CarouCELL – a novel compact submerged perfusion bioreactor for drug screening and tissue engineering

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ABSTRACT

Development of traditional methods of cell growth and manipulation on 2-dimensional (2D) surfaces, have been shown to be far from optimal for new challenges of tissue engineering and drug screening. This has lead to the design of a range of 3D cell culture matrices. However, a heterogeneous cell distribution ranging over a scaffold is still a challenge. We present a new compact 3D submerged perfusion bioreactor. It has a central chamber and eight outlet channels radiating from the central chamber to peripheral cutouts with scaffold cassettes. A magnetic stirrer bar in the central chamber is set to rotate thereby creating a vortex with a pressure minimum aligned with the central inlet hole. The media flow can be regulated via the RPM of the magnetic stirrer. The new design was initially tested with human mesenchymal stem cells (hMSCs) derived from bone marrow. After static seeding for two days cells was either grown in a standard static culture or in the perfusion reactor. DNA and alkaline phosphatase (ALP)/DNA analysis was performed both of the whole scaffold and partitioned scaffolds. It is concluded that i) dynamic culture speeds up osteogenic differentiation and ii) dynamic culture promotes a more disperse cell distribution.

Keywords: perfusion, 3D cell culture, hMSC, drug screening

1 INTRODUCTION

The perfusion bioreactor (PB) improves mass transfer at the interior of three-dimensional scaffolds [1]. This bioreactor type uses a peristaltic pump to perfuse culture media uni-directionally through the interconnected pores and enhance internal nutrient exchange. Perfusion is the most reliable method of dynamically culturing thick scaffolds or small pored scaffolds where internal gradients cannot be mitigated by media convection only outside the scaffold. Perfusion is furthermore a convenient way of providing mechanical stimulation to all seeded cells by means of fluid shear stress.

Peristaltic pumps are available with multiple channels and are capable of very fine regulation of flow rates giving perfusion bioreactors an additional advantage of excellent experimental homogeneity through minimal variation in media feed rates between cultured scaffolds (Figure 3A). The design has been popularized by Bancroft et al. and is commercially available.

Common PBs are disadvantaged by excessive equipment footprint and build height. Furthermore the PB holds culture media in no less than three different compartments: The perfusion chamber, the media reservoir, and the tubing. The connection points in between these compartments all raise the risk of infection and complicate media change and cleaning procedures.

To increase testing throughput of bone tissue engineering scaffolds, we designed a device that addressed the limitations of current dynamic culturing devices and merged the familiarity and size of a 6-well plate with the handling and cost of a spinner flask and the reproducible, uni-directional flow of a PB. Along with a wish to eliminate the use of silicone tubing, a design was achieved in which all the separate components that comprised the PB were integrated into a single unit (Figure 3C).

2 MATERIALS AND METHODS

2.1 CarouCELL bioreactor

The submerged perfusion bioreactor is machined from a a 60 mm wide, 15 mm thick disc of polysulfone (Linatex A/S, Denmark). Eight outlet channels with an internal diameter of 1.5 mm radiate from a central chamber to peripheral cutouts with a width of 12 mm that hold the scaffold cassettes. The device is then placed in a flat-bottomed container and completely submerged in culture media. The fluid circuit from the central inlet to scaffold is closed by the reservoir which acts as a unidirectional (centripetal) fluid conduit (Figure 3D).

A magnetic stirrer bar in the central chamber is set to rotate thereby creating a vortex with a pressure minimum aligned with the central inlet hole. The central chamber effectively becomes a centrifugal pump with multiple radial flow outlets. The media flow rate through the scaffolds can be regulated via the size and shape of the stirrer bar and more finely via the RPM of the magnetic stirrer.

During the culture period, oxygenation of the culture media is facilitated by the large unobstructed air-water interface in conjunction with the centripetal flow pattern from the peripheral cassette outlets to the central inlet.

2.2 Scaffolds

Scaffolds were made from polycaprolactone with a molecular weight of 50 kDa (Perstorp, UK) by fused deposition modeling (FDM) with a BioScaffolder (SYS+ENG GmbH, Germany) as described in [2]. Porous mats were made by a layered deposition of 200µm thick fibers of PCL melt with an edge-edge distance of 800 µm. The height of each layer was set to 120 µm, less than the fiber thickness, to achieve thorough fusing between consecutive layers. The direction of the fibers is changed by 105° between each layer to generate a tortuous yet interconnected internal architecture.

Cylindrical scaffolds with a diameter of 10 mm were punched out from 5 mm thick mats with a biopsy punch (Acuderm, FL).

