UV-Photolithography Fabrication of Poly-Ethylene Glycol Hydrogels Encapsulated with Hepatocytes

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Abstract

The development of biomanufacturing technologies particularly, layered manufacturing has advanced cell encapsulation processes in an effort to mimic the cellular microenvironment for *in-vitro* studies. This paper illustrates an inexpensive UV-photolithographic method for encapsulation of human hepatocytes in three dimensional structures using poly-ethylene diacrylate (PEGDA) hydrogels as candidate substrates. In order to further develop this technology for layered fabrication, we have quantified the long-term effects of the photo-initiator concentration and UV light exposure on the metabolic rates of encapsulated human hepatocytes under a 21 day study. The photoinitator toxicity was observed immediately after polymerization with no significant cytotoxicity on a long term basis. A cellular viability is examined and reported for the UV photopolymerization process. Cell phenotype maintenance was observed by measuring the amount of urea produced over a 1 week time period. This photo encapsulation process may find use in the fabrication of spatially complex 3D scaffolds for tissue engineering applications, elucidation of the 3D structure-pharmacokinetic response relationship and the fabrication of complex multi-compartment liver tissue analog devices for drug screening applications.

1. Introduction

Cell encapsulated hydrogels are increasingly seen as a promising approach to engineer biological tissue for tissue regeneration and human pathophysiological study. These deformable, biocompatible, and hydrated gel environments provide a platform upon which cells can proliferate, aggregate, and be allowed to express the required tissue function. Recent advancements in manufacturing methods such as patterning and deposition technologies have enabled researchers to spatially control hydrogel architectural features through the use of soft lithography [1-3], photolithography [5-6] and solid freeform fabrication [7-9]. These techniques have produced patterned hydrogels with desired chemical and mechanical properties in biomimetic microenvironments. Soft lithographic techniques have produced feature sizes in ranges of 20μm and above but have limited 3D capability. Solid Freeform fabrication technologies enable the production of complex 3D hydrogels due to its CAD integration but are generally limited to a minimum of 100μm feature resolutions. Photolithographic methods have produced hydrogels by the direct exposure of the polymer solution to UV radiation to produce micro-patterned scaffolds with feature sizes in the range of 50μm or more. However, the photolithographic techniques have had limited capability in producing three dimensional scaffolds due to the lack of true three dimensional formation capabilities. With the development of the maskless projection photolithographic and micro-stereolithography, it is now possible to create complex three-dimensional scaffolds with defined architecture and minimum feature sizes

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on the order of 50μm [10,11]. The maskless projection photolithography has the capability of producing hydrogels with well-defined, repeatable and complex 3D features due to its integration with CAD systems. This automated technique can be extended to potentially create multi-material heterogeneous cell constructs.

Given the capability of spatial patterning of cells and the incorporation of growth factors using the photopolymerization process, it is important to understand the detrimental effects of the UV exposure and polymerization on biological molecules. These biological molecules can denature over time under harsh temperature environments. Hence for any viable biofabrication process using UV, the mechanical/chemical environments and their effect on living cells must be studied and optimized. Liu et al proposed a method of designing tissue constructs by three dimensional photopatterning of living cells, where the use of photolithographic techniques is implemented to broaden the capability of photopolymerizable PEG-based biomaterials to include structural features within the hydrogel network. This technique allows photoencapsulation in specified patterns controlled by a photomask of live mammalian cells. Their research has looked into the effect of UV exposure and photoinitiator concentration on both photopatterning resolution and cell viability. They used human hepatoma cell line for testing and observed that while under the exposure of UV light, significant cell death is negligible compared to the detrimental effects of the photo initiator concentration. Therefore, this may be a limitation for creating very small features on the order of few tens of microns, but nevertheless the achievable resolution is sufficient for producing complex 3-dimensional structures that vary on the same length scale of most tissues feature sizes [5].

Expanding on these studies, the objective of this paper is to study the combined effects of the photoinitiator and UV exposure on hepatocyte cell viability and maintenance in PEG based hydrogels on a long term basis. Cell viability is measured using Alamar blue assays and the cellular metabolic activities are quantified as a function of the concentration of photoinitiator and monomer concentration over time. Cell phenotype maintenance is quantified using urea assay kits as a maker of liver function to determine whether the liver cells maintained phenotype during the entire duration of the experiment. Section 2 of the paper describes the materials and methods used in this study followed by results obtained in this preliminary study in Section 3. Conclusions and future research prospects are discussed in Section 4.

