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# Integration of hollow fiber membranes improves nutrient supply in three-dimensional tissue constructs

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ABSTRACT

Sufficient nutrient and oxygen transport is a potent modulator of cell proliferation in in vitro tissue-engineered constructs. The lack of oxygen and culture medium can create a potentially lethal environment and limit cellular metabolic activity and growth. Diffusion through scaffold and multi-cellular tissue typically limits transport in vitro, leading to potential hypoxic regions and reduction in the viable tissue thickness. For the in vitro generation of clinically relevant tissue-engineered grafts, current nutrient diffusion limitations should be addressed. Major approaches to overcoming these include culture with bioreactors, scaffolds with artificial microvasculature, oxygen carriers and pre-vascularization of the engineered tissues. This study focuses on the development and utilization of a new perfusion culture system to provide adequate nutrient delivery to cells within large three-dimensional (3D) scaffolds. Perfusion of oxygenated culture medium through porous hollow fiber (HF) integrated within 3D free form fabricated (FFF) scaffolds is proposed. Mouse pre-myoblast (C2C12) cells cultured on scaffolds of poly(ethylene-oxide-terephthalate)–poly(butylene-terephthalate) block copolymer (300PEOT55PBT45) integrated with porous HF membranes of modified poly(ether-sulfone) (mPES, Gambro GmbH) is used as a model system. Various parameters such as fiber transport properties, fiber spacing within a scaffold and medium flow conditions are optimized. The results show that four HF membranes integrated with the scaffold significantly improve the cell density and cell distribution. This study provides a basis for the development of a new HF perfusion culture methodology to overcome the limitations of nutrient diffusion in the culture of large 3D tissue constructs.



## 1. Introduction

Implantation of a tissue-engineered construct based on scaffolds combined with cells or biological molecules is a potential treatment for tissue defects. However, the creation of constructs of clinically relevant dimensions, homogeneously filled with corresponding tissue is still a challenge. This paradigm requires scaffolds which balance between temporary mechanical support with sufficient mass transport to aid biological delivery and uniform tissue growth.

High-density cell cultures are often limited by inadequate supply of nutrients [1]. More specifically, the transplanted cells compete among themselves for oxygen and nutrient supply, thus limiting tissue formation ~200 μm (only diffusion) to a maximum of 1 mm (diffusion + convection in bioreactor) [2]. Investigators have tried to overcome the diffusion limitations within tissue-engineered constructs by using convective bioreactor culture [3–6]. A spinner flask or rotating flask bioreactor can provide control over the hydrodynamics around the tissue construct, and hence external diffusion limitations can be minimized. Despite this, exponential decrease in cell proliferation and oxygen distribution from the periphery to the center of scaffolds cultured in spinner flask was reported earlier for engineered cartilage [6,7] and cardiac tissue [8]. Although a perfusion bioreactor can offer greater control of mass transfer than other convective systems, there still remains the potential for the flow to follow a preferential path through the construct (particularly for scaffold with wide pore size distribution or non-uniform tissue development) creating regions with nutrient deficiency. The above techniques may also induce shear stress due to fluid flow across the scaffolds.

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The need for vasculature to deliver nutrients and oxygen and to remove waste material is particularly important for three-dimensional (3D) large/thick tissue constructs. Smart scaffold design [9,10] and mechanical loading of constructs [11–13] have proved to be effective tools to increase the histological and biochemical properties of tissue-engineered constructs, partly by decreasing the internal diffusion constraints. Despite the fact that mass transfer is increased within the dynamic systems, considerable gradients are still present inside the tissue-engineered constructs obtained.

This work presents proof of the concept of improving nutrient transport into a scaffold by integration of hollow fiber (HF) membranes, which have shown to supply adequate nutrient to 3D cell culture systems [14]. The fibers are incorporated into the scaffold and act as artificial capillaries providing nutrients and oxygen to the cells during cell culture in vitro. To demonstrate the concept, a scaffold system prepared by free form fabrication (FFF) of poly(ethylene oxide terephthalate) (PEOT) and poly(butylene terephthalate) (PBT) polymer was selected. Others reported limited nutrient and oxygen supply to the cell in its core for such scaffolds [7]. The size of the scaffold is 64 $mm^3$ ($4 \times 4 \times 4$ mm) which is a critical size defect for many vascularized soft tissues. For example Grayson et al. [2] have shown that it is possible to obtain a homogeneous distribution in 3D scaffolds with maximum thickness 1 mm. To the best of the present authors' knowledge, this work is the first to expand these findings to a considerably larger volume with the help of HF perfusion. The present study gives particular emphasis to:

- selection of the best HF for this application: fibers with good biocompatibility, high medium delivery to the cells;
- the optimal integration of the fibers into the scaffold and the numbers of fibers used;
- for the optimal cell culture conditions in vitro, a bioreactor system was developed; for this, static and dynamic cell culture conditions were compared – in fact, for the dynamic studies three different cases were compared: medium flow through the scaffold (FTS) (as typically done in the dynamic experiments), medium flow via the lumen of integrated HF alone, and dual flow (combining the two flows through the scaffold and via the HF).

The proof of the concept of this approach is shown using a combination of techniques: scanning electron microscopy (SEM) and cell staining for showing the cell distribution in the scaffold, and DNA assay for quantitative determination of the cell proliferation within the scaffold.

## 2. Materials and methods

### 2.1. FFF scaffold with integrated HF module

#### 2.1.1. FFF scaffold fabrication

Three-dimensional FFF scaffold (3DF) were fabricated using biodegradable PEOT/PBT block copolymers (PolyVation, the Netherlands). The copolymer chemical composition is represented by the notation aPEOTbPBTc, where *a* is the molecular weight of the starting poly(ethylene glycol) (PEG) segments used in the polymerization process, while *b* and *c* refer to the weight ratio between the PEOT and PBT blocks, respectively. For this study, 300PEOT55PBT45 copolymer was used over a wide range of different copolymer compositions, owing to its balanced hydrophobic nature, high mechanical stability, low swelling (~2% as reported by Woodfield et al. [15]), good cell adhesion and tissue formation [16–19].

3DF scaffolds were produced based on the 3D fiber deposition technique using a Bioplotter device (Envisiontec GmbH, Germany), which is an XYZ plotter scaffold construction device [15,20]. To extrude highly visco-elastic fibers, the bioplotter underwent a few modifications as reported by Moroni et al. [21]. Briefly, the granules of PEOT/PBT polymer were fed into a stainless steel syringe and heated to 190 °C using a thermostat heating unit attached to a mobile arm of the bioplotter. Inert nitrogen gas pressure of 4 bar was applied to the top of the syringe to extrude molten polymer through the nozzle attached at the bottom. The nozzles used to extrude the polymer were stainless steel Luer Lock hypodermic needles with internal diameter 400 μm shortened to a length of ~16 mm. The desired scaffold design was loaded to the CAM program (PrimCAM, Switzerland), and the polymer extruded through the nozzle was deposited layer by layer on a stationary stage (Fig. 1). The deposition speed was set to ~200 mm $min^{-1}$ with fiber spacing of 1 mm and layer thickness 250 μm. A 0–90 fiber deposition scaffold architecture was chosen, where fibers were deposited with 90° orientation steps between successive layers.

Cubic samples of 64 $mm^3$ ($4 \times 4 \times 4$ mm) were punched out from a large fabricated scaffold block ($30 \times 30 \times 4$ mm) for all cell culture experiments. Uniform scaffold made of straight smooth fibers ~300 μm in diameter resulted in 65% porosity (the estimation of the scaffold porosity is described in Supplemental S1) with completely interconnected pores [22] ~600 μm in size in the *x*–*y* plane and 250 μm in the *z* plane (Fig. 2a). These scaffolds were further

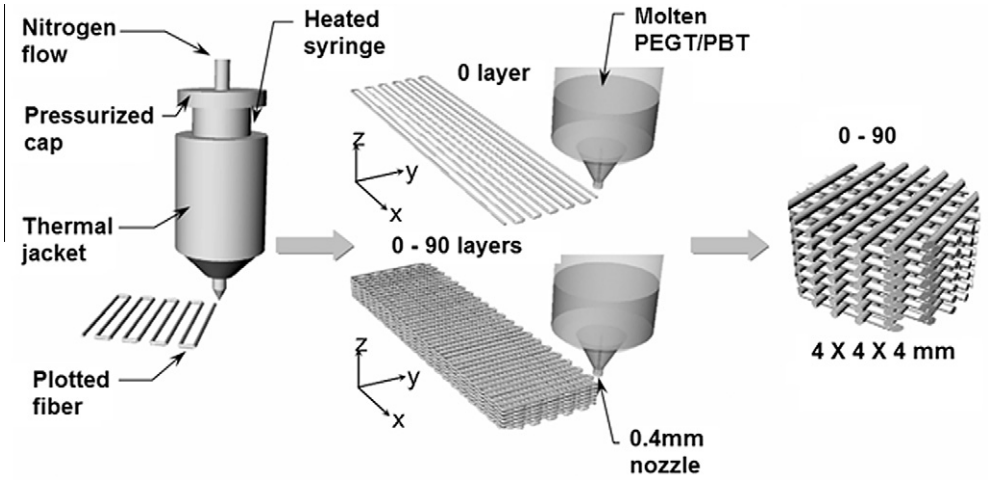


**Fig. 1.** Schematic of a Bioplotter device and 3DF scaffold fabrication process with 0–90 scaffolds architecture in alternate layers are presented. Scheme adapted from Ref. [22].

