Role of HGF- loaded Fibrin Nano constructs in Alginate/Agarose Scaffolds for Liver assist devices

SYNOPSIS OF THE THESIS SUBMITTED TO AMRITA VISHWA VIDYAPEETHAM UNIVERSITY FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

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February 2014
AMRITA SCHOOL OF ENGINEERING
AMRITA VISHWA Vidyapeetham, KOCHI - 682041
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DECLARATION

I Mr. Praveen G (KH.NS.D*NMS 08008) hereby declare that this synopsis of the thesis titled “Role of HGF- loaded Fibrin Nano constructs in Alginate/Agarose Scaffolds for Liver assist devices” is a bonafide record of original work done by me under the guidance of Dr. Krishnaprasad Chennazhi, Associate Professor, Amrita Centre for Nanosciences and Molecular Medicine, Kochi and to the best of my knowledge and belief, it contains no material previously published or written by another person, no material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Place: Kochi
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Chapter 1: Introduction

Modifications of polymers with biomolecules containing cell recognition protein motifs in nanoscale have recently attracted much attention for enhancing the cell adhesiveness of substrates in tissue engineering. For regeneration of complex organs such as liver, besides good biocompatibility and biodegradability, the scaffolds ideally should possess the 3D spatial architecture of the liver as well to promote gene expression related to cell growth and liver-specific functions. Thus, 3D scaffolds embedded with protein nanoconstructs have drawn increasing interest in the biomedical arena owing to their additional ability to deliver drugs at optimal doses, often resulting in increased therapeutic efficacy of the drug, weakened side effects, and improved patient compliance. Biodegradability, bioactivity, drug loading capacity as well as the drug release kinetics of such protein nanoconstructs are also of prime concern in processing them as novel nanocarriers in tissue engineered constructs.

Agarose is a natural biopolymer derived from red algae, which consists of a galactose-based backbone with alternating β-D-galactose and 3,6-anhydro-α-L-galactose units. Fibrin is one such natural bio-polymer, synthesized during the coagulation cascade and is also a less exploited research material particularly in its nano size scale as body’s choice of biomimetic agent for absorption and delivery of growth factors. Initial part of this research work explains the combinatorial strategy adopted for the synthesis and characterization of Agarose based cryogel scaffolds by incorporating growth factor loaded nano fibrin cell recognition moieties for liver tissue engineering applications.

Since a major obstacle in engineering liver tissues for clinical use is the limited availability of human cells, later part of this research work describes the prospect of in vitro differentiation of the readily available human mesenchymal stem cells (hMSCs) from umbilical cord blood into hepatocyte like cells (HLCs) over Agarose-nano Fibrin (AnF) cryogel matrices and thus contributes an executable strategy to overcome liver tissue shortage. But, the practical inability of AnF cryogel matrix in maintaining prolonged hepatic functionalities owing to its weak mechanical sturdiness experimented using a perfusion bioreactor leads us to develop a hydrogel based live cell loaded 3D construct for conducting prolonged perfusion experiments.

A biological liver assisted system was thus designed and developed in the final part of this research work by embedding metabolically active human hepatocytes inside alginate
micro beads and incorporated within a 3D hydrogel matrix framed of Agarose-nano Fibrin milieu. In order to study the prolonged response of embedded hepatocytes to the in situ liver fluid dynamics, the liver assisted system thus designed was positioned in a perfusion bioreactor system by giving a controlled fluid flow of cell culture media and body fluids (blood plasma and serum). Hepatocytes proliferated in this biological liver assisted system spontaneously forms aggregates within the hydrogel matrices and retain their metabolic and detoxification activities for longer durations.

Specific Objectives of the study:
1. Synthesis of biocompatible fibrin nanoconstructs through surfactant free technique and its thorough physicochemical characterization.
3. Development and characterization of Agarose nano fibrin (AnF) cryogel matrix bed and evaluating its potential for slow and sustained release of growth factors from nano Fibrin (nF) moieties.
4. Design and development of a live cell based bioartificial liver support system for prolonged hepatic functionality maintenance.
5. Standardization of in situ hepatic simulation conditions in a perfusion bioreactor followed by the extensive metabolic and detoxification assays to prove hepatocyte functionalities.

