aimed to first establish an experimental setup for the real-time monitoring of heat flow generated by human articular chondrocytes (HAC) during their in vitro proliferation. We next aimed to assess whether HAC proliferation within the IMC-based model system was similar to proliferation under well-established conventional culture conditions, verifying its relevance for predicting results of traditional cell and tissue culture applications. Finally, with the goal of establishing a rapid and real-time calorimetric method to predict the proliferative capacity of HAC under defined in vitro culture conditions, we assessed whether there was a correlation between the growth rate of HAC during the exponential phase of proliferation and the heat increase rate determined by the IMC-based method.

Materials and Methods

Cell Isolation and Expansion

Human articular chondrocytes were isolated from cartilage biopsies obtained post-mortem from 12 individuals (ages 65 ± 13 years) after informed consent and in accordance with the local Ethical Commission. As previously described (Jakob et al., 2001), HAC were isolated using 0.15% type II collagenase (10 mL solution/g tissue) for 22 h and resuspended in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen #10938-025, Basel, Switzerland) supplemented with 10% fetal bovine serum, 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). The isolated chondrocytes were expanded for 1 passage (4–5 doublings) in culture medium supplemented with 1 ng/mL of transforming growth factor-β_1 (TGF β_1), 5 ng/mL of fibroblast growth factor-2 (FGF2) and 10 ng/mL of platelet derived growth factor-BB (PDGF-BB), a cocktail of factors previously shown to increase human chondrocyte proliferation (Jakob et al., 2001). Twelve hours prior to calorimetry experiments, HAC were synchronized with Aphidicolin (1 μg) (Saris et al., 1999); this compound does not affect cell viability or "S" phase duration, does not interfere with the synthesis of dNTPs or DNA polymerases, thus permitting to obtain a population of cells ready to start the replication when the block is removed. Synchronized HAC were then trypsinized, and either replated and cultured under conventional cell expansion conditions (i.e., in 6 wells plates, 10,000 cells/well with 2 mL medium, within a 37°C 5% CO_2 incubator), or seeded in calorimetry ampoules (100,000 cells/ampoule in 3 mL medium). Culture medium in the wells was exchanged twice weekly, whereas the microcalorimetry ampules were sealed, and thus no culture medium or gas exchange could be performed throughout the culture period.

Heat Flow Measurement

A multi-channel IMC (TAM III, TA Instruments, New Castle, DE) equipped with 48 measuring channels was used to monitor the heat production by HAC. The IMC instrument was thermostated to 37°C, 2 days before the start of experiments to achieve maximum stability. The heat flow signal was acquired continuously and resampled to obtain an effective sampling rate of one data point every 300 s, for up to 6 days. Glass ampoules having a total volume of 4 mL, loaded with 3 mL of cell suspension, were introduced into the TAM III channels, using a two step temperature equilibration procedure: the samples were first lowered into an equilibration position, and only after 15 min they were further lowered into the measuring position (von Ah et al., 2009). Data acquisition started 45 min after samples were lowered in the measurement position. Ampoules containing only cell-free culture medium were placed in three channels as controls, in order to assess background heat production resulting from the potential degradation of medium components. All samples were assayed in duplicate or triplicate.

Cell Quantification

The cell numbers were assessed every 24 h (in the first experiment every 12 h) in the calorimetry ampoules and every 48 h in the 6-well plates. Both microcalorimetry ampoules and wells were rinsed with PBS (Gibco, Grand Island, NY), frozen (−20°C) and then washed in 300 μL of 1% SDS to collect the DNA. Aliquots of this solution were analyzed using the CyQUANT Cell Proliferation Assay Kit

(Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Moreover, at the time of each seeding, three aliquots of the seeded HAC cell suspension were collected. Cells were centrifuged to obtain pellets, which, after freezing, were digested with 1% SDS. Since these samples contained a known number of cells they served as a reference to correlate the DNA content with the cell number.

Cell Growth Rate Determination

The growth of HAC, in a defined time window, can be described by means of an exponential law (Barbero et al., 2005)

$$N(t) = N_0 e^{\mu_G t} \quad (1)$$

where $N(t)$ is the number of cells at time t and μ_G is the growth rate of HAC (cell doublings per day). We hypothesized that the growth rate is the same for HAC cultured in the ampoules and in the wells. As a consequence, the experimental data was fit by the use of a model described by three independent parameters: N_{0W}—the initial number of cells in the wells, N_{0A}—the initial number of cells in the ampoules, and μ_G. The Levenberg–Marquardt algorithm from the proFit code 6.2.0 (Quantum Soft, Uetikon am See, Switzerland) served for combined fitting of the two sets of experimental data and for deriving the related error of the three independent parameters. In order to obtain a reasonable estimate of the errors of the measures to be included into the fit, the following procedure was carried out. For both tubes and wells, three specimens were harvested every 24 ± 1 h to determine the cell number via DNA quantification. For each individual experiment the average of the cell number including standard deviation was examined. Subsequently, the coefficient of variation CV, defined as ratio between standard deviation and average value, was calculated. The mean CVs averaged through all days and donors for both data sets were used to estimate the error in cell number quantification.

Heat Increase Rate Quantification

The heat generated over time, $Q(t)$, by a number of various cell types has previously been shown to fit an exponential equation (Kimura and Takahashi, 1985)

$$Q(t) = Q_0 e^{\mu_Q t} \quad (2)$$

where μ_Q will here within be defined as the "heat increase rate." Given that Q is the integration of the heat flow Φ [Watts], which is measured directly by the microcalorimeter, then Equation (2) becomes

$$\Phi(t) = \frac{dQ(t)}{dt} = Q_0 \mu_Q e^{\mu_Q t} \quad (3)$$

This model was applied for $t_{equil} < t < t_{max}$ where t_{equil} was the time needed for the full equilibration (10 h), and t_{max} was the time at which the maximum heat flow was reached. The error of $\Phi(t)$ was 0.2 μW, according to the manufacturer of the microcalorimeter and the error of the measurement time was 2 s. Again, the Levenberg–Marquardt algorithm from the proFit code 6.2.0 was applied to fit the experimental data in order to determine μ_Q.

Results and Discussion

First, we developed an experimental setup to assess the feasibility to apply IMC for real-time monitoring of the heat flow of HAC. As controls, ampoules filled only with culture medium (cell-free) were assessed in the IMC in order to quantify the potential heat flow resulting from degradation of medium components. Measurements of the control samples remained constant at background levels throughout the culture period, indicating that the culture medium per se would have a negligible contribution to the overall heat flow in cell-based experiments. Since no data are currently available on the typical heat flow per cell of HAC in culture, we next aimed to optimize the number of cells in relation to the IMC heat flow measurement sensitivity. When 5E + 04 HAC per ampoule were assessed, a significant lag time was observed before growth related heat flow was sufficient such that measurements could be acquired above instrument background levels. When 5E + 05 HAC were assessed, the heat flow curve reached a plateau rapidly, that is, in several hours, therefore not allowing for the calculation of dynamic parameters. Alternatively, when 1E + 05 HAC were seeded per ampoule, the heat flow quickly rose above the instrument detection limit (baseline), then steadily increased within the first day, reached a maximum between 3 and 4 days, and then gradually decreased. Heat flow curves, shown in Figure 1a, were consistent in shape to those typically reported in the literature for other cell types (Karnebogen et al., 1993; Ma et al., 2007; Murigande et al., 2009; Nedergaard et al., 1977). Integrating the heat flow data over time, sigmoidal heat curves were generated (Fig. 1b), which were also consistent with those previously reported for other cell types (reviewed in Braissant et al., 2010). Therefore, in all subsequent experiments, 1E + 05 HAC were seeded into the ampoules.

Quantifying the number of adherent cells within the ampoules at different time points of culture, we observed that cells reached confluence at day 3, with the number of HAC plateauing at $4.1E + 05 \pm 8E + 04$. Interestingly, the maximum peak in the heat flow curve (8.9 ± 2.3 μW) also corresponded to the time point of 3 days. Therefore, at

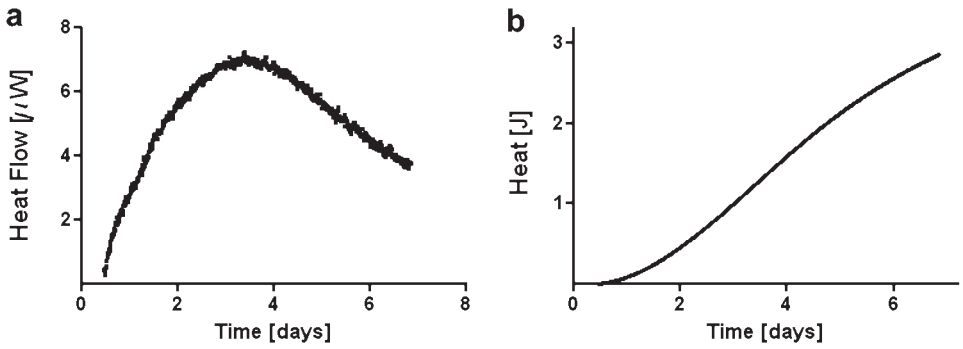

Figure 1. **a**: Real-time monitoring of the heat flow of HAC during in vitro proliferation when an initial cell density of 100,000 cells/ampoule was used. **b**: Heat curves generated by integrating the heat flow data over time. The peak in the heat flow curve, corresponding to the inflection point of the sigmoidal heat curve, occurred in the same time period as when HAC reached confluence in the IMC ampoules.

confluence, the heat flow per HAC was calculated to be 22.0 ± 5.3 pW. This value is in the range of those previously reported for fibroblasts (17 pW per mouse fibroblast, 40 pW per human fibroblast) (Kemp, 1991), which have a size and phenotype resembling in vitro expanded (i.e., dedifferentiated) HAC. Next, given that the heat curve has been directly related to biomass production for several cell types (Barros et al., 2003; Karnebogen et al., 1993; Sharma and Jain, 1990), we compared HAC growth up to confluence with the heat curve up to the inflection point (corresponding to the maximum point of the heat flow curve). Figure 2 shows that HAC heat production and HAC growth appeared to be related, and within this time frame seemed to follow exponential trends.

Based on the relation shown in Figure 2, we next aimed to determine whether there was a quantitative correlation between the heat increase rate and the growth rate during the exponential phase of HAC proliferation. This would allow establishing a rapid and real-time method to predict the proliferative capacity of HAC under defined in vitro culture conditions. However, for the IMC-based method to be relevant for cell and tissue culture applications, HAC proliferation within the ampoule/IMC-based model system must reflect proliferation under well-established conventional culture conditions. This needed to be validated given the different substrate materials (plastic vs. glass) and considering the sealed ampoules do not allow the same gas exchange as well plates. Figure 3 shows that the number of cells quantified in ampoules and in standard multi-well culture plates grow exponentially over time. Although both

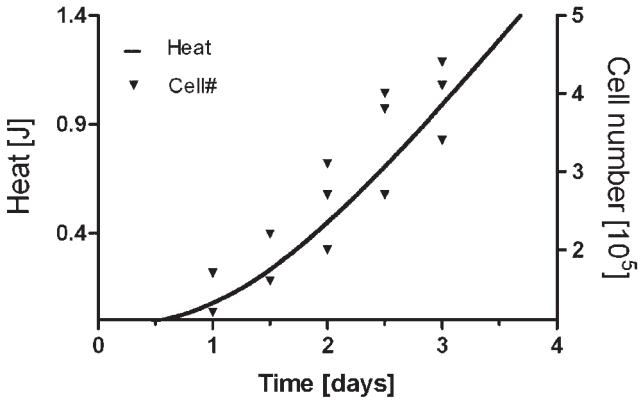

Figure 2. Relationship between heat and cell growth. Triangles in the graph represent the number of cells quantified in ampoules every 12 h, and the solid line represents the heat curve. For this donor, the growth of HAC and the heat both appear exponential and to be strongly related.

