Viability of 3D Alginate Encapsulation Isolated and Aggregated Rat Hepatocytes Through 8-days of Roller Bottle Culture

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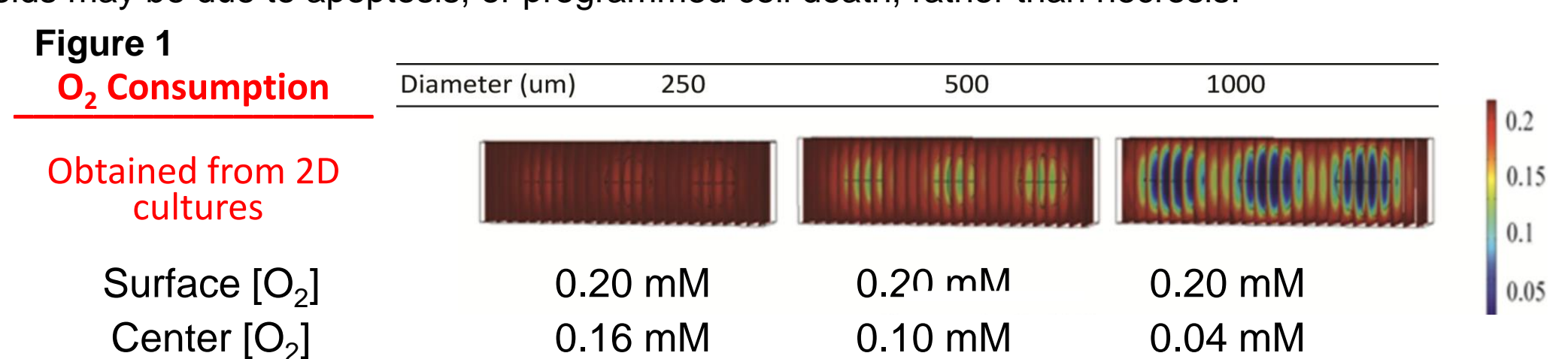Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill
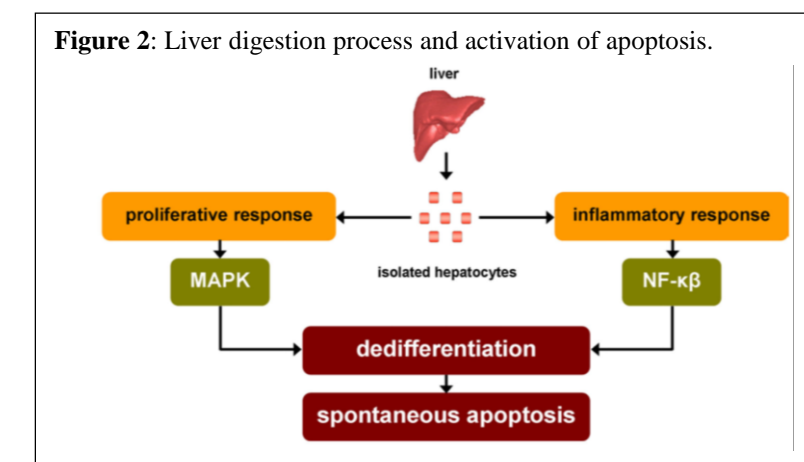
ABSTRACT

This project is one of many experiments in Dr. Macdonald's lab, looking into the viability of 3D alginate encapsulated rat hepatocytes. This is an effort to more effectively model the liver in vitro, for purposes such as improved medicine testing. Previous research has shown that oxygen availability differs based on the size of the encapsulates, therefore, cell viability within encapsulates likely will as well. In this experiment, hepatocytes were isolated into 250 µm and 500 µm diameter encapsulates, along with a coculture sample, which included nonparenchymal cells. The cells were imaged daily over the course of 8 days, and were dyed in order to indicate whether they were living, apoptotic, or necrotic. Based on these results, the cocultured cells appeared to remain living at a much greater rate than either of the two monocultures. Both the 250 µm and the 500 µm diameter samples showed significant apoptosis and necrosis over the 8 day period. Cell viability was also determined throughout the experiment, using an LDH assay. The coculture sample had the best viability, with 94.7% of the cells remaining viable 8 days after encapsulation. The 250 µm diameter sample had 52.7% of the cells remaining viable at the conclusion of the experiment. The 500 µm diameter encapsulates were unable to get an accurate LDH reading, as the amount of LDH present in the sample was too high for the reader.