2.3 Cell culture

A telomerase reverse transcriptase gene-transduced adult human mesenchymal stem cell population, hMSC-TERT cells, was used in this study. Cells from PD level 262 (passage 45) were seeded at a density of 4,000 cells/cm^2 in culture flasks in Dulbecco's modified essential medium containing 10% fetal bovine serum and expanded in a humidified atmosphere of 37 °C and 5% CO_2. After the cells reached PD level 271, passage 47 they were detached with 1.25% trypsin and 5 mM EDTA and seeded at a density of 1x10^6 cells/scaffold and cultured in DMEM/10% FBS with 10^{-8} M vitamin D (Leo Pharma 1α,25(OH_2)D_3). Penicillin (100 U/mL) and streptomycin (100 mg/L) was added to each group for the first week. All scaffolds were statically cultured on agarose coated wells for 48 h to ensure cell attachment before perfusion culture. Statically cultured scaffolds were individually placed in 6-well plates and added 7.5 mL media. Dynamically cultured scaffolds were arranged in groups of eight in each CarouCELL and added 60 mL of culture media which resulted in a distance of 3 mm from the media surface to the bioreactor inlet hole. The culture media was changed bi-weekly. Analyses were made at day 2 (all static), day 7, and 14 (static and perfusion).

2.4 CellTracker staining

To assess cell viability, the cell/scaffold constructs were incubated for 30 min in DMEM containing 10 µM CellTrackerTM Green CMFDA (Invitrogen, Denmark). The staining media was then replaced with fresh DMEM/10 % FBS and incubated for another 30 min at 37°C. Non-fluorescent CMFDA is converted to brightly green fluorescent product when cytosolic esterases cleave off the acetates. The cell/scaffold constructs were then rinsed in pre-warmed PBS and fixed in 10 % formalin. Images were acquired using a laser scanning confocal microscope, 510 Meta (Zeiss Microimaging GmbH, Germany). The confocal settings (excitation, laser power, detector gain, and pinhole size) were identical for all images.

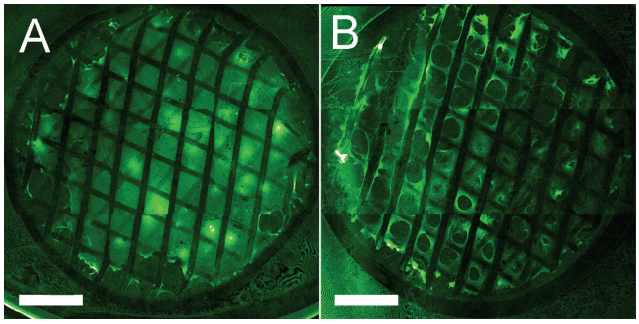

Figure 1: CellTracker staining of cellular confluence on the upper face of the scaffolds at day 14. Perfusion culture prevented the proliferating cells from bridging the pores and closing off the surface with a cell sheet. A) After 14 days of static culture. B) After 14 days perfusion. Bars are 2 mm.

2.5 Histology

Scaffolds were fixed in 70% ethanol and embedded in Technovit® 7100 (Ax-lab, Vedbæk, Denmark). 25 µm sections were made using a Sawing Microtome KDG 95 (Meprotech, Netherlands) and stained with 0.1% toluidine blue (Fluka, Denmark) at pH 7. Sections were viewed and photographed on a BX50 microscope with a Camedia C-5060 digital camera (All products from Olympus, Denmark).

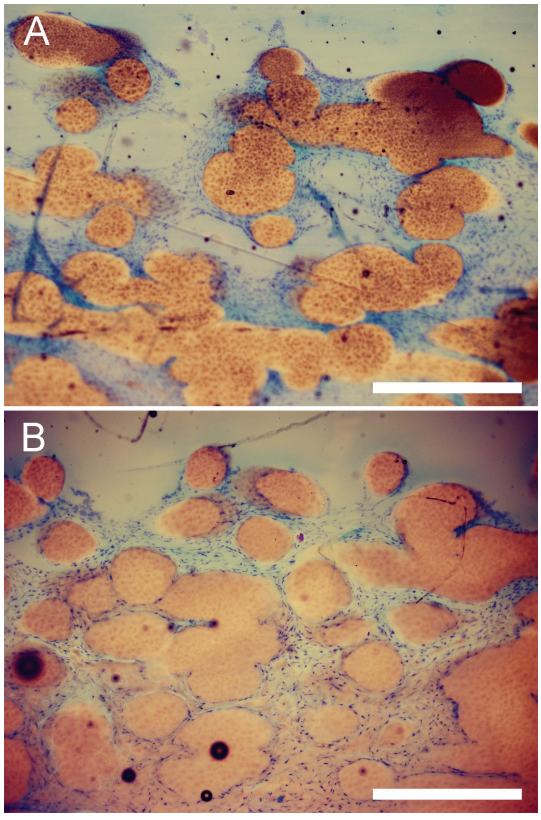

Figure 2: Distribution of cells inside the upper central part of the scaffolds at day 14. A) Static culture showing a

representative uneven cell distribution with clustering around the fiber junctions and large voids in the pores. Dense cellularization is seen bridging the top fibers. B) CarouCELL perfusion culture with a much less clustered, homogeneous cell distribution that spans half the thickness of the scaffold. Toluidine Blue staining. Bars are 500 μm.