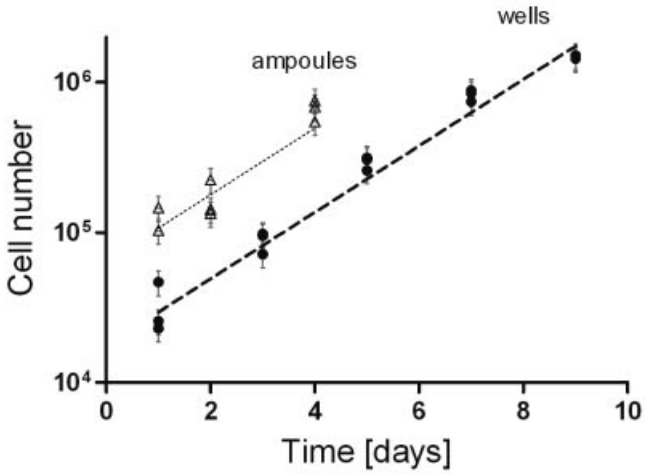

Figure 3. HAC growth within the ampoule/IMC-based model system and under conventional culture conditions in standard multi-well plates. Due to different surface areas available for the adherent cells the initial and final numbers of cells were different in the ampoules and in the well plates, as well as the time to reach confluence. However, the growth rates (i.e., slope of logarithmic plot) appeared similar between the two model systems. (Error bars for cell number are smaller than data points.)

the initial and final number of cells are different in the ampoules and in the well plates, due to the different surface areas available for the adherent cells, Figure 3 shows that the growth rates (i.e., slope in logarithmic plot) of HAC were similar within the two systems. Growth rates of HAC derived from different donors were variable, in the range of 0.2–0.9 doublings/day, consistent with rates previously found for HAC (Jakob et al., 2001). Therefore, sealed IMC ampoules provide a relevant environment and offer the potential to use resultant heat flow data for fast, online, and high-throughput monitoring of chondrocyte growth.

The growth rates (μ_G) of HAC derived from 12 different donors were plotted against the corresponding heat increase rates (μ_Q) in Figure 4. Regression analysis indicated a very weak linear correlation between the two parameters ($r^2 = 0.15$, $P = 0.00063$). As can be seen in Figure 4, the values of μ_Q were larger than the corresponding values of μ_G, highlighting that the rate of heat generated is function of not only the metabolic activities associated with cell proliferation, but of other ongoing biological processes as well.

In its native milieu, HAC have a very slow growth rate since the primary function of HAC in vivo is the gradual degradation and production of extracellular matrix proteins that comprise the cartilage tissue. For tissue engineering applications, the limited number of HAC that can obtained from a small cartilage biopsy must be induced to extensively proliferate in vitro in order to obtain a sufficiently high number of cells, which can then be implanted back into the patient. However, in addition to simply replicating, proliferating HAC may also synthesize ECM macromolecules such as collagen type I and versican (Jakob et al., 2001), which would contribute to overall metabolic heat production by the cells. Interestingly, the specific types of ECM proteins that are produced by HAC are known to be modulated by the stage of differentiation of the cell (Benya and Shaffer, 1982). While established cell lines and cells of animal origin tend to behave highly reproducibly in vitro, articular chondrocytes derived from adult humans have high donor to donor variability in their proliferation rate (Barbero et al., 2004), the amount of ECM produced (Barbero et al., 2004), and gene expression profiles (Grogan et al., 2007). Taken together, it may be that the inter-donor variation in types of ECM proteins synthesized, produced in varying amounts, also tend to confound the relation between cell counts and heat flow data. Although age-related changes have been reported to contribute to HAC variability (Barbero et al., 2004), no trends could be established between the donor age and the relation between μ_Q and μ_G.

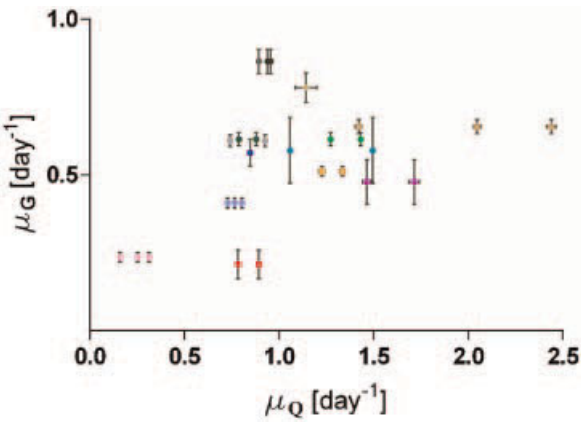

Figure 4. Growth rates (μ_G) of HAC derived from 12 different donors plotted against the corresponding heat increase rates (μ_Q). Although linear relationships have been established between μ_G and μ_Q for organisms such as bacteria with highly reproducible growth patterns, no such relationship could be established for chondrocytes derived from different human donors ($r^2 = 0.15$, $P = 0.00063$). Independent experiments are identified by different colors.

In vivo, HAC are embedded within a dense extracellular matrix and have highly restricted motility. In contrast, during in vitro culture, HAC are highly motile (mean speed of $\approx$10 μm/h) and migrate randomly or in direct response to chemotactic signals (Maniwa et al., 2001), at rates also shown to have age-related variation (Hidaka et al., 2006). While microcalorimetry has been used to assess the heat output associated with the motility of sperm (Antonelli et al., 1991) and parasitic worms (Manneck et al., 2011), no studies to date have attempted to uncouple migration-associated heat flow from other cellular energetic pathways for adherent cell types such as chondrocytes. Considering that IMC simply provides a net signal of heat flow, encompassing all chemical and physical processes, it remains a significant challenge to carefully design hypothesis driven experiments allowing to uncouple the different metabolic processes.

In this study, we established a method for the real-time monitoring of heat production by human chondrocytes during in vitro expansion. For HAC derived from particular donors, thermograms appeared to be related to the growth of the cells. However, likely due to high donor-to-donor variability among other metabolic processes occurring in parallel with proliferation, it was not yet possible to establish a predictive correlation between the heat increase rate and the growth rate, such as those readily shown for organisms with highly reproducible growth patterns (e.g., bacteria). Reproducible chondrocyte cell lines, which mitigate inter-donor variability, could be used within a more limited but more controlled model system to better understand the metabolic heat production associated with specific cell functions before adding the complexity associated with primary human chondrocytes.

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JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med (2011)
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.473

RESEARCH ARTICLE

On-line monitoring of oxygen as a non-destructive method to quantify cells in engineered 3D tissue constructs

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Abstract

Regulatory guidelines have established the importance of introducing quantitative quality controls during the production and/or at the time of release of cellular grafts for clinical applications. In this study we aimed to determine whether on-line measurements of oxygen can be used as a non-destructive method to estimate the number of chondrocytes within an engineered cartilage graft. Human chondrocytes were seeded and cultured in a perfusion bioreactor, and oxygen levels in the culture medium were continuously monitored at the inlet and outlet of the bioreactor chamber throughout the culture period. We found that the drop in oxygen across the perfused construct was linearly correlated with the number of cells per construct ($R^2 = 0.82$, $p < 0.0001$). The method was valid for a wide range of cell numbers, including cell densities currently used in the manufacture of cartilage grafts for clinical applications. Given that few or no non-destructive assays that quantitatively characterize an engineered construct currently exist, this non-invasive method could represent a relevant instrument in regulatory compliant manufacturing of engineered grafts meeting specific quality criteria. Copyright © 2011 John Wiley & Sons, Ltd.

Received 5 January 2011; Revised 6 June 2011; Accepted 5 July 2011

Keywords **bioreactor; sensor; tissue engineering; quality control; perfusion; chondrocyte**

1. Introduction

Regulatory bodies have recently outlined stringent quality control guidelines for the production of engineered tissue products for clinical applications (European Medicines Agency, 2008; FDA, 2008). In particular, these guidelines specify that an engineered product must be thoroughly characterized during the production and/or at the time of release, including quantitative measurements of the tissue's most relevant biological attributes (e.g. cell number, cell phenotype, amount of specific extracelluar matrix components). Considering that essentially all methods that are routinely employed for the characterization of engineered tissues are currently based upon destructive techniques, the new regulations pose significant hurdles for the translation of cell-based tissue products into the clinic, necessitating the development of novel non-destructive methods to quantify key biological parameters.

Monitoring of oxygen in culture media may seem to be an obvious approach to characterize cell/tissue cultures, given its common practice in bioreactors used in other fields of biotechnology (e.g. stirred-tank bioreactor for fermentation). It is quite surprising, then, that on-line monitoring of oxygen is rarely performed during the cultivation of engineered tissues. One tissue-engineering study, which included the acquisition of oxygen measurements, proposed the method as a potential means of estimating the total number of cells within an engineered graft (Janssen *et al*., 2006). However, oxygen measurements were simply related to a theoretical model of exponential cell growth, but no correlations were established with experimentally derived data of the actual number of cells within engineered constructs.

We previously developed a bioreactor system to perfuse culture media directly through the pores of three-dimensional (3D) scaffolds, thereby reducing diffusion controlled oxygen transport, minimizing oxygen gradients

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within the construct, and facilitating control over the oxygen levels applied to the cells (Wendt *et al.*, 2006). On-line micro-oxygen sensors were integrated into the system to continuously monitor the range of oxygen in the culture medium perfused through the 3D constructs throughout the culture period. Using this bioreactor system, we demonstrated that when chondrocytes 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 extracellular matrix. Using our established bioreactor-based model system, we now demonstrate that on-line measurements of oxygen can be used to estimate the number of cells within a 3D construct during culture, thus providing a non-invasive and quantitative method to assess a key quality parameter of engineered tissue grafts.

2. Methods

2.1. Chondrocyte isolation and expansion

Full-thickness human articular cartilage samples were collected post mortem from the femoral lateral condyle of nine individuals, aged 26–70 (51 ± 13) years, with no known clinical history of joint disease, after informed consent of the relatives and approval by the local ethical committee. Chondrocytes were isolated by collagenase digestion and expanded for up to two passages, as previously described (Barbero *et al.*, 2004).