2. Materials and Methods

2.1 PEGDA Solution and Photoinitiator

The preparation of the macromere solution and photoinitiator was based on a protocol previously described by Bhatia et al, 2002. Poly (ethylene) glycol (PEGDA) (Sigma, St. Louis, MO) was dissolved in HEPES solution to form PEGDA concentrations of 20%, 25%, 30% and 35% w/v. The photoinitiator solution was prepared by completely dissolving 2, 2-dimethoxy-2-phenyl-acetophenone (Sigma, St. Louis, MO) in 1-vinyl-2-pyrolidinone (100mg/mL). The photoinitiator solution was added to the pre-polymer PEGDA solution prior to UV exposure at concentrations of 0.6mg/mL, 0.9mg/mL and 1.2mg/mL.

2.2 Cell Maintenance

HEPG2 cells (ATCC, MA) were cultured in 75mL flasks and maintained in Dulbecco's modified Eagle's medium (DMEM, ATCC), supplemented with 10% (w/v) fetal bovine serum

and maintained in the incubator at 5% CO_2 and 37°C. The cells were mixed into the pre-polymer solution prior to UV-exposure and mixed gently to ensure uniform cell distribution. Hemocytometer readings measured the cell concentration at 2 million cells per mL.

2.3 Layered Photolithography Process

A fixture apparatus was designed for the photo-patterning of PEGDA hydrogels via exposure to UV irradiation (UVP BlakRay 100SP, 365nm). A square PDMS mold with dimensions of 10mm by 10mm and 750μm deep was prepared using soft-lithographic techniques. A single layer of pre-polymer solution was injected into the mold to form a 200um thick solution. The desired pattern was prepared using AutoCAD (Autodesk Inc) and printed on standard HP overhead transparencies using a high resolution office laser printer. The first layer was cross-linked by exposing the solution to UV light for 20 seconds at a working distance of 6in. A second layer of the pre-polymer solution was injected into the mold and the desired pattern transparency mask was placed over the solution. After 20 seconds of UV exposure, the printed mask was carefully removed and the PEG hydrogel removed from the mold. The hydrogel was washed with PBS to remove any uncrosslinked polymer solution.

2.4 Reaction Kinetics

The total energy released during the photopolymerization reaction is a function of UV light intensity, I_a, and the exposure time, t as given by (1).

$$E_{total} = I_a\, t \tag{1}$$

Assuming, that the UV light is incident upon the photo polymer and propagates into the polymer depth (x), the UV irradiation is absorbed by the photoinitiator and can be described by the Beer-Lambert law given by (2).

$$I_a = I_o e^{(-x/D_p)} \tag{2}$$

where I_0 describes the peak intensity of the UV irradiation on the surface of the polymer resin, x describes the depth of polymerization and D_p is the penetration depth of the polymer and defined as the depth within the resin where the irradiation drops to 1/e times the irradiation available at the surface (I_0). Assuming that the wavelength of the UV light and time of exposure remains constant, the depth of penetration depends on the amount of photoinitiator present in the solution. The free radicals released by the photoinitiator upon UV illumination are detrimental to the cellular membranes and are the primary cause of cellular death [5]. The exothermic crosslinking reaction increases the temperature of the polymer solution and must be controlled to ensure maximum viability of cells. Lower reaction kinetics is preferred to allow a gradual increase in temperature but must not be at the cost of prolonged UV exposure times. Hence the amounts of photoinitiator concentrations must be optimized to ensure proper gelation and maximum cellular viability during the process. For our experiments we have set a 20sec exposure time limit at 10mW/cm^2 maximum energy intensity.

2.5 Quantification of Metabolism using Alamar Blue Assay

To determine the effects of cell growth and proliferation on the different concentration of photoinitiator to be used for crosslinking, we have tested three different concentrations of 0.6, 0.9 and 1.2 mg/mL. Specifically, 6 samples of 115μl of 20% PEGDA in HEPES solution containing one concentration of photoinitiator and approximately 200,000cells/construct were

exposed to 365nm UV light for 20 seconds to crosslink. The final constructs were submerged in 500ul of DMEM nutrient rich medium, and the samples were maintained in a 37°C incubator. The samples were incubated for a span of 21 days, and the medium was changed periodically every 3 days to ensure sufficient nutrient supply to the cells. Throughout the period of study, Alamar Blue assays were performed at regular intervals to measure the cell metabolic activity. Live-Dead cell assay was performed immediately after cross-linking to assess qualitatively the detrimental effects of the cross-linking process.

