Chapter 2
MATERIALS AND METHODS

2.1 Synthesis and characterisation of fibrin nanoconstructs loaded with candidate chemokines (drugs/growth factors) for their use as controlled delivery agents

2.1.1. Isolation of fibrinogen from frozen blood plasma.
Fibrinogen was isolated from human blood plasma as per the previously reported protocol [1]. Briefly, human plasma bags obtained from hospital blood bank of AIMS through prior written consent and was first frozen at −20 ºC for 24 h and then thawed at 4 ºC for 18 h. Plasma was centrifuged at 6500 g for 20 min using a refrigerated centrifuge (Hermle - Z36-HK, Germany) at 4 ºC. The precipitate was dissolved in 0.9% sodium chloride solution, which was then frozen at −20 ºC for 2 h and lyophilized for 18 h using a freeze-drier (Alpha 2-4 LD plus Christ, Germany) to obtain fibrinogen powder, enriched by the coagulation component FXIIIa.

2.1.2. Synthesis and characterization of bare and candidate chemokine loaded Fibrin nanoconstructs:
A surfactant-free water-in-oil emulsification–diffusion system was designed to induce Thrombin-FXIIIa mediated cross-linking of fibrin molecules. The basis of this new approach involves the simultaneous administration of fibrinogen and thrombin taken separately in individual applicators, allowing its interaction only in the pre-heated vegetable oil system. Another aspect of this method that is different from previously reported approaches is that the particles were collected at the interface of oil and water by density gradient separation. This was followed by probe sonication that enabled good control over the shape as well as size of the resultant nanoconstructs. In brief, 5 ml aqueous suspensions of both Fibrinogen - FXIIIa cryoprecipitate and Thrombin were instantaneously injected into 40 ml of purified vegetable oil. These aqueous suspensions were allowed to crosslink and emulsify in the oil phase, which was kept under constant magnetic stirring at 400 rpm and maintained at a temperature of 60 ºC. The system was allowed to equilibrate under stirring for 6 to 8 hours, wherein the cross-linking of fibrin clot and its
uniform dispersion in oil occurred. The nanoconstructs thus formed in the emulsion were then centrifuged at 10,000 rpm for 15 minutes. This resulted in the formation of a density gradient layer of nanoconstructs at the oil-water interface, which was pooled up and harvested from the emulsion. The resultant constructs were further re-dispersed in water and probe sonicated for 20 min to attain a uniform nanosize distribution and preserved after lyophilizing for 48 hours. For synthesizing Tacrolimus conjugated fibrin nanoconstructs (T-FNCs), Tacrolimus (as monohydrate) was purchased from Concord Biotech Limited (Ahmedabad, India). 2 mg of Tacrolimus was dissolved in 1 ml of Ethanol:PBS (1:2) solution and mixed with 4 ml of aqueous Fibrinogen - FXIIIa cryoprecipitate. For synthesizing VEGF and HGF conjugated fibrin nanoconstructs, recombinant human VEGF and HGF was purchased from PeproTech Inc. (Rocky Hill, USA). 5 µg VEGF or 10 µg of HGF was added independently to 1 ml of Hank’s balanced salt solution and mixed with 4 ml aqueous suspension of Fibrinogen - FXIIIa cryoprecipitate. The procedures further to this step were identical to that adopted for the preparation of FNCs as explained above. The rheological behavior of the oil used for the synthesis was evaluated by measuring the viscosity as a function of temperature in the range 50 to 80 °C using a digitalized viscometer (DV-II Pro., Brookfield, USA).

2.1.2.1. Morphological analysis of bare FNCs.

The hydrodynamic particle size distribution of FNCs was analyzed using the technique of Dynamic Light Scattering (DLS) by measuring the intensity of scattered light as the FNCs underwent Brownian motion [2]. The samples suspended in MilliQ water were analyzed using Nicomp™ 380 ZLS-ZP/Particle Sizer (Santa Barbara, USA) with a laser source emitting at 632.8 nm, and the scattered intensity measured at an angle of 90°. The intensity-weighted Gaussian distribution was recorded as the average of three measurements [3]. Surface charge and thereby the stability of the same system was analyzed from its zeta potential measurements using the Nicomp Zetasizer.
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The size as well as the surface topography of FNCs was analyzed by Scanning electron microscopy (SEM) and Atomic force microscopy (AFM). For SEM analysis, freeze-dried nanoconstructs redispersed in MilliQ water was dropped on aluminium stub and sputter coated with platinum using an Auto Fine Platinum Coater (JEOL JFC-1600) at 10 mA for 120 s before imaging under SEM (JEOL-JSM-6490LA). For AFM analysis, 1 mg/ml suspension of freeze-dried nanoconstructs was prepared and drop casted over an atomically flat mica surface and air dried. The atomic force microscopic images were acquired using an AFM (JEOL-SPM-5200) in tapping mode at a scan size of 2×2 µm at a scan rate of 333.33 µs.

2.1.2.2. In vitro pH dependent and enzymatic degradation of bare FNCs.

The in vitro enzymatic degradation of FNCs was performed by incubating freeze-dried nanoparticles (5 mg/ml) in salined buffer containing 60 µg/mL active Matrix Metalloproteinases (MMP-3) in presence of 10 mM CaCl₂ at 37°C. Enzymatic treatment was terminated at predefined time intervals of 0, 2, 4, 6, 8, 24, 48, 96 and 120 hrs by addition of 25 mM EDTA [4]. The reaction mix was filtered through a Centricon® plus filter device of MW cutoff 300 KDa (Millipore) by centrifugation at 4000 rpm for 5 minutes. 200 µL filtrate sample was analyzed using D-dimer assay [5]. In order to examine the molecular integrity of cross-linked fibrin and to analyze the possible structural alterations that might have occurred between stable and enzymatically degraded FNCs, SDS-PAGE analysis was also carried out with 500 µg of FNCs and 500 µl of enzymatically cleaved FNCs (96th hour). Samples were mixed with sample buffer (0.5 M Tris-HCL, 20 % glycerol, 10 % SDS, 0.5 [6] samples were then analyzed by electrophoresis in 5 % stacking and 8 % resolving SDS-polyacrylamide gel. The gel was stained and imaged using a ChemiDoc™ XRS system (BioRad, CA, USA).