2.6 Alkaline phosphatase (ALP) activity assay

To obtain a coarse quantitative measure of cell distribution, the scaffolds were bisected and grouped by tops and bottoms before analysis. Scaffolds were frozen in 1 mL MEM without Phenol Red. Upon analysis, the samples were thawed, sonicated to disintegrate cells and deposited matrix, and then centrifuged. The supernatant was used in a colorimetric endpoint assay to quantify the rate of which ALP converts p-nitrophenyl phosphate to p-nitrophenol. measured by microspectrophotometry at dual wavelengths 405 and 600 nm in a 96-well plate using a microplate reader, (Victor3 1420 Multilabel Counter, PerkinElmer Life Sciences, Denmark). Technical duplicates were used for each sample. The remaining sample volumes were used for determining DNA amount.

2.7 DNA quantification

To further break down cells and matrix, each DNA sample was added 3 mg collagenase and incubated in a 37˚C water bath for 3 h. 1 mg proteinase K was added and the samples were incubated overnight at 45˚C. The sample volume was diluted 1:10 in Tris–EDTA buffer and vortexed to release DNA from the scaffold. From each sample, 2 × 50 μL was aliquotted, prepared with Quant-iT™ PicoGreen® dsDNA assay incubated 5 min in the dark, and measured in a 96-well plate according to manufacturer's instruction. Technical duplicates were used for each sample.

A human diploid cell contains 6.5 pg DNA and DNA quantification is a direct measure for the number of cells on the scaffolds.

2.8 Statistical analysis

Results are presented as mean ± standard deviation (SD) for n=4 biological replicates. Statistics were assessed using Stata 10.0 (College station, TX). The data of ALP activity, calcium content and gene expression were determined using two-way ANOVA (time × treatment). When significant main effects or an interaction between the main effects were found, specific comparisons were made with student t-tests (variance equal) or Wilcoxon rank-sum test (variance not equal). Differences between means were considered statistically significant when p-values <0.05.

3 RESULTS

3.1 Qualitative effects of perfusion on cell distribution

At day 14, the top face of the statically cultured scaffolds had been enclosed by a confluent dense cell sheet, while the perfusion cultured scaffolds still had open pores (Figure **1**). Vertical sections of the scaffolds likewise showed a more homogenous cell distribution from the top face to the scaffold core. Cells in statically cultured scaffolds were typically clustered into colonies around fiber junctions with cell density quickly decreasing away from the fibers. Perfusion cultured scaffolds still had open pores on the top face at the end of the culturing period and had a much more disperse distribution inside the scaffold pores Figure **2**).

3.2 Quantitative effects of perfusion on cell density and differentiation

DNA results showed no global differences in cell number for between static and perfusion culture for any time point. Locally, there is a statistically insignificant increase in cell number for the bottom parts of the scaffolds in perfusion at day 14 compared to static culture. At the same time, the top parts of the perfused scaffolds have a lower cell number, comparatively. The higher cell count in the bottom is mainly because of the better nutrient transport which in part is due to the prevention of top face cell confluence and in part due to mitigation of nutrient gradients. Cells are also more prone to migrate in the direction of the shear stress; further enhancing cellular distribution homogeneity [3].

For the whole scaffolds, ALP activity was significantly higher in perfusion at day 7. ALP activity was highest in the upper portion of the scaffolds in static culture while there were no significant difference between the top and bottom in perfusion culture (**Figure 4**).

4 CONCLUSION

We have demonstrated the osteogenic efficacy of a new perfusion bioreactor using statically cultured scaffolds as controls. Cell distribution along the scaffold axis was also quantified with a resolution of 2.5 mm. The CarouCELL represents a marked departure from traditional perfusion bioreactor design philosophies and has been deliberately developed with user friendliness, a small footprint, and infection resilience in mind. All important features in this prototype can be implemented using injection molding thus enabling mass production. Lower acquisition and operating costs along with a flat learning curve will help to make perfusion culture available as a routine cell culture method for cell biology, drug development, and biomedical research communities.

