

A Low Shear Stress Modular Bioreactor for Connected Cell Culture Under High Flow Rates

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ABSTRACT: A generic "system on a plate" modular multi-compartmental bioreactor array which enables microwell protocols to be transferred directly to the bioreactor modules, without redesign of cell culture experiments or protocols is described. The modular bioreactors are simple to assemble and use and can be easily compared with standard controls since cell numbers and medium volumes are quite similar. Starting from fluid dynamic and mass transport considerations, a modular bioreactor chamber was first modeled and then fabricated using "milli-molding," a technique adapted from soft lithography. After confirming that the shear stress was extremely low in the system in the range of useful flow rates, the bioreactor chambers were tested using hepatocytes. The results show that the bioreactor chambers can increase or maintain cell viability and function when the flow rates are below 500 $\mu\text{L}/\text{min}$, corresponding to wall shear stresses of 10^{-5} Pa or less at the cell culture surface.

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Introduction

The microwell (MW) plate has become a standard in cell culture. The plates are available in a variety of formats (6–1,536 wells), although for most general cell culture and tissue engineering applications, 12-, 24-, 48-, and 96-well formats appear to be the most common (Chen et al., 2009). However, the complexity of the physiological environment is not replicated in Petri dishes or microplates. All cells are exquisitely sensitive to their microenvironment which is rich with cues from other cells, and from mechanical stimuli due to flow, perfusion, and movement. MWs do not offer any form of dynamic chemical or physical stimulus to cells, such

as concentration gradients, flow, pressure, or mechanical stress. This is a major limitation in experiments investigating cellular responses *in vitro* since the complex interplay of mechanical and biochemical factors is absent (Janmey and McCulloch, 2007). Most researchers and industry now accept that classical *in vitro* experiments offer poor predictive value or mechanistic understanding, and there is a shift to new technologies, generally in the form of bioreactors.

A large number of bioreactor systems for cell culture have been designed and described. They range from commercial bioreactors which apply laminar flow (Fu et al., 2008), membrane systems (Morelli et al., 2007), rotating vessel systems (Martin et al., 2004) to purpose designed devices for specific tissues such as blood vessels (Miyakawa et al., 2008), heart valves (Dumont et al., 2002), and livers (Powers et al., 2002a). In most cases, the bioreactors described are custom designed for specific requirements and necessitate the use of particular seeding methods or scaffolds with narrow dimensional and design specifications. Sizable they can be large-scale bioartificial livers with several billions of cells and several milliliters of fluid (De Bartolo et al., 2000) down to microfluidic systems with a few hundred microliters of medium (Baudoin et al., 2007). In fact, microfluidic microfabricated bioreactors which enable the culture of different cell types in a shear stress controlled environments (Tanaka et al., 2006) are highly popular but remain very much a niche research tool. In a micro-bioreactor, the cell culture surface is generally around 0.5–0.8 mm^2 (Baudoin et al., 2007) and this tiny surface is seeded with a few thousand cells. Such a small number of cells, organized on a tiny surface can be only a rough approximation of an organ and cannot meaningfully predict *in vivo* physiology or pathophysiology (Tingley, 2006). Another problem encountered in micro-bioreactors is the so-called "edge effect." In a micro-scaled surface, the percentage of area close to the edge of the system is higher than in a millimeter-sized surface. A large fraction of the cell population will therefore be found in a peripheral zone of the system. Cell cultures in the edge zone are usually organized differently and have higher

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cytoskeletal tensions (McBeath et al., 2004), and they may also have different viability or activity (Francis and Palsson, 1997). The edge effect is consequently a problem that can directly affect the results obtained in micro cell culture systems as demonstrated in Lundholt et al. (2003). Furthermore, most microfluidic bioreactors are fabricated using polydimethylsiloxane (PDMS) or other elastomeric polymers, which are known to adsorb small hydrophobic molecules (Toepke and Beebe, 2006). In microfluidic circuits, where the surface area to volume ratio is high, surface adsorption can lead to nutrient or ligand depletion so giving rise to experimental artifacts such as increased metabolic consumption rates. Finally, microsystems and microbioreactors are difficult to use and assemble, and the seeding and filling processes are quite complicated. This could lead to increased experimental failure and decreased reliability and limits their usefulness and scope and also puts them out of reach of many cell culture experiments which could benefit from added dynamic stimuli. In fact, for alternative tools to become acceptable as cell culture standards, the transition from wells has to be as smooth as possible. Only then will biologists and technicians adopt and adapt to new culture methods. For this reason, we have developed a “system on a plate” Multi-Compartmental modular Bioreactor (MCmB) with dimensions of the order of a few millimeters, which enables MW protocols to be transferred directly to the bioreactor modules.

The main design criteria for bioreactors are based on maximizing mass transport between the cells and the culture medium and on the application of mechanical, electrical, chemical, or other stimuli. Given that hepatocytes present a major challenge in cell culture (Coleman and Presnell, 2003; Guillouzo, 1998) and are probably the “limiting” cell in an *in vitro* system, the MCmB was designed using hepatocytes as a reference cell type. Hepatocytes are known to rapidly lose phenotypic expression *in vitro* due to the absence of an adequately equipped microenvironment, and they are a particular focus of attention in bioreactor development (Nahmias et al., 2007).