Review of literature
The ability of liver to regenerate is well known although complete regeneration need not take place after liver injury ¹-³. Owing to this inherent potential for self-regeneration restoration of liver architecture and its functional activity is a subject of continued research. This is supported by the fact that hepatocytes are known to be anchorage dependent and can be immobilized on scaffolds ⁴, encapsulated as aggregates or cultured ex vivo to form liver “organoids” and surgically transplanted ⁵. While porous scaffold systems continue to be explored for use in liver constructs, many of these scaffold architectures are essentially two-dimensional surfaces, from the hepatocyte perspective, perhaps limiting their utility toward the full recapitulation of three-dimensional cues ⁶. Hence, fabricating 3D biomimetic scaffolds with modifications of polymers and
incorporation of biomolecules containing cell recognition protein motifs in nanoscale have recently attracted much attention for enhancing the cell adhesiveness of substrates in tissue engineering \(^7\). Cryogelation is the method of choice especially for the preparation of macroporous natural polymeric scaffolds, as it involves the formation of ice crystals inside polymer solution during freezing that eventually act as a porogen during lyophilization resulting in the formation of a porous 3D architecture \(^8\). Tissue engineering of liver over such macro porous polymeric scaffolds thus represents an improvement over the traditional approach of hepatocyte transplantation as this methodology remains largely experimental and must overcome a number of significant hurdles before it will become a viable clinical modality \(^9\). Since hepatocytes are known to be anchorage dependent, they are immobilized on scaffolds, or encapsulated in aggregates, or cultured \textit{ex vivo} and surgically transplanted \(^10\). One of the most promising approaches for treating liver failure patients is the development of extracorporeal support devices as dialysis systems that would process the blood or plasma of liver-failure patients and enabling sufficient regeneration of the host liver tissue or serve as a bridge to transplantation \(^11\). The non cell based artificial devices are intended to remove protein bound and water soluble toxins without any providing synthetic/metabolic functions \(^12\). Extracorporeal Liver Assist Device (ELAD) is the only bioartificial device in which a human hepatocyte cell line (C3A) is used, which is comprised of a dual pump dialysis system and several “metabolically active” cartridges \(^13\).

In this backdrop, it is proposed that bioartificial liver support systems containing metabolically active hepatocytes functioning as extracorporeal bioreactors will be more effective than completely artificial systems which provides only excretory capacity and toxin removal.

**Chapter 2: Materials and Methods**

**Green route synthesis of Fibrin nanoconstructs (FNCs) and its characterization**

A surfactant-free water-in-oil emulsification–diffusion system was designed to induce thrombin–FXIIIa mediated cross linking of fibrin molecules. Aqueous suspensions of both fibrinogen–FXIIIa cryoprecipitate and thrombin 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 70–80 °C for 6–8 h, wherein the cross linking of fibrin clot and its uniform dispersion in oil occurred. Tacrolimus (a candidate drug) and Vascular endothelial
growth factor –VEGF (a candidate growth factor) was conjugated separately within FNCs to study its control release efficiency. FNCs were characterized using DLS and Zeta potential measurements and structural morphology was analyzed using SEM and AFM.

**In vitro & in vivo assays to prove FNCs efficacy as controlled delivery agents**

The *in vitro* and *in vivo* release rate of tacrolimus was analyzed using HPLC. *In vivo* studies are done in Adult Sprague Dawley rats (N = 12) weighing 210–240 g. The net *in vitro* elution rate of VEGF from conjugated fibrin nanoconstructs was performed through ELISA. *In vitro* analysis of VEGF assisted cell proliferation and tube formation of HUVEC was also done.

