Design and Characterization of a Rotating Bed System Bioreactor for Tissue Engineering Applications

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The main challenge in the development of bioreactors for tissue engineering is the delivery of a sufficient nutrient and oxygen supply for cell growth in a 3D environment. Thus, a new rotating bed system bioreactor for tissue engineering applications was developed. The system consists of a culture vessel as well as an integrated rotating bed of special porous ceramic discs and a process control unit connected with the reactor to ensure optimal culturing conditions. The aim of the project was the design and construction of a fully equipped rotating bed reactor, and in particular, the characterization and optimization of the system with regard to technical parameters such as mixing time and pH-control to guarantee optimal conditions for cell growth and differentiation. Furthermore, the applicability of the developed system was demonstrated by cultivation of osteoblast precursor cells. The porous structure of the ceramic discs and the external medium circulation loop provide an optimal environment for tissue generation in long-term cultivations. Mass transfer limitations were minimized by the slow rotation, which also provides the cells with sufficient nutrients and oxygen through alternate contact to air and medium. An osteoblast precursor cell line was successfully cultivated in this bioreactor for 28 days.

Introduction

Several different types of cell culture systems are widly used for the small-scale cultivation of mammalian cells. Petri dishes, well-plates, or T-flasks are used for the cultivation of anchorage-dependent cells under so-called static conditions (*1*). The term static condition refers to the absence of stirring, shaking, or perfusion during the cultivation. The cultivation of cells under static conditions often results in a lack of oxygen and nutrient supply because of external diffusion limitations. The consequence of such a limitation is the formation of a necrotic center coated by viable cells as a result of oxygen deficiency (*2*). Furthermore, controlling environmental parameters including pH, pO_2, and temperature is not possible. For cells cultured in suspension, spinner flasks are used which enable cultivation under dynamic conditions. The term "dynamic condition" refers to stirring or agitation during cultivation, which provides better distribution and allocation of nutrients and also increased oxygen transfer. On the other hand agitation or stirring also causes shear stress forces which can damage cells.

The development of special bioreactors for the generation of tissues represents an emerging research field in biomedicine during the last years (*3*, *4*). To date only rather simple 2D tissues mostly consisting of one cell type can be generated (*5*, *6*). To engineer functional tissue in vitro, special tissue engineering bioreactors have to be designed to provide controlled and reproducible cell proliferation and differentiation. Furthermore, such systems are necessary to perform controlled studies in terms of understanding the basic mechanisms of cell function and biochemical effects in a 3D environment. Moreover, automation and standardization improve the quality of engineered tissue and will undoubtedly improve tissue manufacturing (*4*, *7*, *8*).

The design of a bioreactor for tissue engineering requires the application of a suitable 3D biocompatible matrix that will support cell attachment, thus creating an optimal environment for cell proliferation and differentiation in 3D matrix structures toward the desired phenotype. Depending on the tissue to be replaced the matrix needs to fulfill different requirements with regard to mechanical stability, biodegradability, and porosity (*9*). For a reproducible cell and tissue proliferation the bioreactor must provide controlled cultivation conditions. Variables that need to be controlled include temperature, pH, pO_2, and medium flow rate. Mechanical stimulation is also known to be an important factor for tissue generation (*2*, *3*, *10*). The type of mechanical stimulation and the intensity depend on the cell type or construct that has to be generated. The bioreactor has to provide controlled mechanical stimulation, e.g., fluid flow induced shear stress to ensure optimal and reproducible cell cultivation. Throughout the cultivation optimal nutrient concentrations as well as efficient oxygen supply must be maintained and waste products must be removed (*2*, *11*).

Different types of culture systems and bioreactors have been developed and tested for tissue engineering applications (*3*−*5*, *7*). Mass transfer limitations can be reduced by culturing cell seeded matrices (scaffolds) in stirred or rotating vessels. Under these dynamic conditions the homogeneous concentration of nutrients and oxygen leads to a decrease in the concentration boundary layer on the construct surface.

Two examples for bioreactor designs for tissue engineering in which cells are cultivated under dynamic conditions are the spinner flask and the rotating wall vessel (*12*). In the spinner flask scaffolds are fixed on needles or seeded onto microcarriers

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(*13*−*15*). A well-mixed environment is maintained with a magnetic stir bar at the bottom of the flask. The stirring enhances external mass transport but also generates turbulent eddies, which could be detrimental to the development of tissue (*2*).

In a rotating wall vessel the scaffolds are suspended in the medium and the vessel wall is rotated at a rate that enables the construct to remain in a state of microgravity within the culture medium (*16*−*19*). A dynamic culture environment to the construct is provided.

Both systems provide an increase of external mass transport but a diffusion limitation in the interior of the scaffold remains. This leads to an inhomogeneous distribution of cells. Furthermore, medium agitation in the spinner flask creates intense shear forces. The rotating wall vessel provides a dynamic culture environment with lower shear stress. Nevertheless, increasing the size of the scaffolds leads to collisions of the scaffolds with the reactor wall.

Another bioreactor design with improved supply of nutrients is the perfusions reactor in which the medium is pumped through the pores of the scaffolds (*20*, *21*). Thus, internal mass transport limitations are minimized through continuous replenishment of nutrients. However, the increased number of cells resulting from proliferation may lead to a reduction in the scaffold pore size causing a change of the flow rate and a pressure built up within the scaffold pores. This may lead to uncontrolled shear stress and promotes inhomogeneous proliferation of the cell construct.