As the main orchestrators of endogenous and exogenous metabolism in mammals, hepatocytes are extremely sensitive to oxygen concentration, with high metabolic demands (Balis et al., 1999; Schumacker et al., 1993). One of the main engineering issues in bioreactors for *in vitro* liver models is therefore the balance between high mass transfer and low wall shear stress to cells. Several reports describe the effects of flow and shear stress on hepatocyte cultures (Mufti and Shuler, 1995; Nakatsuka et al., 2006; Powers et al., 2002b; Tanaka et al., 2006). Moreover, many investigators have shown that the viability of hepatocyte cultures under high shear is usually lower than that of static controls, indicating that the cells are under conditions of stress (Park et al., 2007). Hepatocytes are therefore very sensitive to shear, and according to Tilles et al., (2001) hepatocyte function is compromised at wall shear stresses greater than 0.03 Pa.

To realize a generic bioreactor system, a modular chamber with shape and dimensions similar to the 24-MW was designed. The new bioreactor unit is called the Multi-Compartmental modular Bioreactor (MCmB), and it consists of a cell culture chamber made of PDMS, a widely available and reasonably priced biocompatible silicone polymer with excellent self-sealing properties, transparency and flexibility. For this reason, in systems, which have a small surface to volume ratio with respect to microfluidic circuits, its use can be highly advantageous. By plugging together different chambers in different configurations (series or parallel) it is possible to mimic different metabolic pathways in order to investigate and test multi-compartmental biological models *in vitro* without having to design dedicated equipment or culture chambers, but just by connecting together a set of prefabricated chambers. The MCmB stems from a previous multi-compartmental bioreactor (MCB), in which the metabolic circuit has a fixed topology (Guzzardi et al., 2009; Vozzi et al., 2009). To mimic salient features of the glucose consumption pathway, the MCB system was designed with four chambers representing the pancreas, the liver, and the two target organs, respectively. The chambers were connected by channels, and the circuit dimensions were calculated using allometric laws (West et al., 1997).

The MCmB is a further evolution of the MCB system and allows any tissue or organ model to be simulated simply by connecting the modular chambers in a desired configuration. The bioreactor design process started with the analysis of the oxygen concentration and with the assessment of the minimum concentration allowed near the cell surface, using hepatocytes as a reference. Subsequently, we developed a fluid dynamic model of the MCmB chamber in order to investigate the shear stress and to estimate the optimal chamber size to obtain both adequate oxygen diffusion and low shear stress near the cell surface. This article describes the designing, modeling, and dimensioning of the bioreactor chambers, their fabrication using precision machining and “milli-molding,” and testing of the bioreactors using rat hepatocytes.

Materials and Methods

Mass Transport and Flow Modeling

The first model of the MCmB (MCmB 1.0) was based on the dimensions of the classic 24-MW so as to directly compare static and dynamic cell cultures in equal sized chambers. The MCmB 1.0 was designed to allow the use of 12 mm glass or plastic cover slips, commonly used for cell culture, or different types of scaffolds. It is also possible to place a slice of tissue, pregrown cell construct, or pellets directly on the cell culture zone. A finite-element modeling (FEM) model of the cell culture chambers was developed in order to study the oxygen concentration and the shear stress at the cell surface. Cosmos Flowworks, a SolidworksTM (Dassault

Systemes SolidWorks Corp., Concord, MA) extension that allows fluid-dynamic FEM analysis and Comsol Multiphysics (COMSOL AB, Stockholm, Sweden) were used for this purpose. In both the fluid dynamic and mass transport models we used the following system constants: viscosity = 10^{-3} Pa s, fluid density = $1,000 \text{ kg/m}^3$, medium flow rate in the range between 60 and $1,000 \mu\text{L/min}$, pressure = 1 atm or 760 mmHg, temperature = 37°C , and no slip boundary conditions. Water was chosen as a reference fluid, so that the actual values of shear stress on the walls will depend linearly on the density and viscosity of the culture medium used. Initial considerations were focused on a flow rate of $180 \mu\text{L/min}$ for the sake of comparison with our previous tests in the MCB system (Guzzardi et al., 2009; Vozzi et al., 2009).

In order to study the fluid dynamics of the modular bioreactor, we developed a parametric model in which the effect of changes in height on flow parameters (flow speed, shear stress, and stream lines) could be observed. As shown in Figure 1, the first model had a base height H which varied between 3 and 9 mm, and a 13-mm diameter base, with a 12-mm diameter, 160 μm thick cover slip placed on of the bottom of the bioreactor. The distance between the roof and the 1 mm diameter inlet and outlet tubes was fixed at 3 mm, and the FEM model was solved for different heights between the tubes and the cell culture surface: 3, 4, 6, and 9 mm.

Oxygen Concentration Analysis

A steady-state model of oxygen diffusion in the MCmB chamber was developed, in order to investigate if the cells were adequately supplied with oxygen during the experiments. We used Michaelis–Menten (MM) kinetics to model oxygen consumption and Fick's laws to model oxygen diffusion in water.

In the bioreactor circuit, which is described in the following section, air rather than O_2 is used for safety purposes. The O_2 concentration ($C_{[\text{O}_2]}$) in the medium just below the gas–liquid interface is directly dependent on the O_2 partial pressure in air, typically 20% or 159 mmHg, through Henry's law and we can easily calculate $C_{[\text{O}_2]}$ using

tabulated values of the Henry constant:

$$P(\text{O}_2) = K(\text{O}_2)C[\text{O}_2]$$

with $K_{\text{O}_2} = 932.4 \text{ atm}/(\text{mol/L})$ and $(P_{\text{O}_2}) = 0.2 \text{ atm}$ (20%), $C[\text{O}_2]$ below the gas–liquid interface or $C_{[\text{O}_2]} = 214 \mu\text{M} = 0.214 \text{ mol/m}^3$.