2.2. Cell seeding and culture in the perfusion bioreactor

The bioreactor system was designed to first perfuse a cell suspension directly through the pores of a 3D scaffold, to seed cells uniformly throughout the entire scaffold, and subsequently to perfuse culture medium to maintain cell viability within the seeded constructs throughout culture. The bioreactor was placed in a humidified incubator with 5% CO_2, therefore providing an environment with an oxygen concentration of 19% O_2 (as opposed to 21% O_2 in ambient air). In nine independent experiments, 1.0E + 05 human articular chondrocytes (HACs) were perfusion-seeded into fibronectin-coated Hyaff-11 non-woven meshes (6 mm diameter $\times$ 2 mm thick; Fidia Advanced Biopolymers, Italy) at a superficial velocity of 1 mm/s for 16 h, as previously described (Wendt *et al.*, 2003). The cell-seeded meshes remained within the bioreactor and were then further cultured under perfusion at a superficial velocity of 10 μm/s for time points ranging from 3 days up to 2 weeks (Wendt *et al.*, 2006). Perfused constructs were cultured in 20 ml Dulbecco's modified Eagle's medium (DMEM; 10% fetal bovine serum, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.29 mg/ml L-glutamine) supplemented with 1 ng/ml transforming growth factor-β1 and 5 ng/ml fibroblast growth factor-2, factors previously shown to enhance HAC proliferation (Barbero *et al.*, 2003), and the medium changed twice per week. The culture medium, which was recirculated through the system, was re-oxygenated prior to reinfusion at the inlet of the construct by flowing through one meter of gas-permeable platinum-cured silicon tubing (1/32 in i.d., 1/16 in o.d.; Cole Parmer).

2.3. Oxygen sensing

To monitor dissolved oxygen concentrations at the inlet and at the outlet of perfused constructs, disposable chemo-optic flow-through micro-oxygen sensors (FTC-PSt-3; PreSens GmbH, Germany) were connected to the top and bottom of the bioreactor scaffold chambers (Figure 1). The on-line sensors, which are based on the quenching of luminescence by oxygen molecules, do not consume oxygen, are independent of flow rate and maintain long-term calibration stability. Disposable sensors were supplied sterile and pre-calibrated, with the 0% oxygen calibration value determined in nitrogen and the 100% air saturation value determined in ambient air (i.e. 21% O_2). Oxygen measurements were acquired every 10 min throughout the culture period.

In the direct perfusion bioreactor, the mass balance on oxygen can be described by:

$$F \cdot \left(C_{O,\ in} - C_{O,\ out}\right) = N(t) \cdot q_o \tag{1}$$

where F is the volumetric flow rate of the culture medium, $C_{0,in}$ and $C_{0,out}$ are, respectively, the oxygen concentration at the inlet and at the outlet of the scaffold, $N(t)$ is the number of cells at time t, and q_o is the oxygen consumption rate/cell. To calculate the oxygen consumption rate/cell, this can be represented as:

$$q_o = \frac{\Delta O_2}{N(t)} \cdot F \tag{2}$$

where ΔO_2 is the drop in oxygen concentration across the perfused construct (i.e. $\Delta O_2 = C_{O,\ in} - C_{O,\ out}$).

2.4. Cell quantification

Oxygen measurements were compared to the number of cells within the 3D tissue constructs, which were determined by an established but destructive method. At the end of the culture period, constructs were harvested from the bioreactor and digested in 500 μl proteinase K solution (1 mg/ml protease K in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide and 10 μg/ml pepstatin-A) for 15 h at 56 °C, as previously described (Hollander *et al.*, 1994). The amount of DNA in the digested constructs was quantified using a CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. To relate the quantity of DNA to cell number, at the time of each seeding, aliquots of the seeding cell suspension, which contained a known number of cells, were collected, centrifuged to obtain

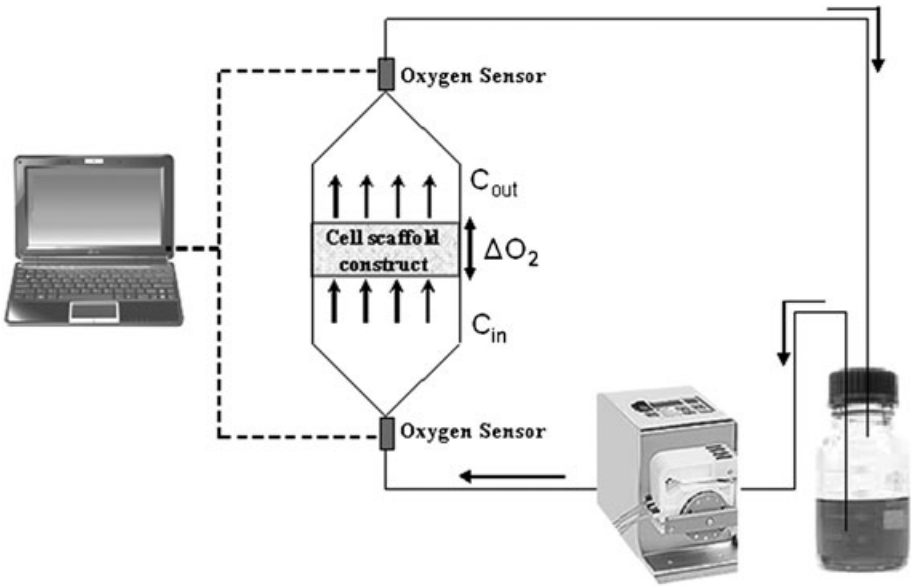

Figure 1. Bioreactor-based model system. The bioreactor system was designed to first perfuse a cell suspension directly through the pores of a 3D scaffold, to seed cells uniformly throughout the entire scaffold, and subsequently to perfuse culture medium to maintain cell viability within the seeded constructs throughout culture. To monitor the oxygen concentrations in the culture medium that was perfused through the construct, disposable chemo-optic flow-through micro-oxygen sensors were connected to the inlet and outlet of the bioreactor scaffold chambers. Measurements were acquired at defined intervals and monitored on a PC throughout the culture period

pellets, digested with proteinase K solution, and assessed using the CyQUANT kit as for the cultured constructs.

2.5. Statistical analysis

Linear regression analysis was performed using Statistica software (StatSoft, Inc., USA).

3. Results and discussion

Representative on-line oxygen measurements during a 2 week culture period are shown in Figure 2. Measurements at the inlet remained quite constant at 90% of ambient air saturation throughout the culture period, confirming that the recirculated culture medium was fully re-oxygenated to incubator saturation levels (i.e. 19% O_2) prior to re-infusion at the inlet of the construct. Therefore, following the first experiment, acquisitions of the inlet sensors were reduced to a continuous 30 min sampling period once per day, but maintaining the 10 min sampling rate at the outlet. Oxygen levels measured at the outlet tended to decrease rather slowly during the initial 2–4 days, then more rapidly until days 10–12, when they reached a plateau.

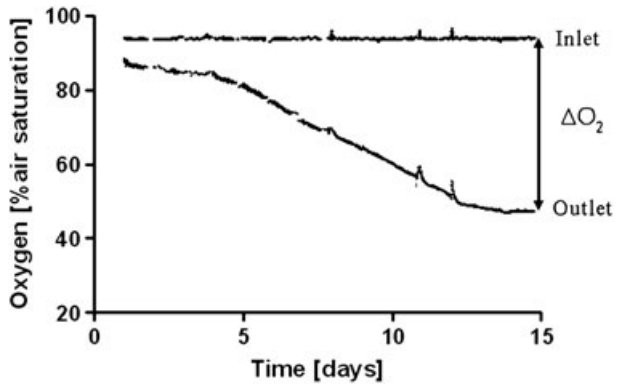

Figure 2. On-line oxygen measurements acquired at the inlet and the outlet of the perfused bioreactor chamber every 10 min during a 2 week culture period in the first experiment. Measurements at the inlet remained constant throughout the 2 week culture period and indicated that the culture medium was fully re-oxygenated within the perfusion loop to incubator saturation levels (approx. 90% saturation in ambient air) prior to re-infusion at the inlet of the construct. Artifacts (small peaks) in the inlet and outlet oxygen measurements could be observed as a result of temporary fluctuations in temperature and gas composition when opening the incubator door. The ΔO_2 across each perfused 3D construct was calculated from the difference between the inlet and outlet measurements at the end of the culture period (e.g. day 14 in this figure)

Growth rates of HACs within the 3D scaffolds were found to vary with cell donor. This is consistent with the donor-to-donor variability that has been reported in the proliferation rates of HACs when expanded in conventional two-dimensional (2D) monolayer culture (Barbero *et al.*, 2003). Nevertheless, on average, cell numbers in the 3D scaffolds tended to increase slowly during the initial 3–4 days, then increase more rapidly until approximately days 10–12, at which time the cell number did not increase appreciably. Figure 3 shows that the oxygen drop (ΔO_2; calculated as the difference between the inlet and outlet oxygen measurements) and the cell number over time have similar-shaped sigmoidal distributions, suggesting that cell growth within the 3D tissues could

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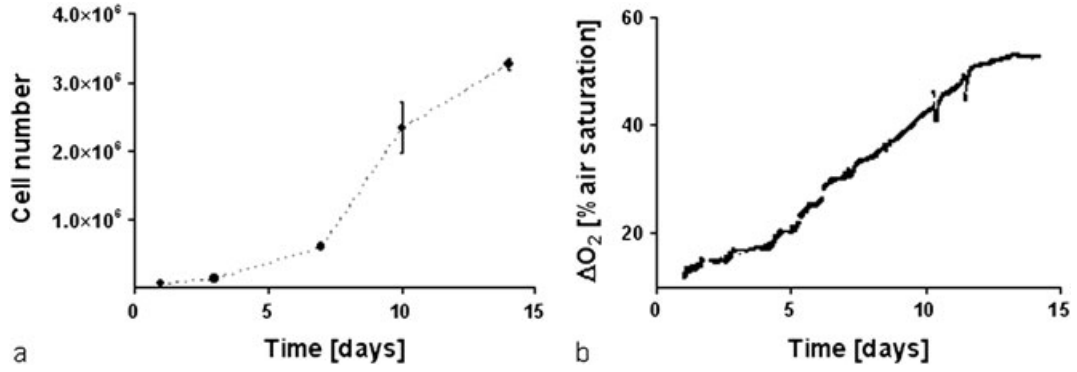

Figure 3. (a) Representative plot of the average cell number ($n = 3$ independent chambers/time point) in constructs that were harvested at different time points (1, 3, 7, 10 and 14 days). (b) Representative plot of the oxygen drop across a construct during a 2 week culture period, in the same experiment corresponding to panel (a). Plots of ΔO_2 and cell numbers over time had similar-shaped sigmoidal distributions, suggesting that cell growth within the engineered 3D tissues could be monitored throughout the culture time via oxygen measurements

be monitored over the time of culture via the oxygen measurements.

The number of cells in each 3D tissue construct was then plotted vs ΔO_2 for the corresponding construct at the end of the specific culture period (Figure 4). Linear regression analysis showed that a significant linear relationship could be established between ΔO_2 and the cell number, with $R^2 = 0.82$ and $p < 0.0001$. Therefore, the oxygen measurements can be used as a tool to non-invasively and quantitatively estimate the number of cells within the 3D tissue constructs throughout a prolonged culture period.