As already stated, existing bioreactors have several drawbacks and limitations. The ideal bioreactor for tissue engineering would allow for efficient, homogeneous mixing with controlled mechanical stimuli and a sufficient nutrient and oxygen supply without mass transport and diffusion limitations in the interior of the scaffold (*11*, *22*−*25*). Furthermore, the bioreactor should provide control of environmental conditions such as oxygen, pH, temperature, or the medium flow rate (*7*).

In addition to the bioreactor types previously mentioned, many other bioreactor concepts for tissue engineering have been introduced. One example is the rotating shaft bioreactor (RSB) published by Chen et al. (*26*). Our new bioreactor concept is the rotating bed system bioreactor (RBS), which is quite similar to the RSB but differs with regard to several special features.

The principle of both reactor types is to supply cells, seeded onto matrices, continuously with nutrients and oxygen. Therefore, discs consisting of, e.g., ceramic matrix material are fixed at a shaft inside the reactor, which rotates continuously during cultivation. The RBS as well as the RSB is half-filled with medium, which provides alternating contact to medium and to gas atmosphere in the headspace of the system. This provides a sufficient oxygen and nutrient supply and allows long-term cultivation with low medium consumption. In comparison to the spinner flask and the rotating wall vessel, diffusion limitations in the interior of the scaffold can be minimized.

In contrast to the RSB system our bioreactor provides (a) a fully equipped design with a control unit, (b) direct pO_2, pH, and temperature control and adjustment within the bioreactor chamber, (c) a new dynamic seeding strategy by the application of a cell suspension through a feeding pipe located above the matrix discs, (d) continuous medium circulation (medium drips out of a feeding pipe directly onto the scaffolds to ensure homogeneous distribution of medium of all discs), and (e) the realization of systematic feeding strategies and, for desired mechanical stimulation, the application of high pressure due to the configuration of the system.

The aim of this work was the setup of the system but also the characterization and optimization of a rotating bed system (RBS) bioreactor prototype. Initial tests comprised of mixing studies and pH-control strategy optimizations were performed to guarantee optimal conditions for cell growth and differentiation.

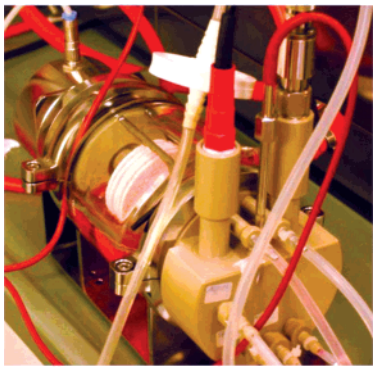

Figure 1. Prototype of the rotating bed system bioreactor for tissue engineering.

Materials and Methods

Chemicals. Deionized water (ARIUM Sartorius AG, Goettingen, Germany) was used for the preparation of media and buffers. Dulbecco's and α-Modified Eagle's Medium (α-MEM) were purchased from Sigma-Aldrich (Steinheim, Germany). Foetal and new born calf serum FCS/NCS, *β*-glycerolphosphate, ascorbic acid, and antibiotics were purchased from PAA laboratories GmbH (Coelbe, Germany). Buffers, salts, phenol red, and other reagents were purchased from Fluka (Buchs, Switzerland) and Sigma-Aldrich (Steinheim, Germany) and were of *per analysis* quality.

Materials. The prototype of the rotating bed system bioreactor was set up with Zellwerk GmbH (Oberkraemer, Germany). The control unit was from Sartorius BBI Systems GmbH (Melsungen, Germany). The sterilization of the reactor and other glass bottles was performed with an autoclave, Tecnomara, from Integra Bioscience (Fernwald, Germany). The pH- and a pO_2-electrode were from Mettler-Toledo (Columbus,). A visual documentation of the mixing experiments was performed with a camera (EOS 300D) from Canon (New York). A photometer (Multiscan Spectrophotometer) from Thermo Labsystem (Waltham) and a flow cuvette (170-Q) from Helma (Müllheim, Germany) were used. Further analytics were performed with the scanning electron microscope JSM-6700F from JEOL (Tokyo, Japan) and the YSI 2700 from Yellow Springs Instruments (Ohio).

Rotating Bed System Bioreactor. A prototype of a rotating bed system bioreactor (Figure 1) was used for the proliferation of adherent cells with regard to the manufacturing of functional tissues like bone and cartilage constructs. Furthermore, it is applicable for recombinant protein production.

The system consists of a motor fitting and a 500 mL borosilicate glass culture vessel. The culture vessel is composed of a temperature control double jacked glass cylinder and a locking cap. The cap is equipped with ports for pH- and pO_2-electrodes, a temperature probe, and the medium circulation. The rotating bed inside the vessel is based on porous ceramic Sponceram discs (Figure 2). The discs are fixed on a shaft and rotate at a rotation speed of 2 rpm during cultivation. The rotation is caused by a magnetic drive coupled to the cultivation vessel. The low rotation speed minimizes the exhaustive shear stress.

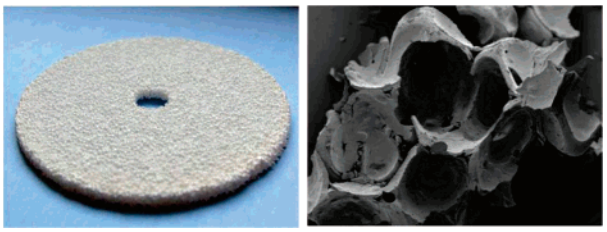

Figure 2. Sponceram discs for bioreactor cultivation (left). These discs are fixed on a shaft inside the reactor and rotate at 2 rpm during the cultivation. Scanning electron micrographs (SEM) of Sponceram scaffold (right).

