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PROJECT COMPLETION REPORT

1	**Project Number**	:	P 8141
2	**Title of the Project**	:	3D printing of liver constructs for in vitro hepatotoxicity testing
3	**Funding Agency Name**	:	Technical Research Centre (TRC): SCTIMST

4 **Project Reference Number provided by the Funding Agency**:

TRC/8141/PSN, Dated 18-04-2016

5 **Principal Investigator (Name & Address) :**

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7	**Implementing Institution**	:	Sree Chitra Tirunal Institute for Medical Sciences and Technology
8	**Collaborating Institutions**	:	Wake Forest Institute for Regenerative Medicine, NC, USA
9	**Date of Commencement**	:	18-04-2016
10	**Duration**	:	36+18 months
11	**Date of Completion**	:	15-08-2021

12 **Objectives as approved :**

1. Establish dedicated 3D Bio-printing facility of approx size of 400 sqft
2. Procurement and training of locally designed customized 3D Printer
3. Selection of polymer and basic cytotoxicity screening
4. Liver ink (BioInk) formulation - Optimization of printing 3D tissues with cell lines
5. Isolation and culture of hepatocytes and 3D printing with hepatocytes
6. Printing 3D tissues with primary human hepatocytes and analysis of tissue function characteristics

13 **Deviation made from original objectives if any, while implementing the project and reasons thereof :**

Not applicable

14 Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs :

14.1 Dedicated 3D Bio-printing facility of approximate size of 300-400 sqft

A dedicated 3D Bioprinting lab was established for carrying out the project. The Bioprinting lab has inbuilt cell culture facility for pre-printing and post printing aspects of the Bioprinting.

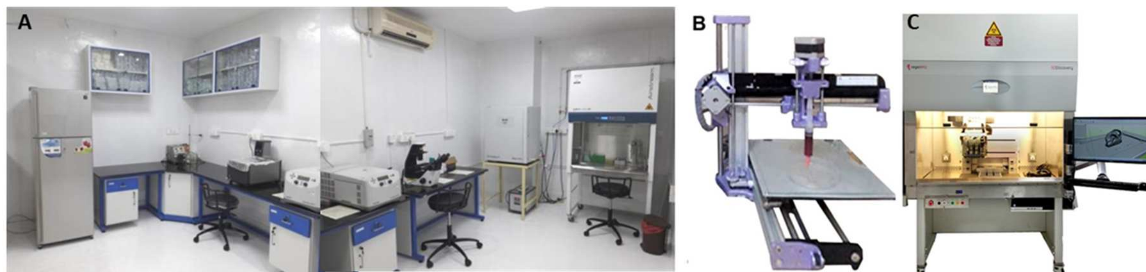

Figure 1 3D Bioprinting facility. A) cell culture lab adjascent to 3D Biopriniting facility, B) The locally procured 3D Bioprinter and 3) Imported RegenHU tissue and organ printing system

14.2 Procurement and training of locally designed customized 3D Printer

14.2.1 Local custom made 3D Bioprinter

Based on the requirements of the project the study was initiated with a custom made 3D bioprinter which is procured from India. This machine has the minimal features that is required for the testing of bioink and printing of liver constructs. All the initial studies on extrudability, printability and cell printing was performed with this printer.

14.2.2 Imported 3D Bioprinter with tissue and organ printing capability

A four head 3D bioprinter was installed in September 2019 for the Bioprinting of liver construct and develop the hepatotoxicity test platform.

14.2.3 Training on 3D Bioprinting

The PI has been trained in 3D Bioprinting under the MoU between SCTIMST and Wake Forest Institute for Regenerative Medicine (WFIRM), North Carolina, USA. The Research Fellowship completed as Research Fellow WFIRM for two years to get basic and advanced training in 3D Bioprinting of tissues and organ so as to establish a 3D bioprinting facility at SCTIMST. The program was successfully completed and the training objectives were fulfilled. The visiting researcher was involved with the core group at WFIRM and have seen how a multidisciplinary group is managed with various research programs. Got training to work with vascular construct, Chip, Kidney construct and bioink printability aspects. The WFIRM training program was well balanced and upto the need of the academic collaboration by involving with the bioprinting and related aspects throughout visiting time.

14.3 Selection of polymer and basic cytotoxicity screening

The base polymer selected was gelatin. Among the various kinds of hydrogels used for extrusion based bioprinting, Gelatin methacrylamide (GelMA) has been widely used for bioprinting owing to its biocompatibility and superior printability. Gelatin methacrylamide is readily photocrosslinkable in presence of photoinitiators like Irgacure 2959 and Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP).

14.3.1 Synthesis of base polymer for bioink

GelMA was synthesized using a modified buffer method. In summary, 10 g of gelatin was dissolved in 100 ml alkaline buffer (carbonate bicarbonate, CB, pH > 10) at a temperature of 50°C. MAA was added sequentially every 30 minutes to the gelatin solution at 50°C for a duration of 3 hours. The pH of the reaction solution was monitored after each MAA addition, and CB buffer was used to maintain a pH greater than 10. Once 3 hours had elapsed, the pH of the reaction was adjusted to 7.3. The reaction mixture was then dialyzed in distilled water for 4 days at 40°C and subsequently lyophilized. The lyophilized polymer (GelMA) was stored at -20°C for later use. To ensure biological analysis, GelMA was sterilized using ethylene oxide gas at 37°C and stored at -20°C until needed. The characterization of

gelatin functionalization was done by 1H NMR analysis and Degree of Functionalization

14.3.2 1H NMR analysis

1H NMR analysis was conducted to confirm the gelatin functionalization with MAA. For NMR analysis, sample were prepared by dissolving 10 mg each of gelatin and GelMA in 1 ml deuterium oxide (D_2O) at 40 °C. The NMR spectrum was obtained using a Bruker Avance 400 MHz FT-NMR spectrometer (Germany). The neutrally charged amino groups effectively reacted with MAA, thereby providing a higher degree of functionalization. The presence of two peaks at 5.5 and 5.7 ppm in the ^{1}HNMR spectra confirmed the presence of methacrylamide moieties onto gelatin. The formation of vinylic protons of the methacrylamide moiety confirmed that gelatin was successfully functionalized with gelatin methacrylamide

14.3.3 Degree of Functionalization

The degree of functionalization (DoF) was determined by utilizing the ninhydrin method, which was previously discussed by Loessner et al. In summary, a mixture of 850 ml ninhydrin solution (consisting of 2.5 mg/ml ninhydrin in a combination of 0.5M sodium citrate monobasic and glycerol at a ratio of 1:2) and 150 ml GelMA solution (with a concentration of 10% wt/V in deionized water) was incubated at 50°C for a duration of 12 minutes. Standard solutions were prepared using linear dilutions of gelatin in deionized water. After cooling the solution to room temperature, the absorbance was measured at 570 nm using a spectrophotometer (Biotek, USA). By extrapolating the absorbance obtained for a gelatin concentration of X% from the standard curve, the concentration of unreacted groups in GelMA was determined. The DoF was calculated as 100-X%, attributing the decrease in absorbance to methacrylamide functionalization

$$\text{Degree of functionalization (DoF)} = 1 - \left(\frac{\text{GelMA}}{\text{Gelatin}}\right) \times 100$$

The functionalization of gelatin was determined by estimating the unmodified free amino groups in GelMA using a previously reported protocol [20]. A standard curve of gelatin was generated, and the percentage of free amino groups was extrapolated from it (Figure 2a). The degree of functionalization was calculated from three different batches of GelMA and was found to be approximately 90 ± 4 % (Figure 2b).