While numerous studies have previously measured oxygen gradients and/or oxygen consumption of 3D tissues, this study establishes a quantitative relationship between cell number and oxygen measurements, based on a non-invasive method that is directly applicable during the engineering of 3D tissues and that can be readily adapted for clinically related applications. Although fluorescence-based sensors integrated into the bottom of multi-well plates (Guarino *et al*., 2004) can be a valuable high-throughput research tool, particularly for suspension cultures, little information can be obtained regarding the oxygen concentrations within 3D tissues. 2D maps of oxygen levels over the external surfaces of tissues have been obtained by placing a fluorescence-based sensor in direct contact with the tissue; however, this method also provides little information regarding oxygen levels within the internal region of a 3D tissue (Kellner *et al*., 2002). A microelectrode in combination with a micromanipulator has been used to physically penetrate engineered cartilage constructs in order to measure local oxygen levels with high spatial resolution (Malda *et al*., 2004a). Although based on an invasive method, and without establishing a direct correlation between cell number and oxygen consumption, this study demonstrated the presence of steep oxygen gradients within statically cultured constructs (20% O_2 at the periphery to 2% O_2 within the interior), reflecting the non-homogeneous distribution of cells. Considering that the phenotype (Murphy and Polak, 2004) and metabolism (Bibby *et al*., 2005; Das *et al*., 2010) of cells can be affected by the level of oxygen supplied, this highlights the limited reliability of assessing oxygen consumption in uncontrolled 3D models suffering from diffusional limitations, such as static culture systems. Instead, when cultured under perfusion, cells throughout a 3D construct can be supplied with more homogeneous and controlled levels of oxygen. Although we cannot confirm that cells within specific local regions of the porous scaffold were subjected to lower oxygen levels than those measured at the outlet, computational models have predicted this to be a negligible phenomenon at high flow rates and to occur in only a relatively minor volume fraction of the scaffold at low flow rates (Cioffi *et al*., 2008).

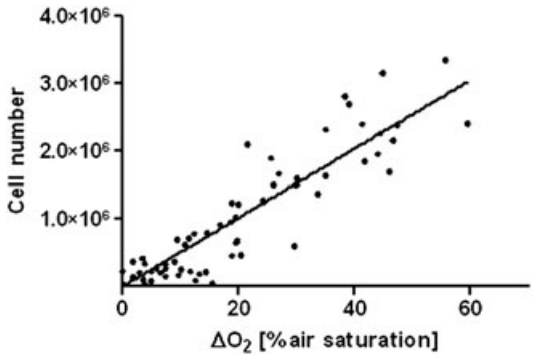

Figure 4. The number of cells in each 3D tissue construct vs the oxygen drop (ΔO_2) for the corresponding construct at the end of the culture period for all nine experiments. Linear regression analysis showed a significant linear relationship between ΔO_2 and the cell number. Cell no. = (5.1E + 04)·(ΔO_2); $R^2 = 0.82$ and $p < 0.0001$

The average specific oxygen consumption rate calculated among all constructs was 86 ± 15 fM/h/cell, which is consistent with rates found for chondrocytes isolated from the growth plate (Haselgrove *et al*., 1993), chondrocytes cultured on ceramic granules (Nehring *et al*., 1999) and chondrocytes expanded on microcarriers (Malda *et al*., 2004b). This rate is one order of magnitude higher than that measured for native cartilage (Stockwell, 1979), although this is not surprising, given the low

proliferation rate and limited metabolic activity of chondrocytes within their native milieu. Our calculated rate is also 10-fold higher than that previously estimated for 3D engineered cartilage constructs (Obradovic *et al*., 2000). However, in the latter study, culture medium was mixed around the specimens and not directly through the 3D tissues, oxygen measurements were obtained during periodic partial media exchanges, and cells were not cultured under proliferative culture conditions.

While we have established a linear relationship between oxygen measurements and the number of HACs under a set of defined culture conditions, the model could readily be applied to other cell types and various other culture conditions. However, the methodology may need to be adapted to each particular application, including the consideration of more complex oxygen metabolism (e.g. greater dependence on oxygen levels; larger oxygen gradients throughout the construct). Moreover, oxygen measurements may need to be combined with the monitoring of a second metabolic parameter (e.g. glucose, pH) (Zhou *et al*., 2008) or used in conjunction with other non-invasive techniques, such as ultrasound (Oe *et al*., 2010), to characterize the engineered graft. In the current model system, we have specifically assessed the expansion of HACs in 3D scaffolds, applying a normoxic range of oxygen (i.e. 19% at inlet and >10% at outlet). Ongoing efforts are aimed at adapting the method to account for HACs' more complex oxygen consumption under hypoxic conditions, and assessing whether oxygen measurements can be used to predict cell numbers when constructs are cultured at high density and under low oxygen (1–5% oxygen tension). This would ultimately be key in the process control of automated bioreactor systems by monitoring the progression of cell growth during culture, and possibly providing feedback to adjust the bioreactor culture parameters or to initiate a subsequent phase of culture when a threshold number of cells is reached (Wendt *et al*., 2011). In this context, bioreactor-based production systems that monitor process parameters and specific properties of the developing/final graft will ultimately provide a higher level of traceability of key manufacturing data related to the quality of the engineered implant (Martin *et al*., 2009).

4. Conclusions

In this study we have established a quantitative relation between on-line oxygen measurements and the number of cells within a 3D engineered tissue during perfusion bioreactor culture. Given that few to no non-destructive quantitative assays currently exist that clearly define the quality of an engineered construct, this non-invasive method could represent a significant element in regulatory compliant manufacturing of engineered grafts meeting specific quality criteria.

Acknowledgements

We would like to acknowledge the European Union for financial support ('STEPS' Project; Grant No. FP6-NMP3-CT-2005-500465; www.stepsproject.com).

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J Tissue Eng Regen Med (2011)
DOI: 10.1002/term

Biomaterials 31 (2010) 8946–8952

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Bioreactor based engineering of large-scale human cartilage grafts for joint resurfacing

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ARTICLE INFO

Article history:
Received 30 July 2010
Accepted 4 August 2010
Available online 25 August 2010

Keywords:
Bioreactor
Cartilage repair
Computational fluid dynamics
Scale-up
Regenerative medicine
Tissue engineering

ABSTRACT

Apart from partial or total joint replacement, no surgical procedure is currently available to treat large and deep cartilage defects associated with advanced diseases such as osteoarthritis. In this work, we developed a perfusion bioreactor system to engineer human cartilage grafts in a size with clinical relevance for unicompartmental resurfacing of human knee joints (50 mm diameter × 3 mm thick). Computational fluid dynamics models were developed to optimize the flow profile when designing the perfusion chamber. Using the developed system, human chondrocytes could be seeded throughout large 50 mm diameter scaffolds with a uniform distribution. Following two weeks culture, tissues grown in the bioreactor were viable and homogeneously cartilaginous, with biomechanical properties approaching those of native cartilage. In contrast, tissues generated by conventional manual production procedures were highly inhomogeneous and contained large necrotic regions. The unprecedented engineering of human cartilage tissues in this large-scale opens the practical perspective of grafting functional biological substitutes for the clinical treatment for extensive cartilage defects, possibly in combination with surgical or pharmacological therapies to support durability of the implant. Ongoing efforts are aimed at integrating the up-scaled bioreactor based processes within a fully automated and closed manufacturing system for safe, standardized, and GMP compliant production of large-scale cartilage grafts.

1. Introduction

While Carticel® and Hyalograft-C® have been well established in the clinic for the treatment of traumatic focal cartilage defects [1–3], no tissue engineered product is currently available to treat large defects or those associated with advanced diseases such as osteoarthritis. Beyond the biological challenges that must be addressed to treat such joint disorders, it remains a significant engineering challenge to generate up-scaled cartilage grafts with dimensions that would be sufficient for the repair of large, advanced, and deep defects.

We previously developed a perfusion bioreactor for seeding and culturing cell-scaffold constructs with a clinically relevant thickness (≈4 mm) and demonstrated that highly viable and homogeneous tissues could be generated [4]. However, the diameter of the engineered tissues, representative of constructs typically described in the literature for research purposes (i.e., ≈8 mm diameter), would not be applicable for the treatment of large defects unless multiple plugs were generated and implanted in a surgical procedure resembling mosaicplasty. Therefore, in this work, we scaled-up our perfusion bioreactor system to engineer large-sized human cartilage grafts, in dimensions that would be sufficient for unicompartmental resurfacing of a human knee joint (50 mm diameter × 3 mm thick). Computational fluid dynamics (CFD) models were developed to assist in the design of a bioreactor that could generate a uniform velocity profile over the surface of the large-diameter scaffold. Experimental validations demonstrated that the developed bioreactor system seeded cells uniformly throughout the large-scale scaffold, and following prolonged culture, supported the generation of viable, homogeneous, and cartilaginous tissue constructs with biomechanical properties approaching those of native cartilage. In contrast, constructs generated by conventional manual production procedures were highly inhomogeneous containing a significant non-viable and void region, highlighting the necessity of a perfusion bioreactor based approach for engineering large-scale cartilage grafts.

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doi:10.1016/j.biomaterials.2010.08.009

2. Methods

2.1. Perfusion bioreactor system

The bioreactor system was designed to first perfuse a cell suspension directly through the pores of a 3D scaffold, to seed cells uniformly throughout the entire scaffold [5], and subsequently perfuse culture media, to maintain cell viability within the seeded constructs throughout culture [4]. The bioreactor chamber (Applikon Biotechnology BV, The Netherlands) was fabricated from electropolished AISI 316L stainless steel and included four inlet and four outlet ports (Fig. 1a) in order to disperse the cell suspension and the culture media uniformly over the large scaffold surface area. Mesh scaffolds were clamped around the outer 1 mm periphery by a 3 mm thick Teflon ring (Fig. 1b) to prevent culture media from flowing around the scaffold, ensuring flow through the scaffold pores. To provide mechanical support to the delicate mesh and prevent deformation against flow induced drag forces, scaffolds were sandwiched between two stainless steel wire grids (Bopp AG, Switzerland). The chamber was integrated within the bioreactor system shown in Fig. 1c, which included a fluid flow pathway for the phase of cell seeding and one pathway for prolonged culture.