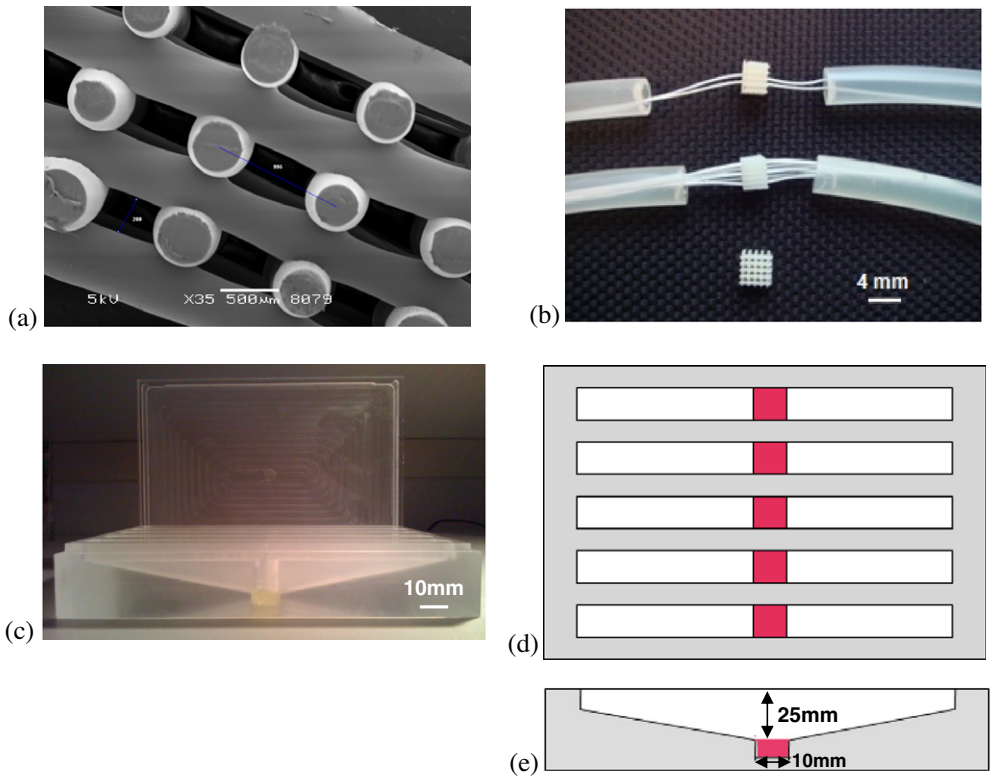


**Fig. 2.** (a) SEM image of 3DF scaffold fabricated using bioplotter. (b) Photographic image of 3DF-HF integrated scaffold module with two and eight HF with HF ends embedded in polyurethane glue inside 6 mm polyethylene tube. (c) Photo of the seeding box. (d), (e) Schematic top and side view of seeding box, respectively.

treated with argon gas plasma by placing them inside a radiofrequency glow discharge chamber (Harrick, US). A vacuum was applied to the chamber (0.01 mbar), which was then flushed four times with argon (purity $\geqslant$ 99.999%). The scaffolds were treated under argon plasma (0.1–0.2 mbar) for 10 min. After cooling, the scaffolds were turned around and again treated for 10 min as described above.

*2.1.2. HF membranes*

Three commercially available porous HF made of biocompatible materials were selected and integrated in the pores of FFF scaffolds: (i) modified polypropylene (PP, Membrana GmbH); (ii) modified polyethersulfone mPES (Gambro GmbH); and (iii) modified polysulfone (mPS, Asahi Kasei Medical Co. Ltd.) which are currently used in plasmapheresis (separation of blood cells from plasma). These membranes are designed to work well (low fouling, high plasma transport) during plasmapheresis treatment of 3–4 h. However, to the best of the authors' knowledge there are no reported performance data with cell culture medium (flux or fouling behavior) for these fibers. To select the best membrane, such studies as well as clean water permeation studies (to evaluate the fiber porosity) and SEM analysis were performed.

*2.1.3. 3D scaffold HF integration: module assembly and treatment*

The scaffold integrated with HF (indicated by 3DF-HF) modules was prepared manually by inserting the HF through the porous scaffold structure. The number of HF and the position within the scaffold was pre-defined to achieve symmetry, as shown in Fig. 3. Both ends of the HF were embedded within 6 mm polyethylene tubing using two-component polyurethane glue, such that 2 cm of HF at the center which pass through the FFF scaffold would be available for perfusion (Fig. 2b). Care was taken to avoid any air bubble entrapment within the embedded portion. The polyurethane was allowed to set for 2 days at room temperature such that solid encapsulation was achieved around the HF, which can be easily connected to the in-house-built perfusion bioreactor. The 3DF-HF modules were first sterilized by pumping 70% ethanol through the HF at low pressure (0.1 bar) in a sterile environment. Further, they were conditioned by pumping phosphate buffer solution (PBS) and proliferation medium correspondingly.

*2.2. Characterization*

*2.2.1. SEM*

SEM images of 3DF scaffold and HF were obtained using a JEOL 5600LV scanning electron microscope at an accelerating voltage of 5 kV. Samples were dried in a vacuum oven at 30 °C overnight and then used for the surface scan, whereas samples for cross-sectional observation were carefully fractured in liquid nitrogen. All the samples were sputtered with gold ($\sim$15–20 nm thick, Balzer-Union SCD-040).

*2.2.2. HF permeability*

*2.2.2.1. Water permeance.* The water permeability of the HF was determined by pressurizing ultrapure water (18.2 MΩ cm, MilliQ) through the lumen of the HF (inside-out permeation). The experiments were carried out in modules containing two HF, each prepared using 6 mm polyethylene tube housing $\sim$8 cm long. The fibers were carefully inserted into the polyethylene tube and sealed with polyurethane glue. A T-junction was placed in the middle of the housing to collect the flowing permeate. Before the clean water transport was measured, the fibers were pre-wetted by pumping water through the modules for at least 30 min. The flux through the membrane was measured at different trans-membrane pressures ranging between 0.1 and 0.3 bar at 20 ± 2 °C. The flux (in kg $h^{-1}$ $m^{-2}$) at each pressure was successively measured

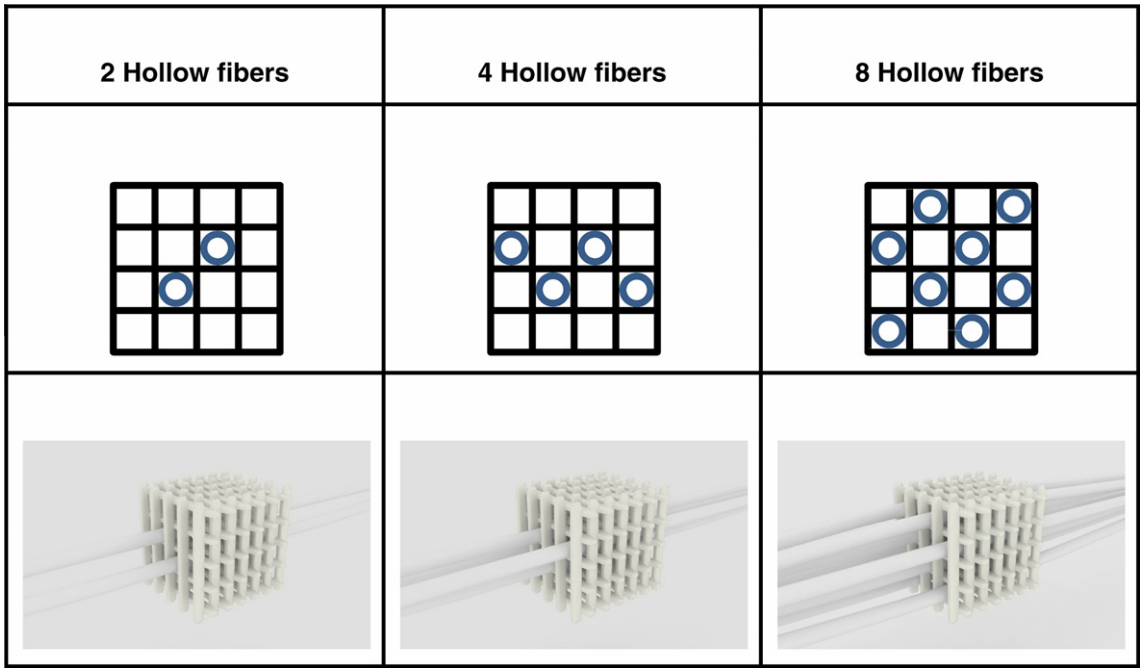


**Fig. 3.** Two-dimensional and 3D illustration of hollow fiber integration architecture within 64 $mm^3$ scaffold.

by collecting the permeating water (kg) for at least 60 min. The pure water permeance (in $kg\,h^{-1}\,m^{-2}\,bar^{-1}$) was calculated using the slope of flux versus pressure plot. The data presented in this work are averages of five different ($n = 5$) HF modules.

