ADA-GELATIN-FIBRIN BASED 3D NICHE FOR HepG2 GROWTH

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DECLARATION

I, ANUSREE K.S., hereby certify that I had personally carried out the work depicted in the dissertation entitled, *"***ADA-GELATIN-FIBRIN BASED 3D NICHE FOR HepG2 GROWTH***"*, under the direct supervision of Dr. Anugya Bhatt, Scientist E, Division of Applied Biology, Department of Thrombosis Research Unit, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Thiruvananthapuram, Kerala, India except where due acknowledgment has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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ADA-GELATIN-FIBRIN BASED 3D NICHE FOR HepG2 GROWTH

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SYNOPSIS

Liver is the largest internal organ and performs many important physiological functions. Because the organ plays a vital role in many detoxification reactions it continuously met with chemicals and toxins. Damage of liver by various diseases/toxins are of major concern due to changes in lifestyle such as alcohol consumption and unhealthy food habits. Liver diseases are the fourteenth leading cause of mortality in the world and the tenth most common cause of death in India. The only treatment for liver diseases is liver transplantation but the treatments are always ineffective due to the shortage of donors. Cell therapy using hepatocytes or other hepatocyte alternatives such as stem cells or other terminally differentiated cell are promising approaches, however success of such treatments have not been proven. The problem with allogeneic hepatocytes is immune rejection and for autologous cells the inefficiency of hepatic cells because they are taken from diseased condition. Another option is stem cells but they are always associated with immune rejection, ethical concerns etc. So the best possible option is use of HepG2 cells they are very similar to hepatocytes in its origin and functions.

Tissue engineering is a newly developing field with the coordination of biology with technology. Tissue engineering is used to create tissue construct that can resembles the physiological atmosphere of body by using conventional 2D culture. But this approach may not be effective due to the restriction of cells to perform like the physiological system. So a new approach known as 3dimensional culture in which the cells are able to migrate into three dimensions which closely mimics the in-vivo. The very popular approach for this is hydrogels using natural or synthetic polymers. Hydrogels behave like aqueous environment exactly like the ECM of cells. Hydrogels are three-dimensional polymeric networks that have the capacity to absorb and retain high amount of water within their network. Natural polymers such as proteins and carbohydrates are used for preparing hydrogels because syntheticpolymersareverydifficulttodegradeandcausesimmunereaction.In the present study ADA gelatin and fibrinogen based hydrogel system is being optimized to promote HepG2 cells growth and viability. This dissertation is divided into 4 chapters. Chapter I provide background knowledge of the topic, review of research works done so far related to the topic, the gap area identified, hypothesis formulated and the objectives of the study. Chapter II compiles all the materials, reagents and equipment's used in this study including the methodologies followed during each of the experiments. The results obtained are compiled in chapter III. The observations are discussed in the light of knowledge from related literature. Chapter IV summarizes the work and conclusive remarks are made. In chapter 1 introduction and review of literature gives idea about tissue engineering and the developments happens during time. The chapter also describes the various approaches of tissue

engineering like 2D culture 3D culture. Hydrogel system is a prominent 3D system where cells can grow exactly like physiological system. The chapter also describes various natural and synthetic polymers used for making hydrogels and their drawback. The chapter also describes the use of liver like cells like stem cells and HepG2 cells for tissue engineering in detail.

Chapter II describes various materials and methods selected for the study. It describes various methods adopted for making and characterize hydrogel and raw material. The ADA was prepared from sodium alginate using sodium meta periodate and characterized by degree of oxidation, aldehyde content, molar mass calculation, FTIR, and NMR. Synthesized hydrogel were characterized by FTIR, gelation time, in-vitro swelling, degradation study, rheological analysis, SEM and micro CT. In-order to find weather the hydrogel support cell culture MTT assay was performed on direct contact for 24h .The biologically characterized hydrogel was then used for seeding of HepG2 cells. The viability of cells are analyzed by live dead staining and actin staining. To characterize the functions of seeded HepG2 on hydrogel indocyanine green uptake assay, LDL uptake assay, Glycogen storage assay, CYPP450 staining, ammonia clearance and albumin assay for 24 hours were done.

Chapter III includes all the results obtained in the work. The synthesized ADA shows appropriate degree of oxidation, aldehyde content and molar mass and expected functional group. The hydrogel constructed by using ADA, fibrin and gelatin shows good cross linking property, low gelation time, better porous structure with unequally distributed interconnected pores. The HepG2 cells seeded on the hydrogel after 24h shows morphological changes, good uptake of indocyanine green dye, LDL, shows positivity on Glycogen storage assay, CYP450 staining, significant ammonia concentration and Albumin concentration

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ABBREVIATIONS

2D	2 Dimensional
3D	3 Dimensional
ADA	Alginate dialdehyde
BSA	Bovine serum albumin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide mg Milligram
ECM	Extracellular matrix
FBS	Fetal Bovine Serum
FTIR	Fourier transform Infrared Spectrophotometer
HBSS	Hank’s Balanced Salt Solution
ICG	Indocyanine Green
Micro CT	Micro-Computed Tomography
Ml	Millilitrelitre
Mm	Millimeter
MSC	Mesenchymal stem cell
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate Buffered Saline
WHO	World health organization

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

The liver, largest organ in the body has a complex architecture and performs a myriad of functions. Even though the liver is highly regenerative, drugs and toxins or viral infections can cause extensive damage to hepatocytes, reducing function and regeneration. End stage liver disease occurs when large parts of the liver become damaged beyond repair and the liver is no longer able to function. Liver failure develops gradually and caused due to many factors. The common causes of chronic liver disease are Hepatitis B & Hepatitis C. In developed countries, drug toxicity and alcoholic liver diseases are of major concern, while in developing countries viral Hepatitis is a major clinical concern. Globalization and immigration have resulted in the global spread of viral Hepatitis and this disease has now become a major world problem. The world health organization (WHO) report shows that 2.5% of total deaths in the world are because of liver disease, and this will become the 14^{th} most common cause of death by 2030. In the UK, liver disease is the 5^{th} most common cause of death, and continues to rise. It was predicted that more than fifty million people in the world would have chance of died due to chronic liver disease in the near future. In 2000s, liver cirrhosis was the cause of death of nearly eight lakh people worldwide. It was 14^{th} leading cause of mortality in the world. In India, deaths due to liver diseases were estimated to be 216,865 as per the data published by WHO in May 2014. The WHO report also points out that around 10 lakh new cases of liver cirrhosis are getting reported each year and that it is the tenth most common cause of death in the

nation. Liver diseases may affect one in every five Indians. Extensive damage to hepatocytes can lead to liver failure. As a result of liver failure, multi-systemic complications arise, leading to neurological impairment, hematological disturbance, renal dysfunction and metabolic abnormalities. Liver transplantation is the only available and, usually, successful treatment option. However, the number of liver transplantations is limited because of the lack of availability of donor organs. Cell-based therapies have been proposed as an alternative to whole organ transplantation, as a temporary bridge to transplantation, and/or an adjunct to traditional therapies during liver regeneration. The three main approaches that have been proposed are: transplantation of isolated hepatocytes, implantable tissue-engineered constructs, and perfusion of blood through an extracorporeal bio-artificial liver device containing parenchymal liver cells called hepatocytes. Despite significant investigations into each of these areas, progress has been stymied due to the propensity for isolated hepatocytes to rapidly lose viability and key liver-specific functions upon isolation from the native microenvironment of the liver. Thus needs for providing microenvironment where liver cells can grow and stay functionally active remain. Different 3D gel based approaches have been used to provide a three dimension niche to the cells to mimic in-vivo and better cell survival.

Different synthetic materials have been used to fabricate scaffolds, but primary limitations with such materials are biocompatibility concerns, inability to support cell attachment, and undesirable degradation rate. The other group of material or natural biopolymers includes protein-based (i.e. Fibrin, collagen) materials which provide binding sites for cell adhesion, or the polysaccharide-based (i.e., alginate, chitosan, and agarose) scaffolds which provide good porosity/strength, but usually require further cell-attachment modification to promote cell adhesion and proliferation .Fibrin gel, a bio polymeric material, provides numerous advantages over others primarily due to its excellent biocompatibility, promotes cell attachment, and can degrade in a controllable manner. In the present study a formulation which can provide stability, good cell attachment and proliferation is being optimized using ADA-gelatin and fibrin system. This formulation was evaluated for supporting liver cells viability and functionality using HepG2 cell lines.

1.2 REVIEW OFLITERATURE

Tissue and organ transplantation is a widely accepted treatment for the millions of patients suffering from the loss or failure of tissues or organs as a result of accidents or diseases. But this approach is always lagged because of the shortage of donors. Fortunately tissue engineering approaches have paved way through. The primary goal of tissue engineering is to develop strategies that regenerate living, healthy, and functional tissues that can be used as tissue grafts or even organ replacements. In order to achieve this, conventional approach is to grow cells in a 2 dimensional niche. However, 2D culture system doesn't mimic the in-vivo system and cannot replicate the native tissue. Thus the preferable approach is 3dimensional culture system that serves as temporary supports for cell growth and new tissue development. The scaffold may be designed as purely a structural support providing passive cues to the cells or with biological cues incorporated into the scaffold to guide cell and tissue growth. There are two main strategies in utilizing scaffolds for tissue engineering: (i) cells are seeded onto prefabricated porous scaffolds or (ii) Cells are encapsulated during scaffold formation. In the former strategy, a wide range of hydrophilic and/or hydrophobic precursors can be used, and the fabrication process may involve harsh solvents and/or reactants, as long as the final product is cell friendly. In cell encapsulation strategies, the process by which scaffolds form must be cytocompatible, which significantly limits the number of suitable materials and formulations. This strategy, however, offers several advantages. Because the encapsulation process is mild, this strategy is often employable as an injectable system where cells suspended in a liquid precursor solution are delivered in vivo to the site of interest. In addition, by curing the hydrogel directly at the site of interest, the precursor solution can diffuse into the adjacent tissue, leading to enhanced adhesion of the scaffold to the tissue without requiring glue or sutures.

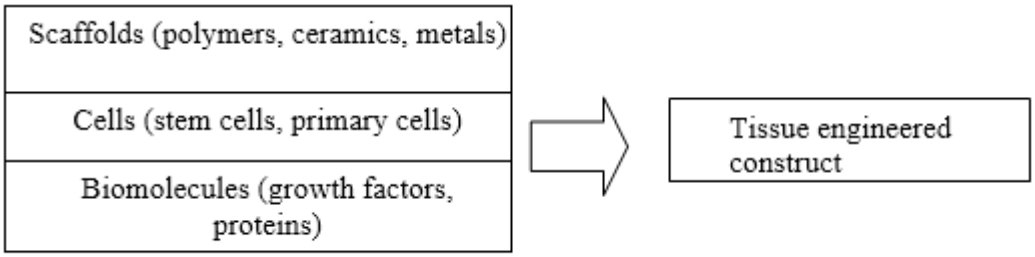


Fig 1. 1 The concept of tissue engineering

1.2.1 BIOMATERIALS; SYNTHETIC AND NATURAL POLYMERS IN TISSUE ENGINEERING

In tissue engineering scaffolds play a crucial role. Biodegradable polymers with processing flexibility are preferred for making scaffolds for tissue engineering. Variety of materials can be used for making scaffolds such as metals, ceramics, and polymers. Of these materials, polymers possess great processing flexibility and biodegradability that can be endowed through structural design**.** Studies show the capacity of synthetic as well as natural polymers for the construction of scaffolds for tissue engineering [Nair et al., 2007]. Therefore, polymers including natural polymers, natural-polymer-derived materials, synthetic polymers, and synthetic polymers made of natural monomers or modified with natural moieties are now widely used as dominant scaffold materials in tissue engineering [Tian et al., 2012]. The synthetic biodegradable polymers that are widely used in tissue engineering, including polyesters, polyanhydrides, polyphosphazenes, polyurethane, and poly (glycerol sebacate) [Baolin et al., 2009]. Aliphatic polymers are used frequently for tissue engineering because these ones usually undergo degradation in their ester backbone. PGA is one of the most widely accepted polymers for scaffolds due to its highly crystalline structure and chain-structural regularity [Cameron et al., 2011]. PLA is also used as a scaffold material because of its biodegradability and high hydrophilic nature compared to PGA [Liu et al., 2010]. Poly (ε-caprolactone) (PCL) is another polymer used in tissue engineering it can degraded by microorganism, hydrolytic enzymatic or intracellular mechanisms under physiological conditions [Chang et al., 2013]. Some disadvantages of these polymers in tissue engineering

applications are their poor biocompatibility, release of acidic degradation products, poor process ability and loss of mechanical properties very early during degradation, immunogenicity etc.

To overcome the limitations of synthetic polymers scientists begin to search natural polymers for the development of tissue engineered constructs because they are cytocompatible and non-toxic biomaterials, comprising adequate mechanical and structural support and able to control cell attachment, migration, proliferation and differentiation [Biondi et al.,2008]. Even though there are enormous research effort during last decades, scientist have not fully developed a new generation of biocompatible biomaterials due to many issues including legal, the need to develop functional blood vessel networks to nourish the new tissues mainly inside scaffolds, inability of the biomaterials to promote the formation of functional tissues [Kohn etal.,2007].

The natural polymers are either protein based or polysaccharide based. The similarity between natural proteins and extra cellular matrix allows the incorporation of natural proteinsinthescaffoldsfortissueengineering.Naturalpolymerssuchasfibrin,fibronectin, collagen,elastin,silk,keratin,chitosan,alginate,amylose/amylopectinandhyaluronicacid are widely used in tissue engineering [Chung. et al.,2007]. Collagen is synthesized by fibroblasts and other cell types such as chondrocytes and osteoblasts and is the most abundant protein in the mammalian body, the primary functions of collagen in tissues is to provide mechanical support and to control cell adhesion, cell migration and tissue repair [lee et al., 2001]. Collagen is used as an important biomaterial due to its non-immunogenicity, mechanical stability etc. Collagen scaffolds have been used for soft tissue repair [Pabbruwe et al., 2010], vascular [Park et al., 2009] and dermal tissue engineering [Helary et al., 2010], bone repair [Lyons et al., 2010]. Even though the rear wide range of applications, collagens matrices lack the mechanical properties required for hard tissue during initial implantation. Fibronectin is another type of extra cellular matrix (ECM) protein helps in cell signaling, migration etc. Fibronectin binds to other biologically important molecules such as heparin, collagen/gelatin and fibrin [Pankov et al., 2002]. Since fibronectin is biocompatible fibronectin or domains of the protein is used to functionalize scaffolds for tissue engineering

[Rexeisen et al., 2010]. Polymeric scaffolds of chitosan [Amaral et al., 2009], collagen [Bush et al., 2009] and hyaluronic acid [Barbucci et al., 2005] have been modified with fibronectin to improve cell adhesion and proliferation. Elastin is another important ECM protein. Due to its characteristics, elastin has been used in the fabrication of hybrid materials in combinations with collagen [Sionkowska et al., 2010], and silk [Huetal., 2010] for the production of vascular grafts hydrogels and for bone repair. Fibrin is another example of a specialized extracellular matrix protein with potential application for tissue engineering and used for skin repair, replacing sutures and staples in fixation of skin grafts promoting a better wound healing [Surg et al., 2010]. Silk is another example of fibrous protein and used in tissue engineering due to its high mechanical properties and being studied for tissue engineering in the form of scaffolds for a range of tissue needs, such as corneal regeneration [Mandal et al.,2010], cartilage repair [Chao et al.,2010].