2.2. Computational fluid dynamics (CFD) simulations

Computational models of fluid flow within two proposed bioreactor designs were developed and analyzed using Ansys Fluent 12.1. The first design was based on coaxial single inlet and outlet ports, and second design with four inlet and outlet ports. The scaffold (50 mm diameter × 3 mm thick) was simulated as a highly viscous fluid, calculated by setting Darcy's Law and the Poisseuille equation equivalent. The value of the apparent viscosity (η_s) depended on the geometry dimension (scaffold surface area A, properties of fluid (density ρ) and morphology characteristics (porosity φ, permeability k).

$$\eta_S = \frac{\pi r^4 \rho g}{8A\phi k} \quad (1)$$

The properties of the scaffold, a Hyaff-11 non-woven mesh (Fidia Advanced Biopolymers; Italy), were obtained experimentally through mercury porosymetric analysis (average pore size = 68 μm, $\varphi = 91\%$, $k = 2.13 \times 10^{-11} m^2$). The continuous phase (culture media), with density approximated as $\rho = 1000\ kg\ m^{-3}$ and viscosity of $\eta = 0.001$ Pa s, was assumed under steady state conditions inside the bioreactor chamber. No slip boundary conditions were applied at the bioreactors walls. Flow rates were 30 ml/min (corresponding to 18 mm/s at the inlet) for cell seeding simulations and 12 ml/min (corresponding to 7 mm/s at the inlet) for culturing simulations. The inlet velocity applied to each of the four inlet ports was divided by four in the multiple-port design as compared to the single inlet port design. Nil pressure was applied at the outlet. An axisymmetric model was developed for the single coaxial port bioreactor whereas a 3D model was developed for the four-port bioreactor.

2.3. Cell seeding & construct culture

Human Articular Chondrocytes (HAC) were isolated from cartilage biopsies obtained from three individuals (age: 26–60 years) after informed consent and in accordance with the local Ethical Commission. HAC were expanded for 2 passages (8–10 doublings) in basic culture medium (DMEM, 10% fetal bovine serum, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamine) further supplemented with transforming growth factor-β1 (TGF-β1, 1 ng/mL) and fibroblast growth factor-2 (FGF-2, 5 ng/mL), factors previously shown to increase the proliferation rate and post-expansion redifferentiation capacity of human chondrocytes [6]. A clinically relevant cell seeding density was determined based on the number of chondrocytes that can be obtained from an average cartilage biopsy size obtained for autologous chondrocyte implantation (ACI) procedures (≈280 mg) [1], the average chondrocyte yield from a human cartilage digest (2.5E+06 cells/g tissue) [7], and the number of cell doublings during expansion with two passages (≈8 doublings). Based on these calculations, 2.0E + 08 HAC were resuspended in basic culture media (90 ml) and perfusion seeded into a Hyaff-11 non-woven mesh (50 mm diameter × 3 mm thick) at a flow rate of 30 ml/min for 16 h within two independent bioreactor systems. Cell-seeded meshes were then either harvested and stained with MTT to assess the distribution of cells seeded throughout the scaffold or were further perfusion cultured in the bioreactor (flow rate of 12 ml/min) for two weeks to generate a cartilaginous graft. Constructs were cultured in 300 ml chondrogenic media (basic culture media supplemented with 10 μg/mL Insulin, 0.1 mM ascorbic acid 2-phosphate, and 10 ng/mL Transforming Growth Factor-β3 [8]) under hypoxic oxygen levels (5%O_2). In parallel, small diameter constructs ($n = 2$ per experiment) were also engineered in our previously described "research-scale" bioreactor system [4] (2.0E+06 chondrocytes seeded and cultured on 6 mm diameter × 3 mm thick Hyaff-11) to serve as controls for the scaled-up bioreactor system. In one experiment, large-scale engineered constructs were also generated by static cell seeding and static culturing methods to compare the bioreactor based approach to conventional manual manufacturing techniques. Engineered constructs were assessed biochemically, histologically, and biomechanically.

2.4. Histological analyses

Following the seeding phase, cell-seeded meshes ($n = 2$) were cut into four sections, rinsed in phosphate buffered saline (PBS), and incubated at 37 °C for 2 h with 0.12 mM MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) to assess the distribution of cells throughout the scaffold. Following two weeks of culture, sections of engineered constructs were rinsed in PBS, fixed in 4% formalin, embedded in paraffin, cross-sectioned (10 μm thick) and stained with Safranin-O for glycosaminoglycans (GAG).

2.5. Biochemical quantification

Engineered constructs were digested with protease K solution (1 mg/ml protease K in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 μg/ml pepstatin-A for 15 h at 56 °C) as previously described [9]. 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 [10].

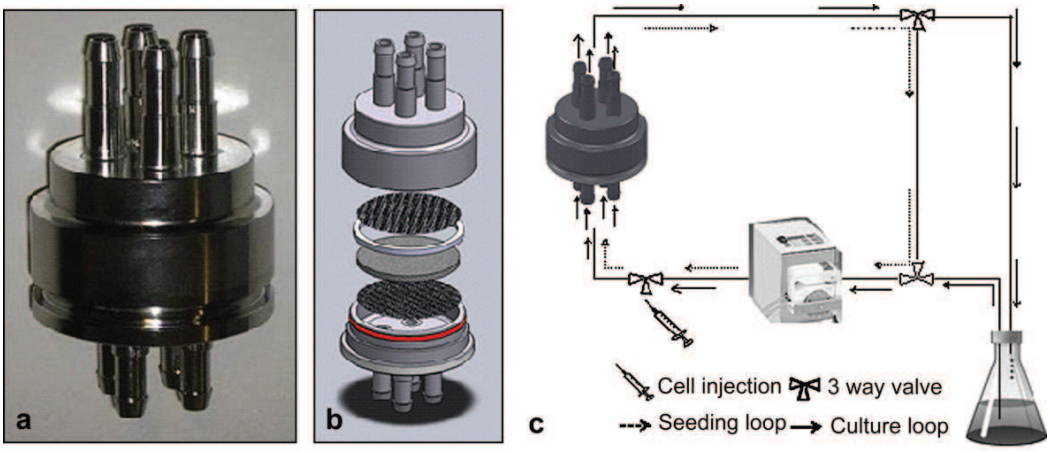

Fig. 1. (a) Scaled-up perfusion bioreactor for engineering human cartilage grafts in a size with clinical relevance for unicompartmental resurfacing a human knee joint. (b) Hyaff-11 non-woven mesh scaffolds (50 mm diameter × 3 mm thick) were clamped at the periphery to ensure flow through the scaffold pores. Stainless steel grids provided mechanical support to cell-scaffold constructs during seeding and perfusion culture. (c) The bioreactor system was designed to first perfuse a cell suspension directly through the pores of a 3D scaffold, to seed cells uniformly throughout the entire scaffold [5], and subsequently perfuse culture media, to maintain cell viability within the seeded constructs throughout culture [4].

2.6. Suture pull-out test

Since the large-scale grafts would likely require fixation with sutures to the surrounding tissue when implanted, pull-out tests were performed to measure the maximal force that could be applied on a suture in the axial direction before pulling out from the construct, as previously described [11]. Sections of the engineered constructs (≈20 mm length × 10 mm wide) were secured at their lower end with a suture (POLYSORB 5-0, coated, braided lactomer 9-1, synthetic absorbable suture with a P-13 needle; Covidien, Mansfield, MA) inserted 1 mm–2 mm above the end of the specimen and fixed with a flying triple knot to a holding hook affixed to the base plate of the mechanical testing instrument (MTS Synergie 100, MTS Systems Corporation, Eden Prairie, MN). The knot from the suture was additionally held by a needle holder to reduce the internal sliding from the knot. The upper end of the specimen was gripped with another suture hung from a rigid metal hook attached to the load cell of the test instrument. The actuator was programmed to apply elongation at a rate of 1 mm/s until the suture pulled out of the specimen. The maximal applied force was normalized to the specimen thickness, measured prior to performing the tests, and is reported as N/mm.

2.7. Indentation tests

Biomechanical properties of the engineered constructs were assessed by both dynamic and impact indentation tests [12]. Three specimens were punched out of each construct and assessed with five repetitions for each test. Dynamic loading was performed with a MTS Synergie 100 using an indenter tip of radius 1.585 mm. Repeated single sinusoidal cycles at 0.1Hz were applied under displacement control to a depth of ≈0.1 mm, separated by 50s pauses, shown to be sufficient to allow dimensional recovery. Impact loading was performed with a single impact microindentation (SIMI) device, mounted in a rigid load frame. The SIMI indenter pendulum (radius = 500 μm) falls freely, with impact force determined by mass (1.7g) and gravity, which provides indentation depths of ≈0.1 mm–0.2 mm in healthy cartilage [12]. The motion of the indenter during indentation and rebound is captured by an electromagnetic coil at a sampling rate of 125 kHz. The aggregate moduli (E^*) were calculated using equation (2):

$$E^* = \frac{3}{4}\left(1-\upsilon^2\right)\left[\frac{P_{\alpha\,\max}}{R^{1/2}\alpha_{\max}^{3/2}}\right] \quad (2)$$

where, υ is Poisson's ratio (with $\upsilon = 0.44$ [13]), α_{max} is the maximum displacement, $P_{\alpha max}$ is the force at maximum displacement, and R is the indenter radius.

3. Results

3.1. CFD simulations

With the goal of obtaining a uniform cell seeding distribution and homogeneous tissue development, we aimed to design a perfusion bioreactor chamber that could generate a uniform velocity profile over the surface of a 50 mm diameter scaffold. Initial concepts for the chamber were based on a simple scaling of a "research-sized" bioreactor, previously designed to house a scaffold 6–8 mm in diameter [4]. CFD simulations predicted this initial design, which was based on coaxial single inlet and outlet ports, to induce significantly higher fluid velocities at the center of the scaffold than towards the periphery, for the range of flow rates applied experimentally in this study (Figs. 2 and 3). Alternatively, simulations based on the four-port chamber showed the flow to quickly disperse within the main body of the chamber, resulting in a uniform velocity profile over the surface of the 50 mm diameter mesh (Figs. 2 and 3). The effect of the bioreactor design on the velocity profile at the scaffold surface was quite prominent at the high flow rate applied during the cell seeding phase. As shown by the velocity distributions plotted in Fig. 2, for the single-port bioreactor, the fluid reaches a high peak velocity of over 6 mm/s near the center of the scaffold and steadily decreases along the radius. In contrast, in the four-port bioreactor, velocity profiles along two different radii (one passing directly under one port and the other passing mid-way between two ports) were similar and quite constant (approximately 1 mm/s), indicating a uniform flow profile over the entire scaffold surface.

3.2. Cell seeding distribution

Consistent with the CFD simulations, which predicted a uniform velocity profile at the surface of the scaffold, MTT staining of the cell-seeded 50 mm meshes showed a homogeneous distribution of cells throughout the scaffold volume (Fig. 4), similar to previous results of perfusion cell-seeded small-scale scaffolds [5]. The absence of staining at the periphery of the scaffold verified that clamping of the mesh was effective in ensuring fluid flow (i.e., the cell suspension) through the scaffold pores and preventing flow around the edges of the scaffold.

3.3. Graft handling properties

Following two weeks of culture, macroscopically, large-scale perfused constructs had a similar appearance as the small-scale perfused constructs, despite the 70-fold scale-up in size (Fig. 5ab). Large-scale constructs were quite stiff such that they could easily be handled with forceps and did not bend under their own weight (Fig. 5c). On the other hand, these constructs were flexible enough to readily be bent when applying force with the forceps (Fig. 5d), suggesting their potential to be molded to the contours of a condyle during surgical implantation.