In the presence of oxygen-consuming cells, the O_2 concentration will decrease as a function of distance from the interface and the metabolic requirements of the cells. The steady-state flux of O_2 in the bioreactor chamber is due to cellular oxygen consumption at the base. The oxygen consumption rate per unit volume is given by the MM equation:

$$\frac{dC}{dt} = \frac{V_m C}{K_m + C}$$

where C is the oxygen concentration in mol/m^3 , V_m is the maximum volumetric consumption rate in $\text{mol/m}^3/\text{s}$, and K_m is the MM constant in mol/m^3 .

The flux at the base of the chamber where the cells lie are

$$J_c = -D \frac{\partial C}{\partial x} = \frac{V_{\max} C}{K_m + C} \frac{1}{\text{cell area}}$$

where J_c is the O_2 mass flow at the cell surface, $(\partial C/\partial x)$ is the O_2 concentration gradient at the cell surface calculated along the axis perpendicular to the cell culture layer, D is the O_2 diffusion coefficient (in water) $D = 3 \times 10^{-9} \text{ m}^2/\text{s}$ at 37°C , and V_{\max} is the maximum consumption rate in mol/s .

The kinetic model was calculated for a medium flow rate of $180 \mu\text{L/min}$, for a comparison with the MCB result, and a cell monolayer of 1.33 cm^2 surface area with 500,000 cells, which represents typical cell numbers observed 48 h after seeding HepG2 cells on a three-dimensional scaffold (Vinci et al., 2009). In order to choose the height of the bioreactor, we fixed a minimum oxygen concentration of the medium at the cell surface; $C_{\min} = 0.04 \text{ mol/m}^3$. Below this concentration we assume that cell function is compromised. In fact, typical oxygen concentrations in the liver are of the order of $0.04\text{--}0.15 \text{ mol/m}^3$ (4–15%), while 0.021 mol/m^3 (2%) is known to inhibit mitosis in hepatocytes (Smith and Mooney, 2007).

Using the data in Patzer (2004), $V_{\max} = 0.048 \text{ nmol/s}$ for 1 million cells, giving a value of 0.024 nmol/s for 500,000 cells ($V_{\max(500,000)}$), and $K_m = 0.5 \text{ mmHg}$.

Figure 2 illustrates that the O_2 concentration in the MCmB is always higher than the minimal threshold and falls rapidly at the edge of the chamber near the outlet. Increasing bioreactor height from 3 to 9 mm, the oxygen concentration decreases rapidly but cell survival is always guaranteed in the central region of the chamber. Above $H = 9 \text{ mm}$, however, in the bottom of the chamber the oxygen concentration is constantly below the C_{\min} threshold of 0.04 mol/m^3 . The maximum oxygen concentration at the base is 0.17 , 0.12 , 0.10 , and 0.007 mol/m^3 for $H = 3$, 4, 6, and 9 mm, respectively.

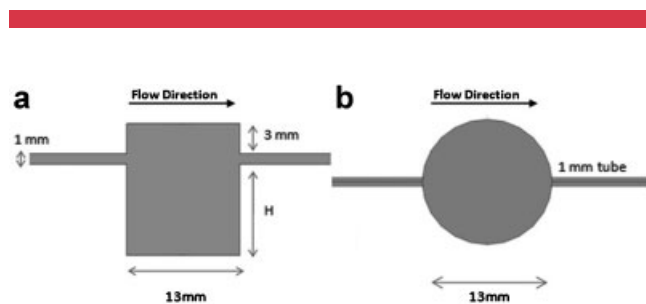


Figure 1. The FEM model geometry for the first modular bioreactor chamber. H is the variable height, (a) side view and (b) top view.

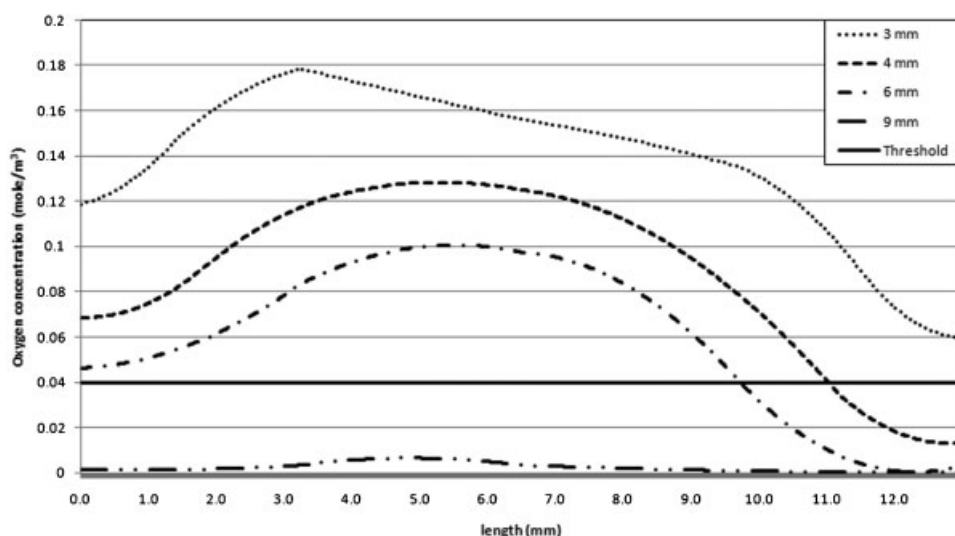


Figure 2. Theoretical oxygen concentration profile across the bioreactor chamber for different heights H , calculated using Michaelis–Menten kinetics and a flow rate of $180 \mu\text{L}/\text{min}$. The minimal concentration threshold of $0.04 \text{ mol}/\text{m}^3$ is indicated by the solid line.

Fluid Dynamic Model

To investigate the fluid dynamics of the modular bioreactor, a parametric model similar to the mass-transfer model was developed. The height H was varied to determine its influence on the shear stress generated at the cell surface, assuming again that the cells are seeded on a 12-mm, $160 \mu\text{m}$ thick cover slip placed in the bioreactor.

