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In-vitro liver model using microfabricated scaffolds in a modular bioreactor

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Keywords: Scaffold, microfabrication, liver model, bioreactor.

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Abbreviations:

PAM: Pressure Activated Microsyringe
MCmB: MultiCompartment modular Bioreactor
3D: 3 dimensional
2D: 2 dimensional
CAD/CAM: Computer Aided Design/Computer Aided Manufacturing
PLGA: poly-DL-lactide-co-glycolide
PLLA: poly-L-lactide
PDMS: Polydimethyldisiloxane
EMEM: Eagle's minimal essential medium
ANCOVA: Analysis of covariance

ABSTRACT

Hepatocyte function on three-dimensional microfabricated polymer scaffolds realised with the Pressure Activated Microsyringe (PAM) was tested in static and dynamic conditions. The dynamic cell culture was obtained using the MCmB (MultiCompartment modular Bioreactor) system. Hepatocyte cell density, glucose consumption, and albumin secretion rate were measured daily over a week. Cells seeded on scaffolds showed an increase in cell density compared with monolayer controls. Moreover, in dynamic culture, cell metabolic function increased three times in comparison with static monolayer cultures. These results suggest that cell density and cell-cell interactions are mediated by the architecture of the substrate, while the endogenous biochemical functions are regulated by a sustainable supply of nutrients and interstitial-like flow. Thus a combination of three-dimensional scaffolds and dynamic flow conditions are both important for the development of a hepatic tissue model for applications in drug testing and regenerative medicine.

Keywords: Scaffold, microfabrication, liver model, bioreactor.

I. Introduction

A great deal of effort is being made to preserve liver specific function *in-vitro*. There are several driving forces for this, perhaps the two most important are drug testing and bioartificial liver devices. For the moment, the prevailing opinion in the literature is that it is impossible to maintain an adequately differentiated hepatic phenotype after isolation from the liver for more than a week [1]. Several parameters are known to play a role in maintaining liver function; among these are the presence of an adequate nutrient supply, an extra cellular environment rich with ligands for adhesion and signalling, and a spatial architecture resembling that of the native liver, as well as a multicomponent medium. These parameters form what we call here the tripartite of cues: a trio of biochemical, biophysical and biomechanical signalling systems which interact synergically to support both form and function in all living tissues [2].

A wide variety of methods have been and are being developed to maintain and study the function of hepatocytes *in-vitro*. Many of these are based on devising complex cocktails of culture medium, which often include inducing agents in order to drive hepatocytes towards expression of P450 cytochromes [3]. Others use engineering based methods such as scaffolds and bioreactors. Several authors have used: three dimensional (3D) structures such as collagen [4], or loofa sponges in static and perfused cell culture conditions [5] and hydrogel encapsulated hepatocytes [6]. In the past few years the use of bioreactors has become very popular because a dynamic cell culture system allows efficient metabolite exchange, and may also provide an indirect physical stimulus through percolative or interstitial-like flow [7]. The bioreactors can be divided into 2 main groups: those for large scale culture, such as flat membrane systems, and microfabricated systems which involve 2 or 3 dimensional cultures and microfluidics. The simplest systems are continuous flow reactors such as that proposed by Catapano and De Bartolo, which are useful for large scale culture, particularly for artificial liver applications [8]. A microfabricated array reactor with deep wells for hepatocyte spheroids was described by Powers *et al.* [9, 10]. In this system, the wells were perfused by the medium and the cells were reported to remain viable for 2 weeks, with high levels of urea and albumin secretion. Hongo *et al* , and Kataoka *et al.* describe the culture of porus scaffolds in a radial flow bioreactor so combining two of the cues in a single experiment [11, 12]. It is also recognised that coculture of hepatocytes and non parenchymal cells augments liver phenotypic function. Bhatia *et al* pioneered the use of two dimensional (2D) micro patterns in mono and co-culture, and they have reported on various combinations of heterotypic cultures as well as a comparative study which highlights the differences in gene expression in different coculture combinations [13,14]. Several reports by the group of Yarmush and Toner on microfabricated grooved susbstrates for hepatocyte

cocultures have been published, showing the advantage of grooves with respect to flat surfaces [15,16]. Finally, sandwich cultures, in which hepatocytes are either supplied with an adhesive protein based roof and floor to recreate a pseudo 3D extracellular matrix environment have also been widely reported to enhance hepatic function [17]. Despite the extensive literature on the subject, it is not clear how strongly each of these parameters influence hepatic phenotype, quite likely the biochemical, biophysical and mechanical stimuli provide multiple cues which act in unison to mimic the *in-vivo* hepatic environment. The aim of this work was to gain a better understanding of the role of a 3D architecture and convective mass transfer on hepatocytes *in-vitro*. We therefore designed an *in-vitro* model of the liver by using a bottom up engineering approach, that is by a step wise increase of the number of cues in the system. For this study we considered only cues which can be controlled externally using mechanical and structural design parameters. Biochemical control of hepatocyte function is far more complex because the cells themselves modulate the chemistry of their environment. Firstly the influence of a 3D open-pore microfabricated scaffoldon HepG2 cell proliferation and metabolic function was assessed using two different biomaterials. The most suitable material was then selected for experiments using a low shear, high flow bioreactor [18], and both cell proliferation and metabolism were analysed and compared with controls.

2 Materials and Methods

2.1 Microfabrication with PAM

The syringe-based deposition method PAM (Pressure Assisted Microsyringe) has been described in several references [19]. Basically it consists of a pressure activated syringe which is controlled by a CAD/CAM (Computer Aided Design/Computer Aided Manufacturing) fabrication system. The syringe is a stainless steel barrel with a glass capillary needle with a diameter of 5 to 20 µm. It is filled with a viscous polymer solution and mounted on a 3 axis micropositioner. Pressurised air is used to extrude the polymer solution through the micro-needle and deposit it on a solid substrate such as a glass cover slip. The CAD/CAM system allows an infinite range of structures with a lateral resolution of 5 µm to be designed and fabricated. 3D polymeric scaffolds are assembled by depositing a water soluble polymeric spacer (Hydrofilm, Lucart, Italy) of about 10-20 µm between the layers. This avoids the collapse of subsequent layers and creates large pores in which cells are able to penetrate and adhere. After the microfabrication step, scaffolds are immersed in water and

during this process the polymer spacer is dissolved away and the scaffolds float off the substrates and can be handled with tweezers.

PAM has the highest resolution of all 3D CAD/CAM microfabrication methods used in tissue engineering, and the fidelity of the scaffolds is about 10% [20]. Apart from the machine dependent deposition parameters such as needle bore size and motor speed and precision, the fidelity depends mainly on the viscosity and surface tension of the polymer.