Like proteins carbohydrate possess some of the characters needed for tissue engineering. Their cyclic structure helps to give an adequate conformational rigidity, the presence of multiple hydroxyl groups provides different positions for linkages, and the chirality provides different orientations of the hydroxyl groups and therefore different directions for the substituent's linked to them. Polysaccharides and some of glycoproteins in the ECM have been used to produce artificial tissue engineered 3D scaffolds. Glycosaminoglycanis the native component of ECM composed of a core protein and one or more glycosaminoglycan are linear, negatively charged polysaccharides made up of repeating disaccharide units that can be sulphated. It is used for create tissue constructs because of its wide physiological functions such as their involvement in cell signaling events, cell– cell interactions and cell–matrix interactions in the ECM and on the cell surface [Esko et al.,2007]. Hyaluronic acid for example is the simplest glycosaminoglycan and important component of cartilage ECM. Hyaluronic acid has reported to increase the retention and ECM secretion of chondrocytes seeded on the scaffolds made up of hyaluronic acid [Goodstone et al., 2004]. Hyaluronic acid based hydrogels have been extensively used for stem cell encapsulation and differentiation [Son et al., 2015].There is works suggest the differentiation of chondrocytes into a 3D hydrogel. Alginate is another carbohydrate obtained from brown sea weed can be formed into stable hydrogels with highly

porous structures via ionic cross linking to create artificial ECMs for the encapsulation of MSCs [Toh et al., 2012]. Chitosan, or poly-(β-1/4)-2-amino-2-deoxyd-glucopyranose, is a natural polysaccharide composed of glucosamine and n-acetylglucosamine. Chitosan is nontoxic and non-immunogenic and is being used as hydrogels and nanoparticles drug delivery vehicles [Ahmadi et al., .2015]. Chitosan-based hydrogel scaffold with a series of growth factors such as bone morphogenic protein-2 (BMP-2), was able to direct neural stem/progenitor cell differentiation [Li et al., 2015]. Dextran is a complex branched glucan of varying sizes, naturally derived polysaccharide from sucrose and cryogels were produced by cryogelation of dextran to provide an inter connective macro porous structure for 3D culture. It is reported that aminin-coatedd extran cryogel was found to be a suitable scaffold that can provide a "niche"-like structure for optimal adhesion and proliferation of human cord blood derived stem cells [Jurga et al., 2011].

1.2.2 THE 3D SYSTEM FOR CELLCULTURE

The cells in our physiological system are embedded in a complex micro environment [Dunn et al., 1989]. The proper functioning of human body needs cell-cell and cell–ECM interactions. In 3 Dimensional systems the ECM provide channels for nutrition and waste removal. There are various types of 3D cell approaches (Fig.1.2) like the spheroid type system provides highest chance of proliferation whereas the interior of the 3D cell body possess the highest number of necrotic cells [Andersion et al., 2004]. A well designed 3D system can promote proliferation, migration, matrix production and differentiation of cells growing in the 3Dsystem.

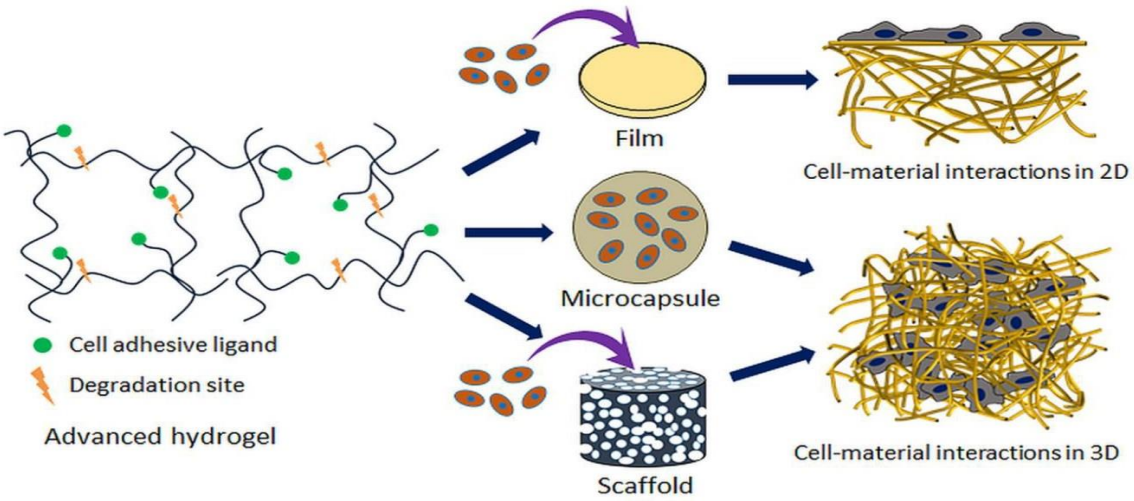


Fig 1. 2 Hydrogel based tissue engineered scaffolds investigated exhibit three different structures and cell-materials interactions in 2D and 3D scaffolding matrices[Technischen et al., 2015]

1.2.3 SCAFFOLDS FOR 3D CELLCULTURE

The main consideration for 3D cell culture system is the choice of scaffold for the creation of the system. Commonly natural polymers like collagen, gelatin, alginate, fibrin, and synthetic polymers or the mix of natural and synthetic polymers are used as 3D system. These polymers can mimic the ECM of cells by means of porosity, mechanical stability, permeability etc. The choice of these materials varies from cells to cells because these compounds determine the fate of cells in the system. More than 100 types of matrices and scaffolds of both organic and inorganic nature are being currently used. There are mainly 3 types of 3D niche they are spheroid culture. Including micro fluidics [Seidell et al., 1996], microchips [Field et al., 1997]. Biopolymer scaffolds by using the techniques to encapsulate cells in 3D using tissue-engineering scaffolds with customized biochemical and biophysical components [Hu et al., 2001]. Another type of 3D niche is hydrogel based one, which is a prefabricated scaffold to which cells are seeded or encapsulated

1.2.4 HYDROGELS AS 3-DIMENSIONALNICHE

Hydrogels are hydrophilic three-dimensional network held together by crosslinked chemical or physical bonds. Hydrogels are water absorbable water like structure and can mimic the natural environment of the body and its properties can be easily tunable. Most hydrogels consist of nanometer-sized mesh that lack the micro-topography needed for controlling cell shape and supporting cell mobility, cell proliferation, and matrix production. To overcome this enzymatically degradable natural polymers have been incorporated into the hydrogel system [Benton et al., 2009]. These hydrogels have components those dissolve gradually and produce internal space to facilitate matrix production and other cell bioactivities. Hydrogels can be created to facilitates the cell growth either by trapping the cells inside the gel [Heywood et al., 2004] or seeded on the top and allowed to migrate to the interior of the gel [Topman et al., 2013]. Self-assembly, ionic cross-linking or radical polymerizations by UV exposure are the methods to encapsulate cells into the gels. Hydrogel derived from natural substances are very useful for the 3D culture because it can stimulate cell migration proliferation and also possess optimum biodegradability with minimum immunogenic properties [Fisher et al., 2007]. Chemical cross-linking is a more versatile method to synthesize hydrogels to optimize mechanical stability and to control degradability [Keeffe et al., 2007]. Its properties can be increased by chemical modification by using compounds like glutaraldehyde and genipin.

1.2.5 FIBRIN

Fibrin gels naturally formed in human body and it is important to maintain the homeostasis following injury [Sierra et al., 1993]. Fibrin gel is a polymer compound formed by the cleavage of fibrinogen by thrombin molecule and forms rigid hydrogel like structure. In the final stage of blood clotting soluble fibrinogen is converted into insoluble fibrin through thrombin mediated proto fibril formation and in the presence of factor XIII to form a stabilized 3D network of fibril known as the fibrin clot. Fibrinogen is a water-soluble glycoprotein of 340 kDA, comprising two sets of three polypeptide chains that are linked together by disulfide bonds. Fibrinogen is

synthesized in liver and its concentration increases after any imbalance of homeostasis. Fibrinogen is isolated by precipitation reaction that starting from the plasma of autologous blood, by a series of freezing and thawing cycles or by using chemicals aimed at decreasing the solubility of the protein [Oseni et al., 2013]. Thrombin catalyzes the cleavage of fibrin peptides leading to fibrin monomers formation .Cleavage occurs in the N-terminal part of fibrinogen leading to the exposition of A and B knobs binding sites [Litvinov et al., 2005]. The knobs interact with the 'a-' and 'b-holes' present at the end of the fibrinogen molecule forming insoluble fibrin fibers. The branching of the fibers results in a three-dimensional fibrin network. Finally, in the presence of calcium the transglutaminase factor XIIIA cross-links and stabilizes the structure (Fig.1.3). Factor 13A is derived from the thrombin-mediated cleavage of factor XIII, a transglutaminase that can promote stem cell adhesion and proliferation.

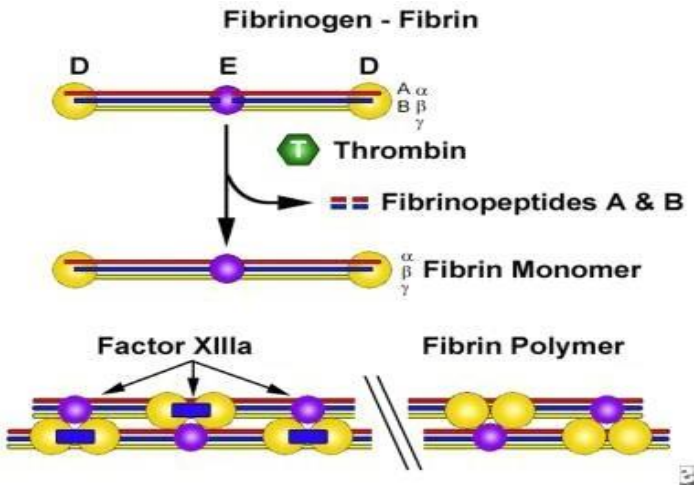


Fig 1. 3 The conversion of soluble fibrinogen into insoluble fibrin [Suchitra et al., 2015]

Formationoffibrinclotsfollowsdegradationoftheclotoverdaysorweeksandarefinally eliminated through kidney as a part of normal fibrinolytic processes mediated by phagocytosis and plasminogen cleavage [Yeetal., 2015]. Fibrin gels are important cell culture matrix as it helps in the cell adhesion and differentiation of the cells. In body fibrin gels are rapidly degraded due to the proteolytic enzymes secreted by the cells [Mol et al., 2005 16]. The ability to form rapid hydrogels

makes fibrin gel as a suitable carrier for tissue engineering. The reare evidence that fibrin and its degradation products could initiate signaling that canin-turn regulates the migration and growth of cells [Wang et al., 1986].The results showed that fibrin gel used in tissue engineering may restrict the growth of some cells like osteocytes, chondrocytes and hepatocytes therefore to attain a better result researches incorporate some of the macromolecules to improve the properties of fibrin [Homminga et al., 1986]. The cell suspensions in fibrin and thrombin can be used as an injectable system can form fibringel in-situ with encapsulated hydrogel and this system can restores myocardial functions [Yamada et al.,2003], bone regeneration and muscle formation [Bhattarai et al.,2005]. However, the use of fibrin glues as scaffolds in tissue engineering remains a challenge due to their highly dense structure that is unfit for cell survival. Fibrin was combined with fibronectin to enhance the biologic properties of fibrin and the results highlighted the increase of cellular migration especially in the skin tissue engineering [Priya et al., 2008]. Therefore the applications of fibrin-based scaffolds in tissue engineering have been limited due to their poor mechanical properties and fast biodegrability. A number of strategies could improve the degradation rate of fibrin networks including optimizing fibrinogen precursor and calcium ion concentrations, conjugating fibrin with synthetic polymers such as polyethylene glycol (pegylation), reducing cell density or using protease inhibitors such as aprotinin [Ahmed et al.,2008]. Fibrin stabilizing factors such as a protinin, factor 13 and e–amino-n-caproicacidetc., Can be used to prevent the degradation of fibrin gels in-vitro and can thereby increase the stability over a long time [Park et al., 2005 &Ye et al., 2000] that makes fibrin a useful injectable biomaterial for tissue repair. There are approaches used to reinforce the mechanical stiffness of fibrin matrix by. Fibrin with polyethylene glycol exhibited improved mechanical properties compared to fibrin alone combine this glycoprotein with a synthetic polymer. Fibrin has been conjugated to several synthetic polymers such as polyethylene glycol, polyurethane and polycaprolactone [Akpalo et al., 2011]. The novel interconnected porous modified alginate-fibrinogen scaffolds with high porosity and mechanical strength were successfully fabricated by freeze-drying procedure. The results showed increased mechanical stability of the fibrin and also the biodegradability of alginate increased when binds with fibrin. Thus a fibrin –ADA based modified system can be optimized for individual tissues in order to meet the mechanical and cellular properties of the native tissues.

1.2.6 ALGINATE & GELATIN

Most commonly used hydrogels are prepared by polysaccharides among them alginate stands the best [Wilson et al., 2014]. Alginate is derived from algae and consisting of mannuronic-and guluronic acid [leeetal.,2012]. Alginate can be easily form gels in the presence of divalent cations. Alginate having adhesive groups can

modified with peptides mostly RGD containing ones are used for cell culture techniques because these modification made alginate more suitable for cell interaction. Additionally, such hydrogels can be applied as 3D scaffolds for the cultivation and differentiation of stem cells for both invitro and invivo applications in soft tissue regeneration [Güntheretal., 2015]. However, alginated not promote cell-material interactions in ceit does not contain any biological moiety [Rowley et al., 1999]. Alginate cannot be completely removed from the body since the average molecular weights of commercially available alginate are higher than therenal clearance threshold of the kidneys [Shamkhani et al., 1995]. Moreover, ALG hydrogels promote minimal protein adsorption due to its hydrophilic nature. Consequently mammalian cells are unable to interact with the hydrogel through serum proteins [Smetana et al., 1993]

Gelatin is the hydroxylation product of collagen. Gelatin constitutes almost all amino acids of collagen except tryptophan and very low amount of methionine, cystine and tyrosine due to the degradation during hydrolysis [Jamilah et al., 2002]. The typical sequence of amino acid singelatinis (gly-x-y) n, where glycine is the most abundant amino acid; x and y are mostly proline and hydroxyproline, respectively, however x and y can accommodate any other amino acid. Gelatin dissolves in water at high temperature usually at 60^0C and forms hydrogel at lower temperature and it is easily biodegradable [Young et al., 2005]. Among other biopolymers, gelatin has been widely used due to its low cost, biodegradability, biocompatibility, and non-immunogenic properties. It is widely used in many fields of biomedical technology as wound dressing materials, microspheres etc. Gelatin shows poor mechanical strength due to this reason it failed to use in many hydrogel systems. Gelatin, in its dry state like porous scaffolds, show brittleness, less flexibility, and extremely fast degradation rate problems [Tan et al., 2007]. However, gelatin in combination with ADA enhances the stability of hydrogel system. ADA or alginatedialdehyde can be modified with gelatin for tissue engineering. ADA is a partially oxidized product of ALG which facilitates the covalent cross linking with gelatin through the Schiff's base formation due to the reaction of free amino groups of lysine or hydroxylysine amino acid residues of gelatin and available aldehyde

groups of ADA[Balakrishnan et al.,2005] . The partial oxidation cleaves the carbon-carbon bond of the cis-diol group in the uronate residue of ALG and alters the chair conformation to an open-chain adduct, which facilitates degradation of the ALG [Bouhadir et al.,2001] .Moreover, the biodegradability of alginate-gelatin cross-linked hydrogel can be tuned by using ADA of different degrees of oxidation which can control the hydrolysis property of ALG and also by changing the ratio of ADA and gelatin. Mixing of ADA with gelatin increase the mitochondrial activity of mg-63 osteoblast like cells compared to pure alginate. The covalent binding of gelatin with ADA provides an excellent platform forcell development [Pawar et al., 2012]. Studies shows that 2D films composed of ADA and gelatin provides an excellent platform for the adhesion and proliferation of primary fibroblasts[Zimmermann et al.,2012].The preparation of microcapsules formed of these two elements opens the chance of 3D printing by using this compounds [Dragetetal.,2005]. The culturing of soft tissues needed a highly porous structure and the scaffold should promote effective cell migration and cellproliferation. Porosity of ADA gels can bealtered based on the degree of oxidation and aldehyde contents. A 3 dimension scaffold which can provide stability, porosity and cellular viability is an ideal system for the tissue engineering approaches.

