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# Overcoming hypoxia in 3D culture systems for tissue engineering of bone in vitro using an automated, oxygen-triggered feedback loop

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**Abstract** Tissue engineering is an attractive approach to heal bony defects. However, three-dimensional cell-scaffold constructs display uneven oxygen supply resulting in inhomogeneous tissue quality. We assessed different strategies to improve oxygen supply in vitro. Scaffolds with differing inner surface were seeded with preosteoblastic cells and cultivated either statically or in perfusion bioreactors. Oxygen concentration and pH were measured in the center of the scaffolds. An inductive feedback mechanism was build to increase bioreactor pump speed according to the oxygen concentrations measured within the scaffolds. While pH remained stable, oxygen concentration decreased significantly under static conditions within the cell-seeded scaffolds. Reducing the scaffolds' inner surface as well as increasing perfusion speeds in bioreactors resulted in improved oxygen supply. We conclude that improving oxygen supply to three dimensional culture systems for bone tissue engineering is feasible in an automated manner. Culture conditions have to be adapted to each cell-scaffold system individually.

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## 1 Introduction

Whenever it comes to bony defects, which exceed the self-healing capacity of bone surgical therapy is required to achieve complete reconstruction. Conventional treatment strategies include autologous or allogenic bone grafting, callus distraction techniques or the application of synthetic bone substitutes [1]. Unfortunately, these techniques exhibit several disadvantages including donor site morbidity, infection, additional scars and chronic pain [2–4]. If allogenic bone grafts are used, there is a risk of disease transmission and typically only small defects can be managed, which is equally true for synthetic bone grafts [1, 4]. Synthetic bone substitutes bear the risk of foreign body reaction and finally, all above mentioned procedures may result in non-unions [1, 5].

Tissue engineering of bone is a promising tool to produce a virtually unlimited supply of bone substitute material [1–3, 5]. It relies on the application of fast-dividing, autologous bone precursor cells such as human mesenchymal stem cells (hMSC) or preosteoblasts seeded on biocompatible scaffold materials to promote bone regeneration [1, 2, 6]. Major recent advances in the field include the osteogenic differentiation of hMSC into osteoblasts in vitro, the generation of a plethora of biocompatible and resorbable scaffold materials and the discovery and production of bioactive molecules, which stimulate endogenous pathways along the osteogenic cascade. However, scaffold-based tissue engineering is not yet routinely used in the clinical setting to treat bony defects. One major problem is hampered oxygen supply in three-dimensional tissue constructs [7, 8]. If oxygen supply merely relies on diffusion, adequate oxygen delivery is possible up to a maximum range of only 200 μm [9, 10]. This fact tremendously limits the potential of tissue engineering as it is currently impossible to generate homogeneous biological bone substitutes of clinically relevant size [7, 11].

We have previously shown that the oxygen concentration within central regions of cylindrical cell-seeded demineralized bone matrix (DBM) scaffolds of 5 × 9 mm drops dramatically under static cell culture conditions. This results in a remarkable gradient of vital cells from the periphery towards the center of the scaffolds [7]. This effect was less pronounced in scaffolds seeded with hMSC, suggesting that some cell types display a certain metabolic flexibility, which helps them to adapt to altered environments [8, 12]. We further demonstrated that perfusion bioreactors improved oxygen supply to central areas of scaffolds, yet they were unable to eliminate oxygen gradients entirely when perfusion speeds of 18 μl/min were used [7]. Given that oxygen supply and pH directly correlate in biological systems via the production of lactate during anaerobic glycolysis it was speculated that pH changes may have had an effect on our results; however, this hypothesis had not been directly tested until now [7].

In the present study we therefore addressed how oxygen supply to 3D cell cultures can be optimized and whether relevant pH changes occur. To this end we directly measured pH values in the center of statically cultured cell-scaffold constructs. Furthermore, we measured oxygen concentrations in static 3D cultures of MC3T3-E1 cells in comparison to a slower growing, hTERT immortalized hMSC cell line, which serves as a model system for hMSC in our lab [13]. We then assessed whether a custom-made, dispense-plotted hydroxyapatite-polyurethane composite scaffold with a reduced inner surface would allow for better oxygen supply to central regions of the scaffolds under static culture conditions [14]. After that we tested whether increasing the perfusion speed to threefold and fivefold would enhance oxygen supply within central regions of the 3D constructs. Finally, as a tentative solution for the oxygen problem in vitro, we designed, built and tested an inductive, oxygen-triggered feedback mechanism to automatically increase the medium flow rate of perfusion bioreactors upon a decrease of the oxygen level measured in the geometric center of the scaffold.