**Synthesis of Agarose-nano Fibrin (AnF) cryogels & its physicochemical characterization**

Agarose solution (6 wt%) was prepared in MilliQ water by microwaving for about 10 min. until the solution became transparent. 1 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 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. This composite gel was then lyophilized for 20 hours to obtain ANF cryogels of desired mechanical strength. The cryogels were characterized using SEM, FT-IR and TGA analysis. Mechanical stability, porosity, swelling ratio, *in vitro* cell viability, attachment and proliferation etc. was also evaluated.

**In vitro differentiation of human Mesenchymal Stem cells (hMSCs) to Hepatocyte Like Cells (HLCs).**

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 1x104 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). 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.

**Design and Development of live cell based bioartificial liver support system**

The live cell based bioartificial liver support system (LC-BALSS) is composed of a core Agarose (6 wt%) hydrogel matrix embedded with approximately $10^7$ hepatocyte cells laden Alginate micro beads (5 wt%) and 10 mg of HGF loaded nanofibrin. This core hydrogel matrix is moulded inside a perfusion chamber made of plexiglass. The perfusion chamber is designed to have two cylindrical O-rings (15 mm in diameter and 25 mm height). The bioartificial liver support system is connected to an external perfusion bioreactor system (Bioflo 310, New Brunswick, USA) via. silicon tubing to ensure a fully controlled cell culture environment.

**Evaluation of prolonged hepatic functionalities in a perfusion bioreactor system**

Dynamic perfusion of cell culture media and body fluids (blood serum and plasma) was given through LC-BALSS for extended duration in bioreactor system. A fluid perfusion at 5 ml/min was performed as continuous and discontinuous perfusion loops. Extended hepatocyte functionality assessment was performed by evaluating ALT and Cytochrome P-450 activities, Albumin and Fibrinogen secretion, Ammonia to Urea conversion and also by evaluating the SLCO1A2 gene and OATP protein expressions. Immuno-fluorescent imaging of hepatocyte specific antigen (OCH1E5) and gap junction protein (Connexin-32) was also done at varied time intervals.

**Chapter 3: Results and Discussion**

**Green route synthesis of Fibrin nanoconstructs (FNCs)**

![Fig. 1](image)

Fig. 1. (A) Schematic chart representation of the main steps involved in the preparation of the surfactant-free water-in-oil emulsification–diffusion system designed for
preparing fibrin nanoconstructs and (B) Scanning electron microscopy representation of fibrin nanoconstructs.

**Fig. 2.** Atomic force microscopy topography images of fibrin nanoconstructs.

**Agarose-nano Fibrin (AnF) cryogels for hepatic tissue regeneration**

The macroporous nature of bare agarose and AnF cryogels was evident from the scanning electron micrographs (Fig.3 A&B). Higher magnification images of the ANF composite cryogel matrix depicted in Fig. 3 B, C & D that clearly reveals the homogeneous distribution of HGF loaded Fibrin nanoconstructs (nF) throughout the matrix. Such a uniform distribution of fibrin moieties over the matrix would permit an easy diffusion of the incorporated growth factor HGF and eventually help in a rapid cell adherence. The uniaxial distribution of pores in AnF matrix further holds the promise of using this construct as an ideal biomaterial that mimics the hepatic micro environment.
Fig. 3. SEM images of the cross-section segment of (A) bare 6 % Agarose and (B) ANF cryogels (C & D) Higher magnifications of the ANF cryogels depicting a uniform distribution of HGF loaded fibrin nanoconstructs (nF) over the cryogel matrices. Magnified regions of interest are indicated in boxes and given as subsequent images.

Matrix assisted differentiation of hMSCs to HLCs
The immunoflorescent imaging of hepatocyte specific antigen (OCH1E5) also revealed the progression in metabolic activity of differentiated HLCs upto 4 weeks of in vitro expansion over AnF cryogel matrix (insets of Fig. 4 B & D). The PAS staining of these functional HLCs (day-28) specifically showed the colony forming ability after proteolytic detachment from ANF matrices (Fig. 4 E). The real time relative expression of solute carrier protein (SLCO1A2) was proved further through gene expression analysis of this transcriptome (Fig. 4 F)