INTRODUCTION

This project is part of Professor Macdonald's on-going research program on identifying physico-chemical and biological parameters that affect the long-term functionality of 3D alginate-encapsulated liver cells, or hepatocytes. Thus far, this project has compared two diameters of alginate-hepatocyte spheroids, 500 µm and 250 µm, each loaded with hepatocytes at one-fourth that of normal liver tissue density. Lactate dehydrogenase (LDH) is an enzyme leaked into the cell medium when cells in culture die. Total LDH per million hepatocytes can be determined and knowing the total number of cells per culture vessel, one can correlate the percent of mortality of cells over time in the culture. Using the correlation, both diameters had a logarithmic increase in the percent mortality, but the larger 500 µm encapsulates had a maximum of 35% mortality versus 20% for the 250 µm diameter spheroids. Computational models of oxygen concentration across 500 µm encapsulates[1] show that it could have a hypoxic core with the elevated hypermetabolism that occurs after hepatocyte isolation[2] creating a necrotic core and resulting in the relatively elevated mortality rate compared the 250 µm diameter (fig. 1). Necrosis is an uncontrolled cell death that is rapid, within hour to a day, while apoptosis is controlled programmed cell death that can occur over the life span of the culture. The 15% lower mortality rate observed with 250 µm diameter spheroids may be due to apoptosis, or programmed cell death, rather than necrosis.

Figure 1

O₂ Consumption
Obtained from 2D cultures

Diameter (um)	250	500	1000
Surface [O₂]	0.20 mM	0.20 mM	0.20 mM
Center [O₂]	0.16 mM	0.10 mM	0.04 mM

In 2001, a seminal publication discovered that as much as 50% of hepatocytes undergo apoptosis after isolation.[3] This is due to the fact that they are initially induced to grow due to lack of contact inhibition during collagenase digestion of the liver, which is then followed by a period of 1 hour or more of hypoxia during processing and purification (**Fig. 2**). Upon re-introduction to oxygen, reactive oxygen species is generated which induces apoptosis.[3] One way of culturing the apoptotic hepatocytes is to create aggregates by placing them in a petri dish whereby the healthy, nonapoptotic ones attach to each other. These aggregates can then be filtered through a series of 200 mm and 70 mm filters to retain only the healthy aggregates.

Figure 2: Liver digestion process and activation of apoptosis.

The dynamics of viability as a function of mortality type has not been performed for 3D alginate-encapsulated hepatocyte spheroids post-isolation. In this study, we will determine mortality rate in isolated hepatocytes from the time of encapsulation to 8 days post-isolation in a roller bottle culture, and compare this to the percent and distribution of hepatocytes dying by necrosis or apoptosis obtained from confocal microscopy.