## 2 Materials and methods

### 2.1 Cells and cell culture

The murine preosteoblastic cell line MC3T3-E1 (DSZM, Braunschweig, Germany) and a human telomerase reverse transcriptase (hTERT) immortalized hMSC cell line named SCP-1 were used for the experiments. The SCP-1 cell line is a robustly growing, well-established hMSC-derived cell line. The complete transduction process and characterization of the SCP-1 cell line was described in detail elsewhere [13]. Cells were cultured in a humidified atmosphere of 95 % air and 5 % carbon dioxide ($CO_2$) at 37 °C and expanded in minimum essential medium alpha with L-glutamine (MEMalpha; Invitrogen, Carlsbad, California) supplemented with 10 % fetal bovine serum (FBS; Sigma, Munich, Germany) and 40 IU/μl penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria). The medium was changed three times a week.

### 2.2 Scaffolds

#### *2.2.1 Scaffold production*

Bovine, cylindrical demineralized bone matrix (DBM; Tutogen Medical, Neunkirchen, Germany) scaffolds of 9 mm in diameter and 5 mm in height served as matrix for most experiments. Additionally, scaffolds of the same size

with a reduced inner surface were custom-made by the rapid prototyping technique dispense-plotting. These scaffolds were composed of a composite of hydroxyapatite (HA) and a biocompatible, polyurethane-based polymer (polyMaterials AG, Kaufbeuren, Germany). The complete scaffold preparation procedure and biological compatibility was published elsewhere [14]. In brief, to produce these scaffolds two precursors of the polymer were thoroughly mixed with HA-powder to form an extrudable paste. The paste was filled into a cartridge and extruded through a 600 µm nozzle by pressurized air. The extruded rods were deposited on the building platform by computer control according to a predefined CAD-dataset. By changing the orientation of the parallel rods by 90° from layer to layer a three-dimensional scaffold with interconnecting porosity was produced. After fabrication the scaffolds were dried several days to gain the final properties. Prior to cell seeding the scaffolds were autoclaved.

#### 2.2.2 *Scaffold characteristics*

The rods of the HA/polymer-composite scaffolds had a diameter of 650 ± 30 µm. with rectangular pores of 510 × 510 (±30) $\mu m^2$ in x/y-direction and 510 × 300 $\mu m^2$ in x/z-direction. Total porosity was measured to be 35 vol %. Compressive strength of the dispense-plotted composite scaffolds was approx. 17 MPa, bending strength approx. 9 MPa. The hardness of the applied material measured by Vickers HV100 was about 30, which was in the same range as porcine bone. The inner surface of these custom-made scaffolds was approximately 708 × $10^6$ $\mu m^2$.

### 2.3 3D culture

For 3D culture cells were trypsinized using trypsin–EDTA (PAA), centrifuged and resuspended in medium. The cells were counted to reach a final concentration of ∼7.5 × $10^4$ cells per ml. Prior to seeding, the scaffolds were centrifuged in MEMalpha medium for 5 min at 500 G to remove air bubbles from the scaffold. Each matrix was transferred to one well of a 48-well dish (NUNC, Wiesbaden, Germany) and 666 µl of the cell suspension (∼5 × $10^4$ cells) were pipetted onto the scaffolds, except for scaffolds for automated feedback mechanism, which were seeded with ∼3 × $10^5$ cells to challenge the system with a high number of cells. During the first 2 h, the scaffolds were turned around and re-seeded with the cell suspension in 20 min intervals to ensure a maximum number of cells attaching to the matrix rather than to the surface of the culture dish. At last, the seeded constructs were either transferred to 24-well dishes (NUNC) for static culture, or alternatively, inserted press-fit into custom-made polycarbonate rings as described previously [7]. They were then placed into bioreactors with cylindrical flow chambers (MINUCELLS and MINUTISSUE GmbH, Bad Abbach, Germany). Seeding efficiency was calculated by counting the number of cells remaining in the 48-well cavity where the seeding had been carried out. With this technique, a seeding efficiency of ∼85–90 % was reached. In static 3D culture, the medium was not changed to avoid alteration of the running oxygen measurements as noted previously [8].

Dynamic cultivation was performed in bioreactors as described previously [7]. In brief, bioreactors were connected to both fresh medium containing 25 mM hepes buffer (PAA) and to waste reservoirs by gas permeable silicone tubes. Multi-channel roller pumps (Ismatec, IPC, Switzerland), with each flow chamber being connected to its own independent pumping system, guaranteed flow through the scaffold from bottom to top at a flow rate of 18 µl/min, 54 µl/min or 90 µl/min respectively. Tilting of the reactors prevented air trapping within the reactors. All perfusion culture systems were assembled under sterile conditions. Systems were placed on a heating plate (37 °C) and covered with an atmosphere heating unit set to 37 °C.