2.1.2.3. Physicochemical characterization of bare FNCs

FNCs were further evaluated for their functional characteristics using Fourier Transform Infrared (FTIR) Spectroscopy. For FTIR analysis, 2 mg of freeze-dried fibrin clot and bare fibrin nanoconstructs were pelletized with 175 mg
KBr and the spectrum recorded in the frequency range 650-2100 cm\(^{-1}\) using an RX1-FT-IR spectrometer (Perkin Elmer, USA).

Mallory’s Phosphotungstic acid hematoxylin (PTAH) staining technique was adopted to specifically stain the core fibrin component of the nanoconstructs [7]. In brief, the freeze dried nanoconstructs were dehydrated in 80% ethanol and prestained with eosin for 5 min. After washing with double distilled water the particles were stained with PTAH, incubated at 60\(^{0}\)C for 30 min and viewed under bright field mode of fluorescent microscope (Olympus-BX-51).

2.1.2.4. Morphological analysis of candidate chemokine loaded FNCs

The hydrodynamic particle size distribution and stability of chemokine loaded FNCs was analyzed using DLS and zeta potential measurements. The average particle size was also measured by SEM imaging.

2.1.3. Isolation, characterization and primary culture maintenance of hMSCs and HUVECs

The isolation of human umbilical cord blood derived hMSCs was performed as per the established protocol [8]. In brief, human umbilical cord blood was collected from Gynecology department after obtaining prior ethical consent of the institutional body of Amrita Institute of Medical Sciences. Cord blood was then diluted with equal volume of sterile PBS and subjected for density gradient centrifugation using half the volume of Ficoll-Paque (GE Healthcare, Sweden). The buffy coat was collected in a 50 mL centrifuge tube (BD Falcon, USA) and washed repeatedly by centrifugation with 25 mL of sterile PBS for 10 min at 3000 rpm. The nucleated cells were plated in a 75-cm\(^{2}\) culture flask (BD Falcon, USA) and incubated by adding 15mL complete cell culture Mesencult™ medium (Stem Cell tech. Canada) at 37\(^{0}\)C with 5% CO2. After 24 h, non adherent cells were discarded, and adhered cells were thoroughly washed twice with PBS and the healthy cells were maintained up to 60 % confluency on passage 3 and taken further for experiments. Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cord samples as per the established protocol [9]. Umbilical cord was collected from the persons who underwent normal delivery at the
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Gynecology department of AIMS with their prior written consent and also the ethical clearance of the institutional body of Amrita Institute of Medical Sciences. The HUVEC cells were detached the umbilical cord by 0.1% collagenase proteolytic cleavage, cells were then immediately washed in sterile FBS and resuspended in complete IMDM (containing 20% fetal calf serum (GIBCO, Invitrogen), 100 U/ml pen-strep antibiotic solutions (GIBCO, Invitrogen, USA) and 150μg/ml ECGF. The HUVECs were grown on 2% gelatin (Sigma Aldrich, USA) coated tissue culture plates in complete IMDM in a humidified atmosphere of 5% CO₂ at 37°C. Unattached cells were removed after 6 hrs and the attached cells were passaged at 80% confluency.

2.1.3.1. In vitro expansion of primary hMSCs and CFU identification.

Isolated hMSCs were characterized by monitoring their ability to form Colony forming units (CFUs). Type I and Type II CFUs were identified [10] by seeding 1 × 10³ early passage cells in a 13 mm. sterile polystyrene petri dish (BD Falcon, USA). Sub-cultured and expanded hMSCs with all Type I colonies at passages 2-3 were characterized and used for further experiments.

2.1.3.2. FACS analysis of isolated hMSCs.

The isolated hMSCs were expanded up to passage 3 prior to analysis for surface markers [11]. The cells were labelled with the following fluorochrome-conjugated mouse anti-human antibodies: CD31-FITC, CD34-FITC, CD44-FITC, CD45-PE, CD73-PE (BD Biosciences, USA), Stro-1 (Invitrogen, USA), and goat anti mouse-FITC (Sigma USA). At least 50,000 events were acquired and analyzed using a fluorescent-activated cell sorting system (FACS-Aria, BDBiosciences, USA) and quantified using FACS DIVA software.

2.1.4. In vitro cell viability analysis of bare FNCs using hMSCs.

About 10,000 cells/well were seeded in a 24-well tissue culture polystyrene and incubated for 72 hrs with varied concentrations of FNCs (1-5 mg/ml) in 10% FBS supplemented Mesencult® medium (Stem Cell technologies, Canada). The cells grown on tissue culture polystyrene treated with and without 20 μl of 10% Triton X-100 (Sigma Aldrich) were considered as
negative and positive controls respectively. Following direct incubation, 50 μl of 10% Alamar blue® reagent (Invitrogen, Bangalore) was added to the culture medium of each well and incubated for 4 hrs. 100 μl of culture reagent was then transferred to a 96 well plate and the absorbance was read at 570nm and 600nm using Powerwave HT- Microplate reader (BioTek, USA).