3.4. Graft composition and structure

Statically-seeded/statically-cultured constructs had an average of 0.24 ± 0.07% GAG per wet weight tissue, but histologically were highly heterogeneous. As shown in Fig. 6, statically-cultured constructs were encapsulated by a dense layer of cells and were intensely stained for Safranin-O along the outer 0.5–1.0 mm periphery, but contained a necrotic internal region that was essentially void of viable cells and extracellular matrix. Due to the highly inhomogeneous structure of these constructs, additional analyses were not performed. Constructs seeded and cultured in the large-scale perfusion bioreactors contained similar fractions of GAG as those cultured in the small-scale systems (50 mm: 0.15 ± 0.03%; 6 mm: 0.43 ± 0.05% GAG per wet weight) and were histologically highly homogeneous (Fig. 6b and c). Cells were uniformly distributed throughout the entire volume of the scaffolds and embedded within extracellular matrix positively stained for Safranin-O. To quantitatively assess the uniformity of extracellular matrix deposition within a large 50 mm construct, samples (5 mm in diameter) were punched from eight different locations across the diameter of the scaffold and biochemically assessed for GAG content. Samples across the diameter were found to have similar GAG fractions, with a coefficient of variation of only 27.5% among the specimens.

3.5. Graft biomechanical properties

Following two weeks of culture, the average suture pull-out force for 50 mm perfused constructs was 0.83 ± 0.10N/mm, which was 20% of the force previously found for native cartilage (4.5N/mm) [11]. Suture retention test were not performed on 6 mm constructs due their small size, which physically precluded their testing. The aggregate moduli under impact testing were similar for 50 mm and 6 mm constructs (50 mm: 1.70 ± 0.19 MPa; 6 mm: 1.88 ± 0.49 MPa), and were approximately 10% of the modulus of native cartilage [12]. The aggregate modulus of 50 mm constructs under dynamic testing was 30% that of 6 mm constructs (50 mm: 0.32 ± 0.13 MPa; 6 mm: 1.12 ± 0.56 MPa) and 5% of the modulus for native cartilage [12]. Cell-free Hyaff-11 meshes were also assessed and found to have a negligible suture pull-out force and negligible aggregate moduli (under both dynamic and impact modes), with values below the sensitivities of the instruments.

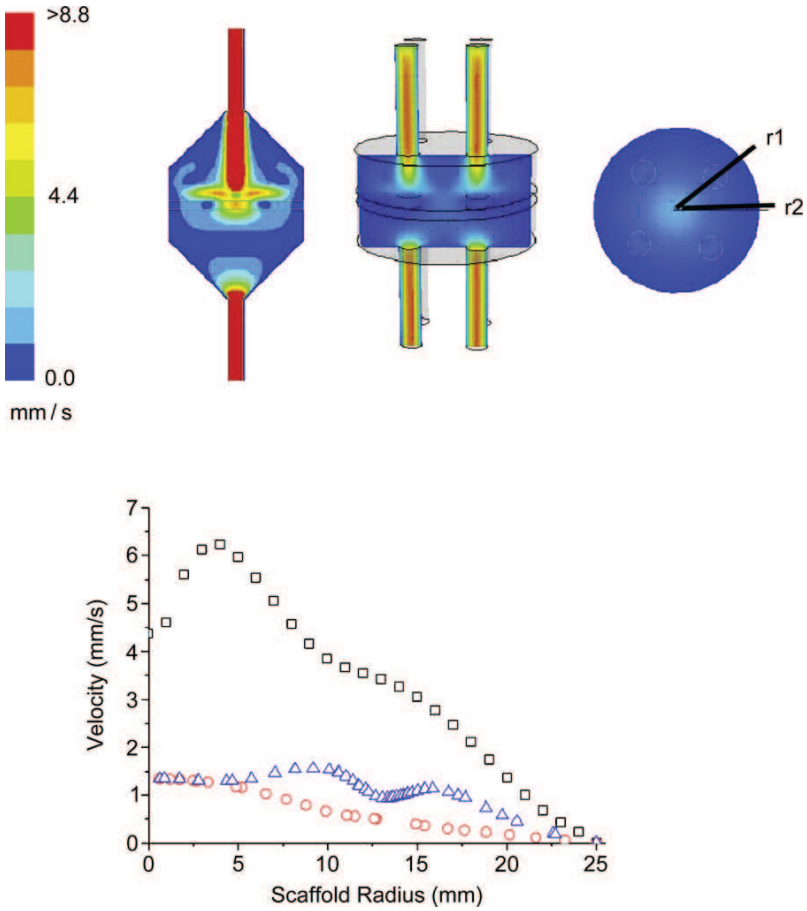

Fig. 2. CFD simulations of fluid flow within the bioreactors under the cell seeding flow rate of 30 ml/min (Top left) In the bioreactor with single coaxial inlet and outlet ports, significantly higher fluid velocities were predicted at the center of the scaffold than towards the periphery. (Top middle) Simulations based on the four-port chamber showed the flow to quickly disperse within the main body of the chamber after exiting each of the four ports, (top right) resulting in a highly uniform velocity profile over the surface of the 50 mm diameter scaffold. (Bottom) Velocity distributions at the scaffold surface along the scaffold radii in the two bioreactor designs. The range of fluid velocities in the single-port bioreactor was quite broad, reaching a peak at over 6 mm/s near the center of the scaffold and steadily decreasing along the radius. The velocity distribution in the four-port bioreactor was assessed along two different radii, one passing directly under one of the four inlet ports (r_1), and the other mid-way between two inlet ports (r_2). The velocity distributions were quite similar along r_1 and r_2 and were quite constant along the radius. (□ velocity in single-port bioreactor, Δ velocity in four-port bioreactor along radius r_1, ○ velocity in four-port bioreactor along radius r_2).

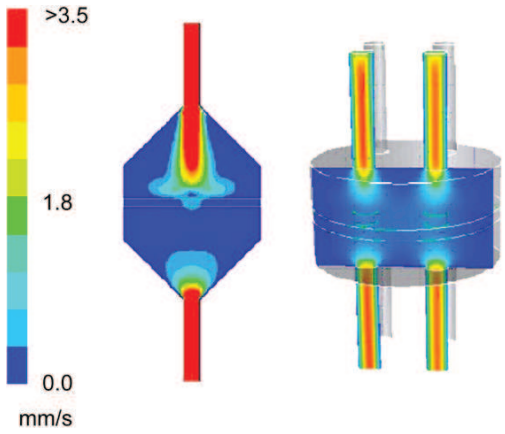

Fig. 3. CFD simulations of fluid flow within the bioreactors under the culturing flow rate of 12 ml/min. Fluid flow within both bioreactors was more uniform at the culturing flow rate than at the cell seeding flow rate, however, the velocities within the single-port bioreactor were clearly still significantly higher at the center of the scaffold than at the periphery.

4. Discussion

In this work, we have developed a perfusion bioreactor system to scale-up engineered human cartilage grafts to a size with clinical relevance for unicompartmental resurfacing of a human condyle. CFD modeling was employed during bioreactor development as part of a rational design strategy, leading to a bioreactor chamber that facilitated uniform cell seeding throughout the large scaffold volume and the generation of a homogeneous cartilaginous tissue construct. To our knowledge, this is the first report of engineered human cartilage constructs generated in this large-scale.

While one seemingly intuitive design to generate uniform flow over the scaffold would be the inclusion of a flow distributor (e.g., perforated plate) within the chamber, it is likely that a significant fraction of the perfused cells would attach/settle on such a device during the cell seeding phase [5], thereby dramatically reducing the efficiency of cell utilization. As opposed to scaling-up the bioreactor chamber through a trial and error approach, CFD modeling allowed for the efficient assessment and refinement of various chamber geometries during the initial conceptual design stage. Therefore, the bioreactor could be optimized, in term of flow profile, prior to

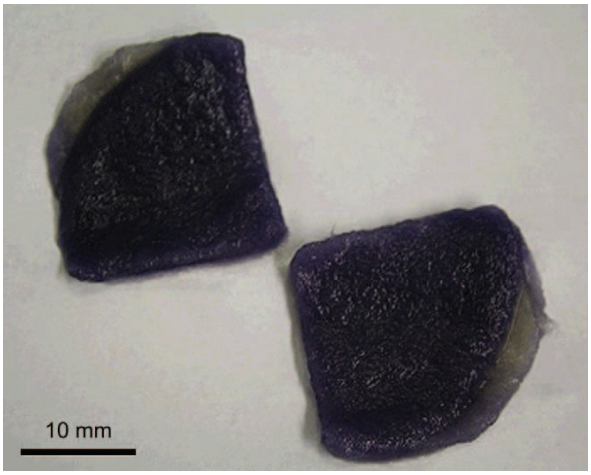

Fig. 4. Two sections of the MTT stained meshes following perfusion cell seeding. Cells were homogeneous distributed throughout the large-diameter scaffolds. The unstained periphery of the scaffold reflects the outer 1 mm region that was clamped in the bioreactor.

manufacturing costly prototypes and performing time-consuming experimental tests, saving time and resources.

Hyalograft-C, a 2 cm × 2 cm × 0.15 cm thick Hyaff-11 based cartilage graft, has been shown to provide a clinical improvement to young patients with focal cartilage defects [2,3,14]. While the current manufacturing process, based on conventional manual static cell/tissue culture techniques [15], may be sufficient to produce Hyalograft-C in its current size, we speculated that a perfusion bioreactor based approach could improve the quality of larger sized grafts. Indeed, the data we have presented here are consistent with our previous study [4] showing that the viability, homogeneity, and quality of engineered tissue constructs that are several millimeters in thickness can be dramatically improved when cultured under perfusion vs conventional static culture methods. Moreover, we have also shown that we could generate 50 mm diameter constructs in the scaled-up bioreactor with similar quality to those generated in our small research-scale bioreactor system, despite the 70-fold up-scaling (5.9 cm^3 vs. 0.085 cm^3).

One of the central questions in cartilage tissue engineering that remains to be answered is "How good is good enough?". In other words, what degree of *in vitro* maturation is required for a cartilage graft to support or induce a successful repair? Based on ectopic *in vivo* model systems, Hyaff-11 based constructs that were pre-cultivated *in vitro* to generate a cartilaginous extracellular matrix had an enhanced capacity to further develop *in vivo* as compared to those constructs that contained only cells attached to the Hyaff-11 meshes [11,8]. Using a bioreactor based *in vitro* model system to simulate aspects of joint loading, Demarteau found that mechanical deformation could increase the synthesis and accumulation of GAG in engineered cartilage constructs, but only if the tissues were sufficiently developed prior to the time of loading [16], suggesting that engineered cartilage tissues may need to possess sufficient mechanical integrity and biological responsiveness prior to implantation.