METHODS

For this experiment, liver cells were taken from rats, or some other test subject, via liver perfusion. After isolating, the hepatocytes were then placed in an alginate solution (1:1 vol:vol), which was used to create the encapsulates. This solution was then pumped through a syringe, with a charge applied to the needle, into a Hank's buffered solution containing 150 mM calcium chloride. As the alginate was pumped through the syringe, a bead, containing hepatocytes, formed on the tip of the syringe before falling into the buffer solution as a spherical encapsulate. The charge on the needle was then manipulated to alter the encapsulate size. Using this method, encapsulates of 250 µm and 500 µm diameter were created. In addition to the encapsulates, a sample of co-cultured liver cells, including hepatocytes (aka PC) and nonparenchymal cells (aka NPC) was also created and mixed with the hepatocyte pellet in a 1:4 NPC-to-PC (vol:vol) ratio before encapsulation. 400 µL of gravity packed beads were then placed in 400 mL roller bottles containing 12 mL of culture medium (DMEM+0.2% BSA for PC +4% FBS – dexamethasone for NPCs) where they were kept for 10 days. The cell media was changed approximately every two days to feed cells and prevent contamination.
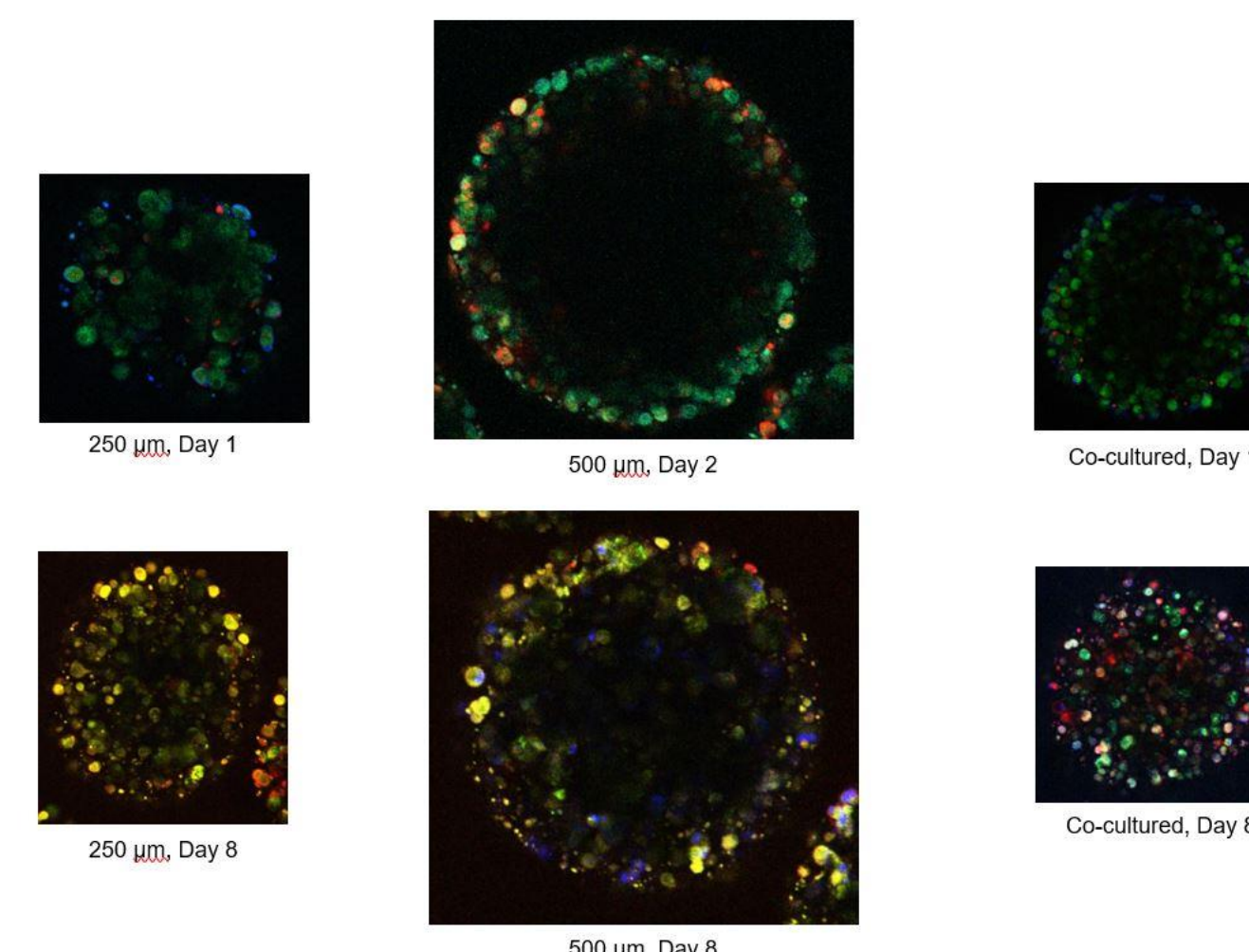
Every day for 8 days, 10 µL of each encapsulates were obtained and imaged on an Olympus Fluoview 1000 confocal microscope. This involved creating three dimensional images out to about 250 µm in thickness to render a three dimensional image. Due to the sizes of these images, only one representative slice will be presented for each sample. To image, an Annexin V imaging kit was used, to allow alive, dead, and apoptotic cells to be differentiated in the images. To use this kit, 10 µL of a sample were placed in 600 µL of Annexin V binding buffer. Next, 6 µL of both Annexin and propidium iodide were placed into the solution. The confocal microscopy scan was then run, taking slices with thicknesses between 3 µm and 6 µm.