1.2.7 LIVER & ITSCELLS

Liver is the one of the most important vital organ in the human body. It performs many important functions of human body and plays a central role in both endocrine and exocrine physiological functions such as formation and secretion of bile, albumin, and urea, metabolism of cholesterol and fat, and detoxifications. Hepatic tissue is created of the cells hepatocytes which are structured in to apolarized epithelium with apical and basaldomains [Schon et al.,2014]. The cells present in the liver can be divided into two classes- parenchymalandnon-parenchymal cells. Hepatocytes are the parenchymal cells. There are mainly two types of cells are present in the liver namely parenchymalandnon-parenchymal cells include kupffercells, hepaticstellate cells, liver sinusoidal endothelial cells and biliary epithelial cell [Agnieszka et.al. 2016]. Of the variety of cells hepatocytes accounts

for 80% of total cells and performs the important functions and non-parenchymal cells accounts for 40% of the total cells.

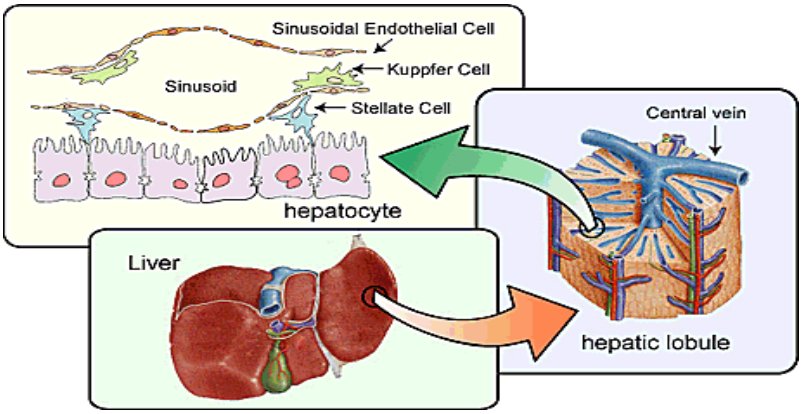


Fig 1. 4. Arrangements of cells of liver[Burgada et al.,2015]

Liver failures as a situation in which large parts of the liver become damaged beyond any possibilities of a repair and the liver is no longer able to perform its native functions. Liver failure occurs gradually over a period of many years. Acute liver failure can also happen which is a comparatively rarer condition that occurs rapidly, even within a time span of 48h and is often difficult to diagnose on time. The main factors which can cause chronic liver failure which include long- term alcohol consumption, hepatitis B, hepatitisC, cirrhosis, hemochromatosis. The causes of a cute liver failure in which the liver fails rapidly are often different from those mentioned above. Some examples are an overdose of drugs like acetaminophen, reactions to certain herbalmedications an dingestion of poisonous wild mushrooms. According to the annual report of the American liver foundation in 2000, hepatitis and other liver diseases affect 25 million Americans. Liver failure is the 8th most frequent cause of death. In the United States, accounting for roughly 43,000 deaths each year[Seidell et al, .2012]. Poor diet and sedentary lifestyle, and obesity contributing the damage of liver, and are responsible for more than 300,000 deaths per year, making it these cond leading cause of preventable death after smoking many diseases like including cardiovascular disease [Field et al., 2012], hypertension

, type-2 diabetes [Manson et al., 2012) and non-alcoholic fatty liver disease (NAFLD) are developed due to obesity. Life style modifications etc. are the treatment for liver diseases. But liver transplantation is the only treatment for endstage liver diseases. However, this mode of treatment is limited due to donor organ shortage, high costs, and the lifelong need for immunosuppression. Researchers worldwide are trying to develop cell-based therapeutic strategies. This includes various approaches like cell transplantation and bioartificial liver devices .so cell therapy by using hepatic cells and stem cells are found to be effective for liver damages. Hepatocytes in vitro culture is an intricate area of research because it is hard to get hepatocytes that can be cultured at high cell densities and demonstrate high liver-specific functions, in an extracorporeal bioartificial liver device, such high- performing hepatocyte like cells can be worn which may offer the essential sustain to a tolerant with an advanced liver disease until he receives a donated liver [Tilles et al., 1998]. Hepatocytes are currently cultured in a 2-dimensional (2D) environment culture of hepatocytes to investigate new treatment approaches in vitro. However, this simplified culture model does not stand for the intricate in vivo liver tissue. Construction of 3D in- vitro models of the liver tissue, therefore, will help understand the characteristics of liver tissue and hence help to develop new treatment strategies. The best solution for this problem is the construction of polymer scaffolds that can support hepatic cell growth polymer scaffolds that can support liver cell growth over a long-term culture period.

The burden of liver diseases liver failure can be considered as a situation in which large parts of the liver become damaged beyond any possibilities of a repair and the liver is no longer able to perform its functions. It is a life-threatening condition and requires urgent medical care. In most of the cases, a liver failure occurs gradually over a period of many years. Acute liver failure can also happen which is a comparatively rarer condition that occurs rapidly, even within a time span of 48h and is often difficult to diagnose on time. There can be several different factors which can cause chronic liver failure which include long-term alcohol consumption, hepatitis B, hepatitis C, cirrhosis, hemochromatosis and even malnutrition can play a role. The causes of acute liver failure in which the liver fails rapidlyareoftendifferentfromthosementionedabove.Someexamplesareanoverdoseof

drugs like acetaminophen, reactions to certain herbal medications and ingestion of poisonous wild mushrooms. Often a failure in liver function can be treated only by a liver transplantation. However the ratio of donor to recipients is huge and demand cannot be met. There is a critical shortage of donors and at the same time the medical procedure is quite expensive. Furthermore there can be additional problems arising due to surgical complications and chances of chronic rejections are also high. Hence alternative approaches are needed to withstand the scenario. Today, researchers worldwide are trying todevelopcell-based therapeutic strategies. This includes various approaches like cell transplantation and bioartificial liver devices [Nussler et al., 2006]. It is even more challenging to maintain the phenotype of primary hepatocytes while in a culture. Thus, what emerges is a critical scarcity of human hepatocytes which continues to remain as a stumbling block to advancement of cell-based therapies.

1.2.8 HepG2 & OTHER LIVER CELLSUBSTITUTES

The stem cells are one of the most useful cells used as an alternative source of human primary hepatocytes. Stem cells like embryonic stem cells, mesenchymal stem cells, induced pluripotent stem cells are found to be promising cell culture technique because they can be differentiate into any other desired organs among them mesenchymal stem cells are found to be the best one because of its low immunogenicity and effective proliferation. But they are always face so many ethical problems and yet to be standardized. So the best method is the culturing of liver cell line with effective growth. HepG2 is an immortalized cell line. The morphology of the HepG2 is epithelial and it consists of 55 pair chromosomes and significantly less responsive to chemotherapy. This cell line has been widely used in biochemical and nutritional studies because it is considered one of the experimental models that more closely resemble the human hepatocyte in culture HepG2 cells can grow effectively in cell culture and secrets many proteins like albumin, fibrinogen etc. It grow like epithelial cells and grow as monolayer and adherent. HepG2 cells lines are widely used in the field of liver metabolism, development, oncogenesis, and hepatotoxicity. HepG2 cell line was established in 1979 [Mauriceetal.,1988]. In 1980 the patent for

human hepatoma derived cell line was filed by the investigators at the Wistar Institute in Philadelphia. Since then HepG2 cell line has been listed on the American Type Culture Collection, Rockvile, MD, USA. HepG2 system is a well-established system used to investigate the liver cell functions in-vitro. In conclusion HepG2 cell line shows almost all the properties of liver cell [Rollier et al., 1993], including secretion of gene products [Maurice et al., 1988], plasma membrane polarity [Tajima et al., 1992), and growth factor regulation of cell mobility. Hepg2 cells perform almost all the functions of the liver cells thus are used for the study of toxicity [Mersch et al., 2002]). In the present study HepG2 cell lines are used to validate the ADA Gelatin Fib based 3D hydrogel system.

1.3 GAPAREA

Though there are numerous studies is being done in the field of tissue engineering using ADA fibrinogen and gelatin in various combination for different celltypes. However, a combination of the three components and validation of this system for hepatic cells are not being tested. All need of a 3 dimensional niche which can be used for the hepatic cell growth and proliferation remains.

1.4 HYPOTHESIS

ADA gelatin and fibrinogen in a optimize formulation may provide a 3-Dimension niche optimum for the hepatic cells viability and proliferation. This System may also be used for 3D bioprinting of liver tissue.

1.5 OBJECTIVES OF THESTUDY

- **Synthesis and Characterization of ADA**

- **Optimization of the formulation using ADA gelatin and fibrinogen**

- **Physical characterization of the synthesized hydrogel**

- **Culturing of HepG2 cell line**

- **Culturing of HepG2 cells in the 3Dconstruct**

- **Evaluation of the functionality of the HepG2 cells grown in the hydrogel**

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS USED

Sodium alginate(Sigma Aldrich),Sodium metaperiodate (Merck),Hank's balanced salt solution (HBSS) buffer, Collagenase NB 4 standard grade (SERVA Electrophoresis), Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) (Gibco), Trypsin, Phosphate-buffered saline (PBS), Thrombin, Fibrinogen, Gelatin, Ammonia assay kit(Sigma Aldrich), H_2SO_4 (Qualigens Fine Chemicals)

2.2 SYNTHESIS OF ALGINATE ALDEHYDE

About 10g sodium alginate was dissolved in 50 ml of methanol and different amount of sodium metaperiodate in 50 ml distilled water was added and stirred at 4℃ for 6 h in dark to obtain alginate dialdehyde of different oxidation. After reaction, solutions were dialysed against distilled water (2.5 L) for 4 days with several changes of water till the dialyzate was periodate free. The absence of periodate was checked by adding a 0.5ml aliquot of the dialyzate to 0.5ml of a 1% solution of silver nitrate and ensuring the absence of any precipitate. The dialyzate was then freeze dried and lyophilized for 4-5 days [Balakrishna et al., 2005].

2.2.1 DEGREE OF OXIDATION OF ADA

The periodate oxidation was determined by iodometric titration of the residual periodate present in the reaction mixture [Guthrie et al., 1962]. A 5ml aliquot of the reaction mixture was neutralized with 10ml of 10% sodium bicarbonate solution. Iodine was liberated by the addition of 20% potassium iodide solution (2 ml). This

was kept under dark for 15 min and liberated iodine was then titrated with standardized sodium thiosulphate solution using starch as the indicator.

2.2.2 DETERMINATION OF DIALDEHYDE CONTENT

About 50 mg of lyophilized ADA was dissolved in 10 ml of 0.25N hydroxylamine hydrochloride –methyl orange solution. The solution was kept at room temperature for 2h and titrated against standard sodium hydroxide solution. The end point was estimated by the change in pH of the solution. Briefly took the initial pH of the solution, and titrated against 0.1M sodium hydroxide solution. End point was reached when the initial pH was changed to 4. Estimation of aldehyde content was calculated based on the volumes of sodium hydroxide used in the titration against the released hydrochloric acid.

2.2.3 MOLECULAR WEIGHT MEASUREMENTS

The molar mass of ALG and synthesized ADA were determined using the viscosity method [198– 200]. Alginate and ADA was dissolved in 0.1 M NaCl solution to get the final concentrations 1% (w/v). The experiment was carried out at 25^0C with micro viscometer (Rolling –ball viscometer, Lovis 2000 M/ME). The viscosity average and molar mass of sodium ALG and ADA were calculated from its measured intrinsic viscosity according to the following Mark-Houwink equation by adapting *a* and *K* values.

2.2.4 CHARACTERIZATION OF ADA BY FTIR

A Fourier transform infrared (FTIR) spectrometer (JASCO FT/IR -4200 SPECTROMETER) was used to evaluate the structure of ADA. Dried films were used to record attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra. Film of ADA was made by casting the corresponding hydrogels into a polystyrene Petri dish and allowed to dry for 3 days at room temperature.

2.2.5 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY ANALYSIS

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to assess the chemical modification of alginate. About 10 mg ml^{-1} of ADA was dissolved in D_2O at a temperature of 50 °C. ^{1}H NMR spectra were recorded with a Bruker AMX 500 spectrometer (NIST, TVM)

2.3 PREPARATION OF HYDROGEL

10% (w/v) ADA was prepared by dissolving adequate amount of lyophilized ADA in 0.1M borax solution. 10% (w/v) gelatin was prepared in distilled water at 40℃. 1 ml of 10% ADA solution was taken in a beaker and to which 0.5 ml of 10% gelatin and 0.5 ml of 30 mg/ml fibrinogen was added slowly by continuously stirring in magnetic stirrer at a speed of 50 rpm for half an hour. After complete mixing the partially cross-linked solution was taken in a 1 ml syringe and to another syringe 1 ml of thrombin at a concentration of 15 IU was filled and the syringes were fixed in an applicator and injected into a Petri dish. The applicator helps in the mixing of the two solutions.

2.4 CHRARACTERIZATION OF HYDROGEL

2.4.1 CHARACTERIZATION OF HYDROGEL BY FTIR

A Fourier transform infrared (FTIR) spectrometer (JASCO FT/IR -4200 SPECTROMETER) was used to evaluate the cross linking between ADA, gelatin and fibrin .Dried films were used to record attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra. Films of ADA, gelatin, and fibrinogen and ADA-GEL-fibrin materials were made by casting the corresponding hydrogels into a polystyrene Petri dish and allowed to dry for 3 days at room temperature and after drying the samples were lyophilized and analysed.

2.4.2 DETERMINATION OF GELLATION TIME

The test tube inverting method was applied to determine the gelation time [Nguyen et al., 2012]. Gelatin and fibrinogen solution was slowly added to ADA solution in a narrow beaker under stirring. The chemical coupling between ADA, gelatin and fibrinogen occurred during the cross linking and the mixing of the second cross linker thrombin provides quick gel formation. The hydrogel was observed by

inverting the beaker and gelation time was recorded when the hydrogel stopped flowing. Gelation time was recorded at room temperature.

2.4.3 RHEOLOGICAL PROPERTY ANALYSIS

The rheological analysis of the ADA–GEL-Fibrin hydrogel was carried out by modular compact rheometer (Anton Paar, MCR 102, and NIST). A cone plate with cone diameter of 24 mm and a cone angle of 2.009 ° was used and the measurement gap was fixed at 0.105mm. A plate with a diameter of 40 mm combined with a thermostat was used to maintain the temperature at 25 °C. The oscillation frequency was set at 10 rad s^{-1} and applied shear stress at 2 Pa. The oscillation time sweep experiment was performed to record the viscosity of ADA–GEL- Fibrin gel and uncross linked gel. The oscillation frequency was set at 10 rad $^{s-1}$and applied shear stress at 2 Pa.