2.1.5. Analysis of in vitro VEGF release and in vitro proliferation of HUVECs.

The net VEGF elution rate from conjugated Fibrin nanoconstructs was performed through Enzyme linked immunosorbent assay (ELISA) using RayBio® human VEGF-ELISA kit protocol (Ray Biotech, USA) [12]. In brief, standard calibration curves were prepared (R²= 0.98) with the known amount of standards provided with the kit. The VEGF present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated antihuman VEGF antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. The equation of each regression line was calculated and expressed in the form y = mx + c, where y = S/P ratio, m = slope of the line, x = ELISA titer (log 2), and c = the intercept value with the y-axis.

To assess the VEGF assisted in vitro cell proliferation of HUVEC, Pico Green® dsDNA Assay Kit (Invitrogen) was used as per the standard protocol [13]. In brief, HUVECs were plated in a density of 2 x 10^3 cells/well on gelatin coated 24-well tissue culture grade polystyrene plates. 500 μl MEM containing 10 % FBS with no ECGF was added. Cells treated with 10% Triton X-100 were considered as the negative control. In experimental samples, the cell culture media was supplemented additionally with 3mg/ml of FNCs and V-FNCS and incubated at 37°C. The media change was given every alternate day. The total cells per well were trypsinized and proliferation was evaluated at 24 hrs, 3rd day, 5th day and 7th day.
2.1.6. Chemotaxis of HUVECs in response to the VEGF release from FNCs.

For evaluating the chemotaxis of HUVEC cells in response to the VEGF released from FNCs, the HUVECs proliferated up to third passage having a typical cobble stone morphology were seeded over gelatine coated micro slides (IBIDI, Martinsried, Germany) [14]. Initial cell sprouting of HUVECs were visualized by SEM at 24 and 72 hours of experiment. Cells were also imaged on 3rd day and 7th day of experiment using confocal microscope TCS SP5 –II (Leica, Germany). The cells were stained with Texas red-labelled anti CD31 (Santa Cruz, USA).

2.1.7. In vitro and in vivo analysis of Tacrolimus release from FNCs.

Initially, the solubility of tacrolimus in Ethanol: PBS (1:2 ratio) buffered solutions were assessed at two different pH conditions (7.4 and 1.5). pH of the buffered solutions was adjusted using HCl / KCl. The freeze-dried FNCs (5 mg) conjugated with (1mg) Tacrolimus were soaked in 10 ml each of the buffered solutions with different pH. Determination of encapsulation efficiency (EE) and in vitro release of T-FNCs were performed in a shaking incubator (RivoTek, Chennai, India) maintained at 37°C. The suspensions were filtered through a Centricon® plus filter device of MW cutoff 300 KDa by centrifugation at 4000 rpm for 5 minutes. Filtrate sample (100 µL) was pipetted to a 96 well UV-transparent microplate - Costar® (Corning Life sciences, NY, USA) and analyzed for its absorbance maximum using a microplate spectrophotometer at wavelength of 213 nm in predetermined time intervals [15]. The in vivo drug absorption of tacrolimus was studied in adult Sprague-Dawley rats (N=12) weighing 210-240 g. In vivo studies are designed to look at the drug absorption of bare tacrolimus and tacrolimus loaded FNCs administered to SD rats via oral route. The approval of Institutional Ethical Committee (IEC) was acquired for performing the in vivo studies in rats and NIH guidelines were followed for care and use of laboratory animals. The rats were housed in separate cages and kept at a controlled temperature (23± 2°C) and relative humidity (45±5%). The animals were exposed to 12h light/dark cycle, fasted over night and had free access to sterile drinking water. The animals were divided into two groups (A
and B) of three each. Drug administration was performed through oral gavage. Rats of Group A were supplied with T-FNCs suspension 1mg/rat (4 mg/kg) through oral route, while Group B was administered with bare tacrolimus 1 mg/rat (4mg/kg). Further to the administration, blood samples (200µl) were drawn from the rats through retro-orbital sinus bleeding using non-heparanized capillary tubes. The blood was collected in 0.5 ml micro centrifuge tubes at predetermined time intervals. Serum from the blood samples was separated out by centrifuging at 12000 rpm for 20 minutes. The separated serum was then mixed with equal volume of 80 % methanol for precipitating serum proteins [16]. The samples were then analyzed for its drug content using HPLC system (LC-2010 A HT, Shimadzu) under the following conditions: Mobile phase - 100 % Acetonitrile, Column - Luna 5u C18, 250 x 4.6 mm (Phenomenex), Flow rate – 0.5 ml/min. A standard calibration curve was initially generated for tacrolimus concentrations between 6.25 and 500 µg/ml that yielded a good linear correlation (R²=0.995). Partial medium replacement was performed after each analysis by adding 100 µL of fresh buffered solutions to the suspension. Total drug absorption (mg/whole blood) was calculated assuming a blood volume of 15ml per rat.

2.1.8 Statistical analysis
All data were expressed as mean ± standard deviation. Statistical analysis of the data was performed via two-way analysis of variance (ANOVA) and Student's t test using Microsoft® Excel software; a value of p < 0.05 was considered significant (n = 6).

2.2 Development of 3D Agarose nano fibrin (AnF) cryogel matrix bed for differentiation and maintenance of human hepatocytes through sustained release of hepatocyte growth factor.

