TISSUE ENGINEERING OF CARTILAGE: MATRIX MEDIATED EFFECTS ON CHONDROGENESIS

A Thesis Presented

By

NEETHU MOHAN

To

The Division of Laboratory for Polymer Analysis
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy of

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM – 695 012

January, 2008

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
LIBRARY
THIRUVANANTHAPURAM

Declaration

I, NEETHU MOHAN, hereby declare that I had personally carried out the work depicted in the thesis entitled **"TISSUE ENGINEERING OF CARTILAGE: MATRIX MEDIATED EFFECTS ON CHONDROGENESIS"** under the direct supervision of Dr. Prabha.D.Nair, Scientist G & SIC, Laboratory for Polymer Analysis, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

Date:

NEETHU MOHAN

Tele: 0471-2340801
Fax : 0471-2341814

Grams: CHITRAMET
Telex : 0435-6290

श्री चित्रा तिरुनाल आयुर्विज्ञान तथा प्रौद्योगिकी संस्थान
बायो मेडिकल टैक्नोलॉजी विंग
पूजापुरा, तिरुवनन्तपुरम-695 012, इन्डिया

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
BIO MEDICAL TECHNOLOGY WING
POOJAPPURA, THIRUVANANTHAPURAM-695 012, INDIA
(An Institute of National Importance under Govt. of India)

Ref:

Date: 28.1.08

CERTIFICATE

This is to certify that Ms. NEETHU MOHAN, in the division of Laboratory for Polymer Analysis of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to her thesis entitled "**TISSUE ENGINEERING OF CARTILAGE: MATRIX MEDIATED EFFECTS ON CHONDROGENESIS**" was carried out under my direct supervision.

Dr. PRABHA D NAIR
(GUIDE)

The Thesis

Entitled

TISSUE ENGINEERING OF CARTILAGE: MATRIX MEDIATED EFFECTS ON CHONDROGENESIS

Submitted

By

NEETHU MOHAN

for

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM

Evaluated and approved
by

Dr. Prabha D Nair
(Guide)

(Thesis examiner)

Acknowledgements

I would like to express my deep and sincere gratitude to my guide, Dr. Prabha D Nair, for her valuable guidance and constant encouragement that provided a good basis for the present thesis. Her enthusiasm, high expectations and trust pushed me towards the successful completion of my work.

I am greatful to Director SCTIMST and Head BMT Wing for providing facilities to carry out my work in this institute. I also acknowledge for the Institute Fellowship.

I am deeply grateful to Dr. K. Sreenivasan for his detailed and constructive comments, and important support throughout this work.

I express my sincere thanks to my DAC members, Dr. Lissy Krishnan and Dr. Maya Nandakumar, for their valuable suggestions.

I acknowledge the Jawaharlal Nehru Memorial Fellowship for funding part of my work in University of Kyoto, Japan.

My sincere gratitude to Prof. Yasuhiko Tabata, Institute for Frontier Medical Sciences, University of Kyoto, Japan, for giving me an opportunity to understand and work on stem cells.

On a personal note, the friendships that I have shared with many of my colleagues Ms. RadhaKumary, Mr. Rowsen Moses and Mr.P.R.Hari have enriched my experience and their kind support has been of great value in this study. Heartfelt thanks to all my colleagues especially Ms. Lynda Thomas, Mr. Sudhakar and Mr.Arun for their timely help and kind co-operation for the successful completion my work. I warmly thank all other colleagues for their care and concern.

My special thanks to Dr.Umashankar, Dr. Sachin, and all colleagues, Department of Vivarium, for their sincere effort in successful isolation of articular joints for my studies.

I warmly thank Dr. Roy Joseph, Polymer Processing Laboratory, for providing facilities for the analysis of mechanical property of my samples.

My warm thanks are due to Mr. Sreekumar for Scanning electron Microscopy images.

I also wish to thank Dr. Kalyanakrishnan for the Micro CT imaging studies.

I thank the Registrar SCTIMST, my colleagues from other departments of BMT and Hospital Wing, for their good will and valuable help.

With great pleasure, I record the encouragement, support and prayers received from my Parents, Husband and Daughter for always being by my side, for their love, sacrifices, and continued encouragement throughout the years.

Above all I am deeply indebted to the Almighty who always remains as a spiritual hand behind me in all my crisis and have showered me with innumerable blessings.

Neethu Mohan

CONTENTS

LIST OF TABLES

LIST OF FIGURES

SYNOPSIS

Hyaline cartilage at the articular joint helps in the friction free movement of the joints by distributing and transferring the load applied at the joints and resisting compression. Diseases such as osteoarthritis, trauma associated with injury, chondrosarcoma and other congenital diseases form a defective cartilage where the complex metabolic balance of the cartilage is disrupted leading to the loss in integrity of tissue and biomechanical function. These disease conditions affect almost all people above the age of sixty and according to the World Health Organization, the prevalence of a damaged joint is going to increase drastically in the coming years. Currently available treatment options provide short term relief with poor tissue regeneration. Tissue engineering is an emerging alternative treatment using autologous cells that are isolated from patient, expanded and cultured in vitro in three dimensional (3D) scaffolds which can be implanted back into the patient. Appropriate combination of 3D polymeric scaffolds, cells and signaling molecules are required for successful invitro regeneration of the tissue.

Tissue engineering of cartilage generally utilizes a natural or synthetic matrix to serve as a 3D template for growing cells. The two dimensional cultures are usually inadequate to model the complex cellular interactions compared to the 3D nature of tissue. 3D culture in scaffolds on the other hand, promotes high cell-cell and cell-matrix interaction, provides more surface area and is a better representation of actual tissue. It serves as an artificial extra cellular matrix providing support for the cells during invitro culture. The overall morphology of the 3D scaffolds, inbuilt properties of the materials and chemical composition may directly or indirectly influence the behavior of cells cultured on the scaffold. Considering the major role of 3D scaffolds in invitro engineering of tissue, the first hypothesis evaluated in this thesis is that physicochemical properties and 3D

structure of the scaffold play a significant role in determining the cell morphology and the extra-cellular matrix production by cells cultured in these scaffolds.

The next important factor in invitro engineering of tissue is the requirement of high density culture of chondrocytes in scaffolds to produce a 3D tissue like construct. It has been established by several earlier reports that chondrocytes have limited expansion potential and lose phenotype on long term culture in monolayer. So in this study the problem is addressed using mesenchymal progenitor cells which serves as an alternative source with a high expansion potential capable of supplying large quantities of cells to form a 3D tissue like structure. These cells have the differentiation potential to specific lineages upon proper signaling. The differentiation of mesenchymal stem cells is influenced by growth factors, cell-cell interactions and cell-matrix interactions. In this study the invitro chondrogenic differentiation of mesenchymal stem cells is facilitated by supplying an array of growth factors in the appropriate concentration and combination to obtain a proper phenotype. Several previous reports have shown that in native tissue the differentiation process takes place by the combined or simultaneous action of different growth factors. So in this study the second hypothesis evaluated is that supplement of a single growth factor may not elicit all the necessary molecular signals for the differentiation process during invitro studies and a combination of growth factors along with a chosen 3D scaffold is necessary to attain appropriate differentiation. The effect of 3D scaffolds and role of signaling molecules in the differentiation of mesenchymal stem cells is investigated in this study, both in presence of growth factors supplied individually and in various combinations. One hybrid composition of 3D scaffold and a cocktail of growth factors have been identified to promote better chondrogenesis than others.

This thesis has been divided into four chapters, the first introductory chapter briefly discusses the composition, physiology and pathology of articular cartilage. The currently available treatment options and its drawbacks are discussed

in detail. The concept and role of the three major components of tissue engineering namely, 3D scaffolds, cells and signaling molecules are discussed focusing on to chondrocyte culture in 3 D scaffolds. A literature review of the polymeric scaffolds currently used for chondrocyte culture and their drawbacks are given. Hence the necessity to find a hybrid scaffold with better properties is pointed out in this section. One major problem in tissue engineering of cartilage is the nonavailability of autologous cells in sufficient number to form a 3D structure. Hence the requirement of mesenchymal stem cells and differentiation process is dealt in this chapter along with information regarding the signalling molecules that cue the differentiation process.