2.4.4 IN VITRO SWELLING

Hydrogels (n=3) were prepared and placed in PBS at 37ºC for 24 hrs. Samples were removed from PBS and blotted with tissue paper to remove the residual liquid and the swollen weight was recorded. The percentage swelling was calculated from the following equation:

S% = [(Mt – M0)/M0] x 100

Where, M0 is the weight of the dry gel at time 0 and Mt is the weight of the swollen gel after 24hrs.

2.4.5 DEGRADATION STUDY

For the degradation studies the ADA-Gelatin-Fibrin Gels were prepared as described above and incubated with 5ml distilled water to which 0.1% sodium azide was dissolved. At 37° C and the degradation of gels was followed for one week by aspirating the medium followed by freeze-drying the gel to dryness and normalising the weight obtained to their initial values in different time point [Balakrishna et al., 2005].

The weight loss was calculated as:

*Weight loss = Initial weight-Final weight/Initial weight *100*

The Extract of the degraded extract after incubation was analysed by SDS-PAGE electrophoresis.10% SDS polyacrylamide gel was used separate the proteins. The stacking gel and resolving gel were prepared using the standard protocol and the samples with equal protein concentrations (20µl; estimated with the help of standard plotted from Lowry assay) were loaded on the gel. The samples were run on 100V until it crossed the stacking and the voltage was increases to 120V upon reaching the resolving gel. Once the dye front has run out the electrophoresis was stopped and the gel was carefully removed from the glass plates and the stacking gel was cut out. The gel was washed with distilled water and incubated in freshly prepared coomassie stain for 3 hours. The gel was destained with destaining solution.

2.4.6 MORPHOLOGY ANALYSIS BY SEM

The surface topography of the fabricated films was investigated using a scanning electron microscope. Lyophilized gels were placed on double-sided tape, sputter coated with gold and examined in the microscope (Hitachi, Model S- 2400, Japan). The images were acquired at different magnifications.

2.4.7 POROSITY MEASUREMENT BY MICRO CT

The porosity and microstructure analysis of lyophilized hydrogels samples were conducted using micro-computed tomography (Micro CT 40, Scanco, Switzerland). A PMMA tube was used as a sample holder and the lyophilized samples were placed on the sample holder for X-ray attenuation. The scanning was done with a high X-ray energy (45kV) and an intensity of 177µA having 10µm resolution (2D slice thickness). The cone beam algorithm was used to prepare the 2D reconstructions. The 3D image was also collected from the machine by setting appropriate threshold values.

2.5 BIOLOGICAL CHARACTERIZATION OF HYDROGEL

2.5.1 CYTOCOMPACTABILITY TEST BY MTT ASSAY

Cytotoxicity evaluation of ADA-cross-linked gelatin gel was carried out by the MTT assay of hydrogel by direct contact assay and MTT assay with a monolayer of L929 mouse fibroblast cells. Gels were prepared as described above. Briefly, L929

cells were sub cultured from stock culture (National Centre for Cell Sciences, Pune, India) by trypsinization and seeded onto multi-well tissue culture plates. Cells were fed with Dulbecco's minimum essential medium (F12) supplemented with 1% bovine serum and incubated at 37 ^{0} C in 5% carbon dioxide atmosphere. When the cells attained a monolayer, the gel (0.75 cm2) was kept in contact with the cells in triplicate for direct contact assay. After incubation at 37 ^{0}C for 24 h, cell culture was examined for cell viability by MTT assay which measures the metabolic reduction of 3-(4, 5-dimethylthiazol-2yl)-2, 5, diphenyltetrazolium bromide to a coloured formazan by viable cells. MTT dissolved at a concentration of 1 mg/ml in sterile PBS .After culturing with gel and gel extract and 0.2 ml of MTT working solution was introduced and the plates were wrapped with aluminium foil and incubated at 95% humidified atmosphere at 37^{0}C for 6 h. After removing the reagent solution and rinsing with PBS, 0.2 ml of DMSO was added to each well and incubated for 20 min at 37^{0}C. The absorbance of the resulting solution in each well was recorded immediately at 595 nm in Bio-Rad iMarkmicroplate absorbance reader using automated micro plate reader. The resulted were calculated as noted below

*Percentage viability = Absorbance of Test/Absorbance of control *100*

2.5.2 CULTURE OF HepG2 CELL LINE

HepG2 cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 1% non-essential amino acids, 1 mM pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin (all reagents were obtained from Gibco). Cells were maintained in a humidified incubator at 37°C in a 5% CO2 atmosphere. When monolayer attained, trypsinized the cells with 0.25% trypsin (Invitrogen, Gibco) and seeded on to another flasks and maintained.

2.5.3 SEEDING OF HepG2 CELLS ON THE HYDROGEL

The 10 % ADA and gelatin was sterilized by UV sterilization and fibrinogen and thrombin was sterilized by filtration through filter (0.22 μm) .HepG2 cells were used for the study. The gel was prepared as described before with cells. Cells at a density of 10,000 cells were seeded on the surface of the hydrogel placed in the multi

well plate and filled with DMEM medium with 10% FBS.Cells with medium alone serves as control. The hydrogel with cellsseeded on the top was maintained in a humidified incubator at 37°C in a 5% CO2 atmosphere. Morphology was analysed by phase contrast microscopy (Leica, Germany)

2.5.4 LIVE/DEAD STAINING OF THE CELL ENCAPSULATED HYDROGEL

Fluorescence based live/dead assay was used to evaluate the viability of the cells encapsulated inside the hydrogel after 24 hrs. Live / dead staining was performed with calcein AM and ethidium homodimer-1 respectively. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). The assay was performed according to the manufacture's instruction after 24 hours of incubation at standard culture conditions. The fluorescent imaging was done using confocal laser scanning microscope (Olympus Flouview FV3000).

2.5.5 ACTIN STAINING

For analyzing two dimensional cell growth, HepG2 cells were seeded onto the top of the hydrogel discs placed on 35mm culture plates to evaluate cell attachment on the hydrogel and incubated for 24 h at 37 °C, 5% CO_2 in a humidified atmosphere. After 24 h. the hydrogel was stained for Actin staining by Rhodamine phallodine stain (Invitrogen, A12379, ex/em ~495 nm/~518 nm). Briefly, the hydrogel seeded with cells were washed with PBS after removing media, fixed with 3.7% formaldehyde, permeated by 0.2% triton x 100, washed and stained with rhodamine phallodine for 45 minutes. Stained cells were washed with PBS and then viewed under confocal microscope (Olympus, Fluoview, FV3000).

2.6 FUNCTIONAL EVALUATION OF HEPG2 CELLS IN THE HYDROGEL

2.6.1 INDOCYANINE GREEN UPTAKE ASSAY

The Indocyanine green (cardiogreen) dye (Sigma Aldrich) was dissolved in sterile water to make a 1mg/ml solution. The cells with hydrogel were submerged in this solution for 1 hour at 37^{o} C. After the incubation, the cells were washed thrice

with PBS. The cellular uptake of the dye was observed under a confocal microscopy (Olympus, Fluoview, FV3000) and phase contrast microscopy.

2.6.2 LDL UPTAKE ASSAY

LDL uptake of HepG2 cells were evaluated using LDL uptake assay kit (abcam, ab133127). Briefly 5µl LDL was diluted in 500µl media. Then the diluted samples were added into the 4 well plate. The samples were kept in dark for 4 hours. After incubation the samples were wash with HBSS and visualize under green filter in phase contrast microscope ((Leica, Germany)and the images were captured at different magnification using LAS software (Leica, Germany).

2.6.3 GLYCOGEN STORAGE ASSAY

The glycogen storage by the cells was assayed using Periodic acid –Schiff staining (PAS).Cells were permeabilized with 0.1% Trition X-100 for 10 minutes followed by fixation in 4% formaldehyde. Cells were stained with 1% periodic acid for 5 minutes at room temperature. Cells were rinsed thrice with distilled water and then incubated with Schiff's solution for 15 minutes at room temperature. Cells were counter stain with haematoxylin for 1 to 2 minutes and then were washed thoroughly with distilled water. Cells were observed using light microscopy (Leica, Germany) and the images were captured at different magnification using LAS software (Leica, Germany).

2.6.4 IMMUNOFLUORESCEN STAINING OF CYP450

The cells grown in the four well plate was assayed by immunofluorescence stain of CYP450.The cells were fixed in 3.7% formaldehyde for 30 minutes. After incubation the cells were washed thrice with PBS buffer and added 1% BSA solution to the wells. Then added diluted antibody(1:2000) and incubated in dark for 1 hour .After incubation the wells were washed thrice with PBS buffer and add secondary antibody(Antimouse antibody tagged with Alexa 488) and incubated 1 hour for dark and observed using light microscopy(Leica, Germany) and the images were captured at different magnification using LAS software(Leica, Germany).

2.6.5 AMMONIA CLEARANCE ASSAY

The capacity of the cells to metabolize the ammonia was calculated by using Ammonia assay kit (Sigma Aldrich) by using the manufactures protocol. The absorbance of the samples were evaluated by using UV spectrophotometer at 340 nm. Briefly, ammonia reacts with a-ketoglutaric acid (KGA) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of L-

glutamate dehydrogenase (GDH) to form L-glutamate and oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), as follows:

GDH KGA + NH4 + + NADPH L-Glutamate + NADP+ + H2O

The decrease in absorbance at 340 nm, due to the oxidation of NADPH, is proportional to the ammonia concentration. L-Glutamate dehydrogenase reacts specifically with ammonia.

2.6.6 ALBUMIN ASSAY

The synthesised albumin by HepG2 cells were analysed by SDS-PAGE analysis. 10% SDS polyacrylamide gel was used separate the proteins. The stacking gel and resolving gel were prepared using the standard protocol and the samples with equal protein concentrations (20µl; estimated with the help of standard plotted from Lowry assay) were loaded on the gel. Human serum albumin was used as standard. The samples were run on 100V until it crossed the stacking and the voltage was increases to 120V upon reaching the resolving gel. Once the dye front has run out the electrophoresis was stopped and the gel was carefully removed from the glass plates and the stacking gel was cut out. The gel was washed with distilled water and incubated in freshly prepared coomassie stain for 3 hours. The gel was destained with destaining solution.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. SYNTHESIS OF ADA

Sodium Alginate was oxidized by sodium metaperiodate in an ethanol–water (1: 1) mixture at lower temperature for 6 hours under dark. Periodate oxidation results in the cleavage of vicinal glycols of sodium alginate to form ADA. Each α-glycol group consumes one molecule of periodate. The sodium metaperiodate oxidize hydroxyl group on carbon 2 and 3 (Fig: 3.1) leading to the formation of two -CHO in oxidized monomeric unit by cleaving the carbon carbon bond.

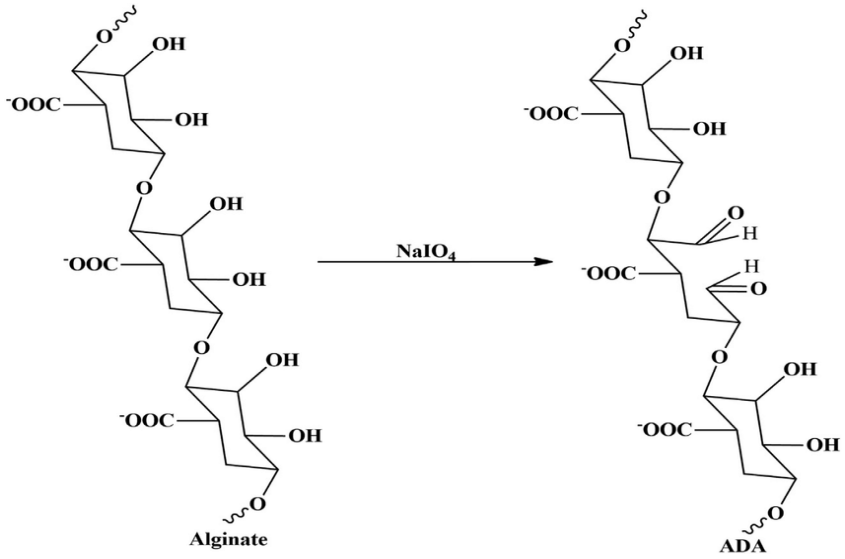


Fig 3. 1Scheme for synthesis of ADA from alginate by periodic oxidation [Balakrishna et al., 2005].

ADA with different degree of oxidation was synthesized and the respective degree of oxidation, yield, dialdehyde content and molar mass was calculated (Table 3.1)

Table 3. 1Effect of periodate content on the properties of ADA

No:	Wt. of sodium alginate (g)	Wt. of meta periodate (g)	Degree of oxidation (%)	Yield (%)	Dialdehyde content (%)	Molar mass (g/mol)
3	10	5.4	45.3±0.4	86.7±3.3	40.3±3.1	19447±360
3	10	6.5	54.6±0.3	83.2±5.7	48.0±4.5	7501±199
3	10	8	66.1±0.4	60.5±5.8	55.80±4.5	8600±200

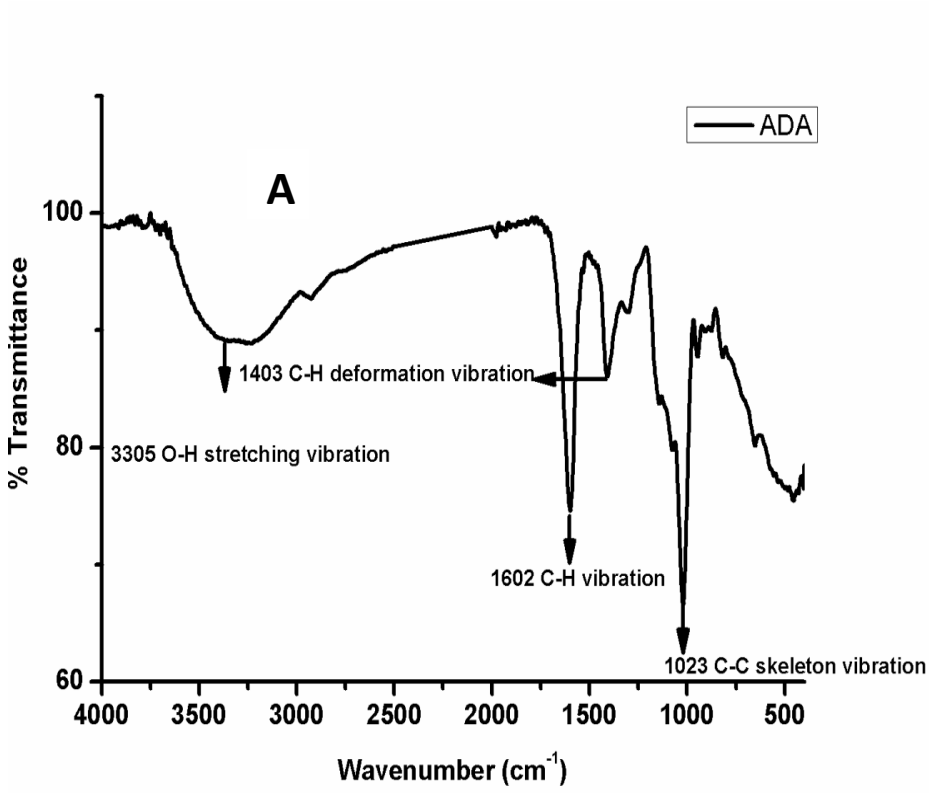
The degree of oxidation was found to increase with increase in the concentration of periodate. Dialdehyde content was estimated by hydroxylamine hydrochloride method. When hydroxylamine hydrochloride in methyl orange indicator reacted with aldehydes in ADAs at pH4, alginate polyoxime is produced, releasing HCl equivalent for each aldehyde group. By titrating the solution against standard sodium hydroxide, the dialdehyde content was estimated. The dialdehyde content was found to increase with the increasing concentration of sodium metaperiodate. Molecular mass of ADA was measured by intrinsic viscosity method. Molecular mass of ADA reduces with increase in the amount of periodate used and thus increase in the degree of oxidation. This is due to increase in the extent of oxidation and associated depolymerization. However, in the present study we observed that there was a decrease in molecular mass when weight of metaperiodate increased from 5.4 g to 6.5 g, but increase in molecular mass was observed when metaperiodate was increased further to 8g.This may be due to the remaining of un reacted polymers. For further studies hydrogel was prepared using 55% oxidized ADA. It is reported that the gelling time decreases with increase in the degree of oxidation. This can be attributed to the increase in the reactivity due to increase in the number of available aldehydic groups to react. In our study we observed increase in the molar mass at higher oxidation (66.10%) which may affect the gelation time and reflects the partial reaction. Therefore for further study ADA with 55 %(Fig.3.2) oxidation was selected. The optimized hydrogel was prepared by mixing ADA (dissolved in borax solution), fibrinogen and gelatin with thrombin as cross linker.

