

Review on Patents for Mechanical Stimulation of Articular Cartilage Tissue Engineering

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Abstract: To repair articular cartilage defects in osteoarthritic patients with three-dimensional tissue engineered chondrocyte grafts, requires the formation of new cartilage with sufficient mechanical properties. The premise is that mechanical stimulation during the culturing process is necessary to reach this aim. Therefore, mechanical stimulation systems have been integrated in aseptic bioreactors for *in vitro* cultivation of tissue engineered cartilage. These vary from simple unconfined compression systems to advanced bioreactors in which deformation and loading are fully controlled. Fluid handling in these devices is another decisive parameter for the success of cartilage tissue engineering.

Over the last decades bioreactor developments have resulted in the filing of many patents. The aim of this paper is to review these patents, categorize them according to their possibilities for mechanical stimulation and fluid handling systems and finally to discuss them in the context of the demands of a functional tissue engineered cartilage from a mechanical perspective.

Keywords: Cartilage, chondrocytes, tissue engineering, three-dimensional, ACT, bioreactor, mechanics, compression, pressure, shear, perfusion, fluid handling, computer model.

INTRODUCTION

Osteoarthritis is a degenerative joint disease characterized by pain and disability. It involves all ages and by the age of 60, 85% of all people have some degree of osteoarthritis. Severe osteoarthritis is the prime cause for joint-replacement surgery. The annual costs of treatment in the US were estimated at \$60 billion. Natural cartilage repair is limited because chondrocyte density and metabolism are low and cartilage has no blood supply. The results of joint-preserving treatment protocols such as debridement, mosaic plasty, perichondrium transplantation and autologous chondrocyte implantation vary largely and the average long-term result is unsatisfactory [1-5]. One reason for limited clinical success is that most treatments require new cartilage to be formed at the site of a defect, under circumstances that previously induced cartilage damage. Therefore it is unlikely that healthy cartilage would form at these locations.

The most promising method to circumvent this problem is to engineer mechanically stable cartilage *ex vivo* and implant the three-dimensional (3D) matrix-associated graft/transplant (MACT) into the damaged tissue area [6-8]. One clinical study based on chondrocyte loaded hyaluronic acid scaffolds regenerated hyaline cartilage in 40% of the patients after 16 months [9], illustrating the potential of the method. The main problem with tissue engineered cartilage in general, is that the load-bearing capacity is insufficient to cope with the severe loading conditions in an articular joint. If the load-bearing capacity, i.e. the mechanical properties, of the tissue engineered cartilage could be enhanced, the

clinical success would dramatically increase and replacing damaged articular cartilage with healthy tissue engineered implants may become the primary method of clinical treatment of focal cartilage damage. The premise in the area of tissue engineering of articular cartilage, like for other load bearing tissues, is that mechanical loading of the developing cartilage prior to implantation will enhance the load-bearing capacity and therefore increase both the survival rate and the scores for post-operative cartilage repair quality.

The importance of mechanical stimulation for the development of tissue-engineered cartilage is evident. Chondrocyte metabolism and synthesized matrix proteins can be modulated with mechanical loading [6,7,10-14]. Similar results are found with bone-marrow derived mesenchymal stem cells from various species which undergo increased chondrogenesis in three-dimensional cultures when appropriate mechanical stimulation is applied [15-17]. In addition, dynamically loaded tissue-engineered cartilage has superior properties compared to unloaded tissue with the same amount of matrix [18].

In view of the beneficial effects of mechanical loading for cartilage tissue development, several groups have put effort in developing bioreactors for the tissue engineering of articular cartilage, which incorporate the possibility to apply mechanical loading to the tissue engineered cartilage under development during *in vitro* culture. Techniques for including a system for such mechanical stimulation can be implemented in various ways and with different views. Extended reviews on bioreactor systems and culture conditions for articular cartilage tissue engineering have recently been published [19-21]. Some of these bioreactor developments have resulted in the filing of patents. The aim of this paper is to provide an overview of patents that have emerged on bioreactors with mechanical stimulation for

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tissue engineering of articular cartilage transplants and chondrocyte loaded scaffolds.

BIOREACTORS AND MECHANICAL STIMULATION

“Bioreactor” is a general term, defined by the European Commission as “a contained vessel or other structure in which chemical reactions are carried out (usually on an industrial scale), mediated by a biological system, enzymes or cells. A bioreactor can range in size from a small container to an entire building”. Bioreactors for tissue engineering, however, are relatively small. They are designed to engineer biological tissues, which are in the order of few centimeters, maximum. The size of a disc-shaped implant for articular cartilage that is generally used in scientific research is in the range of 10-15 mm diameter and 1-5 mm thick, while for clinical application, this is at the low range of the required sample size [22]. To culture 10-15 mm constructs, bioreactors range in size between five cm and one meter and the diversity is enormous. Bioreactors can be designed to fit in a standard incubator for cell cultures, or they can be fully self-supporting desktop systems that control biophysical and biochemical environmental conditions such as pH, pO₂ and temperature. One bioreactor can house between one and one hundred samples, depending on the rationale of the design and the required control per sample.

Over the last years, research groups and biomedical companies developed and patented bioreactor models for the automated production of autologous cartilage transplants (ACT) as a tissue-engineered product for therapeutic use in osteoarthritic patients [19]. It is claimed that these bioreactors allow for industrial cultivation of patient-specific cartilage implants using autologous chondrocytes or mesenchymal stem cell in aseptic, almost decentralized manufacturing facilities, under full control of general culturing conditions such as temperature, pH, and partial gas pressures. Bioreactors comprise all stages of the tissue engineering process, including cell isolation, harvesting, freezing, storage, seeding or inoculation, proliferation and differentiation phases. Such systems try to meet the legal requirements for manufacturing a patient specific medication according to current good manufacturing practices (GMP), which can be ensured through a quality control/quality assurance program (QC/QA). Current clinical concepts for the GMP-adherent production of autologous transplants for OA diseases are cartridge-based systems, described and applied by Cannon *et al* [23] and by the industrial developers Olympus [24], Millenium Biologix [25] and Aastrom Biosciences [26,27].