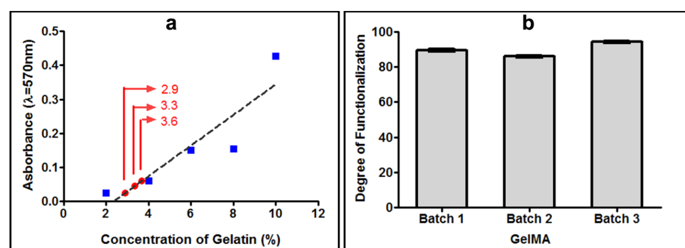

Figure 2 Ninhydrin a) Free amino groups in GelMA extrapolated from free amino groups of gelatin; b) DoF calculated for three different batches of GelMA showing modification efficiency of 89±0.6, 86±0.5 and 94±0.5%.

14.4 Liver ink (BioInk) formulation - Optimization of printing 3D tissues with cell lines

14.4.1 Formulation of base bioink - GelMA

Gelatin methacrylamide hydrogels were prepared by crosslinking methacrylamide groups by photoinitiated chain polymerization. GelMA (10 % w/v) solution was prepared in phosphate-buffered saline (PBS) or serum-free MEM containing 1% Irgacure as a photoinitiator at 60°C until fully dissolved. A requisite amount of this solution was exposed to 365 nm UV light at an intensity of 8-12 mW/cm^2 to initiate crosslinking. The exposure time was varied from to 2-7 min depending on the thickness of the prepared gels.

14.4.2 Liver ink formulation

Photo-protective GelMA was prepared by adding various concentrations of FRS(X) (X representing 1, 1.5, and 2. 25 or 3.4 mM) to 10% GelMA in serum-free medium containing 1% Irgacure. The effectiveness of FRS in protecting HepG2 cells was assessed by measuring cell viability in a sandwich culture model using GelMA and GelMA-FRS.

14.4.3 Biological evaluation of GelMA with liver cells

14.4.3.1 Photoprotective effect

Hepatocelluar carcinoma cell line (HepG2) was cultured in MEM supplemented with 10% FBS and 1% penicillin-streptomycin antibiotics in a CO_2 incubator at 37°C with 5% CO2 and >90% relative humidity. The cells were harvested, trypsinized, and then cultured in a sandwich model. This sandwich method, similar to cell encapsulation, allows for the efficient analysis of cell-based assays and imaging by placing the cells on a single plane. GelMA-FRS(X) systems with different concentrations of FRS (X = 0, 1, 1.5, 2.2, or 3.4 mM) were prepared and added to a 48 well plate (100 µL/well) and cross-linked using UV light. Approximately 1×10^5 HepG2 cells were seeded onto the cross-linked GelMA-FRS hydrogel and allowed to adhere for 2 hours. A second layer of 100 ml GelMA-FRS system was overlaid on the cells and cross-linking was repeated to trap the cells in a sandwich arrangement. The cells were incubated for 24 hours in an adequate amount of cell culture medium. Cells sandwiched in GelMA (with FRS concentration = 0 mM) served as the control.

The number of viable HepG2 cells in the GelMA-FRS sandwich increased with increasing FRS concentration (Figure 3). The increase in viable cell numbers with increasing FRS concentration is encouraging, as it is a direct testimony of the photoprotective effect of FRS. The photo-protective effect of FRS observed in the cells, however, did not affect the physical cross-linking efficiency of GelMA. Furthermore, GelMA sandwich model the layers adhered to each other without any sign of delamination.

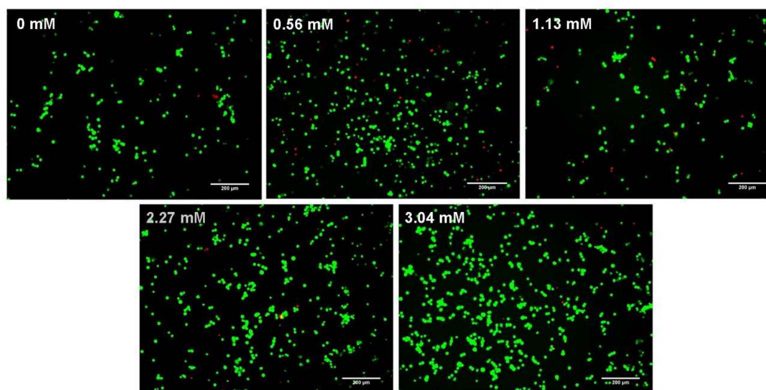

Figure 3 Viability of HepG2 cells in GelMA with various concentrations of FRS, analyzed by sandwich culture method. Values shown in the figure shows the concentration of FRS present in the base 10% GelMA. The number of viable cells (green) increases with increase in concentration of FRS.

14.4.3.2 Cell proliferation

The proliferation of the cells while sandwiched in GelMA was estimated from the cell activity expressed in a standard curve plotted with known concentrations of cells. HepG2 cells at different cell densities (0.5×10^6, 1×10^6, 2×10^6, 2.5×10^6 cells/well) were seeded in triplicate into a 24 multi-well plate, incubated for 24h and CCK-8 assay was carried out according to the manufacturer's instructions. Briefly, the culture medium was removed and the cells were incubated with 500µl CCK-8 reagent (1:10 dilution in serum-free MEM) for 2h. The medium was then transferred to a clear 96 multi-well plate and the absorbance was measured at 450 nm using a multi-well plate reader (BioTek, USA). Approximately 1×10^5 HepG2 cells were sandwiched in the GelMA-FRS(X) system. The cell suspension used for plotting the standard curve was used to estimate the cell number in the sandwich culture within GelMA and GelMA-FRS(X). The cells were cultured for 7 days and proliferation was analyzed on every alternate day (1, 3, 5, and 7 days) using the CCK-8 assay as described above. The number of cells at various concentrations of GelMA-FRS was calculated from the standard curve. The GelMA-FRS(X) system, which supported maximum cell viability and proliferation, was considered for further experiments.

The proliferation of HepG2 cells sandwiched between GelMA and GelMA-FRS(X) was quantified using the CCK-8 assay. Cells embedded in GelMA-FRS(1), GelMA-FRS(1.5), and GelMA-FRS(2.2) did not show any increase in proliferation over a period of 7 days (Figure 4). However, the HepG2 cells sandwiched in GelMA-FRS(3.4), where 3.4 mM of FRS was employed, showed a linear increase in cell number with time. Thus, it was concluded that the minimum concentration of FRS required in the bioink

for photoprotective effect is 3.4 mM, without compromising the crosslinking efficiency.