The second chapter deals with the materials and methodology in fabrication of two sets of novel 3D hybrid scaffolds used in this work. First the semi interpenetrating network (IPN) of polyvinyl-alcohol and polycaprolactone (PVA-PCL) made from synthetic polymers and second a blend of natural polymers made from gelatin and albumin. The novel hybrid scaffolds are characterized for its physicochemical properties and invitro cytotoxicity. The physicochemical properties of the novel hybrid materials are evaluated using techniques like thermal analysis, Fourier transform IR spectroscopy (FTIR) and contact angle measurements. The suitability of hybrid scaffolds to serve as a 3D scaffold is confirmed by analyses of its surface morphology, pore characteristics, swelling and mechanical properties. The feasibility of modifying the scaffolds with peptides to make it more biomimetic is examined by coupling representative amino acid to the functional groups in the polymers. Both these scaffolds are evaluated for the initial cytotoxicity using L929 fibroblast cells as per ISO standards. Further in this chapter the isolation and in vitro culture of chondrocytes and mesenchymal stem cells in monolayer and its characterization are described. Initially the morphology and viability of chondrocytes in 3D scaffolds is determined. After this study cells are seeded at an appropriate seeding density and cultured in chondrogenic medium for a period of two and four months. The invitro cultured constructs are evaluated

for overall cell distribution, cell morphology and for production of cartilage specific matrix molecules using various biochemical and histological techniques. Thus the suitability of these scaffolds for chondrocyte culture is analyzed. Further the differentiation of mesenchymal stem cells to chondrocytes is also attempted in these scaffolds. The seeding methodology of mesenchymal stem cells in this scaffold is standardized initially and further the in vitro conditions for differentiation to chondrocytes is established by culturing in chondrogenic medium supplemented with individual or combination of growth factors. The one month construct is evaluated for chondrogenesis to compare the effect of different signaling molecules in the differentiation process. The effect of 3D scaffold in the differentiation process is evaluated by conducting this study in two different hybrid scaffolds.

The third chapter on results and discussion is segregated into four parts. The first section discusses in detail the selection of polymers for fabrication of 3D scaffold. The scaffold is expected to show biocompatibility, provide mechanical strength during the regeneration process and act as a support to the ingrowth of cells and matrix. Considering the fact that scaffolds made of a single polymer does not match the properties of extra cellular matrix, new hybrid scaffolds with better properties were fabricated for this work. Synthetic scaffolds have the advantage that its properties are finely tunable according to the requirement while the natural polymers are more biomimetic. So scaffolds fabricated from both synthetic and natural polymers were used in this work. Two hybrid compositions, a Semi IPN of synthetic polymers PVA-PCL and blend of natural polymers, gelatin-albumin was fabricated to serve as 3D scaffolds. Physicochemical properties of the synthetic hybrid compositions vary depending on the concentration of parent material, which was studied by fabricating three different compositions of PVA-PCL. The Semi IPN scaffolds were evaluated for their physicochemical properties by thermal analysis, mechanical compression, swelling studies, FTIR spectroscopy and contact angle measurements. Based on the physicochemical properties like

mechanical stability, high swelling behavior and long term stability in culture, one composition of PVA-PCL was selected for further characterization and invitro studies. Surface morphology, porosity and pore structure play a significant role in determining the cell behavior during invitro culture. The hybrid scaffolds characterized for their 3D morphology using SEM, Micro CT and Liquid extrusion porosimetry was identified to be porous with open interconnected pores. The scaffolds were found to be chemically modifiable by coupling the moieties to the free functionable groups available on the polymers. The hybrid scaffolds made of natural polymers were also characterized for their properties in a similar manner and was found to be favorable to serve as a 3D scaffold. Both the scaffolds were found to be non cytotoxic using standard invitro tests.

The second section deals with results on isolation, standardization of culture conditions and characterization of both chondrocytes and mesenchymal stem cells in monolayer. The cell morphology plays a significant role in maintaining the chondrocyte phenotype. Cell morphology and the viability of cells in 3D scaffolds were characterized using evidence from SEM and confocal microscopy analyses. The results showed that scaffold material and pore structure of 3D scaffolds determine the morphology of chondrocyte in 3D culture.

In the third section the results of long term culture of chondrocytes seeded on hybrid scaffolds is discussed. Considering the fact that the material properties and the scaffold structure directly or indirectly influence the chondrocyte behavior and matrix metabolism, chondrocytes culture was performed in both the hybrid scaffolds. The uniform distribution and morphology of cells in the construct revealed that the hybrid scaffolds have favorable properties to promote the cell phenotype. Chondrocyte cultured in the semi IPN has been found to maintain their phenotypic expression during long term culture as manifested in synthesis of matrix containing cartilage specific collagen and proteoglycans like aggrecan. The scaffolds appeared to promote the synthesis of extra cellular matrix molecules with

a uniform deposition of matrix components as evidenced in microscopic analysis, throughout the surface that completely masked the pores of the scaffold. The synthetic semi IPN was found to promote more secretion of extra cellular matrix components than the other hybrid composition.

The fourth section on results and discussion evaluates the role of scaffold as well as the external signals in the invitro differentiation of mesenchymal stem cells to chondrocytes. Previous studies have reported that members of transforming growth factor beta (TGFβ) super family induce chondrogenic differentiation, but the optimal invitro conditions within which the mesenchymal stem cells can achieve the most chondrogenic response have to be explored in depth in these scaffolds. Chondrogenesis in native tissue is a complex process regulated by the combined action of an array of growth factors. A single growth factor is usually employed for most of the invitro studies on chondrogenesis in 3D scaffolds. Supplement of a single growth factor will not provide all the necessary signals for the differentiation process. In this study the invitro chondrogenic differentiation of mesenchymal stem cells on 3D scaffolds supplemented with individual growth factors and in combination was evaluated. TGFβ1, TGFβ3 and bone morphogenetic protein-2 (BMP2) were selected for this study due to their widely known chondrogenic differentiation potential and two combination of growth factors were identified with some insight into the molecular signaling process. The one month construct grown in different culture supplement cocktails were evaluated for chondrogenesis which identified one combination of growth factors to be better than others in the differentiation process in the selected scaffolds. Based on all the results on overall distribution of cells, matrix molecules, cell morphology and secretion of cartilage specific molecules it was concluded that a combination of TGFβ3 and BMP2 promotes better chondrogenic differentiation of mesenchymal stem cells than other growth conditions used in the study. It has been reported earlier that isoforms of TGFβ and BMP2 regulate gene expression via two

Smad signaling pathways. Results of this study hypothesize that two pathways of Smad signalling might have been simultaneously activated in the combination of TGFβ3 and BMP2 which resulted in enhanced chondrogenesis whereas a single pathway might be activated in the case of TGFβ1 and β3 combinations. The Semi IPN was found to promote better differentiation of mesenchymal stem cells indicating the role of scaffold in the differentiation process.

In the fourth chapter results are summarized and it is concluded that the scaffolds and signaling molecules used in invitro chondrogenesis play a significant role in tissue engineering of cartilage. Future aspects of the investigation are also proposed.

Chapter 1
Introduction

1. BACKGROUND

Articular cartilage is an avascular and aneural tissue which has the important functions to assure the freedom of movement of the joints, and to bear loads and dissipate stresses. Its smooth and frictionless surfaces, combined with viscoelastic properties, allow a stable movement of our skeleton. During the last century, the life expectancy of human population has increased at a pace, leading to the formation of age related diseases. The prevalence of disorders linked to damaged cartilage has increased accordingly. Cartilage degeneration caused by congenital abnormalities or disease and trauma, is of great clinical consequence, due to the limited intrinsic healing potential of the tissue. The lack of blood supply and subsequent wound-healing response, results in an incomplete repair. Full-thickness articular cartilage damage, results in inferior fibrocartilage formation. To prevent progressive joint degeneration in diseases, surgical intervention is often the only option. In spite of the success of total joint replacement, treatments for repair of cartilage damage are often less than satisfactory, and rarely restore full function or return the tissue to its native normal state. The goal of tissue engineering is to surpass the limitations of conventional treatments based on organ transplantation and biomaterial implantation. It has the potential to produce a supply of immunologically tolerant 'artificial' organ and tissue substitutes that can grow with the patient.