2.2 Polymer and scaffold treatment

The polymers used in this study were: PLGA, poly-DL-lactide-co-glycolide 75:25 and PLLA (poly-L-lactide) (Lactel, NPPharm-F, Bazainville, France). PLGA and PLLA are biodegradable polymers widely used in tissue engineering applications. Our group has tested these polymers in the form of spin-coated films and PAM scaffolds and their suitability for cell adhesion and tissue engineering has been evaluated [21]. Solutions of 20% (w/v) PLGA and 10% (w/v) PLLA in chloroform respectively have excellent deposition characteristics in terms of their viscosity and surface tension, and these concentrations were used for all experiments. Hexagonal unit cell structures were microfabricated with 50 µm line width and 500 µm unit side length, (Figure 1A illustrates the definition of these terms) and were composed of three layers with 70 hexagons each (Figure 1A). Each layer was laterally offset by 250 µm so that the hexagons were not piled flush but formed small open pores in the scaffold. The dimensions were chosen because they mimic the characteristic size of hepatic lobules [22]. Figure 1B shows an example of a PLGA 3D scaffold. Polymer films were realized by spin coating (Delta 10TT, SÜSS MicroTec) at 7500 rpm for 15 seconds on 13 mm diameter glass cover slips. These films were used as a monolayer static control in order to investigate how cell function is influenced by a 3D topology and to compare and to normalize the experimental data with respect to the polymer's intrinsic characteristics rather than laboratory tissue culture plastic.

Before cell seeding to remove all traces of solvent, the scaffolds and films were placed in a dessicator under vacuum for at least a week, and then washed extensively with deionised water and dried in an oven at 50°C. Following this, the structures were placed in 24 well plates (Sarstedt, Verona, Italy) and sterilized using a standard hospital H_2O_2 Gas-Plasma protocol available at our clinical facilities. In order to promote cell attachment, 5 µg/cm^2 purified collagen, PureCol^{TM} (INAMED, Leimuiden, Netherlands), was pipetted over the structures and after one hour's incubation at 37°C, they were washed with PBS three times and equilibrated with fresh medium overnight in the incubator.

2.3 Bioreactor design and assembly

The MCmB bioreactor was developed to enable cell and tissue culture in a variety of configurations [18, 23]. Its main characteristics are high flow rates and low wall shear stresses. In its simplest form, as used in this work, it is made of PDMS (Polydimethyldisiloxane, Dow Corning, Italy), and has the same dimensions as a 24- microwell plate (15 mm diameter and 2 ml volume). A patented sloping roof ensures the absence of bubbles in the chamber. A FEM (Finite Element Modeling) model of the cell culture chamber was developed in order to study the shear stress at the cell surface. Cosmos Floworks, a Solidworks™ (Dassault Systèmes SolidWorks Corp. Concord, MA, USA) extension which allows fluid-dynamic FEM analysis as well as 3D CAD was used for this purpose. In the fluid dynamic model we used the following system constants: viscosity=10^{-3} Pa·s, fluid density =1000 kg/m^3, medium flow rate=250 µL/min, pressure= 1 atmosphere or 760 mmHg, temperature=37 °C and no slip boundary conditions. Figure 2A shows a three dimensional representation of the chamber, and in Figure 2B the velocity streamlines modelled using SolidWorks™ are drawn.

Three MCmB bioreactors were connected in parallel to a pump (Instec P720, Instec, Boulder, Colorado, USA) and a mixing chamber which has a volume of 2 ml and serves to facilitate assembly of the flow circuit, allows gaseous mixing, and acts as a bubble trap. A flow rate of 250 µl/min was used in all experiments, and the average shear stress in the cell culture zone was calculated by fluid dynamic simulation. The maximum wall shear stress at the flow rate used was about 10^{-5} Pa, and .

In our experience, even small flow rates may damage hepatocytes. Therefore all the cultures were protected with a thin coating of alginate as described in the section on cell culture. In the bioreactor experiments the medium was not changed since sufficient nutrients were present in the 10 ml closed loop circuit (2 ml in each bioreactor, 7 ml in the mixing chamber and about 1 ml in the tubing).

2.4 Cell culture

Since primary hepatocytes *in-vitro* rapidly lose liver-morphology and differentiated functions, in this study we used the HepG2 cell line as an alternative model cell. HepG2 cells maintain the main synthetic and endogenous functions of primary hepatocytes, as well some exogenous metabolic functions [24]. Furthermore, the HepG2 cell line is simple to culture and by virtue of its immortality, it is possible to make standard comparisons which remain valid over time and for all experiments.

HepG2 human hepatoblastoma cells were a kind gift from Dr. S. Quarta, University of Padova. The cells were grown in Eagle's minimal essential medium (EMEM, glucose 1g/liter) supplemented with 5% fetal bovine serum, 1% non essential aminoacids, 1% EMEM vitamins, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomicyn in a humidified incubator at 37°C, 5% CO_2 (Hereaus SpA, Milan, Italy). The cells were all used at the same passage (# 22 after receipt) and passaged using 0.05 % trypsin with 0.02% EDTA in PBS (all reagents from Eurolone, Milan, Italy). 100,000 cells/cm^2 were seeded on the films or scaffolds using 2 ml of complete medium per well. After 24 hours the structures were moved to a new microwell plate to eliminate interference from non adherent cells.

Both scaffolds and films were coated with an alginate film consisting of 250 µl 1% sodium alginate dissolved in serum free medium, cross linked with 50 µl of 1% $CaCl_2$ (both from Sigma-Aldrich, Milan, Italy). Excess alginate was removed with a pipette. The resulting film had a thickness of a few tens of microns as measured by an optical profilometer (Opto NCDT, model ILD1400-10, UK), having a nominal resolution of 1 µm. The profilometer uses a small laser spot (0.7 mm×0.5 mm) which is reflected off a surface. Displacements were calibrated using an uncoated glass slide as a reference. The coating was not uniform and flat as observed by an optical microscope, and this was also reflected in the scatter of the profilometer readings across the slide (40 to 100 µm). At the low alginate concentrations used, the diffusion coefficient of oxygen and of small solutes is similar to that in water [25, 26, and 27].

Finally 2 ml of fresh medium was added to each well to begin the experiment. Cells were maintained on the structures for a week and cell counting and medium collection was performed daily. The experiment was performed in triplicate using 21 structures, three samples were sacrificed for counting per day, and the culture medium was changed every third day.

2.5 Bioreactor Culture

For the dynamic experiments, cells were seeded as described for the static cultures. 24 hours after seeding, the scaffolds were transferred to a glass slide, placed in the MCmB (one scaffold per bioreactor) and coated with an alginate film. The alginate coating was used to protect the cells from direct mechanical stress, whilst allowing adequate nutrient diffusion. The coating also stops the scaffolds from floating off the slides. Medium was perfused and re-circulated through the chambers at a flow rate of 250 µl/min. A total of 21 scaffolds were used for the bioreactor experiments. In particular, three bioreactors with three scaffolds were used per time point, and at the end of each time point the bioreactors were disassembled for cell counting and a final sample of medium

collected. In addition up to 200 µl of media (50 µl per assay) was withdrawn from the mixing chambers for analysis at fixed intervals. All cell culture experiments were carried out in the same incubator.