2.6 Quantification of Urea Synthesis

A 7 day preliminary study was performed to investigate the differential hepatocyte-specific function of urea synthesis in the photopolymerized 3D PEG hydrogel encapsulated hepatocytes discs. The aim of this study was to evaluate the effects of the UV radiation and photoinitiator cytoxic effects on cellular functionality as a measure of hepatocyte-specific function, namely urea synthesis. Medium was collected every week and stored at -20C until the point of termination of the experiment (after 7 days). After collection of medium samples for urea analysis, the remaining medium was aspirated and the samples were submerged in fresh 500µl DMEM medium to remove all previous traces of urea content. At the end of the experiment, 5µl of the samples were mixed with the reagents of the urea analysis kit (BioAssay Systems, Hayward, CA). The samples were pipetted onto a 96well plate (Fisher Scientific) and the fluorescence reading was measured using a cytofluorometer (Tecan Inc, Durham, NC) equipped with a 492nm absorbance filter. The fluorescence reading was measured for each sample and was converted to the corresponding urea amount based on available urea standards.

3. Results and Discussion

Figure 1 shows the variety of patterns that can be created using transparency-based photolithography. Minimum feature sizes are in the range of 80um and lower feature sizes can be obtained using high resolution printers with glass masks. Figure 2 depicts the uniform dispersal of cells during encapsulation and eventual photopolymerization. Due to the use of non-collimated light, the UV light irradiation on the polymer surface is not uniform and hence can result in substantial errors in feature resolution. The feature resolution and uniformity can be improved through the use of optics.

