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Functionalization of Chitosan Based Microparticles for In Vitro 3D Culture of Human Liver Cells

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Abstract

Previous work involving 3D culture of human liver cells with fluorinated chitosan based microparticles has shown that their incorporation provides needed structural cues that the culture of cells alone does not provide, such as increased gas transport. However, using bare microparticles to grow large 3D cellular structures is not practical as they tend to collapse before any meaningful research can be done on them. It is proposed that this is because of the lack of extracellular matrix (ECM)components within pure cell culture *in vitro*, which in their absence cannot adequately facilitate biochemical communication and adhesion between cells. It is possible to utilize protein linkage techniques, namely carbodiimide chemistry, to immobilize proteins derived from the liver cellular environment to the surface of these microparticles and grow stable 3D cell aggregates known as spheroids.

I hypothesize that the surface functionalization of fluorinated methacrylamide chitosan (MACF) microparticles with liver ECM adhesive ligands and their culture with liver cells will increase formation and biological function of spheroids. It was found that through the modification of MACF microparticles with native extracellular matrix proteins, particularly variations of laminin native to the *in vivo* liver environment, the overall functionality of cells increased over a 5 day culture time, as shown by higher levels of both albumin and urea secretion when compared to controls ($p<0.05$).

1 Introduction

The liver is an important organ to study in clinical trials, as adverse effects by new drugs can cause serious bodily harm due to the wide range of functions the organ serves. Because of the great risk involved with testing these new pharmaceuticals on human test subjects, commonly

preclinical studies involve the use of animals to assess the effects of developed drugs, but obviously the liver of an animal model is not anatomically and physiologically identical to that of a human. It is difficult to extrapolate that a drug found to be safe and effective in animal test subjects will yield the same results in a later human trial due to interspecies differences. The use of animals in medical testing brings about ethical concerns as well, as the inhumane treatment of animals in the lab is an issue that has been hotly debated for decades. These issues could be minimized using human-based cell model studies that can be done quicker and cheaper than animal studies.

3D cell aggregates or spheroids are expanding in popularity especially as a platform to study human disease and drug development, these models can be made using immortalized cell lines, tissues, and primary cells from the species of interest, which aim to replicate an *in vivo* environment.[1] Early models of these cell systems were typically small in size, which although easy to control and culture, do not provide a great replication of *in vivo* environment. These simplistic models are typically comprised of single cell-lines found within the organ of interest, and when grown in 3D setting do not include ECM components.[1] Studies of *in vitro* cell aggregates have shown that spheroid formation is a multi-phase process which can initiate without the presence of ECM. Upon initial contact between cells adhesion is mediated by specific molecules such as cadherins and cell receptor binding interactions, and it is not until this initial aggregation that ECM components begin to form complex structures with themselves and cytoskeleton proteins which allow for a more tightly packed structure.[2] Further advances in this technique led to the development of synthetic cellular environments for 3D cell culture methods to capture and maintain specific functionalities in vitro which is a key priority in the field.[1,2]

While liver cell spheroids are understood to accurately represent *in vitro* conditions, the main two challenges involved with this technique currently are limited mass transport and lack of biochemical cues in the culture environment. Within the body, cells contain extracellular matrix components which are vital to tissue assembly and architecture and promote a number of important cell functions such as adhesion, migration, differentiation, and proliferation. However, 3D microtissue models do not effectively maintain these extracellular matrix components by themselves.[3] Within the human body, the liver is not made up of a single cell type, but of many which perform different functions and can be classified in to 2 major groups, parenchymal and non-parenchymal cell types. Parenchymal cells typically perform major cell functions and make up the majority of the total cells within the population of the organ, while non-parenchymal cells usually serve more regulatory roles, assisting in functions of the major cell lines and maintaining a viable environment within the organ.[4] Because of the vast number of cell lines present in an *in* vivo environment, it would be a near impossible task to completely recreate the native human liver environment within a well plate due to sophistication of the organ and limited accessibility to cell cultures. However, by mixing a few key native cell lines a three dimensional cell culture with enough similarity *in vitro* can be achieved that meaningful experimental results may be obtained through their use.

In vivo, the liver is highly vascularized which readily supplies oxygen and nutrients. However, with regard to *in vitro* spheroids, the structure tends to fall apart after extended culture times because cells at the innermost area of the sphere do not receive sufficient oxygen and nutrient delivery by diffusion, especially as spheroid size and density increase. This leads to decreased chances of extended culture needed for clinical drug trials.[5] One way to combat this issue is by using synthetic oxygenating biomaterials based on fluorinated chitosan.