External medium circulation as well as the rotating bed provide the mixing of the media. During the circulation the medium drips out of a feeding pipe, installed in the head space of the reactor, and directly located above the Sponceram discs to ensure homogeneous distribution of medium for all discs. This configuration also allows for a dynamic seeding procedure. To ensure a good gas supply the medium as well as the overlay atmosphere in the headspace is aerated with compressed air via a ceramic sparger in the medium and a medium pipe in the headspace of the reactor. The pH-value is controlled by CO_2 to pH 6.5−7.5, which is conducted into the compressed air and entered directly via a sparger into the medium. To guarantee controlled cultivation conditions, a control unit was used. Figure 3 shows the schematic experimental setup of the bioreactor.

Sponceram. Sponceram (Zellwerk GmbH, Eichstaedt, Germany) consists of a unique doped zirconium dioxide based material with macro and micro pores (Figure 2). Detailed information about technical data on Sponceram is summarized in the publication of Suck et al. (*27*). The cultivation in the bioreactor system was performed with Sponceram (pore size: 600 μm) used as carrier discs (65 mm in diameter, 3 mm thickness) for the cultivation of osteoblast precursor cells in the prototype of the rotating bed system bioreactor.

Mixing Time Experiments. Mixing experiments were performed by dye tracer experiments (0.001 g/L phenol red) to visualize the mixing effect with and without Sponceram discs from clear to pink. Images were taken to document the dye distribution inside the reactor. The reactor was filled with 300 mL of an aqueous solution of 0.001 M NaOH. Further tracer experiments where carried out with 5 M NaOH. In these experiments the reactor was filled with 50 mM phosphate buffer at pH 7 supplemented with phenol red.

pH-Control. The pH-value was measured with a pH-electrode inserted into the front cap of the reactor and via a flow cuvette in the external medium circulation (Figure 3). To simulate cell culture conditions, cell culture medium (α-MEM) + 10% NCS was used. The pH-value was controlled via CO_2-supply by a PID-controller (control unit, Sartorius BBI Systems GmbH, Melsungen, Germany) and was detected with the pH-electrode and a photometer every 5 min. The color change of the pH indicator dye was measured every 30 s with a photometer at a wavelength of 557 nm.

Cell Cultivation. ***Cultivation of MC3T3-E1.*** The mouse preosteoblastic cell line MC3T3-E1 was obtained from DSMZ (ACC 210). MC3T3-E1 cells were cultured using DMEM containing 10% FCS and antibiotics as proliferation medium. For osteogenic differentiation the medium was supplemented with 1 μM Dexamethasone, 80 μM ascorbic acid, 10 mM β-glycerolphosphate, and 10 ng/mL bone morphogenetic protein 2 (BMP-2). Medium was changed three times a week and cells were subdivided when they reached near-confluency.

Cultivation in the Rotating Bed System. The bioreactor can be equipped with up to 20 thin Sponceram discs (Figure 2, left). In our study the reactor was equipped with two Sponceram (pore size: 600 μm) carrier discs and respective spacers for the cultivation. Cell inoculation was carried out with a total volume of 2 mL cell suspension/disc (seeding cell number: 2.5×10^7 cells/disc). Another RBS cultivation was started 7 days later with a higher cell seeding density (5.5×10^7 cells/disc) and was run in parallel. The cell suspension was injected through a feeding pipe onto the carrier discs (Figure 3). To distribute the cell suspension homogeneously onto the discs a rotation speed of 4 rpm during cell seeding was applied. To allow adhesion onto the Sponceram surface, the reactor was filled with 300 mL of medium 30 min after cell inoculation. The following cultivation was performed at 37 °C, 2 rpm, and a pH of 7.3 for 28 days in differentiation medium. During cultivation the medium circulates continuously out of the cultivation vessel and drips out of the medium pipe back into the vessel. In the first cultivation experiment the MC3T3-E1 cells were kept in proliferation medium for 28 days. The following experiment was started 7 days afterward and the cells were cultured in proliferation medium for 20 days and subsequently in differentiation medium with BMP-2 for an additional 11 days. After the cultivation the disc shaped scaffolds were used for investigation of matrix mineralization and scanning electron microscopy.

Assays. ***Glucose Assay.*** The biomass can indirectly be determined by measuring glucose consumption in the culture medium assuming that the metabolic rates of the measured substrates correlate with cell density. Cell growth during the bioreactor cultivations was determined daily by the estimation of glucose consumption using the YSI 2700.

***Von Kossa Staining* (*Matrix Mineralization*).** After cultivation the Sponceram disc was briefly washed with PBS and fixed in ice cold 100% ethanol for 20 min at room temperature. For *von Kossa staining* fixed cells were washed with deionized water and incubated for 30 min in 5% $AgNO_3$ in the dark, washed with deionized water, and exposed to ultraviolet light for 2 min. Cells were fixed with 5% sodium thiosulfate for 2 min and washed 3 times with deionized water.