*2.2.3. Medium permeance*

The proliferation medium permeability experiments were performed in a sterile cross-flow setup by pumping Dulbecco's Modified Eagle's Medium (D-MEM, Gibco) supplemented with 10% fetal

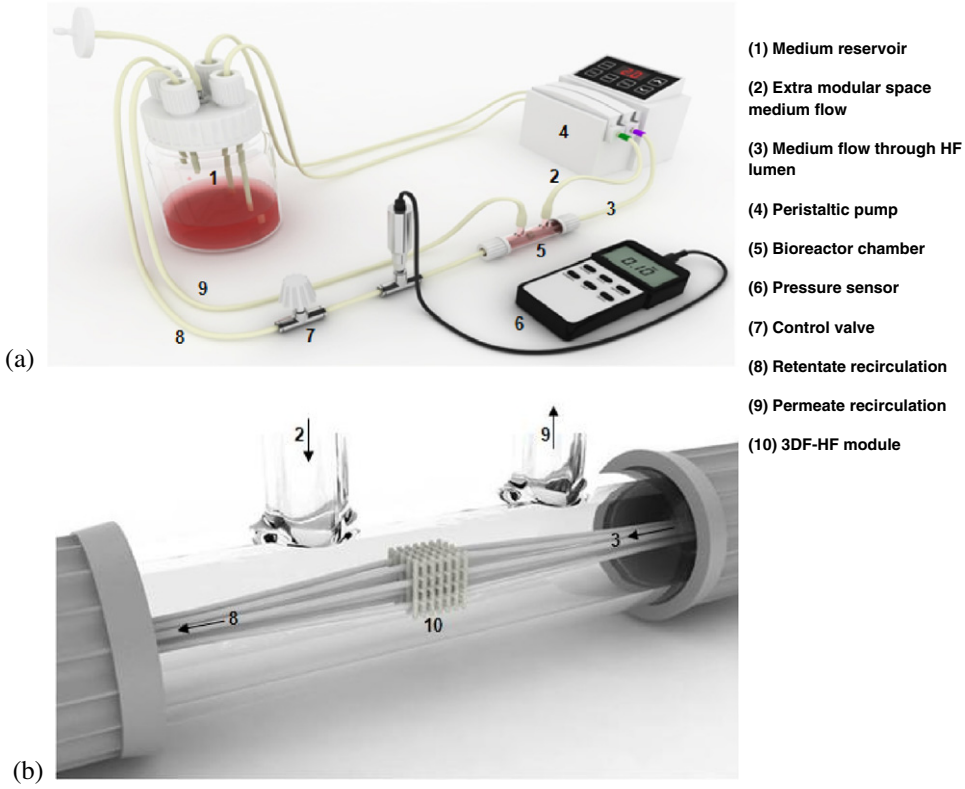


**Fig. 4.** Schematic of dynamic perfusion cell culture system illustrating (a) perfusion bioreactor setup with various components and (b) 3DF-HF integrated module mounted within glass bioreactor with side ports.

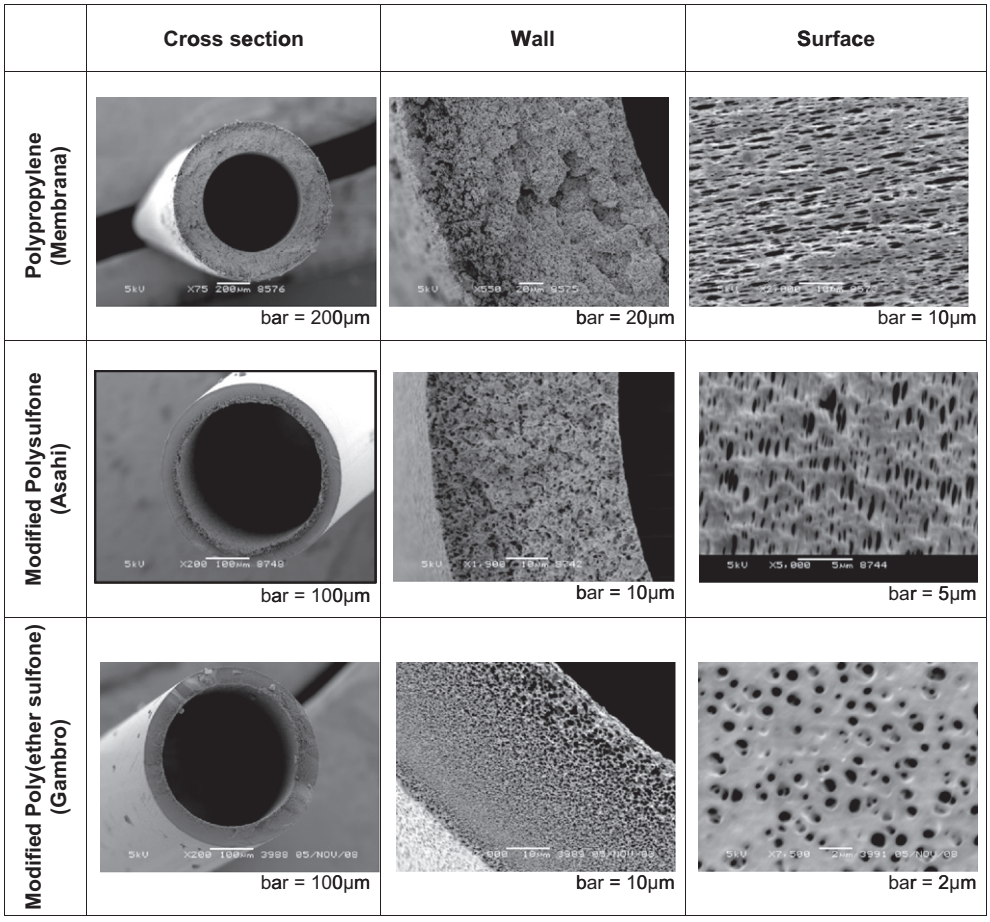


**Fig. 5.** SEM images of biocompatible HF screened to be used to integrate with 3DF scaffold.

bovine serum (FBS, Cambrex), 100 U ml$^{-1}$ penicillin (Gibco) and 100 μg ml$^{-1}$ streptomycin (Gibco) through the lumen of the HF. The maximum allowable pressure in a bioreactor system for tissue engineering application mimicking the physiological condition for cell culture is between 0.1 and 0.15 bar, corresponding to human diastolic and systolic blood pressure. In this study, the authors chose to perform medium permeation experiments at 0.1 bar trans-membrane pressure (the lower margin of the physiological conditions) at 20 ± 2 °C. During the experiment, the medium reservoir (feed) was stirred and pumped to the membrane module using a peristaltic pump with recirculation. The collected permeating medium and unfiltered medium were also used to statically culture mouse pre-myoblast (C2C12) cells in a T-flask for comparison of cell proliferation rate (see details later).

### 2.3. Cell culture

#### 2.3.1. Static cell culture on HF

Fibers 2 cm long were first sterilized in excess of 70% ethanol and iso-propanol. After evaporation of the alcohols inside a sterile flow-hood, the fibers were washed three times with PBS and finally neutralized in proliferation medium. Five sterile fibers were kept inside the tissue culture plate (non-treated) and seeded with C2C12 cells at 4000 cells cm$^{-2}$. These samples were cultured for 3 and 7 days in a humidified incubator (Sanyo, kept at 37 °C, 5% $CO_2$, relative humidity >95%) with proliferation medium containing D-MEM (Gibco) supplemented with 10% FBS (Cambrex), 100 U ml$^{-1}$ penicillin (Gibco) and 100 μg ml$^{-1}$ streptomycin (Gibco), being exchanged every other day.

#### 2.3.2. Dynamic cell culture on HF

HF to be tested for dynamic perfusion cell culture were potted at both ends using polyurethane inside 6 mm silicon tubing, such that the surface of 2 cm long fibers were exposed for cell adhesion and proliferation in the middle section. The HF modules were sterilized by pumping 70% ethanol and then conditioned by pumping PBS and proliferation medium correspondingly at low pressure (~0.1 bar). The sterilized fiber modules were seeded with C2C12 cells and cultured in a recirculation perfusion bioreactor for 3 and 7 days (see details above, as explained for static culture). Trans-membrane pressure of 0.1 bar was maintained across the cell seeded fiber module with an average cross flow velocity of 1 ml min$^{-1}$.