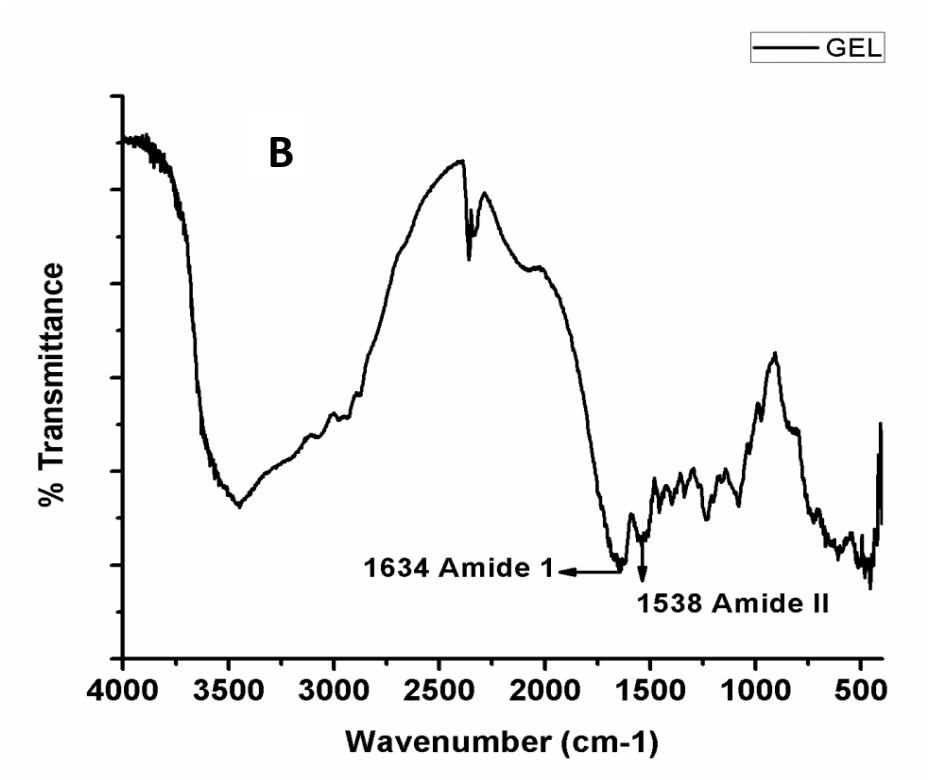
Fig 3. 2. Lyophilized ADA

3.2. CHARACTERIZATION OF RAW MATERIALS BY FTIR

Sodium Alginate shows its characteristics alkyl C-H stretching at 2922 cm^{-1}, -COO asymmetric stretching bands at 1613.16 cm^{-1}, COO symmetric stretching bands at 1418.39 cm^{-1} and C–O–C stretching at 1031.73 cm^{-1}[Balakrishna et al.,2005]. FTIR spectrum of ADA showed 3305 O-H stretching, 1403 C-H peak whereas the symmetric vibrations of aldehyde at 1734 cm^{1} could not be detected in the FTIR spectrum (Fig: 3.3. A), this was may be due to the hemiacetal formation of free aldehyde groups in ADA [Sarker et.al. 2014]**.**

Gelatin shows the characteristic absorption bands at 1622.52 and 1538.21 cm^{-1} which correspond to the stretching vibrations of peptide C= O groups and N-H stretching vibrations of amide I and amide II respectively (fig: 3.3. B). The FTIR spectra of fibrinogen (figure 3.3.C) Shows its characteristics amide I, II and III peaks at 1528, 1636 and 1350 cm-1 respectively.


ADA
A
100
80
60
% Transmittance
1403 C-H deformation vibration
3305 O-H stretching vibration
1602 C-H vibration
1023 C-C skeleton vibration
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)


GEL
B
% Transmittance
1634 Amide 1
1538 Amide II
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

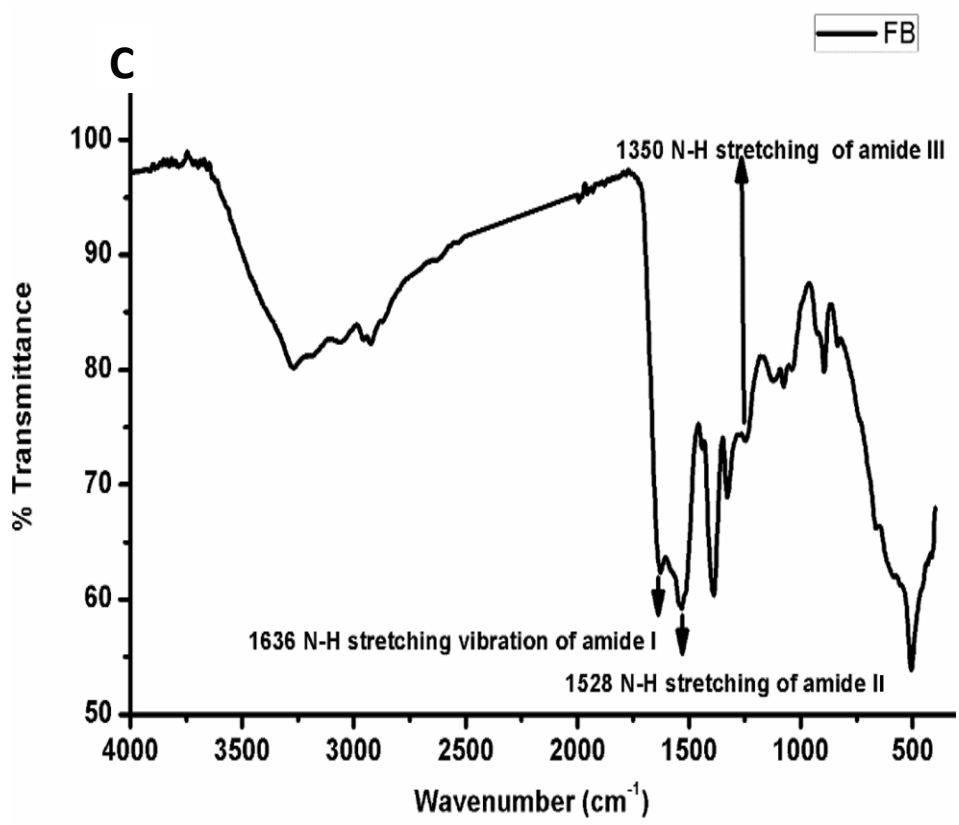


Fig 3. 3A) FTIR spectra of ADA, B) FTIR spectra of Gelatin & C) FTIR spectra of Fibrinogen

3.2.1. NMR SPECTROSCOPY ANALYSIS OF ADA

To confirm the formation of reactive aldehydic groups on synthesised ADA, Sodium alginate and ADA of 55% oxidation were analysed by NMR spectroscopy(Fig 3.4 A & B) .From the results of ^{1}H NMR spectroscopy of sodium alginate and ADA, the signals observed at 5.18 correspond to peak of H^{1}-G. Signals observed at 3.60 and 4.13 ppm was assigned for H_3-M and H_3-G, respectively. In the spectrum of ADA, the proton signal modification of the H^{1} and H^{5} signals for M and G units at 5.39 and 4.93 ppm confirm the oxidation of sodium alginate. Moreover, the appearance of a new signal at 5.66, which would correspond to a hemiacetalic proton formed from aldehyde and neighbors hydroxyl groups, also confirm the proposed modification. The results are matched with another works done with same compounds [Bajpai et al., 2016].

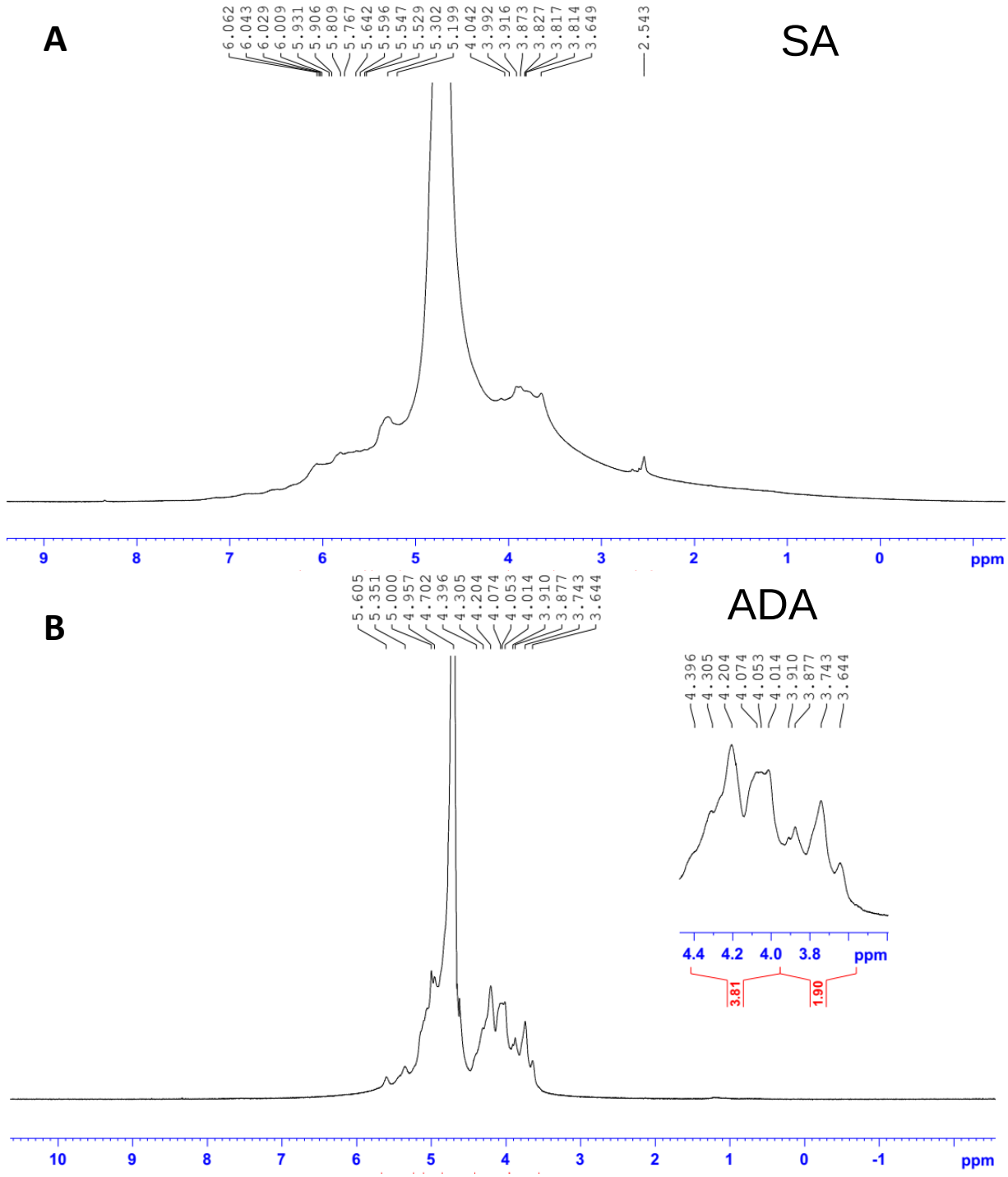


Fig 3. 4 A) NMR analysis of Sodium alginate and B) NMR analysis of Alginate dialdehyde

3.3. PREPARATION AND OPTIMIZATION OF ADA FIBRIN HYDROGEL

Different formulation by varying ratio of ADA to gelatin and fibrinogen were prepared (Table 3.2). The hydrogel was prepared by mixing the individual components. Cross-linking of ADA and gelatin is due to Schiff's base formation between the ε-amino groups of lysine or hydroxylysine side groups of gelatin and the available aldehyde group in ADA. Fibrinogen having the same group may react to the ADA in the same manner if free aldehyde groups are available. ADA forms Schiff's base immediately at alkali pH. Thrombin acts as a cross linker for fibrinogen that binds with ADA.

Table 3. 2Gelation time of hydrogel with respect to different ratio of components.

Sl. No	ADA wt%	Borax (M)	Gelatin wt%	Fibrinogen (mg)	Thrombin (IU)	Gelation time (Minutes)
F1	5(1)	-	5(1)	-	-	>30
F2	8(1)	-	8(1|)	-	-	>30
F3	10(1)	-	10(1)	-	-	15
F4	10(1)	0.1	10(1)	-	-	10
F5	10(1)	-	10(0.5)	30(0.5)	15	10
F6	10(1)	0.1	10(0.5)	30(0.5)	15	3

Lower concentration (5% and 8%) of ADA and gelatin without borax did not form hydrogel. This was may be due to the lesser availability of -CHO and NH2 group in ADA and gelatin respectively. As the concentration of ADA and gelatin increased gelation occurred within 15 minutes without borax. Gelation time reduced to 10 min when 0.1M borax was also added in 10% of ADA and gelatin solution. But the stability of hydrogel was poor when stored at 37˚C. As per the literature, concentration of fibrinogen between 20-40 mg and thrombin 10-20 IU gives mechanically stable gel with good cell proliferation. In the present study 30 mg of fibrinogen and 15 IU thrombin (F6) was added to 10% ADA and 10% gelatin solution with 0.1M borax. There was a significant reduction in the gelation time (3-4 minutes) which is optimum for in-vitro culture system. Gelation time was reduced due to the availability of more amino group from fibrinogen which also forms Schiff base reaction with ADA similar to gelatin. Thus F6 was used for the further characterization studies.