The other portion of this experiment involved measuring the viability of the cells throughout the study. To do this, first the number of cells in each sample type needed to be determined. The cells were counted using a Life Technologies Automated cell counter, the Countess II. Then, cell viability could be tracked over time by monitoring the amount of LDH present in each sample. At every media change, 1 mL samples were taken from the culture media of the roller bottles containing 400 mL of 250 µm encapsulates, 500 µm encapsulates, and co-cultures. A Sigma Aldrich Tox 7 LDH assay kit was used in a 96 well format to analyse LDH levels in each sample. Each sample was diluted in a 1 to 3 ratio by combining 10 µL of the sample with 10 µL each of the three solutions in the kit (Substrate, Cofactor Preparation, and Dye solutions). The reactions were quenched after 30 minutes with the addition of 15 µL of 1M hydrochloric acid. The plates were then read by a 96 well plate reader to obtain the concentrations of LDH in each sample. In order to determine the number of dead cells represented by the amount of LDH present, a calibration curve was obtained using 10 ul and 20 uL of hepatocytes that were taken from the cell pellet and freeze thawed in same culture medium. This sample contained a known amount of dead cells. Therefore, by taking LDH readings at different dilutions, a calibration curve could be constructed, allowing for the number of dead cells in an unknown sample to be calculated using its LDH concentration. Then, using the dead cell amounts and the total cell amounts, percent viability could be calculated for each sample at each measuring point. The entire procedure above was repeated two times.

RESULTS

From previous studies, the maximum cell death appeared to be on day 1-2 and then mortality leveled off by day 5. *Figure 3* shows representative images from about 150 µm deep of the three types of encapsulates on day 1 and 8. By day 8, the cocultured encapsulates are the only ones with green and blue features (live cells), although there are many red features (dead cells) as well. The monoculture 250 µm and 500 µm encapsulates appear yellow, which is likely a mix between dead and apoptotic cells.

Figure 3: Sample Confocal Slices

250 µm, Day 1 — 500 µm, Day 2 — Co-cultured, Day 1 — 250 µm, Day 8 — 500 µm, Day 8 — Co-cultured, Day 8

These confocal microscopy images, in Figure 3, above, show encapsulates that are stained so that living cells are blue, dead cells are red, and green cells are apoptotic. The yellow cells are mixture of red and green cells, meaning that they are a combination of necrotic and apoptotic cells. The fact that there was less color in the 500 µm sample by day 8 means that the sample was mostly cell debris or dead. Each image depicts one slice taken from a stack of images for every encapsulate. In the 250 µm mono-culture sample and coculture sample, the amount of red and green is about even, on Day 1. However, by day 8, there were more dead cells in the mono-culture 250 µm sample than the coculture sample.

Table 1: Number of Cells per Milliliter of Cell Pellet

Cell Category	Number of Cells (per mL)
Countess Number- Live	1.43*10⁶
Countess Number- Dead	7.86*10⁵
Countess Number- Total	2.2*10⁶
Total After Accounting for 50x Dilution	1.1*10⁸
Total After Accounting for 0.5 mL Sample	5.5*10⁷

Table 1 gives the results of the cell counts taken from the PC pellet on day 1 using the Countess II cell counting instrument, which by using propidium iodine will give live/dead numbers. The viability was 65%, but by day 3 of storage in at 4°C, the viability was 88%, which was not predicted. The overall cell density of the pellet was 55 million PC per mL.