The statically or dynamically cultured fibers were analyzed for cell adhesion and proliferation using a light microscope after staining the cells with methylene blue stain. Cells were first fixed using freshly prepared formaldehyde (4%) then washed with PBS. Samples were then stained with 1% methylene blue staining solution for 30 s and rinsed with PBS.

#### 2.3.3. Cell seeding on 3DF-HF scaffold module

Mouse pre-myoblast, C2C12 cells were cultured in proliferation medium containing Dulbecco's Modified Eagle's Medium (D-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Cambrex), 100 U ml$^{-1}$ penicillin (Gibco) and 100 μg ml$^{-1}$ streptomycin (Gibco). Cells were initially plated in a T-flask for expansion at 2000 cells cm$^{-2}$ until they reached 70–80% confluence, after which they were trypsinized using 0.05% Trypsin contained in 1 mM ethylene-diamine-tetra-acetate (Gibco).

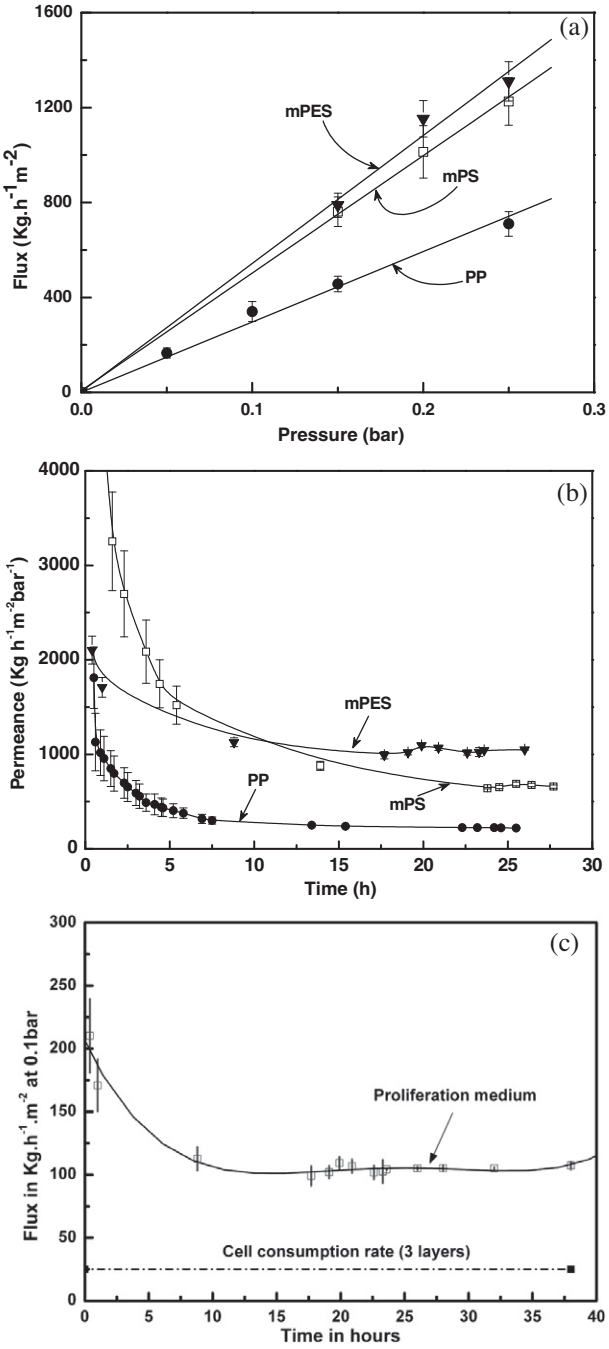


**Fig. 6.** Plot of different biocompatible HF tested for (a) clean water flux, (b) proliferation medium permeance and (c) flux of proliferation medium at trans-membrane pressure of 0.1 bar across mPES HF (Gambro) in comparison with cell consumption rate (error bars indicate standard deviation, $n = 5$).

The C2C12 cells obtained were aggregated and re-suspended in 50 µl medium subsequently seeded onto the scaffold module at $3 \times 10^6$ cells in a special seeding box (Fig. 2c) which accommodated the 3DF-HF modules. The seeding box was made by engraving or selective milling of a polystyrene block. To avoid cell adhesion, the polystyrene surface was treated with Sigmacote® to make it super hydrophobic. The effect of this treatment was tested by cell culturing for 7 days, showing no cell adhesion to the polystyrene surface (results not shown). Then 50 µl of cell suspension corresponding to the pore volume of the scaffold were carefully filled into the scaffold. After allowing the cells to attach to the scaffold for 3 h, 10 ml medium was slowly added and cultured statically in the incubator. The seeding box was specially designed to accommodate the scaffold at 25 mm below the horizontal plane, so that the cells could be cultured without the module floating in the culture medium (Fig. 2d and e). The cells were allowed to attach there for 3 days with 10 ml proliferation medium refreshed every day before the static or dynamic culture experiments were performed. The bottom of the seeding box was coated with non-tissue culture coating, so that the cells could only adhere to and proliferate on the scaffold.

### 2.3.4. Cell culturing on 3DF-HF scaffold module

3DF-HF scaffold modules seeded with cells were statically cultured in sterile non-treated petri dishes. The samples were cultured for 3, 7 and 14 days in a sterile incubator with 25 ml of proliferation medium refreshed every 2 days. The resulting samples were analyzed using SEM and light microscopy for spatial distribution and were quantified by total DNA assay.

The dynamic cell culture within a glass bioreactor was performed using a perfusion system consisting of a medium reservoir (Schott AG), Food and Drug Administration Agency (USA) approved platinum-cured silicon tubing (Masterflex), a multi-channel precision flow peristaltic pump (Watson-Marlow), click connectors and a pressure sensor (see Fig. 4a). All the various parts of the bioreactor were sterilized by autoclaving before each experiment. The whole perfusion bioreactor system was built within a sterile incubator controlled for temperature, humidity and $CO_2$ (Sanyo, kept at 37 °C, 5% $CO_2$, relative humidity >95%). Fresh medium was perfused in single-pass, and the stream leaving the bioreactor was collected as waste until approaching the steady state. When the system reached steady state, the stream leaving the bioreactor was recycled. Before the dynamic cell culture experiments were started, the medium used in the reservoir was recirculated for at least 12 h via gas permeable tubing to ensure that the medium attained the incubator environment and to remove any air bubbles entrapped within the closed circuit (tube length ~1.5 m). A peristaltic pump with a different tube diameter was used to control medium volumetric flow rate at same pump speed. A pressure sensor was used to regulate the input trans-membrane pressure of the medium across the HF.

A reservoir containing 100 ml of proliferation medium was recirculated to feed cells for 84 h before refreshing. Media samples were collected from the outlet stream to evaluate the glucose depletion and lactate production using Vitros-DT60 diagnostic slides (Ortho-Clinical Diagnostics). The medium was maintained with minimum glucose at 17 ± 0.5 mM and maximum lactate at 6.5 ± 0.7 mM concentration before refreshing in the reservoir for both static and dynamic culture (compared with fresh medium

**Table 1**
Screening of biocompatible HF based on water and medium permeability (data presented are averages of five different HF tested for each fiber type, standard deviation $n = 5$).

| HF type | Material | Water permeance (kg $m^{-2}$ $h^{-1}$bar$^{-1}$) | Medium permeance (kg $m^{-2}$ $h^{-1}$bar$^{-1}$) (at steady state) |
|---|---|---|---|
| Membrana | PP | 3010 ± 37 | 300 ± 26 |
| Asahi | Modified PS | 4990 ± 84 | 662 ± 17 |
| Gambro | Modified PES | 5410 ± 71 | 1040 ± 32 |

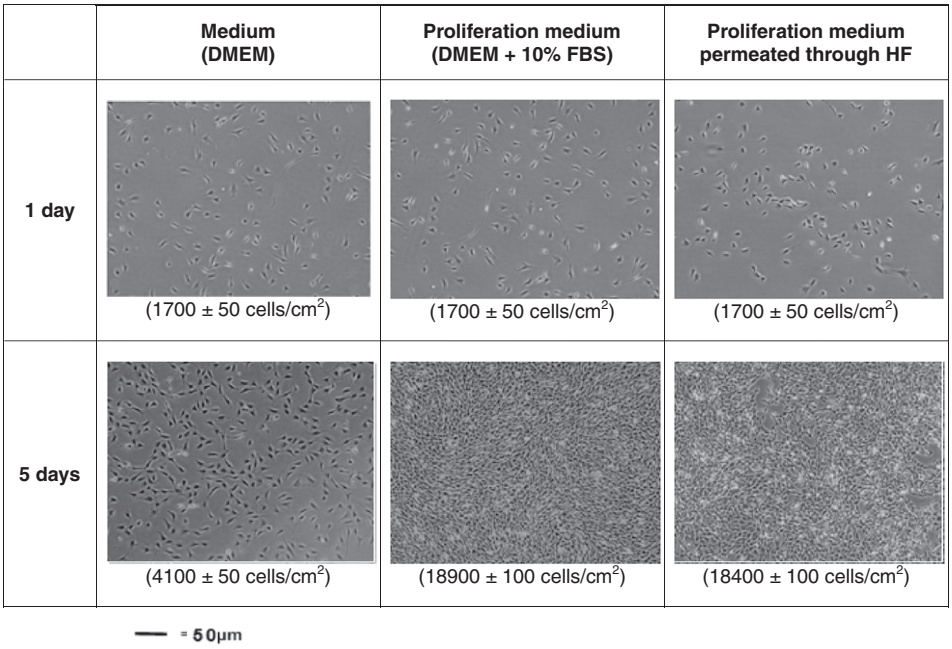


**Fig. 7.** Light microscopic image of C2C12 cells cultured for 1 and 5 days with DMEM, proliferation medium and HF permeated medium (Gambro) (seeding density ~2000 cells $cm^{-2}$, $n = 5$).