3.4. CHARACTERIZATION OF HYDROGEL

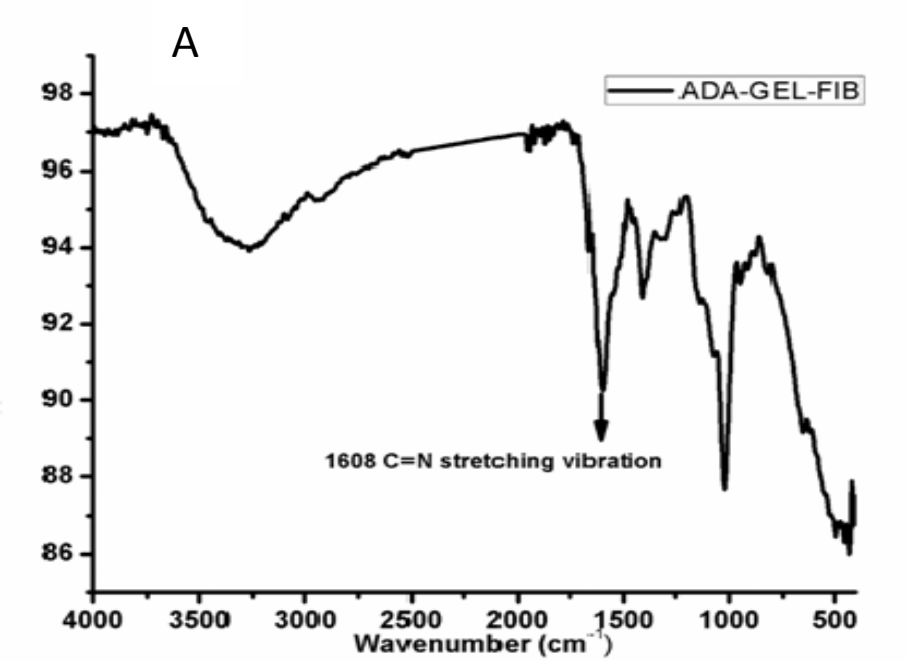
3.4.1. FTIR ANALYSIS

The hydrogel was prepared by ADA, gelatin and fibrinogen with cross linker thrombin. The nature of the interaction between ADA and gelatin can be clearly elucidated from the spectra of the hydrogel. From the hydrogel spectrum (fig 3.5. A) ,it can be found

that the aldehyde peak of ADA at 1738.51 cm^{-1} is absent due to the cross-linking with gelatin and fibrinogen. Although the C=N stretching of Schiff's base seems to be present between 1615-1650 cm^{-1}[Ye, Xiong, & Sun, 2012] a shift in the peak position to 1608.53 cm^{-1} was observed in the hydrogel spectrum. This may be due to the overlapping of the band at 1622.05 cm-1 of amide I of uncross linked gelatin. The band at 1538.21 cm^{-1} disappeared showing the involvement of amide II group in the hydrogel formation (Fig. 3.5 B).

In hydrogel crosslinking mainly occurs due to Schiff's base formation through the interaction between the 3-amino groups of lysine or hydroxylysine of gelatin and the available aldehyde groups of ADA. Since fibrinogen contains the same lysine or hydroxylisine it can bind to ADA as Gelatin (Fig .3.5.B).

A



B

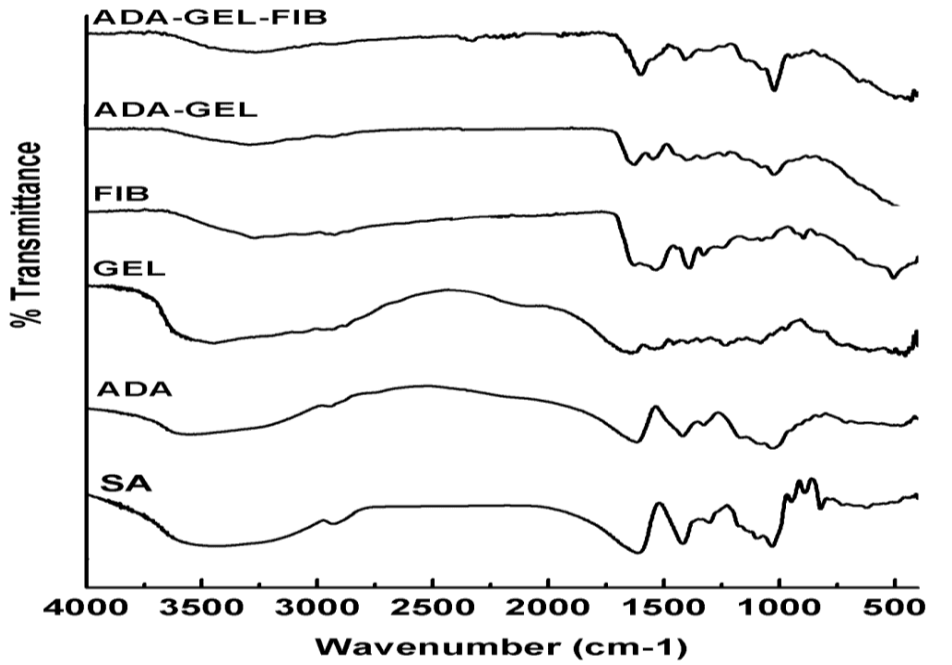


Fig 3. 5 FTIR spectra of A) Hydrogel and B) overlay Image

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3.4.2. GELATION TIME BY TUBE INVERSION METHOD

Gelation time is defined as the time required for complete cross linking reaction. Gelation time for the optimized formulation was analyzed. It was observed that F6 forms the gel by 3 min(Fig 3.6)

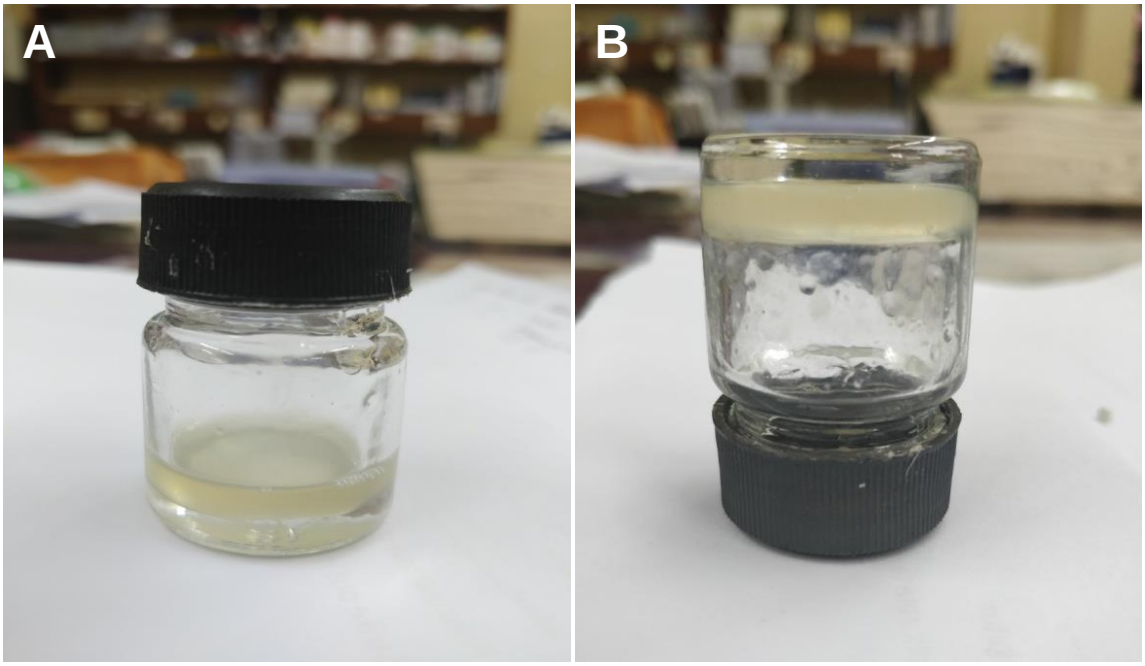


Fig 3. 6Gelation time determination by Tube Inversion method

3.4.3. RHEOLOGICAL ANALYSIS

The Rheological properties of the individual components, components without cross linker and components with cross linker were analyzed. The graph was plotted against viscosity and time is shown in Fig 3.7. The results shows that the mix of components without any cross linker shows increase in viscosity with time while in the presence of cross linker the viscosity shows a sudden initial decrease and then maintain a static viscosity because of the immediate formation of hydrogel.

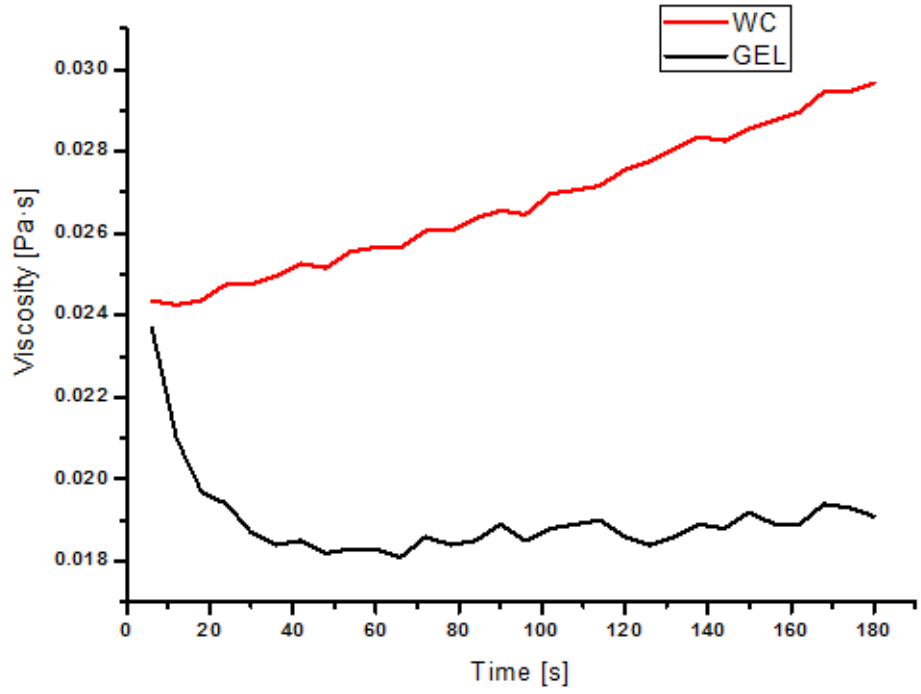


Fig 3. 7Rheological analysis of Gel without cross linker(WC) and with cross linker(Gel)

3.4.4. *IN VITRO* SWELLING

The in vitro swelling study was carried to find out the water absorption character of hydrogel. The swelling ratio was calculated from initial dry weight and final weight of hydrogel after 24h incubation in PBS. The swelling percentage was obtained as 95.92. From the data it is clear that the hydrogel is highly hydrophilic. The hydrogel become hydrophilic because each component used for making the hydrogel is a natural polymer. As the network is swollen by the absorption of solvent, the chains between the cross linker assume elongated

configuration. There are studies where the swelling ratio of ADA-gelatin hydrogel was found as 90% because of high hydrophilic nature of the polymers used for hydrogel construction [Yin et al., 2014].

3.4.5. DEGRADATION BEHAVIOUR OF HYDROGEL

The degradation profile of hydrogel was examined under in vitro conditions in distilled water at 37˚C as shown in Fig 3.8.The data was shown as graph by plotting Weight percentage obtained by using the formula (Initial weight-Final weight/Initial weight*100) for one week. From the data it was noted that there is a reduction in weight of hydrogel incubating in PBS with respect to increase in time period. 70% reduction was observed in the weight of hydrogel by 7th day. This is because the fibrin and gelatin degraded fatly when kept 37 ^{0} C.

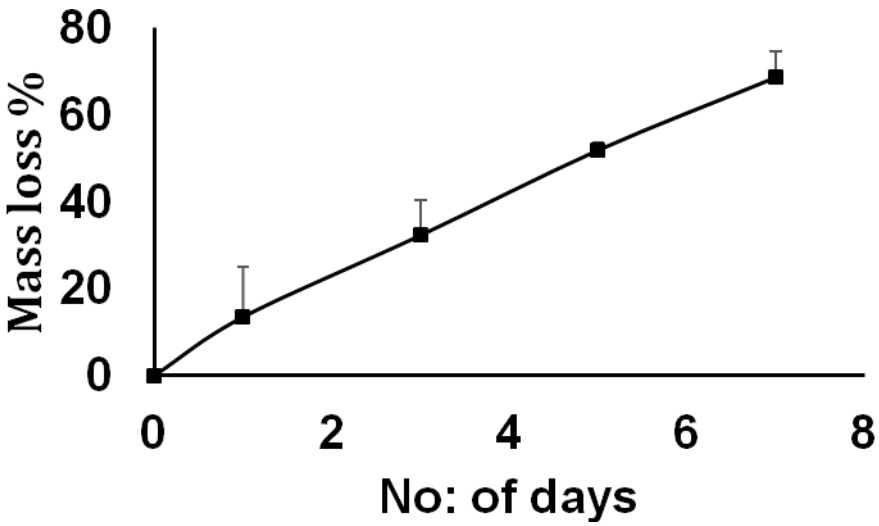


Fig 3. 8 Graph showing the weight loss behavior of hydrogel for 7 days

The degradation behavior of the hydrogel is again confirmed with SDS-PAGE analysis using the extract of the incubated hydrogel of three different time period (Fig 3.9). Gelatin hydrogel was taken as standard and crude gelatin, fibrinogen and thrombin was used to confirm the degraded product. The results shows that the gelatin is firstly degraded in the sample and the bands correspond to fibrin couldn't find out in the SDS-PAGE.

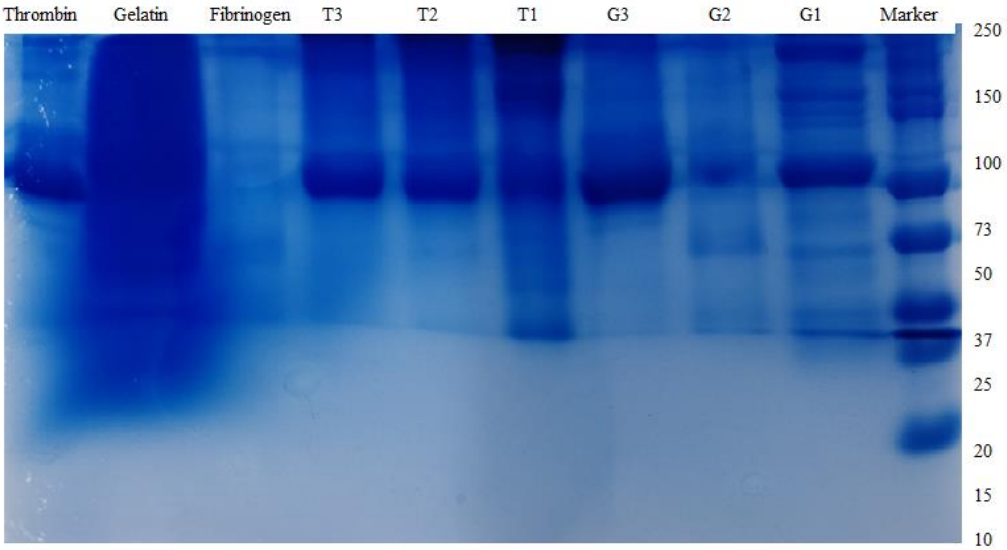


Fig 3. 9SDS-PAGE of degraded hydrogel

3.4.6. SURFACE TOPOGRAPHY OF HYDROGEL- SEM EVALUATION

The surface topography of optimized hydrogel was investigated by SEM (Fig: 3.10. A. Top view of gel with magnification 500 as and, B. Cross section of hydrogel with 500x magnification). SEM image clearly shows the interconnecting pores in hydrogel. Randomly oriented fibrillar morphology of gelatin was also seen in the image, when cross linked with ADA. Fibrin forms a uniform network on ADA- gelatin hydrogel, shows in arrow on the image which gives a further confirmation on the cross linking of individual components in hydrogel. Highly interconnected porous structure on hydrogel facilitates proper cell seeding, attachment and migration [Mikos et al., 1992]. Fibrillar morphology of hydrogel resembles ECM which also provides a friendly environment for cells seeded on hydrogel.