Table I summarizes the results, showing how the shear stress at the cells surface decreases rapidly with increasing H . However, the laminarity of the flow is compromised by increasing the height of the bioreactors. This is a consequence of the separation of flow streamlines caused by the difference in height between the inlet tube and the base.

After evaluating the results from the fluid dynamic and mass transport modeling, we choose to realize a modular bioreactor chamber with tubes placed 6 mm over the cell surface (H). A distance of at least 1 mm between the wall of the tube and the top of the chamber is necessary to ensure mechanical stability of the tube/chamber junction. Since standard silicone tubing with an inner diameter of 1 mm has an outer diameter of 3 mm, the total height of the chamber is 10 mm. This is the best compromise between shear stress, flow, oxygen diffusion, and mechanical feasibility.

Figure 3 shows the three-dimensional FEM model of the $H = 6 \text{ mm}$ MCmB with an internal diameter of 13 mm, sufficient to place a slice or a scaffold of 12 mm in diameter. This bioreactor has a peak shear stress value of $6.85 \times 10^{-6} \text{ Pa}$ and a velocity peak of $10^{-6} \text{ m}/\text{s}$ near the center of the base.

Chamber Fabrication

A first prototype of the bioreactor chamber (MCmB 1.0) was fabricated using “milli-molding” in order to investigate the performance of the system. Milli-molding is similar to micromolding (Xia and Whitesides, 1998), in that PDMS is used as a mold, but the master is machined using a mill or rapid prototyping rather than lithographic methods, and the features of the mold are of the order of tenths of millimeters, rather than microns.

The chamber is composed of two separate parts which are plugged together through a friction fit system. In the friction fit system two complementary geometries form a seal when brought together thus avoiding the use of o-rings or additional parts (Mazzei et al., Improved Bioreactor Chamber, GB Patent Application No. 0814034.5). PDMS (Sylgard 184, Dow Corning, Silverstar, Italy) is used to fabricate the chambers. Along with the friction fit system, the use of PDMS, which is self adhesive and deformable, ensures that the seal is watertight.

The two parts of the chamber are made by casting and curing PDMS as per the manufacturer’s instructions on aluminum masters purposely machined in our workshop. Each master is made of two separate pieces which allow easy removal of the chambers after polymerization. The two parts are held together using an aluminum frame with four screws as shown in Figure 4a.

Turbulence Tests

The first prototype of the MCmB 1.0 cell culture chamber had severe problems with the build-up of air bubbles during the

Table I. Fluid dynamic FEM model results for a fixed flow rate of 180 $\mu\text{L}/\text{min}$ for the first MCmB model as a function of height H .

Height (H)	3 mm	4 mm	6 mm	9 mm
Shear stress peak (Pa) at 180 $\mu\text{L}/\text{min}$	3.217×10^{-5}	1.195×10^{-5}	6.856×10^{-6}	8.747×10^{-7}
Shear stress profile (log Pa)				
Velocity peak (m/s) at 180 $\mu\text{L}/\text{min}$	4.429×10^{-6}	1.665×10^{-6}	9.887×10^{-7}	1.389×10^{-7}
Velocity vector	<p><i>Parallel to the cell surface</i></p>	<p><i>Parallel to the cell surface</i></p>	<p><i>Slightly sloped</i></p>	<p><i>Sloped</i></p>
Stream line	<p><i>Parallel to the cell culture</i></p>	<p><i>Parallel to the cell culture</i></p>	<p><i>Nearly parallel to the cell culture</i></p>	<p><i>High impact angle on the cell culture surface</i></p>

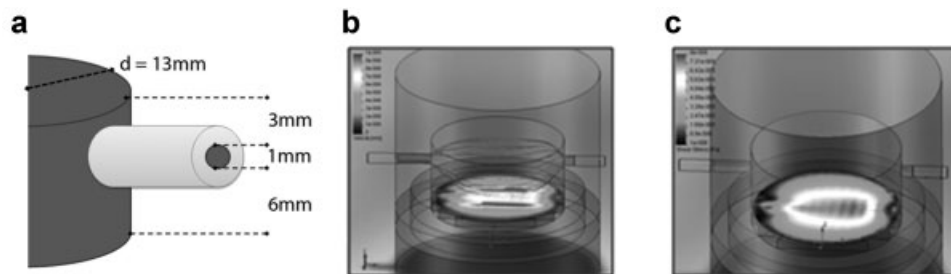


Figure 3. a: FEM model of the $H = 6$ mm MCmB 1.0 indicating the tubes position and dimensions, b: velocity profile and c: shear stress profile. The analysis takes into account a 160-mm thick glass cover slip placed on the base of the chamber.

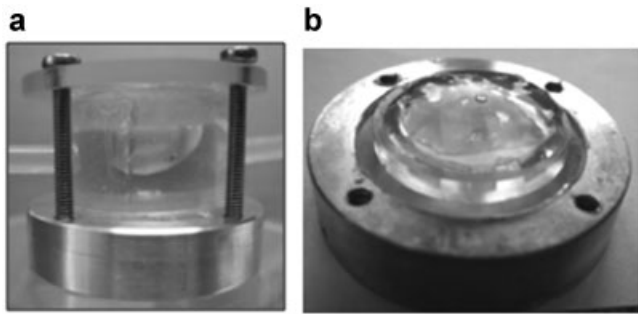


Figure 4. a: Bubble formed at the top of the first MCmB 1.0 chamber, and (b) alginate drop used for turbulence tests placed on the bottom half of the chamber.