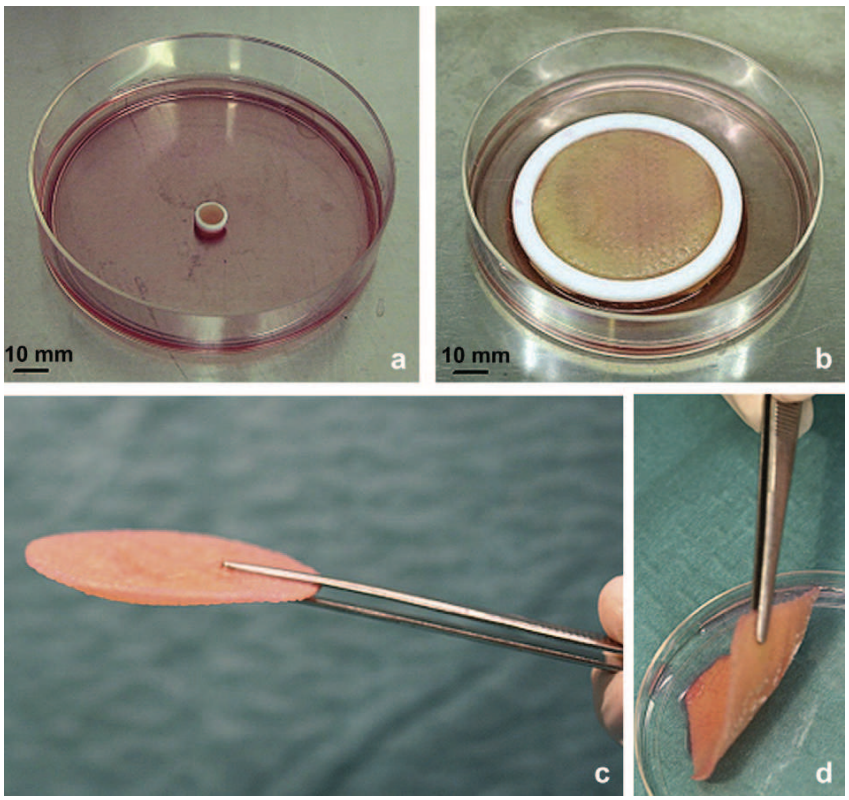

Fig. 5. Macroscopic views of (a) small-scale perfused cartilage constructs (6 mm diameter) compared to (b) large-scale constructs generated in the bioreactor (50 mm diameter) after 2 weeks of culture. (c) Large-scale grafts generated in the bioreactor were quite stiff, could be easily handled with forceps, and did not bend under their own weight. (d) However, the grafts were flexible enough to be bent with forceps and could potentially be molded to the contours of a human knee joint.

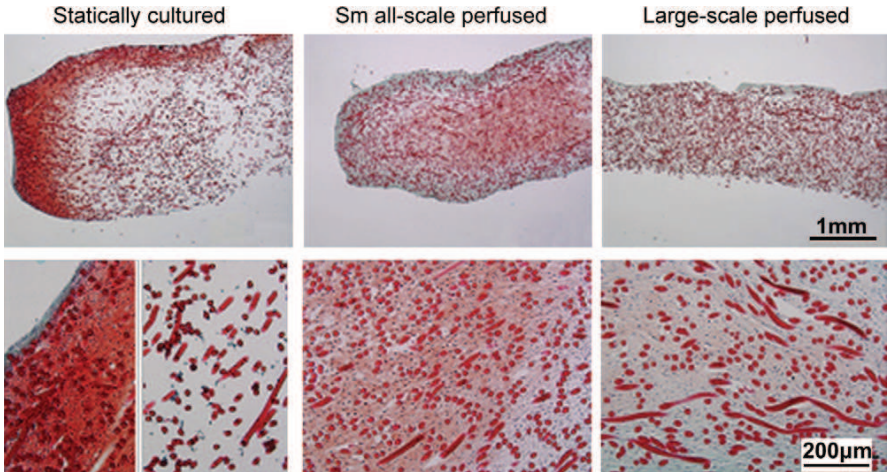

Fig. 6. Safranin-O staining for GAG following 2 weeks of culture. (Left) Large-scale constructs generated by conventional production methods (static seeding and static culture) had a dense layer of cells and GAG along the outer periphery but contained a necrotic internal region void of cells and matrix. (Middle) Small-scale constructs (6 mm diameter × 3 mm thick) generated in our previously described "research-scale" perfusion bioreactor [4] contained cells and Safranin-O stained matrix that were uniformly distributed throughout the scaffold. (Right) Large-scale constructs generated in the up-scaled perfusion bioreactor were histologically similar to small-scale perfused constructs, with cells and GAG homogeneously distributed throughout the cross-sections.

At the time of its implantation, the Hyalograft-C graft contains chondrocytes attached to the Hyaff-11 mesh fibers, but little to no extracellular matrix, and thus, maturation of the implanted construct (biochemically and mechanically) occurs within the repair site [17], typically taking more than 18 months [18]. While a graft with negligible mechanical properties may be sufficient for filling focal defects, in which the surrounding healthy native cartilage may be sufficient to support joint loading, a graft for resurfacing the lateral or medial condyle would likely need to be at a more advanced stage of maturation to provide a higher degree of functionality. Interestingly, recent mid-term follow-up studies have shown poorer clinical outcomes, in terms of defect filling and repair, of larger vs smaller defects treated with Hyalograft-C [14,3].

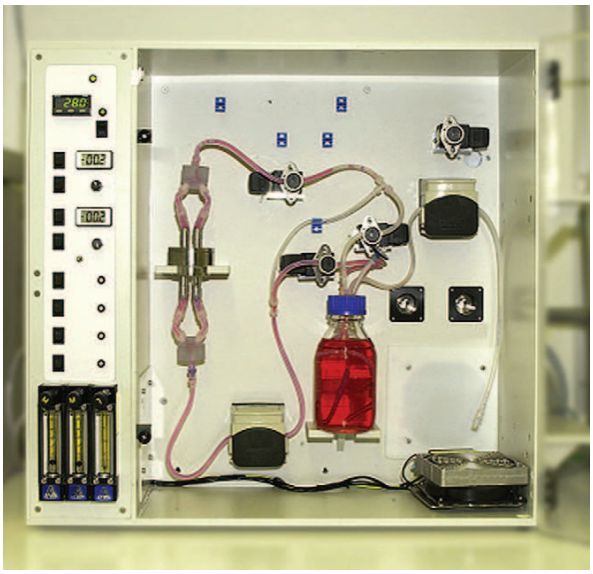

Fig. 7. Ongoing efforts are aimed to integrate the perfusion bioreactor within a prototype (Applikon Biotechnology BV, The Netherlands) of an automated and closed bioreactor system, with the ultimate goal of safe, standardized, cost-effective and GMP compliant production of large-scale cartilage grafts [20].

Based on a short culture period of only two weeks, the 50 mm constructs engineered in our scaled-up bioreactor had 20% of the suture retention and 5–10% of the aggregate moduli of native cartilage tissue. If deemed necessary, the culture time could simply be prolonged or culture conditions further optimized (e.g., culture media supplements, flow rate, oxygen tension) to further increase construct maturity. However, a more developed tissue with higher mechanical properties could potentially be challenging to mold to the contours of a knee and may therefore require the engineering of a graft of pre-defined shape. In that case, the design strategy outlined in this work could serve as a foundation to design a bioreactor for seeding and culturing constructs of anatomically shaped grafts.

We have previously reported on the engineering of thin small-scale Hyaff-11 based cartilage constructs, generated by conventional labor-intensive and manual static cell/tissue culture techniques [19]. In the current study, we have generated cartilaginous constructs with comparable GAG staining and GAG contents, which were 140-fold larger in size and which were produced by bioreactor based methods that are amenable to process automation and control. In this context, ongoing efforts are aimed at integrating the up-scaled bioreactor within a fully automated and closed manufacturing system (Fig. 7) with the ultimate goal of safe, standardized, cost-effective and GMP compliant production of large-scale cartilage grafts. In conjunction with surgical or pharmacological therapies, which may aid in the durability of the implanted graft, the engineering of human cartilage tissues in this large-scale paves the way for grafting functional biological substitutes for the clinical treatment of extensive cartilage defects.

Acknowledgements

We would like to acknowledge the European Union for financial support ("STEPS"; FP6- #NMP3-CT-2005-500465, www.steps project.com) and the Deutsche Arthrosehilfe e.V., Saarlouis, Germany.

Appendix

Figures with essential color discrimination. Figs. 1–7 in this article are difficult to interpret in black and white. The full color

images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.08.009.

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Conclusions and future perspectives

Tissue engineering products promise to be a viable and competitive alternative to upcoming off-the-shelf innovations in regenerative medicine if they are manufactured with reproducible properties, using processes compatible with regulatory and commercialization requirements. Implementation of automation, monitoring and control of the manufacture process are thus critical steps towards a broad clinical translation.

In this work a method was first studied to predict chondrocyte proliferation rate. Then a technique for on line monitoring of 3D growth was validated and finally, a controlled model system was developed to reproducibly generate clinically relevant size cartilaginous grafts. Figure 1 shows a possible application, in which all the techniques developed during this thesis work could be integrated, synergistically leading towards a more controlled and automated engineering of the final graft. Indeed, Figure1-A represents the traditional tissue engineering approach for the generation of a graft, in which all the manufacture processes are manual. In this process, after isolation, chondrocytes are plated and expanded on plastic dishes, then trypsinized and reseeded manually on the 3D scaffold; the construct maturation is performed under static culture. A valuable alternative is the streamlined bioreactor based approach described in Figure 1B: cells isolated from the tissue biopsy are expanded directly within the porous 3D scaffold and cultured in the same environment until the graft is sufficiently mature for implantation. The use of a bioreactor system combined with a streamlined approach would, indeed, benefit in minimizing the process and product variability. By bypassing the operator/handling-dependent procedures, through monitoring and controlling the culture parameters, the bioprocesses and resulting engineered graft could be standardized, thus ensuring compatibility with regulatory and commercialization requirements (Martin et al., 2009).

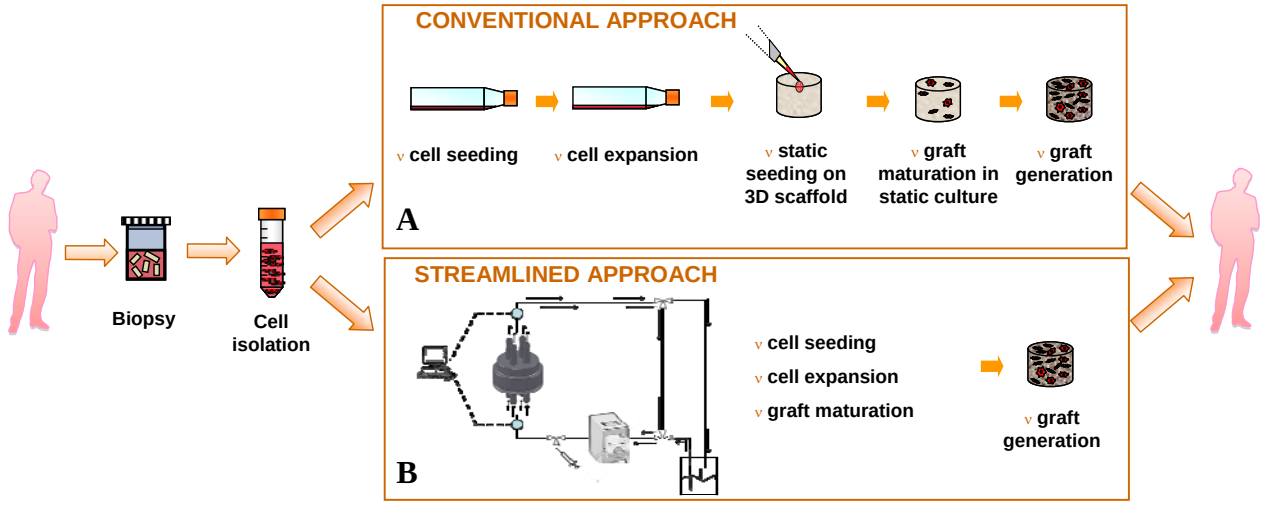

Figure 1. Production of cartilage tissues by conventional methods or the simplified bioreactor-based streamlined process. In the streamlined process, primary chondrocytes are seeded, expanded, & differentiated directly in a 3D scaffold to generate a cartilaginous tissue within a single closed & monitored bioreactor system.

