Development and Validation of a Novel Bioreactor System for Load- and Perfusion-Controlled Tissue Engineering of Chondrocyte- Constructs

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ABSTRACT: Osteoarthritis is a severe socio-economic disease, for which a suitable treatment modality does not exist. Tissue engineering of cartilage transplants is the most promising method to treat focal cartilage defects. However, current culturing procedures do not yet meet the requirements for clinical implementation. This article presents a novel bioreactor device for the functional tissue engineering of articular cartilage which enables cyclic mechanical loading combined with medium perfusion over long periods of time, under controlled cultivation and stimulation conditions whilst ensuring system sterility. The closed bioreactor consists of a small, perfused, autoclavable, twin chamber culture device with a contactless actuator for mechanical loading. Uni-axial loading is guided by externally applied magnetic fields with real-time feedback-control from a platform load cell and an inductive proximity sensor. This precise measurement allows the development of the mechanical properties of the cultured tissue to be monitored in real-time. This is an essential step towards clinical implementation, as it allows accounting for differences in the culture procedure induced by patient-variability. This article describes, based on standard agarose hydrogels of 3 mm height and 10 mm diameter, the technical concept, implementation, scalability, reproducibility, precision, and the calibration procedures of the whole bioreactor instrument. Particular attention is given to the contactless loading system by which chondrocyte scaffolds can be compressed at defined loading frequencies and magnitudes, whilst maintaining an aseptic cultivation procedure. In a "proof of principle" experiment, chondrocyte seeded agarose gels were cultured for 21 days in the bioreactor system. Intermittent medium perfusion at a steady flow rate (0.5 mL/min) was applied. Sterility and cell viability (ds-DNA quantification and fluorometric live/dead staining) were preserved in the system. Flow induced shear stress stimulated sGAG (sulfated glycosaminoglycan) content (DMMB assay) after 21 days, which was confirmed by histological staining of Alcian blue and by immunostaining of Aggrecan. Experimental data on mechanotransduction and long-term studies on the beneficial effects of combined perfusion and different mechanical loading patterns on chondrocyte seeded scaffolds will be published separately. Biotechnol. Bioeng. 2008;101: 714–728. © 2008 Wiley Periodicals, Inc.

KEYWORDS: chondrocytes; cartilage; bioreactor; tissue engineering; biomedical engineering; physical stimulation

Introduction

Articular cartilage has a high load bearing capacity (Cohen et al., 1998) and a smooth joint surface with low friction due to its structure and composition (Buckwalter...
and Mankin, 1998a). Native articular cartilage contains a highly organized extracellular matrix (ECM) (Buckwalter et al., 1988) which is maintained by chondrocytes, the only cell-type in cartilage (Buckwalter and Mankin, 1998b). Due to the limited regenerative capacity of articular cartilage, damage is often progressive. Therefore, the prevalence of osteoarthritis is high. Due to the socio-economical impact (Buckwalter, 2002), there is a pressing requirement for innovative strategies to repair damaged hyaline cartilage (Hunziker, 2002). One promising approach is to replace damaged cartilage with tissue engineered grafts (Langer, 2000; Langer and Vacanti, 1993; Lindahl et al., 2001).

To create such grafts involves the in vitro cultivation of chondrocytes (Kuo et al., 2006; Marlovits et al., 2006; Nesic et al., 2006) in a suitable matrix material (Behrens et al., 2006) in an appropriate culture system (Martin et al., 2004). These chondrocyte grafts in the shape of matrix-coupled autologous cartilage transplants (MACt) must be biocompatible and have appropriate mechanical properties in order to be clinically useful (Andereya et al., 2006; Maracchi et al., 2005). Many approaches are now being taken to enhance the quality of these cartilage substitutes through the application of bioreactor devices with different working principles (Donkelaar and Schulz, 2008; Schulz and Bader, 2007). Bioreactor technologies should be automated to become of economical interest, allow up scaling, ensure reliable, reproducible outcomes with low contamination risk and high product safety. Such automated systems should be able to monitor, control and regulate operational (e.g., media supplementation, nutrient supply, oxygen tension), mechanical, biochemical and biophysical (e.g., pH, exchange of gases, humidity, and temperature) environmental conditions, and perform aseptically several bioprocess operations (e.g., scaffold seeding, feeding, and sampling). Ultimately, clinically used automated bioreactors are equipped with feedback controlled operation procedures to cope with expected patient-variability. The specific requirements for an extracorporeal cultivation system can be distilled from the results of the pioneering experiments, as recently reviewed (Schulz and Bader, 2007).

In addition to the above-mentioned global biotechnological needs, two key requirements for three-dimensional (3D) cartilage tissue engineering include the ability for mechanical loading and the delivery of nutrients to and removal of waste products. Application of mechanical loading to chondrocytes is essential for developing cartilage constructs with sufficient mechanical properties (LeBaron and Athanasiou, 2000). Dynamic mechanical stimulation of chondrocytes in vitro can modulate chondrocyte metabolism, improve ECM synthesis, prevent loss of chondrocytic phenotype (Chowdhury et al., 2003; Davisson et al., 2002a; Lima et al., 2006; Mauck et al., 2000, 2003; Risbud and Sittinger, 2002), and enhance the load bearing capacity of the graft (Kelly et al., 2006). Chondrocytes rapidly dedifferentiate in static culture and cease to synthesize the appropriate matrix in the absence of appropriate mechanical loading (Domm et al., 2002; Vunjak-Novakovic et al., 1999).