Chitosan is the second most abundant polysaccharide after cellulose that can be produced from crustacean, squid and insects. The presence of protonatable amino groups in chitosan as well as structural similarity to glycosaminoglycans (GAGs) are two important properties of the material. However, very low solubility of chitosan in water due to its strong hydrogen bonding hampers its wide application especially in spheroid formation.[6] Chitosan can be blended with other polymers to further improve the physiochemical properties with the aim of resembling native tissue as well as increasing its solubility.[6] Methacrylic anhydride is a commonly used methcacrylating agent that has been utilized to modify chemical structure of various natural polymers including chitosan. Methacrylated chitosan, a water-soluble derivative, is produced by coupling methacrylate group to the polymer backbone. In addition, methacrylate groups are the most accepted photopolymerizable groups to produce photo-crosslinking hydrogels for drug delivery and tissue engineering.[7] The Leipzig group has extended the biomedical usefulness of chitosan via the creation of fluorinated methacrylated chitosan or MACF, which is synthesized via nucleophilic substitution reaction where fluorine groups are conjugated to the free amines of chitosan as shown in Figure 1.

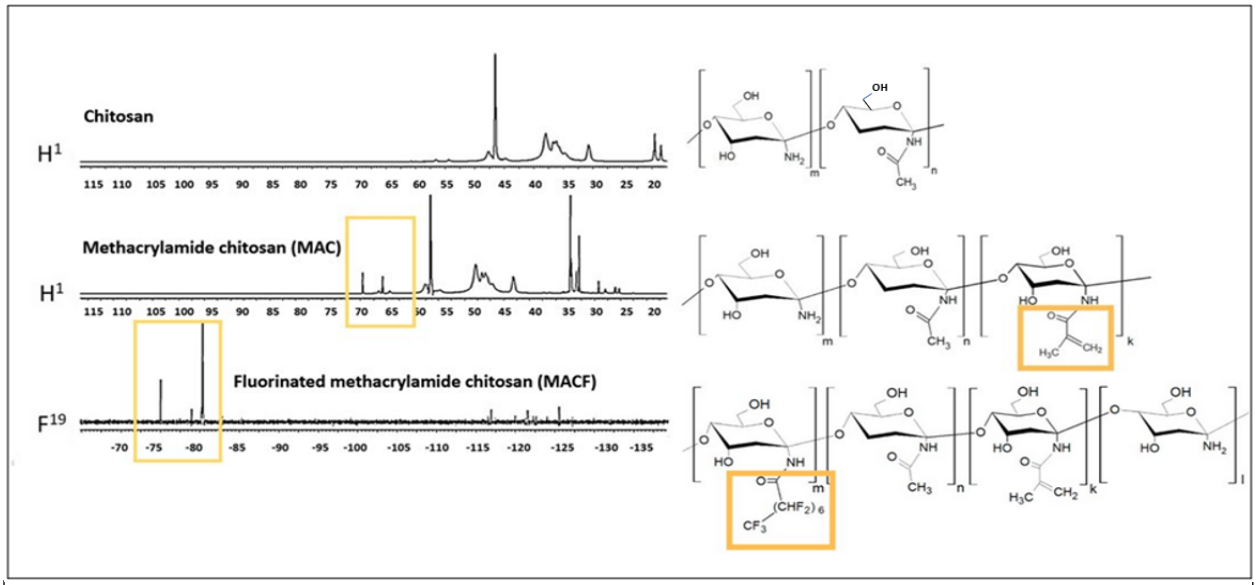

Figure 1: Chemical modification of chitosan using methacrylic anhydride and PFC.

Furthermore, they were able to show that the type and size of PFC substitution affected oxygen transport properties. *In vitro* studies conducted in this lab on MACF hydrogels have shown beneficial results, including showing that MACF can be saturated and release oxygen to improve cellular functions *in vitro* using fibroblast cells.[8] Furthermore, *in vivo* studies conducted in a rat and pig excisional wound healing model showed that oxygenating hydrogel sheets of MACF improved re-epithelization, collagen metabolism and deposition.[8]

More recent work done by the Leipzig group suggests that oxygenating fluorinated microparticles can be beneficial in decreasing mass transfer limitations, able to assist in some of the functions of vasculature in vivo.[9] In a recent study, perfluorocarbon (PFCs) -conjugated chitosan-based microgels (~20 mm diameter) added during spheroid agglomeration drive oxygen transport internally. Highly electronegative fluorine groups in PFCs dissolve O_2 according to Henry's law. Because there is no chemical bond between O_2 and PFC molecules, this allows for the efficient release of gases when sinks (e.g., metabolically active cells) are present, thereby enhancing the gradient/driving force for oxygen transport.