Scanning Electron Microscope. Sponceram discs for scanning electron microscopy were fixed in Karnovsky buffer at 4 °C overnight. Samples were then dehydrated in solutions containing increasing percentages of acetone (10%, 30%, 50%, 70%, 90%, and 100%) and subsequently imaged with a JEOL JSM-6700F scanning electron microscope.

Results

To make tissue engineering products clinically accessible, a detailed characterization of the rotating bed bioreactor by means of important parameters including mixing behavior is required for the transition from the laboratory to industrial scale bioreactors. Therefore, mixing experiments were performed in the newly developed rotating bed system bioreactor. Moreover, studies were carried out to optimize the control of the pH-value via CO_2-supply. Furthermore, cultivation of an osteoblast precursor cell line was performed in the same system after the optimization of process parameters to examine if the reactor can create an environment that allows the cells to proliferate and differentiate into proper tissue structures.

Mixing Time Experiments. All experiments in the rotating bed system bioreactor were performed in the reactor setup shown in Figure 3. The reactor can be equipped with Sponceram used as carrier discs. The discs are fixed on a shaft inside the reactor

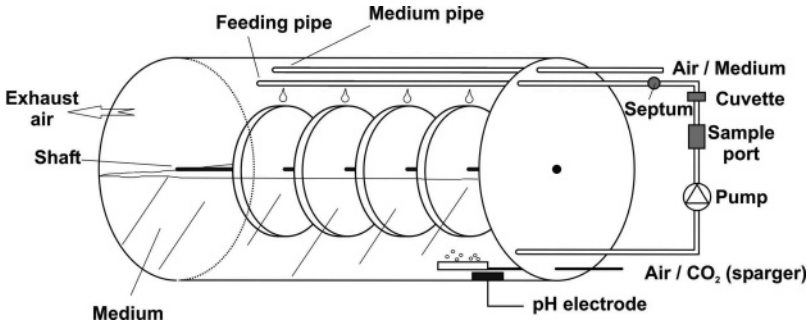

Figure 3. Schematic experimental setup of the rotating bed system bioreactor (RBS).

and rotate continuously during the experiments at 2 rpm. Externally circulating medium and the rotating bed provide the mixing of the media. The medium is continuously pumped through the reactor system as indicated in Figure 3. Thereby, the medium drips out of the feeding pipe directly onto the discs. A maximum circulation flow rate of 60 mL/min (300 mL total volume) can be achieved with this reactor setup and is used for all experiments. A pH-electrode is located in the front cap of the reactor and is always placed in the same position for all experiments. For further experiments regarding the pH-control and the cultivation in this bioreactor setup the medium as well as the overlay atmosphere in the headspace is aerated with compressed air via a ceramic sparger in the medium and via a medium/air pipe in the headspace of the reactor. The pH-value is controlled by CO_2, which enters directly via a sparger in the medium.

The characterization of the mixing behavior in the rotating bed system bioreactor was initially performed by dye tracer experiments. The reactor was filled with 300 mL of an aqueous solution of 0.001 M NaOH. The solution was continuously pumped out of the vessel and dripped back into the cultivation vessel through the feeding pipe. A constant circulation flow rate of 60 mL/min was used. One milliliter of a dye indicator (phenol red) was added through a septum at the front of the feeding pipe.

Mixing time was defined as the time required to observe homogeneous dye distribution from clear to pink and was measured manually. Images were taken to document the distribution of the dye inside the reactor. The experiments were initially carried out without discs. The experiment was repeated with 3, 6, and 12 discs to investigate the influence of the number of discs. The discs were rotated at 2 rpm. The mixing characteristics for the dye tracer experiments with and without discs are depicted in Figure 4A−D .

An inhomogeneous distribution of the dye independent of the disc number is obvious in the beginning. After 3 min distribution of the dye in the experimental setup without discs could be observed. An increase in the number of discs enhances homogeneous dye distribution and results in a shorter mixing time of 1.5 min compared to the experimental setup without discs. Increasing the number of discs from 3 to 12 has no significant effects on mixing time.

To gain more detailed and accurate information about the mixing time of this reactor setup further tracer experiments were performed. Therefore, the pH was changed stepwise by the addition of NaOH aliquots and was measured until homogeneous distribution. The experiment was performed with three discs fixed at the center of the shaft rotating at 2 rpm. The reactor was filled with 300 mL of phosphate buffer. The phosphate buffer was additionally colored with a dye indicator (phenol red), to visualize the change in pH. The solution was continuously pumped out of the vessel and dripped back into the cultivation vessel through the feeding pipe. A constant circulation flow rate of 60 mL/min was used.