### 2.4 Oxygen measurements

Oxygen measurements were performed as described previously using needle type oxygen microsensors with fixed sensor tip [7] (NFSx, PreSens, Regensburg, Germany). These oxygen sensors are mounted on optic fibers with a tip diameter of 50 µm. To protect these fragile sensors they are fixed within a standard hollow needle of 0.4 mm diameter. To continuously monitor oxygen partial pressure the needle sensor was inserted in the geometric center of the freshly seeded scaffold. To monitor oxygen within the perfusion setup, the probes were inserted rectangularly from the side, halfway from the top to a depth of 4.5 mm of the scaffold. Oxygen measurements in the medium were carried out by inserting additional oxygen probes directly in the bioreactor, one in the afferent and one in the efferent medium reservoir. Oxygen measurements within the static setup were performed by inserting the probe from the top of the scaffold into its center as well as in the medium next to the scaffold. A two point calibration was performed before each measurement using ambient air as the 21 % oxygen reference and a 100 % $CO_2$ atmosphere as the 0 % oxygen reference. Oxygen was measured every hour over a period of 7 days.

### 2.5 pH measurements

pH measurements were performed analogously to the oxygen measurements. Needle-type micro sensors with

fixed tip (NTH pH Microoptode, Presens, Regensburg, Germany) were calibrated ex factory and inserted into the geometric center of scaffolds as well as in the surrounding medium. As no pH changes were observed in the static 3D cultures, there was no rationale to perform pH measurements under dynamic conditions.

### 2.6 Live-dead-assay

To assess survival of cells a vitality stain was performed. It was based on fluorescence microscopy after incubation of cells with fluoresceindiacetate (FDA) and propidium iodide (PI), both from Fluka/Sigma, Munich, Germany. After staining with FDA and PI, viable cells appear green, nonviable cells are red. A stock solution of FDA was prepared by freshly dissolving 10 mg of FDA in 2 ml of pure acetone and by diluting this mix 1:500 with phosphate buffered saline (PBS). To obtain the final FDA/PI staining solution, the two components were mixed at a ratio of 1:1.

Prior to evaluation by fluorescence microscopy, scaffolds were cut in halves with a scalpel using a self-designed cutting fixture. For staining, medium was removed from either the wells (2D culture) or the petridish and samples were washed with 0.5 ml PBS. PBS was removed and each sample was then incubated for 1 min with 500 µl FDA/PI staining solution. After discarding the dye, the wells were washed with 0.5 ml PBS again. Subsequently, samples were analyzed by fluorescence microscopy using an Axiovert 100 microscope equipped with a 75 W mercury lamp (Zeiss, Munich, Germany). To detect red and green fluorescence, the Zeiss filter sets #10 and #15 were used. Pictures were taken with a Zeiss black and white digital camera (AxioCam MRm) and processed with the Zeiss Axiovision software.

### 2.7 Automated, oxygen-triggered feedback loop

In order to automatize the positive correlation between central oxygen content and pump speed, an oxygen-triggered inductive feedback of a dynamic culture system mechanism was constructed. Using LabView (National Instruments, Munich, Germany), the peristaltic roller pump of the bioreactor was programmed to increase pump speed upon an oxygen concentration below 15 % within the center of 3D cultures (Fig. 5). The feedback-loop was programmed to respond to oxygen measurements produced by the PreSens software. Details about the exact programming will be published elsewhere.

### 2.8 Statistical analysis

Statistical analysis was performed using SigmaPlot version 8 (SPSS, Munich, Germany). Significances were calculated using student's $t$ test. A value of $P < 0.05$ was considered significant. For all experiments, a minimum of two independent experimental runs was performed.

## 3 Results

### 3.1 Static 3D culture results in rapid oxygen depletion

When cultured statically, the oxygen concentration measured in the center of murine preosteoblast-seeded DBM scaffolds dropped to 0 % over the course of 7 days (Fig. 1a). In an attempt to improve oxygen maintenance of in vitro model systems for bone tissue engineering we seeded DBM scaffolds with an hTERT immortalized hMSC cell line SCP-1 and measured oxygen concentrations (Fig. 1b). In a similar manner, the oxygen concentration dropped to 0 % within 7 days of static culture (Fig. 1b).

### 3.2 pH is stable in static 3D cultures independent of the prevailing oxygen concentration

To assure that changes in pH are not the cause for cell death previously noted in static 3D cultures [7] we directly measured the pH in the centres of seeded DBM scaffolds. There was no significant change of pH in scaffolds seeded