In their next attempt, the Leipzig lab incorporated the fluorinated microparticles into liver cell spheroids. In this subsequent work done, they found that providing only physical cues, such as increasing gas transport via fluorinated microparticles, is not enough for the culture of large and long-lasting liver spheroids due to lack of suitable ECM. It was shown that spheroid integrity and viability tended to start to deteriorate after about 5 days. This was shown through simple light microscopic techniques where spheroid could be visibly seen breaking apart.[5] Therefore, they hypothesized that by modifying microparticle surfaces with proteins to closer mimic native environmental conditions, cell spheroids would grow more successfully. In this study, we used heterogenous population of immortalized human hepatoma (HepG2) as parenchymal cell and

hepatic stellate cells (HSCs) as non-parenchymal cell (at an initial cell ratio of 4:1 HepG2s to HSC) to form the liver model which has been shown to produce suitable spheroids.[10]

The proteins of interest for the current study were ones which are known ECM components that affect signaling processes and tissue structures *in vivo*. Particularly fibronectin and laminin were of interest to be studied, both are critical in many functions within the liver extracellular matrix, but most importantly in cell adhesion and growth.[11,12] Both are known to be important basement membrane proteins which provide a structural layer for cells to grow as well as aid in cell signaling. It has been shown in previous studies that laminin plays an important role in the regeneration of liver tissue, as wound samples in mice show elevated laminin levels in areas currently undergoing proliferation.[13] Specifically, two variations of laminin, laminin-511 (LM-511) and laminin-521 (LM-521), have been demonstrated as effective growth substrates for liver cell culture.[14,15] Similarly, matrices incorporating fibronectin have been shown to increase adhesion in studies using rat hepatocytes.[16] In theory the growth of hepatic liver cells amongst microparticles which exhibit these scaffolding proteins may increase viability of the overall spheroid.

One benchmark used to assess spheroid viability was shown through assay analysis of key enzymes and proteins produced by growing spheroids, particularly albumin and urea. Albumin is the most plentiful protein found in human blood plasma, which is produced in the liver, and detecting the levels of this important enzyme is helpful in culture as it can be an indicator of the viability of cells.[17] Similarly, urea production can be measured as a means of tracking the health of cells, as urea is the compound produced when cells remove toxic ammonia from the *in vitro* environment.[18] Analysis of these analytes provides a more quantitative method for determining spheroid viability as biochemical assays for them are commercially available.

In this study, we coated MACF microparticles with different conjugated ECM proteins. After confirming and quantifying the attachment via BCA kit, we produced liver spheroids using u-bottom well plate and force aggregation using centrifuge. We studied morphology and protein secretion from the spheroids in 5 days and found that this technique has the ability to direct the cellular response by means of ECM ligands. By taking the next steps in order to functionalize these particles to mimic the native environment more closely, the hope is that cell adhesion and proliferation will be more successful bringing the *in vitro* model closer to *in vivo* conditions for longer periods of time. If this is true, this technique could eventually be implemented alongside or replace testing of new pharmaceutical products which involve animals, leading to better estimations of drug side effects and efficiency before going to *in vivo* human trials.

2 Materials and Methods

2.1 Microparticle synthesis and surface functionalization

MACF MPs were synthesized using a T-shaped PDMS droplet generator as previously described.[10] Briefly, using oil (span80, Sigma Aldrich, cat# 1338-43-8) as a continuous phase in the two side channels and polymer solution as the dispersed phase in the middle channel, we produced a steady stream of homogenous MACF MPs. These MPs were passed through a glass tube and polymerized under the irradiation of red lamp (600-700 nm) since the photo initiator Lithium phenyl-2,4,6-trimethylbenzoylphosphinate **(**LAP, 0.1wt% in distilled water, Sigma-Aldrich, cat# 85073-19-4) was included. The protocol for covalent grafting of proteins on MACF MPs was similar to that reported in previous studies.[10] The surface activation was achieved by dispersing the MACF MPs for 1 h at room temperature in a solution containing 2mM *N*-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma Aldrich, cat# E7750) and 5mM N-hydroxysuccinimide esters (NHS) (Sigma Aldrich, cat #130672) in 0.1M 2-(N-morpholino)