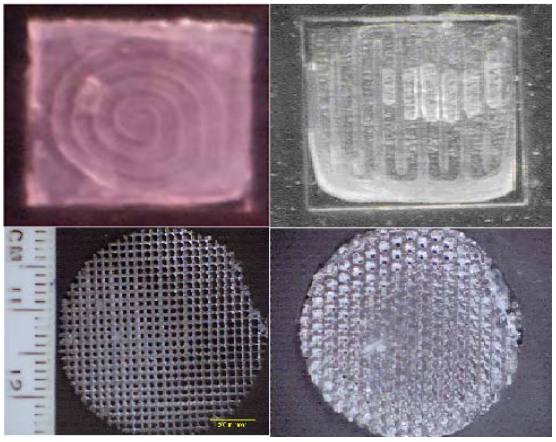


Figure 1: Patterned PEG hydrogel through UV photolithography process

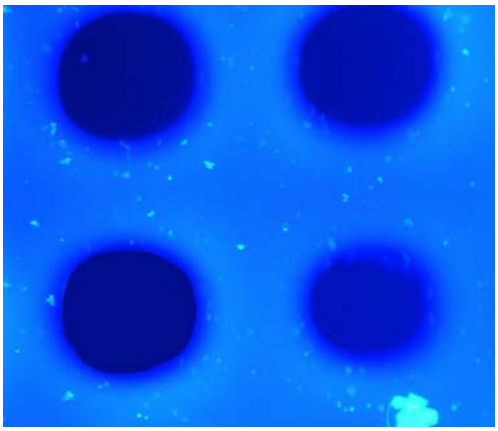


Figure 2: Biz-benzamide staining of encapsulated hepatocytes in PEG hydrogel

3.1 Effects of Photoinitiator concentration on Cell Viability

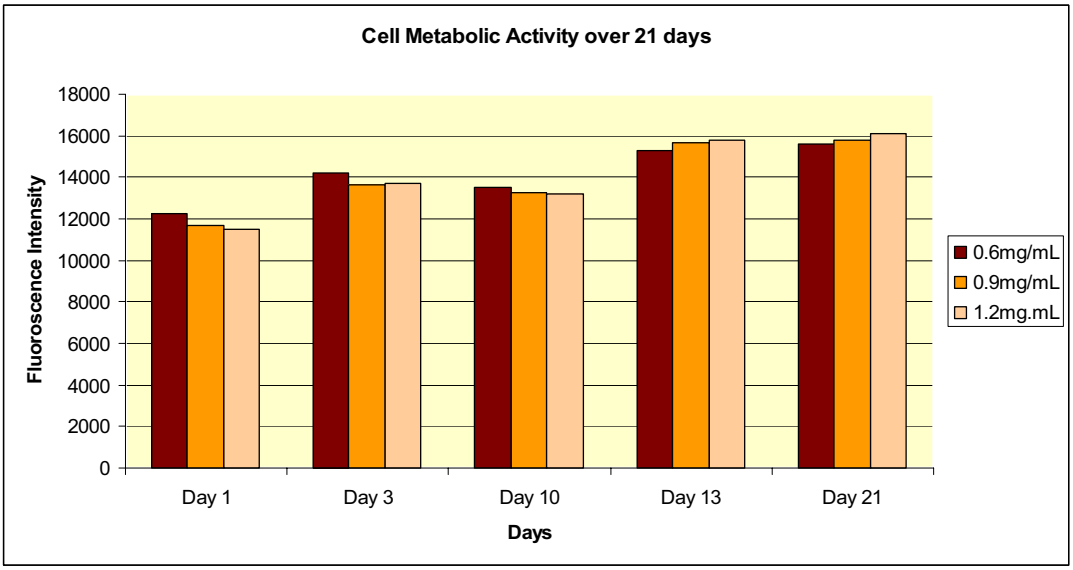
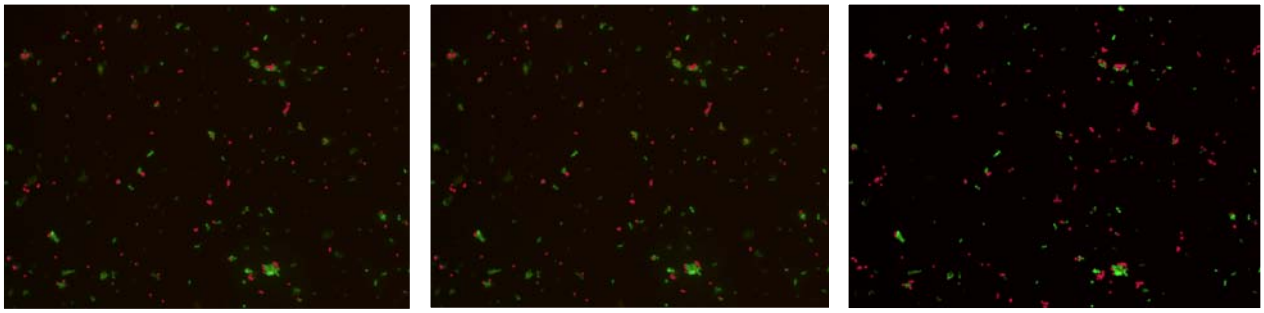


Figure 3: Cellular Metabolic Activity over a period of 21 days for the hydrogels photopolymerized under different photoinitiator concentrations

Figure 3 indicates the proliferation readings from the different samples tested. Each data point is an average of 3 samples taken over a period of 21 days. Samples from all concentrations showed low rates of metabolic activity during the first 3 days but picked up slightly for the rest of the experiment. On Day 1, there were slight differences in fluorescence intensity relative to the amount of photoinitiator used. As expected, concentrations of 0.6mg/ml and 1.2mg/ml showed the highest and least intensity respectively. Due to the high concentration of the photoinitiator, the reaction kinetics dictates a rapid increase in temperature during the exothermic reaction culminating in cell death



(a)0.6mg/mL (b)0.9mg/mL (c)1.2mg/mL

Figure 4: Live/Dead Qualitative Assessment of Cellular Viability During Photopolymerization

A qualitative assessment of cellular viability during the photopolymerization process with various photoinitiator concentrations was performed using Live/Dead Assay kits (Molecular Probes Inc) at Day 0. A two-color fluorescence viability assay (L-3224; Molecular Probes) was used to demonstrate the viability of encapsulated hepatocytes in various photoinitiator

concentrations. Immediately after polymerization (Day 0), the hydrogels were incubated for 60min in a combined 2 mM calcein AM and 2 mM ethidium homodimer solution at a 1:4 volumetric ratio in sterile phosphate-buffered saline. The calcein AM lights up the live cells as green (excitation, 495 nm; emission, 515 nm), while the ethidium homodimer stains the dead cells as red fluorescence (excitation, 495 nm; emission, 635 nm). The samples were viewed in a Leica fluorescence microscope under 10X magnification. Figure 4(a) qualitatively shows that the lowest concentration of photoinitiator in the polymer solution produces better cellular viability ratios. This is also confirmed by the Alamar blue tests shown in Figure 3. To achieve similar levels of cross-linking gelation, higher concentration of photoinitiator in the pre-polymer solution need reduced UV exposure time. The reduced UV exposure time on a layer by layer basis leads to faster fabrication times. However, it has been reported by Liu et al. that the cell viability was mostly affected by the concentration of the photoinitiator in the solution than the exposure to UV radiation. Still, the long term affects of the UV irradiation on cellular function are yet to be studied.