In general, bioreactors designed for (fundamental) research have different requirements. Rather than more standardization, more flexibility is desirable to vary experimental conditions. Depending on the research aim, particular requirements for control of the tissue engineering process are essential. To study mechanobiological effects, such as mechanotransduction pathways in chondrocytes requires extensive control over the mechanical conditions to which the tissue is subjected during culture. Such bioreactor systems normally house fewer samples than bioreactors in production facilities. This review focuses on bioreactors for tissue engineering of articular cartilage transplants and

chondrocyte loaded scaffolds that have integrated methods for mechanical stimulation. A generalized representation of possible mechanical stimulation systems used in bioreactors is provided in Fig. (1).

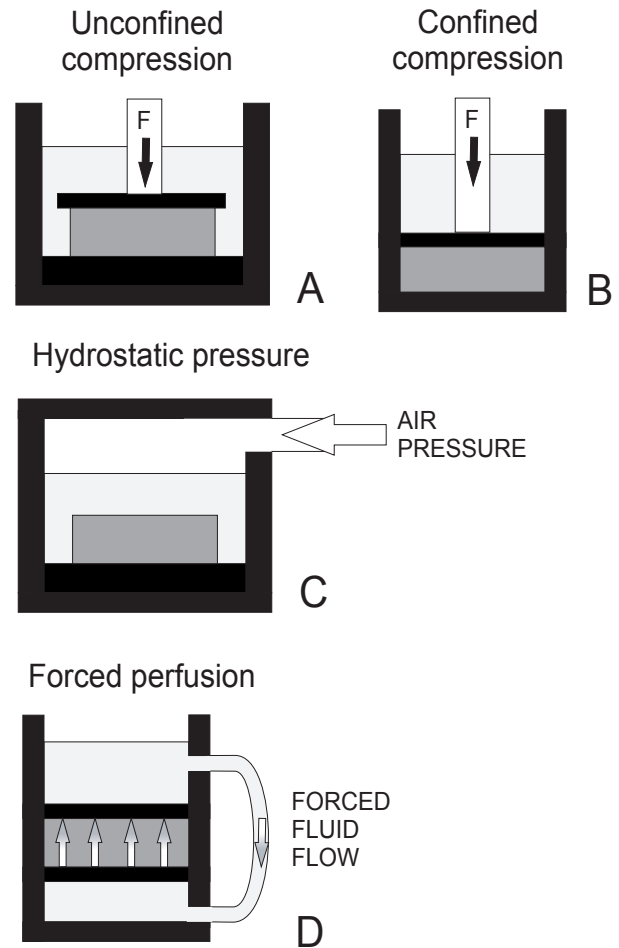


Fig. (1). Schematic drawings illustrating the main loading principles used in cartilage bioreactor systems: unconfined compression (A), confined compression (B), hydrostatic pressure (C) and forced perfusion (D). These principles have been implemented in different ways, and many patented bioreactors incorporated combinations of these loading possibilities. Black: bioreactor walls; dark-gray: tissue engineering construct; light-gray: culture medium; striped: porous (or non-porous) compression and supporting plates. Mechanical force (F), air pressure or forced fluid flow directions are indicated with arrows.

In this context, “mechanical stimulation” requires further explanation. For the engineering of load-bearing tissues, tensile and compressive loading are the first regimes that come in mind, tensile loading being more relevant for fibrous tissues such as tendons, and compressive loading for bone and articular cartilage. Compressive loading can be further categorized. Uniaxial compression is probably the most frequently used stimulation method for cartilage tissue engineering. In confined uniaxial compression (Fig. 1B), the tissue is compressed in one direction, while deformation of the tissue in any other direction is prohibited. This occurs when a sample perfectly fits a container, while it is compressed by a piston at one side. In unconfined compression

(Fig. 1A), the tissue is allowed to expand freely in the direction perpendicular to the axis of compression. In both compression types, particularly in a confined setup, the porosity of the container, the supporting plate and/or the piston are important boundary conditions for the applied mechanical loading. Time-dependent tissue deformation and fluid pressurization depend on fluid expression from the tissue during compression and on rehydration of the tissue upon relaxation.

In addition to uniaxial compression, it is possible to compress a tissue from all sides at the same time, for instance by imposing a hydrostatic pressure via the surrounding fluid (Fig. 1C). Less frequent are compression regimes where compression is induced through an indenter with a size smaller than the size of the sample itself, or compression with platens that are shaped according to the desired, patient-specific *in vivo* geometry.

Finally, shear loading of cells and tissues has been shown to stimulate cells and to induce formation of particular tissue types. Shear can be applied by grabbing two sides of a construct and sliding the grips in opposite directions in parallel planes. A specific type of such shear loading is applied by rotating the grips. Rather than by grips, however, shear loading on cells in culture is more frequently induced by forced fluid flow. Drag forces due to the flow of culture medium over the sample or the cells are known to stimulate cell metabolism. For some cell types, such as vessel wall endothelial cells, these fluid flow patterns are of ultimate importance. This likely holds for superficial zone cells in articular cartilage as well, which have shown to express surface zone protein (SZP/lubricin), a protein determining the mechanical functionality of articular cartilage, in response to shear loading [28,29].