initial phases in which the bioreactor is filled with medium. This resulted in the formation of a bubble of air at the top of the chamber (Fig. 4a), which disturbed the flow profile, causing turbulence and more importantly unpredictable values of shear stress. In order to evaluate the effects of the bubble induced turbulence, a small drop or blob of alginate was introduced in the chamber as a “turbulence sensor.” An alginate blob is easily disaggregated under turbulent or high impact flow, and for this reason it is a good indicator of the presence of altered flow

profiles. Moreover, cross-linked alginate at the concentrations used here has approximately the same elastic modulus as the liver (~ 10 kPa) (Constantinides et al., 2008; Tirella et al., unpublished work). The alginate blobs are made placing $166 \mu\text{L}$ of 2% sodium alginate (Sigma, Milan, Italy) dissolved in MEM (Sigma) on top of a glass cover slip in the chamber and cross-linking in situ with $104 \mu\text{L}$ of 0.1 M CaCl_2 (Fig. 4b). The volume of the blob was chosen in order to form a thin uniform coating of the glass cover slip, without interfering with the inlet tube. A peristaltic pump (Ismatec, Glattbrugg, Switzerland) was attached to the inlet, and keeping the flow rate constant at $180 \mu\text{L}/\text{min}$, the consistency of the alginate blob after 24 h in the MCmB 1.0 at 37°C was analyzed. The alginate drop was completely disaggregated, showing that the bubble induced a turbulent environment.

Design Improvements: MCmB 2.0

In order to eliminate bubble entrapment and turbulence in the bioreactor chamber, a second prototype (MCmB 2.0) of the modular bioreactor was designed. The new bioreactor is slightly larger in diameter (15 mm) to enable 13 or 14 mm slides to be easily inserted and its top surface is sloped along and perpendicular to the axis of flow, so that bubbles are collected and conveyed to the outlet tube (Fig. 5a).

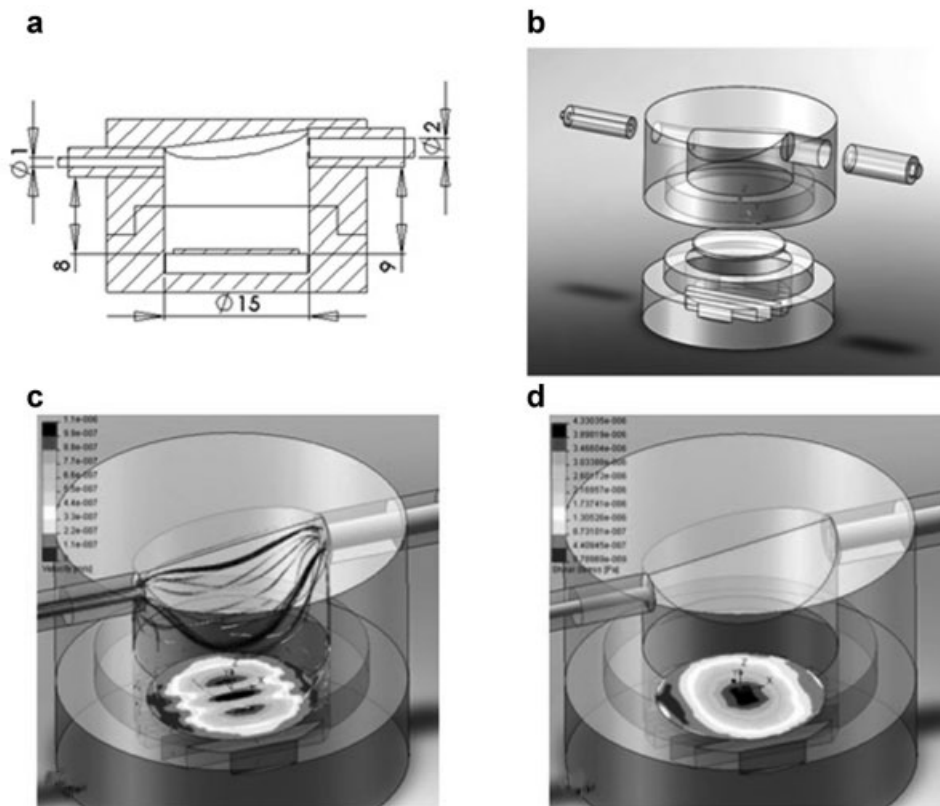


Figure 5. a: Dimensions of the new chamber, (b) three-dimensional representation of the sloped roof and ridged base, (c) MCmB 2.0 velocity profile, showing stream lines, and (d) shear stress at the base.

Furthermore, the diameter of the outlet tube was increased to 2 mm to facilitate the removal of bubbles. This also reduces the impact angle on the cell culture surface and the recirculation near the outlet wall as shown in Table II. The final height of the MCmB 2.0 is 11 mm at the inlet side and 13 mm at the outlet side, with the tubes positioned, respectively, at $H=9$ and 10 mm from the cell surface (Fig. 5b).

Through FEM modeling a tenfold decrease in fluid velocity and shear stress on the cell surface with respect to MCmB 1.0 was observed with the introduction of the sloping roof and the increased outlet tube diameter.

The MCmB 2.0 is 3 mm higher than MCmB 1.0, we choose to increase the height of the new chamber design because this allows a further reduction in shear stress in comparison with MCmB 1.0 (6 mm in height) while maintaining the oxygen concentration over the 4% threshold.

Table II shows the velocity profile and the streamlines in the MCmB 2.0.

The base of the bioreactor chamber was also modified to ensure easy removal and support of coverslips, since the slides and thin substrates tend to stick to the flat silicone base. The three small rectangular ridges, shown in

Table II. Fluid dynamic FEM model results for the MCmB 2.0 at two different flow rates, the average height (H) of the MCmB 2.0 is 9.5 mm.