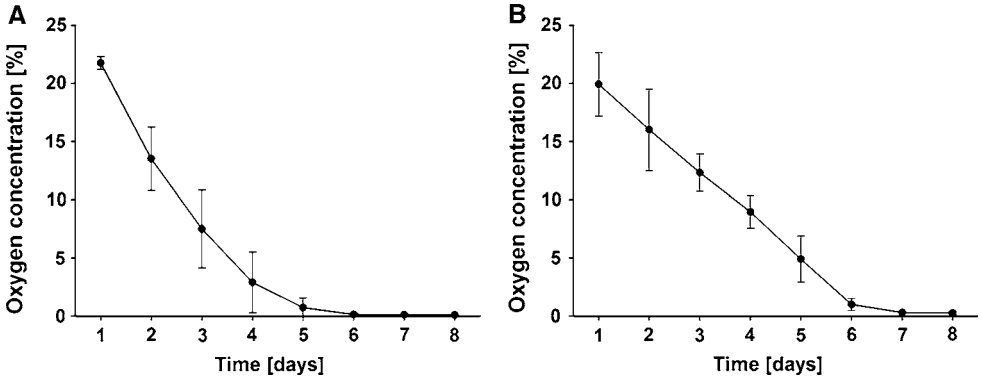


**Fig. 1** Oxygen concentration in the center of 5 × 9 mm DBM-scaffolds seeded with 50,000 MC3T3-cells (**a**) and 50,000 hTERT immortalized hMSC (**b**) under static cell culture conditions. There is a similar drop of oxygen concentration in the center of the scaffolds towards 0 % after only 5 days of static culture (n = 2, mean, standard deviation)

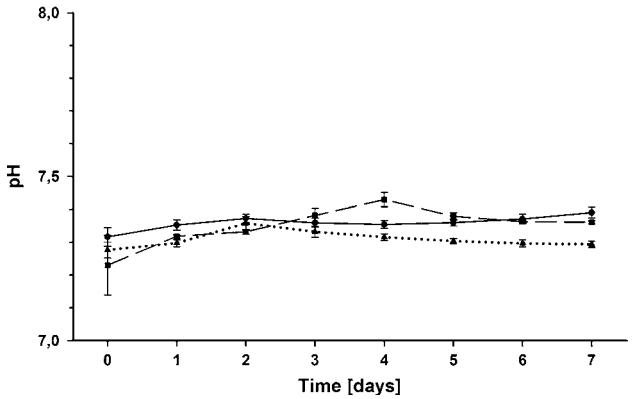


**Fig. 2** pH-measurements in the center of statically cultured DBM-scaffolds seeded with 50,000 (*dashed line*) and 100,000 (*dotted line*) MC3T3-E1 cells and in the surrounding medium (*solid line*) of a scaffold seeded with 50,000 cells. There was no significant change in pH over time in any of the measurements (n = 2, mean, standard deviation)

with MC3T3-E1 cells (Fig. 2, dashed line) compared to the start values and the surrounding medium (Fig. 2, solid line). Even doubling the number of cells seeded on the scaffold to 100,000 did not result in relevant changes of pH over the course of 7 days (Fig. 2, dotted line). All values remained within a range of 7.2–7.5.

### 3.3 Reducing scaffold surface improves oxygen maintenance in 3D cultures of fast growing cells

Our previous studies had shown that seeding DBM scaffolds from bovine origin, which have a huge inner surface due to their trabecular structure, resulted in a rapid depletion of oxygen [7, 8]. We hypothesized, that decreasing the inner surface of the scaffolds may reduce the total number of cells growing on the scaffold thus improving oxygen supply. To test this hypothesis, we designed a scaffold with a reduced inner surface and measured central oxygen concentrations in static culture (Fig. 3). After 7 days we found homogeneously growing cells throughout the scaffolds (Fig. 3a) without the appearance of dead cells in their centres (Fig. 3b). To the more, we detected oxygen concentrations well above 15 % over the entire period of cultivation (Fig. 3c).

### 3.4 Increasing the bioreactor's perfusion speed results in improved oxygen supply to central areas of DBM scaffolds

We previously showed that perfusion bioreactors improved central oxygen concentrations in 3D cultures, yet the oxygen concentration decreased to as little as 2 % if the standard perfusion speed of 18 µl/min was used [7] (solid line; Fig. 4a). To further progress with optimizing central oxygen concentrations in 3D cultures we tested the effect of increasing medium perfusion speed in perfusion bioreactors on central oxygen concentrations. Increasing the perfusion speed to three-fold (54 µl/min) resulted in a distinctive improvement in oxygen supply in the center of the cell-seeded scaffolds: the oxygen concentration did not drop below 9 % in any of the measurements within the usual measuring interval (solid line; Fig. 4b). A further increase in perfusion speed to five-fold (90 µl/min) resulted in an additional but discrete enhancement of oxygen supply to the center of the scaffolds (solid line; Fig. 4c). Oxygen concentrations did not drop below 12 % within the cultivation period.