The primary reason for inducing fluid flow in bioreactors for tissue engineering of articular cartilage, however, is often not mechanical, but rather to enhance transport of nutrients and other metabolites. Chondrocyte metabolism relies on nutrition [30-32], which evidently is not uniform throughout a cartilage tissue engineering construct. Chondrocyte viability in the center of tissue engineering constructs of a few mm thick suffers from limited nutrition, due to the slow diffusion process in combination with nutrient utilization by peripheral cells [33]. Limited nutrition in the center of chondrocyte-seeded construct explains the low viability and matrix synthesis in the center and enhanced proliferation and matrix synthesis along the edges [34-36]. In bioreactors that mix the medium around constructs, for instance in the well-known rotating wall vessel (RWV) bioreactors [37,38], nutrient supply is enhanced and waste-product accumulation is prevented in the deeper areas of the construct [39]. This results in more homogenous tissue distributions [19,37]. To further enhance nutrient delivery to cells in the center of constructs, bioreactors have been designed by which perfusion is forced through the constructs (Fig. 1D) [40-49]. This forced perfusion not only delivers nutrients, but induces shear forces to the seeded cells as well. Although, the effect of the individual contribution of these effects is hard to distinguish, both are likely to contribute to the development of tissue engineered articular cartilage.

In addition to the method of loading, it is evident that the loading regime, i.e. the frequency and magnitude, and whether the load is continuously or intermittently applied, is of utmost importance for the development of the tissue (for instance [10,13,50-52]). The loading experienced by the cells further depends on the properties of the scaffold material. Many studies have shown that scaffold stiffness and texture affect cell behavior immediately, and that properties such as scaffold porosity and permeability are important in tissue engineering. The latter has special relevance in relation with forced perfusion and nutrition. Such effects have been extensively discussed in reviews on tissue engineering [19-21,53] and are not addressed in the present overview.

This review focuses on the implementation of loading devices in bioreactors for articular cartilage tissue engineering. For the above reasons, special attention is given to fluid handling, i.e. whether or not fluid is refreshed around the tissue, or forced through the construct. The loading regime (frequency, strain magnitude, etc.) to which the samples are subjected, and aspects of the scaffold material that influence the transmission of the loading to the cells, are beyond the scope of this review.

ANALYZING PATENTS

For our patent search, we made use of the United States Patent office search engine (available through www.uspto.gov). A search on "cartilage and bioreactor" results in 215 hits, "cartilage and "tissue engineering"" gives 171 hits. Of these patents, 90 are included in both searches, leaving 296 unique patents. The combination of "cartilage and bioreactor and mechanical" produces 137 patents, indicating the importance of mechanics in cartilage tissue engineering studies.

When "cartilage and bioreactor" is combined with keywords "mechanical stimulation" or "mechanical loading", the number of hits remains 15 and 10, respectively, including 20 different patents. Combining "cartilage and bioreactor" with "fluid flow" or "perfusion" results in 23 and 85 hits, respectively, of which 98 patents are different. Ten patents include the keywords "mechanical stimulation" or "mechanical loading", as well as "fluid flow" or "perfusion".

All initial 296 patents were screened to determine whether they suited the criteria for inclusion in this study. It turns out that cartilage is mentioned in several introductions as one example of tissues that respond to mechanical loading. Other patents mention cartilage as one of the tissues that could be engineered with variations of the design of the patented bioreactor. However, whether this is truly a possibility remains questionable in several designs, to the opinion of the authors.

We identified those patents that were designed for the purpose of culturing cartilage, or were well suitable for this purpose. Subsequently, we selected those emphasizing the possibility to mechanically stimulate the constructs. We also included patents on bioreactors focusing at fluid perfusion such that fluid would have a significant interaction with the tissue. From these bioreactors, we included those that can be used for culturing three-dimensional cartilage grafts. This criterion excluded bioreactors designed for stimulation of single cell layers, for instance of cells cultured and

stimulated by tensile forces on flexible membranes [54] as used in many studies [55,56], and stimulation devices such as the cone viscometer which induced shear forces to chondrocytes cultivated on coverslips [57,58].

Bioreactors designed for 2D cultures, but which can also be used to culture 3D constructs in the presence of mechanical stimulation, remain included. All bioreactors are categorized according to mechanical-loading mechanism or fluid-handling approaches.

A final remark is that we searched for patents worldwide. Whenever possible, US patent numbers are referred to in this study. Second choice was the use of WO-numbers. Few interesting German patents remained without US or WO-filing (patent number starts with DE). For these patents, the official German titles are mentioned as well as an English translation.

PATENTS DEDICATED TO MECHANICAL LOADING SYSTEMS IN BIOREACTORS FOR TISSUE ENGINEERING OF ARTICULAR CARTILAGE

Confined and Unconfined Compression

Patents that include compression with a piston or compression between two platens do not distinguish between confined and unconfined compression (indicated as 'CC' and 'UC' in Table 1). We derived from the text or images published with the patent that several bioreactors do not allow to apply confined compression to the sample, because the piston size is smaller than the container and maximum sample size equals that of the piston [59-64]. One reason for designing the bioreactor this way can be to ensure limited resistance. Other designs seem to allow both unconfined and confined compression, because the piston diameter matches the diameter of the container [41,42,65-67]. If in these cases the sample fits the container, loading is confined. If the sample diameter is smaller than the diameter of the container, loading is unconfined. One design appears to be dedicated to confined compression only [68].

In general, patents do not specify whether the compression plates are of porous material. This information is important for the applied loading conditions to the cells, and in particular to the time-dependent response, as mentioned before. Most likely, the porosity of the piston and supporting plates is often adjustable by using different materials. Only when these are inherently part of the container wall itself, this is not possible.