ethanesulfonic acid hemisodium salt (MES) buffer (Sigma Aldrich, cat#M3671). Afterwards, 10μg/ml of each respective ECM protein solutions were added to the particles to react with the amino groups available on the surfaces. The mixture was kept under mild shaking for 3 h at RT and dialyzed for 3 days using SnakeSkin™ Dialysis Tubing (3.5K MWCO, Fisher scientific, cat#PI68035) against distilled water and then freeze-dried. The amount of protein conjugated to MPs was measured by the standard Bicinchoninic acid (BCA) assay (Thermofisher, cat# PI23235) for proteins.

2.2 Preparation of liver spheroids incorporated with functionalized MACF MPs

HepG2 and HSC cell lines were used for *in vitro* experimentation. The media for expansion of HepG2 cells was Dulbecco's Modified Eagle's Medium - low glucose, with L-glutamine, and sodium bicarbonate (DMEM; Sigma Aldrich, cat#D6046) supplemented with fetal bovine serum (10%v/v, FBS, R&D system, cat# S11150) and penicillin/streptomycin (1%v/v, P/S, Thermofisher, cat# 15070-063). For HSC cells, DMEM with high glucose containing L-glutamine, sodium pyruvate, and sodium bicarbonate (Sigma Aldrich, cat#D6029) was used. For the fabrication of cell spheroids with MPs, HepG2, and HSC cells were mixed in 4:1 ratio and then added at a certain cell to particle ratio (150:1) and transferred to the 96-well cell repellant plates (CELLSTAR® Plates with Cell-Repellent Surface, cat#: 650970) at a seeding density of 20k/well. In this paper the letter “k” refers to multiples of 1000. Cells were overlaid with a serum-free and defined hepatocyte culture media (Corning®, cat# 355056). Using a centrifuge, the well plate was spun at 300 × *g* for 5 min, forcing cells to move to the bottom of the well to form cell aggregates. Morphology of spheroids were monitored according to size and compactness for up to 5 days using brightfield microscope (Objective: 4X).

2.3 Functional characterization of liver spheroids

Levels of albumin were determined using a commercially available kit (QuantiChrom™ BCP Albumin, BioAssay Systems, cat# DIAP-250,). The experiment was performed according to the manufacturer's instructions. Briefly, the medium was collected on days 1 and 5. A 20 µl aliquot was used to measure albumin secretion in the medium. 200 µL of working reagent was added and after 5 min incubation at room temperature, the optical density at 590-630 nm (peak absorbance at 610 nm) was read by a plate reader. Urea was also measured by QuantiChrom™ Urea Assay Kit (BioAssay Systems, cat# DIUR-100,) using a similar procedure. 5 µL of sample was mixed with 200 µL of working reagent, incubated for 20 min, and absorbance was read at 520 nm by plate reader.

2.4 Statistical Analysis

All findings were confirmed by a minimum of 3 independent experiments (3 spheroids per condition). Data processing and displaying were performed using GraphPad Prism 5 (GraphPad software, USA). Mean and standard deviation were displayed for all data sets. Statistical significances were determined using either Student's t-test or ANOVA followed by a Tukey's pairwise post hoc test ($p < 0.05$). Groups with significant differences were shown with alphabetic letters to signify ranking of means. The groups indicated with the same letters were not found to be significantly different.

3 Results and Discussion

3.1 Cell Lines Used in Culture

As mentioned previously, a plethora of native cell types are needed *in vivo* for the liver to complete its functions, however, the incorporation of all of these cell lines into an *in vitro* model is simply not feasible or controllable for practical experimentation. In this study two

immortalized human liver cell lines were mixed together. HepG2 cells are an immortalized hepatic cell line originally isolated from the cancerous liver tissue of a 15-year-old Caucasian male yet are nontumorigenic. They are characterized by their high proliferation rates but are known to express lower levels of metabolism than typical hepatocytes due to phenotypic changes attributed to their cancerous origin.[18,19] HepG2 cells serve as a parenchymal cell model, accounting for the majority of the makeup in liver cell culture. Cultured alongside the HepG2 cells in this study were hepatic stellate cells (HSC) obtained through a partnership with The Cleveland Clinic, a non-parenchymal cell line known to promote cell adhesion and support vital organ functions.[20] For the purposes of this experiment, any cell culture mixtures made in preparation for spheroid aggregation were done in a 4 HepG2: 1 HSC ratio to simulate the ratio of parenchymal: non-parenchymal cells found *in vivo*.

