Encapsulated HepG2 cells in a pilot-scale bioreactor for a bioartificial liver

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Introduction

Bioartificial liver support systems are a possible solution to the worldwide shortage of donor organs for transplantation in cases of hepatic failure acting as a bridge to transplantation, or 'buying time' for a patient's own liver to recover, reducing demand on donor organs.
Existing designs can be divided into two categories: mechanical devices relying on filtration, molecular adsorption or dialysis to detoxify a patient's plasma, and bioartificial liver devices that include a cellular component (BAL) to replicate the functions of the liver (A. J. Wigg, 2005). For the wide scope of liver function, the bioartificial liver approach seems preferable. We previously published an alginate encapsulated cell culture system using HepG2 cells that we are developing as the biological component of a bioartificial liver (S. M. Coward, 2005; M. Khalil, 2001). We have tested it *in vitro* on a small scale and now report the issues of scale up to a clinically useful size that will be compatible with human plasma rather than simple culture medium, and demonstrate stable function of the encapsulated HepG2 cells during prolonged perfusion in toxic liver failure plasma. We use alginate encapsulated HepG2 cells in a fluidised bed bioreactor (FBB), with proven efficiency *in vitro* in terms of mass transfer and biological functions (B. David, 2004).

Methods

Small scale alginate bead preparation: HepG2 cells were used at 1.5×10^6 cells/ml in 1% alginate/culture medium. 10-30μm glass beads were added to the mixture at 2% w/v, to increase the density of beads relative to plasma. The solution was transferred to an Inotech IER-20 encapsulator. The cell suspension was passed at 5ml/min through the encapsulator using a 200μm nozzle vibrating at 1295Hz, yielding spherical beads of average 400 ± 17 μm diameter. The beads fell into stirred 0.204M $CaCl_2$ in 0.15M NaCl, were gelled for 15 min, washed with DMEM prior to transfer to microgravity culture. **Large scale alginate bead preparation:** to enable volumes of up to 3L of beads to be prepared a different approach was required. This utilised a different principle of bead making: cutting a jet of liquid into small pieces. A "Jet-cutter" from GeniaLab (Germany), was custom built for our purposes. The alginate/cell mixture is delivered under pressure through a 350um nozzle. For volumes up to ~120ml beads a similar batch gelling procedure was utilized; for large volumes a calcium "feed and bleed bath" was developed. Initial experiments explored uniformity of size, density and shape. **Perfusion with human plasma in a fluidised bed bioreactor:** Prior to inoculation into the fluidised bed bioreactor encapsulated cells were cultured for 8 days under microgravity culture conditions. 70ml of beads containing 1.5×10^9 cells were transferred to a 5 cm diameter fluidised bed bioreactor (12 cm column FBB) and perfused for up to 8h with 300ml of plasma. To study detoxificatory function 7ml beads containing 1.5×10^8 cells were perfused with 30ml of plasma in a column 10 cm high with a diameter of 1.2 cm. Initially beads settle in a packed bed. Plasma is perfused through the column vertically from the base and the frictional force exerted by the plasma flowing past the beads causes them to rise, leading to expansion of the bed to a maximum. Then a decrease in particle concentration causes a decrease in the linear velocity of the fluid and the force exerted by gravity causes them to sink back before rising again back to the final bed height. Under fluidised conditions there is an environment of low shear stress, high mass transfer and minimal dead space making it ideal for a bioartificial liver device. Samples were collected hourly. Plasma from patients with acute liver failure was obtained with ethical approval from clinical Hepatology centres in Copenhagen, Denmark; Kings College Hospital, London and the Royal Free Hospital, London. **Assessment of function: Cell viability:** Beads pre- and post-perfusion were stained with fluorescein diacetate (FDA) and propidium iodide

(PI) and examined using a Nikon Eclipse fluorescence microscope. **Measurement of cell toxicity:** Lactate dehydrogenase was measured using a Cytotox assay using a standard LDH solution of 1000U/ml for quantitation. Transaminase activity in plasma samples was measured in a clinical analyser. **Measurement of metabolic and synthetic capacity:** Glucose consumption and lactate production were calculated from hourly measurements of glucose or lactate concentrations in the plasma using an Analox GM-7. Alpha-fetoprotein (AFP) was measured using a clinical analyser. **Bilirubin determination:** Alginate beads containing HepG2 cells were transferred to a 4 FBBs and perfused for 8h with healthy human plasma containing 300µM bilirubin, to reflect levels seen in patients with liver failure. One FBB was set-up with empty alginate beads to act as a control. Concentrations of total, conjugated and unconjugated bilirubin were measured using a Vitros system. **Restoration of Urea cycle function in HepG2 cells by genetic modification:** Our recent work demonstrated that HepG2 lack a full complement of urea cycle enzymes and are unable to detoxify ammonia to urea via this route (D. Mavri-Damelin, 2007). A multiple gene transfer approach was taken to restore urea cycle function in HepG2 cells. Dual transfectants (DT) containing Ornithine Transcarbamylase and arginase I cDNA were cloned. **Quantification of urea and *de novo* urea synthesis:** Encapsulated HepG2 dual transfectant cell lines were perfused for 8h in a FBB with culture medium spiked with 1mM $^{15}NH_4Cl$. Samples of medium were taken at 0 and 8h and processed for analysis by GC-MS (D. Mavri-Damelin, 2007a).