Discussion

Many inventors claim that their bioreactors can apply physiological loading conditions to the tissues in their bioreactors. Unfortunately, none of them specifies what they mean by that. In fact, physiological loading conditions are probably not met in both confined and unconfined compression setups. *In vivo* each small piece of cartilage is supported by the adjacent cartilage. Deformations of the tissue in the direction perpendicular to the loading direction therefore depend on the sideways deformation of the adjacent tissue. Thus, the true compression regime is likely to be in between confined and unconfined. Since the adjacent tissue is likely to receive a similar loading pattern, the nature of cartilage compression is probably more similar to

confined than to unconfined compression. However, for two reasons a confined compression setup is far less favorable than an unconfined setup in bioreactors for tissue engineering.

First, in an unconfined compression setup, culture medium surrounds the sides of the construct. In a confined compression setup however, no space is available between the construct and the bioreactor (Fig. 1A,B). Hence, confined setups are more vulnerable to hampered nutrient supply and reduced viability compared to unconfined setups. To allow exchange of nutrients and waste products between the culture medium and the scaffold, porous materials must be used, which are often combined with the use of flexible membranes that physically separate the loading plates from the constructs to ensure sterility. None of the designs allowing for confined compression however, uses porous side-walls of the container to enhance nutrition.

Second, a perfect fit of the tissue and the piston in the container are necessary for true confined compression. A perfect fit ensures that the material cannot dilate in the direction perpendicular to the loading direction, and that tissue cannot bulge in the space between the piston and the sidewalls of the container. This requirement is not easy to meet, and at the same time have a flexible system that can easily be worked with. For instance, installing a perfectly fitting tissue in a container and installing a loading plate on top of the tissue, can be difficult. Also, friction between the piston and the container may become an issue, especially when force-controlled and dynamic compression is aimed for.

One underexplored drawback emerges in tissue engineering protocols that use (dynamic) unconfined compression. When allowing for deformation in the plane perpendicular to the loading direction, the deformation of the construct depends largely on the resistance against sliding between the construct and the loading and supporting plates. The difference in deformation between frictionless contact Fig. (2B) and maximum friction conditions Fig. (2C) is large. In the frictionless situation, cells in the periphery and in the center of the construct experience the same compressive strain in the direction of loading. However, when friction is involved, the deformation changes with distance from the center of the construct. In fact, due to bulging of the construct, peripheral cells may actually experience tension rather than compression. Noteworthy, porous plates often used to stimulate nutrition, generally have higher friction coefficients and therefore are likely to induce the bulging effect Fig. (2C). Obviously, knowing the deformation in response to the applied loading is of importance in studies focusing on the stimulatory effects of mechanical compression regimes on the development of tissue engineered cartilage.

An additional complication arises especially in long-term cultures, when the tissue expands as a result of matrix synthesis. In that situation, adjustments to the loading regime may become necessary to ensure that the desired loading conditions (for instance 10% compressive strain) persist. This requires continuous control of the position of the piston and the applied force, which is a feature that only few bioreactors have incorporated [41,59,61]. More important

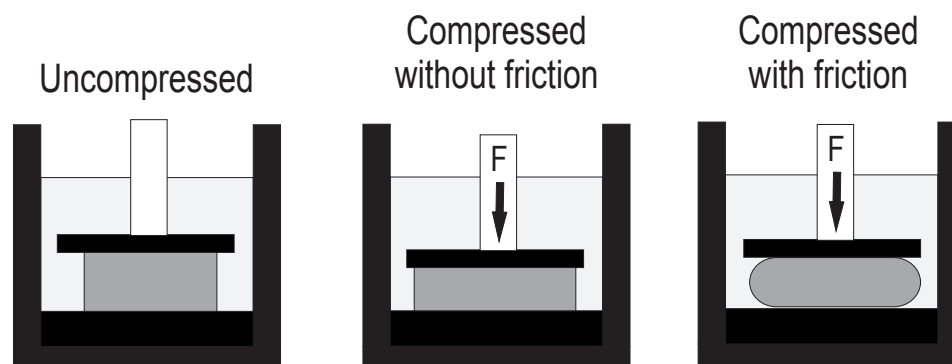


Fig. (2). Illustration of the deformation of a construct that is loaded in an unconfined compression system (A), when the friction between sample and loading plates is negligible (B) or dominant (C).

size changes occur when the tissue shrinks. For instance it is known for a long time that widely used collagen type I gels, which are often used in tissue engineering studies, compact significantly after a few days culture [69,70]. Updating piston height is required to continue loading such construct, and these scaffolds are not suitable for use in confined compression setups as the construct will not stay in contact with the container. One bioreactor patent makes use of collagen gel compaction: by preventing gel compaction to occur stresses are introduced in the tissue in the direction of clamping [71].

A final remark regards the use of scaffolds that are pre-shaped such that the geometry matches the ultimate geometry of the defect that is to be repaired. The few bioreactor designs that allow for such patient-specific or joint-shape-like geometries, indicated in Table 1 as 'GEOM', all use unconfined compression setups [45,47,59,67]. It should be noted that the deformation or mechanical loading of the material in these systems is probably not homogenous and it will therefore be even more challenging to obtain reproducible results with these systems, compared to regular bioreactor systems. Also, it seems unfeasible to generate bioreactors with containers for the tissue engineering construct that exactly match the shape required for the individual patient, although the advances in clinical imaging modalities such as CT and MRI in combination with rapid prototyping techniques may enable such developments in the future.

Hydrostatic Pressure

Hydrostatic pressure has been shown to affect cell metabolism, with static hydrostatic loading inhibiting matrix synthesis and dynamic hydrostatic loading stimulating proteoglycan synthesis by chondrocytes, depending on the characteristics of the pressure wave. The easiest way to apply a hydrostatic pressure to a sample in culture is by pressurizing the culture medium around the sample in a closed container, for instance by increasing the pressure that is developed by the pump that circulates culture medium, by increasing the air-pressure in the container above the culture medium (Fig. 1C), or in a more controlled way through a dedicated piston in direct contact with the fluid. In all patents in which fluid flow is controlled, it is possible to increase the hydrostatic pressure simply by occluding the outlet. Irrespective of the method, the effect on the construct is the same: it is equally pressurized from all sides at the same

time. In many patents, hydrostatic pressure is directly feasible and it is often mentioned as one way of stimulating tissue development. All such patents are marked 'HP' in Table 1.