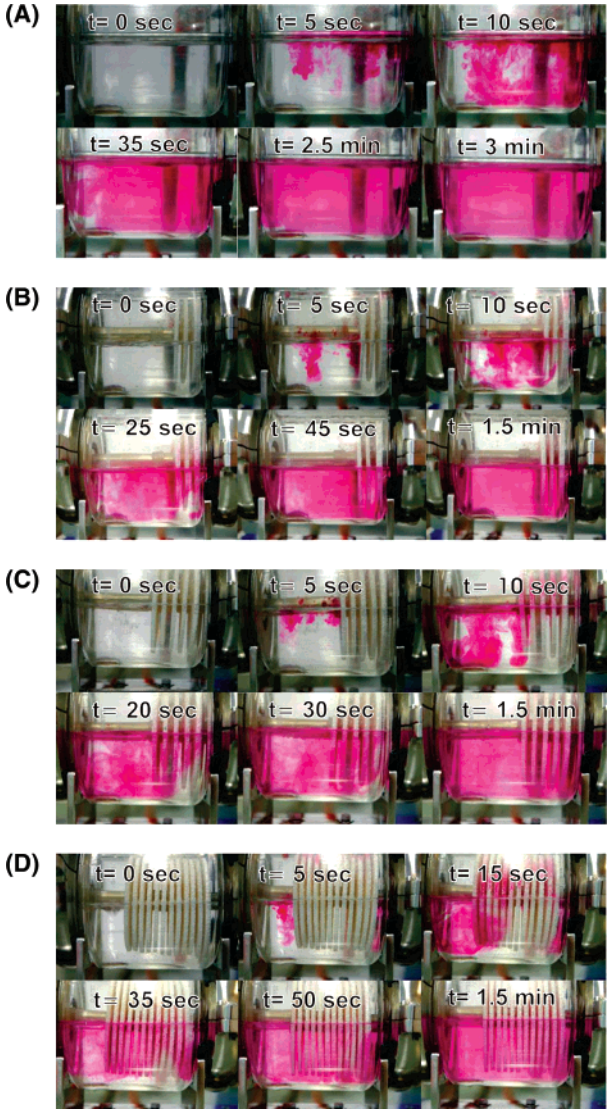

Figure 4. Mixing characteristics inside the reactor without discs (A), with 3 discs (B), with 6 discs (C), and with 12 discs (D) visualized with phenol red as a dye indicator. The picture shows the mixing effect at different times.

For tracer experiments the pH was increased by the addition of 1 mL of 5 M NaOH via a septum in front of the feeding pipe. The change in pH during circulation was measured every 5 min with a pH-electrode inside the reactor. In the external medium circulation of the colored phosphate buffer passed additionally through a flow cuvette and the color change of the dye indicator was measured with a photometer at a wavelength of 557 nm every 30 s. Before starting the next addition of

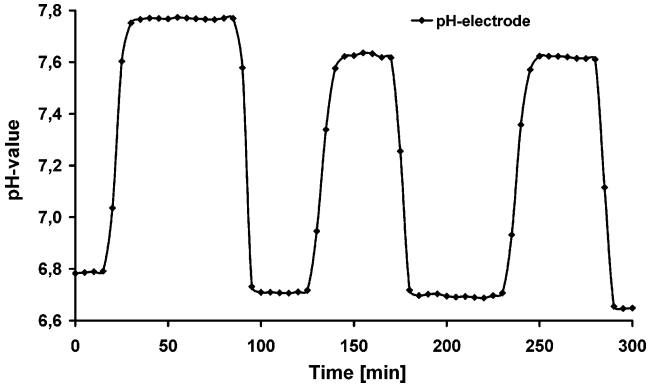

Figure 5. The change of the pH-value during tracer experiments by the addition of 5 M NaOH/HCl aliquots via septum in front of the feeding pipe of the rotating bed bioreactor. The change of the pH-value was measured every 5 min with a pH-electrode inside the reactor. The experiment was done in triplicate.

1 mL of 5 M NaOH the pH was lowered by the addition of 1 mL of 5 M HCl. The experiment was carried out in triplicate. Due to the insignificant differences in curve progression between the photometer data and the measured pH-values only the pH-value is depicted as a function of time (Figure 5).

To characterize the mixing behavior of the reactor a simplified theoretical model was applied, which treats this process as a first-order dynamic system. For the evaluation of these pulse experiments the typical response signal of a first-order dynamic system to a pulse was fitted to the measurement data separately for each pulse. In eq 1 the applied response signal for one pulse is presented.

$$\mathrm{pH}(t) = \begin{bmatrix} \mathrm{pH}_0 & \text{if } t \le t_\mathrm{p} \\ \mathrm{pH}_0 + (\mathrm{pH}_\infty - \mathrm{pH}_0)(1 - \mathrm{e}^{-t-t_i/\tau}) & \text{if } t > t_\mathrm{p} \end{bmatrix} \quad (1)$$

The time-depended pH response values pH(t) are calculated using the initial pH value of pH_0 before the pulse is applied. The final pH value (new stationary value) is represented by pH_∞, the time t_i, at which the tracer pulse is injected, as well as the typical time constant τ of a first-order dynamic system. The fit is performed using the least-squares method of Gauss.

As an example, in Figure 6 the measured as well as the theoretical values for one pulse experiment are presented. Using the fitted parameters a characteristic mixing time t_{90} is calculated, which is the time required to attain 90% of the final pH value ($\mathrm{pH}(t_{90}) = 0.9\ \mathrm{pH}_\infty$). In Table 1 the time constant τ, as well as the mixing times t_{90}, is presented for the three pulse injections. Due to the low measurement frequency as well as the oversimplified model, the mixing time is not determined very precisely, with an average mixing time of 9.4 min with a standard deviation of 1.6 min.

pH-Control. Further experiments targeting pH-control in the rotating bed system bioreactor were performed. In tissue engineering pH-control is typically performed via bicarbonate−CO_2 equilibrium. Thus, CO_2 is added into the reactor via a ceramic sparger directly into the medium.