2.6 Cell Analysis and Assays

Cell Viability and Density

At the end of each time point the cells adhered on the structures were trypsinised off the scaffolds and membranes and counted using a Burker chamber. Cell viability was evaluated using trypan blue exclusion, and this was always greater than 95%. To ensure that all cells had been accounted for, the scaffolds were also stained with trypan blue and typically only a few cells were observed on the scaffolds after trypsinization. Cell morphology was assessed using an optical microscope (AX70, Olympus Italia, Milan).

Because the 3D scaffolds are composed of 3 layers of hexagons, the "true" area available for cell adhesion is difficult to estimate. According to the CAD model shown in Fig 1A the total surface area of one layer of the scaffold is about 0.06 cm^2, however a 3D scaffold may also have adhesive surfaces in more than one plane. Therefore we use the term "nominal area" to refer to the overall area of the scaffold or film. Cell densities are expressed as the total number of cells counted divided by the nominal 2D-surface area of the samples. The PAM scaffolds have a nominal surface area of 0.8 cm^2 and a large percentage of this is free space of the pores, while the spin-coated films have an area of 1.33 cm^2.

Biochemical assays

Albumin production, which characterizes the specific functional activity of liver cells, was measured by an enzyme-linked immunosorbent assay (ELISA) (Bethyl Laboratories, Montgomery, TX USA). A standard curve was made using purified human albumin under the conditions recommended by the manufacturer. Species specificity of the anti-human albumin antibodies was verified using fetal bovine serum. Glucose consumption and urea production were quantified using commercial enzymatic kits according to the manufacturers' instructions (Megazyme International Poncarale, Italy and Urea Kit,Sigma-Aldrich, Milan, Italy respectively). In order to compare data from different experiments with different cell numbers and media volumes the glucose, albumin and urea data are expressed as quantities consumed or produced per cell per day, and therefore as rates, not cumulative quantities.

Statistical analysis

Statistical analysis was performed using analysis of covariance - ANCOVA for comparison of trends (Matlab Statistics Toolbox, The MathWorks Inc.); a *p* value of less than 0.05 was considered statistically significant. Each data point is represented as the mean and standard deviation of 3 samples and at each time point 3 scaffolds were analyzed destructively (by trypsinization) for cell counting.

3. Results

3.1 Comparison between 2D films and 3D scaffolds in static culture

As shown in Figure 3, the cell density was higher on the scaffolds than on films particularly from day 4 onwards (ANCOVA, $p<0.01$). The cells seeded on 3D structures proliferated throughout the duration of the experiment and the rate of increase was modulated by the media changes (day 3 and 6), whereas the cells seeded on 2D films proliferated until the fourth day and the rate of increase did not appear to be influenced by the media changes (day 3 and 6). This could be due to removal of loosely adherent cells when the medium was aspirated.

Figure 4 shows the glucose consumption rate. Glucose was consumed at a constant rate (5.2±0.3 ng/(cell/ day), $p=0.004$, ANCOVA) by cells seeded on 3D scaffolds and the consumption was not influenced by the continuous cell proliferation in 3D. The cells on the 2D films consumed a higher quantity of glucose than 3D scaffolds in the first two days. After this period, the glucose consumption per cell was constant and similar to that of the 3D scaffolds. The initial high glucose consumption by cells seeded on polymeric films does not seem to depend on the rate of proliferation but could represent increased energetic requirements due to cell-matrix interactions. In fact 2D films present a large surface area to the cells for adhesion whereas the 3D scaffold is more porous and there are fewer adhesive sites per unit area. Once the adhesion process is over, all cells converge to a constant glucose consumption rate.

Albumin is one of the functional marker proteins of the liver. Human albumin was produced in similar quantities by 3D scaffolds and 2D films during the experiment (Figure 5), and the production rates are similar to those reported in [24, 28]. An analysis of the production rates using ANCOVA indicated that the albumin production rate was higher in the first two days than on subsequent days (days 1 to 2: 0.82±0.32 pg/(cell/day), days 4 to 7: 0.49±0.05 pg/(cell/day), $p<0.05$). Although the cell proliferation rate was higher on the 3D scaffolds than on the 2D films, we found no differences cell behaviour with respect to the two polymers. PLGA and PLLA are known to have different surface and mechanical properties, and this result implies that in our experiments only the

microtopology influences cell proliferation and not the differences in physico-chemical properties between the polymers.

3.2 3D scaffolds in dynamic conditions in the MCmB

Having established that 3D scaffolds can increase the cell density consistently for up to 7 days, the next step was to select the polymer to be used. Since there were no significant cell metabolic and proliferative differences between the 2 polymers, PLGA was selected for successive experiments because of its deposition characteristics. The choice was based on the fact that 20% PLGA is slightly more viscous than 10% PLLA, and the scaffolds obtained using the PAM system are of higher fidelity than the corresponding PLLA scaffolds [29].

For each time point three bioreactors were set up in parallel with one 3D scaffold per chamber. In the bioreactors, the cell density increased smoothly over time, as the peaks due to media changes were absent, and density values were similar to those in static conditions (Figure 6).

Glucose consumption was slightly higher in the MCmB with a constant consumption rate of 7.6 ± 0.1 ng/(cell/ day) ($p=0.0001$ ANCOVA) with respect to the 3D scaffold in static conditions (5.2±0.2 ng/(cell/day), $p=0.001$, ANCOVA) (Figure 7). Therefore the cell energy requirements are greater in the bioreactor, and glucose is used as a substrate. This may be due to an upregulation of liver-specific synthetic function in the bioreactor, as shown in Figure 8. A large increase in albumin production rate per cell was observed in dynamic culture. In the first 2 days; the albumin production rate was 10 times higher in the MCmB than in static conditions and then decreased progressively maintaining a value 4~5 times greater than in static culture. In fact, the average albumin production rate in the bioreactor over the 7 days was 3.12±0.43 pg/(cell/day), compared with 0.65± 0.16 pg/(cell/day) ($p=0.02$, ANCOVA) in the scaffolds in static conditions .

To ensure that the increased albumin production rate was not due to the increased volume of medium in the bioreactor, a final experiment was performed using 3D scaffolds cultured in 50 mm Petri dishes in which the total medium volume was 10 ml. The albumin production rate was 0.50 ±0.14 pg/(cell/day) over the 2 day period of analysis, indicating that the volume of medium does not influence the secretion rate of this protein in static conditions.

Figure 9 shows the that urea production rate, indicative of an intact nitrogen metabolism pathway, is significantly enhanced in the dynamic 3D culture with respect to both the 2D and 3D static conditions. In static conditions, the 2D films and 3D scaffolds converged to the same production rate towards the end of the culture period (136±101 pg/(cell/day)), while the average urea production rate in the bioreactor was significantly higher throughout the experiment (559±71

pg/(cell/day) for the 3D scaffolds in static conditions with respect to 1248±71 pg/(cell/day) for the 3D scaffolds in dynamic conditions p<0.00001, ANCOVA).