The rapidly emerging field of tissue engineering holds great promise for the generation of functional tissue substitutes, by engineering tissue constructs *in vitro* for subsequent implantation *in vivo*. The basic principle is to utilize a biocompatible, structurally and mechanically sound 3 dimensional scaffold, seeded with appropriate cell source, and cultured in presence of bioactive molecules to promote cellular differentiation and/or maturation. The scaffolds are used to facilitate cellular attachment while providing superior mechanical properties. In order to support an adequate cell attachment, proliferation and differentiation, it

should comply with numerous defined requirements regarding its architecture, chemical, mechanical and surface properties. The natural biopolymeric scaffolds have shown promise in invitro regeneration of cartilage but have lot of inconsistency and pose potential immunogenic problems. This has prompted investigators to focus mainly on synthetic polymer-based scaffolds. The ability to promote chondrocyte proliferation, maturation, and differentiation, and the superior mechanical properties has shown applicability of synthetic biodegradable polymers to the repair of cartilage defects. Studies are still continuing to identify the most suitable material combinations and appropriate physico-chemical properties required to fabricate a 3 dimensional scaffold that can mimic the extra cellular matrix of cartilage.

Another objective of tissue engineering is to identify the most suitable cell source to provide sufficient number of cells for invitro tissue regeneration. An orderly chain of highly regulated processes, involving cell proliferation, migration, differentiation, and maturation leads to the production and sustenance of most cell lineages, in adult organisms. The earliest cell type on this chain has been called a stem cell. Together with their extensive capacity for self-renewal, stem cells display a broad potential for giving rise to diverse differentiated progenies. The multipotential of mesenchymal stem cells, their easy isolation and culture, as well as their high *ex vivo* expansive potential make these cells an attractive therapeutic tool capable of playing a role in a wide range of clinical applications. *In vivo,* bone marrow has been considered as the site of residency of the uncommitted mesenchymal stem cell, with high capacity of self-renewal and multidifferentiation potential. They have the capability of continuous replenishing of a given supply of mesenchymal cells during the entire lifespan of an organism, both at steady-state and altered conditions. Bone marrow stroma is the most recurrent tissue source utilized in growing mesenchymal progenitors. Maintenance of stem cell compartment depends on cell autonomous regulators modulated by external

signals. The extrinsic signals that control stem cell fate collectively make up the stem cell microenvironment or niche.

Growth factors are proteins involved in the cellular communication system which modulate stem cell activity in a concentration and time dependent fashion. With regard to cartilage, several growth factors have regulatory effects on cartilage metabolism among which the most investigated are transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs). These molecules play a significant role in the maintenance of the chondrocyte phenotype and the differentiation of progenitor cells towards cartilage phenotype. For successful tissue regeneration it is necessary to identify an appropriate growth factor cocktail and the 3 dimensional scaffolds to direct the stem cell differentiation to a chondrogenic phenotype.

The first introductory chapter briefly discusses the composition, physiology and pathology of articular cartilage. The currently available treatment options and its drawbacks are discussed in detail. The concept and role of the three major components of tissue engineering namely, 3D scaffolds, cells and signaling molecules are discussed focusing on to chondrocyte culture in 3 D scaffolds. A literature review of the polymeric scaffolds currently used for chondrocyte culture and their drawbacks are given. Hence the necessity to find a hybrid scaffold with better properties is pointed out in this section. One major problem in tissue engineering of cartilage is the nonavailability of autologous cells in sufficient number to form a 3D structure. Hence the requirement of mesenchymal stem cells and differentiation process is dealt in this chapter along with information regarding the signalling molecules that cue the differentiation process.

1.1. ARTICULAR JOINT

Joints are highly specialized organs that allow pain-free and frictionless movements of the body. Joints are complex composites of different types of

connective tissues including bone, cartilage surfaces, ligaments and the joint capsule. The knee joint is made up of four bones, the femur, tibia, fibula and patella. Ligaments connect the bones of the upper and lower leg. They are made up of strong groups of collagen fibers that help to provide stability to the knee. They allow joint movement and prevent excessive or abnormal motion. The two main groups of ligaments are called the collateral ligaments, at the sides of the knee, and the cruciate ligaments which cross in the centre of the knee. Tendons attach muscles to bones. The end surfaces of each bone are covered with smooth articular (joint surface) cartilage. The anatomy of the knee joint is represented in Fig 1.

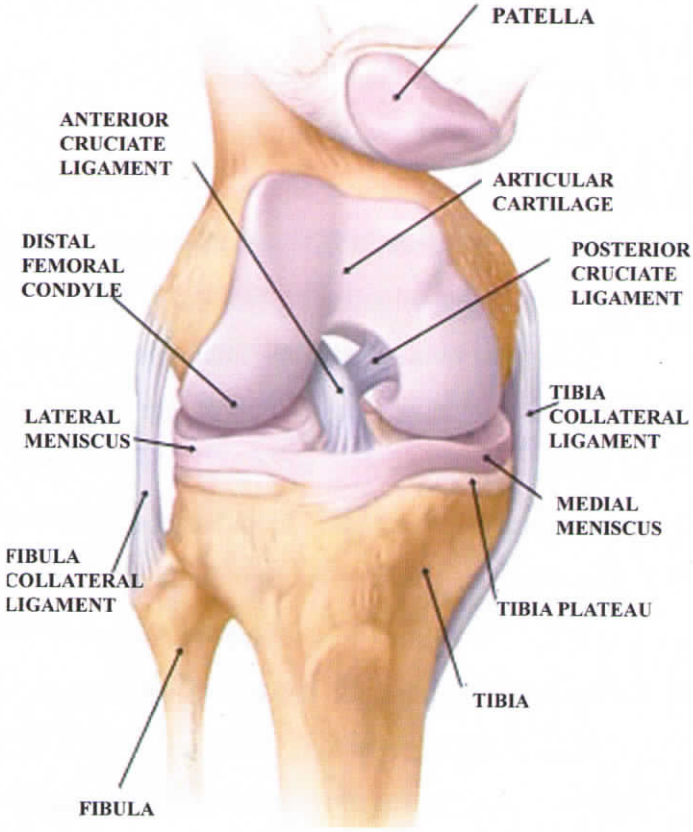


Fig 1. The anatomy of the knee joint

The articular cartilage is a 2-5 mm thick smooth white glistening tissue that is uniquely designed to cover the gliding surface of all the diarthrodial joints of the body. Articular cartilage functions as a wear-resistant, smooth, nearly frictionless and load-bearing surface. This makes it ideally suited for placement in joints. Its composition and architecture permits it to achieve and maintain proper biomechanical function over the majority of a human lifespan. Articular cartilage is an avascular, aneural and alymphatic tissue. The functional property of articular cartilage depends on its extracellular matrix, which is synthesized and maintained by chondrocytes. (*Buckwalter 1998*)

1.2. ARTICULAR CARTILAGE: STRUCTURE

The articular cartilage consists of cells called chondrocytes, dispersed in a highly specialized extracellular matrix. The extra cellular matrix is a complex network of several forms of collagens, proteoglycans as well as a heterogeneous group of noncollagenous proteins. *(Heinegard et al 1992)* Most of the physiological wet weight (70-80%) of articular cartilage comes from interstitial fluid bound to the proteoglycans. Besides water, the interstitial fluid contains gases, metabolites and a large amount of cations to balance the negatively charged glycosaminoglycans in the ECM. The physicochemical properties of articular cartilage, like the intrinsic mechanical properties of the extracellular matrix, the fluid transport and diffusional properties all depends on the fundamental organization of the collagen network and proteoglycans. These characteristics help articular cartilage to perform its normal functions of lubrication, wear, and load-bearing. The ECM is also involved in the regulation of cellular behaviour by providing signals to entrapped cells through receptors and by binding, storage, release and presentation of growth factors. ECM is the natural medium in which cells proliferate, differentiate and migrate, and therefore is the gold standard for tissue regeneration (*Meredith et al 1993, Bosman et al 2003).* Cells synthesize, assemble and degrade ECM components responding to specific signals and, on the

other hand, ECM controls and guides specific cell functions. This continuous cross-talk between cells and ECM is essential for tissue and organ development and repair. In physiologic conditions, a fine dynamic balance exist in regeneration, differentiation and programmed cellular death (apoptosis), which continuously remodel ECM through protein breakdown and synthesis . Natural ECM is a condensed matrix mainly composed of locally secreted proteins and polysaccharides, arranged as a molecular network.