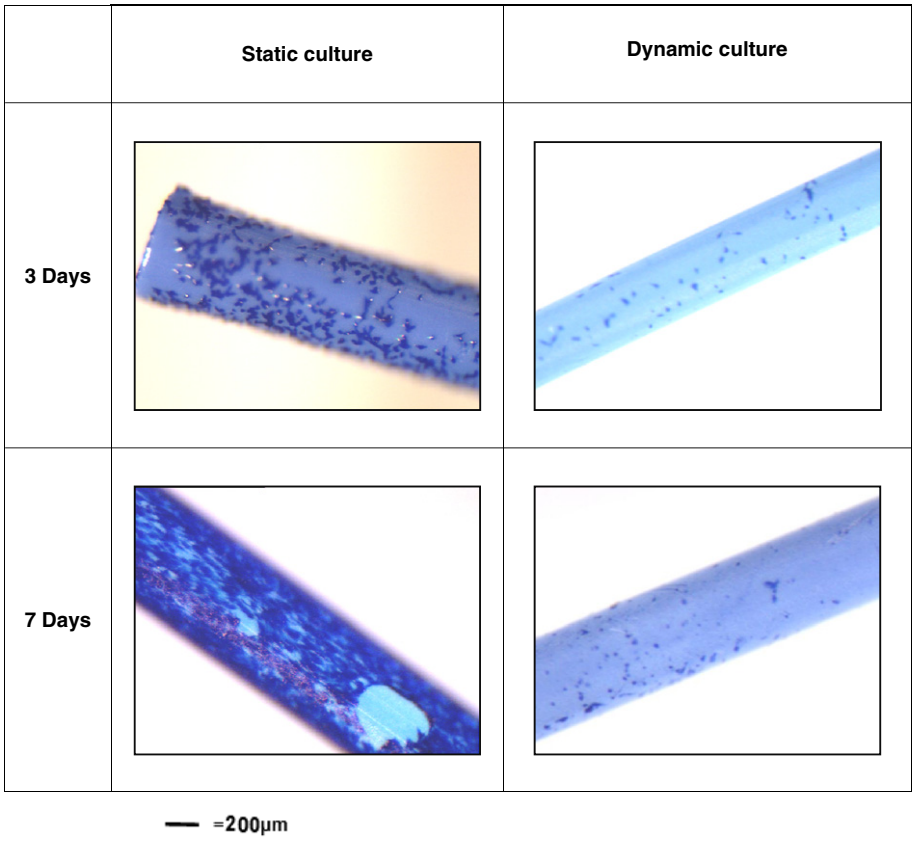


**Fig. 8.** Light microscopic images of C2C12 cells cultured statically and dynamically for 3 and 7 days on mPES (Gambro) fibers and stained with methylene blue stain (seeding density, 4000 cells $cm^{-2}$, $n = 5$).

concentration of 25.1 ± 0.4 mM and 0.9 ± 0.3 mM, respectively) to avoid the risk of recirculating toxic waste to the proliferating cells.

The glass bioreactor itself consists of scaffold-fiber module housed in a glass tube reactor 1 cm in diameter and 5 cm long, with two side ports for medium flow and recirculation (Fig. 4b). The module with cell-seeded scaffolds was held freely in the center of the glass reactor, using a screw cap and silicon sealant rings mounted on the 6 mm tubing at both ends of the scaffold module

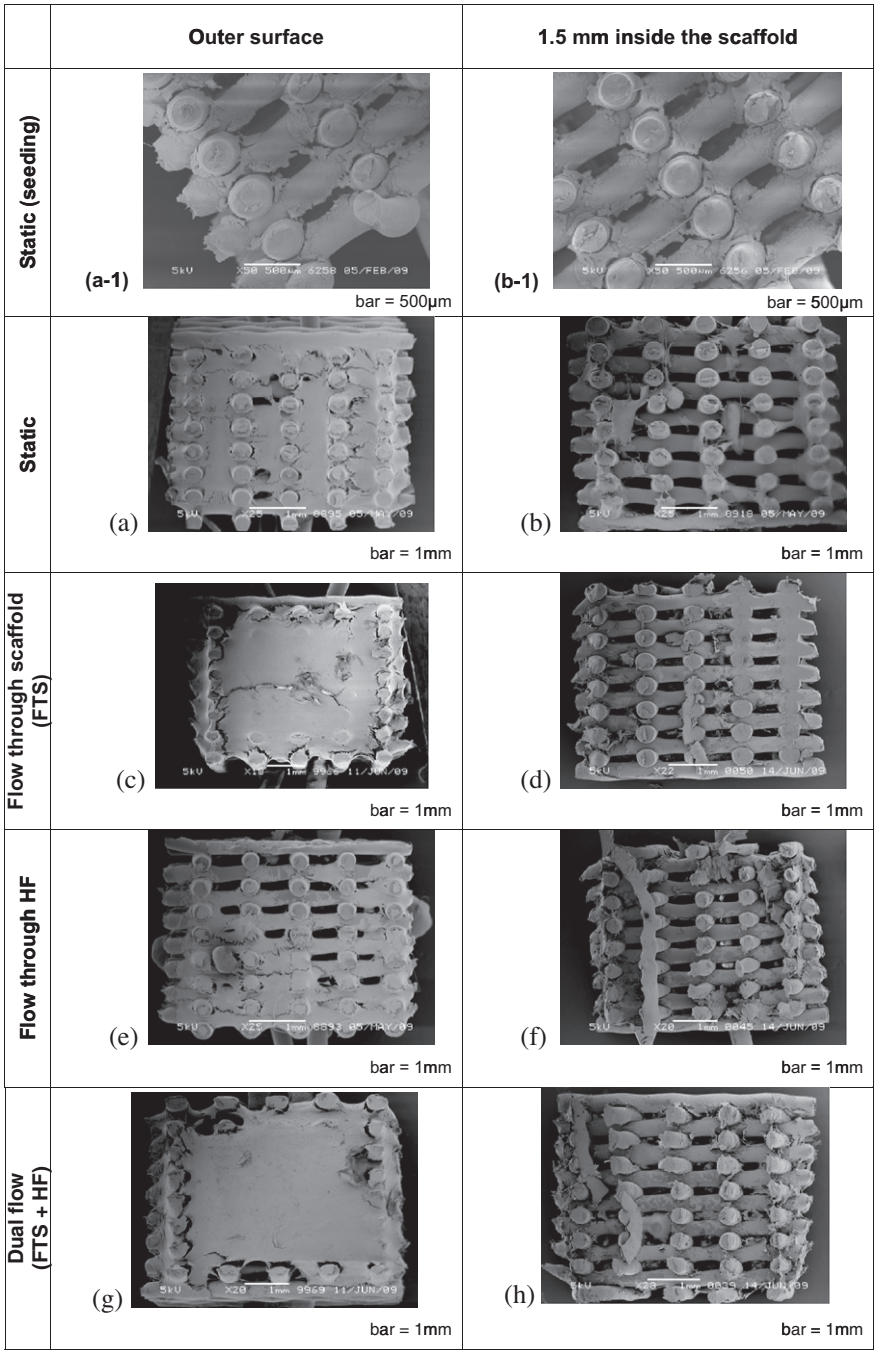


**Fig. 9.** SEM images of cells cultured for 7 days on 3DF scaffold integrated with 2 HF in static and dynamic culture conditions (with different bioreactor flow conditions) ($n = 4$).

(Fig. 4b). In this study, three different medium flow conditions were investigated:

(1) *Medium FTS*: the medium was pumped through the scaffold (as in conventional dynamic experiments). An average flow rate of ~0.26 ml $min^{-1}$ was maintained.
(2) *Medium flow through HF lumen*: the medium was pumped only through the lumen of the HF. The trans-membrane pressure across the HF was monitored and kept constant at 100 mbar, corresponding to human diastolic blood pressure, with an average cross flow velocity of ~1 ml $min^{-1}$. The permeating medium through the HF refreshes the medium in the bioreactor and exits through the side ports to the reservoir. (The details for optimizing the average cross flow velocity are given in Supplemental S2).
(3) *Dual FTS and the HF lumen (FTS + HF)*: the medium was pumped simultaneously through the scaffold (FTS) and through the HF lumen in a counter current direction, using the flow rates described earlier (combination of conditions 1 and 2). The flow rates were maintained using a single peristaltic pump with a different tube diameter. The same proliferation medium reservoir was used for both flows in the bioreactor.