3.2 Microparticle Formulation

Microparticles were developed using a procedure from Monsouri et al., 2022, where a T-shaped microfluidic emulsifier was used to form fluorinated methacrylamide chitosan (MACF) microparticles. Chitosan which is the basis polymer for the synthesis of MACF, is a polycationic polymer that has two hydroxyl groups in the repeating glycosidic residue and one amino group. The primary amino groups present on the polymer backbone provide reactive sites for a variety of side-group attachments by employing only mild reaction. Surfactant (span80) is used as a continuous phase in the two side channels, and polymer solution as the dispersed phase in the middle channel. Resultant microparticles (~20μm in diameter) were washed and dried before modification.

3.3 Microparticle Modification

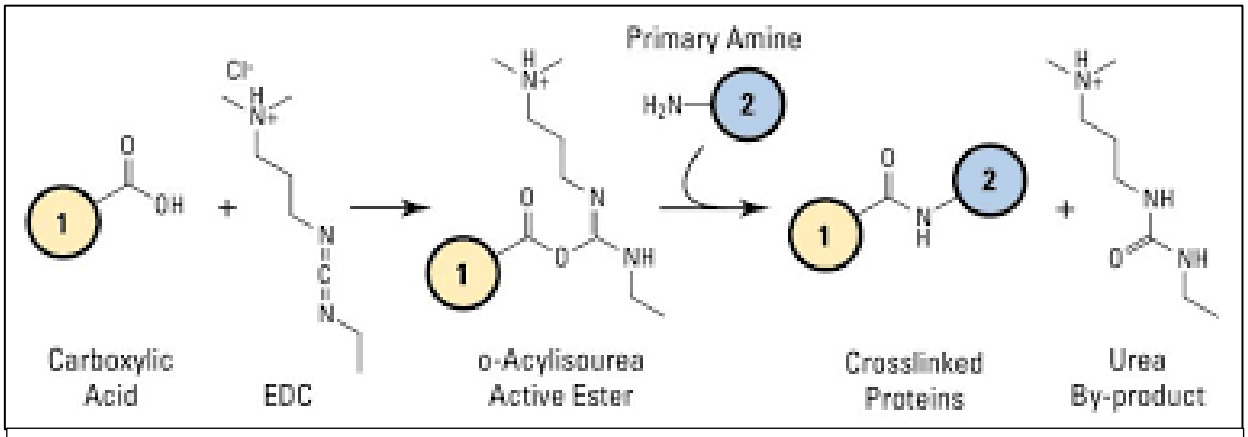

Figure 2 EDC/NHS reaction depicting the crosslinking of two proteins by means of peptide bond. Free amine groups on the surface of microparticles were utilized to achieve the same effect to modify microparticles with proteins of interest, native to the human liver ECM. [21]

Using the coupling reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxy succinimide (NHS), the carboxyl group of the proteins of interest can be readily attached to the amine groups of the chitosan backbone of the microparticles, immobilizing them as seen in Figure 2.[21,22] No previous work was found using the EDC/NHS coupling method for fluorinated chitosan microparticles, so two experimental groups were created to compare subsequent levels protein immobilization by the following procedure:

	Experimental Group 1	Experimental Group 2
EDC Concentration	2mM	24mM
NHS Concentration	5mM	70mM

Table 1 Experimental concentrations tested in EDC/NHS reaction to determine optimal conditions for protein crosslinking and immobilization.

Dispersed MACF microparticles were prepared by sonification in 0.1M MES buffer (pH=4.5-5) at a concentration of 10mg/ml. 200mM stock solution of EDC was made by

combining 19.2mg EDC to 500uL Milli-Q water. Stock solution of Sulfo-NHS was made by adding 21.7mg of Sulfo-NHS to 500uL of activation/coupling buffer resulting in a 200mM concentration. Two experimental conditions were tested, in one reaction 2mM EDC and 5mM NHS were added to the dispersed microparticles, and in the second 24mM EDC and 70mM NHS. In both experimental groups the reaction was allowed to proceed for 30 minutes with shaking before microparticles were dispersed in 1mL fresh PBS (pH=7.4) and protein of interest was added at a concentration of 10µg/mL. Reaction proceeded on shaker for 4 hours. After analysis using BCA assay with bovine serum albumin (BSA) as a control, it was determined that the experimental concentrations of 2mM EDC and 5mM NHS were more efficient in functionalizing microparticles with protein of interest and it resulted in more conjugation of proteins to the surface of microparticles ($p < 0.05$). Therefore group 1 was selected going forward when modifying microparticles. Microparticles were dialyzed after modification against DI water for 3 days and dried.