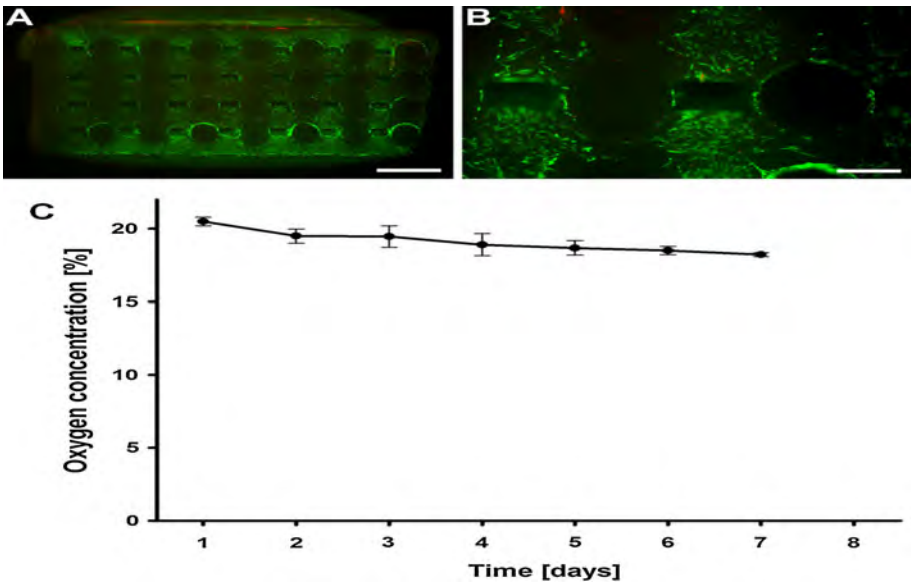


**Fig. 3** Performance of cylindrical HA-scaffolds displaying a reduced inner surface of 708 × $10^6$ µm$^2$. The viability stain (vital cells appear *green* and dead cells *red*) reveals that cells are homogeneously growing within the entire scaffold (**a**) after a period of 7 days in static culture. There are virtually no dead cells detectable in the center of the scaffold (**b**). Oxygen measurement in the center of the scaffold over a time period of 7 days (n = 3, mean, standard deviation) shows that there is only a discrete decrease in oxygen concentration. *Scale bar* 1,000 µm (**a**) and 500 µm (**b**) (Color figure online)

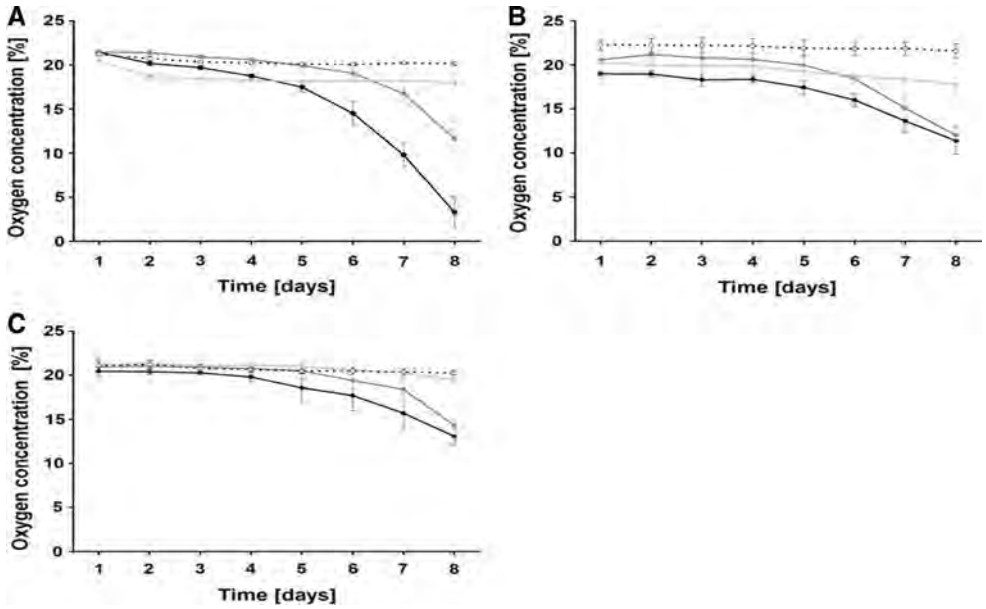


**Fig. 4** Oxygen-concentration in the center of DBM-scaffolds seeded with 50,000 MC3T3-E1 cells cultured under dynamic conditions with (**a**) standard perfusion speed (18 µl/min), with (**b**) three-fold perfusion speed (54 µl/min) and with (**c**) five-fold perfusion speed (90 µl/min). There was an increasing and significant improvement of oxygen supply in the center of the cell-seeded scaffolds over time with increasing perfusion speed (n = 2, mean, standard deviation). In all figures, *dotted lines* represent control measurements in ambient air; *solid lines* represent measurements within the center of cell-seeded scaffolds; *short-dashed lines* represent oxygen-measurements within medium inflow; *long-dashed lines* represent measurements within medium outflow