Flow rate ($\mu\text{L}/\text{min}$)	180	300
Shear stress peak (Pa)	4.295×10^{-6}	6.456×10^{-6}
Shear stress profile (log Pa)		
Velocity peak (m/s)	5.939×10^{-7}	9.048×10^{-7}
Velocity vector		
	<i>Parallel to the cell surface</i>	<i>Parallel to the cell surface</i>
Stream line		
	<i>Parallel to the cell culture</i>	<i>Parallel to the cell culture</i>

Figure 5b, provide support and also allow medium to flow under the support, supplying nutrients to the lower surface of tissue slices or scaffolds.

Control System

The bioreactor chambers are part of a complex gas and fluid circuit, which enables individual chambers to be connected in series or in parallel to mimic different metabolic pathways. Experimental variables such as oxygen concentration, pH, pressure, and medium flow rate through the MCmB chambers can all be controlled through a purposely designed electronic and software control system (Mazzei et al., 2008). A series of MCmB chambers plugged together in series and parallel are represented in Figure 6. Culture media is moved by a peristaltic pump through to a mixing chamber and from here to the MCmB chambers in a closed-loop circuit. The mixing chamber acts as a medium reservoir, a sampling port, a gas exchanger, and a flow rectifier to convert peristaltic flow into a smooth flow. The controlled injection of an appropriate combination of air and CO₂ through a dedicated algorithm enables the oxygen concentration, pH, and pressure of the medium to be tightly modulated. A microcontroller board is used to read the data collected by a sensing unit placed in the mixing chamber. The MCmB bioreactor can also be used in a classic cell culture incubator, without need for the control unit. In this case, the mixing chamber is equipped with a single gas inlet with a 0.2 μm syringe filter which allows gas exchange without risk of contamination.

Cell Culture

All cell culture reagents were all purchased from Sigma, unless otherwise specified. Primary rat hepatocytes were used to test the performance of the MCmB 2.0 bioreactor at various flow rates. Hepatocytes were isolated from adult male Wistar rats weighing between 250 and 300 g as described by Seglen (1979) and Papeleu et al. (2006). Isolated cells were assessed for vitality by trypan blue

exclusion (vitality routinely greater than 90%), and then seeded on glass cover slips (2×10^5 cells per sample) pretreated with 0.1 mg/mL collagen extracted from rat tails according to standard procedures (Rajan et al., 2006), placed in 24-MW plates and left at 37°C and 5% CO₂. About 5 h after seeding, the medium was removed and replaced with FBS-free William's E complete medium. The following day, the hepatocyte seeded coverslips were placed into the MCmB 2.0 chambers and coated with 250 μL collagen gel prepared by mixing ice-cold collagen solution and acetic acid 0.2 N (to a final collagen concentration of about 1.1 mg/mL) and 10× M199. The collagen coating used in these experiments has a Young's modulus similar to the alginate blob used to evaluate the turbulence in the chamber (Wu et al., 2005). It also provides an adhesive roof to the cells and shields the cells from the effects of direct flow.

MCmB Assembly

Prior to assembly the bioreactors with hepatocyte seeded and collagen coated slides were left in the incubator for 40 min to allow the collagen to set. The MCmB system was then assembled by connecting two chambers in series to a peristaltic pump and the mixing chamber. Finally, the system was filled with 10 mL FBS-free complete William's E, and run for up to 24 h. The first experiment was run at a flow rate of 180 μL/min. At 2, 4, 6, and 24 h a 100-μL sample of medium was withdrawn for analysis of rat albumin. A second set of experiments was carried out at various flow rates (60–100–180–250–300–500–1,000 μL/min) to determine cell viability as a function of flow rate. The bioreactors were run for 24 h after which the cover slips were removed and assessed for cell viability. Control experiments with cells from the same rat liver were run using glass slides seeded with the same number of cells, coated with collagen, and placed in 10 mL Petri dish plate multiwell (BD Biosciences, Milan, Italy).

Viability and Albumin Testing

Albumin

Albumin, which is an important marker of hepatic function, was measured using a commercial ELISA kit (Bethyl Laboratories, Montgomery, TX), according to the manufacturer's instructions. Rat albumin production is expressed as total quantities of albumin per seeded cell. Each experiment was repeated at least three times, and comparisons were only made between cells extracted from the same liver.

Viability

The viability kit used was CellTiter-Blue™ Cell Viability Assay (Promega, Madison, WI), a resazurin-based fluorescent compound metabolized by mitochondrial cytosolic

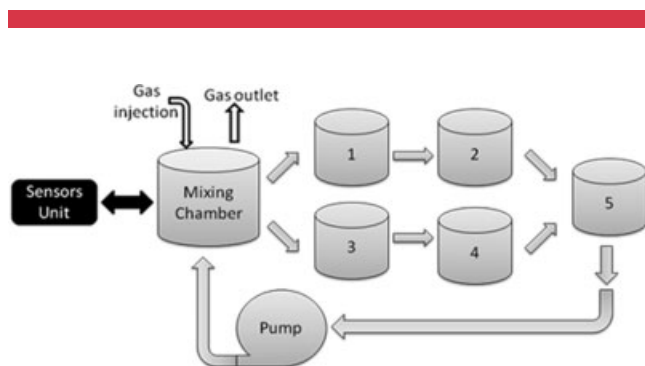


Figure 6. Scheme of the bioreactor medium and gas flow circuit.

and microsomal enzymes to resorufin which can be detected with a fluorimeter (FLUOstar Omega, BMG Labtech, Offenburg, Germany) at 573 nm. Viability was assessed both in control static cultures and in the MCmB 2.0, and the results are expressed as ratio between hepatocyte viability in dynamic and static conditions after 24 h. The static control was a hepatocyte seeded and alginate-coated cover slip placed in a bioreactor base filled with medium and left open in order to allow oxygen exchange without medium flow. Each experiment was repeated at least three times, and comparisons were only made between cells extracted from the same liver.