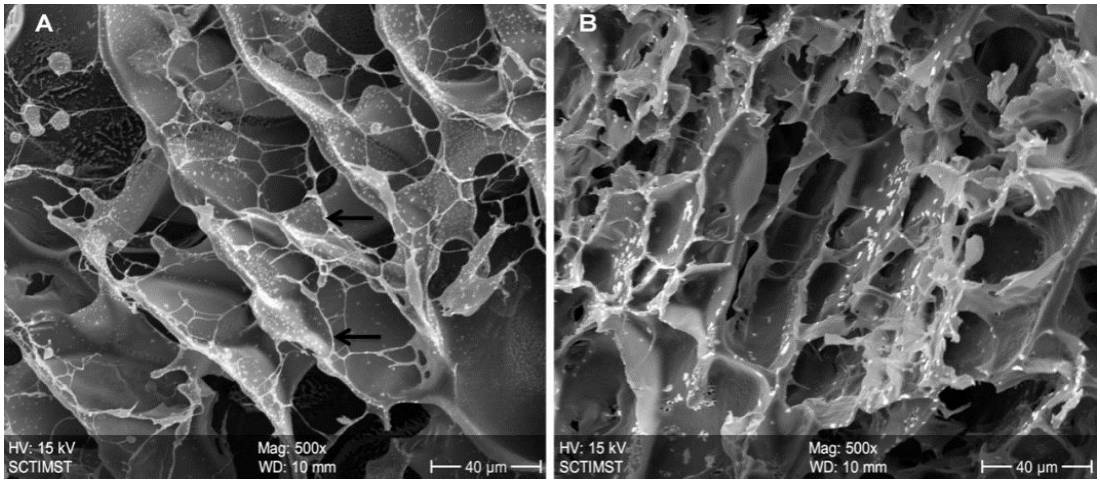
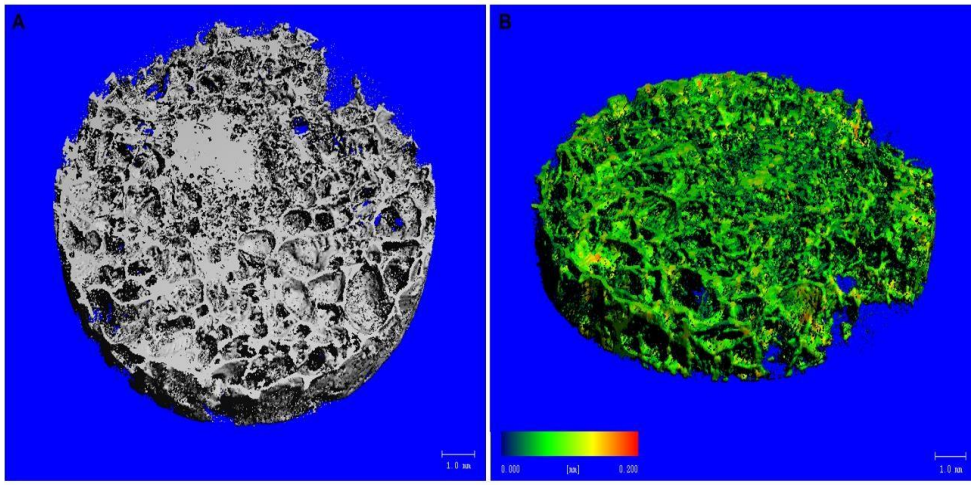


Fig 3. 10Topographical morphology of hydrogel. A) Top view of hydrogel B) Cross section. Arrow represent fibrin network.

3.4.7. POROSITY AND 3D MICROARCHITECTURE BY MICRO-CT

The micro CT images were obtained for analyzing porosity and pore volume of hydrogel. From the 3D morphology (Fig: 3.11. A&B) it is clear that the hydrogel is highly porous. The quantative analysis of pore size distribution was depicted in figure. 3.11. C by plotting the pore size on X axis and pore volume in Y axis. Form the graph it is clear that the lyophilized hydrogel shows high porosity with uneven distribution of pores. The maximum size of pores on the hydrogel lies in between 0.2 to 0.4mm with a wide range between 0.2 to 0.7mm. Thus it makes the hydrogel is highly suitable for cells with varying sizes.



C

Fig 3. 11A) & B) 3D morphology of hydrogel and C) pore size distribution in hydrogel

3.5. BIOLOGICAL EVALUATION OF HYDROGEL

3.5.1. CYTOCOMPACTABILITY OF HYDROGEL BY MTT ASSAY

To confirm the non-toxic nature of the hydrogel, a preliminary cytotoxicity evaluation using mouse fibroblast L929 cells was carried out using direct contact as well as MTT assay. The morphological analysis by direct contact assay for 24 h shows no morphological changes thus confirm that the cells are viable when contact with the hydrogel (Fig.3.12 A) & B). Quantitative assessment of the cytotoxicity by MTT assay of cells after contact with the material showed 80 % viable cells for 24h (Fig 3.13). There are studies where the viability of the fibroblast for ADA-Gelatin gel shows 93% viability for 24 hours [Balakrishna et al., 2005] .However the result obtained from our results also shows similar viability with previous works. But viability of cell by MTT assay in long term culture condition is not evaluated.

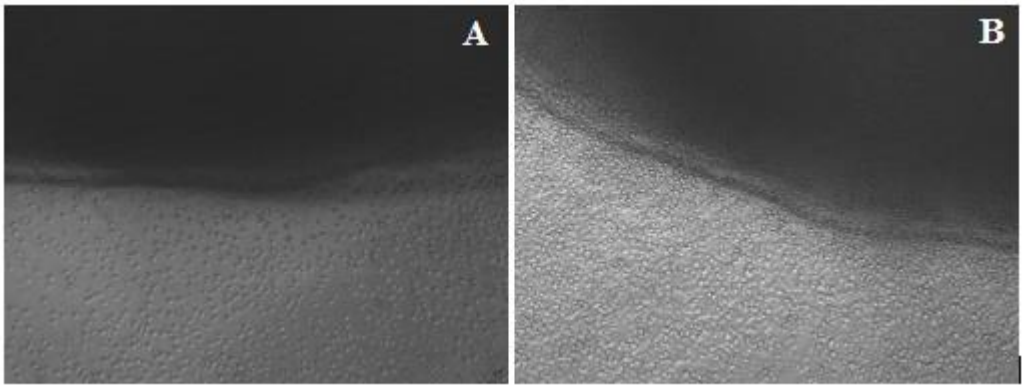


Fig 3. 12 Direct contact assay of A)L929 cells at 0h B) L929 cells at 24h

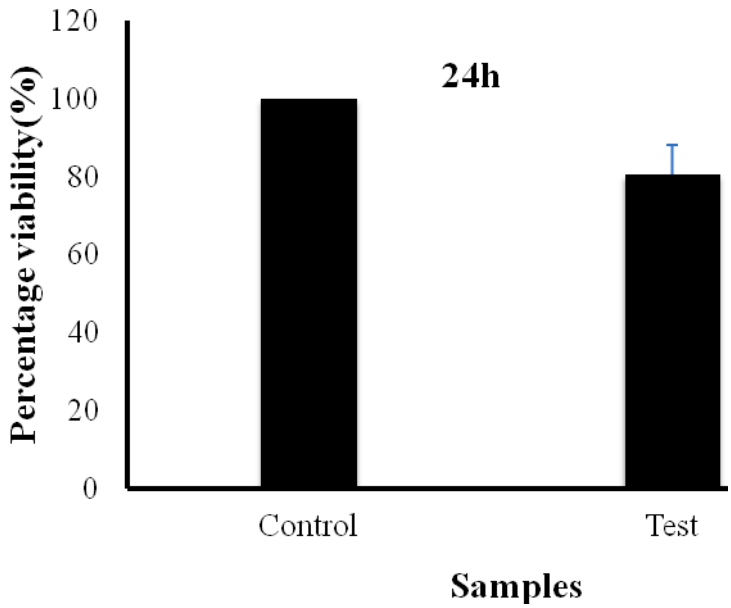


Fig 3. 13Graph showing the viability of cells by MTT assay for 24h

3.5.2. HEPG2 VIABILITY ON HYDROGEL

The HepG2 cells are seeded on the top of the hydrogel to see whether the system can support liver cells .Morphological analysis of HepG2 cells reveled viable cells in gel at 24h. Fig 3.14.A and D represents the cells in control conditions (Cells without hydrogel) whereas Fig 3.14 B and E represents cells in hydrogel. Fig 3.14 C and F represents hydrogel without cells. It's clear from the images that cells are proliferating well in hydrogel, probably due to the 3D porous structure of hydrogel which allows cells to migrate and proliferate and mimic the in-vivo microenvironment as well as due to the presence of fibrinogen. It is known that fibrin supports cell growth and proliferation. Thus present system provides a good micro-environment for HepG2 cells growth and proliferation. Further confirmation analysis live dead assay will be done.

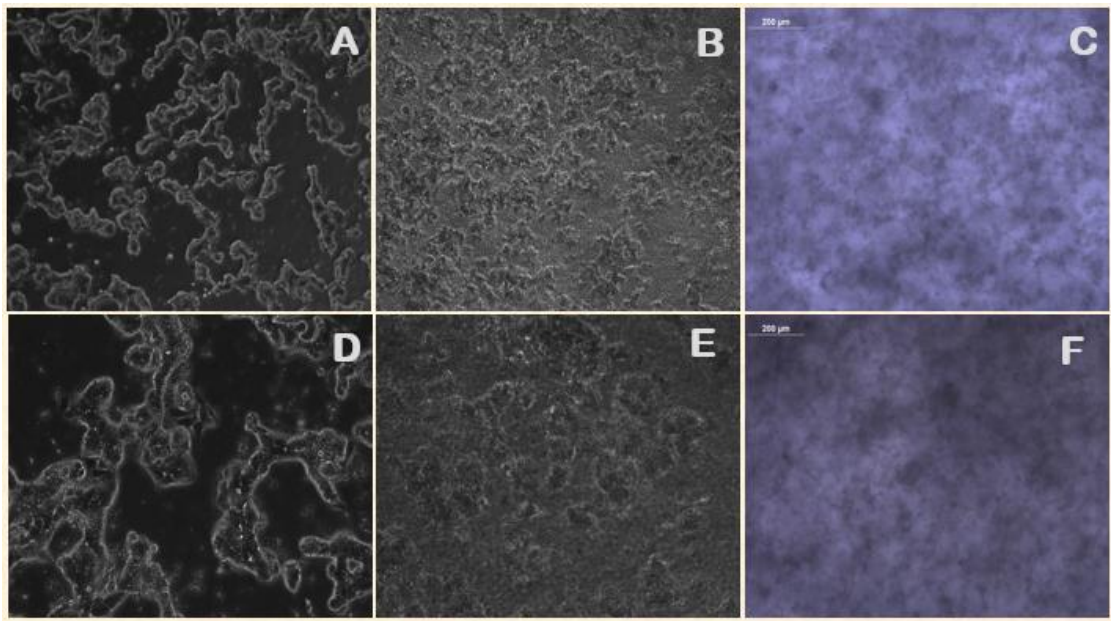


Fig 3. 14Phase contrast image of A) Control (10X), B) HepG2 on hydrogel (10X),C) Hydrogel alone (10X) D) Control (20X), E) HepG2 on hydrogel (20X) & F) hydrogel (20X).

3.5.3. CELL VIABILITY STUDIES USING LIVE DEAD AND ACTIN STAINING

The viability studies of cells growing in hydrogel were studied by live dead staining and actin staining. The viability and morphology of living cells can be evaluated using calcein AM that stains the cytoplasm of living cells. Esterases present in the cytoplasm of the living cells break down calcein, resulting in a fluorescent green product which is impermeable to cell membranes. Apart from the cell viability, calcein staining further provides information about the cell shape and membrane integrity which are the hallmarks of normal cell equilibrium. From the image it is clear that the cells are viable on the hydrogel after 24h (Fig.3.15). Moreover, cells were found to be attached and spread after 24h of incubation. As expected, the number of adherent cells increased when seeded on the ADA-Gelatin-Fibrin hydrogel. This phenomenon can be attributed to the

availability of cell-binding peptides of gelatin and fibrinogen in the hydrogel. These hydrogels therefore possess more cell adhesion peptides which enhance their cell-material interaction.

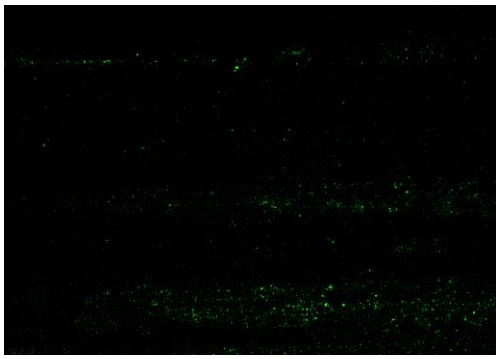


Fig 3. 15 Live dead staining of cells in hydrogel (10x)

To visualize cell morphology and cell spreading onto the hydrogels, actin cytoskeleton staining of the cells was performed using rhodamine phalloidin. Cell morphology plays an important role in the general cell hemostasis. The F-actin cytoskeleton staining showed that cells were having round morphology and the number of cells in the hydrogel is more compared with control with cells alone (Fig 3.16.). As expected, on the hydrogel, there were markedly higher cells as compared to the control at day 1. Moreover, cellular morphology was disturbed and cell shape was rounded, which is in accordance with the results observed by calcein staining. HepG2 cells normally develop spheroid like morphology when seeded on the hydrogel.

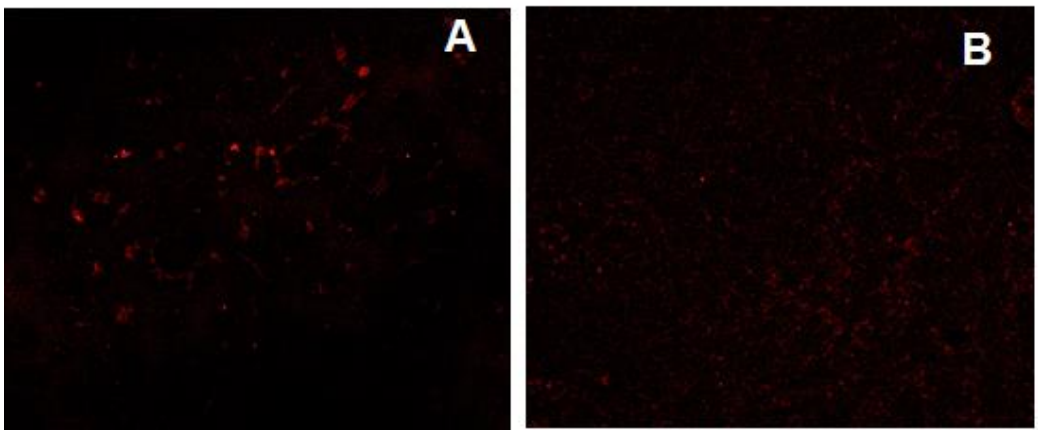


Fig 3. 16 A) Actin staining on Control cells(10X) & B) Actin staining on cells in gel(10X)

3.5.4. INDOCYANINE GREEN UPTAKE ASSAY

Studying the clearance of dyes which are removed from the circulation mainly by the liver is one of the best methods for evaluating hepatic function. Indocyanine green is a cyanine dye generally used for clinical diagnostic applications. It is used as an indicator substance to assess liver functionality. The uptake of Indocyanine green/cardiogreen by the HepG2control cells grown in medium and in the hydrogel at 24h was observed by confocal microscopy as well as phase contrast microscopy (Fig3.17 A, B &C).The ICG uptake pattern shown by cells grown in hydrogel conditions was notably high compared to the control groups. There

are studies that growth factors induced ADMSC shows maximum uptake than the one which is control group with cell culture medium alone[Ji et el., 2012]. The cells grown in hydrogel did uptake more dye than those cultured in medium alone which is an indicative of support of hepatic cells like cells by the 3D hydrogel niche. Some studies have evaluated ICG elimination by the cells after a time period of six hours [Cai et al., 2007]. Hence future experiments can be conducted so as to examine if the cells are eliminating the dye after 6h and the differences in the elimination pattern by cells grown in different experimental conditions can be compared to arrive at better conclusions.