We also monitored the macroscopic morphology of the cells on the 3D scaffolds and 2D films (Figure 10). Although we found little difference between cell morphology and spreading on scaffolds in the dynamic and static cultures, the cells grown for 5 days on 2 and 3D surfaces were quite different. On the films cells were spread evenly and typically had larger dimensions than on the 3D substrates, and even after 5 days, free spaces between cells were apparent. This indicates greater cell-substrate interaction than cell-cell contact on the films. Cultures grown on 3D scaffolds spread and migrated into the structure so that even the voids were colonised. The cells were closely packed, and smaller, forming aggregates. This is indicative of greater interactions between adjacent cells. Therefore even though the quantity of solid substrate is minimal, the cells seeded on the 3D scaffolds assemble in the pores to maximize cell-cell interaction and cell density.

4 Discussion

Hepatocytes, like all cells, are dramatically affected by the physical and chemical nature of their micro-environment. This is one of the reasons why *in-vitro* cell culture experiments are so difficult to compare from laboratory to laboratory. The hepatic cell habitat comprises 3 main features, or cues, which are known to influence cell behaviour; the biochemistry, the architecture and the supply of nutrients to meet the metabolic demands of the cells. In this work we investigate 2 of the main cues which are known to influence hepatocyte function, a 3D topology and convective flow. Several investigators have shown that cell culture in 3D represents a more physiologically relevant environment. However the extent to which this influences hepatocyte function with respect to the provision of oxygen and other nutrients is unclear. Moreover, the relationship between the topological features of a scaffold and hepatic function has not been studied in depth. As far as HepG2 cells are concerned, our results show that the main contribution of the scaffold is to increase cell density and to promote the formation of aggregates which allow greater cell-cell contact with respect to cell-substrate interactions. Thus, we observe a large increase in the number of cells per unit area, but the expression of albumin per cell is the same as on a monolayer, as is the glucose consumption rate per cell, while the urea production rates also converge to similar values after 5 days in culture. Similar results have also been reported by Glicklis R *et al.* [30] and Bokhari *et al.* [31].

On the other hand, convective transport is often mandatory in 3D scaffolds, especially if they are thicker than a few hundred microns. Indeed, bioreactors for hepatocyte culture are generally

designed for allowing adequate mass transfer to high density 3 dimensional cultures in porous sponges or encapsulated spheroids. One of the main engineering issues in bioreactors for *in-vitro* liver models is the balance between high mass transfer and low wall shear stress to cells. There appears to be some debate on the effects of shear stress on hepatocytes. Some papers cite extremely high values of shear (0.5 Pa [32]) and some also report on enhancement of cytochrome expression in conditions of fairly high shear stress [33]. Other investigators cite somewhat lower values of maximum shear stress that these cells can support, even in the presence of non-parenchymal cells which afford support and protection (0.03 Pa [34]). The application of direct fluid flow parallel to the cell surface is certainly not physiological, since hepatocytes are not in direct contact with flowing blood in capillaries. In this context, Wang and Tarbell's model [35] is often cited to justify direct shear stress on cells of the order of 0.1 Pa [9]. These values should be applied with caution as some models predict even lower values of wall shear from porous flow [36]. Here, we effectively shield the cells from the effect of direct flow, although the highly permeable alginate coating allows low velocity porous or percolative flow perpendicular to the convective flow provided by the pumps. This flow is similar to interstitial flow [7], and allows the passage of essential nutrients and catabolites thanks to concentration gradients established between the scaffold and the moving fluid. In the bioreactor then, the cell density on 3D scaffolds remains unchanged with respect to static cultures, but the metabolic and synthetic function in the form of albumin and urea production rates per cell are greatly enhanced, as reported in Figures 6 to 9. Similar results have also been reported for 2D cultures in dynamic and static conditions [28, 37] suggesting that these observations can also be generalized to 2D systems.

The objective of this work was to recreate a biomimetic environment for hepatocytes and to determine, first singly and then together, the influence of a 3 dimensional porous architecture and enhanced mass transport on liver cultures. HepG2 cells were initially studied on 2D polymeric membranes and 3D scaffolds. The results of this first stage demonstrated that the 3D scaffolds promote cell proliferation, such that final cell densities are significantly higher than on 2D surfaces. Other metabolic parameters were unchanged, indicating that the baseline metabolic cell function was unaltered. While the endogenous synthetic and metabolic functions (glucose consumption rate, albumin and urea production rates) of hepatocytes do not appear to be significantly related to cell morphology and 3D organization, this does not necessarily mean that all functions are unaltered in 3D. In fact, Bokhari *et al.* [31] observe an increase in exogenous metabolic function in HepG2 cells on 3D scaffolds with respect to 2D cultures, while the endogenous functions are unaltered. In the second phase of experiments the scaffolds were placed a bioreactor and culture medium was

pumped through the system in a closed-loop. Neither cell proliferation nor glucose consumption rate per cell increased significantly with respect to the scaffolds in static conditions, but protein synthesis rates increased dramatically about 4 to 10 fold, and urea production rates by 5 to 2 fold. These experiments established then that 3D HepG2 cultures in a dynamic environment are metabolically more efficient than static monolayer hepatocyte cultures and that the critical factor which drives endogenous metabolism in our experiments is the interstitial-like flow.

Our results demonstrate that, in order to more closely approximate functional hepatic tissue, it is important to give to cells two important stimuli, the topographical and the physical stimulus. The micro-topology and porosity of the three-dimensional scaffolds increases cell density due to the formation of cell/cell aggregates, thus maximizing cell-cell interactions. The second stimulus is produced by the flow of culture medium in the bioreactor which contributes to an increased metabolic turnover. Although the hepatocytes are protected from direct open channel flow by a thin coating of gel, the flow allows a sustainable supply of nutrients to the cells, as well as a physical stimulus in the form of indirect interstitial-like flow.

Legends

Figure 1: A) CAD design of one layer of the hexagonal scaffold showing approximate total dimensions which define the nominal area. On the right, the terms unit side length and line width are defined for one unit hexagon, B) optical micrograph of a 3D PLGA scaffold.

Figure 2: A) A three dimensional representation of the MCmB cell culture chamber, B) velocity streamlines at a flow rate of 250 µl/min.

Figure 3: Cell density (defined as cell number/ nominal surface area in cm^2) for 2D PLGA and PLLA films, 3D PLGA and PLLA scaffolds (n=3 per data point).

Figure 4: Glucose consumption rate in static conditions in 2D films and 3D scaffolds of PLGA and PLLA, (n=3 per data point).

Figure 5: Albumin production rate in static conditions for 2D films and 3D scaffolds of PLGA and PLLA (n=3 per data point).

Figure 6: Cell density (defined as cell number/ nominal surface area in cm^2) for PLGA scaffolds in the bioreactor and in static conditions (n=3 per data point).

Figure 7: Glucose production rate in static and dynamic conditions for PLGA 3D scaffolds (n=3 per data point).

Figure 8: Albumin production rate in static and dynamic conditions for PLGA 3D scaffolds (n=3 per data point).

Figure 9: Urea production rate in static conditions for PLGA 2D films and 3D scaffolds and in dynamic conditions for PLGA 3D scaffolds (n=3 per data point).

Figure 10: Micrographs of HepG2 cells on A) PLGA film B) 3D PLGA scaffold after 5 days in culture. The bar indicates 50 µm.