Statistical Tests

Statistical analysis was performed using the Student's *t*-test when comparing between albumin production in static ($n = 3$) and dynamic ($n = 3$) experiments; a *P*-value of less than 0.05 was considered statistically significant. At least three dynamic experiments and three static controls were performed per flow rate for viability tests.

Results and Discussion

Oxygen Consumption and Shear Stress in the MCmB 2.0

Figure 7 shows the oxygen concentration in the two bioreactor designs; for a given flow rate, the mean value is slightly lower in the new MCmB 2.0 design. As far as oxygen consumption is concerned, the difference between the two chambers is the value of H (6 mm in MCmB 1.0 and 9 mm in MCmB 2.0) and their respective volumes. The MCmB 2.0 chamber has a slightly larger volume so the total amount of oxygen available is greater. Therefore, despite the greater distance between the tubes and the cell surface in the new chamber the minimal oxygen concentration is still guaranteed except at the outer edge of the base closest to the outlet. To minimize oxygen depletion at the edges, the bioreactor could be placed on a low-frequency oscillator or shaker. It should be noted however that the number of cells used in the model was more than twice that used in the experiments, and that the oxygen concentration is highly dependent on the flow rate and metabolic requirements of the cells.

We also characterized the Graetz number which is a dimensionless number indicating the ratio between the characteristic diffusion time and the convection time, respectively, perpendicular and parallel to the direction of flow. The Graetz number is greater than 100 even for low flow rates (60 $\mu\text{L}/\text{min}$) showing that the fraction of oxygen consumed per reactor is insignificant and so downstream chambers do not suffer from any input oxygen depletion.

Using the model we established empirical equations for the wall shear stress and peak fluid velocity at the cell surface

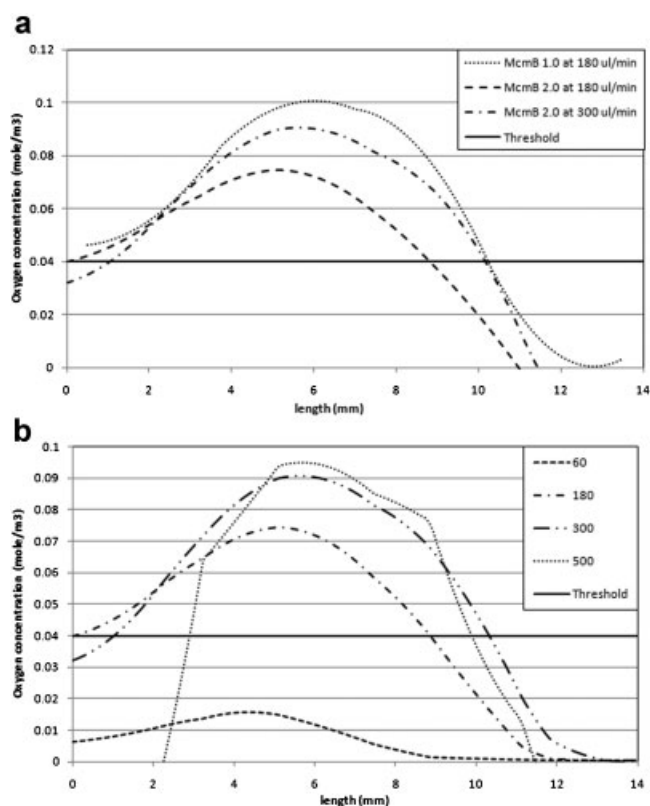


Figure 7. a) Oxygen concentration in the MCmB 1.0 for a flow rate of 180 $\mu\text{L}/\text{min}$ and MCmB 2.0 for a flow rate of 180 and 300 $\mu\text{L}/\text{min}$, and (b) oxygen concentration in the MCmB 2.0 for different flow rates between 60 and 500 $\mu\text{L}/\text{min}$.

as a function of flow rate

$$\text{Shear (Pa)} = \text{Flow}(\mu\text{L}/\text{min}) \times 1.8 \times 10^{-8} + 1.1 \times 10^{-6}$$

$$\text{Flow Speed (m/s)} = \text{flow}(\mu\text{L}/\text{min}) \times 2.6 \times 10^{-9} + 1.3 \times 10^{-7}$$

Therefore, the wall shear stress at 180 and 300 $\mu\text{L}/\text{min}$ is, respectively, 4.34×10^{-6} and 6.5×10^{-6} Pa and the peak flow velocity is 5.98×10^{-7} and 9.1×10^{-7} m/s.

Bubble and Turbulence Testing

As shown in Tables I and II, the MCmB 2.0 has lower values of shear stress, and the streamlines are parallel to the base of the chamber, even though its height (H) is 3 mm greater than MCmB 1.0. At a flow rate of 1,000 $\mu\text{L}/\text{min}$ the gel showed signs of disaggregation after 24 h, due to the direct high impact of fluid on the base of the chamber as well as the high wall shear (not shown). The MCmB 2.0 was bubble free, as the sloped roof and the position and respective diameters of the inlet and out tubes forced bubbles to be conveyed out of the chambers. In the

turbulence tests carried out with the alginate blob, the gel showed no signs of wear even after 48 h under a flow of 500 $\mu\text{L}/\text{min}$. The alginate blob turbulence test is not only a simple method for investigating turbulent, high impact, or disturbed flow in the system but can also be used to create a cell culture environment more similar to the *in vivo* liver environment. As shown in Vozzi et al. (2009) and Guzzardi et al. (2009), coating the cells in an alginate or collagen gel increases hepatocyte viability and phenotypic stability during the culture.