### 2.4. Cell culture: analysis

#### 2.4.1. Critical point dried sample for SEM

For the SEM analysis, cell-cultured scaffold samples were washed with PBS to remove culture medium prior to fixation by incubating for at least 1 h with freshly prepared 4% para-formaldehyde (Merck).

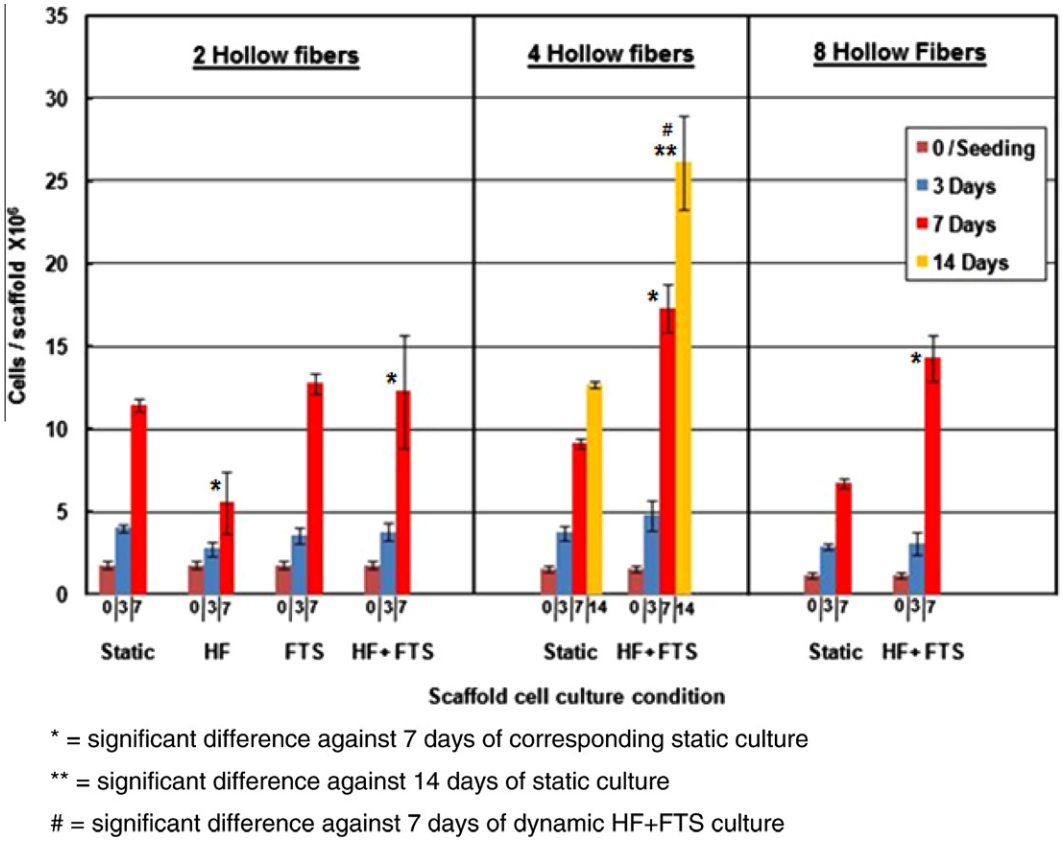


**Fig. 10.** C2C12 cell proliferation within 3DF-HF scaffold module with two, four and eight HF integrated module cultured statically and dynamically for 0, 3, 7 and 14 days (error bars indicate standard deviation, $n = 5$).

Further, the samples were dehydrated in 50%, 70%, 80%, 90%, 98% and 100% ethanol for at least 4 h for each step. Dehydration in 100% ethanol was performed for two additional times to remove all the water. Dehydrated samples were dried using liquid carbon-dioxide with a Blazers CPD 030 Critical Point Drier. SEM images were taken as described above.

*2.4.2. Histology: embedding and staining for light microscopy*

Cell-scaffold samples were fixed for 6 h in 10% neutral buffered formalin and dehydrated (as explained above) before embedding in a two-component glycol methacrylate acrylic resin (GMA) (Merck) mixed according to the protocol for crosslinking specified by the provider. The samples were placed in an embedding plate (non-adhering) into which the two-component glycol methacrylate acrylic resin (GMA) was poured and allowed to polymerize for 24 h (avoiding any air bubble entrapment). A microtome specific holder was glued to one end of the embedded sample, using fast-acting glue. Transverse microtome sections were made to yield 5 μm thick sections, which were placed on silanized slides and dried on a hot plate before staining with hematoxylin and eosin (H&E) (Sigma–Aldrich) according to the manufacturer's protocol. The resulting stained samples were stored dry until further use for light microscopic analysis to visualize cells and tissue formation. The slides were examined under a light microscope (Nikon Eclipse E400) and representative images were captured using a digital camera (Sony Corporation, Japan) and Matrix Vision software (Matrix Vision GmbH, Germany).

*2.4.3. Cell proliferation assay*

The amount of DNA per scaffold sample (at different culture times of 0, 3, 7 and 14 days) was used as an indication for cell proliferation. The samples to be assayed were washed with PBS and stored at −85 °C for at least 1 day. The samples were allowed to attain room temperature and sliced to small pieces such that they could be immersed in a small quantity (1 ml) of cell lysis buffer before performing the DNA assay. Quantification of total DNA per sample was measured according to the manufacturer's protocol (CyQuant Cell Proliferation Assay Kit, Invitrogen/Molecular probes) using a fluorescent plate reader (Perkin–Elmer). The total cell number was calculated using C2C12 cell-based standard curve experimentally measured (total DNA vs. cell number, data not shown).

## 3. Results and discussion

*3.1. Fabrication and characterization of 3DF scaffold*

It has been demonstrated that the effective diffusion within porous scaffolds is proportional to the porosity and inversely proportional to the square of the geometrical tortuosity of the path that the substrate encounters through the pores [23]. Hence, the scaffold was fabricated based on the fiber deposition technique to minimize the pore tortuosity. This rapid prototyping technology has already been demonstrated to produce 3D scaffolds with completely open and interconnected pores [7]. The 300PEOT55PBT45 copolymer was chosen over a wide range of different copolymer compositions because of its balanced hydrophobic nature and low swelling property (~2% as reported by Woodfield et al. [15]).

*3.2. HF selection*

*3.2.1. SEM*

Fig. 5 shows the SEM images of all the HF used in this study. The fiber surface images demonstrate that the pores on the outer surface of the HF are between 0.5 and 3 μm. Thus, the fiber wall acts

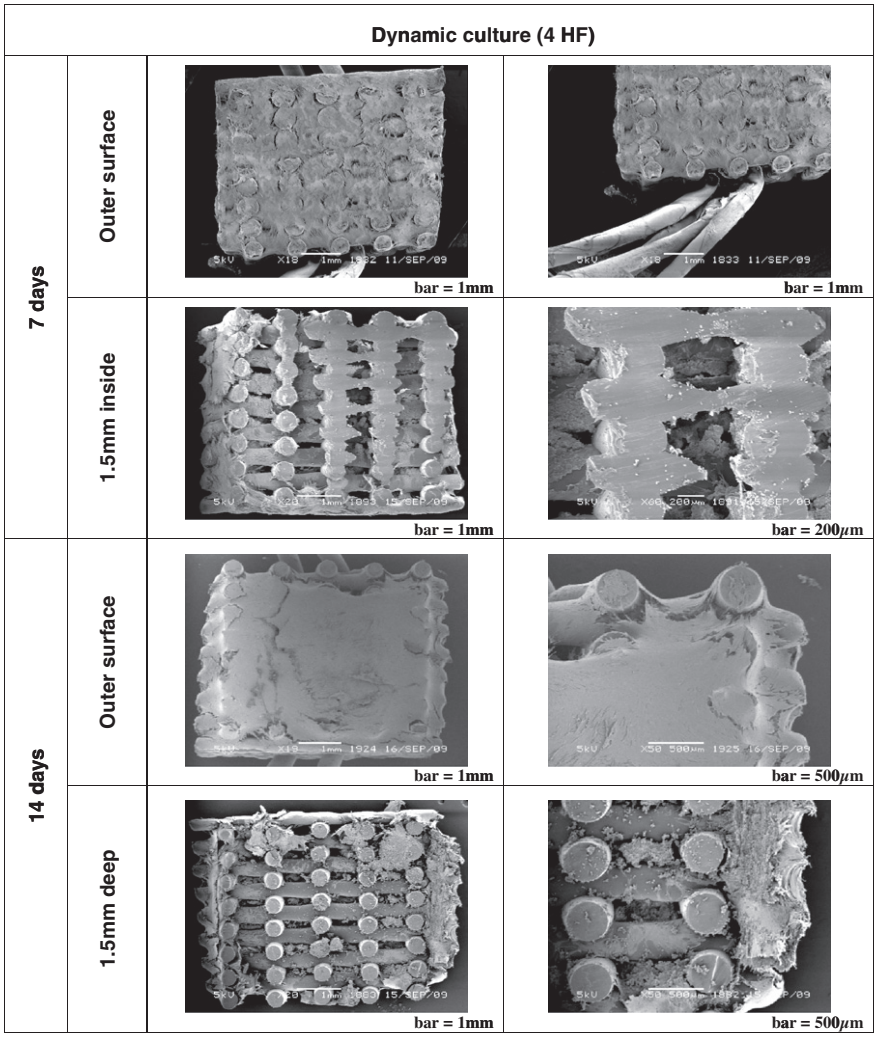


**Fig. 11.** SEM images of C2C12 cells cultured for 7 and 14 days on 3DF scaffold integrated with four HF in dual perfusion bioreactor system with medium flow through FTS and HF lumen ($n = 4$).

as a barrier and inhibits cell infiltration into the HF pores, as the C2C12 cell dimensions are typically >5 μm.