Discussion

Applying hydrostatic pressure to the fluid is much more direct than applying it via compliant air. Hence, controlling the pressure wave is easier and fast pressure changes are possible, which can be advantages in particular studies. In some setups, applying hydrostatic pressure via the air above the culture medium has the advantage that it is easier to assure sufficient surface area for gas-exchange (O_2 , CO_2 , etc.) between culture medium and air.

As a result of the biphasic nature of scaffold materials used for cartilage tissue engineering, confined and unconfined compression setups also induce hydrostatic pressure in the construct. However, in these systems the pressurized fluid is expelled from the tissue. The hydrostatic pressure decreases in time when the fluid fraction reduces and more of the loading is carried by the solid content of the construct. Under sustained (un)confined compression between porous plates, the hydrostatic pressure theoretically decreases to ultimately reach 0 Pa throughout the construct. Under dynamic loading conditions however, hydrostatic pressure may remain present in (un)confined compression loading regimes, depending on stimulation frequency and on (poro) elastic and osmotic properties of the scaffold material.

It should be noted that most systems in which forced perfusion of the construct is possible (see next paragraph), the construct is located between two fluid reservoirs. In these systems, forced perfusion is induced by increasing the fluid pressure at one side of the construct. This creates a gradient in hydrostatic pressure over a tissue engineering construct, with the high pressure at the pressurized side. Obviously, by increasing the pressure in such systems at both sides at the same time, or by occluding the outflow, these systems generate a true hydrostatic pressure.

Forced Perfusion / Fluid Flow Induced Shear

An interesting aspect of many bioreactors for cartilage tissue engineering is the possibility to apply a hydrostatic pressure difference between two sides of a tissue engineering construct. Due to the pressure difference, fluid is forced through the construct [40-49]. Although mechanical forces

Table 1. Overview of Bioreactor Patents with Mechanical Actuators. Detailed Explanation can be Found in the Body Text

Number, Inventor and year of Patent	Patent title	Mechanical loading	Fluid handling	System character
(Un-)Confined compression devices				
US2001043918, Masini and Bancroft, 2001 [59]	<i>in vitro</i> Mechanical loading of musculoskeletal tissues	UC, HP	D	3D, GEOM
US2005106720, Nagel-Heyer and Pörtner, 2005 [79]	Device and method for cultivating tissue cells	CC, HP	MIX	2D, 3D (thin)
WO05087912, Nehring <i>et al.</i> , 2005 [65]	Inventive bioreactors and bioreactor systems	CC, UC	D	3D
DE19808055, Adamietz <i>et al.</i> , 2007 [68]	Method and apparatus for manufacturing of three-dimensional tissue cultures [Verfahren und Apparatur zur Herstellung von dreidimensionalen Gewebezellkulturen]	CC	D, MIX	3D
US20077163825, Gault, 2007 [60]	Bio-reactor for tissue cultivated in form of a thin layer and uses thereof	UC, HP	D, MIX	2D, 3D (thin)
Compression systems with additional stimuli				
US5406853, Lintilhac and Vesecky, 1995 [61]	Instrument for the application of controlled mechanical loads to tissues in sterile culture	UC, O (tension, bending)	D	3D
US20006121042, Petersen <i>et al.</i> , 2000 [62]	Apparatus and method for simulating <i>in vivo</i> conditions while seeding and culturing three-dimensional tissue constructs	UC, HP	MIX, FP	3D
DE19962456, Wilke and Nehring, 2001 [63]	Method and apparatus for manufacturing, analysing and mechanical stimulation of three-dimensional tissue cultures [Verfahren und Apparatur zur Herstellung, Untersuchung und mechanischen Stimulation dreidimensionaler Gewebezellkulturen]	UC, HP	FP, MIX	3D
US2004208761, Bader, 2004 [42]	Device for pressurized perfusion, especially for cultivating and/or treating cells	CC, UC, HP	FP	3D
US2005084954, Bader, 2005 [66]	Device for raising or cultivating cells in a container-like receptacle	CC, UC, HP	D, MIX	2D, 3D
WO2005123905, Nakata, 2005 [67]	Cell culturing method by biomechanical stimulus load and its device	UC, CC, FIS	D, MIX	3D, GEOM
US2006293724, Kronberg <i>et al.</i> , 2006 [64]	Methods for modulating osteochondral development using bioelectrical stimulation	UC, HP, FIS, O (tension)	D	2D
US20070026517, Schulz and Bader, 2007 [41]	Method and bioreactor for the cultivation and stimulation of three-dimensional vital and mechanically-resistant cell transplants	UC, CC, FIS	MIX, FP	3D
Hydrostatic pressure devices				
US6060306, Flatt <i>et al.</i> , 2000 [40]	Apparatus and method for sterilizing, seeding, culturing, storing, shipping and testing replacement cartilage tissue constructs	HP	FP	3D
US2006216822, Mizuno <i>et al.</i> , 2006 [44]	Method for preparation of neo-cartilage constructs	HP	D, FP	3D
WO07045619, Jagodzinski and Krettek, 2007 [45]	Tissue implant and process for its production	HP	FP	3D, GEOM

(Table 1) Contd....