1.2.1. CHONDROCYTES

Normal articular cartilage consist of a single type of cell called the chondrocytes, that occupies less than 1% of wet weight of cartilage. Chondrocytes vary in size, shape and metabolic activity in different zones of the cartilage tissue. Chondrocytes surround them selves with their extracellular matrix and do not form cell-cell contact .Individual cells are metabolically active but the total metabolic activity of the tissue is low due to low cell density. Chondrocytes play a critical role in the secretion of appropriate matrix components in the required amounts and then assemble and organize them into a highly ordered framework. Adult chondrocytes derive their nutrients from the synovial fluid by diffusion. They primarily depend on anaerobic metabolism due to low nutrients and thick extra cellular matrix. Chondrocytes proliferate and secrete the extra cellular matrix in response to the signalling molecules stored in the matrix.

1.2.2. PROTEOGLYCANS

Hyaline cartilage is characterized by the high content of large aggregating proteoglycans *(Hascall 1988)* which constitute about 15 -30% of dry weight of cartilage. *(Freed et al, 1998)* Proteoglycans consist of a core protein to which repeating units of glycosaminoglycans (GAGs) are attached. Hyaluronan, chondroitin sulphate, dermatan sulphate, and keratan sulphate *(Buckwalter and Mankin 1997a)* are the GAGs found in the articular cartilage. Aggrecan is the

major proteoglycan present in cartilage, along with minor proteoglycans like decorin, biglycan, fibromodulin and lumican. The GAG attachment provides the high anionic charge density needed for the unique osmotic properties of aggrecan. *(Roughley et al 2006)*. The proteoglycan aggregates form gels which occupy a large volume relative to their mass. These hydrophilic gels draw in considerable quantities of water that confer high compressive strength properties to the tissue *(Bryant et al 2001)*. The flow of interstitial fluid in an out of the proteoglycan matrix helps to balance the external load by regulating the interstitial fluid pressure and osmotic pressure. Thus the physicochemical characteristics of the proteoglycan gel trapped within the collagen meshwork enable cartilage to resist compression. *(Mow et al 1988)*

1.2.3. COLLAGEN

Collagen constitute about two-third of dry weight of adult articular cartilage. The material strength and biological properties of articular cartilage depends heavily on its uniquely and extensively cross-linked collagen network and characteristic fibrillar organization. Type II collagen forms around 75 % of total fetal collagens in cartilage and >90% in adults.*(Eyre et al 2006)* The nascent type II collagen is a heteropolymer, which forms an interconnected network of fibrils along with minor collagens like type IX and XI which ultimately provides the required tensile strength to cartilage. Collagen type XI is located in the core of the collagen type II fibrils and is thought to be involved in fibril initiation and limiting fibril diameter *(Brukner et al 1994, Mendler et al 1989)* Collagen type IX is a fibril-associated collagens and is involved in crosslinking the collagen network with itself and also to the non-collagenous matrix (*Rest et al 1988, Mayne et al 1989*)

1.3. FUNCTIONS OF EXTRA CELLULAR MATRIX IN CARTILAGE

The major function of the extra cellular matrix is to provide mechanical integrity to the cells embedded in it. They also have important signalling and

regulatory functions in the development, maintenance and regeneration of tissues. ECM components, in synergy with soluble signals provided by growth factors and hormones, participate in tissue specific control of gene expression through a variety of transduction mechanisms. *(Jones et al 1993, Juliano et al 1993)* The ECM is a dynamic structure that is actively remodeled by the cell with which it interacts. *(Birkedal et al 1995)* In case of the cartilage, the extra cellular matrix play very important role in performing the tissue function. The composition and physicochemical properties of articular cartilage, the fundamental organization of the collagen network, and the molecular organization of collagen and proteoglycans all have profound effects on the intrinsic mechanical properties of the extracellular matrix and the fluid transport and diffusional properties of the cartilage. These characteristics provide articular cartilage with its normal function, lubrication, wear, and load-bearing features. The load bearing functions of cartilage mainly depends on the properties of collagen and the proteoglycans in the extracellular matrix. Collagen forms a network of fibrils, which resist the swelling pressure generated by the proteoglycans. Proteoglycans forms huge aggregates that become trapped in the collagen network. Because of their numerous negatively charged sulfate groups, these proteoglycan aggregates attract cations, which in turn bring in water to minimize differences in osmotic pressure. Thus, type II collagen and proteoglycans create a swollen, hydrated tissue that resists compression. The lubrication of cartilage is due to the secretion of lubricin, a protein. Hyaluronic acid also helps in the lubrication. A second mechanism of lubrication of the cartilage is effected by fluid being squeezed out of the cartilage onto the surface when load bearing occurs. When the load bearing ceases, the fluid is absorbed back into the cartilage, and becomes ready for a next cycle of load bearing.

When an external load is placed on the cartilage surface, immediate deformation is produced primarily by a change in the proteoglycan molecular domain. This external load can also make the interstitial fluid pressure in the porous solid matrix exceed the osmotic swelling pressure; therefore, the interstitial

fluid begins to flow and exudation occurs. After exudation occurs and the load is removed, GAGs function hydrophilically, pulling the fluid back into the cartilage, similar to the action of a sponge soaking up water, in preparation for the next load. With a decrease in the interstitial fluid, the proteoglycan concentration within the solid matrix increases, this in turn increases the osmotic swelling pressure, charge-charge repulsive force, and bulk compressive stress until they are balanced with the applied external load. In this manner, the physicochemical characteristics of the proteoglycan gel trapped within the collagen meshwork enable cartilage to resist compression. This mechanism supplements the role played by collagen fibers, which are strong in tension but can easily fold under compression *(Mow et al 1988)*

1.4. DAMAGE TO ARTICULAR CARTILAGE:

1.4.1. PATHOLOGY & STATISTICS

There are three main types of cartilage injury: matrix disruption, partial thickness defects, and full thickness defects. Matrix disruption occurs from blunt trauma, such as dashboard injuries in automobile accidents. The ECM is damaged, but if the injury is not extreme, the remaining viable chondrocytes will increase their synthetic activity to repair the tissue. Partial thickness defects demonstrate disruption of the cartilage surface but this does not extend to the subchondral bone. Immediately following the injury, nearby cells begin to proliferate, but the defect cease before it is repaired. Full thickness defects arise from damage that transverses the entire cartilage thickness and penetrates the subchondral bone. In this case, the defect is filled with a fibrin clot and a classic wound healing response ensues. With this type of injury, there is access to a population of progenitor cells from the bone marrow which can migrate to fill the defect. These cells usually cause replacement of the fibrin clot with tissue intermediate between hyaline and fibrocartilage. Chondrocytes have a relatively low metabolic activity and except in the case of full-thickness defects, there is no direct access to progenitor cells,

(found in bone marrow) and the resident chondrocytes may be impeded by the ECM from migrating to fill the defect.

One of the major causes of articular cartilage damage is osteoarthritis (OA), leading to long-term disability in major population of people over 65 years of age. *(Praemer et al 1999).* Osteoarthritis is a severe disease of the joints, in which articular cartilage is degenerated and eventually, worn away. This is a multifactorial disease, involving age, alignment of the joint, and the weight of the patient which affects people of all ages. The early changes in cartilage tissue, associated with OA, include loss of proteoglycans and degradation of the collagen fibril network *(Buckwalter and Mankin, 1997).* This results from an imbalance between degradation and de novo synthesis of matrix components. Enhanced levels of many metalloproteinases have been reported to accompany the increased matrix degradation in osteoarthritic cartilage. *(Ohta et al 1998, Shlopov et al 1997, Freemont et al 1997)* The developing OA increases pain, restricts exercise and limits physical capability. When OA progresses to its terminal point, cartilage tissue is almost completely worn away exposing the subchondral bone.

Blunt trauma, penetrating injuries, frictional injuries associated with sports and accidents in young children and adults also cause damage to articular cartilage. Osteochondritis dissecans is another rare pathological condition affecting the teenagers and young adults. In osteochondritis dissecans, fragments of cartilage and bone separate from the surface of the joint and give rise to loose bodies, producing locking, pain, and swelling. *(Twyman et al 1991).* Currently, there is no efficient way to re-establish eroded cartilage and, therefore, only palliative treatment or arthroplasty can be used to relieve patients.