Table 2: Number of Cells per Milliliter of Gravity-Packed Encapsulates.

Sample Type	Number of Cells (per mL)
250 µm Encapsulates	1.7*10⁷
500 µm Encapsulates	2.3*10⁷
Co-cultured Liver Cells	7.5*10⁶

Table 2 gives the results of the cell counts taken from gravity packed encapsulates taken on day 1 using the Countess II cell counting instrument. Assuming a 1:1 mixture of 55 million PC per mL with alginate and a 64% optimal packing efficiency, the theoretical PC density per mL would be (55/2 x 0.64) 17.6 million cells per mL, which is nearly what the 250 µm diameter encapsulates reached.

LDH Concentrations Within Samples

Figure 4: LDH Concentration for Each Sample Over Time

Amount of LDH Present Over Time

Table 3: Amount of Dead Cells per Milliliter Using LDH

Sample Type	Day 1	Day 3	Day 5	Day 6	Day 8
250 µm Encapsulates	6.9*10⁵	5.6*10⁵	33000	1.0*10⁵	32000
500 µm Encapsulates	3.2*10⁷	1.8*10⁸	33000	2.6*10⁶	33000
Co-Culture	32000	59000	2.1*10⁵	66000	31000

The data shown in **Table 3** depicts the number of dead cells in each sample type at each medium change. This data was acquired by using a calibration curve, and converting the LDH amount in each sample to a value for the number of dead cells.
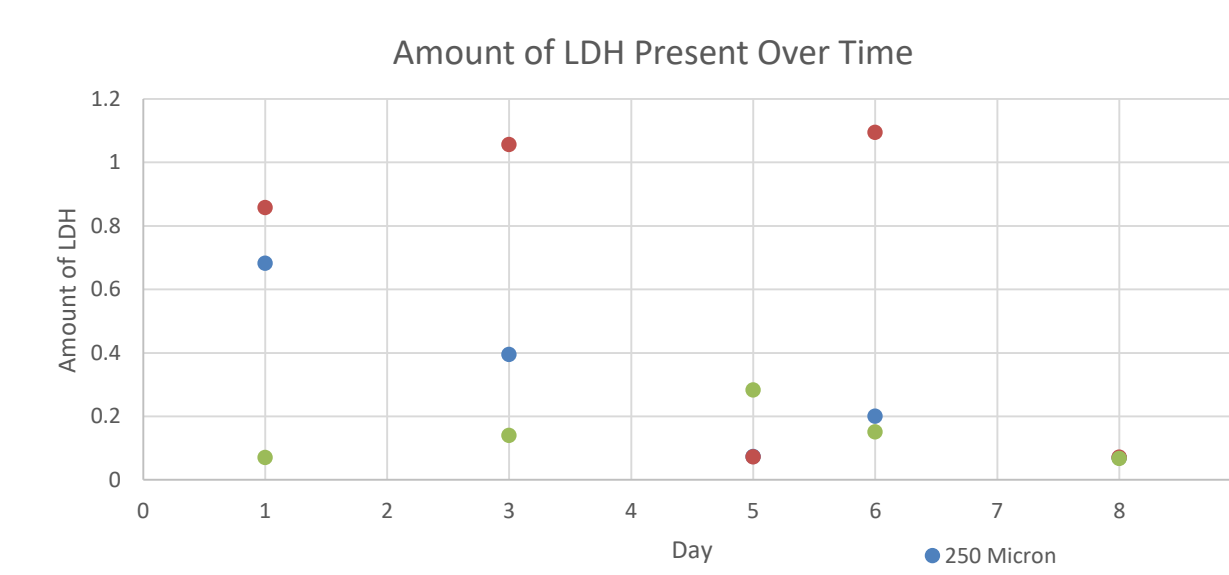
Figure 4 shows the amount of LDH present in each sample at each medium change, over the course of the experiment. The 500 µm encapsulates appear to have the highest fluctuations from day to day, with LDH measurements ranging from less than 0.1 up to about 1.1.