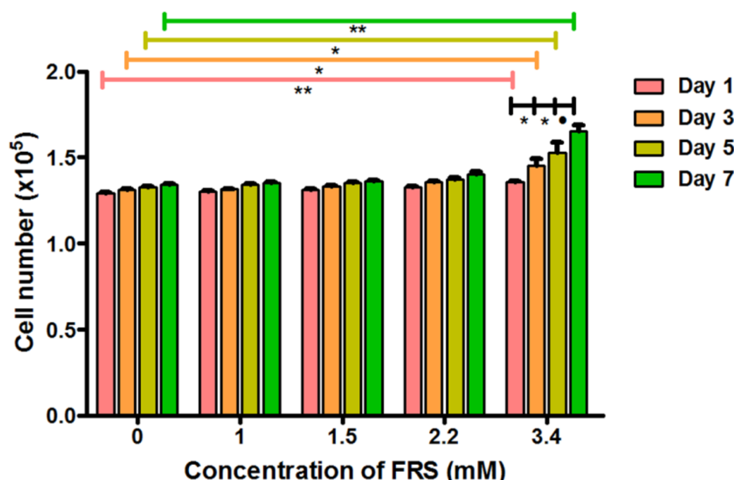

Figure 4 Cell proliferation analyzed by CCK-8 assay. GelMA alone (0 mM) and FRS at concentrations 1 mM, 1.5 mM, 2.2 mM and 3.4 mM showed a steady increase in cell number. The values are normalized to cell number of cells in 2D monolayer of the respective days.
• = (P > 0.05), ∗ = (P ≤ 0.05), ∗∗ = (P ≤ 0.01)

14.4.3.3 Photoprotective effect of Gelatin Methacrylamide -Free radical scavenger system

Based on the cell proliferation data, GelMA-FRS(3.4) was selected for further evaluation of the photoprotective properties. The reactive oxygen species (ROS) generated within cells suspended in GelMA FRS during UV crosslinking was estimated. The GelMA and GelMA FRS solutions were prepared using HepG2 cells at a concentration of 5 × 10^5 cells/mL and were dispersed in PBS. The solution (200 μL) was transferred to multi-well plates and irradiated with UV light at 365 nm for 3 min to form a hydrogel. Cell monolayers adhered to similar multi-well plates formed from the same cell number were used as controls. Cells exposed to UV were considered as a positive control and the cell monolayer without UV treatment was treated as a negative control. Cells in controls and hydrogels were harvested by incubating with 200μL of 0.3 % collagenase – A in calcium containing HEPES buffer (100mM HEPES buffer with 20 mM calcium chloride) for 20 min at 37°C. The cells were transferred to a centrifuge tube and centrifuged (Eppendorf 5430R, India) at 1500 rpm for 2 min to pellet the cells. The supernatant was discarded, and the ROS activities of the cells were determined per product sheet (Lot# AB113851, Abcam). The cell suspension was transferred to a black-bottom-clear 96 well plate and 2′, 7′ – dichlorofluorescein diacetate formed due to ROS activity inside the cells was quantified by measuring the fluorescence at excitation/emission of 485 nm / 535 nm in a spectrofluorimeter (Synergy H1, Biotek, USA). The relative fluorescence unit (RFU) obtained for the cells from GelMA, GelMA-FRS(3.4), and the positive control was compared with that of the negative control using the following equation:

$$\text{ROS activity } (\%) = \frac{\text{Test}_{\text{RFU}}}{\text{Negative}_{\text{RFU}}} \times 100$$

The intracellular ROS activity of HepG2 cells embedded in the GelMA/FRS system (following UV irradiation) was compared with that of the bare GelMA hydrogel (following UV irradiation), cell monolayer exposed to UV (positive control), and cell monolayer unexposed to UV (negative control) (Figure 5). It can be seen that while ROS activity of cells embedded in GelMA/FRS system was comparable to that of negative control, the ROS activity associated with cells embedded in GelMA was closer to positive control. The ROS values associated with the negative control (cells that were not exposed to UV) represent the normal amount of ROS produced inside the cells. It was encouraging to see that cells embedded in GelMA/FRS (77 ± 42 %) showed values closer to this. On the other hand, the cells embedded in bare GelMA showed ROS activity of 212±30%, which was closer to that of the positive control (200±28 %). It is hence clear from these values that the GelMA/FRS system induced protection against the radicals generated during photopolymerization. This is an interesting finding and is particularly relevant when clinically relevant/larger structures are bioprinted and stabilized using

photocrosslinking.

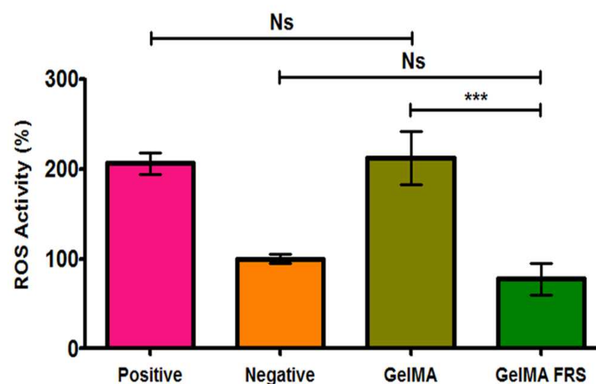

Figure 5 The comparison of intracellular ROS activity of HepG2 cells due to phtocrosslinking of GelMA and GelMA-FRS bioink to normal cells (Negative) and UV exposed (Positive) cells. [Ns = Non significant, *** = $p \leq 0.0001$]

14.4.3.4 Functional evaluation of HepG2 Cells in sandwich culture

The effect of the photoprotective nature of GelMA-FRS(3.4) hydrogel in preserving liver-specific functions was determined by analyzing albumin and urea synthesis in HepG2 cells sandwiched within the hydrogel. The HepG2 cells were sandwiched in GelMA-FRS(X) systems and maintained for 7 days with medium changes on days 1, 3, 5, and 7. The spent medium was collected and stored at -80°C until analysis.

Albumin synthesis by HepG2 cells was analyzed using an ELISA kit (Bethyl Laboratories, USA) according to the manufacturer's instructions. The ammonia detoxification ability of HepG2 cells was analyzed by estimating urea in the spent medium using a urea assay kit (Biochain, USA).

The effect of FRS on the functional ability of hepatocytes, such as albumin and urea synthesis, in the GelMA-FRS hydrogel was also evaluated over a period of 7 days. The variations in albumin and urea synthesis at various concentrations of FRS at different time points are shown in Figure 6. The albumin secretion by cells embedded in GelMA was 60±2 ng/ml (Day 1) and increased linearly to 86±4 ng/ml at the end of 7 days (Figure 6a). A similar increase in albumin secretion was observed in the cells sandwiched in GelMA with 1, 1.5 mM and 2.2 mM FRS concentrations up to day 5. The albumin secretion in cells sandwiched in GelMA with 2.2 mM FRS was 111±7 ng/ml on day 7. The cells embedded in the gel with FRS 3.4 mM showed albumin synthesis of 94±4, 100±5, 131±16, and 172±11 ng/ml on days 1, 3, 5, and 7, respectively. This was very similar to albumin synthesis by the control cells on days 1, 3, and 5. However, the albumin synthesis of control cells on 7th day was 212±18 ng/ml, which was significantly higher than that of the cells in GelMA/FRS 3.4 mM.