In Figure 5, urea synthesis is quantitatively detected with a cell-based colorimetric assay, allowing comparison of time-dependent phenotype for cell-encapsulated hepatocytes. Cell medium was replenished after every reading to remove all previous traces of urea from the well plate. Each data point is an average of 4 samples taken over a 7 day time period. This data suggests that hepatocytes encapsulated in a 3D PEGDA hydrogel tissue construct do synthesize considerable urea amounts during a 7 day time period to an observed maximum of up to 4mg/dl. The amount of urea content present in the medium significantly increased over the studied time period. However, there is no significant difference in urea amounts among the different hydrogels produced with different photoinitiator concentrations. Lower amounts of urea on the first day indicate that not all cells have recovered from the photopolymerization process. The amounts of urea increase in the remaining days attributed to the increased cellular activity of the liver cells within the hydrogel.

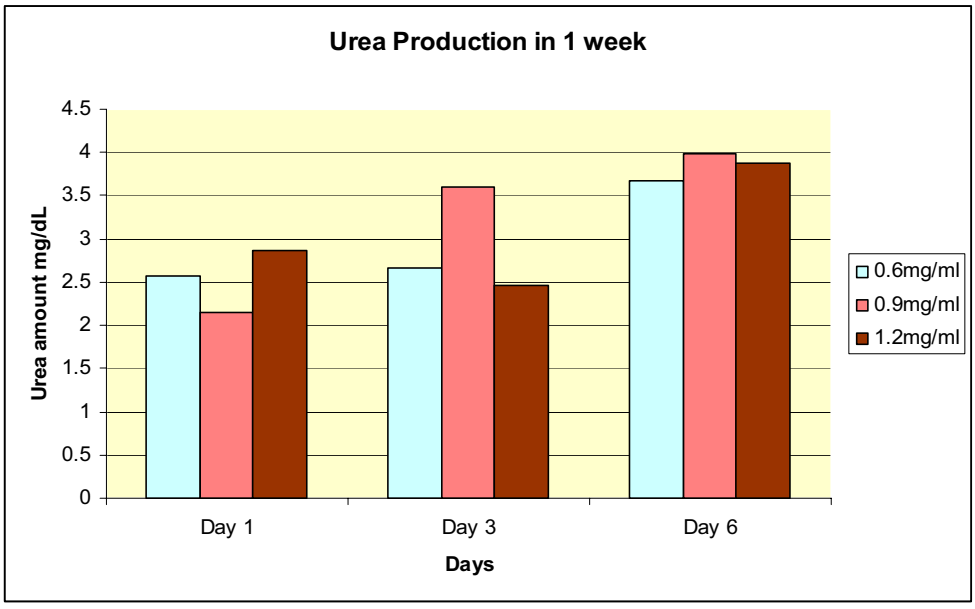


Figure 5: Urea Production for PEG hydrogels photopolymerized under different photoinitiator concentrations of 0.6mg/ml, 0.9mg/ml and 1.2mg/ml