Active tissue perfusion is often considered to ensure the constant supply of nutrients and removal of waste products (Fermor et al., 2005; Galban and Locke, 1999; Grimshaw and Mason, 2001; Hofstaetter et al., 2005; Sengers et al., 2005). Another beneficial effect of perfusion is that the fluid-flow induced shear stress stimulates the expression of cartilage specific matrix markers (Darling and Athanasiou, 2003; Heath and Magari, 1996).

A number of cultivation systems are geared towards applying mechanical stimuli by compressive loading (Buschmann et al., 1995; Cassino et al., 2007; Davisson et al., 2002a; Demartea et al., 2003; Lee and Bader, 1997; Mauck et al., 2002; Torzilli et al., 1997), bending or tensile stress (De Witt et al., 1984; Fukuda et al., 1997; Millward-Sadler et al., 2000; Wright et al., 1997), shear stress (Frank et al., 2000; Jin et al., 2001; Waldman et al., 2003), or hydrostatic pressure (Carver and Heath, 1999a; Ikenoue et al., 2003; Parkkinnen et al., 1993). Others have developed fluid flow culture systems that are intended to provide the appropriate biochemical environment, via direct medium perfusion through cell seeded scaffolds (Davisson et al., 2002b; Kim et al., 1994; Pazzano et al., 2000; Sittinger et al., 1994; Wendt et al., 2003), by inducing fluid motion around the scaffolds (e.g., using spinner flasks (Freed et al., 1994; Vunjak-Novakovic et al., 1999), orbital shakers (Martin et al., 1998), or rotating wall vessels (Freed et al., 1998; Obradovic et al., 2001), or which enable the constructs to be transferred between separate perfusion and loading systems (Carver and Heath, 1999b). Surprisingly, only two devices (Demartea et al., 2003; Mizuno et al., 2002) allow cartilage substitutes to be cultivated in an environment in which both fluid-handling and compressive loading are controlled.

Once the above conditions are met, the final requirement before clinical application is feasible is to make the culture system compliant to cGMP regulations. Ideally, chondrocytes would be aseptically delivered to the decentralized production system with the bioreactor then remaining completely closed, and they would be processed through the entire tissue manufacturing (cell isolation, scaffold seeding or inoculation, sampling, proliferation and differentiation phases) according to cGMP regulations using a respective quality control/quality assurance (QC/QA) program until implantation when the resultant cartilage would be aseptically transferred from the device to the recipient within the operating theatre (Martin et al., 2004). During the past few years biomedical companies, for example, Aastrom Biosciences (Armstrong et al., 1997), Millenium Biologix (Smith et al., 2005), and Olympus (Hibino et al., 2005) have claimed and developed the so-called cartridge-based bioreactor systems for the cGMP-compliant manufacture of mainly autologous chondrocyte transplants as a tissue-engineered product for therapeutic use in osteoarthritic patients (Donkelaar and Schulz, 2008; Schulz et al.: Bioreactor System for Bioengineered Cartilage Grafts 715 Biotechnology and Bioengineering
However, to the best of our knowledge, none of these industrial concepts allows mechanical loading. Probably the most important reason for this drawback is that current concepts of load transmission are based on pistons that are attached to non-sterile actuators on the one side, and connect to the sterile cultivation area on the other side. This is a potential contamination risk contradicting the present guidelines of GMP.

We set out to develop a new bioreactor system that allows controlled mechanical loading and simultaneous perfusion to be applied in a closed aseptic bioreactor equipped with an accurate, integrated mechanical loading device which guarantees for product safety and system sterility. These are the principal requirements to develop a system that operates under GMP conditions. This bioreactor is to be used for the tissue engineering of articular cartilage equivalents in both research and clinical applications. The purpose of this technical article is to describe the resulting novel bioreactor design, which uses a magnetic actuator for contact-free load transmission, controlled by an integrated sensing concept for monitoring applied force and construct deformation in real-time. This allows the use of advanced feedback control for a fully automated stimulation process under cGMP conditions. The technological developments, calibration procedures and validation experiments were specifically designed to expose a model scaffold of chondrocyte-seeded agarose gels (3% (w/w); outer diameter (OD) of 10 mm, and height (H) of 3 mm) to a controlled regime of compressive deformation. The applicability of the bioreactor was evaluated in a 3-week experiment in which porcine articular chondrocytes in a 3D agarose hydrogel are subjected to forced perfusion. Cell viability and deposition of extracellular matrix were compared between perfused and control samples. The biological “proof of principle” of the complete bioreactor system in experiments on mechanotransduction and in long-term studies on the beneficial effects of combined perfusion and mechanical loading lie in the scope of subsequent articles.

**Materials and Methods**

**Bioreactor Device**

**Bioreactor Construction**

The bioreactor (Fig. 1) is constructed from a 32 mm long, 28 mm diameter polycarbonate (Makrolon, Bayer AG, Leverkusen, Germany) cylinder (I). A 30 mm long and 15 mm diameter hole was centrally drilled. In the remaining 2.0 mm depth, a 1.5 mm central hole of 7 mm diameter was drilled, which will be referred to as the lower chamber. The remaining 0.5 mm polycarbonate wall is highly polished in order to serve as an effective (98%) transparent surface to examine constructs inside the bioreactor with an inverted microscope. The vessel (I) is closed with a cylindrical polycarbonate lid (II) and a 1.5 mm Viton O-ring. The inside of the lid contains a central hole 10 mm in diameter and 10.5 mm in depth to fit the mini-actuator (V). Constructs (III) are precisely located in the bioreactor with the help of a perforated locating stage (IV) such that they are centrally positioned and with the entire lower surface of the construct being situated 0.75 mm above the collar, covering the lower chamber. The inner wall of the location stage limits the movement of the actuator such that the applied compressive strain by the mini-actuator (V) cannot exceed a predefined magnitude. The construct and the locating stage divide the bioreactor into two chambers. The narrower lower chamber serves as a pre-mixing compartment. It contains two diametrically opposed ports (1 mm OD, 0.7 mm inner diameter (ID)) which can serve as fluid in- or outlet. Two additional ports are located on the circumferential wall of the upper chamber. One meets the circumferential inner wall tangentially 6.5 mm above the collar, the other is located radially 12.5 mm above the collar.