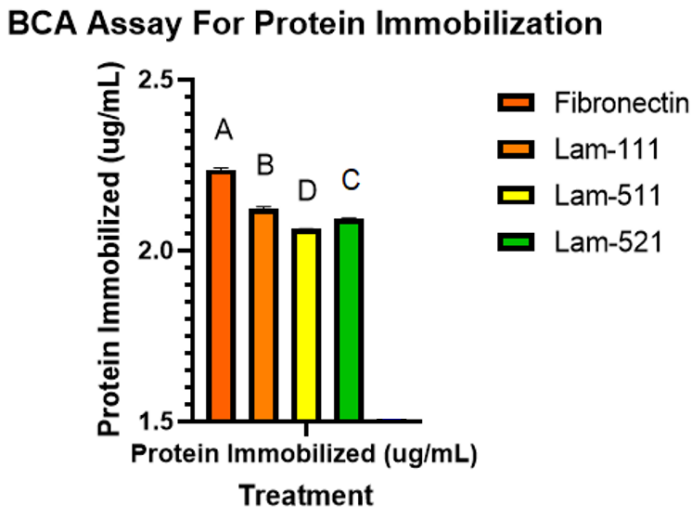

Figure 3 Results standard colorimetric BCA assay which represents concentration of each protein immobilized on MACF microparticles through the use of EDC/NHS reaction (n=3). Groups with significant differences were shown with alphabetic letters to signify ranking of means.

Proteins of interest tested were laminin-511, laminin-521, laminin-111, and fibronectin. Laminin-111 was used to represent a variation of laminin not found abundantly in the liver to test if the presence of general ECM components itself is enough to promote cell-cell adhesion or if tissue specificity of proteins used for modification also plays a role in spheroid viability. To determine a baseline for how well the EDC/NHS process immobilized these proteins onto microparticles, a coupling reaction was run using the determined most efficient conditions and subsequent protein immobilization was analyzed using standard BCA procedure. The experimental group that saw the greatest attachment through the EDC/NHS coupling reaction were those microparticles modified with fibronectin as shown in Figure 3. Because all proteins were added to the reaction mixture at a concentration of 10μg/mL, in the best case of this experiment (fibronectin) a percent yield of 22.4% was immobilized on the microparticles, and in the worst case of laminin-521 a percent yield of 20.6% was achieved. Although these numbers may be lower than initially expected, it is shown that at the conditions used each protein was attached with relative consistency, and even at these small concentrations the supplemented ECM proteins may be enough to promote cell-microparticle adhesion thus increasing viability of the overall culture.

3.4 Spheroid Formation

Using cell repellent U-bottom well plates with concave wells which promote cell-cell adhesion, multiple cell cultures were prepared with microparticles modified with different proteins to assess which offered the most viable and functional spheroids. Full list of additions to each experimental group of spheroids can be found in Table 2.

Experimental Group	1 (Fibronectin)	2 (LM-511)	3 (LM-521)	4 (MACF)	5 (None)
Spheroid Media (μL)	4475	4475	4475	4475	4475
HepG2 (μL)	300	300	300	300	300
HSC (μL)	35	35	35	35	35
Microparticles (μL)	5	20	10	40	0

Table 2 Outline of ingredients added in the case of each experimental condition as well as control group. Note experimental group 4 which served as a control through the addition of unmodified microparticles, as well as group 5 which contained pure cell culture and no microparticles.

After the addition of all necessary components the well plate was spun down at 300g for 5 minutes using a centrifuge which allows cells to aggregate, at which point spheroids were placed in a 37C incubator and allowed to grow. After 5 days of growth liver spheroids were observed and photographed using brightfield light microscopy shown in figure 4 where they were found to have formed spherical cell aggregates. Through measurements with ImageJ all groups were found to appear stable and were consistently around 500μm in diameter which was constant between groups as shown in Figure 4.