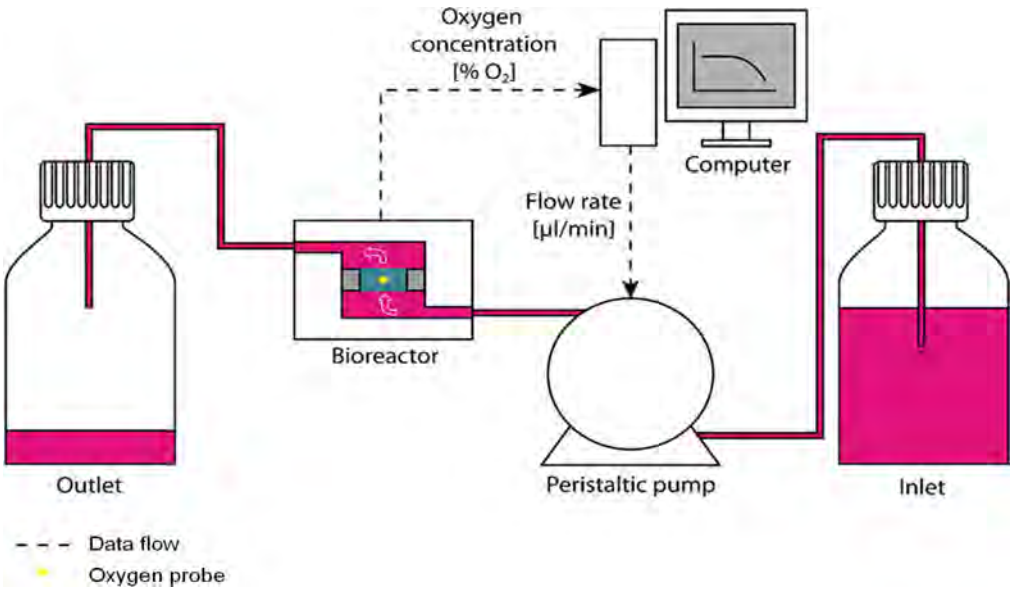


**Fig. 5** Schematic view of the oxygen-triggered feedback mechanism. A computer-steered (LabView) peristaltic pump drives fresh medium from the reservoir (*inlet*) through the perfusion bioreactor into the waste reservoir (*outlet*). The bioreactor houses the cell-laden scaffold (*green*) in the polycarbonate carrier cassette (*gray*). The oxygen sensor (*yellow diamond*) constantly senses the oxygen concentration prevailing in the center of the scaffold to the computer, which increases the pump speed whenever the oxygen concentration drops below 15 %. The maximum pump speed was set to 140 µl/min (Color figure online)

### 3.5 Automated feedback loop

Increasing the perfusion speed within the bioreactors successfully prevented hypoxic conditions in our scaffolds. However, the pump's speed was set independently of the prevailing oxygen concentration within the scaffold. We therefore designed an oxygen-triggered feedback mechanism to increase pump speed in response to dropping

oxygen concentrations in the scaffold's center (Fig. 5). Using LabView, we steered the peristaltic pump of the bioreactor system. Whenever the oxygen concentration in the center of the scaffold dropped below 15 % the pump speed was automatically increased, up to a maximum speed of 140 µl/min.

To challenge the new system, we seeded DBM scaffolds with 300,000 MC3T3-E1 cells before introducing them into the bioreactor. In the control groups, we ran the pump with a constant rate of 18 µl/min. After a cultivation period of 72 h, cells had evenly grown within the entire scaffold (Fig. 6a). When looking at the center of the scaffold using a higher magnification, some dead (red) cells were detectable (Fig. 6b). In this setting, the oxygen concentration measured in the center of the scaffold declined to 0 % within 72 h (Fig. 6c, solid line). Similarly, cells grew abundantly in scaffolds that were cultivated using the oxygen-triggered feedback mechanism (Fig. 6d). Even in central areas of the scaffolds cells were vital throughout (Fig. 6e). Analogously to the control groups, central oxygen concentrations dropped while the pump ran at 18 µl/min (Fig. 6f, solid line). As soon as an oxygen concentration of 15 % was reached, however, the pump speed was automatically increased. The pump's flow rate rose until the maximum speed of 140 µl/min was reached (Fig. 6f, dashed line) thereby stabilizing the oxygen concentration to the pre-set value of 15 %.

## 4 Discussion

Limitations of scaffold-based tissue engineering arise from uneven oxygen supply from the periphery of the cell–matrix constructs towards their centres, which is reflected by significant gradients in tissue quality [7, 8, 11, 15, 16]. Perfusion bioreactors running at standard medium flow rates were shown to improve oxygen supply to central areas of scaffolds, nonetheless they were unable to eliminate oxygen gradients entirely [7, 8]. It was speculated that pH changes may have influenced these results yet this hypothesis had not been directly tested in cell seeded scaffolds [7]. In the present study we therefore assessed how oxygen supply may be homogenized and whether relevant pH gradients exist in 3D cell cultures.