Table 4: Percent Viability Using LDH

Sample Type	Day 1	Day 3	Day 5	Day 6	Day 8
250 µm Encapsulates	58.1%	53.7%	53.5%	52.9%	52.7%
500 µm Encapsulates	-*	-*	-*	-*	-*
Co-Culture	99.6%	98.8%	96.0%	95.1%	94.7%

By tracking the amount of dead cells, the percent viability of each sample could be calculated, which is shown above, in **Table 4**. This was done by comparing the number of dead cells to the total number of cells in each sample. The values for the 500 µm samples fell outside of the calibration curve, and therefore, were not valid.

DISCUSSION

In observing the results, it is difficult to draw any strong conclusions from this experiment, however, certain parts did line up with the expected results. For instance, in viewing the confocal images in *Figure 3*, it can be observed that in each of the three sample types, there did appear to be a decrease in the number of blue, or alive, cells. On top of that, there was also a noticeable increase in green (apoptotic) cells, and red (dead) cells. *Table 1* shows the amount of cells found to be in a 1 mL sample of the cell pellet, which is significantly larger than the number of cells that were observed in each of the three experimental samples, who's values are displayed in *Table 2*. The cell pellet had over 3 times as many cells as the 250 µm encapsulates, over twice as many cells as the 500 µm encapsulates, and over 7 times as many cells as the co-cultured sample. The calibration curve constructed using dilutions from a sample of a known amount of dead cells appears fairly reasonable, since the concentration of LDH increases in a logarithmic manner as the number of dead cells increases. The data displayed in Figure 4, on the other hand, appears to lack consistency, as the concentration of LDH in every sample varies wildly from day to day. There are no apparent trends that can be made out from this graph. Using the calibration curve and LDH data, Table 3 shows the amount of dead cells that were calculated to be in each sample. As expected after observing Figure 4, the number of dead cells fluctuates greatly from day to day. There are even some instances where the number of dead cells for one day's sample is less than the number from the previous day's. Table 4, shows the percent viability of cells in each sample, using the LDH data. The results for the 250 µm sample and the coculture samples show a small decrease in the number of viable cells from day to day. The coculture cells appear to be more viable, however, as they remain above 90% viable for the entirety of the experiment, as opposed to the 250 µm samples which showed viabilities just about 50%. This may be due to the fact that the coculture samples contain other cells found in the liver, such as kupffer cells and other nonparenchymal cells. These other cell types may help to inhibit apoptosis after isolation, thus improving the vitality rate. The 500 µm samples did not produce reliable results. This is due to the fact that the amount of LDH in the samples was too high, thus maxing out the LDH reader, causing it to give inaccurate readings.

FUTURE RESEARCH

In future experiments, there are a few changes that can be made to the methods in order to improve the procedure. For starters, more dilutions could be used on the calibration curve in order to improve its accuracy. At the moment, only five points were used, meaning that there was a relatively high degree of error in using the curve to determine cell viability from LDH measurements. Also, further dilutions should be made in the 500 µm samples. The amount of LDH present in the samples from this study were too high for the LDH reader. Therefore, in order to get an accurate measurement, the samples must be diluted further before measuring.

Another addition to this experiment would include further analysis of the confocal images. Currently, the images obtained via confocal microscopy have been used in solely qualitative analysis. Quantifying the images, by obtaining cell counts for alive, necrotic, and apoptotic cells, could greatly increase the accuracy of this experiment. Because the coculture samples appeared to show much greater viability in comparison to the 250 µm monoculture sample (Table 3 and Table 4), further trials may be required to figure out the exact reason. As shown in Figure 1, oxygen availability should not be an issue in the 250 µm samples, therefore, the presence of the nonparenchymal cells must lead to an increase in viability of the hepatocytes after encapsulation.