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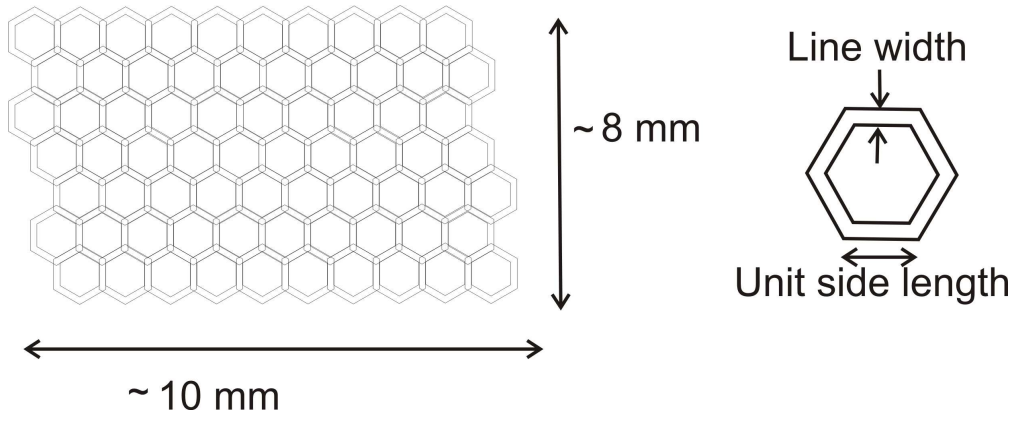
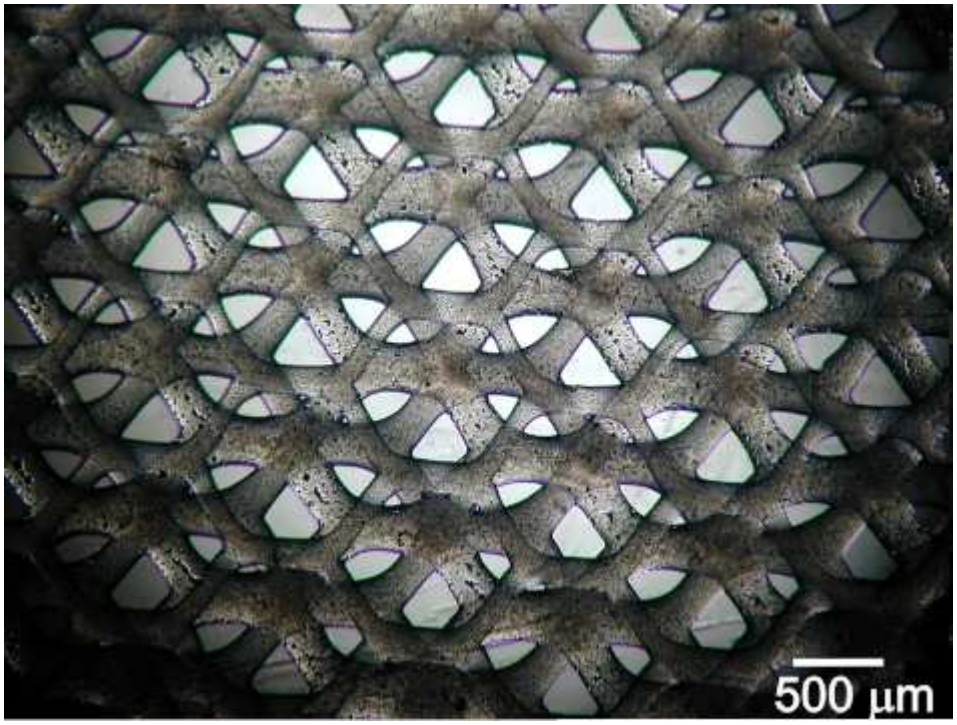


Figure 1: A) CAD design of one layer of the hexagonal scaffold showing approximate total dimensions which define the nominal area. On the right, the terms unit side length and line width are defined for one unit hexagon, B)optical micrograph of a 3D PLGA scaffold.



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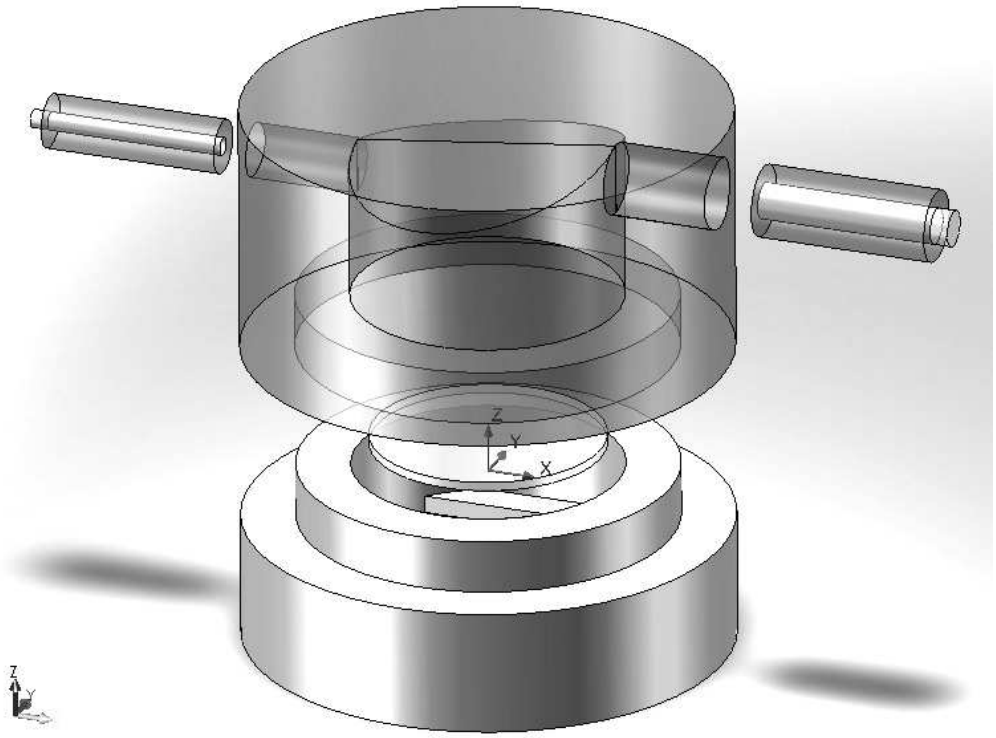
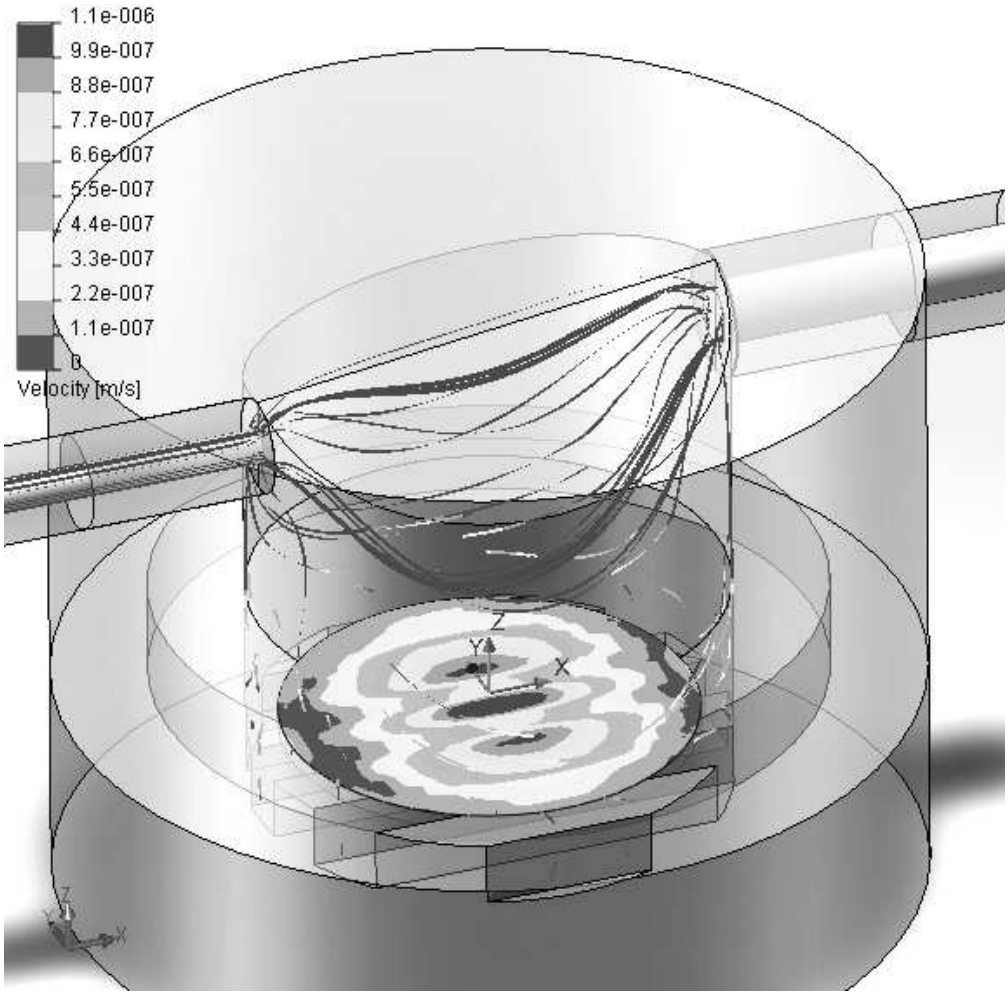