The medical statistics point out the huge burden created by osteoarthritis on the society. In India the osteoarthritis affected people are still higher. Clinical indications have focused on athletic trauma to the knees, osteochondritis dissecans,

and revision of silicon nasal implantation, which account for approximately 1000 operations a year internationally.*(Marlovits et al 2006, Yanaga et al 2006)* To broaden the indication range to severe osteoarthritis, congenital anomaly of craniofacial areas, and other major cartilage diseases cover more than 100,000 patients annually.(*Weng et al 2006*)OA affects people in all geographic locations, it develops in both men and women, more commonly in women. *(Buckwalter et al 2000).*The World Health Organization (WHO) estimates that 10% of the world's people over the age of 60 years suffers from OA, and that 80% of people with OA, have limitation of movement and 25% cannot perform major daily activities *(WHO 2001).* The percent of people with evidence of OA in one or more joints increases from less than 5% of people between 15 and 44 years, to 25% to 30% percent of the people 45 to 64 years of age, and to more than 60% and as high as 90% in some populations, of the people over 65 years of age. *(Buckwalter et al 2006).* A recent report points out that about 46 million people have a damaged cartilage. *(Hootman et al 2006)* In 2003, the total cost attributed to arthritis and other rheumatic conditions in the United States was 128 billion dollars, up from 86.2 billion dollars in 1997. (*MMWR 2007)* Of persons aged 18–44, 7.9% (8.7 million) report doctor-diagnosed arthritis. Of persons aged 45–64, 29.3% (20.5 million) report doctor-diagnosed arthritis. Of persons aged 65+, 50.0% (17.2 million) report doctor-diagnosed arthritis. *(MMWR 2006)* Another survey projects the number of people with arthritis in the coming years, and is found to be around 67 million by year 2030. (*MMWR 2007, Arthritis & Rheumatism 2006).* The adverse effects of OA on the quality of life of millions of people, the costs of health care, and the costs of lost economic productivity continue to present major challenges.

1.5. CURRENTLY EMPLOYED TREATMENT OPTIONS

The damaged cartilage has very poor regenerative capacity. Osteoarthritis is a degenerative disorder that generally starts off relatively mild and escalates with

time and wear. Nonsurgical treatment methods are generally opted for patients in an early stage of the disease. As the disease progress, surgical treatments like chondrectomy, microfracture, chondroplasty and mosaicoplasty are often employed depending on the nature and degree of damage and the age of the patient. Autologous chondrocyte implantation is another commonly available treatment that has attained some success. Total joint replacement is reserved for the most severe and recalcitrant forms of cartilage damage. When other forms of treatment fail or when patients are unlikely to succeed with lesser therapies, the last option to treat defective cartilage is total joint replacement. The different treatment modalities and their disadvantages are discussed in this section.

1.5.1. NONSURGICAL TREATMENTS

The use of braces and drug therapies has been shown to alleviate the pain caused by cartilage deficiency, improve physical function, and slow the progress of the disease. The use of braces reduces the amount of load on the defective compartments of the knee, resulting in decreased pain and increased mobility. Drugs like anti-inflammatories, COX-2 inhibitors, and cortisone shots all aim at providing relief from the symptoms of defective cartilage by suppressing an immune response. Non steroidal anti inflammatory drugs (NSAIDs) work by blocking the effect of the enzyme cyclooxygenase. This prevents the formation of prostaglandins, the lipid compounds that cause swelling and pain during arthritis or bursitis. Glucosamine sulfate and chondroitin sulfate are dietary supplements intended to slow down and reverse the degenerative mechanism of cartilage wear. Even though these treatments give a relief to pain at the joints, they have some disadvantages.

NSAID causes the unwanted side-effects like stomach irritation, headache, drowsiness, easy bruising, high blood pressure, fluid retention, and dyspepsia. Cortisone injections should not be repeated more than few times a year, as they can weaken tendons and soften the cartilage, increasing the risk of potential

problems. Cortisone also acts to suppress the body's immune system. Glucosamine and chondroitin sulfate may prove to be useful, but further investigations of larger cohorts of patients for longer time periods are needed before they will be accepted into the primary treatment plan for cartilage damage.

1.5.2. INDUCTION OF THE FIBROCARTILAGE GROWTH

Shaving (chondrectomy) and debridement belong to the treatments that remove the diseased and undermined cartilage, with an aim to temporarily relieve the autoimmune inflammation and pain. These are used in clinics for palliative purposes, as temporarily it relives the pain associated with arthritic inflammation. Chondrectomy is mainly focused in the reduction of the inflammation. In chondrectomy, the damaged cartilage is removed to level the cartilage surface through operation debridement. Statistics show that although originally 74% of patients improve, within 5 years 66% of patients have still a pain in the knee and 99% have a restriction of activity.

A range of abrasive procedures, like drilling, microfracture, chondroplasty, and spongialization are used to trigger the cartilage production. They all rely on the phenomenon of the spontaneous repair of the tissue, following the vascular injury to the subchondral plate of the bone. Drilling is used with the greatest success on younger patients. It is an open operation on the knee, but faces all problems associated with it like longer recovery period, and a bigger probability of complications. Microfracture can be performed arthroscopically reducing the complications associated with normal open surgery. The patients had postsurgery complications like locking of the knee, pain and a discomfort in the knee. Often there is tissue over growth in response to the procedure in the trochlear groove. In laser assisted cartilage reshaping (chondroplasty), heat is used to cause change in the physical matrix. These change causes relaxation, leading to the change of shape of the cartilage resulting in stress reduction. There is a need for this therapy to be more spatially selective to avoid excessive damage. Another method is thermal

chondroplasty using radiofrequency energy (RFE). In general the undesired consequences of the thermal treatment (laser and RFE chondroplasty) encompass thermal damage, air bubble formation, tissue necrosis, reactive synovitis, chondrolysis and an acceleration of articular cartilage degeneration. Fibrocartilage is the most common form of repair tissue achieved. Cells produce a matrix with a poor content of glycosaminoglycans and with abundance of type I collagen. All these techniques typically lead to the formation of fibrous scar tissue, which has poorer durability, load transfer, and strength characteristics when compared to native cartilage. Also, these treatments are effective for a limited number of years, after which the patient experiences a reoccurrence of symptoms. Often, repeated surgical intervention is required and patients are restricted of high activity levels.

1.5.3. CHRONDRAL GRAFTING

Articular cartilage surface grafting, or chondral grafting, is used to transplant cartilage from a nonessential region from the same knee or opposite knee to plug the cartilage defect and hopefully return the patient to near-normal functioning. However, unlike microfracture, osteochondral grafts are not always amenable to arthroscopic technique and may also require an arthrotomy. Mosaicplasty, a form of chondral grafting, is a therapy designed to stimulate growth of articular cartilage on the surface of the knee joint that has been damaged by trauma or arthritis by implanting osteochondral plugs. Mosaicplasty costs on average $14,000 in the United States. An autologous graft is often associated with donor site morbidity and non availability of a healthy tissue from a damaged joint. Allogenic transplantation is another option. This often faces the nonavailability of a suitable graft and also the pose the problem of immune rejection and risk of pathogen transfer. *(Convery et al 1991, Beaver et al 1992)*Given the low long-term success rates, perichondrial grafting alone has not been clinically accepted as a particularly excellent therapy. Clinical experiments with human patients have yielded disappointing results, as complete restoration of the hyaline articular

cartilage layer and long-term stability of the formed repair tissue have not been achieved. Also, graft detachment and uncontrolled calcification of grafts have been observed as practical problems.

1.5.4. AUTOLOGOUS CHONDROCYTE IMPLANTATION

Autologous chondrocyte implantation (ACI) is a dual stage operation consisting of the following stages. A biopsy of cartilage must be taken from a non-load bearing portion of the knee. The chondrocytes are isolated by enzymatic digestion of tissue. The cells are amplified in vitro for four to six weeks. Implantation of autologous cultured chondrocytes consists of multiple steps- Defect preparation, periosteum procurement and fixation, cell implantation, and closure. Carticel is the only commericially available autologous chondrocyte implantation treatment offered by Genzyme Biosurgery. Since the patients use their own cells, disease transmission risk is minimized. There is a wealth of literature defending autologous chondrocyte implantation as an effective method of cartilage replacement. Critics suggest that biopsy result in long term damage. Some common problems reported with the Carticel treatment is graft overgrowth. Chondrocytes grow too much, resulting in joint asymmetry and bulging of the osteochondral patch. Other problems are graft delamination, sticking or locking of the knee, meniscus lesions/tears, chondromalacia (softening of the cartilage) and joint malalignment. After ACT it may take a long time for the repair tissue to reach the stiffness of the adjascent cartilage. (*Vasara et al 2005)* Therefore the use of the porous scaffolds or hydrogels to fill the lesion is a tempting approach to provide the support for the area and to keep the cells on the defect. (*Capito et al 2003, Sharma et al 2004)*The treatment also requires a significant amount of training and manual skill for orthopedic surgeons. The carticel implantation treatment costs approximately $17,600-38,400. Cell Culture cost is approximately $14,000. The cost of the carticel treatment is high relative to other cartilage repair techniques.