2.2.1 Synthesis and characterization of bare Agarose and nano Fibrin (nF) incorporated cryogel matrix beds.
Agarose (Low EEO; gelling temperature 38–40 ºC) was purchased from Qualigens Pvt. Ltd, India. The homopolymeric scaffolds of 6 to 8 wt. %
agarose have shown a rigid gel structure with considerable mechanical stability as equivalent to native healthy human liver tissue [17]. Agarose solution (6 wt %) was prepared in MilliQ water by microwaving for about 10 min until the solution became transparent. Growth factor loaded fibrin nanoconstructs was prepared as cryoprecipitate [18] based on the methodology explained in the previous section. To prepare AnF cryogels, 10 mg of freeze-dried nF was mixed with pre-heated agarose solution (6 wt %) and poured into 12 well-culture plates and allowed to solidify at room temperature. The Agarose-nano Fibrin (AnF) composite gel was then lyophilized for 20 hours to obtain AnF cryogels of desired mechanical strength [19]. Bare agarose cryogels were also prepared as controls as described above.

2.2.2. **Morphological analysis of AnF matrix beds and experimental controls**

The morphology of cryogels was analyzed by Scanning electron microscopy (SEM). For SEM analysis, sections of cryogels were sputter coated with Platinum using an Auto Fine Platinum Coater (JEOL JFC-1600) at 10 mA for 120 s before imaging under SEM (JEOL-JSM-6490LA).

2.2.2.1. **Contact angle measurements.**

Net hydrophilicity of bare agarose and AnF cryogels was obtained by measuring the contact angle of spreading sessile drops, with distilled water as the contacting solvent [20]. A drop shape analyzing system (DSA 100 EasyDrop Contact Angle Measuring System, KRÜSS, Germany) was used to determine the surface contact angles.

2.2.2.2. **Spectroscopic analysis**

To analyze the incorporation of nano fibrin into the AnF cryogel, Fourier Transform Infrared (FTIR) Spectroscopy was carried out. For FTIR analysis, 2 mg of bare Agarose, nano Fibrin and AnF composite cryogel were pelletized with 175 mg Potassium bromide (KBr) in a hydraulic pellet press and the spectrum was recorded in the frequency range 400- 4000 cm\(^{-1}\) using an RX1-FT-IR spectrometer (Perkin Elmer, USA).
2.2.2.3. Thermal, Mechanical, Porosity & in vitro degradation profiles of the cryogels matrix beds.

Thermal stability of bare Agarose, nano Fibrin and AnF cryogel was analyzed by the thermogravimetric analysis (TGA) using Exstar TG/DTA- 6200 system (SII NanoTechnology Inc., USA). 2 mg each of the samples was subjected to thermal degradation studies in the temperature range spanning 35-450°C at a progressive rate of 10°C min^{-1} under inert nitrogen environment [21]. Mechanical properties were evaluated by measuring the compressive strength of both 6% bare Agarose and AnF cryogels for which cylindrical cryogel pieces (5 cm x 5 cm) were prepared [22]. The compressive moduli of cryogels were measured by a UTS (Instron; Model 3365) with a load cell of 5 kN and the crosshead speed of 15mm/min at room temperature. Measurements were run six times for each sample and the average value was taken. The cryogel porosity was determined by the liquid displacement method [23]. Briefly, the scaffold pieces (both composite and control) were immersed into the dehydrated alcohol for 48 h until it got saturated. The porosity of the sample was calculated according to the formula:

\[
\text{Porosity} = \left(\frac{W_2 - W_1}{\rho V_1}\right) \times 100 \%
\]

where, \(W_1\) and \(W_2\) represent the weight of the sample before and after immersing respectively, \(V_1\) is the volume of cryogels before immersing in alcohol and \(\rho\) is the density of dehydrated alcohol, which is a constant. Volume of the cryogels was determined using the formula, \(V = \pi r^2 h\); where \(r\) is the radius and \(h\) is the height of the cryogel and are determined by using a vernier caliper. In vitro biodegradation study of the cryogels was carried out according to a previously reported method [24]. The cryogels were incubated in PBS solutions at three different pH (3, 5 and 7), with 1 x 10^4 U/ml of lysozyme concentration, in 15 ml tubes at 37\(^\circ\)C in a shaking incubator (Rivotek, India). The scaffolds were taken out at predetermined intervals, washed with distilled water and air dried. The degradability rate was calculated using the formula:

\[
\text{Rate of degradation} = \left(\frac{W_o - W_t}{W_o}\right) \times 100 \%
\]
where $W_o$ denotes the original weight and $W_t$ is the dry weight at day $t$ after lyophilization. The experiment was done with three samples and the average value is taken as the percentage of degradation.

2.2.2.4. **Biochemical staining of cryogels using PTAH (Phosphotungstic acid - haematoxylin)**

In brief, thin sections ($2\times2\times1$ cms.) of cryogels were sliced off and dehydrated in 80% ethanol for 30 min. and stained with eosin for five minutes. The excess stain was washed off with double distilled water (three times). The sections were then incubated with PTAH at $60^\circ$C for one hour [25]. After incubation, the sections were washed three times with double distilled water and dehydrated rapidly through three changes each of 80% and 100% ethanol. The dehydrated sections were then blot dried with filter paper and viewed under the bright field mode of a fluorescent microscope (Olympus BX-51).