Number, Inventor and year of Patent	Patent title	Mechanical loading	Fluid handling	System character
Fluid flow-induced shear / Forced perfusion				
US5928945, Seliktar, 1999 [46]	Application of shear flow stress to chondrocytes	FIS	FP	2D, 3D
US5891455 Sittinger and Bujia, 1999 [47]	Process for producing an implant from cell cultures	FIS	FP	3D, GEOM
DE10249903, Winkler <i>et al.</i> , 2004 [43]	Mechanical bioreactor [Mechanischer Bioreaktor]	O (Confined indentation, bending)	MIX	2D, 3D
WO05085429, Martin <i>et al.</i> , 2005 [48]	Reverse-flow perfusion of three-dimensional scaffolds	FIS	FP	3D
US2006234372, Donahue, 2006 [49]	Cell culture method and apparatus for mechanically stimulating cells	FIS	FP	3D
Other stimuli				
US5153136 Vandenburgh, 1992 [102]	Apparatus for growing tissue specimens <i>in vitro</i>	O (stretching)	ND	2D, 3D
US5700688 Lee and Huang, 1998 [71]	Method for producing oriented connective tissue cells	O (restricting compaction)	ND	3D

Mechanical loading: CC = confined compression; UC = unconfined compression; FIS = flow-induced shear; HP = hydrostatic pressure; O = other.

Fluid handling: FP = forced perfusion through tissue; D = nutrition through diffusion, with static medium around the construct; MIX = nutrition through diffusion, but medium is mixed or continuous flow is applied.

System characteristics: 2D: bioreactor can only mechanically stimulate cells in monolayers; 3D: cells are seeded scaffolds with standard, predefined 3D geometries, mostly disc-shaped; GEOM: specific claims involving cells seeded in scaffolds that have specific geometries, adjusted to the site of implant.

(pressures) are the actuators of this fluid transport, forced perfusion ('FP') is captured in the 'fluid handling' column in Table 1. There are two principal reasons for inducing forced perfusion through the tissue. As explained below, the first is to enhance nutrition; the second is to induce shear forces on the chondrocytes.

Appropriate nutrient supply to cells inside the tissue in culture is the first requirement to make the formation of new tissue possible. Articular cartilage is avascular; nutrition naturally occurs through diffusion. However, cells in developing cartilage, for instance in tissue engineering situations, are much more demanding than those in healthy, relatively inactive adult cartilage. Nutrient transport through diffusion is thought to be insufficient in many cases, as explained before. In particular, the cells in the center of the construct will suffer from lack of nutrition, and the viability in these areas is low. Consequently, new tissue formation occurs predominantly in the peripheral parts of the samples and not in the center. To overcome the problem of low nutrition in the center, forced perfusion induced by a fluid pressure difference over the construct is aimed for in several bioreactor designs [40-47]. Some bioreactors have explicitly been designed to exploit particular variations on the regular forced perfusion setups, by allowing for bidirectional [48] or oscillating flows [49]. However, oscillating or bidirectional flow patterns can be induced with many other bioreactor setups as well, even though this is not explicitly mentioned in the patent.

In addition to enhancing nutrient supply, forced perfusion has been proposed as a method to mechanically stimulate cells. The drag that comes along with the transport of fluid theoretically imposes shear forces on the cells in the construct. Since cells are known to respond to shear loading, in some patents fluid-flow-induced shear ('FIS' in Table 1) as a result of forced perfusion is actually mentioned as a method to stimulate the metabolism of cells [44,46-49]. In fact, all aforementioned forced-perfusion bioreactors, including those that did not mention flow-induced shear in the application, actually belong to this category as well. Few bioreactor patents explicitly mention that flow-induced shear in their systems will stimulate cell metabolism, without forced perfusion. These bioreactors rather induce shear by floating the medium over cells in monolayer [46,64], or by moving or shaking the culture receptacle [67] such that fluid-flow-induced shear forces exist on the outer surfaces of the scaffold only.

Discussion

Whether forced perfusion is included with the aim of enhanced nutrition or with the aim of inducing shear forces, both effects automatically occur at the same time and will interact with each other. Optimal flow for nutrition, however, may differ from optimum flow for stimulation of cells by shearing. Interestingly, nutrition depends merely on the volume of fluid, while the shear forces depend on the velocity of the fluid. In principle, both can be tuned at the same time by choosing the appropriate scaffold porosity.

However, optimization is difficult for many reasons. First, it is not known what the optimum values are for both shear stimulation and nutrition. Second, the choice for the material properties of the scaffold depends on many more aspects than on fluid perfusion alone. Third, the porosity of the construct changes with time, when the development of tissue proceeds. It is not easy to monitor this, let alone to adjust perfusion settings accordingly.

Another important issue in forced-perfusion setups is that the local flow through the tissue largely depends on the distribution of the permeability. The fluid, driven by a pressure gradient, follows the path with the least resistance. Two aspects are of particular importance. First, the majority of all fluid that is meant to pass through the construct may actually pass through a small leakage between the construct and the container wall. Bioreactor design is critical to prevent such alternative pathways for fluid flow. Second, low-resistance pathways through the material will develop in time, making both the volume and the velocity of the fluid flow very non-homogenous. Hence, local tissue perfusion depends largely on the quality and homogeneity of the scaffold material [72]. However, even when initially homogeneity is taken care of appropriately, the tissue is likely to develop into a non-homogenous construct during culture and preferential perfusion pathways will develop. These heterogeneities prevent proper tuning of nutrient supply and shear loading throughout the tissue. Thus, although nutrient supply will on the average be enhanced under forced perfusion conditions as compared to free diffusion, care must be taken when using this technique and interpreting data.