*3.2.2. Clean water and medium permeance*

Clean water flux through the fiber gives an indication of HF porosity, pore connectivity and tortuosity. Fig. 6a shows that the clean water flux is linear with no compaction (several cycles of increasing and decreasing pressure flux were tested) at different trans-membrane pressures (0.1–0.25 bar). Table 1 shows the water permeance (slope of Fig. 6a) of the HF tested. For the mPES (Gambro) and mPS (Asahi) fibers, it is $\sim 5200 \pm 200$ kg m$^{-2}$ h$^{-1}$bar$^{-1}$, which is much higher than that of PP (Membrana) fibers ($3010 \pm 37$ kg m$^{-2}$ h$^{-1}$bar$^{-1}$).

Fig. 6b shows that the medium permeance of all HF membranes decreases in time and reaches a plateau (steady state) owing to deposition of medium proteins in the membrane pores. For all membranes, despite their credentials (used in plasmapheresis applications), the transport of the medium decreases in time due to membrane fouling. Nonetheless, their pores do not seem to block completely, and medium transport can be maintained at lower levels after a steady state is reached. Table 1 shows the individual medium permeance of the HF tested after attaining steady-state flux. Based on these results, the mPES (Gambro) fibers were chosen for all cell culture experiments because they attain the plateau of constant medium permeation faster (10 h) and have the highest medium steady-state permeance ($1040 \pm 32$ kg m$^{-2}$ h$^{-1}$bar$^{-1}$). Fig. 6c compares the actual medium flux through the mPES (Gambro) fiber at a trans-membrane pressure of 0.1 bar (this flux stays constant at this level for more than 15 days; results not shown) and the theoretical medium glucose consumption by three layers of cells cultured in confluence over the surface of the HF (calculated using literature data for glucose consumption by the cells [24,25]). These results suggest that the medium flux through the mPES (Gambro) fiber would be sufficient for maintaining multi-layer tissue culture of ~12 cell layers in confluence per unit active HF surface area.

The medium permeated through the mPES (Gambro) HF at steady state was also tested for C2C12 cells culture in a T-flask, and the results were compared with cell culture with proliferation medium and DMEM without FBS (as a control) to check whether there is any difference in proliferation rate due to HF permeation. Fig. 7 shows the light microscopic images of C2C12 cells and the numbers of cells obtained in culture after 1 and 5 days. The proliferation of cells cultured with medium after HF filtration and standard proliferation medium is identical, proving that the mPES (Gambro) fiber indeed delivers to the cells all the valuable nutrients present in the medium.

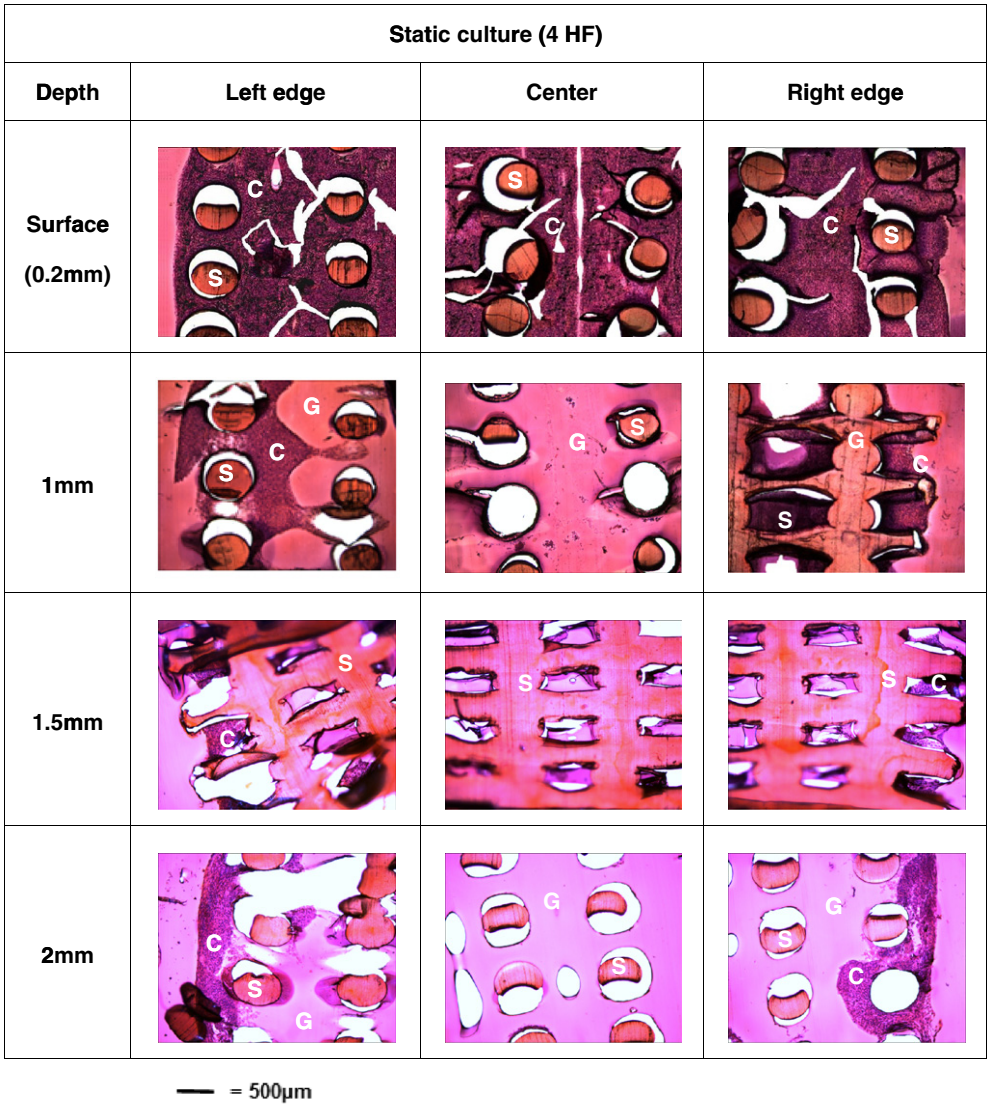


**Fig. 12.** Light microscopic images of H&E stained microtome sections of scaffold embedded in GMA of cell cultured statically for 7 days on 3DF-HF module integrated with 4 HF (G, GMA embedding resin; S, scaffold polymer; C, cells).

### 3.2.3. HF biocompatibility

Fig. 8 shows light microscopy images of C2C12 cells stained with methylene blue after static or dynamic culture on the mPES (Gambro) fibers for 3 and 7 days. The cells attach and proliferate well on the fiber surface, thus confirming the good biocompatibility of the HF. When dynamically cultured in a perfusion bioreactor, very few cells are observed on the fiber surface. This indicates that the permeating medium restricts adhesion of proliferating cells on the fiber surface.

## 3.3. Bioreactor experiments

Perfusion bioreactor cell culture experiments were carried out on 3DF-HF scaffold containing two, four and eight HF with symmetric distribution along the horizontal axis within the scaffold (Fig. 3).

### 3.3.1. Comparison of flow cases

The three different cases of medium flow through the bioreactor were assessed by carrying out cell culture experiments on the 3DF-HF module with two HF. Medium FTS, flow through the HF alone and dual flow (FTS and through the HF combined) were tested. For comparison, cell-seeded 3DF-HF scaffolds were also cultured statically.

Fig. 9 shows SEM images confirming histological observations that cells attach well on the 3DF-HF scaffolds cultured for 7 days under static and dynamic conditions. The images show that the cells and extra-cellular matrix (ECM) produced are present ~1.5 mm deep inside the scaffold. Fig. 10 depicts the total cell number per scaffold cultured for 0 (seeding), 3, 7 and 14 days in corresponding culture conditions. Although the cell number for static culture increases in time (see Fig. 10), the cells tend to proliferate only on the outer surface. Fig. 9a and b shows that the scaffold surface is completely covered with cells and ECM, but the scaffold interior has almost no cells. This observation is in accordance with literature studies, where a decrease in cell number and oxygen concentration was reported in the interior of 3D scaffolds under static culture conditions [26,27].