3.2 Effect of PEGDA concentration on Cell Viability

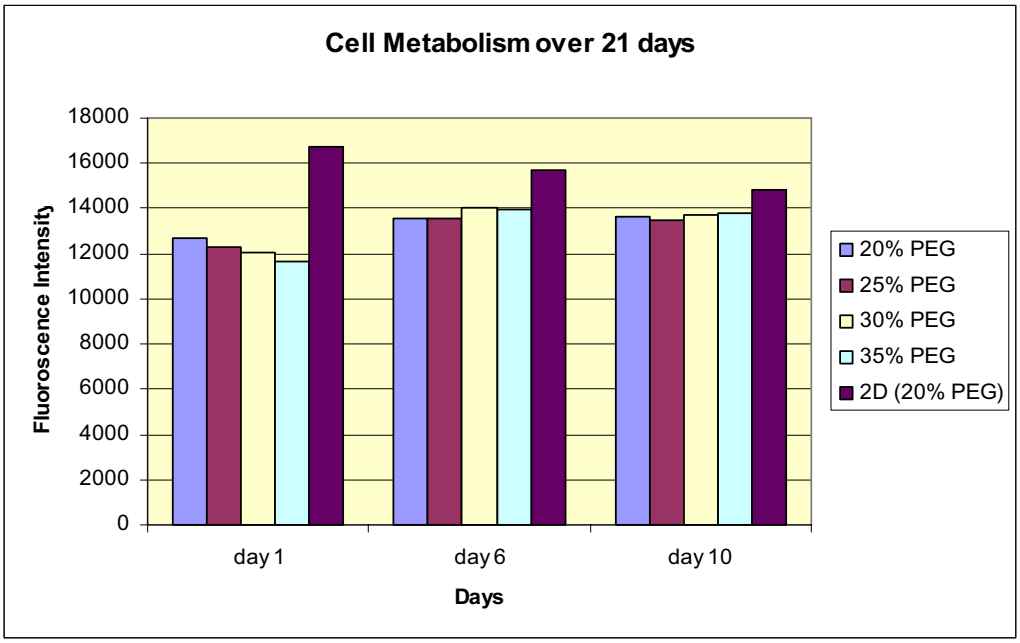


Figure 6: Cell Metabolism as a function of PEGDA concentration.

The effect of the PEGDA concentration in relation to cell viability has also been studied. Four different concentrations of 20%, 25%, 30% and 35% PEGDA solution samples were photopolymerized and encapsulated with liver cells. The same amount of pre-polymer solution and cell density used for photopolymerization was plated onto a cell culture plate and used as a control in this study. The hydrogels were studied over a period of 10 days for cellular metabolic activity using the standard Alamar blue assay test. It is observed that the rate of cellular metabolism did not have any significant differences between the different PEGDA concentrations over a period of 10 days. On Day 1, after the photopolymerization process, about 75% of the cells survived the process. After 10days, the reduction in intensity for the control samples is attributed to the continuous change of medium that may have reduced the number of cells adhering to the well plate.

4 Conclusions

The growth of tissue engineering technology research have spawned the growth of cellular patterning and encapsulation strategies for applications in drug discovery and testing. By encapsulating living mammalian cells in three dimensional hydrogels, it is envisioned that the cellular microenvironment can be reproduced to simulate *in-vivo* conditions in the laboratory. In this paper we have used the photolithographic process to encapsulate liver cells within a PEG hydrogel network expanding on work that has been previously studied. We have found that the detrimental effects of the photointiator and the UV exposure are observed soon after the photopolymerization process. The metabolic activity accelerates after 3 days of culture, indicating that the cells have survived the process. Since we have used a low molecular weight PEG (0.374KDa) in the process, the liver cells are encapsulated within the PEG chains and have limited mobility within the hydrogel. This is observed by the relatively low increase in metabolic

activity over the 21 day period of study. We speculate that the use of high molecular weight PEG monomers will enable the rapid growth of cells within the engineered microenvironment. It is also concluded that the varying concentrations of the photoinitiator had no significant effect on the cellular metabolic activity soon after the process. Cell viability ratio of 75% is observed immediately after the photopolymerization process. The death of the cells is primarily caused by the release of free radicals during the polymerization process which attack cell membranes leading to their death. Furthermore, urea analysis assays demonstrated phenotype maintenance for a period of 1 week without the addition of any liver-specific growth factors or proteins. This is attributed to two reasons namely, the provision of a three dimensional matrix of the encapsulated cells and the high density packing of the cells within the hydrogel enabling them to form aggregrates or spheroids. Future studies will be undertaken to include more tests for liver-specific function such as the albumin production rate capacity and drug metabolic function of the encapsulated liver cells.

Over the last four years, the development of integrated devices that combine cell culture and microfabrication makes the possibility of commercial applications to pharmaceutical evaluation a real possibility. By combining the advantages offered by microfluidic devices with cell encapsulated hydrogel matrices, an *in-vitro* system may serve to improve our ability to predict animal and human response to drug and chemical exposure [12]. The advances made in layered biomanufacturing can aid in the development of 3D matrices that serve as a man-made extra-cellular network. The photopolymerization process can easily be integrated to a viable biocompatible manufacturing process through the use of the Texas Instruments DMD™ technology [13]. The advantages offered by the DMD technology are threefold: 1) Reduced layered curing time, making the process relatively faster when compared with the SLA. 2) No requirement for the use of complex tool path patterns such as in the SLA with only a binary image is required for an instant polymer exposure to produce the desired pattern, and 3) Ease of producing complex 3D patterns that include gradient material structures and multi-material tissue constructs. Ongoing research and study are being done to enhance the feasibility of 3-dimensional structural formation with reproducibility, integration of the polymerized cell construct onto a microfluidic device with no leakage, improved cell viability and maintenance of cell-specific function.

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