The detoxification ability of hepatocytes sandwiched within GelMA and GelMA-FRS hydrogels was analyzed by urea synthesis (Figure 6b). The average urea synthesis of cells embedded in GelMA with 0, 1, 1.5 mM and 2.2 mM FRS was similar and did not show any significant variation. However, the cells embedded in GelMA with 3.4 mM FRS showed a significant increase in urea synthesis and had values comparable to those of the control cells. This clearly indicates that when GelMA was loaded with 3.4 mM FRS, the functional ability of hepatocytes was improved and was comparable to that of control cells even after entrapping in GelMA and UV crosslinking.

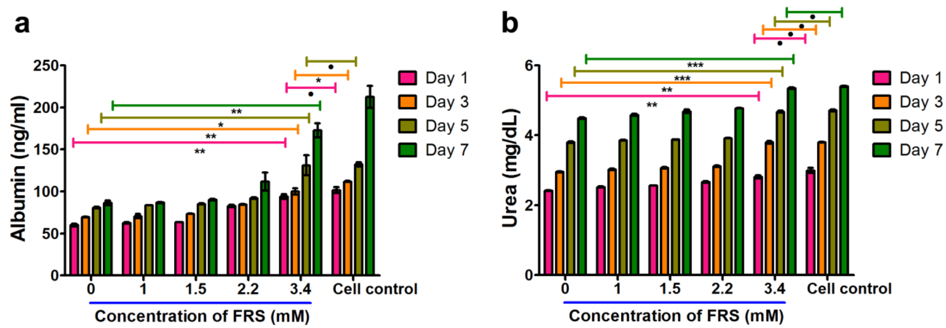

Figure 6 Functionality assessment of HepG2 cells sandwiched in GelMA and GelMA FRS hydrogels. (a) Albumin secretion by cells sandwiched in GelMA and various concentration of FRS containing GelMA showing 3.4 mM FRS as a formulation that is similar to normal culture system. (b) Urea synthesis indicating the detoxification function did not show any dose related response. GelMA with and without FRS express same urea secretion. • = ($P > 0.05$), ∗ = ($P \leq 0.05$), ∗∗ = ($P \leq 0.05$), ∗∗∗ = ($P \leq 0.005$)

14.4.3.5 3D Printability of GelMA

To assess the extrudability of GelMA bioink, a filament drop test was conducted. A freshly prepared bioink solution was placed in a 3ml cartridge and stored at either 25 °C or 18 °C for 5 minutes to undergo thermal gelation. The cartridge was connected to a conical-shaped printing nozzle with an inner diameter of 410 µm. The cartridge was then attached to the extrusion head of a 3D bioprinter (3D Discovery, RegenHU, Switzerland), and pneumatic pressure was gradually increased until a continuous flow of bioink was achieved (Figure 7). The 3D Bioprinting parameters were optimized as follows

Cell concentration – $6x10^5$ per construct of size 10x10x5 mm

Pressure – 80 kPa

Temperature – 19-21 °C

Axis Movement – 10 mm/min

Nozzle diameter – 410 µm

Design – Grid

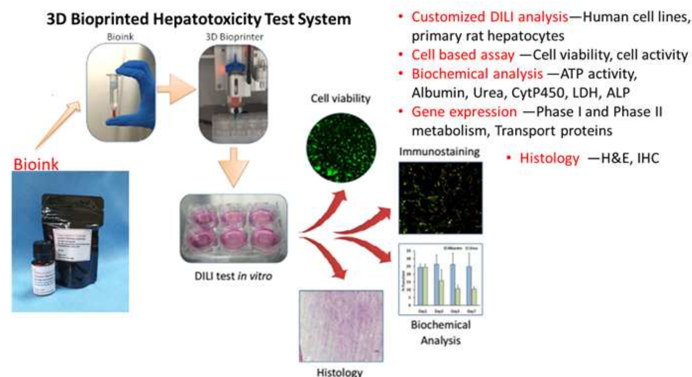

Figure 7 The work flow of 3D Bioprinted Hepatotoxicity test system

14.4.3.6 Bio ink formulation - Optimization of printing 3D tissues with living cells

Three-dimensional bioprinting was carried out on a 3D bioprinter (3D Discovery, RegenHU, Switzerland) using GelMA formulation (10% GelMA, 3% gelatin, 10 % glycerol, and 1% Irgacure 2959) and GelMA-FRS(3.4) formulations. To prepare the bioinks, approximately 3×10^6 HepG2 cells were mixed with 1 mL of the respective GelMA systems, equilibrated at 20°C, and loaded into the extrusion print head of the 3D bioprinter. A tapered plastic nozzle with a 410 µm orifice was also connected. A 3D model square grid pattern (final size of $10 \times 10 \times 5$ mm) with straight lines and cross-overs was designed using BioCAD software (RegenHU, Switzerland), and the construct was bioprinted at a printing speed of 10 mm/s at 20°C into a non-cell-adherent culture plate. The bioprinted constructs were irradiated with 365 nm UV light for 90s. Sufficient culture medium was added to the bioprinted constructs and maintained in a CO_2 incubator at 37°C for 7 days.

The design used for bioprinting is suitable for efficient mass transfer. The grid pattern enabled the diffusion of metabolites effectively, which could be measured in a static culture medium (Figure 8). The bioprinted construct did not show any swelling property, which is a unique characteristic expected in design-based biofabrication. The bioink formulation was selected based on the outcome of the printed construct using the CAD design. The bioink was extruded with very smooth filaments with a width of 410 μm. The final printed construct was exactly the same size as the input design and was structurally stable before and after UV curing.

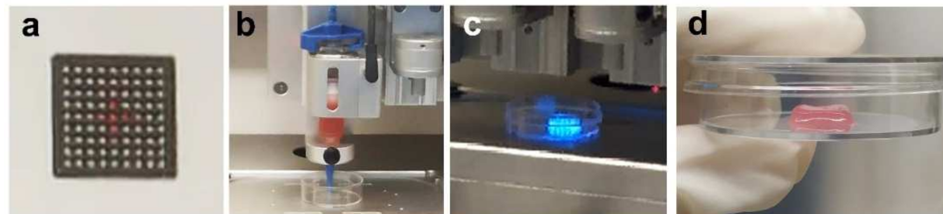

Figure 8 3D bioprinting of liver construct using GelMA-FRS(3.4) bioink. (a) The CAD design showing the dimensions of the construct. (b) Customized 3D Bioprinter for extrusion of hydrogels, (c) Image showing 3D bioprinting in action, (d) Bioprinted liver construct

14.4.3.7 Functional evaluation of 3D Bioprinted Paranchymal Liver Construct using HepG2 cells

The proliferation of HepG2 cells in the 3D bioprinted constructs (fabricated using GelMA and GelMA-FRS (3.4) formulations) was compared. The bioprinted constructs were cultured for 24 h and cell proliferation was analyzed by CCK-8 assay on days 1, 3, 5, and 7. The culture medium was replaced with CCK-8 medium (1:10 dilution of CCK-8 reagent in serum-free MEM) and incubated for 2 h. The medium was then transferred to a 96 multi well plate and the absorbance was measured at 450 nm using a multi-well plate reader. The same constructs were further maintained to repeat the CCK-8 assay on the following days.