1.5.5. JOINT REPLACEMENT

Total joint replacement is used to treat the most severe and recalcitrant forms of osteoarthritis. When other forms of treatment fail the last option to treat defective cartilage is to replace all or part of the joint. Implants are designed so the frictional interface is between plastic and metal. The femoral component is metal, generally produced from titanium and cobalt-chromium alloys. The tibial component is a metal plate with a plastic joint. The most common plastic used is ultra-high-molecular-weight polyethylene. Sometimes a metal plate is added to replace the patella. There are more than 300,000 total knee replacements in the United States each year. Based on data from the year 2000, the number of TKR worldwide (estimated at 900,000 in 2006) is growing at a rate of 5% per year. On average, the actual implant costs around $3000. The first year medical costs for the device, surgery, and rehabilitation is approximately $30,000 for a unilateral surgery and $40,000 for a bilateral surgery. Maintenance costs following the implantation are close to $1,000 per yea. These implants have limited lifespan. The gradual wear and tear and poor integration with surrounding tissue result in loosening and failure of the implant. *(Chandler et al 1981, Dorr et al 1983)*

Tissue engineering approaches to cartilage repair offer potentially important advantages over the modern generation of metal and plastic joint prostheses. The total joint prostheses has been successful in providing pain relief and joint function for many individuals with arthritis, but the non living prostheses wear and generate problematic debri, and are not sufficiently durable for physically active individuals. There are currently no widely applicable and long lasting methods for resurfacing damaged articular cartilage. Thus, development of techniques to stimulate or augment biological healing methods is needed to restore mobility to damaged articular surfaces.

1.6. TISSUE ENGINEERING

Tissue engineering is an interdisciplinary field that incorporates and applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue/organ function. The approach to tissue engineering is to recreate an invivo environment that embodies the biochemical and mechanical signals that regulate tissue development and maintenance in vivo. The major advantage of tissue engineering approach is that tissues can be reconstructed to closely match the needs of the patient's requirements and can be transplanted into the patient's body with a minimal surgical intervention, which eventually conquers several limitations encountered in the traditional tissue transplantation approaches. The prime concept of tissue engineering approach is to harvest a small biopsy of specific cells from the donor site, seed them on a scaffold to culture a specific tissue, and transplant the cultured tissue into the defective site of the patient's body that needs tissue regeneration. The triad of invitro engineering system has three major components. The principal components is polymeric scaffolds that provide a three dimensional structure for cell attachment and tissue growth (into which the extra cellular matrix is secreted and organized), metabolically active cells that are able to express the differentiated phenotype and signaling molecules and culture conditions that provide an invitro environment in which the polymer-cell constructs can develop into a functional tissue. An appropriate combination of all the three factors is required for the invitro engineering of the tissue.

1.6.1. 3 DIMENSIONAL (3D) SCAFFOLDS FOR TISSUE REGENERATION

Tissue engineering aims to create biological substitutes that might restore, maintain or improve tissue functions. For a successful tissue engineering approach, an appropriate cell source must be identified, isolated and amplified on 3D-scaffolds. A scaffold serves as a temporary ECM, which supports cells and enhances the subsequent 3D tissue regeneration. These cells then undergo

proliferation, migration, and differentiation in three dimensions, which eventually leads to the formation of a specific tissue with appropriate functions. Thus, a critical step in this process is the mimicking of some ECM characteristics to provide cells with an adequate mechanical stability and biological environment for tissue growth and integration. The correct synthetic or natural material to be used as a substrate should be chosen and manufactured in the desired shape and dimension (*Langer 2000, Hutmacher 2001*). A variety of biomaterial scaffolds have been introduced in tissue engineering in an effort to induce tissue repair or regeneration. The microstructure and the chemical composition of the scaffolds dictate the formation of tissues by regulating cell infiltration and cell activities. The metabolically active cells that express the differentiated phenotype and signaling molecules and culture conditions that provide an in vitro environment makes the polymer-cell constructs to develop into a functional tissue.

The general features of 3D scaffolds are that the scaffolds should be biodegradable, chemical composition and surface characteristics should promote cell adhesion to the matrix. The material and the degradation products should be nontoxic. The fabrication methodology should be appropriate to have reproducible three dimensional structures. It should have high porosity which increases the surface area for cell- matrix interactions. The high porosity with open interconnected pores should allow cell migration and deposition of extra cellular matrix. The scaffold has to support diffusion of nutrients and gas exchange during invitro culture. The rate of matrix production should balance the rate of degradation of scaffolds. The matrix should have sufficient mechanical properties to withstand the load imparted by the cells during invitro culture and should promote cell migration sufficient to achieve a good integration with the surrounding. Overall the scaffold should promote the secretion of matrix components.

Both natural and synthetic polymers have been used for scaffold fabrication. Natural polymers have the advantage that they are more cell friendly and promote better cell material interactions. Several natural polymers have been found to be favourable for chondrocyte culture as well as for stem cell differentiation to chondrocytes. The major disadvantage in case of natural polymers is the lack of mechanical stability, uncontrolled degradation and non-reproducible properties. There is also concern for the purity of these polymers during isolation and the risk of pathogen transfer. Biodegradable synthetic polymers offer a number of advantages over other materials for developing scaffolds in tissue engineering. They are less biomimetic but are more flexible and have reproducible properties. The key advantages include the ability to tailor mechanical properties and degradation kinetics to suit various applications. Synthetic polymers are also attractive because they can be fabricated into various shapes with desired pore morphologic features conducive to tissue in-growth. Furthermore, polymers can be designed with chemical functional groups that can induce tissue in-growth. Also hydrogels are very attractive candidates in tissue engineering applications because they can fill irregularly shaped defects, and incorporate cells and other bioactive materials (*Drury et al 2003).* Several natural and synthetic polymers have been used for cartilage tissue engineering but so far no scaffold have been identified to be the best for this purpose. Some of the merits and demerits of commonly employed polymers used for cartilage tissue engineering are pointed out in this section.

1.6.2. NATURAL POLYMERS FOR CARTILAGE REGENERATION

It has been reported that the 3D culture systems with hydrogel materials like agarose, (*Benya et al 1982)* alginate, *(Stevens et al 2004, Gerard et al 2005)* collagen type I gel, *(Chaipinyo et al 2004)* collagen type II gel, *(Malemud et al 1994*) fibrin glue, *(Ting et al 1998)* revert the dedifferentiated chondrocytes in monolayer back to a chondrocyte phenotype when seeded at high density.

Alginate is a biodegradable polysaccharide derived from brown seaweed and bacteria with repeating units of mannuronic and guluronic acid. Alginate gels have been mostly used for drug delivery, cell entrapment and as injectable matrix for tissue engineering. Alginate gels are found to stimulate the differentiation of bone marrow and adipose tissue derived stromal cells toward a chondrogenic phenotype. *(Shoichet et al 1996)* This possibly may be due to the lack of cellular interactions or limited oxygen and nutrient supply inside alginate hydrogels. However, unpredictable degradation and purity remain problematic. (*Lawson et al 2004).* Alginate gels are found to quickly lose their mechanical properties in vitro (approximately 40% within 9 days), presumably due to an outward flux of ions into the surrounding medium, (*Shoichet et al 1996*) with uncontrolled dissolution of the gels over time. More over it has been reported that human and animal cells behave differently on alginate surfaces. (*Lawson et al 2004*)

Hyaluronic acid gels are obtained by esterification of biocompatible and biodegradable hyaluronate. Hyaluronates are cell friendly as they are a component of the proteoglycans in the extra cellular matrix. A commercially available hyaluronate (Hyaff) is produced in various 3D configurations, including sponges, and are used as carriers of growth factors and seeded cells in tissue engineered repair of bone, cartilage, and ligament. (*Radice et al 2000, Lisignoli et al 2002, Cristino et al 2005)* However, the gels often contain impurities and remain mechanically weak, despite the introduced crosslinks, compromising their use in skeletal tissue engineering.