![Figure 1. Mechanical drawing (A), isometric sketch (B) and image of the bioreactor seeding procedure with chondrocyte-agarose constructs of 3 mm height and 10 mm in diameter (C). Annotations: I = vessel, II = lid, III = constructs, IV = locating stage, V = mini actuator.](image-url)
A cylindrical neodymium iron boron (NdFeB) magnet (Fig. 2A) (4 mm OD, 25 mm length) (S-04-25-N, Webcraft GmbH, Uster, Switzerland) is placed in a stainless steel tube (5 mm OD, 30.3 mm length) that is located inside the bioreactor. A PTFE cuff (5 mm ID, 10 mm OD, 20 mm length) surrounds this cylinder at one end which is then inserted in the lid as described above. The opposite side of the tube holds a stainless steel construct loading plate (15 mm OD, 1 mm thick). This plate is perforated with a series of 0.5 mm diameter holes to enable medium transfer. The lower surface of this plate which is in contact with the tissue engineering construct is coated with an ultra-thin layer of PTFE to prevent adhesion. This construction allows for a vertical displacement of the entire stainless steel shaft of 0.9 mm, that is, its position varies between 0.15 mm above a 3.0 mm cartilage construct, and can induce a maximum compression of 0.75 mm, or 25% axial compression. As mentioned before, the location stage can be used to limit the maximal imposable strain. The weight of the entire mini actuator is 7.5 g, the PTFE cuff weighs 2.6 g. The friction-coefficient ($\mu$) between PTFE and stainless steel is only 0.04 under dry conditions, and less when lubricated. Even when the horizontal force $F_n$ reaches half the applied vertical stimulation force $F_S$, the maximal frictional force $F_t$ will reach maximal 2% of $F_S$.

$$F_t = \mu F_n = 0.04 \cdot \frac{1}{2} F_S = 0.02 F_S$$  \hspace{1cm} (1)
to the SFC-DC-VC-3-E-HO-IO controller with an implemented CANopen interface (both Festo AG & Co. KG, Esslingen, Germany).

**Inductive Proximity Sensor \( S_{\text{Pos}} \) and Platform Load Cell Sensor \( S_{\text{Force}} \)**

The aim of tissue engineering is to improve tissue properties with cultivation time (Kelly et al., 2006; Mauck et al., 2002). The possibility of monitoring the change of tissue properties in time would therefore be extremely valuable. It is possible to derive this information from the relation between applied load and associated strain. Dynamic protocols such as those used during tissue engineering are particularly suitable as they can provide information on both the elastic and the viscous behavior of the tissue. To enable this requires components to independently monitor the position of the loading plate and imposed load. The position of the loading plate in the bioreactor is detected in a contactless manner by an inductive, analog displacement sensor (BAW-M18MG-UAC80F, Balluff GmbH, Neuhausen, Germany), positioned below the bioreactor. The sensor reports distances between 2 and 8 mm (repeat accuracy of ±0.012 mm, response time of 1.5 ms) with the metal loading plate. The imposed load is determined using a platform load cell (\( S_{\text{Force}} \)) PW4KRC3-MR (HBM GmbH, Darmstadt, Germany), maximal load 5 or 30 N, situated between the bioreactor and the fixed loading structure. Analog output signals of the displacement and force sensors are digitized using CANopen analog input modules (CAN-CBM-AI4, esd GmbH, Germany).

**Complete Bioreactor System**

Figure 3A illustrates how the described bioreactor components are assembled. The bioreactor (BR) and proximity sensor (\( S_{\text{Pos}} \)) are coupled to the fixed platform load cell (\( S_{\text{Force}} \)). The rotating magnet system which determines the mechanical stimulation frequency (\( A_{\text{SF}} \)) is directly linked to the actor for its vertical positioning to control loading magnitude (\( A_{\text{VD}} \)). This setup uncouples the actuating system from the sensory system. In practice, the system can be an individual bioreactor (Fig. 3B), or one with several systems running in parallel.

Figure 3C shows a station with six bioreactors where each culture device is directly connected to its own individual inductive distance sensor (\( S_{\text{Pos}} \)) that itself is fixed to the rack via a single load cell (\( S_{\text{Force}} \)) with nominal load 30 N. The six control magnet suspensions are connected to an adjustable bar which is belt-driven by one motor. Both actors (\( A_{\text{VD}} \), \( A_{\text{SF}} \)) were directly connected to the rack, and thus did not affect the sensors. This principle minimizes the amount of sensor and actor components, thus increasing cost-effectiveness whilst allowing for appropriate monitoring of the mechanical conditioning of, for example, six hydrogels descending from one batch. Figure 3D shows an example of a station where six separated bioreactors are integrated, and with a separate perfusion periphery consisting of its own pump, reservoir, tubing, and sterile filter. This particular station is used in the “proof of principle” perfusion experiment explained below.