Cell Culture Tests

Figure 8a illustrates the viability ratio between the MCmB dynamic culture at different flow rates and a static control.

The figure shows that below 180 $\mu\text{L}/\text{min}$ the viability is compromised, this could be due to the lower oxygen concentration at the bottom of the bioreactor chamber for these low flow rates (shown in Fig. 7b). For flow rates in the range between 180 and 500 $\mu\text{L}/\text{min}$ the viability is very close to the control and the viability peaks at 300 $\mu\text{L}/\text{min}$, this could be due to the high oxygen concentration as demonstrated in Figure 7b. A further increase in flow rates above about 500 $\mu\text{L}/\text{min}$ causes a significant reduction in

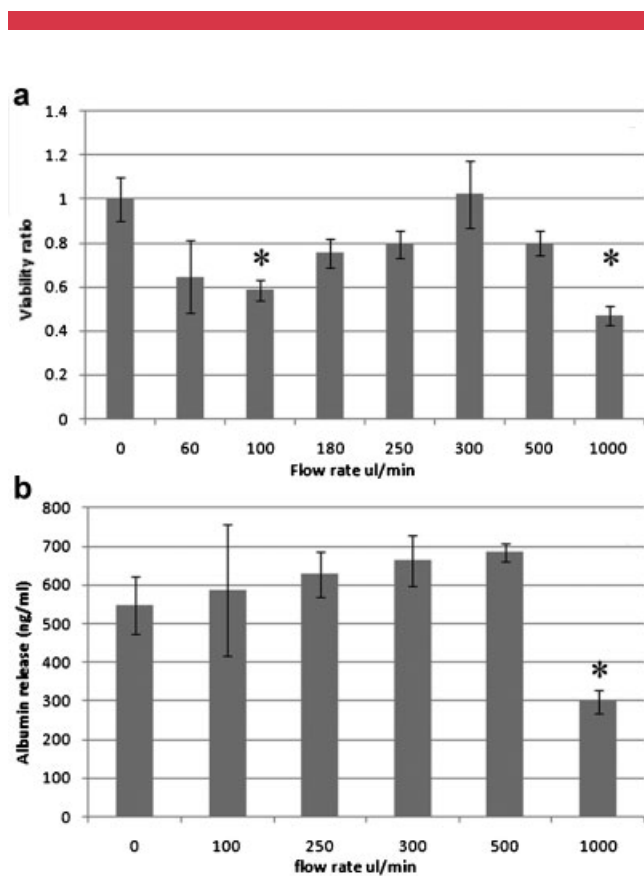


Figure 8. a: Hepatocyte viability, expressed as the ratio between viability in the MCmB 2.0 and controls after 24 h at different flow rates, and (b) rat albumin production after 24 h in the MCmB 2.0 and in the control. * $P < 0.05$.

viability. At higher flow rates, despite the increased availability of oxygen, the cells suffer, and this is due to the high shear and impact angle of flow on the collagen coating and cells.

Figure 8b shows the albumin production after 24 h in the MCmB for different flow rates in the range 100–1,000 $\mu\text{L}/\text{min}$, and the static control. Albumin in the bioreactor chamber is slightly upregulated except for the 1,000 $\mu\text{L}/\text{min}$ flow rate, where the cells are damaged by the shear stress as previously discussed. These results are confirmed by several reports describing dynamic hepatocyte cultures (De Bartolo et al., 2009; Powers et al., 2002b; Tilles et al., 2001) where the albumin production is maintained at control levels even in the presence of shear. We have suggested that this induction is due to two factors: the circulation of medium which provides a sustainable supply of nutrients, as well as efficient removal of metabolic products, and the mechanical stimulus due to the presence of a low velocity porous or percolative interstitial-like flow which is established through the collagen coating (Vinci et al., 2009). In fact, in the human liver, hepatocytes are never subject to direct or tangential flow; they receive nutrients through a rich capillary network and consequently only through interstitial flow driven by concentration and pressure gradients (Rutkowski and Swartz, 2006; Swartz and Fleury, 2007). Not surprisingly a large number of reports on liver bioreactors use co-cultures of hepatocytes with non-parenchymal cells, typically fibroblasts (Allen et al., 2005; Park et al., 2005, 2007; Tilles et al., 2001). These supporting cells not only provide heterotypic signals but also secrete collagen which likely forms a protective coating over the cells shielding them from direct fluid flow.

Conclusions

The objective of this study was to design, realize, and test a modular bioreactor chamber for culture of hepatocytes and other cells, which do not support high shear stress, with dimensions similar to a MW. The various steps of modeling and design of the modular bioreactor, as well as its fabrication using “milli-molding” are described. The main features of the final design are the complete absence of bubbles, due to the particular design of the top part of the chamber, and the very low wall shear stress in the cell culture region. In addition, the bioreactor chambers can be connected together in series or in parallel as desired, using different cell types, tissue slices, or scaffolds in order to recreate *in vitro* models of metabolism or diseases. Two chambers were connected in series and tested over 24 h using primary rat hepatocytes.

At flow rates below about 500 $\mu\text{L}/\text{min}$ the maximum wall shear stress at the base of the chamber chamber is of the order of 10^{-5} Pa or less. Cell viability is maintained at these flow rates, while the expression of albumin is increased. The chambers are easy to assemble and use and can be

employed as a generic dynamic cell culture tool, in place of static multiwells.

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