The reactor was equipped with three discs fixed to the center of the shaft rotating at 2 rpm. To simulate cell culture conditions the experiment was carried out with 300 mL of cell culture medium supplemented with 10% serum at 37 °C. The medium was continuously pumped out of the vessel and dripped back into the cultivation vessel through the feeding pipe. A constant circulation flow rate of 60 mL/min was used. The medium, as well as the overlay atmosphere, was aerated with compressed air via a medium/air pipe in the headspace of the reactor and

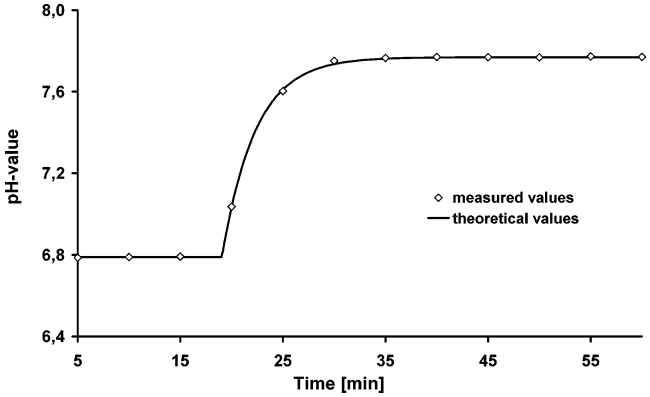

Figure 6. Measurement values as well as theoretical values of one pulse experiment.

Table 1. Time Constant (τ), as Well as the Mixing Time (t_{90}), for the Three Pulse Injections

injection	time constant, τ [min]	mixing time, t_{90} [min]
1	3.3	7.5
2	4.4	10.1
3	4.6	10.5
		9.4 (average)[a]

via ceramic sparger in the medium. The initial pH-value of the medium was about 7.6. For pH-control, a set point of pH 7.2 was chosen. The change in pH was measured every 5 min with a pH-electrode inside the reactor. The measured values during the experiment are presented in Figure 7. The set point of pH 7.2 was not achieved. High oscillation in a range of 0.5 pH units in total can be observed that results from over control of the CO_2-supply.

Furthermore, several experiments were performed to ensure more precise pH-control inside the reactor and to guarantee stable cultivation conditions. Thus, we rearranged our experimental setup to reduce the excessive CO_2 pressure. Under controlled pressure conditions an appropriate pH-control could be realized, which is depicted in Figure 8.

After achieving accurate pH-control, further experiments were performed to demonstrate the reactor's suitability for cell proliferation and differentiation. To show applicability of this newly developed system, two cultivations of an osteoblast precursor cell line will be presented in the following sections.

Cell Cultivation. The growth of MC3T3-E1 cells during cell cultivation experiments was monitored by determining glucose consumption. The glucose consumption per day referring to the culture medium (300 mL) is presented in Figure 9. Cells were cultured on Sponceram using standard proliferation medium or BMP-2 containing differentiation medium. Glucose consumption of the cells cultivated in standard proliferation medium was in total 8.4 g over 28 days, whereas cells cultivated in BMP-2 containing medium consumed 9.1 g of glucose in 21 days. These data show that more cells were grown under BMP-2 conditions in a shorter time period.

Correspondingly, glucose consumption increased more slowly in standard proliferation medium. After 19 days glucose consumption in standard proliferation medium reached a plateau of 560 mg/day. The second cultivation showed a different glucose consumption curve. This cultivation was started in standard proliferation medium for the first 11 days and the medium was changed to BMP-2 containing differentiation medium for another 10 days. Glucose consumption increased much more during the first 11 days compared to the first cultivation, since the reactor was inoculated with more cells.

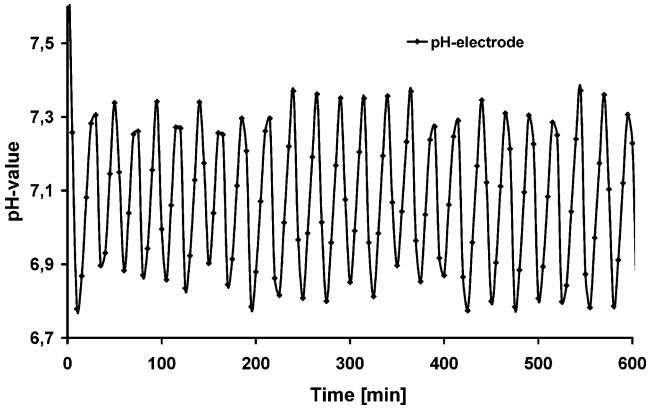

Figure 7. The pH-control by CO_2-supply in the rotating bed bioreactor. The pH was measured every 5 min with a pH-electrode inside the reactor. Set point was pH 7.2.

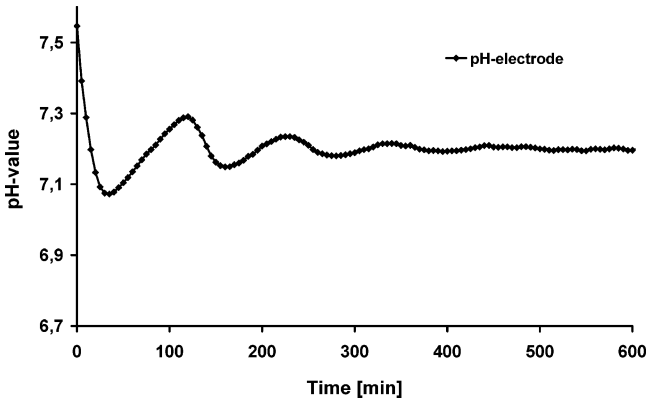

Figure 8. The control of the pH-value by a CO_2-supply under controlled pressure conditions. The pH was measured every 5 min with a pH-electrode inside the reactor. Set point was pH 7.2.