Results

Large scale bead production: in a 5 L pressure vessel the density difference between alginate collected at the start and end of the process was no more than ~2% (Figure 1). Altering the jet wire motor speed had the greatest effect on bead size; a jet-wire ratio of 0.31 and a motor speed of 3500rpm gave beads of 433+/-33um at a flow rate of ~20ml/min. Using a feed and bleed system of calcium chloride we maintained a molarity of 0.17M with no loss of viability over 60 minutes.

	Exp. 1	Exp. 2	Exp. 3
Motor speed (rpm)	3300	3100	3500
Drop size (µm)	559	571	549
Shrinkage	0.75	0.76	0.76
Bead size (µm)	419	434	417
Obtained bead size (µm)	448.78	457.55	432.96
Standard deviation	52.44	34.78	32.89
Jet-wire ratio	0.33	0.36	0.31

Table 1 : average sizes of beads collected at different jet wire motor speeds

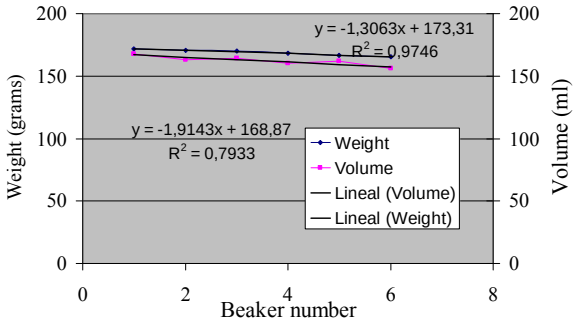

Figure 1: Measurement of alginate volumes and weights over time to derive density

Appearance of alginate beads and viability of HepG2 spheroids in plasma experiments: Alginate beads in all experiments maintained a spherical shape and broken beads were not observed after 8 days of culture and 8h perfusion with plasma. Viability of HepG2 spheroids, assessed by fluorescence of FDA/PI stained cells, was maintained after perfusion. **Functional toxicity:** The initial AST and ALT levels for the liver failure plasma experiments were higher compared to the plasma from healthy individuals reflecting the transaminase release from the patient's own failing liver. However, over the perfusion period there were no significant increases in AST or ALT activity in any experiments. Lactate dehydrogenase levels, representing cell damage in alginate encapsulated HepG2 cell cultures were also not significantly altered during the perfusion period. **Metabolic activity (glucose consumption and lactate production)** Glucose consumption was observed over the period of perfusion (Fig.2a). The decreases in glucose were mirrored by an increase in lactate (Fig.2b) confirming the known energetics of HepG2 cells (W.G. Blackard, 1990).

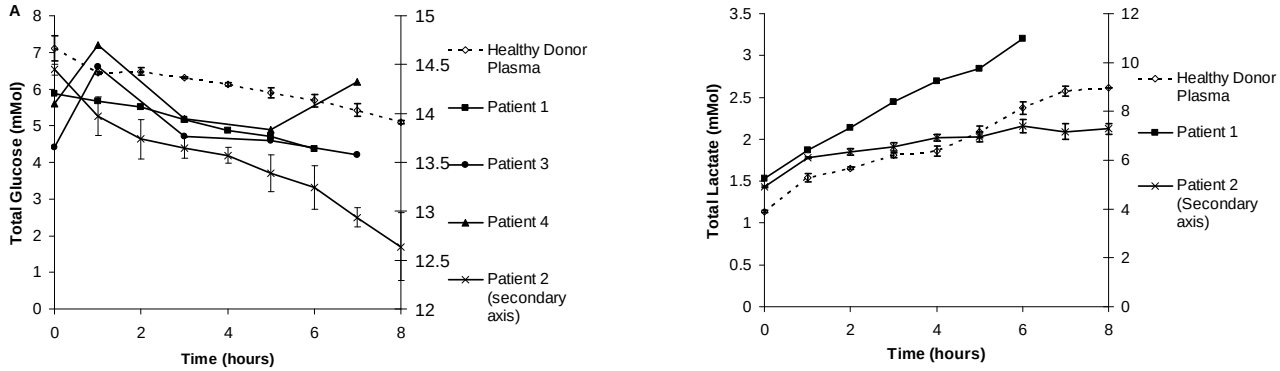

Figure 2a,b: Time course of metabolic activity (glucose consumption and lactate production) of alginate-encapsulated HepG2 cells perfused with liver failure plasma from five patients.