The metabolic activity of HepG2 cells in constructs 3D bioprinted with GelMA and GelMA-FRS(3.4) formulation was evaluated at two time points (day 1 and day 7) using the CCK-8 assay. The metabolic activity of HepG2 cells in GelMA-FRS(3.4) bioink was considerably higher than that of HepG2 cells in GelMA (Figure 9). At the end of 7 days, cell activity in the 3.4 mM FRS bioink formulation was significantly higher than that printed using GelMA alone. This clearly shows that the presence of FRS in the bioink maintained and improved the cell activity after crosslinking with UV.

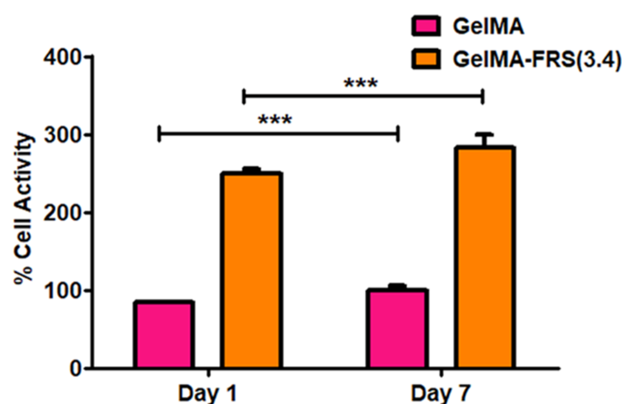

Figure 9. Cell proliferation of HepG2 cells assessed by CCK-8 assay in 3D bioprinted in GelMA and GelMA-FRS(3.4) bioink formulations. The values are normalized to cell activity of construct in GelMA bioink. [*** = $p \leq 0.001$.]

The functional evaluation of the 3D bioprinted liver construct was confirmed from the amounts of albumin and urea secreted from the construct into the culture medium. Culture medium was collected at the end of 1, 3, 5, and 7 days and stored at -80°C until used for estimation. The albumin and urea secreted from the 3D bioprinted construct were quantified using the Human Albumin ELISA Kit and Urea Assay Kit, respectively

The liver-specific functions expressed by the 3D bioprinted constructs showed that cells printed in FRS containing GelMA were more functionally capable than those printed using GelMA alone (Figure

10). Albumin synthesis in the construct bioprinted with GelMA FRS was more than that in constructs printed in GelMA bioink alone (Figure 10**a**). The protein synthesis on day 1 in GelMA-FRS(3.4) was 50±2 ng/ml and increased up to 128±3 ng/ml on 7th day in a linear fashion. A similar trend of increase was observed in the construct printed using GelMA bioink, but a lower albumin synthesis of 32±3 ng/ml and increased up to 91±8 ng/ml on 7th day. The percentage increase in albumin synthesis relative to day 1 in the GelMA-FRS(3.4) construct was 27±17%, 86±18%, and 154±5% on days 3, 5, and 7, respectively. On the other hand, the percentage increase in albumin synthesis by cells in GelMA on days 3, 5, and 7 was 22±6%, 98±5%, and 185±24%, respectively. On the other hand, the urea showed 12±2, 30±5, 40±0, and 33±3 percentage increase in GelMA-FRS(3.4) compared to GelMA bioink (Figure 10**b**).

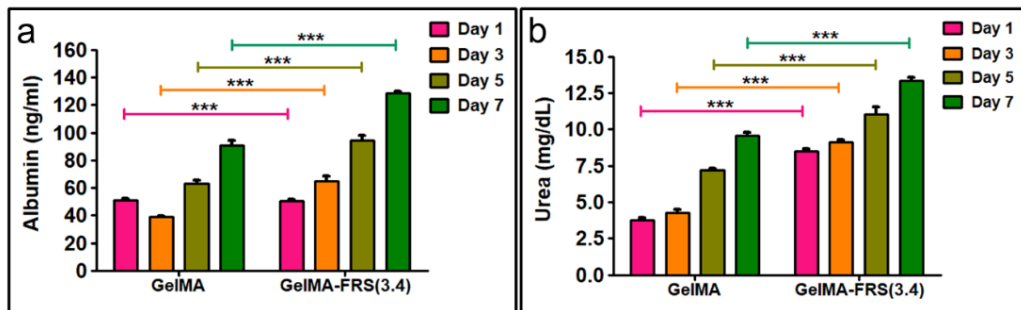

Figure 10. Liver specific function exhibited by the Liver construct 3D bioprinted in GelMA-FRS (3.4 mM) compared with GelMA (0 mM). (a) Albumin synthesis and (b) Urea synthesis was more in GelMA-FRS(3.4) than hepatocyte printed in GelMA. [*** = $p \leq 0.001$.]

14.5 Isolation and culture of hepatocytes and 3D printing with hepatocytes

14.5.1 Isolation and culture of rat hepatocytes

Male Wistar rats weighing 150-200 g were used to isolate hepatocytes. The isolation process was conducted using a modified two-step perfusion method as described by Palakkan et al. (2013). Prior approval was obtained from the Institutional Animal Ethics Committee.

To begin the process, the rats were anesthetized with intramuscular ketamine (10 mg/100 g body weight) and xylazine (1 mg/100 g body weight). Heparin (0.25 mL/100 g body weight) was administered intraperitoneally to prevent blood clotting. The rat's fur was thoroughly cleaned with 70% ethanol and the rat was placed on a bed inside a biosafety cabinet (ESCO, Labculture Type A2, Singapore). A laparotomy was performed, and a 20-gauge blunt cannula was inserted into the portal vein, which was connected to a perfusion system set at 37 °C. The liver was then perfused with an oxygenated calcium-free perfusion buffer at a flow rate of 20 ml/min for 15 minutes. Without delay, the perfusion was switched to a calcium-containing perfusion buffer containing 0.5 mg/mL Collagenase Type IV (Merck-Sigma, cat#.C4-BIOC, Germany), and the perfusion continued at a flow rate of 30 ml/min with continuous oxygenation for 10 minutes. Following cannulation, the rat was euthanized with an overdose of sodium thiopentone (25 mg/ml). The liver was removed from the carcass and placed in a sterile petri dish containing cold (4 – 8 °C) serum-free Iscove's Modified Dulbecco's Medium (IMDM, Merck-Sigma, cat#.56479C, Germany) supplemented with 2% Foetal Bovine Serum and 100 µg/ml Penicillin and 100 IU/ml streptomycin. The Glisson's capsule was gently peeled off, and the liver cells were dislodged into the medium by gentle shaking in a 150 mm sterile Petri dish. The released cells were then passed through a sterile nylon mesh (70 µm) to remove tissue fragments and cell clumps. The cell suspension was incubated at 37 ºC inside a CO2 incubator for 30 minutes and then transferred to 50 ml centrifuge tubes, which were immediately placed on an ice bath for 10 minutes.