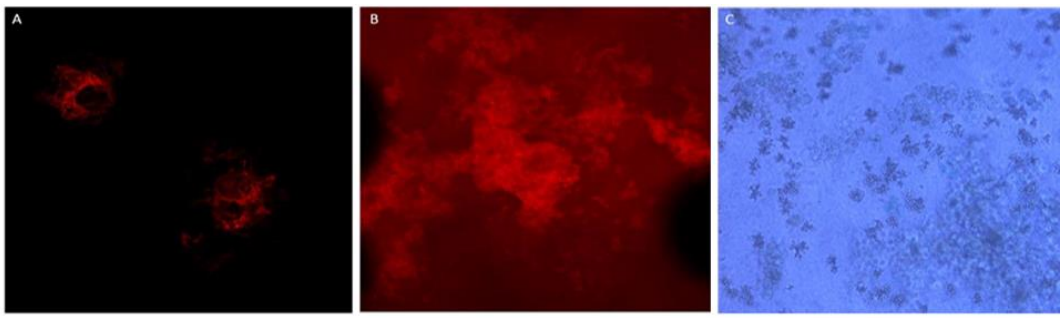


Fig 3. 17Confocal image of A)Control ICG (10X) , B) Cells seeded on hydrogel (10X) & C) phase contrast image of ICG stained cell seeded on hydrogel(10X)

3.5.5. LDL UPTAKE

Fluorescent labeled LDL uptake in HepG2 cells are assed in order to find out the functionality of HepG2 cells grown in the Hydrogel (Fig 3.18). LDL receptor are exclusively synthesized in liver cells and are responsible for the clearance of LDL from the blood circulation by specifically bind to the LDL and hence internalization into the liver cells. Hence HepG2 cells are liver origin they also possess the same property if they are healthy grown in the culture condition. From the LDL uptake assay it is clear that the HepG2 cells grown in hydrogel intake LDL same as that of cells grown in control (Cells without hydrogel).

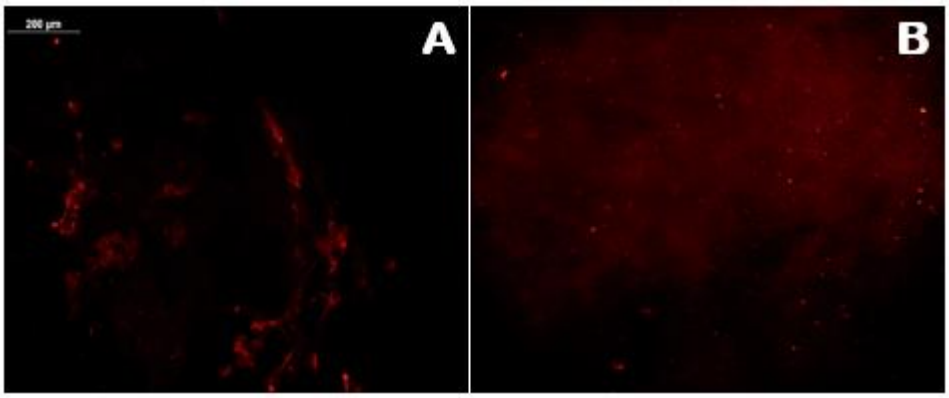


Fig 3. 18LDL uptake of A) Control cells(10X) & B) Cells in hydrogel(10X)

3.5.6. GLYCOGEN STORAGE ASSAY

The liver is one of the major sites of glycogen storage in the human body. The synthesis and degradation of glycogen are regulated by the hepatocytes to maintain blood-glucose levels of the organism. PAS staining was done for HrpG2 cells grown in hydrogel and without hydrogel and glycogen storage within the cells was evaluated with the help of a light microscope (Fig 3.19).The control cells grown in tissue culture plate without any hydrogel support shows glycogen granules to higher extent than cells grown on hydrogel. Because the hydrogel are opaque and shows blurred image while imaging the glycogen granules present in the cells grown on the hydrogel couldn't show clear granules compared to control. But the PAS positivity shown by the cells on the hydrogel confirmed that the functions of liver like HepG2 cells are supported by the hydrogel.

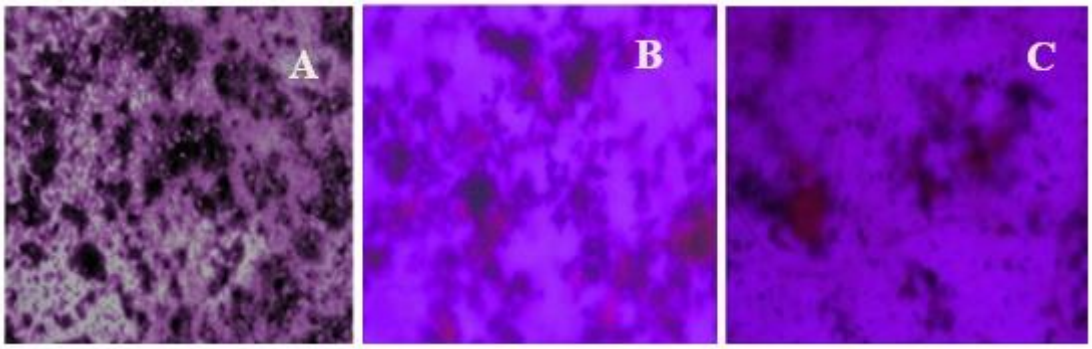


Fig 3. 19PAS staining of A) HepG2 without gel (10X), B) HepG2 in hydrogel (10X) & C) HepG2 in hydrogel (20X)

3.5.7. IMMUNOFLUORESCEN STAINING OF CYP450

Cytochrome P450 (CYP450) is the enzyme synthesize exclusively by the liver cells plays important role in xenobiotic reaction. Xenobiotic reactions are the one through which the toxic hydrophobic drugs and toxins are converted to non-toxic hydrophilic substance. The immunofluorescence of CYP450 (Fig 3.20) was done to confirm whether the HepG2 cells grown on the hydrogel possess the activities like liver cells. From the image it is clear the HepG2 cells grown on the hydrogel shows the presence of CYP450 activity as compared to control cells.

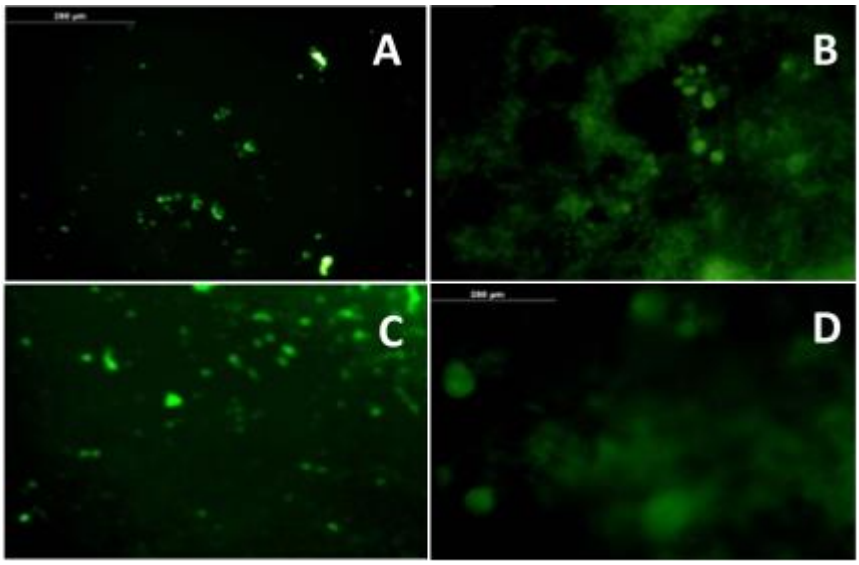


Fig 3. 20 CYP450 immunostaining of A) Control cells(10X), B) Cells in hydrogel(10X), C) Control cells(20X) & D) Cells in hydrogel(20X)

3.5.8. AMMONIA ASSAY

Liver is the major organ involved in maintenance of amino acid levels and its conversion to the final metabolite urea. The media in which the cells the cells were cultured was collected and analyzed for ammonia clearance.HepG2 cells shows almost all the gene expression of normal liver cells, hence it is assumed that the HepG2 cells can effectively convert ammonia into urea by specific enzyme L-Glutamate dehydrogenase[Bergmeyer et al.,1985]. The optical absorbance was taken at 340 nm before and after adding the enzyme shows the effective conversion of ammonia into urea because the OD decreased after adding the enzyme for both control and test. Test (HepG2 in Fibrin-ADA gel) shows clearance same to control that is HepG2 in medium (Fig 3.21). Hence from the assay it is concluded that the HepG2 in hydrogel shows almost similar properties of normal liver cells.

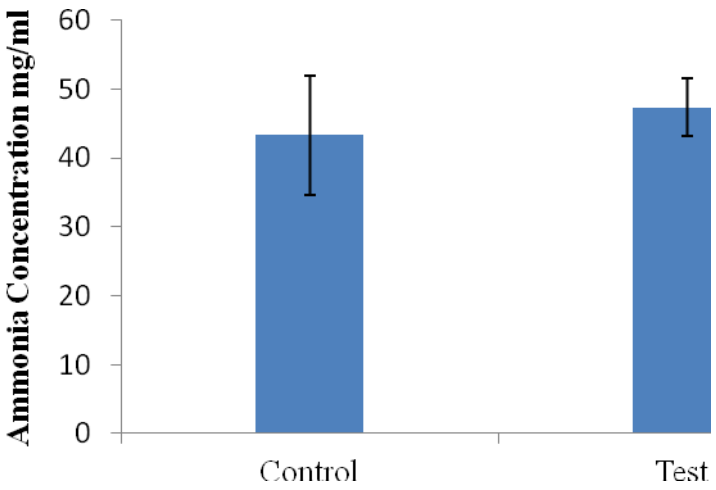


Fig 3. 21Graph showing the ammonia assay concentration in test and control

3.5.9. ALBUMIN ASSAY

Liver is the major organ involved in the synthesis of Albumin which is a blood protein. To confirm the functionality of the HepG2 cells in the hydrogel, SDS-PAGE was done (Fig3.22). It was already shown that HepG2 cells synthesize albumin same as that of control cells [Nakamura et al., 2011]. Which shows that the Control as well as test shows the same band in the region of 73-50 corresponds to molecular weight of albumin. Thus from the results it can concluded that the HepG2 cells grown in the hydrogel synthesize same amount of albumin.

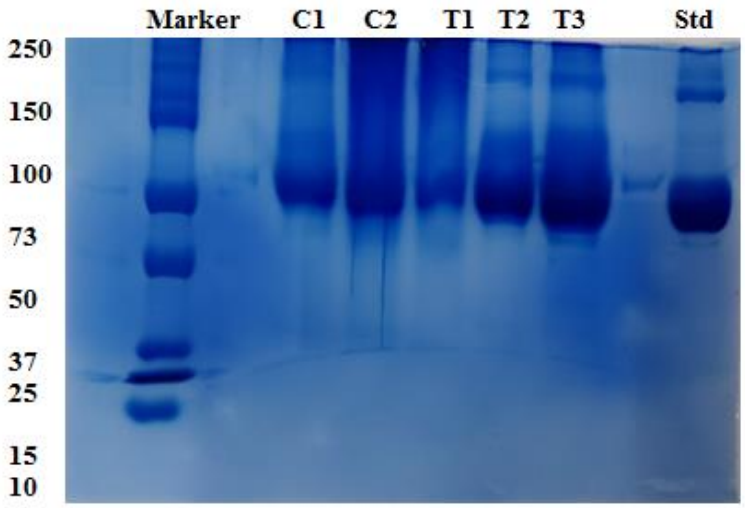


Fig 3. 22 SDS-PAGE of albumin

CHAPTER 4

SUMMARYANDCONCLUSIONS

4.1. SUMMARY

ADA was obtained by the oxidation of Alginate dialdehyde with sodium metaperiodate. The synthesized ADA was characterized using FTIR, NMR, molar mass calculation, aldehyde content and degree of oxidation. Optimized ADA was further used for the preparation of hydrogel with Fibrin and gelatin in-order to improve the existing ADA-Gelatin gel. The hydrogel obtained from the above components are then characterized by FTIR, Micro CTRheological analysis, SEM, Degradation and swelling studies. Results obtained from the characterization lead to the use of this hydrogel for cell culture. In order to access the cytocompactability of hydrogel, MTT assay was done using the direct contact of hydrogel by L929 mouse fibroblast cells for 24h. The results showed that the hydrogel is cytocompatible. In order to investigate whether the hydrogel support liver like cells HepG2 cells were seeded on the hydrogel and observed for viability using Live dead staining and actin staining and its functionality using different functional characterizations such as indocyanine green uptake assay, LDL uptake assay, Glycogen storage assay, CYP450 staining, ammonia clearance assay and albumin assay.

4.2. CONCLUSIONS

ADA of different oxidation was successfully synthesized in laboratory using sodium alginate and sodium metaperiodate. The oxidation generates reactive aldehydic groups on the ADA makes it more reactive and degradative. The FTIR and NMR analysis shows that ADA possesses appropriate groups similar to sodium alginate with extra aldehydic group. Synthesized ADA of different degree of oxidation is then analyzed for molar mass analysis,

degree of oxidation and aldehyde content. Since it is shown in literature that lower degree of oxidation doesn't possess the property of low mechanical stability and higher possess almost same properties of ADA with middle oxidation, the ADA with 55% oxidation is selected for the study. The ADA is then allowed to react with gelatin and fibrinogen with double cross linking using borax and thrombin to form a stable hydrogel. The synthesized hydrogel is then used for physical characterization. The results from FTIR show the peak shifting on hydrogel which is an indicator of cross linking. Gelation time analysis with hydrogel shows the low gelation time within 3 minutes make it use as a bioink in future studies. The rheological property also points out the shear thickening property it is an indication of its use in cell culture and 3D printing application. The morphology

Analysis by SEM shows numerous interconnecting pores on lyophilized hydrogel it is an important property essential for cell culture. The micro CT results shows that the there are numerous interconnecting pores in hydrogel with unequal distribution. The biological evaluation of hydrogel with MTT assay using mouse fibroblast cells shows that the gel is nontoxic for cells even for 24h. The culturing of HepG2 cells on the hydrogel shows that the cells can migrate into the hydrogel and behave like they are in its own ECM. This was confirmed by live dead staining and Actin staining. The functional characterization using Indocyanine green uptake by HepG2 cells shows that the cells in the hydrogel can take dye similar to the control cells. The LDL uptake assay also shows the uptake of LDL by the cells on hydrogel like control cells. The glycogen storage assay also shows the presence of glycogen granules in the cells grown in the hydrogel same as that of control cells. Immunostaining of CYP450 shows that the cells are able to synthesize CYP450 enzyme like control cells, the quantitative estimation of ammonia clearance assay by the cells shows that there is a reduction of ammonia concentration in cells on hydrogel compared to control cells and albumin assay by SDS –PAGE shows that the cells in control and hydrogel are able to secrete albumin in same manner. The results showed that the HepG2 cells in the hydrogel are functionally active. Thus to conclude the constructed hydrogel can support the growth and function of liver like cells.

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