Synthetic function: Synthetic capacity was demonstrated by production and release of AFP, a specific marker of HepG2 cells not normally secreted by adult human liver and therefore not present in the liver failure plasma pre-perfusion (Fig.3a). Linear increases in AFP levels over the course of the perfusion were observed; liver failure plasma did not affect the rate of AFP synthesis. **Detoxification: bilirubin conjugation:** Samples of plasma spiked with bilirubin taken during perfusion of the FBB showed an increase in conjugated bilirubin with a concomitant decrease in unconjugated bilirubin (Fig.3b). Bilirubin conjugation when beads without cells were perfused was insignificant. Conjugated bilirubin production when corrected for cell number and volume of bioreactor circuit was 532 ± 160 mg/ 1×10^{11} cells/ day. **Ammonia detoxification by encapsulated**

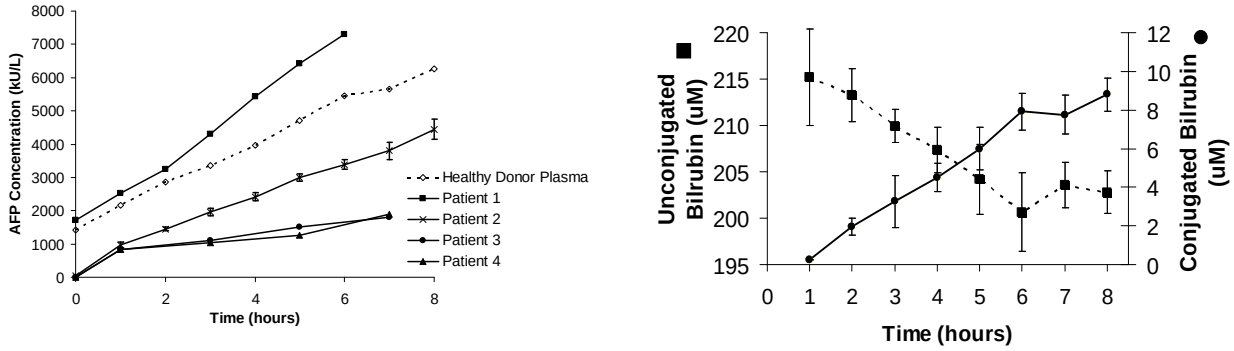

Figure 3a (left): timecourse of AFP production by alginate-encapsulated HepG2 cells perfused with liver failure plasma from five patients. Figure 3b (right): bilirubin conjugation by encapsulated HepG2 cells, data points show mean and standard deviation for 4 experiments.

dual transfected HepG2 cells: GC-MS data showed whilst HepG2 cells in the FBB produced minimal amounts of urea over 8 hours, the dual transfectants containing both OTC and Arginase I cDNA produced >10-fold more, and about 20% of urea produced by primary human hepatocytes.

Discussion

Great strides have been made addressing the technical challenges involved in the design and production of bioartificial liver devices. However, so far the performance of those in clinical trials has been disappointing (M.P. van de Kerkhove, 2004). There are three principal failings. Firstly the principles of design; several previous devices have utilised hollow fibre technology, thereby separating the cells from the plasma to be "improved". Secondly, the number and source of cells used. Finally, there is little data on the status of the bioartificial liver itself, under conditions which simulate use in the clinical setting. In contrast, our approach has established that the device proposed has sufficient sustainable functional capacity to fulfill the role for which it is designed.

Maximising mass transfer is key to the design of any bioartificial liver machine. Accurate modelling of the conditions to which a prototype bioartificial liver device will be exposed in the clinical setting will facilitate a more successful transition from bench to bedside.

In our study, despite the potentially damaging environment of 90% LF plasma, there was no significant damage to the encapsulated cells. Furthermore, carbohydrate metabolism, as shown by maintained glycolysis, showed metabolically active cells. Synthetic function showed no diminution of rate with increased exposure to LF plasma. Conjugated bilirubin production when corrected for volume of bioreactor circuit equated to 532 ± 160 mg/ 1×10^{11} cells/ day, compared to 5mg/day of conjugated bilirubin excreted in the urine of healthy individuals, indicating that the device should have sufficient functional capacity to clear elevated bilirubin levels seen in deeply jaundiced patients. A crucial aspect of liver function for the survival of patients with acute liver failure is ammonia detoxification. We have established stably transfected HepG2 cells expressing the missing enzymes (D. Mavri-Damelin, 2007b). Using this cell line urea synthesis was restored.

In conclusion, we have successfully scaled up thus far to a volume of 1200ml alginate beads, a volume close to that required in a clinical situation. Moreover, our biological data provides evidence that a full scale version of our encapsulated cell system can perform adequately as the biological component of a bioartificial liver, in the setting of acute liver failure.

Acknowledgements
Work was supported by the Liver Group Charity, and the Wellcome Trust. We thank Dr M Thomas (Royal Free Hosp) and Dr Y Foo (Great Ormond Street Hosp) for chemical pathology analyses.

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