Changing the medium resulted in an initial decrease of glucose consumption. Cells obviously needed to adapt to the new medium and subsequently continued their high glucose consumption. After 21 days of cultivation no plateau phase was reached and glucose consumption was over 800 mg/day.

Whether these differences in glucose consumption are related to the modifications of cultivation medium is not directly verifiable. The second cultivation was conducted with a greater inoculum, since the cultivation was started 7 days later. Moreover, temperature variations were detected in the first cultivation, which did not occur under differentiation conditions (data not shown). Even slight differences in pH or oxygen supply can affect cell proliferation and cellular metabolism giving rise to discrepancies in glucose consumption curves.

After the cultivation the disc shaped scaffolds were used for investigation of matrix mineralization and scanning electron microscopy. Matrix mineralization was qualitatively determined by histochemical staining according to von Kossa. In comparison to the control, matrix cells showed positive von Kossa staining in standard proliferation medium and highly intense staining in BMP-2 containing medium indicating the presence of a mineralized extracellular matrix (Figure 10). Interestingly, von Kossa staining showed extracellular matrix calcification related to osteogenic differentiation even in standard proliferation medium, which did not have any differentiation stimuli in the medium. The difference between standard medium and BMP-2 conditions might be due to enhanced osteogenic differentiation induced by the growth factor BMP-2. On the other hand, glucose consumption was higher in BMP-2 medium suggesting a higher cell number. Thus, the more intense von Kossa staining cannot

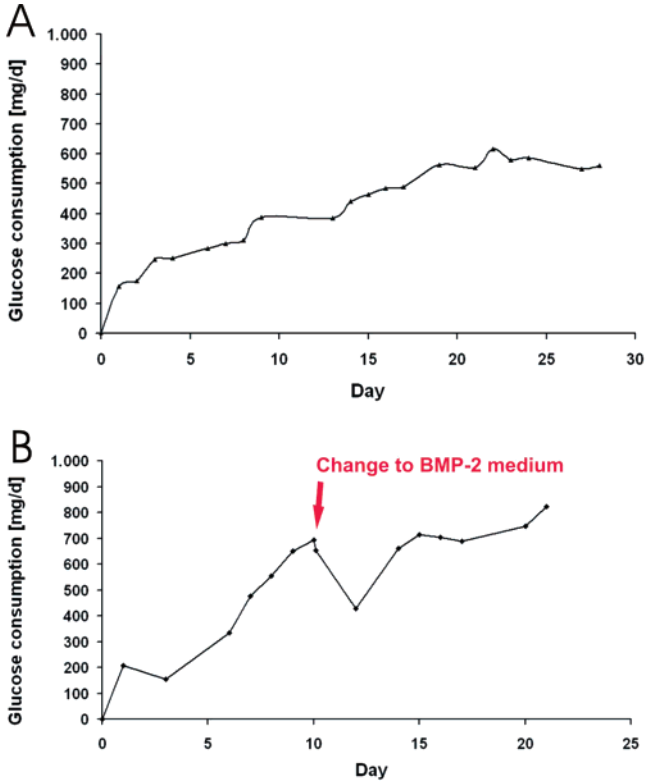

Figure 9. Glucose consumption per day during the bioreactor cultivation of osteoblast precursor cells: (A) standard proliferation conditions and (B) BMP-2 conditions.

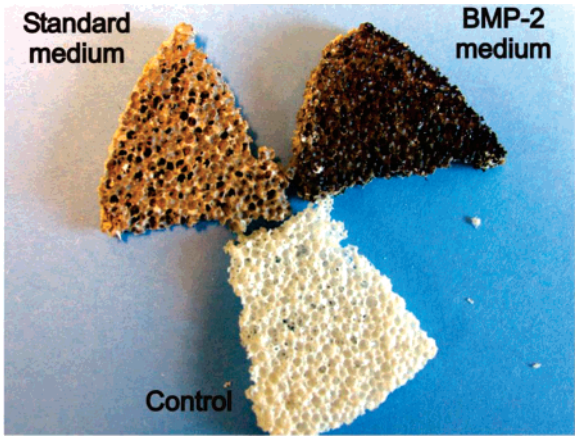

Figure 10. Specific staining of mineralized extracellular matrix (ECM). Von Kossa staining on Sponceram after bioreactor cultivation of osteoblast precursor cells in standard proliferation and BMP-2 medium. Cells cultured in BMP-2 showed a highly intense von Kossa staining. Cells cultured in standard medium also showed mineralization of the ECM in comparison to the control matrix.

directly be correlated with osteogenic differentiation status but might be due to exalted cell numbers.

The scanning electron micrographs show a thick layer of extracellular matrix covering the Sponceram scaffold (Figure 11). The scaffold material itself could not be seen underneath the cell layer. No singular MC3T3-E1 cells were detectable under the thick ECM cover. The observed fibrous ECM structures presumably mainly consist of type I collagen. Moreover, the formation of mineral nodules on the surface was identified. No differences between standard and differentiation conditions were observed.