The hepatocytes were separated from the cell suspension by centrifugation at 50 g for 2 minutes at 4 ºC. The supernatant containing the NPCs was collected, and the hepatocyte pellet was washed twice in IMDM by centrifugation at 20 g, 4 ºC for 2 minutes. Cell viability was assessed using the trypan blue exclusion method, and only cells with viability greater than 85% were used for the experiments.

14.5.2 3D Bioprinting of rat hepatocytes

3D Bioprinting of rat hepatocytes was performed as described above. The cell number per construct was $6x10^5$ cells/construct. The construct was incubated in IMDM supplemented with 2%

Foetal Bovine Serum and 0.1% Penicillin – streptomycin antibiotic.

The bioprinted liver constructs were cultured for three days to stabilize them. A solution of the initial drug, Ketoconazole, was prepared by dissolving 150mg in 0.75ml of DMSO. This solution was then prepared in the culture media to achieve a final concentration of 1mg/ml. The DMSO used to dissolve the drug was considered as the vehicle control at the highest concentration. After three days of incubation, the culture media was replaced with a medium containing the drug and vehicle. The constructs were treated continuously for seven days, with the media being changed every day. To retrieve the cells from the bioprinted constructs, they were treated with Collagenase A (1mg/ml in Calcium-containing HEPES buffer). Lysate samples were collected on day 3, day 5, and day 7, with a total of three samples taken.

CytP450 analysis showed that the monolayer hepatocytes did not show any CYP450 3A4 activity, Whereas the 3D Bioprinted constructs exhibited almost 5 times.

14.6 Printing 3D tissues with primary human hepatocytes and analysis of tissue function characteristics

3D Bioprinting of human hepatocytes was performed as described above using commericially procured cells. The cell density was $6x10^{5}$ cells/construct. The construct was incubated in IMDM supplemented with 2% Foetal Bovine Serum and 0.1% Penicillin – streptomycin antibiotic.

14.7 Hepatotoxicity system - *In vitro - In vivo* trend

The propose of the study was to establish three-dimensional culture system using GelMA hydrogel as an alternative to hepatotoxicity testing. The pilot experiment was designed to collect the first level of information on the trend of metabolic ability of 3D bioprinted construct to in vivo condition.

The study requirement of toxicologist to perform hepatoxicity experiment in animals was essential. Subsequently, Dr. PV Mohanan, Scientist G, Division of Toxicology was included as the co-investigator for the preliminary in vitro and in vivo investigations.

The bioprinted liver constructs were incubated for three days to stabilize the culture. Initial drug (Ketoconazole) concentration was prepared by dissolving 150mg in 0.75ml DMSO and final concentration of 1mg/ml was prepared in culture media. The vehicle (DMSO) used for dissolving the drug was considered as vehicle control at highest concentration. After 3 days of incubation, culture media was replaced with drug and vehicle containing medium. Constructs were continuously treated for 7 days with media change on every day. The cells were retrieved from the bioprinted constructs by treating with Collagenase A (1mg/ml in Calcium containing HEPES buffer). Lysate samples (n=3) were collected on day 3, day 5 and day 7.

The *in vivo* experiments were conducted in rodent model. Rats with 150 to 200 g body weight was used for the study after the approval from Institutional Animal Ethics Committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology. Animals housed in well ventilated cages with stable temperature and relative humidity. Acute toxicity study was performed in test group at definite time interval (Day 3, 5 and 7) with limited number of rats (n=3) per group. Rats were administrated orally with Ketoconazole at a dose of 20 mg/kg body weight daily for 7 days [Khoza *et al*, 2017]. A vehicle group received diluted Dimethylsulphoxide (DMSO) similar to the concentration used in preparing test compound. At each time point, the animals were euthanized and liver biopsy was collected. A small piece of liver was homogenized with lysis assay buffer and the lysate collected was stored at -20°C for cytochrome p450 analysis.

Cytochrome 3A4 activity was estimated by using CYP3A4 activity assay kit (Abcam Cat No. ab211076). The total protein of the cells was also estimated using Takara BCA protein assay kit (Cat No. T9300A) as per product instructions mentioned. The activity of CYP3A4 normalized to total proteins was compared between vehicle and ketoconazole and the percentage loss of CYP3A4 activity was calculated and plotted.

15 Detailed analysis of results :

1. Establish dedicated 3D Bio-printing facility of approx size of 400 sqft

   A State of the Art 3D Bioprinting facility was established. Faculty was trained at WFIRM USA

for 3D bioprinting

2. Procurement and training of locally designed customized 3D Printer

   The project was initiated with a locally procured extrusion 3D Bioprinter. An imported multihead multitechnology 3D bioprinter with tissue and organ printing capability was installed. The users of many other divisions who will used the facility was also trained in designing and printing.

3. Selection of polymer and basic cytotoxicity screening

   The appropriate ECM for cell printing is collagen. To address the physical gelling, printability at room temperature and preparation of bioink at Physiological pH, Gelain, the hydrolysed form of gelatin was selected. Gelatin harbours the cell adhesion sites similar to collagen and can be modified without disturbing the cell binding moieties.

4. Liver ink (BioInk) formulation - Optimization of printing 3D tissues with cell lines

   GelMA is a photo-crosslinkable polymer that can be readily cross-linked by irradiation at a specific wavelength range in the presence of a photoinitiator (PI), which initiates rapid crosslinking at physiological temperature, pH, and ionic environment and allows for the incorporation of living cells by *in situ* gelation. During photo-crosslinking, PIs generate free radicals upon exposure to a specific wavelength of light and initiate polymerization by cross-linking, which leads to the formation of hydrogels. The free radicals formed during photo-initiated cell encapsulation interact within the cell membrane, proteins, and deoxyribonucleic acids, causing serious cellular damage by affecting many biochemical and physiological activities

   In this study we developed a novel photo-protective bioink formulation for bioprinting functional liver constructs. The findings demonstrate that radical scavenging during photocrosslinking enhances the viability and functionality of the bioprinted constructs. The liver construct created with the photoprotective GelMA bioink offers a simple and effective method to improve liver-specific functions in bioengineered liver tissues. By encapsulating hepatocytes within the novel bioink formulation and exposing them to UV irradiation, intracellular ROS is effectively neutralized and liver-specific functions are maintained. Compared to normal GelMA, the cells in the 3D bioprinted construct exhibit over 150 times greater activity and improved cell functionality. Future studies will explore the physiological capabilities and structural organization of 3D liver tissues bioprinted with a variety of primary cells. If further evaluated according to regulatory standards, this bioink formulation holds promise as a realistic approach to develop transplantable liver constructs, addressing the issue of organ shortage in the future.