Fibrin hydrogels, is formed using enzyme-catalyzed crosslinking of fibrinogen at room temperature in the presence of thrombin, calcium chloride, and factor XIII a, are readily available from patient's blood and have been approved for clinical use. Human chondrocytes embedded in fibrin Hydrogels and beads produce tissue with high GAG content. (*Peretti et al 2006)* The major drawback of fibrin gels is their low mechanical quality, limiting their role in load-bearing in

vivo applications to a cell-carrier function. Their use for load bearing applications need to be usually in combination with stiff polymer or ceramic scaffolds. (*Yamada et al 2003, Schantz et al 2003, Spitzer et al 2002*) Fibrin gels have been used for chondrocyte culture, but fibrin chondrocyte constructs have been observed to degrade,before proper formation of cartilaginous tissue. (*Fusseneger et al 2003*)

Agarose is a galactose polymer extracted from red seaweed that gels by virtue of hydrogen bond formation. Agarose is mechanically stabile, non-toxic, and well suited for 3D chondrocyte encapsulation. (*Benya et al 1982*) Cells have not yet been included in the deposition process because of the high temperature of agarose during dispensing. (*Landers et al 2002 a, b*) Studies have showed that agarose facilitates differentiation along the chondrogenic lineage and also redirects osteoblasts to become chondrocytes. (*Bahrami et al 2000*) Despite the fact that agarose strength initially falls 25% after cell incorporation, (*Shoichet et al 1996*) chondrocytes form mechanically functional ECM in 1 month (in vitro). (*Buschmann et al 1992*) BMP2-transduced MSCs form more cartilage- like tissue in agarose gels than in other matrices, such as collagen or alginate. This indicates that the agarose gel, in addition to its tendency to resist blood vessel invasion (resulting in low oxygen tension), is especially suited for cartilage Tissue engineering. (*Gruber et al 2006*) MSCs produced high levels of proteoglycans inside agarose.

Collagen is a component of the natural extra cellular matrix. Collagen from animal origin generates the risk of pathogen transfer. Collagen is expected to provide the most suitable conditions for chondrocytes. Comparison of collagen type I and II matrices indicated greater chondrogenic activity of the cells in collagen type II gels. (*Bosnakovski et al 2006*) However, the use of collagen gels remains limited because of qualitative batch variations and loss of shape and consistency through shrinkage. (*Diduch et al 2000*) The easily gelling gelatin is derived from denatured collagen. Gelatin has been widely used for cell and drug

delivery. Although used successfully as a cell vehicle in chondrogenesis studies and in bone tissue engineering, (*Awad et al 2004, Ponticiello et al 2000)* Gelatin hydrogels are weak at physiological temperature, (*Landers et al 2002*) limiting their use in tissue engineering of cartilage and will be better in combinations with stiffer solid scaffolds. COL2, which is normally present in healthy cartilage, could enhance proteoglycan synthesis in the dedifferentiated chondrocytes by embedding the chondrocytes or by keeping contact with the cells via surface coating of the scaffold.(*Yang et al 2006*) However, that kind of gel was weaker in gel formation properties than COL1 gel.

Several studies have compared the effectiveness of these hydrogels as matrices for skeletal tissue engineering. Initial differences in performance of alginate, collagen, and agarose with respect to cartilage formation tend to decrease over time, with agarose performing particularly well. (*Awad et al 2004*) In alginate, embedded chondrocytes synthesize ECM with a high GAG content, although sporadic tissue formation was suboptimal. (*Mouw et al 2005*) Cells imbedded in collagen I matrices exhibit high proliferative potential and ECM formation despite the sometimes observed fibroblastic morphology. (Yamaoka et al 2006)

1.6.2.1.GELATIN AND ALBUMIN

Gelatin is a protein produced by partial hydrolysis of collagen extracted from skin, bones, cartilage, and ligaments. Collagen being a polymer from animal source, even though known to have wide biomedical applications, expresses antigenicity in physiological condition, while gelatin is known to have no such antigenicity. (*Kuijpers et al 2000*) Thermal, physical or chemical denaturation of collagen involves breaking of triple helix structure to give gelatin, a biodegradable, biocompatible and non immunogenic product, suitable for medical applications. The biomimetic structure and biocompatibility of gelatin has led to its use in wide range of biomedical applications such as controlled drug delivery systems, *(Adhirajan et al 2007, Tseng et al 2007, Lu et al 2007, Liu et al 2007*) wound

dressings, (*Pal et al 2007, Ulubayram et al 2001*) and scaffolds for tissue engineering. (*Kang et al 1999, Jones et al 2006*) Hydrogels of gelatin have been used widely to serve as cell carriers but they lack the mechanical stability required for long term load bearing applications. (*You et al 2007*)Gelatin and their blends with other polymers have been used for tissue engineering applications of soft tissues. (*Liu et al 2007*) Polymer like chitosan is blended with gelatin to improve the biological activity. (*Jiankang et al 2007, Machado et al 2007*) Since gelatin contains RGD like sequences that promote cell adhesion, the scaffold has been used for tissue engineering of skin (*Mao et al 2003*) and cartilage (*Xia et al 2004*). Some of the limitations of 3D gelatin scaffolds is its extensive swelling and lack of stability in culture medium for long term applications. Albumin also served as a non toxic foaming agent which helped to attain adequate porosity during the fabrication. (*Berthold et al 2007, Battacharjee et al 2007*) Albumin is a protein that has been widely used for coating vascular grafts to improve its blood compatibility. (*Domurado et al 1978, Ro et al 1985, Benslimane et al 1986)* Albumin from egg white has been reported earlier to serve as foaming and consolidating agent for the design of smart porous bioceramics. (*Lemos et al 2004*) Microcapsules of albumin have been used for drug delivery.

1.6.3. SYNTHETIC POLYMERS

Most widely used synthetic degradable polymers comprise a class of polyesters like poly (lactic acid) (PLLA), poly (glycolic acid) (PGA) and their copolymer poly (lactic-co-glycolic acid). Poly(glycolic acid) is a long standing, well studied scaffold for engineering stem-cell-derived cartilage, but degrades at a faster rate and is weaker than most synthetic scaffolds (*Grande et al 2003, Fuchs et al 2003)* They are approved by the Food and Drug Administration (FDA) and have been widely applied in bone and cartilage repair. (*Athanasiou et al 1998, Uematsu et al 2005, Sherwood et al 2002)* One of their major advantages is that their degradation properties can be tailored by fabricating copolymers of these

polyesters. Despite the extensive use of these synthetic polymers they have some deficiencies that there surface properties do not readily support cell attachment. (*Wong et al 1997*) The accumulation of the degradation products at the implant site lead to drastic decrease in pH which affects the viability of the cells. This is a major concern in orthopaedic applications where implants with considerable size would be required. Another concern is the release of small particles during degradation, can trigger an inflammatory response. It has been shown that as the material degrades the small particles that break off are phagocytized by macrophages and multinucleated giant cells (*Gibbons et al 1992*).

Poly (ε- caprolactone) (PCL) is another FDA approved biocompatible polyester that have been widely explored for tissue engineering applications. PCL based scaffold matrices have been studied for skin replacement and other tissue engineering applications (*Hutmacher et al 2001, Li et al 2005, Htay et al 2004*). PCL have sufficiently long degradation time of 2 years but the degradation can be tailored by blending with other polymers. Furthermore, novel degradable PCL networks, PLGA/PCL/PLGA tri-block copolymers and PCL-chitosan matrices are more hydrophilic, degrade faster and possess desirable mechanical properties as compared to PCL (*Kweon et al 2003, Choi et al 2001, Sarasam et al 2005*). PCL have been found to have good mechanical properties for load bearing applications. Poly (vinyl alcohol) (PVA) is a synthetic hydrogel that have been employed in various biomedical applications. This hydrophilic polymer exhibits excellent water retention properties. (*Peppas et al 1977*). Some of the most important biomedical applications of this biocompatible polymer include contact lenses, drug delivery systems, (*Paradossi et al 2003)* and cartilage and tendon repair. (*Kobayashi et al 2001*) PVA hydrogels are mostly crosslinked using noncytocompatible freeze-thaw processes. However these Hydrogels have less mechanical stability. The mechanical properties of PVA can be tailored by varying crosslinking density of the gels. (*Schmedlen et al 2002, Nuttelman et al 2002)*

In general the biocompatible synthetic polymers have been generally acceptable for load bearing applications. PCL with high mechanical stability is found to be very favourable for cartilage tissue engineering than the commonly employed polyesters like PLA, PGA and PLGA. Hydrogels like PVA have already been proved to be useful to serve as artificial cartilage due to its excellent water holding capacity and mechanical stability.