**Control Program**

Magnet rotation velocity (\( A_{\text{SF}} \)) and position (\( A_{\text{VD}} \)) control are automated based on the output signals from the platform load cell (\( S_{\text{Force}} \)) and the inductive proximity sensor (\( S_{\text{Pos}} \)). Figure 4 provides a block diagram of the closed-loop control system. The control unit, a CBS637 embedded system (Cogent, Computer Systems Inc., Smithfield, RI), works as a CANopen node through a USB/CAN converter (PCAN USB-ISO, PEAK System Technik GmbH, Darmstadt, Germany). The control software provides continuous feedback of the process and allows at any time to adjust parameters of the otherwise fully automated process. Additional sensors or actuators, for example pumps, flow sensors and the incubator, can easily be integrated in the system.

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**Figure 3.** The developed bioreactor system is composed of actuating and sensing components for control of mechanical loading of three-dimensional constructs inside the cultivation device in a contact-free manner. The illustration depicts the concept of the established control scheme (A) whereas the image shows the technical realization of a measuring station for an individual bioreactor (B). The technical sketch depicts the adaptation of this monitoring principle into a rack for six separated bioreactors (C) that itself were implemented into an individual perfusion environment (D). The models shown here are equipped with a manual motorized height adjuster. Abbreviations in this figure: \( BR = \) bioreactor, \( A_{\text{VD}} = \) actuator vertical distance, \( A_{\text{SF}} = \) actuator stimulation frequency, \( S_{\text{Pos}} = \) sensor position measurement, \( S_{\text{Force}} = \) sensor force measurement. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]
Sensor Calibration

Calibration experiments with the load (S\textsubscript{Force}) and position sensor (S\textsubscript{Pos}) focused on accuracy, reproducibility, and potential zero point drift. Following standard calibration, the load cell was calibrated in the bioreactor system by positioning standard weights (varying between 5 and 500 g) onto the locating stage or into the cultivation chamber of the bioreactor. Linear regression analyses between applied and measured loads correlate well ($r^2 = 0.9989$); the standard deviation of force variations from the linear fit shows a relative full-scale error of 0.48%.

Similarly, the inductive proximity sensor S\textsubscript{Pos} was calibrated on the assembled bioreactor system. Using a micrometer head (164–161, Mitutoyo Messgeräte GmbH, Neuss, Germany), with a measurement range of 0–50 mm and accuracy of 1.0 μm, the bioreactor was displaced several micrometers at a time over the whole range of 5–8 mm, while the positioning of the system was recorded with the inductive proximity sensor. The imposed and measured positions, averaged over eight measurements, correlated linearly ($r^2 = 0.9963$), with a relative full-scale error of 1.04% (data not shown).

Prior to an experiment, the position sensor was calibrated using the lower and upper reversal points of the actuator. At the lower reversal point it touched the inner wall of the locating stage, which has an exactly known height. The upper point equals the top of the container.

Subsequently, a 3% agarose construct (10 mm diameter, 3 mm high) was installed and the bioreactor was filled with culture medium. By adjusting the vertical position of the magnet and using feedback by the force-sensor, the actuator plate was positioned such that it was freely floating in the culture medium without touching the construct. Under this condition the force sensor was zeroed. Finally, the magnets were lowered until the force sensor was loaded 0.02 N. Sample height was now determined from the actual position and the previous position where the actuator touched the inner wall of the locating stage. The displacement sensor was zeroed at sample height.

Assessment of Drift

The fully calibrated system was permitted to run continuously without process control intervention in order to quantify drift. Software instructions were issued with the following input parameters: Maximal compressive strain 15% s 15 (0.45 mm), compressive load frequency 1 Hz, cyclic load duration 120 min, rest time 120 min. This load-rest cycle was run for 2 days followed by a rest period of 2 days. This was done twice under a constant environment (temperature, relative humidity). No signs of drift of both S\textsubscript{Force} and S\textsubscript{Pos} sensors were observed after these 8 days.

Perfusion Experiment

Six parallel bioreactor devices are integrated in one system, each with its own closed loop perfusion circuit containing an individual medium container, pump, flow sensor and sterile filters (see Fig. 3C and D). Medium is delivered to the lower compartment of each bioreactor via one of the inflow ports connected to a 20 mL medium reservoir via gas permeable silicone tubing (inner diameter 1 mm). A micro annular gear pump (MZR 2521) and a control unit (S-KD, both HNP Mikrosysteme GmbH, Parchim, Germany) are used to regulate medium flow. The pump also serves as a dead volume-free, self-closing valve. The flow rate is set at 0.5 mL/min, as monitored using a CMOS thermal mass flow sensor (ALS1430-24, Sensirion AG, Staefa, Switzerland). Medium
exiting the bioreactor is returned to the reservoir via the same type of tubing through the radial outlet port in the upper chamber. The medium in the reservoir is oxygenated (95% air, 5% CO₂) by means of additional tubing connection and a single gassing pump. The entire system is placed in an incubator (Thermo Electron, GmbH, Oberhausen, Germany) which records temperature, relative humidity, carbon dioxide and oxygen partial pressures (see Fig. 4C).