5. Isolation and culture of hepatocytes and 3D printing with hepatocytes

   A full organ perfusion setup has been developed for the isolation of viable and functional rat hepatocytes. The protocol were standardized to get 99% hepatocytes with >95% viability. The metabolic ability of the cells in drug metabolism was preserved with isolation, 3D Bioprinting and Post printing analysis.

6. Printing 3D tissues with primary human hepatocytes and analysis of tissue function characteristics

   Results showed that there is species specific difference in the response of bioprinted construct in the expression of albumin synthesis and urea synthesis. This indicate that the test system developed will be useful for the screening of drugs that have different metabolic potential in human and rodent species.

16 Summary sheet of not more than 2 pages under following heads :
(Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential)

The most commonly used in vitro format for toxicity assessment is monolayer culture of primary hepatocytes. However, it is severely hindered by the lack of 3D organization, non-parenchymal cells, and thus cell-cell interactions via contact or paracrine effects. 3D bio-printing technology can create three-dimensional liver tissues like (organoids) constructs with human cells that can closely mimic in vivo tissue functions. 3D printed liver constructs can be used for drug screening at initial stages to save huge investment otherwise will go useless after post market release.

The study was successfully completed meeting all the proposed objectives for the development of

3D bioprinted hepatotoxicity test system. The product aimed in the project is an in vitro test system for which a bioink was developed for 3D Bioprinting. The bioprinted construct showed liver specific functions and responded to drugs in a dose depended manner. The bioink developed has been considered to be included in the institute compendium to identify potential industry partners. The test system is now open for internal and external customers as non-accredited non-validated test for research. Targeted niche will be pharmaceutical companies involved drug discovery and screening. Only international companies are currently involved providing hepatoxicity testing using 3D printed liver constructs. Hence no potential competitors currently in India.

17 Contributions made towards increasing the state of knowledge in the subject :

17.1 Invited talks in conference

1. Dr. Anil Kumar PR, Advanced Research in Regenerative Medicine – Biofabrication of Tissues, International Conference of SciCon Series on In sync with Next Generation Biosciences (INGB) – 2019 (6-8 November 2019), SciCon Series (Plenary lecture)
2. Dr. Anil Kumar PR, Three Dimensional Bioprinting: Customized Tissues and Organ using Multiple Cell Printing, International training workshop in vitro toxicity: Concept and practice with an Emphasis on hepatotoxicity, enterotoxicity and multiple organ toxicity with IdMOC (11th December 2019) Dept. of Zoology, University of Kerala Kariavattom, Thiruvananthapuram (Invited Talk)
3. Dr. Anil Kumar PR, Biofabrication of tissues: The road ahead, Indo-US Conference on Bioengineering and Regenerative Medicine (27-29 Feb 2020). School of Biochemical Engineering, IIT (BHU), Varanasi (Plenary talk)
4. Dr. Anil Kumar PR, Three dimensional Bioprinting in neural tissue engineering, International Brain Research Organization APRC School 2019 on 02-05-2019, BMT Wing, SCTIMST (Invited speaker)
5. Dr. Anil Kumar PR, Biofabrication of Tissues: The Big Picture, International on Research Interventions and Advancements in Life Science (RIAL-2018), Pune (Plenary Lecture)
6. Dr. Anil Kumar PR, Three Dimensional Bioprinting: A Closer Step Towards Creating Customized Tissues and Organs, International Training Workshop on In vitro Toxicology with Emphasis on Integrated Discrete Multiple Organ Co-Culture (IdMOC) during 22 - 26 November 2018, Dept. of Zoology, University of Kerala, Kariavattom (Invited talk)
7. Dr. Anil Kumar PR, Evaluation challenges in Biofabrication of Tissues and Organs, International Conference on "From Health to Well-being: An Interdisciplinary approach from Fundamental Sciences to Translational Medicine, St. Xavier's College, Mumbai (India) (Plenary Lecture)
8. Dr. Anil Kumar PR, Challenges in Biological Evaluation of 3D Bioprinted Constructs, First Indian Materials Conclave and 30th Annual General Body Meeting of Materials Research Society of India (12-15, February 2019), Indian Institute of Science, Bangalore (Invited talk)

17.2 Invited talks as resource person

1. Dr. Anil Kumar PR, "Stem cell research for tissue engineering", Workshop on Tissue Engineering for Medical Applications – Special Session for SC/ST students (5 – 6 December 2019), IIPC, SCTIMST, Trivandrum (Resource person)
2. Dr. Anil Kumar PR, "Biofabrication – 3D bioprinting & Bioreactors", Workshop on Tissue Engineering for Medical Applications – Special Session for SC/ST students (5 – 6 December 2019), IIPC, SCTIMST, Trivandrum (Resource person)

17.3 Oral presentation in conference

1. Roopesh R Pai, Microvalve-based Biofabrication of Three-Dimensional Liver Parenchymal Microtissues, Tissue Engineering and Regenerative Medicine International Society - Asia Pacific Chapter Meeting & 7th Asian Biomaterial Congress (14 - 17th Oct 2019), Brisbane Exhibition and Convention Centre, Brisbane, Australia
2. Mr. Roopesh R Pai, Spheroid Sandwich Culture as an In Vitro Model System for Evaluation of Drug-Induced Hepatotoxicity, International conference on BioMaterials, BioEngineering, and BioTheranostics (BIOMET 2018), Vellore Institute of Technology, Vellore, TN, 24th-28th July, 2018

3. Ms. Shilpa Ajit, A Gel Casting Device to Establish Consistent Three Dimensional Sandwich Cultures for In Vitro Cytotoxicity Analysis, International on Research Interventions and Advancements in Life Science (RIAL-2018), Pune

17.4 Poster presentation in conference

1. Roopesh R. Pai and Anil Kumar P R, Development of ECM based bioink for 3D Bioprinting of Liver Construct, International conference on Biomaterials, Regenerative medicine an Devices (BIO-Remedi) 2022, Organized by SBAOI at IIT Guwahati, Assam, during 15-18, Dec 2022 Secured Best Poster Pitching Award from the American Chemical Society
2. Ms. Shilpa Ajit, Optimization of Photo Crosslinkable Bioink with UV Protective Molecules for 3D Bio-printing, International conference on BioMaterials, BioEngineering, and BioTheranostics (BIOMET 2018), Vellore Institute of Technology, Vellore, TN 24th-28th July, 2018

18 Conclusions summarising the achievements and indication of scope for future work :

Main technology development program is 3D bioprinted Liver test system developed in the project are Bioink and the 3D Bioprinted Hepatotoxicity test system. The hepatotoxicity test system using 3D bioprinted constructs arised from the project is a test system which is now open for internal and external customers.