### 4.1 pH is stable in static 3D culture

Because cells were exposed to anoxic conditions in the centres of statically cultivated scaffolds, it was assumed that significant local acidification might occur as a result of

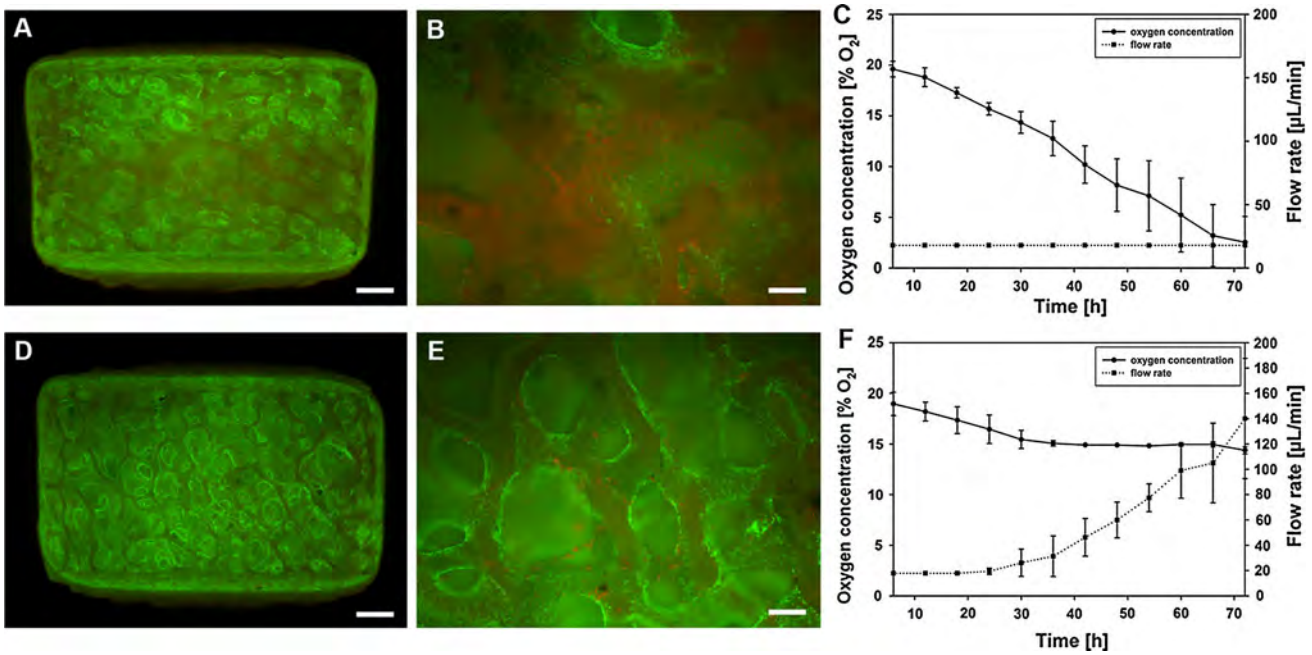


**Fig. 6** To challenge the perfusion bioreactor system, scaffolds were initially loaded with 300,000 MC3T3-E1 and dynamically cultured for a period of 72 h. The viability stain reveals a homogeneous growth pattern after 72 h of culture (**a**, ×5 magnification) with only minimal cell death (*red cells*) in the center of the scaffold (**b**, ×10 magnification) when cultured within the perfusion bioreactor running at the standard perfusion speed of 18 µl/min (**c**, *dashed line*). The oxygen concentration is constantly decreasing over time (**c**, *solid line*). An oxygen-induced increase of the medium flow rate set to kick in below 15 % of oxygen yielded an improved cellular growth in central areas of the scaffolds (**d**) with a reduced number of dead cells in the center of the scaffold (**e**). *Scale bar* 1,000 µm (**a**) and 500 µm (**b**) (Color figure online)

glycolytic production of lactate [7]. In our experiments, however, the pH remained stable in the centres of 3D constructs as well as in the medium surrounding the scaffolds, suggesting that standard medium buffering is sufficient to maintain stable pH even in static 3D cultures. Even doubling the number of initially seeded cells to 100,000 did not significantly influence the pH-values. These findings go in line with results published by Ye et al. [17] who demonstrated that pH was stable in hollow fiber membrane bioreactors over a 1 week period. While it is tempting to speculate that hypoxia may be the only reason for cell death, we believe that other waste products may influence cell survival in static 3D cultures.