To analyze the cellular osteogenic differentiation status after bioreactor cultivation, RT-PCR for osteogenic markers (type I

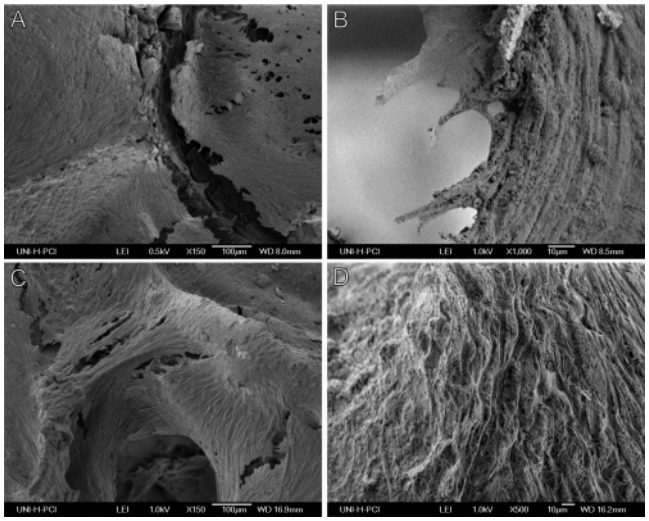

Figure 11. Scanning electron micrographs (SEM) showed the cell morphology of MC3T3-E1 cells cultured on Sponceram after bioreactor cultivation in standard proliferation conditions (A/B) and after bioreactor cultivation in BMP-2 conditions (C/D). A thick layer of extracellular matrix as well as mineral nodules on the surface were identified.

collagen, Osteocalcin and BSP-2) was performed. All three osteogenic markers were detected after both standard condition cultivation and BMP-2 cultivation. These results are discussed in the publication of Suck et al. (*27*).

Discussion

To design and operate bioreactors for tissue engineering, optimal, controlled, as well as reproducible cultivation conditions are necessary. External mass transport limitations due to inefficient mixing leads to an insufficient nutrient and oxygen supply. This fact causes inhomogeneous cell proliferation and inhibits the development of functional tissue. To guarantee optimal cultivation conditions for such bioreactors a characterization of the mixing inside the reactor is of great interest to ensure sufficient amounts of nutrients and an adequate oxygen supply.

The aim of this study was the evaluation of the mixing effect in a new rotating bed system bioreactor and the determination of the mixing time. Furthermore, the regulation of the pH-value via CO_2 was investigated. Based on these results an improvement in the pH-control was performed to ensure optimal conditions for further 3D cell cultivations.

For a good and homogeneous distribution of nutrients and oxygen in bioreactors, the method of mixing is important. External medium circulation with a flow rate of 60 mL/min combined with a rotating bed (2 rpm) consisting of porous ceramic discs provides good mixing of the media. The porous structure of the scaffolds fixed to a shaft inside the culture vessel maintains the mixing.

Further tracer experiments were performed to gain more detailed information about the mixing of this reactor setup. Therefore, the pH was changed stepwise by the addition of NaOH aliquots and was measured until homogeneous distribution. The experiments were carried out with 5 N NaOH to observe a clear shift in the pH-value. To characterize the mixing behavior of the reactor a simplified theoretical model was applied, which treats this process as a first-order dynamic system. The typical response signal of a first-order dynamic system to a pulse was fitted to the measurement data separately for each pulse. Using the fitted parameters a characteristic mixing time t_{90} was calculated, which is the time required to attain 90% of the final pH value. An average mixing time of 9.4 min is obtained with the reactor setup. For deeper characterization of hydrodynamics of the system, computational fluid analysis must be performed.

Controlled cultivation conditions in dynamic cultivations are necessary for reproducible tissue generation. Homogeneous mixing of the medium, including nutrients and metabolics, is advantageous in dynamic cultivations. Furthermore, mammalian cell cultures are sensitive to high pH oscillations. Therefore, a sufficient gas supply and a stable pH-value must be ensured. The modification of the gas supply showed improvement in cell growth and guarantees a stable pH-value for the cultivation of the osteoblast precursor cell line MC3T3-E1.

Further experiments were performed to prove the reactor's applicability for cell proliferation and differentiation. The matrix material used for tissue engineering applications should provide tissue specific environments and architecture. Biocompatibility is essential and the scaffold should promote or enhance cell proliferation and differentiation. To guarantee the development of functional tissue the used culture system should mimic the in vivo environment. In this study preosteoblastic cells were successfully cultivated on a Sponceram carrier disc for 28 days in the bioreactor system in standard proliferation medium and BMP-2 containing differentiation medium, respectively. The course of glucose consumption showed no limitations in cell proliferation during the cultivation. As an indicator of generated bone-like tissue cells, strong mineralization in both standard and differentiation medium was shown. These findings are supported by scanning electron micrographs, where the formation of mineral nodules on the surface could be identified. Moreover, bone markers were detected with RT-PCR (*27*). Interestingly, our experiments showed that osteogenic differentiation of MC3T3-E1 cells can occur even in standard proliferation medium without any osteogenic supplements. Obviously shear stress due to medium flow over the Sponceram discs and the movement of the discs through the medium in combination with a good oxygen and nutrient supply in the bioreactor, and probably some other not examined effects, give an osteogenic stimulus. Static experiments in our laboratory have shown that three-dimensional growths on Sponceram alone did not give rise to osteogenic differentiation of MC3T3-E1 cells. Currently, efforts are being made toward the cultivation of primary human osteoblasts.

In summary, this study demonstrated that this newly developed culture system is appropriate for the generation of bone-like tissue derived from a preosteoblastic mouse cell line. The ultimate shape of the used scaffold and cultivation procedure provided efficient proliferation and formation of the extracellular matrix.

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