The above remarks hold for relatively porous materials. In cartilage tissue engineering studies however, cells are regularly seeded in agarose or alginate gels. The fluid flow that can be forced through such gels is very low. For instance, the Darcy permeability at zero pressure is only $6.61 \times 10^{-13} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}$ for 2% agarose gels [73]. Thus, when a standard 2 mm thick sample is subjected to 10 kPa fluid pressure difference between top and bottom, fluid flux is only 3.3 $\mu\text{m/s}$. In reality, perfusion is even less, because permeability is strain dependent. The permeability of 2% agarose gels decreases to 70% at 10% compression [73]. Given the aggregate modulus of only 6 kPa [11,12], 2% agarose gel deformation will be excessive at constant 10 kPa fluid pressure. It is questionable whether this low flow meets the flow-rate necessary to enhance cell metabolism. Also, the beneficial effects of forced perfusion on nutrition may be limited. In a disc-shaped cell-seeded agarose construct of 10 mm diameter, it takes approximately 15 minutes to force one ml water through an undeformed construct. The transport of (larger) solutes will be even less. In comparison with the above mentioned fluid flux of 3.3 $\mu\text{m/s}$ for water, the diffusivity of large albumin proteins in 3% agarose is around 40 $\mu\text{m}^2/\text{s}$ [74]. Hence, for the relatively small nutrients that are in danger of depletion in the center of a construct, such as oxygen and glucose, the additive effect of forced perfusion over simple diffusion only, will probably be limited.

Yet, forced perfusion has proven to be very effective as it enhances the synthesis of cartilage matrix proteins [75-77] and improves the distribution of matrix within the scaffold

[78], as recently reviewed [20,53]. Part of the beneficial effects of forced perfusion setups may in fact result from mixing of the culture medium around the construct due to fluid flow in the container, rather than from the forced perfusion itself. This mixing effect has been shown very important [33,37] and many bioreactor systems have therefore included a method for mixing the culture medium ('MIX' in Table 1) [41,43,60,62,63,66-68,79]. This way the tissue is kept in place and additional mechanical stimulation by compression plates is enabled. Alternatively, the tissue can be moved through the medium as is for instance done in the already mentioned RWV-bioreactor type (many RWV-related patents exist, an early example is [38]).

Other Stimulation Methods

There are some other methods of stimulation that are of interest for cartilage tissue engineering in this review, yet are not captured by the above descriptions. One of those is a bioreactor by which the tissue is indented with a small indenter through a flexible membrane [43]. Obviously, indentation is possible in most bioreactors that allow for confined or unconfined compression, by just changing the size of the indenter. Yet, none of these patents mentions indentation as a method of stimulation.

Another interesting patent induces tissue stress by restricting the normal compaction of collagen gel [71]. This interesting method induces tension as well as orientation in the tissue. Hence, it may be of more interest to the engineering of tissues with aligned fiber orientations such as tendons and ligaments, rather than to articular cartilage.

Finally, only one of the mentioned patents has the interesting ability to assess tissue growth, using a linear variable displacement transducer (LVDT) [61]. In addition, this bioreactor design can actually measure both force and displacement at the same time, a feature that is only possible in two other patents [41,59].

CURRENT & FUTURE DEVELOPMENTS

After reviewing these patents, the question emerges which of the developed bioreactors has proven successful in the literature. Beneficial effects of principles that are being used in several of the reviewed bioreactor designs have recently been reviewed [19-21]. Surprisingly, to our best knowledge, the biological efficacy of only one patented bioreactor has been evaluated in scientific journal publications. This is the bioreactor developed by Mizuno *et al*, for which the patent was accepted in 2006, but first claims were already filed in 2002 [44]. In 2005, the authors thoroughly tested and validated the performance of their hydrostatic pressure and perfusion culture system [80]. Previously, it had been shown that both cyclic and static hydrostatic fluid pressure of 2.8 MPa, applied within this bioreactor-system, enhances the synthesis of cartilage-specific matrix components in chondrocyte-seeded collagen sponges three-fold after 15 days of culture [81].

A major issue for bioreactor development remains the difference in requirements between the desired control on the tissue engineering process on the one hand and cost-effectiveness on the other hand. Cartilage tissue is a well-organized structure, dedicated to resisting mechanical loa-

ding. The premise is that regenerating such structure requires simulation of 'physiological and mechanical conditions' in culture. To optimize such conditions requires complete control over the mechanical stimulation, i.e. simultaneous and accurate measurement of force and displacement of the actuator. One of the additional benefits of this possibility is that it provides information about the current mechanical properties (e.g. stiffness) of the tissue and the changes thereof with time of culture. This can for instance be used to determine the end-point of culture, or to make sample-specific adjustments to the stimulation protocol. Controlling tissue development more rigorously may become particularly important when considering that future applications require the use of cells from individual patients. Cells from each patient respond differently due to differences in genes, age, harvest location, stage of osteoarthritis, etc. To optimize the tissue engineering process, protocols will have to be updated per patient during culture, based on the response of the cells. This requires more accurate information on the current development of the tissue.

The drawback is that the demands for such mechanical sensors in cartilage tissue engineering are high: compressing a 2 mm thick cartilage tissue by 10 % with 10 % accuracy, which is the minimum requirement, requires a displacement transducer with an accuracy of well-below 20 μm . The force to apply 10% compression to a 10 mm diameter cartilage disc with a modulus of 1 MPa is approximately 8 N. Starting properties of widely-used agarose scaffolds however, are 100 times weaker [11,12], requiring force sensors with accuracies in the mN range. Such delicate force sensors are costly, prone to damaging and it is difficult to implement them in a bioreactor while assuring sterility, user-friendliness and cost-effectiveness. Because each sample may develop differently, for instance due to unequal cell distributions or scaffold heterogeneities, full control it is only possible if force and displacement are measured per sample. This importantly limits the possibilities to run experiments in parallel with various experimental conditions and statistically sufficient group sizes at a time. From a commercial viewpoint, important to ultimately make clinical applicability feasible, bioreactors should be easy to handle, culture should not be labor-intensive (preferably fully automated), the process must be inexpensive and the risk for infections should be minimized/excluded by using disposable containers and culture bags. Since these requirements for commercial production and highly-controlled setups do not match, we anticipate future developments in two opposing ways. On the one hand, bioreactors will be developed with more control over the mechanical conditions and loading regime than presently available. Such bioreactors, which can handle only few samples at a time, are used in research laboratories with mechanobiological expertise, presumably in biomedical engineering groups at universities. On the other hand, cheap disposable bioreactors for culturing of multiple samples at a time will be developed. Some automated closed-loop bioreactor systems that meet GMP criteria are already available [41,62]. We anticipate that the eventually clinically-used commercial bioreactors will have to include possibilities to apply dynamical mechanical loading, but a full feedback-controlled setup will not be feasible. Which loading regime to implement, can only be derived from insights that emerge