2.2.3. **In vitro cell viability assay and proliferation studies.**

For evaluating the cell viability potentials of 3D cryogels, a direct proliferation assay was performed using human umbilical cord blood derived mesenchymal stem cells (hMSCs) [26]. In brief, the cryogel pieces ($2\times2\times0.5$ cms.) were sliced, sterilized by EtO gas and transferred to a 12-well cell culture plate inside a sterile biosafety cabinet. About $5 \times 10^3$ cells/cm$^2$ were seeded and incubated for 4 hours, then additional 2 ml of Mesencult® medium ( Stem Cell technologies, Canada) supplemented with 10 % FBS was added to each well and kept in a humidified 5% CO$_2$ incubator at $37^\circ$C for required time intervals (24 and 72 hours). After incubation with cells, the scaffolds were washed with PBS and placed into fresh sterile 12-well culture plates. 2 ml of Mesencult® media containing 1% (v/v) FBS and 10% (v/v) Alamar Blue® reagent (Invitrogen) was added to each well containing scaffolds, and the plates were incubated for 4 h at $37^\circ$C [27]. The cells treated with 10% Triton X-100 were taken as negative control. 100 μl of culture media was then transferred to a 96 well plate and absorbance was measured at 570nm and 600nm using Powerwave HT- Microplate reader (BioTek, USA). For imaging the cells proliferated over the cryogels,
samples were fixed with 2 % paraformaldehyde, dehydrated using alcohol gradient of 30, 60 and 100% ethanol for 10 min each. The samples were then placed over an Aluminium stub and sputter coated with Platinum before imaging under SEM. The proliferated cells were also visualized by nuclear fluorescent imaging by staining the cell nuclei with DAPI - 4′-6-Diamidino-2-phenylindole (Sigma, USA) and viewed under fluorescent microscope (Olympus-BX-51) with a UV laser filter at excitation of 358 nm.

2.2.4. Particle size and surface charge analysis of HGF loaded nano Fibrin constructs:

The size as well as the surface topography of HGF loaded nanofibrin constructs was analyzed by SEM (JEOL-JSM-6490LA). The hydrodynamic particle size was analyzed using the technique of Dynamic Light Scattering (DLS) by measuring the intensity of scattered light as the nano Fibrin underwent Brownian motion. The samples suspended in MilliQ water were analyzed using Nicomp™ 380 ZLS-ZP/Particle Sizer (Santa Barbara, USA) with a laser source emitting at 632.8 nm, and the scattered intensity measured at an angle of 90°. Surface charge and thereby the stability of the same system was analyzed from its zeta potential measurements using the Nicomp Zetasizer.

2.2.5. ELISA estimation of eluted HGF from nano Fibrin and AnF matrix bed.

The elution of HGF from within nano Fibrin as well as AnF cryogels was performed through enzyme linked immunosorbent assay (ELISA) using RayBio® human HGF-ELISA kit protocol (Ray Biotech, USA). In brief, the HGF present in a sample is bound to the wells by the immobilized antibody [28]. The wells were then washed and a secondary biotinylated antihuman HGF antibody was added. After washing away unbound biotinylated antibody, HRP conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate solution was added to the wells, wherein color developed in proportion to the amount of bound HGF. The stop solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.
2.2.6. In vitro hepatic differentiation of human Mesenchymal Stem cells (hMSCs).

AnF and bare agarose cryogel slices (2×2×1cms) were sterilized by EtO gas and transferred to 12-well cell culture plates inside a sterile biosafety cabinet. hMSCs at early passage (P4) were seeded over these cryogel slices at a density of 1x10^4 cells/cm² and maintained by incubating with Mesencult® medium supplemented with 10% FBS, 0.5µM dexamethasone (Sigma), 50 mg/ml ITS⁺ premix (25 mg/ml insulin, 25 mg/ml transferrin, and 25 µg/ml sodium selenite, Sigma) [29]. No additional growth factors were used in the medium, but the HGF loaded nanofibrin (nF) at a HGF concentration of 1 μg embedded within AnF cryogels. Medium was changed every fourth day and the cells grown over the matrices were regularly inspected for morphological changes under an inverted microscope equipped with CCD camera.

2.2.7. Confirmation of hepatic differentiation.

2.2.7.1. RT-PCR analysis: Total RNA was isolated from hMSCs control and HLCs using TRizol reagent (Invitrogen Life Technologies, USA) according to the manufacturer’s instructions. Total cDNA was generated using Power SYBR® Green RNA-to-Cₜ™ Kit (Ambion®, Life technologies, USA) and amplification was carried out using ABI 7300 Real-Time PCR System (Applied Biosystems, CA). Expression levels of gene encoding soluble organic anion transporter family, member 1A2 (SLCO1A2 - Entrez Gene ID: 6579) was analyzed [30] in HLCs proliferated over the cryogel matrices for 7, 14 and 28 days as well as hMSCs as control. Human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH - Entrez Gene ID: 2597) is used as housekeeping gene.

2.2.7.2. Periodic acid-schiff’s reagent (PAS) staining: PAS staining technique was used to determine the functional activity of differentiated hepatocytes [31]. Briefly, the hMSCs seeded and incubated over ANF cryogel matrices were taken at different time intervals, fixed with 4% paraformaldehyde at room temperature for 15 minutes. It was then oxidized with 1% Periodic acid for 5 minutes, rinsed with distilled water and treated with Schiff’s
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stained with haematoxylin (Sigma) for 30 seconds and observed under light
microscope. These HLCs underwent hepatic differentiation after 28 days,
and were trypsinised, seeded in T-25 culture flasks for attachment and were
also analyzed through PAS staining technique to visualize the colony
forming ability of HLCs.