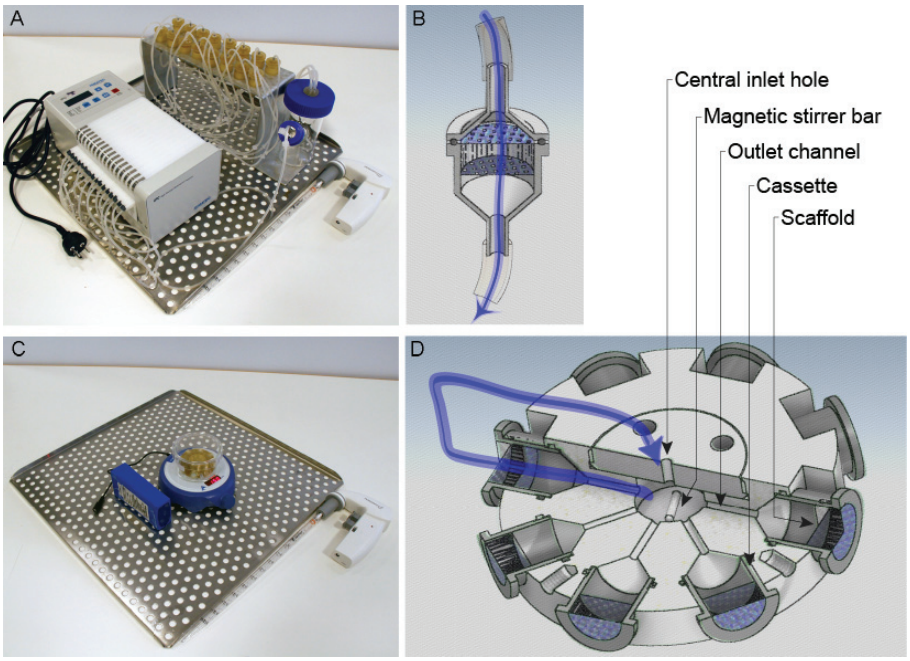

Figure 3: Evolution of the perfusion bioreactor. A) 16-sample perfusion bioreactor assembled on a 50×50 cm incubator tray as used by Bjerre et al. [4]. B) Sectioned view of the working principle of perfusion culture with a porous scaffold placed in a tight fitting cassette with tubing attached to both ends. Arrow indicates media flow. C) A fully assembled 8-sample CarouCELL placed in a crystallizing dish. The magnetic stirrer is powered by rechargeable 12 V, 9000 mAh Li-ion battery for a run-time of ~6 days. D) Quarter-section view of the CarouCELL outlining the internal features that generate and un-directional fluid flow through the peripheral scaffolds residing in cassettes. As the bioreactor is fully submerged, the fluid circuit (arrow) is closed by the container.

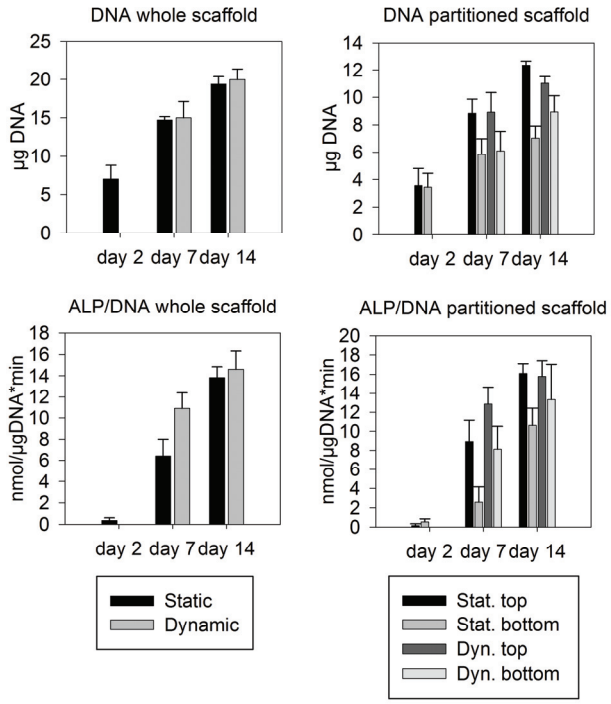

Figure 4: Top left: DNA assay of the whole scaffolds. Top right: DNA content of partitioned scaffolds grouped according to seeding orientation and culture method. Bottom left: ALP/DNA ratio for whole scaffolds. Bottom right: ALP/DNA ratio for the partitioned scaffolds. Dynamic culture facilitates a more even distribution of cells and accelerates osteogenic differentiation evenly throughout the scaffold.

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