Cell Isolation, Construct Preparation, and Culture Conditions

Unless otherwise stated chemicals were obtained from Sigma–Aldrich (Taufkirchen, Germany). Chondrocytes were isolated and seeded into agarose hydrogels as previously described (Schulz et al., 2006). Briefly, articular cartilage samples were aseptically dissected from the metacarpophalangeal joint from porcine feet (n = 3) obtained from the local abattoir. The isolation of the chondrocytes was performed by mechanically mincing the cartilage samples, followed by enzymatic digestion with 2 mg/mL Collagenase A in Dulbecco’s modified Eagle’s medium (DMEM) (Biochrom KG, Berlin, Germany) at 37°C for 24 h. After enzymatic isolation, the primary porcine chondrocytes were counted and the viability was determined using Trypan-blue. Viability greater than 95% was usually achieved. Base medium containing sodium pyruvate (110 mg/L), L-glutamine (580 mg/L), fetal bovine serum (10%) (Invitrogen, Karlsruhe, Germany), ascorbic acid (0.05 mg/mL) and gentamycin (50 μg/mL; PAA Laboratories, Pasching, Austria) was used for the culturing. For cell seeding, 1 mL of chondrocyte suspension (3 x 10⁶ viable cells) was thoroughly mixed with 10 mL of 3.3% agarose type VII in a pipette. Individual 250 μL aliquots of the agarose chondrocyte suspensions (containing 0.75 x 10⁶ primary cells) were seeded into individual wells of a mould filled with 2 mL medium, and incubated under static, free swelling conditions for 24 h at 37°C, 5% CO₂. The constructs were then either placed in the bioreactor to be subjected to perfusion or maintained as controls in Petro dishes with the identical medium volume (20 mL) as used in the perfusion system. For 21 days, an hourly intermittent perfusion regime was applied in the bioreactor with a flow rate of 0.5 mL/min for 10 min followed by a rest period of 50 min. The chambers were disconnected at days 7 and 21, to aseptically remove the constructs for subsequent analysis. Three independent experiments were carried out.

Cell Viability and Cell Content

Cell viability in the agarose gels was assessed using a calcein AM (green) and ethidiumhomodimer (red) fluorometric staining kit (Mobitec, Göttingen, Germany). The number of chondrocytes per construct was indirectly determined by quantification of DNA content from aliquots of papain digests of the hydrogels using the ds-DNA Quantiations Kit (Mobitec). To obtain papain digests, the agarose specimens were mixed with 750 μL papain digestion buffer (5 mM l-cystein, 5 mM EDTA, 100 mM Na₂HPO₄) and melted at 70°C for 1 h, cooled down to 45°C, followed by a terminal 16 h digestion step in 5 μL papain-solution (50 μg) and 10 μL agarase-solution (1.66 U). Afterwards, all samples were stored at −20°C until analysis. After preparation of a Lambda-DNA-standard in a range of 0.02–2 μg, the 100 μL samples were incubated with 100 μL PicoGreen dye for 5 min and measured in a fluorescence spectrometer at 480 nm excitation and 520 nm emission. To convert between DNA content and cell number, 7.7 pg DNA per chondrocyte was assumed.

Determination of Sulfated Glycosaminoglycan Content

Samples of each of the papain digests were also analyzed for the proteoglycan content by quantifying the sGAG content using the DMMB (1,9 dimethyl-methyleneblue) dye binding assay (Roche, Basel, Switzerland). The absorbance was determined in a photometer at 595 nm, the concentration of sGAG was extrapolated from a master curve based on chondroitin sulfate with a range of 10–100 μg/mL. All samples and standards were done in duplicate. sGAG was measured in μg/mL and reported as weight per DNA or weight per wet weight of construct.

Histological Analysis and Immunocytochemistry

Agarose gels were shock frozen, embedded in Tissuetek and sliced with a cryotom (both Leica, Bensheim, Germany). The 6 μm thick sections were processed for histological analysis with Alcian blue staining to verify proteoglycan accumulation. Immunostaining of aggrecan was performed according to a two step indirect method. The sections were treated with monoclonal mouse antibody against aggrecan (1:200; DPC Biermann, Bad Nauheim, Germany). After washing with PBS, the secondary antibody of POD conjugated goat anti-mouse IgG (1:50; Jackson Immuno Research, Cambridgeshire, UK) was added for 1 h at 37°C. Immunostaining used AEC (3-amino-9-ethyl-carbazol) substrate.

Statistical Analyses

All results were expressed as mean ± standard error of the mean of at least three separate experiments (n = 3) and analyzed with statistical software (Origin, Friedrichsdorf, Germany). The data from different animals are pooled together. For pairwise comparisons between the culture conditions collected data were analyzed by one-way analysis of variance (ANOVA) using the Bonferroni method and statistical significance was accepted at P < 0.05.
Results

Maximum Stimulation Force, Frequency, Strain Rate, and Sample Geometry

The determination of maximal applicable uni-axial forces to tissue grafts within this bioreactor construction depends on the magnetic flux density $B(s)$ of the utilized magnets and their material properties, that is, remanence and geometry as formulated in Equation (2) and illustrated in Figure 5A.

$$B(s) = B_r \left[ \frac{L + s}{\sqrt{R^2 + (L + s)^2}} - \frac{s}{\sqrt{R^2 + s^2}} \right]$$  \hspace{1cm} (2)

Figure 5B shows the resultant metered values of compressive forces $F$ (N) or rather pressures $p$ (kPa) depending on the distance $s$ (mm) between two variants of encapsulated loading plate magnets and both variants of externally arranged magnet suspension. For the standard agarose gel with a diameter of 10 mm a force of 1 N corresponds to a pressure of 12.7 kPa. The cylindrical disc magnet S-04-25 in the actuator equipped with NE105 and NE201 external control magnets can deliver forces between 0 N and maximum forces of 2.0 and 2.8 N, depending on the vertical distance ($A_{VD}$) between the magnets and the actuator. Due to the high proportional influence of magnet geometry on the resulting repulsion force another mini-actuator prototype containing a larger (NE105) magnet was utilized in the measuring station. The determined maximum repulsion forces against both control magnets, NE105 and NE201, were found at 7.1 and 9.6 N, respectively. The achievable maximum uni-axial stimulation force, that is, minimum proximity, is spatially limited by the control magnet suspension at 2 mm (NE 105) and 3 mm (NE201), respectively.