from the highly-controlled systems. Hence, close interaction between biomedical industry and research groups at universities is of major importance to ultimately reach the aim of developing a fully automated and clinically applicable bed-side bioreactor for articular cartilage tissue engineering.

Tissue engineering culture protocols merely result from labor-intensive trial and error experiments. The main reason is probably that the diversity in possible mechanical and biochemical loading regimes is enormous, and that metabolic responses are distinct. With regard to the mechanical conditions, differences exist between loading magnitude, frequency and rate. Also, the duration of dynamic loading periods and intermittent non-loading periods affect the response of the cells, and these effects are again modulated by the (temporal) addition of particular biochemical additives. In addition, boundary conditions such as porosity of loading plates, mixing or refreshment of culture medium and fluid pressurization or forced perfusion are relevant. Some growth factors and particular mechanical loading regimes have extremely non-linear interactions. As a result, it is hard to predict the consequence of specific culture conditions. In accordance with this variety of conditions, the mechanical stimulation devices in bioreactor patents are very diverse. What precisely is the best stimulation protocol is yet unknown and hard to determine. Computer-guided study designs can be helpful in this context, and using computers to interpret and extrapolate experimental data may turn out to be the only way to break through the trial-and-error-like approaches that are adopted nowadays. In the past few years many different theoretical models for cartilage mechanics have been developed [82-89]. These models form the basis for computer models that are dedicated for describing cartilage tissue engineering [33,72,90-97]. Unfortunately, computational models have not yet been integrated in tissue engineering studies. We speculate that computer-guided tissue engineering approaches will in the future show extremely valuable. These models can be applied to design loading protocols for tissue engineering and to discriminate between promising protocols and those with poor potential, prior to labor-intensive experiments.

Some bioreactors contain particular features that have not been mentioned before, yet are worth mentioning. Such features relate to the control of the tissue engineering process in distinctive ways. For instance, some bioreactors allow for visual inspection of the tissue through a glass window [41,68]. Such possibility may be very helpful to follow the tissue engineering process in time. An obvious limitation is that visual inspection using a microscope requires the glass to be relatively thin and therefore fragile. Hence, such glasses cannot be used as supporting plates for a mechanically loaded construct. Also, working distances may be relatively large, allowing only for modest magnifications with a microscope, and it is only possible to observe the outside of the developing construct, whereas most problems occur in the center. Finally, microscopic inspection requires some sort of staining, presumably with fluorescent dyes in several mm thick constructs. Some fluorescent dyes, such as the CNA35 for collagen [98], can be used during culture. However, the use of such probes is limited to *in vitro* cultures and cannot be used in implants. A second and probably more versatile example is the use of other measure-

ment systems such as MRI or ultrasound elastomicrography imaging to follow tissue development [99-101]. Although, these techniques may provide ample information of the development of matrix throughout the construct, it has obvious drawbacks related to usability and cost-effectiveness. Alternative options that are more likely to be implemented as feedback for the tissue development include (real-time) measurements of other metabolites such as oxygen, glucose or lactate (pH). Several bioreactors already facilitate such measurements, such as the one by Millenium Biologix [25]. On the actuator side, other types of stimulation have also received attention. For instance, in one bioreactor patent electrical stimulation of the tissue is aimed for, with mechanical loading being optional [64]. Also, it has been shown that rather than actively stretching or compressing a tissue, a well aligned tissue develops when compaction of the cell-seeded collagen gel is prohibited. This mechanical loading mechanism has also resulted in the filing of a bioreactor patent [71].

A final remark is that we have not reviewed the very important area of biomaterials. Suitable chondrogenic materials are of ultimate importance to the success of cartilage tissue engineering. To stimulate chondrocyte metabolism and at the same time prevent differentiation into a fibroblastic phenotypes remains difficult. The scaffold material clearly plays a key-role here. For this reason agarose and alginate gels are still widely used in the literature. Even though they are weak and not degradable, they still perform best in preventing chondrocyte differentiation and stimulating cartilage matrix synthesis. Many studies and patents are dedicated to the development of more suitable, biodegradable scaffold materials for cartilage tissue engineering. This, however, is beyond the scope of this review.

In conclusion, although it is possible to generate a tissue engineered construct that resembles articular cartilage, we are still unable to generate a tissue that is able to withstand the severe mechanical conditions in a joint. Therefore, one of the most important challenges in cartilage tissue engineering is to elucidate by which loading regimes the mechanical quality of the tissue is optimized. Studying this requires bioreactors that incorporate loading devices. Many developments in this area have resulted in the patents reviewed in this paper. These bioreactors will enable researchers to propose loading protocols for the development of a mechanically stable tissue engineered cartilage with clinical potential. These loading regimes will then lead to the development of automated, easy to operate and cost-effective bioreactor systems that can work under GMP conditions. Only when this stage is reached, tissue engineered cartilage implantation will become the primary choice of treatment for millions of patients suffering from osteoarthritis.

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