In the FTS case, it seems that the medium is only refreshed well around the scaffold, resulting in a similar cell attachment and ECM deposition compared with static culture. Although the medium is refreshed constantly at 0.26 ml $min^{-1}$, the ECM on the surface acts as a barrier for nutrient transport into the center of the scaffold. Similar cell numbers compared with static culture are obtained (see Fig. 10).

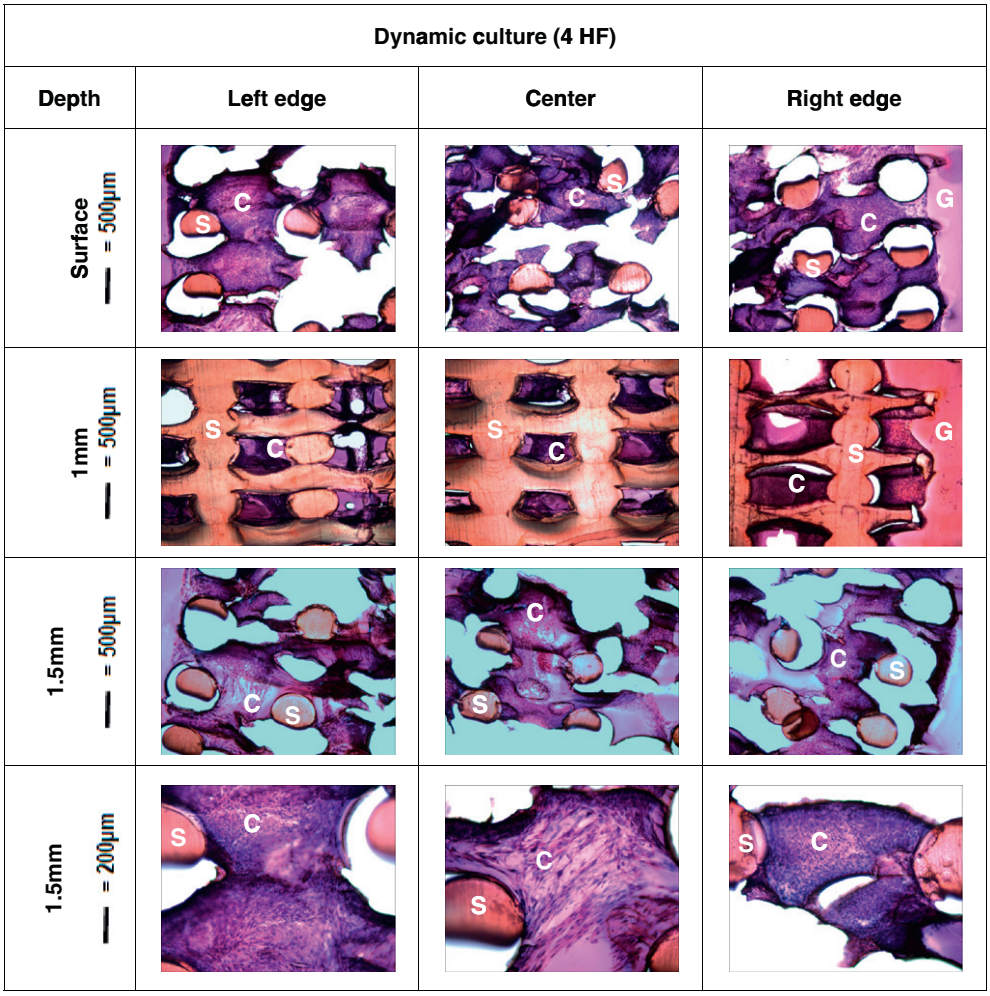


**Fig. 13.** Light microscopic images H&E stained microtome sections of scaffold embedded in GMA, of dynamically cultured for 7 days on 3DF-HF module integrated with four HF in a dual perfusion bioreactor system with medium flow through FTS and HF lumen (G, GMA embedding resin; S, scaffold polymer; C, cells).

In the case of perfusion via the HF lumen only (Fig. 9e and f), a more homogeneous cell and ECM distribution can be appreciated in the interior of the scaffold compared with statically cultured scaffolds. ECM deposition is enhanced in proximity to the HF in the scaffold and reduced on the scaffold surface, probably owing to improved refreshing of medium close to the HF membrane. Yet a decrease in cell number (50% reduction) is measured with respect to the static culture after 7 days' culturing (see Fig. 10). Although the overall cell density is low, the significant improvement in cell and ECM homogeneous distribution inside the scaffold is encouraging.

Fig. 9g and h depicts the ECM deposited by the cells when medium perfusion occurs through the scaffold (FTS) and the HF. In this case, the outer surface of the scaffold is entirely covered by ECM, while the scaffold interior contains ECM deposited with a gradient from the periphery to the center of the scaffold. Cell numbers for this dual flow (FTS + HF) (Fig. 10) are slightly higher, with large standard deviation compared with static culture.

In conclusion, the results suggest that dual flow cell culture with two HF may have a beneficial effect on cell distribution and ECM production inside the scaffold. However, more fibers should be integrated to obtain enhanced cell proliferation in the scaffold.

*3.3.2. Optimization concerning number of integrated HF*

Since the integration of only two HF was not sufficient to improve cell proliferation significantly and achieve better cell distribution into the scaffold, the integration of four and eight HF was also investigated.

*3.3.2.1. Integration of four HF.* For 3DF scaffolds integrated with four HF, the total cell number cultured in dual flow (FTS + HF) bioreactor is substantially increased compared with scaffolds with two HF (Fig. 10). The SEM images of the scaffold integrated with four HF and cultured for 7 and 14 days in the dual flow bioreactor also show a large amount of ECM deposition both on the surface and in the interior of the scaffold (Fig. 11). These results clearly demonstrate the ability of four HF fibers to achieve sufficient long-term perfusion.

Cell distribution within the scaffold with four HF was further analyzed after 7 days in static or dynamic culture by histology. Figs. 12 and 13 show light microscopic images of 5–8 µm thin microtome sections of scaffolds embedded in GMA and stained with H&E. Fig. 12 shows the microscopic image of statically cultured samples with four HF integrated within the scaffold and cultured for 7 days with medium refreshed every alternate day. The images clearly show that the cells (indicated by C) are mostly located at the outer surface (0.2 mm deep). At 1, 1.5 and 2 mm depth in the scaffold, cells cluster at the periphery of the scaffold, forming a barrier for medium diffusion. The images at the center of the scaffold have no cells, and the empty space of the porous scaffold (S) is filled with GMA embedding material (indicated by G). This further confirms that nutrient diffusion to the interior of the scaffold is limited in static culture conditions. Fig. 13 presents the cell distribution within the scaffold integrated with four HF

and cultured in a dual flow perfusion bioreactor system for 7 days. A high cell density is observed at the periphery, as well as in the interior of the scaffold, and the pores are completely filled with cells and ECM. In fact, the cell number there is so high that only very small amount of GMA embedding material could infiltrate into the pores and therefore some of the images have cracks and holes. At a depth of 1.5 mm from the scaffold surface, it can be observed that cells stretch in between the porous space of adjacent scaffold fibers, indicating cell viability and tissue organization through the produced ECM (Fig. 13).

*3.3.2.2. Integration of eight HF.* When eight HF are integrated in the pores of a 3DF scaffold, cell proliferation in a FTS + HF flow configuration is significantly higher compared with static culture, but significantly lower compared with dynamic culture with four HF. This difference in cell proliferation might be due to a decrease in scaffold porosity due to space occupied by the eight HF. In fact, the porosity of the scaffold decreases by 25% when eight HF are integrated. The seeding efficiency or the initial cell number seeded on the scaffold also decreases with increasing number of HF, because the HF themselves block the free straight-through pores of the 3DF scaffold. Obviously, integration of eight fibers to the specific scaffold is detrimental to achieving the present goals and should be avoided.

## 4. Conclusion

This study demonstrated the proof of the concept of using polymeric HF as artificial capillaries for nutrient delivery to relatively large 3D tissue constructs. For the specific type and size of scaffolds, integration of four HF and dual flow perfusion is the optimal configuration to achieve high cell proliferation and homogeneous cell distribution within the scaffold.

SEM analysis showed clearly that only integration of fibers achieves homogeneous cell distribution. The histology confirmed the cell distribution visualized by SEM and showed in more detail the local cell distribution, proving the advantage of the concept. The total DNA assay showed quantitatively the cell proliferation within the scaffolds, proving that four HF combined with FTS achieves the best result.

Future work will focus on the incorporation of biodegradable porous HF such as PLLA or PLGA [28] for permeation of nutrients in vitro and implantation of the construct in vivo. Thereby, the HF could be connected to the host vascular system until the host vascular network infuses into the tissue-engineered construct.

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## Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 2, 3, 4, 8, 10, 12 and 13, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.06.012.

## Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2011.06.012.

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