Observation of spheroids by brightfield light microscopy after 5 days in culture incubation shows dense and intact spheroids for all experimental groups and controls. This is important to determine that spheroids are remaining structural integrity visually and have not

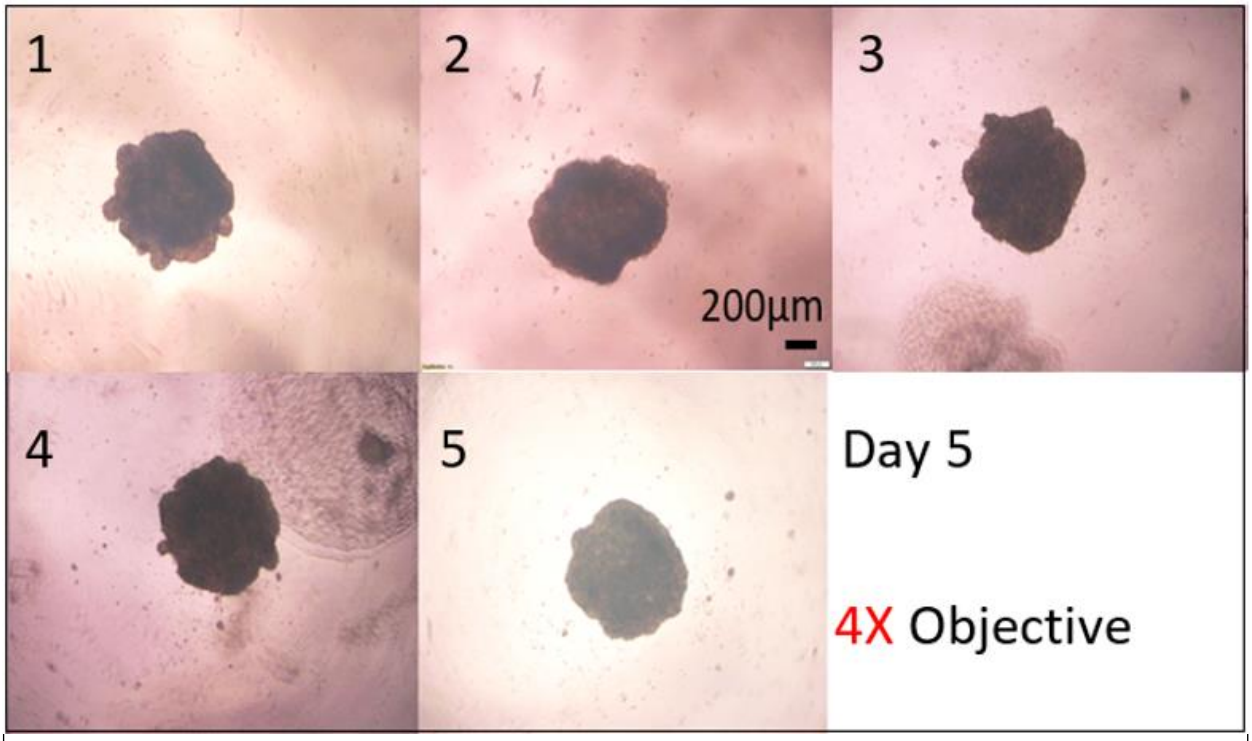

Figure 4 Liver spheroids from each experimental group observed by brightfield light microscopy 5 days after initial spheroid aggregation. Group 1=Fibronectin, 2=Lam-511, 3=Lam-521, 4=MACF, 5=Control.

begun to deteriorate. However, the problem these spheroids encounter after extended culture time is that cells at the innermost portions of the sphere are not receiving enough oxygen by mass transport, and so even though spheroids may look intact the internal cells may no longer be viable at this stage, a limitation that we hope to solve with our oxygenating microparticles. To test this, protein secretion from the cells in culture with different modified microparticles was measured.