### 4.2 Optimizing oxygen supply to 3D cultures for tissue engineering of bone

We next tested several methods to improve central oxygen supply to 3D cultures. First of all, we evaluated the impact of the cell type used. We therefore compared central oxygen concentrations in static 3D cultures of MC3T3-E1 cells to an hTERT immortalized hMSC cell line (SCP-1), which serves as a model system for hMSC in our lab [8, 13]. Even though SCP-1 cells grow somewhat slower than MC3T3-E1 cells, the central oxygen concentration in either 3D culture dropped equally fast to 0 %. These findings suggest that hTERT-immortalized hMSC, similar to MC3T3-E1 cells, may have lost their "metabolic flexibility" during the in vitro selection process, a trait that was earlier attributed to primary hMSC [12]. These findings are in accordance with data from a previous study, where we had seeded identical scaffolds with SCP-1, yet used 1 million cells instead of 50,000 at the initial seeding [8]. In that setting, the margins of the scaffolds were literally overgrown with cells whereas in central areas, all cells had died as a result of hypoxia and potentially other waste products. The use of hMSC, despite using equally high amounts of cells, resulted in a decrease of oxygen to minimally 2 %, however without subsequent cell death [8]. In conclusion, improving central oxygen conditions by selecting specific cell types is possible, but cell lines like the ones tested in this study are not suitable for this purpose.

We next assessed whether the use of scaffolds with a smaller surface would result in better oxygen supply to central regions of the scaffolds. We demonstrated that there were virtually no oxygen gradients in the dispense-plotted scaffolds even though cells were growing abundantly [14]. Dispense-plotted hydroxyapatite-polymer composite scaffolds had the same dimensions as the DBM scaffolds, however the inner surface was considerably smaller. The inner surface was calculated from the rod diameter and pore dimensions and is approx. $0.708 \times 10^6$ mm$^2$. These findings indicate that scaffolds from metaphyseal bone like the DBM used here, which have an approximately tenfold greater inner surface of $7.0 \times 10^6$ mm$^2$ due to the ample trabecular structure, may provide too much space for cells to attach resulting in unnaturally high cell densities within the scaffolds [18]. Another explanation may be that cells grow better on collagen scaffolds such as DBM than they do on artificial materials such as the hydroxyapatite-polymer composite. It is thus advisable to adapt the inner surface of the scaffolds to the growth behavior of the cells on the specific scaffold material that is used.

Another way to gradually improve oxygen supply within central regions of the 3D constructs was to increase the medium flow speed in perfusion bioreactors to either threefold or fivefold. When increasing perfusion speeds, care has to be taken as it was shown that increasing medium flow rates may result in wash out of cells [19]. However, Sikavitsas et al. [20] showed that moderately increasing medium flow even induced osteogenic differentiation by exerting shear stress to the cells. Taken together, it was expectable that oxygen concentrations in central regions of DBM scaffolds would rise upon increased medium flow. Nonetheless, this was a proof of principle to subsequently develop the inducible oxygen-triggered feedback mechanism.

### 4.3 Maintaining stable oxygen concentrations in 3D

As a tentative solution for the oxygen problem in 3D cultures in vitro we built an inductive, oxygen-triggered feedback mechanism, which automatically increased the pump speed of the perfusion bioreactors upon a decrease of the oxygen level measured in the geometric center of the scaffolds. To challenge this system, DBM scaffolds were seeded with 300,000 cells instead of 50,000. This resulted in rapidly declining oxygen concentrations until the pump speed increased at the pre-set level of 15 % oxygen. As cells grew, the pump speed rose to the maximum rate of 140 µl/min after 3 days. During this time, the oxygen level was kept constant at 15 %. We thus managed to tune the oxygen delivery to the amount of oxygen prevailing within central regions of the scaffolds.

We are well aware, that there are several limitations to this study. First of all, it is evident, that the value of 15 % of oxygen is arbitrarily chosen. Probably, oxygen levels around the naturally prevailing 2–5 % would be closer to the true oxygen requirements of cells [8]. Nevertheless, we showed that it is technically feasible to adjust oxygen delivery to the assumed requirements creating the possibility to adjust these bioreactor settings to any individual tissue engineering application. Another weakness of this study may be that the cell lines used in this study are growing unnaturally fast, probably as a result of a selection process that has taken place over the time of cultivation.

This imposes abnormally high challenges to a bioreactor system, as primary hMSC were shown to adapt to low oxygen conditions faster [8, 12]. On the other hand, this model system gave us the opportunity to sound out the limits of the new feedback system. We believe that this invention opened a door towards applying oxygen-triggered feedback systems according to the cell-scaffold combination that is desired. Finally, while in vitro the oxygen problem seems now to be solved, this study has not addressed the issue of hypoxia in 3D constructs after transplantation in vivo, which represents one of the major future problems to be solved in tissue engineering before this technique will be established in clinical practice. Potential strategies include prevascularisation techniques [11] and hypoxic preconditioning of cells [8]. However, these questions will have to be answered by future studies as they exceed the scope of the present study.

## 5 Conclusions

This study revealed that pH is not the cause of cell death in static 3D cultures of DBM scaffolds if regular, buffered medium is used. Oxygen gradients, which occur even in dynamic 3D cultures, can be reduced by either reducing the inner surface of a scaffold or by increasing medium flow rates of perfusion bioreactors. The use of an automated, oxygen-triggered feedback loop results in stable and predictable central oxygen concentrations in cell-scaffold constructs.

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