Mechanical stimulation frequency is defined by the rotation velocity of the magnet disc which ranges between 0 and 6.66 rotations per second, and the number of magnets in the disc, that is, 2 for NE201 and 3 for NE105. Hence, for the NE105 magnet system, maximal stimulation frequency equals $6.66 \times 3 = 20.0$ Hz. Rotation velocity of the control magnet system ($A_{SF}$) is applied by a DC miniature servo motor in combination with one of three fitting planetary gear heads GP16A, which have specific reduction ratios of 369:1, 84:1, and 19:1, enabling 0.33, 1.4, and 6.66 revolutions per second under maximum power, respectively.

The bioreactor was used to stimulate individual hydrogels with a construct dimension of 3 mm thickness and a diameter of 10 mm since this geometry is widely used in studies dedicated to investigating chondrocyte mechano-biology (Chowdhury et al., 2003; Lee and Bader, 1997; Mauck et al., 2003). The modular setup of the system and the scalability of the components allowed the existing vessel, lid, locating stage, and mini actuator to be easily adapted to these agarose construct sizes. Such adaptations can be made at any time, depending on for instance the geometry of the construct or the actuator magnet, without the necessity to modify the core bioreactor setup.

Example Stimulation Protocol

Force and position were recorded for the 3% agarose construct in the bioreactor, with three- (Fig. 6A) and two-magnet embodiments (Fig. 6B), rotating with uniform velocity of 0.33 and 0.5 Hz, respectively, which leads to a periodic loading time of 1 s. The recorded curves for the NE105 magnet arrangement show that at repulsive stimulation forces of 2 N the agarose construct reaches the maximum displacement of 450 mm (i.e., 15% strain) that is allowed by the inserts at a stimulation frequency of 1 Hz. The compression profiles differ between the two- and

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Figure 5. Magnetic flux density $B(s)$ (mT) on the axis of the implemented NdFeB circular disc magnets at a distance $s$ (mm) (A). The forces $F(N)$ of repulsion along with pressures $p$ (kPa) generated between the encapsulated magnet of the mini actuator and both types of external control magnets (NE105 and NE201) show a typically strong proximal dependence (B). Both continuous lines were measured for the actuator containing the standard circular disc magnet (S-04-25-N) whereas both dotted lines were metered when NE105 was encapsulated into the PTFE cuff of the actuator. The pressure $p$ is indicated for a surface area of 78.54 mm$^2$ which corresponds to the geometry of the agarose gels (10 mm OD).
three-magnets systems, wherein the two NE205 magnets generate a more trapezoidal profile.

The dotted line in these figures shows the displacement of the actuator without the presence of an agarose construct. The difference between these lines is determined by the properties of the tissue under evaluation. In fact, the recorded curves can be displayed as a stress–strain curve (Fig. 7) from which material properties of the construct can be derived. Differences in stiffness (slope), nonlinear behavior (increasing slope with strain) and viscoelasticity (hysteresis) between gels with different percentages of agarose are apparent from the example (Fig. 7).

The different material properties used in this experiment imitate the well-known fact that dynamic deformational loading significantly increases the Poisson’s ratio (Kelly et al., 2006) and Young’s modulus (Lima et al., 2006; Mauck et al., 2002) of chondrocyte-seeded agarose hydrogels in long-term studies.

The metered stress–strain curves show the exemplary course of five complete loading and unloading cycles. Obviously, during the tissue engineering process, the cartilage tissue develops and as a result the material properties increase with time. This is represented by increasing slopes of these curves. Hence, this bioreactor system allows the development of the mechanical properties of tissue engineered cartilage to be monitored in real-time.

Cell Viability, Cell Content, and sGAG Accumulation in Static and Perfused Constructs

Figure 8 depicts the microscopic images of the fluorometric viability kit and the staining for cartilage specific extracellular matrix components in agarose constructs assessed by Alcian blue histology and by immunostaining for proteoglycan and aggrecan at 1 and 3 weeks of static well plate culture and perfusion culture in the novel bioreactor system, respectively.

The live/dead images of Figure 8 (top row) demonstrate that viable primary porcine chondrocytes were dispersed throughout the specimens and displayed a phenotypic spherical morphology under the absence of flattened cells in both culture conditions at both examination days. Furthermore, nearly 100% of the chondrocytes survived after 3 weeks in all specimens. Thus, increased cell apoptosis—for example, caused by system non-sterility—was not detectable in samples that were cultured in well
plates as well as in the bioreactor device with the outlined perfusion environment.

The relative changes of cell numbers in the hydrogels for the respective culture condition at both examination days (see Figure 8) were obtained by quantifying the ds-DNA content and the cell amount showed to be 1.52 times in perfused agarose constructs after 7 days, whereas dynamic cultured gels had fewer cells after 3 weeks in vitro when compared to

Table I. Results of the biochemical assays for the chondrocyte-seeded agarose constructs at 1 and 3 weeks of static well plate culture and dynamic perfusion culture in the bioreactor.

<table>
<thead>
<tr>
<th></th>
<th>Well plate</th>
<th>Bioreactor</th>
<th>Well plate</th>
<th>Bioreactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number</td>
<td>761,000 ± 29,000 (^1)</td>
<td>1,156,000 ± 72,000 (^*)</td>
<td>1,495,000 ± 216,000 (^*)</td>
<td>1,248,000 ± 172,000</td>
</tr>
<tr>
<td>sGAG (µg)</td>
<td>91.14 ± 11.80 (^1)</td>
<td>91.51 ± 8.92 (^2)</td>
<td>242.08 ± 24.87</td>
<td>384.55 ± 32.02 (^*)</td>
</tr>
<tr>
<td>sGAG/wet weight (µg/mg)</td>
<td>0.38 ± 0.05 (^3)</td>
<td>0.38 ± 0.05 (^*)</td>
<td>1.01 ± 0.10</td>
<td>1.60 ± 0.13 (^*)</td>
</tr>
<tr>
<td>sGAG/DNA (µg/µg)</td>
<td>15.16 ± 2.18 (^4)</td>
<td>10.02 ± 1.3 (^*)</td>
<td>20.50 ± 1.78</td>
<td>39.00 ± 8.03 (^*)</td>
</tr>
</tbody>
</table>

Asterisks (*) indicate \( P < 0.05 \) versus other culture condition at the same time point, and daggers (\( \dagger \)) at day 7 indicate \( P < 0.05 \) versus the day 21 value for the same culture condition.