The necessity to determine whether a newly developed test method using a new model is superior to the existing one gives rise to a need for validation. As stated in the Organisation for Economic Co-operation and Development (OECD) document ENV/JM/MONO(2005)14, validation is the process through which the reliability and relevance of a specific approach, method, process, or assessment is established for a defined purpose. The OECD test guidelines also emphasize that the test method must satisfy certain essential criteria for validation, including Sensitivity, Specificity, Robustness, Reproducibility, Repeatability, Reliability, Relevance, Interlaboratory repeatability, Interlaboratory reproducibility, Intralaboratory repeatability, Intralaboratory reproducibility, and accuracy. A typical validation process is carried out through five different stages, mainly perform pre-validation, assess scope of the assay, prepare validation protocol, execute the validation protocol and prepare the validation report. This would be the next level for the completed project utilizing internal or external funds.

19 Science and Technology benefits accrued :

a. List of research publications with complete details :

Peer reviewed journals

1. Roopesh RP, Muthusamy S, Velayudhan S, Sabareeswaran A, Anil Kumar PR. High-throughput production of liver parenchymal microtissues and enrichment of organ-specific functions in gelatin methacrylamide microenvironment. Biotechnol Bioeng. 2022 Mar;119(3):1018-1032.
2. Shiny Velayudhan, Shilpa Ajit, Roopesh R. Pai, Ramesh Babu V, Sabareeswaran A and Anil Kumar PR, A gel casting tool for fabrication of three dimensional sandwich in-vitro culture system for cytocompatibility evaluation of hydrogels, IJPSR, 2021; Vol. 12(9): 4799-4706.
3. Mohamed Ali, Anil Kumar PR, James J. Yoo Faten Zahran, Anthony Atala and Sang Jin Lee, A Photo-Crosslinkable Kidney ECM-Derived Bioink Accelerates Renal Tissue Formation. Adv Healthc Mater. 2019 Apr;8(7):e1800992 [Impact Factor: 5.76].
4. Gao T, Gillispie GJ, Copus JS, PR Anil Kumar, Seol YJ, Atala A, Yoo JJ, Lee SJ, Optimization of gelatin-alginate composite bioink printability using rheological parameters: a systematic approach, Biofabrication. 2018 Jun 29;10(3):034106. [Impact Factor: 6.838]
5. Mohamed Ali, Anil Kumar PR, Sang Jin Lee and J. D. Jackson, Three-dimensional bioprinting for organ bioengineering: promise and pitfalls. Curr Opin Organ Transplant, 2018. 23(6): p. 649-656.

6. Shiny Velayudhan and Anil Kumar PR. Three Dimensional Bioprinting – A Closer Step towards Creating Customized Tissues and Organs, In book: Spinco Biotech Cutting Edge, Publisher: Spinco Biotech, November 2018, 34-38
7. P. R. Anil Kumar, M. Ali, S. J. Lee, J. J. Yoo, A. Atala, 3D Bioprinting of Human Kidney Construct: A Step Towards Solid Organ Printing, Tissue Engineering: Part A, Vol 23(S1), 2017. [Impact Factor: 3.508]

Book chapters

1. Gregory J. Gillispie, Jihoon Park, Joshua S. Copus, Anil Kumar PR, James J. Yoo, Anthony Atala, Sang Jin Lee, "Three-Dimensional Tissue and Organ, 3rd Ed. Printing in Regenerative Medicine", In: Principles of Regenerative Medicine, Eds Anthony Atala, Robert Lanza, Aotnios G. Mikos and Robert Nerem
2. Senthilkumar Muthusamy, Shiny Velayudhan, and Anil Kumar PR, Hydrogel Systems for Tissue Engineering in: Handbook of Research on Nano-Drug Delivery and Tissue Engineering: Guide to Strengthening Healthcare Systems Eds: Rajakumari Rajendran, Hanna J. Maria, Sabu Thomas, Nandakumar Kalarikkal, Apple Academic Press, USA
3. Three-dimensional bioprinting of tissues and organs, Roopesh R. Pai, J. Anupama Sekar, Shilpa Ajit, D. Shiny Velayudhan, Naresh Kasoju and Anil Kumar P.R. In: Biomedical Product and Materials Evaluation Standards and Ethics, Ed P.V. Mohanan, Woodhead Publishing, UK

b. Manpower trained on the project :

i. Research Scientists or Research Fellows : 1

ii. No. of PhD's produced : 2

iii. Other Technical Personnel trained : Project Assistant (Cell culture) : 3
Project Assistant (Chemistry) : 1
Project Attendant : 1

c. Patents taken, if any : 2

d. Products developed, if any : 2

20 Abstract: (In 300 words for possible publication in ...... Bulletin)

a. Background:

Biofabrication of tissue for 3D in vitro alternative test systems and tissue engineered construct for transplantation. Major challenge in 3D Bioprinting is development of specific bioinks to produce functional liver constructs.

b. Materials:

A novel hydrogel system has been developed by functionalizing gelatin for biofabrication of paranchymal liver construct using hepatocytes. The hydrogel system when mixed with animal or human hepatocytes and its growth supplements forms the bioink for 3D printing of liver construct.

c. Results:

The bioink has unique properties such as Non-cytotoxic, promotes cell adhesion, cell proliferation and printability. The bioink also protects the cells during photocrosslinking and preserves the liver specific functions such as albumin, urea and cytochrome p450 expression.

d. Conclusion:

The knowhow on synthesis and use of bioink for liver bioprinting is available for technology transfer. Being a 3D culture system, bioprinted test system also offers information on histoanalysis. The test system is in the process of validation into an *in vitro* hepatotoxicity testing platform.

21 Procurement/Usage of Equipment:

a. Details of Equipment:

No.	Name of Equipment	Make/ Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown
1	3D bioprinter for tissue & organ printing	regenHU Discovery	1,55,72,000	11/07/2018	100%	Working
2	3D bioprinter	Alfatek	443888	24/11/2016	50%	Working
3	Refrigerated centrifuge	Eppendorff	440311	23/02/2017	100%	Working
4	Biosafety class 2A cabinet	Esco	346915	21/04/2017	100%	Working
5	CO2 incubator with UPS support	Eppendorff	315340	23/02/2017	100%	Working
6	Inverted phase contrast microscope with imaging facility	Nikon TS100	286163	04/03/2017	100%	Working
7	Laminar flow bench vertical	Mark	87765	24/01/2017	100%	Working
8	Magnetic stirrer with hot plate	ANM MS203	81543	12/10/2020	100%	Working
9	Water bath with external circulator	Grant	68080	30/01/2017	100%	Working
10	UPS 3KVA online with SMF battery	APC	49560	14/12/2020	100%	Working
11	Double door refrigerator	Sharp	43000	23/12/2016	100%	Working
12	Air conditioner and stabilizer	Onida	25000	10/01/2017	100%	Working

b. Suggestions for disposal of equipment(s):

As per institute rules

15-11-2023

Dr. Anil Kumar PR

Routing: Signed copy of “Project completion Report” by PI → root@sctimst.ac.in,