3.5 Protein and Enzyme Analysis

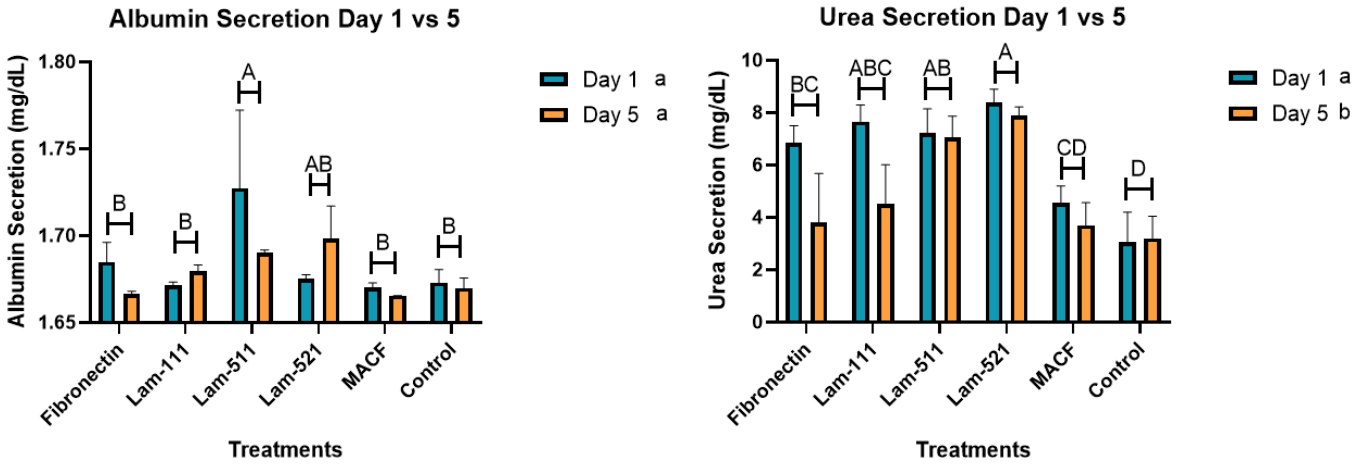

Figure 5 Assays completed to detect levels of two important circulating enzymes secreted into culture media by healthy cells. Each group was assessed at both day 1 and day 5 of culture growth to see how levels would change between the two timepoints in each group. Group labelled MACF contained spheroids with non-modified microparticles and control group contained no microparticle incorporation in culture. An asterisk denotes significance between groups (n=3). All results are presented as mean ± SD of 3 independent spheroids. Groups with significant differences were shown with alphabetic letters to signify ranking of means. The groups indicated with the same letters were not found to be significantly different ($p>0.05$).

Free urea concentration within media is an important metric to consider because it is the byproduct made when cells remove toxic ammonia from culture which is a common reason for cell necrosis and decreased spheroid viability.[23] It has been shown that sustained elevated levels of ammonia *in vivo* have cause an apoptotic effect in hepatocytes and so its removal from culture via the urea cycle is necessary.[23] Similarly albumin has been used as a benchmark enzyme to correlate its concentration in serum to cell growth, as it is a byproduct of the processes undergone during cell proliferation and spheroid growth.[17]

After 1 day of spheroid growth most treatment groups showed similar levels of albumin and urea secretion when compared to unconjugated MACF and no microparticle controls.

However, after 24 hours from initial culture time the LAM-511 treatment group exhibited significantly higher levels of urea ($p=0.0416$) and albumin ($p=0.0302$) when compared to the control and MACF treatment groups respectively. This was shown to stay consistent after 5 days of culture in terms of urea concentration where the urea secretion was shown to be highly significant in the LAM-511 treatment group when compared with the control group ($p=0.0011$). It is also important to note that after 5 days albumin secretion was seen to be significantly higher than the MACF control in the LAM-521 treatment group ($p=0.0270$). These results are promising to the hypothesis that the incorporation of these conjugated ECM protein modified microparticles within culture, specifically those proteins native to the *in vivo* liver environment, will show benefits to the culture overall.

4 Conclusions

It was hypothesized that if MACF microparticles could be altered with liver ECM components to closer reflect native conditions *in* vitro, the formation and biological function of spheroids would be increased. Using common liver ECM proteins, particularly variations of laminin and fibronectin, this modification was achieved via EDC/NHS crosslinking approach. After observation at 5 days by light microscopy all groups including controls were observed to have generated spherically shaped and stable spheroids, however analysis of urea and albumin secretion confirmed that there were differences in protein secretion levels present between groups. After 5 days of spheroid culture, it is promising to see that in the treatment groups containing microparticles modified with laminin-511 and laminin-521, there were significant increases in albumin and urea secretion when compared with control groups. Both of these biological molecules are when estimating spheroid viability beyond what can be see through microscopy as the innermost cells of the sphere cannot be seen. Confirming that these treatments

caused significant change in outcome is a first step in the application of this technique to improve three dimensional cell culture in future work. In continuing this study, the reason behind upregulation of cell function in response to microparticles modified with laminin variants native to the *in vivo* liver environment will be examined through in depth study of expression of proteins involved in cell-cell and cell-ECM interactions. All of these efforts are taken in order to replicate native liver conditions as closely as possible *in vitro*, with the hope that one day this technology may be used to aid in research important to human health, and help elucidate the complexities with which these hepatic pathways operate.

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