Fig.4. Confocal microscopic DIC images of (A) undifferentiated hMSCs of Day-1 and (C) in vitro differentiation induced to HLCs - day 28 adhered on plastic surface (B & D) Corresponding immunoflorescent images of hMSCs and HLCs stained with Texas red-conjugated hepatocyte Specific anti OCH1E5 antibody (E) Photograph of PAS stained day-28 hepatocyte colonies in T-25 flask. (F) Relative expression of SLCO1A2 mRNA analysed through quantitative real-time PCR. (Microscopic images are at 40× magnification) (*p < 0.05, Anova).
Design and development of Live cell based bioartificial liver support system (LC-BALSS)

Fig. 5. Pictorial representations depicting the stepwise procedure of developing live cell based bioartificial liver support system (LC-BALSS).

Extended hepatocyte functionality assessment of LC-BALSS was done and the quantitative estimation of net Albumin secretion (Fig 6 A) as well as the Ammonia to urea conversion (Fig 6 B and C) was done during three days continued plasma perfusion.

Fig. 6. Quantitative estimations of (A) Albumin secretion and Ammonia to Urea conversion (B, C) during 3 days continued plasma perfusion through LC-BALSS

Immuno-fluorescent imaging of time dependent expression of gap junction protein (Connexin-32) on functional hepatocyte cell wall was imaged up to three weeks (Fig 7 A) and proteomic expression of Organic anion transported polypeptide (OATP) was done
through immune blotting (Fig 7 B) showing the continued expression of these metabolically active markers in embedded hepatocytes within LC-BALSS.

![Figure 7](image)

**Fig. 7.** Progressive expressions of (A) Gap junction protein (Cx-32) and (B) Organic transporter peptide (OATP) analyzed among embedded hepatocytes subjected for three weeks cell culture media perfusion in LC-BALSS.

**Chapter 4: Conclusions**

Fibrin nanoconstructs synthesized through surfactant free green chemistry approach was proved to be an excellent biodegradable nanocarrier with remarkable potential for delivering specific drugs/growth factors in tissue engineering scaffolds. The AnF cryogels that mimic hepatic microenvironment developed through this study could be a promising 3D platform for inducing matrix assisted differentiation of multipotent stem cells to hepatocytes under static *in vitro* cell culture conditions. Such approaches would be feasible to generate a small, but functional liver tissue systems. The ultimate focus of this research work was to generate an implantable liver tissue that can extend a long-term sustenance by replenishing and supporting the hepatocyte proliferation. Parallel focus was also given to develop an artificial live cell based liver support system for *in situ* therapeutic and drug screening applications. The fundamental success attained through the development of LC-BALSS can further be translated from ‘bench to bedside’ directly as described strategy or through modifications using similar combinatorial cell-supportive biomatrices in an extracorporeal bioreactor setup to further enhance the metabolic and detoxification hepatic functionalities through successful liver organoid growth.
REFERENCES

LIST OF PUBLICATIONS, AWARDS & ACHIEVEMENTS

Patent:

Publications in peer reviewed Journals:

Publications in Conference proceedings:
**Awards/Achievements:**

- First author manuscript included as a "Research Highlight of 2012" under the Biology & medicine section of IOP Nanotechnology *Highlights 2012* and "Research highlight - Chemistry" of Nature India *Archive 34(3).*

- Recipient of visiting researcher fellowship of Indo-US Science and Technology Forum (IUUSTF) under Indo-US joint R&D network *(95-2009/2010-2011)* to undergo research training at Dept. of Material Science and Engineering, Stanford University, USA.

- Recipient of Senior Research Fellowship (SRF) by Council of Scientific and Industrial Research (CSIR), Govt. of India. Award No: *9/963(0019)2K12-EMR-I* dated 24-02-2012.

- Recipient of Junior Research Fellowship by DST *(Nanomission)* - Project under Nanoscience & Technology Initiative. Govt. of India. *(June 2008- April 2011).*

- Won first prize for the poster presented in Amrita Endocrine and Diabetic Foot Conference 2012 (AEDFC ’12)