The interaction between the techniques developed during this PhD program in a streamlined, upscaled and controlled bioreactor based culture, is illustrated in Figure 2. The upscaled bioreactor system described in Chapter 2 would be functional to a homogeneous seeding of freshly isolated human chondrocytes on the scaffold. As already discussed, in a clinically relevant scenario, due to the limited size of the available cartilage biopsy (only 100-250 mg of tissue, 2.5E+06 cells/gram of cartilage (Brittberg, 2008), the number of cells obtained from the digestion would be insufficient to start in vitro chondrogenesis without an initial in vitro expansion phase. Therefore, following seeding, constructs would remain in the bioreactor system, perfused with "proliferating" culture medium (DMEM supplemented with 10% FBS, 1ng/ml TGFß1, and 5ng/ml FGF-2) to expand the primary cells directly within the scaffold. Due to the donor variability of the proliferation rate, it is critical to monitor the number of cells present in the scaffold, to define when the optimal cell density on the scaffold is reached. Therefore, the in-line micro-oxygen sensors described in Chapter 3 could be integrated in the upscaled bioreactor system and the quantitative correlation between the oxygen measurements and the number of cells previously developed could provide a non invasive, online, real time monitoring of cell proliferation. The number of cells in the scaffold

would become the trigger to proceed to the next culture phase, in which the constructs, still within the same bioreactor, will be perfused with differentiation medium (DMEM, FBS10%, TGFβ3 10ng/ml, Ascorbic acid 0.1mM, Insulin 1UI/ml) in 5% O_2 tension. This graft maturation could be maintained for some weeks, and the oxygen in line sensor measurements would be used to better monitor the experimental conditions. In order to judge the maturation level of the graft at release, nowadays the standard techniques, such as biochemical, biomechanical and histological analyses have the evident disadvantage of being destructive and/or not in line. Indeed, without a quantitative, in line, established technique to evaluate this parameter during perfusion culture, adapting the culture time to the effective developmental stage of the construct is next to impossible. A possible future perspective to overcome this limitation could be to monitor physical or chemical biomarkers in the medium (i.e. GAG concentration), or to directly evaluate the biomechanical properties of the construct within the bioreactor (Buschmann et al., 1995; Davisson et al., 2002). Also testing and adapting the oxygen consumption based technique, described in Chapter 2, to the differentiation culture conditions, could provide an interesting feedback on the cell vitality at graft release. Finally, the feasibility of reaching the target of a clinically usable graft is challenged by the intrinsic variability in the behavior of human cells from different batches or donors (Barbero et al., 2004). The microcalorimetry based technique described in Chapter 1, if further studied and developed, could provide a predictive tool to discriminate between donors eligible to be treated with TE techniques, to better schedule the graft production, and, potentially, to optimally target the culture conditions, based on the reactivity of the specific donor, thus optimizing process cost vs. benefit.

In conclusion, all the technologies proposed in this study aimed to provide tools and methods to standardize the generation of more reproducible cartilaginous grafts. The integration of the upscaling technique with the oxygen consumption based control has already successfully been tested, and a paper describing the results obtained is in preparation. Moreover, the tools and technologies that have been developed within this PhD program will serve as the foundation for a recently approved Collaborative Project funded by the European Union, coordinated by Professor Martin. The goal of this project will be to produce cartilage tissue grafts, engineered to possess functional properties, with the aim of establishing a tissue therapy to reduce initial rehabilitation time and support a durable repair in the long-term. In order to reach this goal the research based technologies and processes described in this dissertation will be adapted for GMP compliance and conformance to regulatory guidelines for the production of engineered tissues for clinical use, which will be tested in a clinical trial. The project will exemplify the

roadmap for a bioreactor-based translation of tissue engineering strategies into clinical products, in which the proof-of-principle in the context of cartilage repair will have an impact on the broad utilization and commercialization of cell-based grafts as therapeutic solutions for a variety of other indications (e.g., bone repair, epithelia reconstruction, etc.).

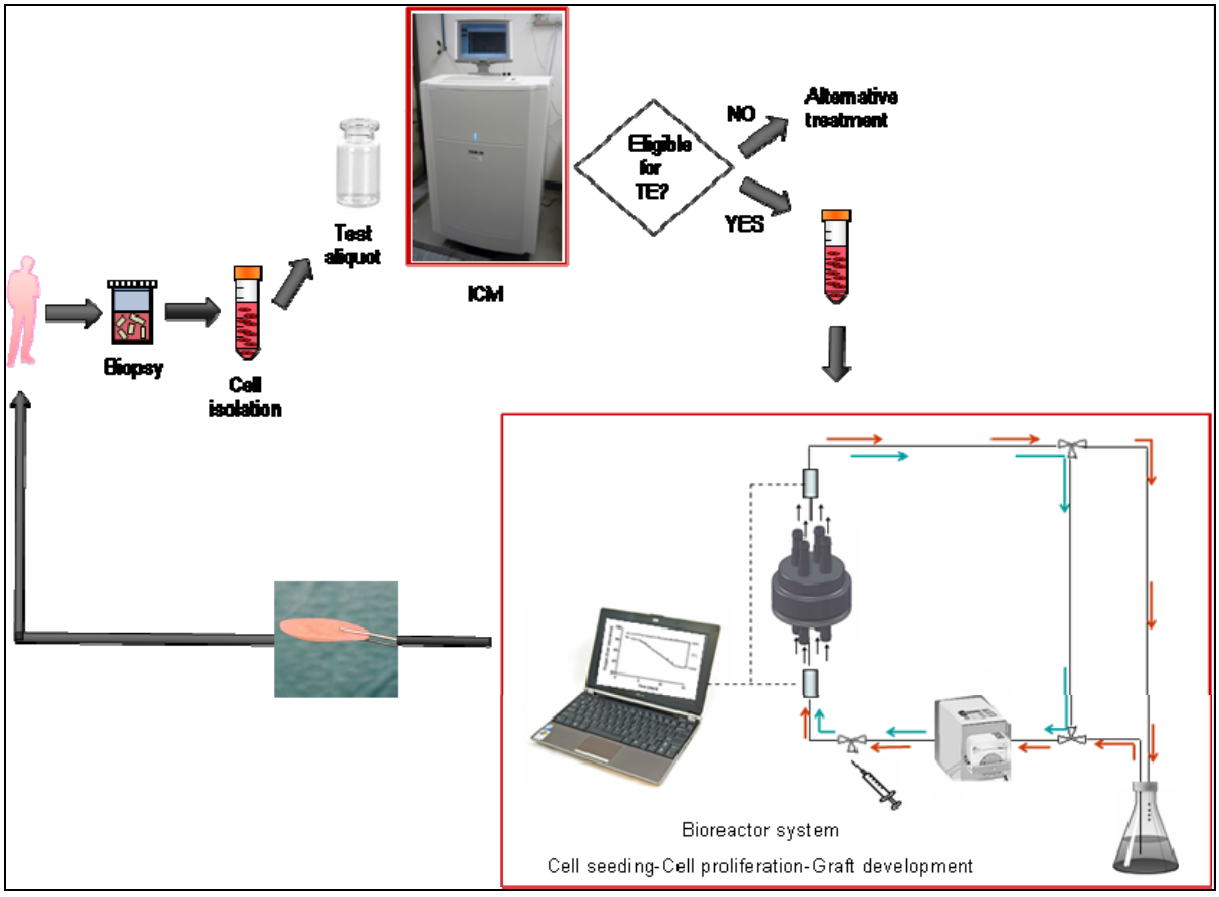

Figure 2. Possible future integration of the techniques developed in this work to a streamlined bioreactor based production of cartilaginous grafts. Microcalorietry (*Chapter 1*) provides a tool for the quality screening of the isolated cells; if the donor is judged eligible for the treatment from the ICM based test, the cells are seeded and cultured within the scaled up bioreactor system described in *Chapter 3*. Finally, by modifying the culture conditions based on the in line oxygen measurements (*Chapter 2*) a feedback controlled automation system can be set.

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Santoro R*, Tonnarelli B*, Martin I, Wendt D, Streamlined engineering of large-scale human cartilage grafts. Paper in preparation. *authors equally contributing

ABSTRACTS AT INTERNATIONAL CONGRESSES

Santoro R., Brans G., Olivares A.L., Lacroix D., Jakob M., Martin I., Wendt D.,Bioreactor based engineering of large-scale engineered cartilage grafts for joint resurfacing. Orthopaedic Research Society (ORS 2010) 2009- Oral presentation

Santoro R, Krause C, Martin I, Wendt D, Online oxygen monitoring for cell quantification during the perfusion-based manufacture of engineered cartilage grafts. European Orthopaedic Research Society (EORS) 2010-Oral presentation

Güven S, Mehrkens A, Saxer F, Schaefer D, **Santoro R**, Martinetti R, Martin I, Scherberich A, Perfusion culture of human adipose-derived progenitors to engineer vasculogenic, large osteogenic grafts. TERMIS 2010- Oral presentation

Santoro R, Krause C, Martin I, Wendt D, Quantification of cells in engineering constructs by non-invasive monitoring of oxygen. Orthopaedic Research Society (ORS 2010)- Poster presentation

Santoro R., Martin I. Wendt D., Online monitoring of oxygen as a non-destructive method to quantify cells in engineered tissue constructs. Tissue Engineering and Regenerative Medicine International Society (TERMIS) 2010- Poster presentation

Martin I, Tonnarelli B, **Santoro R**, Barbero A, Wendt D. A 3D-culture model for the streamlined engineering of large-scale human cartilage grafts. Invited talk at the 9th World Congress of the International Cartilage Repair Society (ICRS) in Sitges/Barcelona, September 2010.

Santoro R., Tonnarelli B, Martin I, Wendt D, Streamlined engineering of large-scale human cartilage grafts. Orthopaedic Research Society (ORS) 2011-Oral presentation

REVIEW FOR SCIENTIFIC JOURNALS

In 2011 I have been an independent reviewer for the journal “JTERM”. Apart from this, I have assisted my supervisors in reviewing scientific manuscripts in other peer-reviewed journals.