2.2.7.3. Immunofluorescent imaging: Distinct morphological changes in cell
phenotype occurring during in vitro differentiation of hMSCs to HLCs were
visualized upto four weeks using a laser confocal microscope (Leica, Model
SP 5 II). In brief, the differentiation of cells was induced over bare agarose
and AnF cryogel matrices. Cells that underwent differentiation within four
weeks of incubation were trypsinized from the matrices, seeded on micro
slides (IBIDI, Martinsried, Germany) and fixed with 4% paraformaldehyde
for 20 min [32]. Permeabilization was done by adding 0.5% Triton X-100 for
5 min. Cells were then blocked by adding 1% FBS and washed with buffered
saline, incubated in room temperature with 50 μl of hepatocyte specific
OCH1E5 primary antibody (1:50 dilution)-mouse monoclonal IgG1 (Santa Cruz biotech, USA) for 45 min in a shaking platform followed by
immunostaining with 50 μl of Texas red-conjugated anti OCH1E5 antibody
(1:100 dilution)-chicken anti mouse IgG (Santa Cruz Biotech. USA) [23]. The
images were taken in DIC and fluorescent mode of a laser confocal
microscope.

2.2.7.4. FACS analysis of hMSCs and differentiated HLCs: Following 2 weeks of
in vitro differentiation of hMSCs to HLCs, the expression of surface markers
for true-to-type hMSCs (CD 73 & CD 44) and that for differentiated HLCs
CD 135 (Flt-3) & CD 117 (C-kit) was determined by FACS analysis [33].
The cells were permeabilized with 0.2% Triton X-100 for 5 min and
incubated with specific fluorophore conjugated antibodies for 20 min [PE-
Anti CD73, FITC-anti CD44, FITC-anti CD 135, APC-anti CD 117] (BD
Biosciences, San Jose, USA). Cells were blocked by adding 1% FBS and
washed with PBS, and FACS analysis was performed using a BD flow
cytometer (FACS Aria II).
2.2.8 Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis of the data was performed via two-way analysis of variance (ANOVA) and Student's t test using Microsoft® Excel software; a value of p < 0.05 was considered significant (n = 6).

2.3 Design and development of a Live Cell based Bioartificial Liver Support System for prolonged hepatic functionality maintenance.

2.3.1. Synthesis and characterization of high density hepatocytes laden Alginate Micro Beads (AMBs).

2.3.1.1. Cell culture of differentiated human hepatocytes

Isolation and in vitro culture maintenance of human Mesenchymal Stem cells (hMSCs) and its in vitro differentiation towards hepatocytes were done as per the reported protocols [34]. The cells committed differentiation towards hepatocyte lineage were taken for the experiments demonstrated from here onwards after maintaining them till passage 3 in Mesencult® medium (Stem Cell technologies, Canada) supplemented with 10% FBS, 0.5μM dexamethasone (Sigma), 50 mg/ml ITS+ premix (25 mg/ml insulin, 25 mg/ml transferrin, and 25 μg/ml sodium selenite, Sigma).

2.3.1.2. Synthesis of cell laded AMBs.

For developing high density cell laden hydrogel micro beads, Alginate was used as a cell supportive matrix and explained as Alginate Micro Beads (AMBs). AMBs were produced by means of simple extrusion – polymerization technique. In brief, 5% w/v Alginate solution was prepared by dissolving low viscosity Alginic acid-sodium salt (Sigma-Aldrich, USA) in 9 ml of 0.9 % sterile saline solution (PAN Biotech, Germany). Sterile cell suspension (1 ml.) containing approximately $1\times10^7$ of pre-differentiated human hepatocytes in complete cell culture media was mixed uniformly with 5% Alginate solution and extruded using a 10 ml. sterile syringe holding 18 guage needle (BD Luer-Lok™, USA). The cell laden alginate droplets were collected and allowed to polymerize in a flotation bath containing 10ml of sterile aqueous solution of 5% w/v calcium chloride.
(Sigma-Aldrich, USA) kept for stirring at 200 rpm. for 15 min. resulted in
the formation of cell laden Alginate Micro Beads (AMBs) of desired
dimensions. The AMBs thus polymerized were immediately sieved off from
the set-up and washed twice in 5 ml. Hank’s balanced salt solution (Gibco®,
USA). AMBs were then soaked immediately in 1 % sterile gelatin solution
and thus stored for short durations for analysis and rest of the experiments.

2.3.1.3. Analysis of surface porosity of cell laden AMBs.
The size as well as the surface porosity of cell laden alginate micro beads
was examined by scanning electron microscopy. Immediately after
synthesis, AMBs were fixed using 2.5% glutaraldehyde in PBS solution for
60 min at room temperature. Samples were then rinsed in 0.1M phosphate
buffer and then dehydrated in 50%, 70%, 95% and 100% ethanol for 5 min,
four times for each respective ethanol change, air dried overnight, placed
over carbon-tape mounted aluminum stub and gold sputtered before imaging
by SEM (JEOLJSM-6490LA).

2.3.2. Design and development of perfusion cassettes.
Perfusion cassettes with three different dimensions are designed for holding
the 3D matrix for perfusion experiments. The outer jacket of cassettes is
made of plexiglass and the inner chamber is designed to have two
cylindrical stainless steel O-rings. Single perfusion inlet and outlet for the
cassettes are provided as metallic nozzles of 8 mm. diameter.

2.3.3. Synthesis and characterization of 3D hydrogel matrix bed.

2.3.3.1. Synthesis of 3D hydrogel matrix bed.
Agarose solution (6 wt%) was prepared in MilliQ water by microwaving for
about 10 min. until the solution became transparent. 10 μg / ml solution of
hepatocyte growth factor (HGF) in Hank’s balanced salt solution was added
with 4 ml aqueous suspension of fibrinogen–FXIIIa cryoprecipitate and
stirred overnight to produce HGF loaded fibrin nanoconstructs (nF). To
prepare AnF hydrogel matrix, 50 mg of freeze-dried nF was mixed with pre-
heated agarose solution (6 wt%) and poured into the cylindrical O rings of
perfusion cassettes and allowed to solidify at room temperature obtain 3D
hydrogel matrix bed of desired mechanical strength. These AnF hydrogel
matrix embedded with high density hepatocytes laden Alginate Micro Beads (AMBs) constitute the live cell based hydrogel matrix of LC-BALSS. Incorporation of cell laden AMBs were done manually in accordance to the dimension of perfusion cassettes.