Values are given as mean ± standard error of the mean (SEM).

Figure 8. Cell viability, histological and immunohistochemical evaluation of chondrocyte seeded 3% agarose constructs that were cultivated as control group in standard well plates (columns 1 and 3) or were perfused in the bioreactor device (columns 2 and 4) and subsequently examined at days 7 and 21. Analysis of chondrocyte viability in agarose gels as determined by fluorometric viability assay (top row, A–D, 50 \(^*\)). Assessment of cartilage specific matrix markers by histological staining with Alcian blue (middle row, E–H, 200 \(^*\)) and by immunostaining of the most abundant proteoglycan Aggrecan (lower row, J–M, 100 \(^*\)) in cryosections of hydrogels. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]
hydrogels of well plate control. Static cultured constructs exhibited no increase in DNA content by day 7, followed by a substantial increase (97%) in DNA content between weeks 1 and 3, resulting in a net increase superior to that seen in the other cultivation condition. Perfused agarose constructs had greatly significant increases in DNA content by day 7 (54%) followed by a slightly insignificant increase (8%) during the subsequent 2 weeks. At day 21, agarose constructs of static and perfused culture had reached approximately 2.0 and 1.66 times the original seeding density, with statistically significant differences among these scaffolds.

No differences were observed in the distribution of the cells or in the deposition of articular cartilage specific matrix markers between Alcian blue (middle row, Fig. 8) and Aggrecan immunostaining (lower row, Fig. 8) in specimens taken after 7 days culture. Both construct types contained uniformly distributed cells surrounded by a less intense pericellular matrix. Specimens of both culture models acquired after 21 days displayed more intense matrix staining surrounding single chondrocytes and cell clusters. However, enhanced staining of pericellular matrix components was observed in cryosections of perfused agarose gels.

To determine the efficiency of both culture models, the measured total accumulated sGAG was expressed per ds-DNA content and construct wet weight (see Table I). The absolute sGAG content in static and perfused agarose gels was similar after 7 days, while significantly increased sGAG values were detected in hydrodynamic stimulated specimens after 3 weeks when compared to static constructs. For static and perfused cultures, sGAG deposition substantially increased between weeks 1 and 3 2.66- and 4.2 fold, respectively. At week 3, perfused agarose gels exhibit 1.59 times higher absolute sGAG content than well plate control constructs. Due to equal agarose seeding volumes in both groups the normalization of sGAG against wet weight demonstrated similar ratios for both conditions after 1 and 3 weeks.

The patterns of matrix accumulation and cell content changes resulted in small differences in sGAG/DNA ratio among cultivation conditions at both time points. Similar to the pattern of sGAG deposition, the relation of sGAG/DNA demonstrates for both cultivation modes a continued substantial increase in accumulated extracellular matrix between days 7 and 21. At day 7, static agarose gels had significantly higher sGAG/DNA than the perfusion group, while at day 21 perfused hydrogels had a substantially increased sGAG/DNA ratio. During the terminal 2 weeks of static and perfused cultivation the sGAG/DNA ratio increased 1.35- and 3.89-fold, respectively. At the final examination time point gels of the perfusion group had a 1.90 times higher sGAG/DNA content than the statically cultured well plate gels.

**Discussion**

This article presents a new bioreactor for articular cartilage tissue engineering that allows for highly controlled mechanical loading and tissue perfusion while ensuring system sterility or rather product safety. The combination of these features in one bioreactor system is new and opens possibilities to culture 3D tissue substitutes such as MACTs, controlled by the development of the viscoelastic tissue properties. This article explains the construction of the bioreactor, the implemented sensors, and the automated control processes in detail. Subsequently, calibration of the system was performed. As an example of the applicability, we finally showed compression profiles in different agarose gels, a widely used carrier material for tissue engineering of articular cartilage (Buschmann et al., 1995; Kelly et al., 2006; Lee and Bader, 1997; Lima et al., 2006; Mauck et al., 2000).

In the bioreactor, the tissue substitute is located on a stage at the boundary between two compartments in a cylindrical bioreactor. The smaller lower compartment serves as a distributor for incoming medium and supplements, the larger upper chamber of the vessel consists of two outflow ports and contains the most culture medium and therefore fulfills a nutritional role during static culture periods (Schulz, 2003). This system can be used to force medium perfusion through the construct as investigated in the presented "proof of principle" experiment.

In this study we examined the effects of intermittent perfusion at a single flow rate of 0.5 mL/min on the viability and the extracellular matrix deposition in chondrocytes seeded into agarose constructs, while static cultured hydrogels act as control group.

The designed bioreactor device with the perfusion system allowed for automated cultivation and automated, intermittent forced medium perfusion of 3D cell-loaded hydrogel scaffolds for a mid term duration of 3 weeks whilst maintaining system sterility. Hence, product safety during culturing is assured in this system.