Figure 2: A) A three dimensional representation of the MCmB cell culture chamber, B) velocity streamlines at a flow rate of 250 µl/min.



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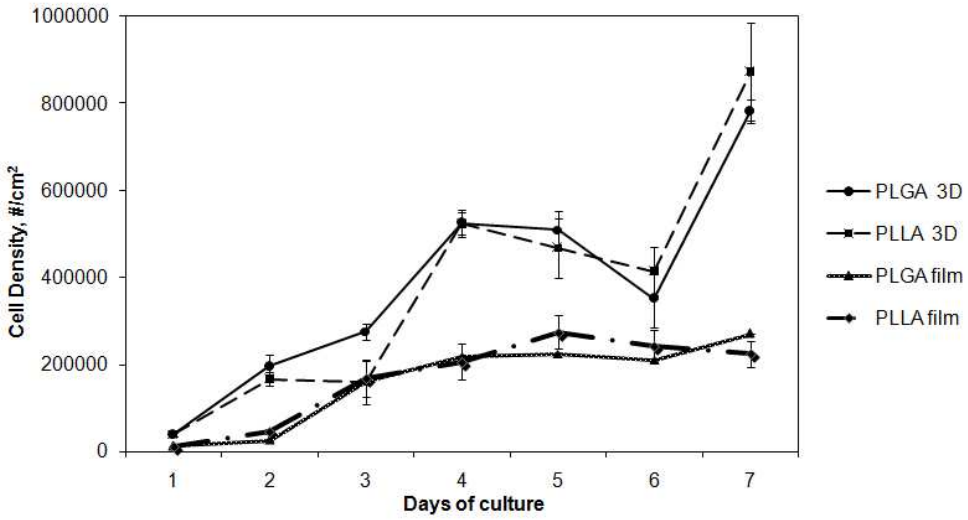


Figure 3: Cell density (defined as cell number/ nominal surface area in cm2) for 2D PLGA and PLLA films, 3D PLGA and PLLA scaffolds (n=3 per data point).

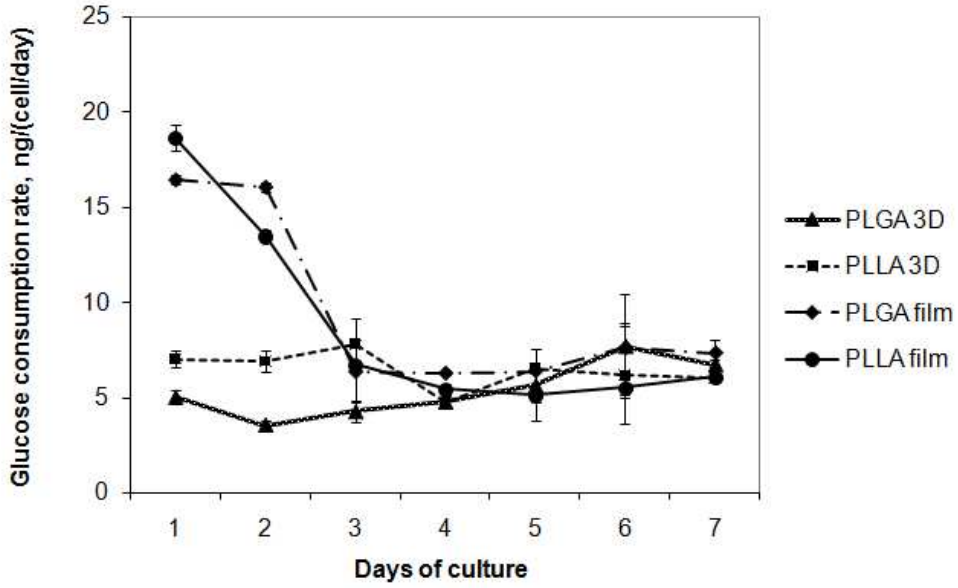


Figure 4: Glucose consumption rate in static conditions in 2D films and 3D scaffolds of PLGA and PLLA, (n=3 per data point).