2.3.3.2. FT-IR spectroscopy of hydrogel matrix and its components.

The individual components and composite hydrogel matrix were evaluated for their chemical integration using Fourier transform infrared (FT-IR) spectroscopy. For FT-IR analysis, 2 mg of freeze-dried composite scaffold and their individual components were mixed thoroughly and pelletized with 175 mg. KBr and the spectrum was recorded in the frequency range 400–4000 cm⁻¹ using RX1-FT-IR spectrometer (Perkin Elmer, USA).

2.3.3.3. Strain-sweep analysis.

Dynamic strain sweep analysis of hydrogel was performed using a stress controlled Kinexus pro+ rotational rheometer (Malvern, UK) using 25 mm diameter parallel plate geometry at a gap distance of 0.5 mm and analysed in linear viscoelastic regime to observe the additive effect of individual hydrogel components on the mechanical stability of composite hydrogel matrix.

2.3.3.4. Elastic modulus measurement.

Mechanical strength of the control and composite hydrogel matrices were examined using a material testing system (Instron UTM - 3365, USA). Hydrogel matrix sections of 2 × 2cm were dissected and the elastic modulus was calculated by measuring the average stress and strain exerted by a load of 5 kN to the hydrogel matrices.

2.3.3.5. Analysis of fluid distribution through hydrogel matrix bed and AMBs

For analysing the uniformity of fluid distribution, the hydrogel matrix bed positioned in individual perfusion cassettes were subjected to a continuous fluid perfusion of 2 L. sterile deionized water having 1% crystal violet dye concentration for 24 hours at a rate of 5 ml/min. The composite hydrogel matrix as well as the individual AMBs after fluid perfusion was taken out from the perfusion cassette. Relative intensity of crystal violet dye distribution through hydrogel matrix bed and the AMB matrix was imaged.
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and analysed using ChemiDoc™ XRS system and Quantity One® software (Bio Rad, USA).

2.3.4. Perfusion bioreactor set-up and experiments to analyze the efficacy of cell laden 3D hydrogel matrix as LC-BALSS.

A custom made bench top perfusion bioreactor (Bioflo 310, New Brunswick, USA) with a segmented stirring media reservoir of 5 L. capacity was attached to the main perfusion system in which pH and pO2 was controlled via aeration with clean air and N2 (for pO2), and CO2 (for pH). The pH was kept at 7.4 and the pO2 at 25 %. The temperature of circulating media was maintained at 37°C by sterile water recirculation through media reservoir jacket. The automated bioreactor controller units were used to monitor and control pH, pO2 and temperature at every stage of experiment. The media perfusion through 3D hydrogel matrix loaded cassettes was established through platinum-cured silicone tubing (0.125 in. I.D × 0.25 in.O.D) (Nalgene).

2.3.4.1. Perfusion trials using non-flourescent dye and cell culture media.

The total blood flow through human liver represents approximately 25% of the cardiac output, and it is reported that a healthy human liver weighing about 2.6 kg can withstand a fluid flow of about 1500 ml / min. [35] The fluid flow for perfusion trials was standardised as 5 ml/min based on this knowledge as the average weight of cell laden hydrogel matrix designed for the experiment was approximately 9 g. Initially, for studying the response of embedded biomass withing the hydrogel matrix of LC-BALSS, the perfusion was carried out for extended durations (upto 28 days) using cell culture media. The perfusion of body fluids (human plasma and pooled serum) was experimented later and quantification of metabolites and detoxified products were carried out.
2.3.4.2. **Perfusion experiments using cell culture media.**

Complete MEM cell culture media supplemented with 10% FBS (Gibco®, USA) was prepared in sterile Milli-Q water and filled up to 3L. capacity of the bioreactor media reservoir. Complete perfusion circuit was established through platinum-cured silicone tubings connecting the perfusion cassette and media reservoir with a fluid flow rate of 5 ml/min. The entire experimental setup was maintained in a sterile clean room. Known volume of cell culture media after perfusion for specified intervals were collected through outlet and subjected for analysis.

2.3.4.3. **Perfusion experiments using human blood plasma.**

Fresh Frozen Plasma (FFP) bags containing approximately 400 ml. of human plasma was obtained from hospital blood bank of AIMS through prior written consent. Perfusion of human blood plasma was done by connecting the individual FFP bags to the perfusion chamber. Complete perfusion system with a fluid flow rate of 5 ml/min was maintained and an additional inlet for dissolution and monitoring of gaseous oxygen at a reported rate [35] of 0.2 ml / min was also provided in the circuit.
2.3.4.4. Perfusion experiments using human blood serum.

Human blood serum was synthesized by collecting approximately 200 ml. of whole blood from healthy individuals through the haematology department of AIMS by prior written consent. The whole blood was allowed to clot by leaving it undisturbed at room temperature for 15-30 minutes. The blood clot thus formed was separated by centrifugation at 2000 g for 10 minutes in a refrigerated centrifuge. 100 ml of fresh serum thus pooled was collected and taken for continuous perfusion experiment at a flow rate of 5 ml/min.