Moreover, in this work, positive effects of direct perfusion on both cell proliferation and cell differentiation were observed. Our results show initially increased cell numbers in perfused hydrogels after 7 days in vitro. Absolute sGAG contents at day 7 are comparable between static and perfused conditions. The chondrocytes in the perfused specimens appear to stop this early proliferation after 7 days of cultivation and proceed to differentiation, since no further increase in ds-DNA was detectable after 7 days, whereas a significant increase in sGAG accumulation was determined between weeks 1 and 3 of dynamic cultivation.

Similar increases in sGAG synthesis and ds-DNA content were demonstrated by other groups with well-accepted perfusion bioreactors in which medium flow is directly forced through a three-dimensional scaffold (Davisson et al., 2002b; Pazzano et al., 2000). Nevertheless, a contrary outcome of a perfusion experiment was reported by Mizuno et al. (2001) when bovine articular chondrocyte loaded collagen sponges were perfused at 0.33 mL/min for 15 days. Currently, the mechanism of the effect of directed perfusion on chondrocyte differentiation is not well understood; differences between reports may for instance relate to the type of scaffold used. However, it is well accepted that flow-
through bioreactor apparatuses provide a controlled culture environment that can promote the development of tissue engineered articular cartilage constructs. It was shown that such a direct perfusion bioreactor system enhances mass transfer and ensures homogenous distribution of nutrients, supplements and oxygen, which are likely to improve chondrocyte metabolism and viability (Grimshaw and Mason, 2000; Ysart and Mason, 1994).

Currently, none of these well-acknowledged direct perfusion devices offer the (bio-)technological opportunity for the implementation of an additional mechanical stimulus, which is also known to have a significant effect on metabolism of articular chondrocytes embedded in scaffolds (Kim et al., 1995; Sah et al., 1989). This enhances the perspectives of the presented bioreactor design, which, in addition to direct perfusion, has an innovative non-contact loading mechanism integrated in its upper compartment. This construction ensures a closed system and therefore aseptic operation, in the identical way as presented in the present article. The promising concept of a scaffold loading technology based on magnetic repulsion was also utilized by Schumann (Schumann, 2004). Drawbacks of their setup and that of others, such as the inability to simultaneously handle culture medium and apply (complex or simple) compression regimes, have been solved in the present design.

In addition, the present bioreactor design is the first system that combines non-contact loading with a reliable real-time measurement of effective load and displacement, therewith enabling the monitoring of tissue development in real-time while culturing, and without compromising the sterility of the graft in the test system. This offers significant benefits over other mechanical testing devices used in tissue engineering studies where the mechanical properties are generally assessed off-line in samples that are taken out of the experiment at various time-points of culture. This makes the present bioreactor particularly useful as part of a QC/QA program where the end-product after the cultivation procedure is required to meet predefined conditions.

The bioreactor system is capable of automatically controlling a complex regime of loading to individual soft tissues. Stimulation frequencies up to 4 Hz is possible, with forces up to 2.8 N. Stronger magnets can increase maximal vertical forces to 9.6 N, without major revisions of the peripheral equipment. For our test specimen geometry, 2.8 and 9.6 N forces are equivalent to pressure values of approximately 36 and 122 kPa, respectively. In addition, a variety of sample thicknesses and strain rates can be used with our device by changing the geometry of the locating stage. This flexibility in the conditions is eminent in scientific research where specific geometries may be required to answer scientific questions.

Alternatively, the fully automated control system digitizes in real time all related bioprocess activities via a user interface (Lintouch client), including time scheme and flow rate of medium perfusion, the regimes of mechanical conditioning and other incubator settings. Thus, it allows the bioreactor system also to be used in standard production facilities or clinical settings.

Measuring the individual progress of the mechanical properties in time is anticipated to become a key feature in the production of patient-specific MACTs. The behavior of autologous cells embedded in a scaffold differs per patient due to variations in cell pool, -age, -constitution, etc. Hence, the response to their biochemical and mechanical environments cannot be predicted. To cope with such different responses requires thorough feedback systems during the culturing. The present bioreactor allows for such real-time feedback.

The geometry of our agarose test specimens with approximately 0.8 cm$^2$ is significantly smaller than the recommended 4–10 cm$^2$ for clinically useful MACT grafts (Behrens et al., 2004). Constructs of the size used in this study are standard in pre-clinical, in vitro research. Smaller constructs allow multiple experiments to run in parallel with cells from single biopsies. To accommodate for the larger MACT scaffolds only requires up-scaling the individual culture chambers with the respective components and the size of the external magnet.

An additional extension of the bioreactor system that we are currently investigating is feedback control of the culture medium. Automated medium supplementation or replenishment upon depletion or particular nutrients may be done with similar feedback control systems as the one described in this article for mechanical loading. To replenish medium requires implementation of miniaturized pumps and valves, control requires input from for instance sensors for pH, $pO_2$, $pCO_2$, glucose- and lactate concentrations. This major biotechnological challenge will be presented in subsequent articles.

### Nomenclature

- $\mu$: frictional coefficient (N)
- $F_S$: stimulation force (N)
- $F_f$: frictional force (N)
- $F_h$: horizontal force (N)
- $B(t)$: magnetic field on axis of round magnet (mT)
- $B_r$: remanence (T)
- $R$: magnet radius (m)
- $L$: magnet length (m)
- $s$: distance (m)
- $p$: pressure (kPa)
- $F$: force (N)
- $s_{15}$: compressive strain of 15% on 3 mm thick gels (equivalent to a displacement of 450$\mu$m) (m)

### Abbreviations

- BR: bioreactor
- $A_{VD}$: actor vertical distance
- $A_{SF}$: actor stimulation frequency
- $S_{pos}$: inductive proximity sensor
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References