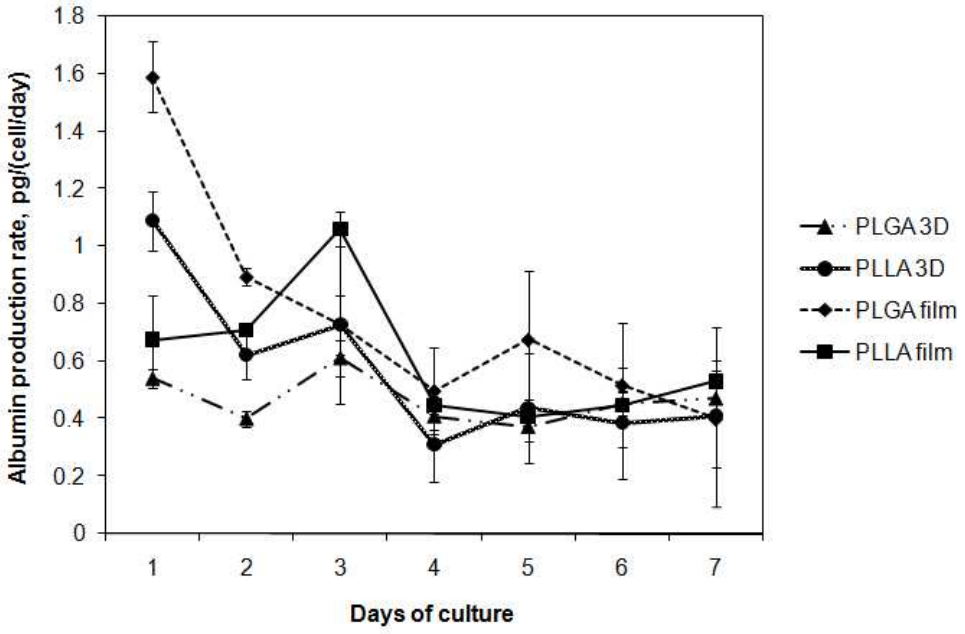


Figure 5: Albumin production rate in static conditions for 2D films and 3D scaffolds of PLGA and PLLA (n=3 per data point).

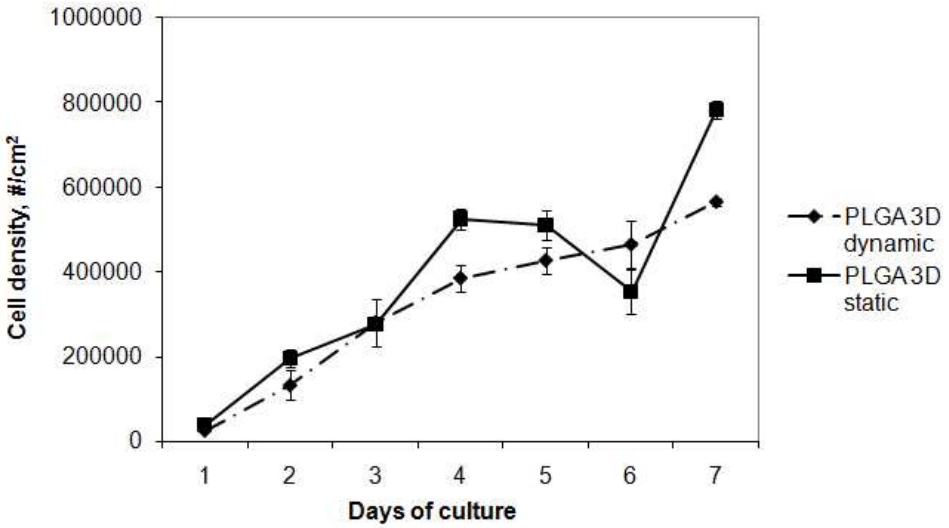


Figure 6: Cell density (defined as cell number/ nominal surface area in cm2) for PLGA scaffolds in the bioreactor and in static conditions (n=3 per data point).

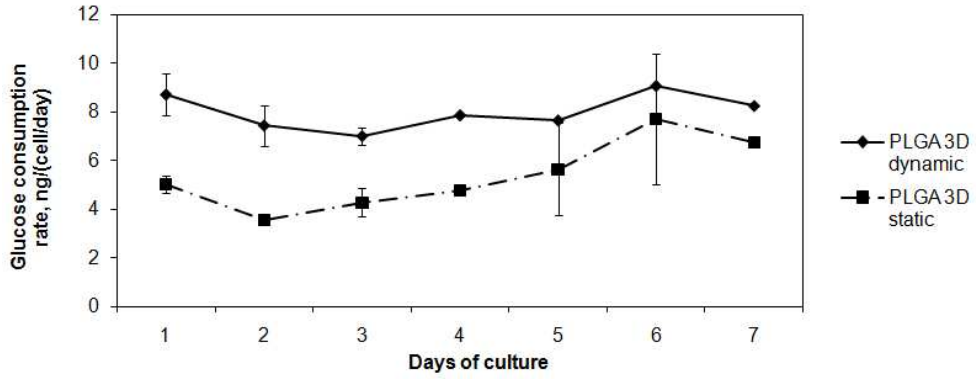


Figure 7: Glucose production rate in static and dynamic conditions for PLGA 3D scaffolds (n=3 per data point).

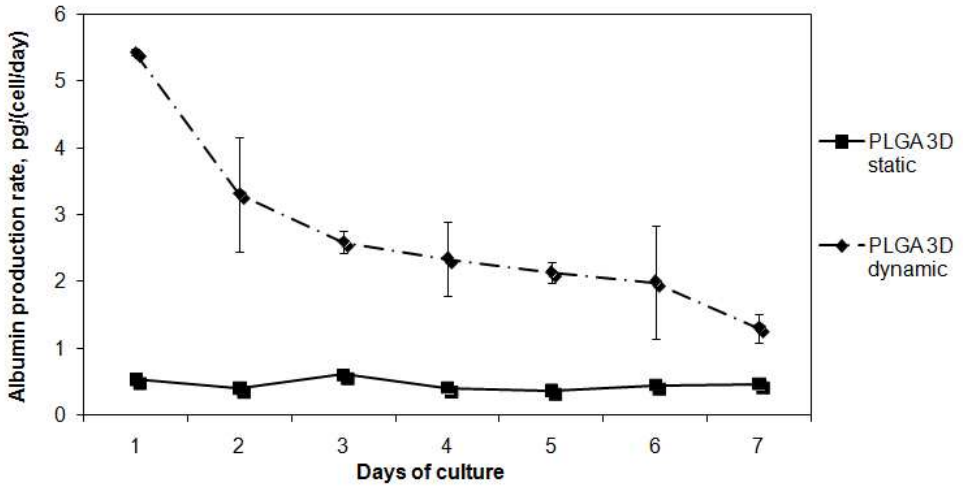


Figure 8: Albumin production rate in static and dynamic conditions for PLGA 3D scaffolds (n=3 per data point).