2.3.5. Quantitative and Qualitative estimations of hepatocyte functionality within 3D hydrogel matrix.

2.3.5.1. Analysis of hepatocyte proliferation through SEM and confocal microscopy

Longitudinal sections (2×2×1cms) of cell laden 3D hydrogel matrices were taken at specified intervals during cell culture media perfusion experiment kept for 28 days. Regions of interest having embedded cell laden AMBs were further dissected as microsections using a cryostat (Leica CM1510s). The progressive cell proliferation within the AMBs was visualized on 3rd, 14th and 28th day through SEM after fixing and processing the matrix sections [36]. Respective immuno stained microscopic images were also captured using laser confocal microscope (Leica, Model SP 5 II) by fixing and staining the hepatocytes with anti OCH1E5 antibody (1:100 dilutions) and Texas red-conjugated chicken anti mouse IgG (Santa cruz Biotech. USA) [37].

2.3.5.2. Immuno blotting analysis - expression of Organic Anion Transporter Polypeptide (OATP)

The progressive expression of Organic Anion Transporter Polypeptide (OATP) during the cell proliferation within the AMBs loaded with hMSCs and Hepatocytes were visualized on 3rd, 14th and 28th day through immuno blotting analysis. In brief, the cells grown over the AMB matrices were lysed in CelLytic™ reagent (Sigma-Aldrich, USA). The cell lysates were cleared by centrifugation at 15,000 rpm for 10 min at 4° C. The separated proteins were then electrophoretically resolved and transferred to a PVDF membrane
by applying 30 mV overnight at 4° C. The resulting PVDF membrane was then washed twice with PBS containing 0.1% Tween 20 (PBS-T) and subsequently blocked overnight with PBS-T containing 5% non-fat dry milk (Sigma-Aldrich, USA). Membranes were then incubated overnight at 4° C with one of the following primary antibodies OATP-A (E-7) and anti-GAPDH (1:5000) (Santa Cruz Biotech. USA). Blots were then washed 3–4 times with PBS-T and incubated for 1 h. with respective HRP-conjugated secondary antibodies (Santa Cruz Biotech. USA). The membranes were then washed 3 times with PBS-T, incubated with an enhanced chemiluminescent substrate (Millipore, USA) and imaged under epi-white illumination using ChemiDoc™ XRS system and Quantity One® software (Bio Rad, USA)

2.3.5.3. Estimation of localized Gap junction protein - Connexin-32 (Cx32) expression.

The progressive localization of gap junction protein - Connexin-32 (Cx32) in the proliferating hepatocytes was visualized on 3rd, 14th and 28th days by analysing the AMB matrix sections through immunofluorescent staining [38]. The cell laden AMB matrices were fixed and stained with Connexin 32 Antibody (E-17) (1:100 dilution) and FITC conjugated donkey anti-goat IgG- (Santa Cruz Biotech. USA). The immuno stained images were captured using a fluorescent microscope (Olympus-BX-51).

2.3.5.4. Alanine Transaminase activity estimation.

Measurement of the Alanine Transaminase (ALT) activity was made during cell culture media perfusion experiment kept for 28 days. ALT assay kit (Cayman chemical, USA) was used as per manufactures instructions [39]. The evaluation of NADH oxidation to NAD⁺ in the cell culture media perfused for specified intervals was monitored at 340 nm. using Powerwave HT- Microplate reader (BioTek, USA).

2.3.5.5. Quantification of Ketamine to DNK conversion.

Metabolic conversion of known quantity of Ketamine (anaesthetic drug) to Dehydronorketamine (DNK) which is a measure of the activity of hepatocyte microsomal cytochrome P450 enzyme (CYP₃B6) was performed during cell culture media perfusion experiment. 500ng/ml of ketamine and its
subsequent demethylation to DNA was estimated in the eluted media using Ketamine ELISA kit (Randox Laboratories, UK)

2.3.5.6. Quantification of Albumin synthesis-media and plasma perfusion.
The quantification of total albumin during perfusion of cell culture media (long duration experiment) and human blood plasma (short duration experiment) was performed using Micro-Albumin competitive ELISA test (Orgentech) as per manufacturer's instruction [40]. The concentration of albumin eluted to the media at specified intervals was determined photometrically at 450 nm.

2.3.5.7. Ammonia to Urea conversion- plasma perfusion.
The metabolic conversion of Ammonia to Urea by functional hepatocytes is typically reported to be like Berthelot reaction [41] and was analyzed by perfusing human blood plasma. The absorbance of 2 mg. of ammonia in the plasma perfusion system was measured at 580 nm and its subsequent conversion to urea was measured at 630 nm. The assay procedure was standardized as per the manufacturer’s protocol of Urea assay kit (Cell Biolabs, USA).

2.3.5.8. Cytochrome P450 activity analysis.
The hepatocytes subjected for short duration plasma perfusion was analysed for the activities of cytochrome P450 sub family enzymes (CYP1A1 and CYP2D6). The specific fluorescent assays involving the metabolic conversion of Alkylxoyresorufin and Propafenone to their respective fluorescent products was estimated at 585 nm and 315 nm, respectively using Cytochrome P450 assay kits (Sigma-Aldrich, USA).

2.3.5.9. Fibrinogen synthesis - blood serum perfusion.
Progressive fibrinogen synthesis by the functional hepatocytes during continuous serum perfusion for 24 hours was estimated at 450 nm. The assay procedure was standardized as per the manufacturer’s protocol of Human Fibrinogen ELISA kit (Abcam, USA).

2.3.7. Statistical Analysis.
All results were obtained from triplicate samples. Quantitative results are represented as mean± standard deviation. Student’s t-test was performed to
determine statistical significance in results. p value of less than 0.05 (p < 0.05) was considered to be statistically significant.

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