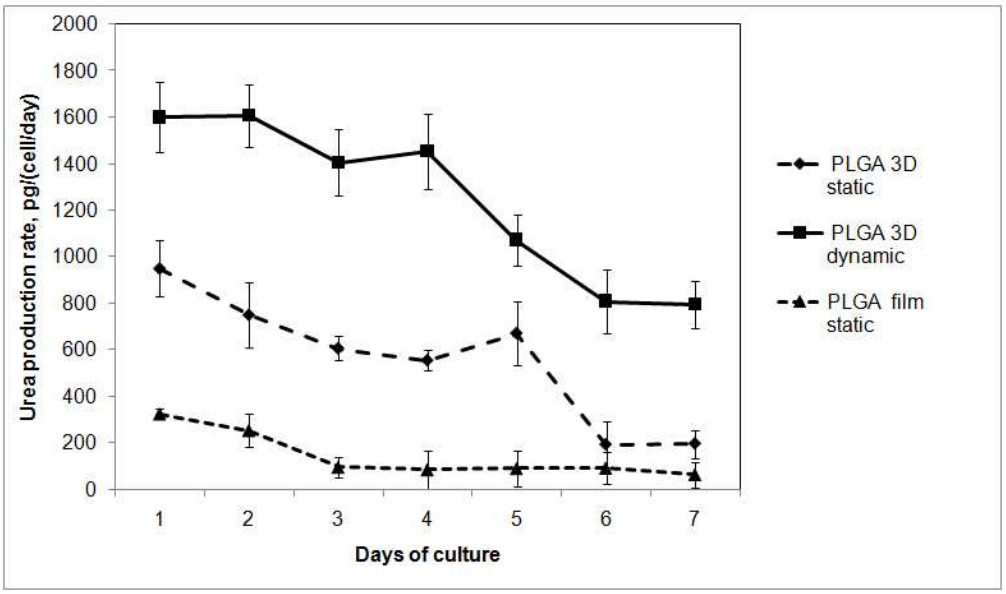
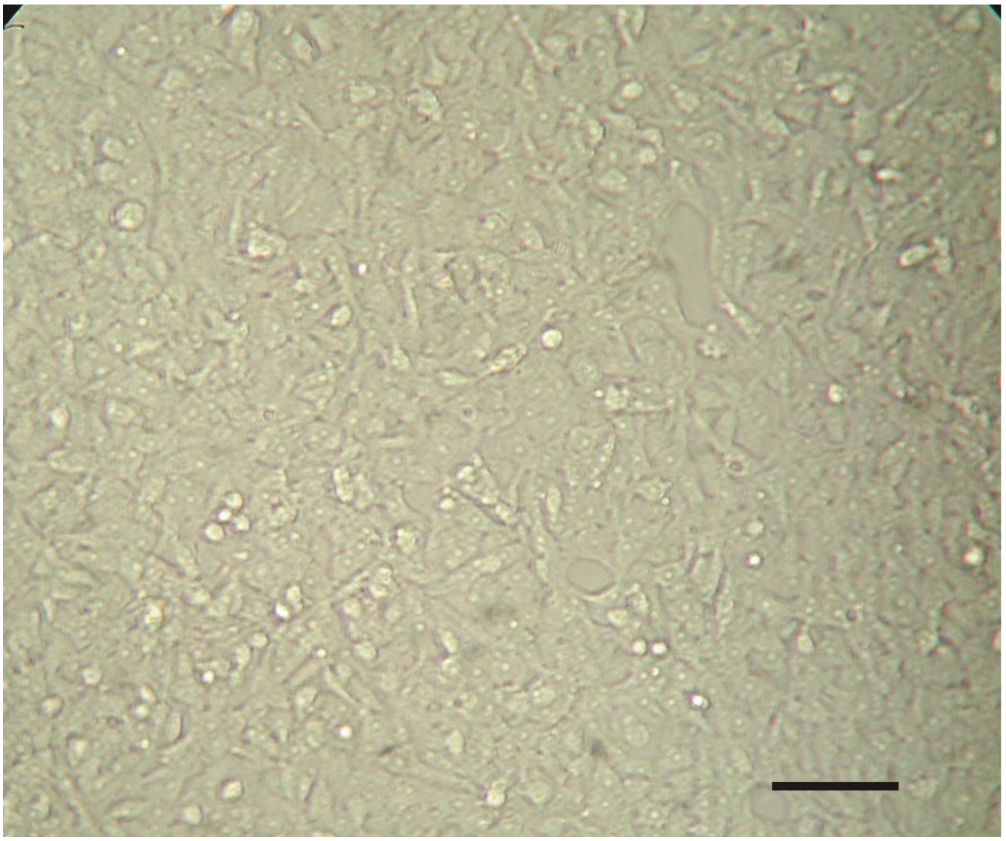


Figure 9: Urea production rate in static conditions for PLGA 2D films and 3D scaffolds and in dynamic conditions for PLGA 3D scaffolds (n=3 per data point).



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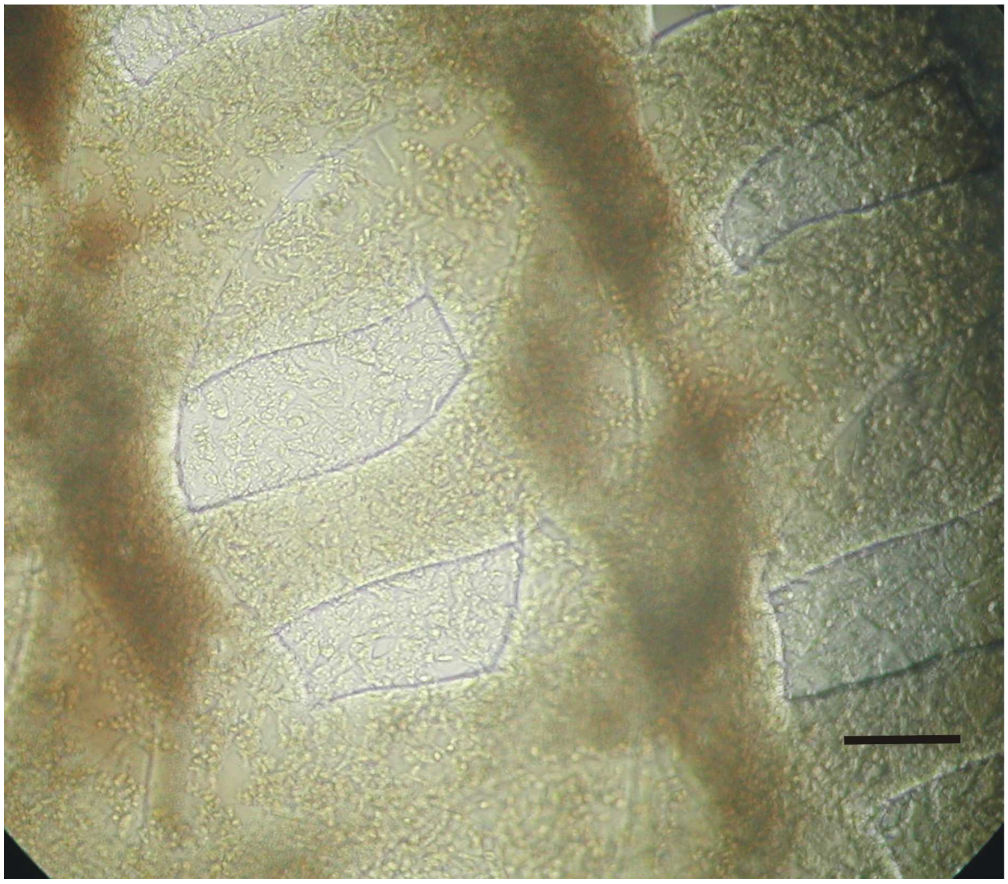

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