1.6.3.1. POLY(VINYL ALCOHOL)

Polyvinyl alcohol is a water-soluble synthetic polymer. PVA is prepared by partial or complete hydrolysis of polyvinyl acetate to remove acetate groups. The properties of PVA are influenced by the molecular weight and the degree of hydrolysis. PVA has a melting point of 230°C and decomposes rapidly above 200°C. It is a highly hydrophilic polymer with free hydroxyl (-OH) groups in every repeating units as represented in Fig 2.

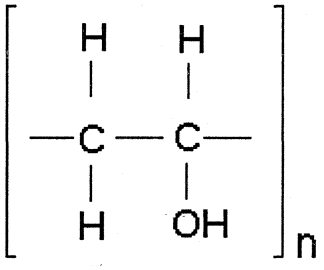


Fig 2. Chemical structure of poly(vinyl alcohol)

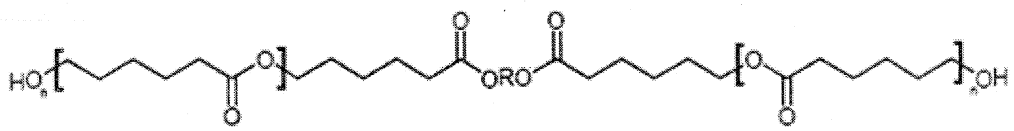
The hydrophilic nature of PVA is due to the interactions of the free hydroxyl groups of PVA with water molecules through hydrogen bonding. PVA undergoes degradation by hydrolysis. The swollen hydrogels of PVA have high water holding capacity. PVA hydrogels are mostly crosslinked using freeze-thaw processes and chemical crosslinking with aldehydes. The stability can be improved by chemical crosslinking. PVA is itself biocompatible and any residual polymer should not induce an inflammatory response. (*Oh et al 2003, Burczak et al 1996*)

PVA is approved by FDA (USA) and regulatory organizations in many countries and widely used as a coating material for other polymers or as a supporting material in cell culture. *(Li et al 1998, Grisel et al 2003)* It may facilitate diffusional exchange of nutrients and waste products with the surrounding environment. It has good pH stability and excellent mechanical properties. These properties made it appropriate to be used in innumerable biomedical applications either alone or in combination with other polymers.

PVA has been employed in several biomedical applications including contact lenses, ophthalmic materials *(Gobbels et al 1991)* tendon repair (*Kobayashi et al, 2001)* and drug delivery (*Uhlich et al 1996, Nguyen et al 2007, Francois et al 2007, Berkland et al 2007)*. PVA and blends of PVA have been fabricated in the form of microcapsules, nanoparticles and films for various biomedical applications. (*Pal et al, 2007, Nicoli et al 2006, Dailey et al, 2006).* Its excellent properties make it ideal to serve as an artificial cartilage. (*Qiu et al 1998, Noguchi et al 2004*) Hydrogels of PVA have already been found to be favorable to serve as artificial cartilage and meniscus. (*Kobayashi 2004*)Two year outcome of PVA as artificial meniscus was found to be very suitable.(*Kobayashi et al 2005*) Different biocomposites of PVA have been used as tissue scaffold for craniofacial and joint defects and bone tissue engineering .(*Pereira et al 2005*) Blends of PVA with other polymers have also been used to serve as artificial cartilage. (*Nakashima et al, 2004, Zheng et al 2007, Lu et al 2005)*

1.6.3.2. POLY(CAPROLACTONE)

Poly (caprolactone) (PCL) is FDA approved biodegradable polyester that is commonly used for tissue engineering applications. Poly (caprolactone) is prepared by the ring-opening polymerization of the cyclic monomer ε -caprolactone. (*Storey and Taylor, 1998*).The chemical structure of polycaprolactone is represented in Fig 3.



Polycaprolactone

Fig 3. Chemical structure of poly(caprolactone)

This is a semicrystalline polymer with a glass transition temperature of about –60°C. The polymer has a low melting temperature (59 to 64°C). This makes it suitable for fabrication into various shapes for biomedical applications. This biocompatible synthetic polymer has good mechanical properties. (*Woodward et al 1985, Pitt et al 1981, Kweon et al 2003)* However, degradation kinetics of PCL is slow due to its hydrophobic and semi crystalline nature which makes its resorption time longer than 2 years.

PCL is degraded by hydrolysis of its ester linkages in physiological conditions and has therefore received a great deal of attention for use as an implantable biomaterial. It may undergo enzymatic degradation through hydrolysis of its ester bonds by lipase, cholesterol esterase, and carboxyl esterase. (*Gan et al 1997, Labow et al 2002)* The main advantage of using PCL for implant application is due to its nontoxic degradation products. The degradation product is caproic acid, a natural fatty acid of human skin. These degradation products enter the tricarboxylic acid cycle in the cells and are eliminated without any harmful effects. The homopolymer has a degradation time of the order of two to three years *(Kronenthal 1975, Holland and Tighe 1992, Middleton and Tipton 2000).* The rate of hydrolysis can be altered by copolymerization or blending with other polymers. Copolymers of caprolactone with dl-lactide have been synthesized to yield materials with more rapid degradation rates (e.g., a commercial suture MONOCRYL, Ethicon) (*Middleton and Tipton, 2000*).

PCL is considered a non-toxic and a tissue compatible material (*Kronenthal, 1975).* The in vivo degradation of poly (ε-caprolactone) (PCL) was observed for 3 years in rats. The distribution, absorption and excretion of PCL were traced in rats by radioactive labeling and the results indicate that the material did not cumulate in body tissue and could be completely excreted. (*Sun et al 2006*). The FDA approved PCL has been widely used for many biomedical applications like drug delivery device, suture (sold under the brand name Monocryl or generically), adhesion barrier and is being investigated as a scaffold for tissue repair via tissue engineering. PCL has been used for the tissue engineering of different tissues like skin, (*Woei et al 2001*), axonal regeneration, and scaffolds for supporting fibroblasts. Its hydrophobicity, limited bioregulatory activity, neutral charge contribution, and long degradation time of up to 2 years have limited its use as a suitable biomaterial for tissue engineering. The greatest advantage of this polymer is its inherent mechanical stability in culture medium for long periods. However the limitations are overcome by blending or copolymerizing with other polymers. Blending and copolymerization have enabled to regulate its degradation, hydrophobicity and biomimeticity. (*She et al 2007, Oliveira et al 2007, Wang et al 2006, Zhao et al 2004).* Scaffolds have been fabricated in various structures using different techniques. (*Oh et al 2007, Yang et al 2006, Lieshout et al 2006*)The low melting point and its solubility in volatile solvents have promoted its fabrication into nanofibres. Electrospun scaffolds with PCL and its blends have been evaluated for tissue engineering applications. (*Sell et al 2007, Klinkhammer et al 2006, Zhang et al 2006)*

The good mechanical property of the polymer favours its use in load bearing applications. Most of the recent reports have shown PCL to be more suitable for bone and cartilage tissue engineering. (*Shor et al 2007, Rai et al 2007*) Blends of PCL with fibrin, alginate and other polymers have been used for cartilage tissue engineering. (*Oliveira et al 2007, Eyrich et al 2007, Bunaprasert et al 2006, Li et al 2003, Johnson et al 2006).* Since PCL have been found to be

favorable for both chondrocyte and osteoblast culture, studies have been conducted to explore its suitability for repair of osteochondral defects.(*Cao et al 2003, Shao et al 2006, Hsu et al 2007*) PCL scaffolds have also been reported to favorable for tissue specific differentiation of stem cells. (*Schantz et al 2007, Kang et al 2007, Shao et al 2006, Santiago et al 2006)*

1.7 HYBRID POLYMERS

Hydrogels are water swollen polymeric materials that maintain a distinct three dimensional structure. Some of the inadequacies of traditional hydrogels are poor mechanical properties and slow delayed response time to external stimuli. The mechanical stability of the hydrogels can be improved by crosslinking, blending with other polymers and by forming interpenetrating polymer networks (IPNs) with other mechanically strong polymers. This will result in good mechanical properties and high degree of swelling without collapse. Hybrid hydrogels are hydrogels systems that possess components of at least two polymers that are interconnected covalently or noncovalently. Compared to synthetic hydrogels, hydrogels of protein molecules have well defined and homogeneous structure. The chondrocytes under physiological conditions are individually embedded in a large amount of firm cartilaginous matrices and maintain their isolation from each other. (Kessel et al *1998*) 3D hydrogel embedding of chondrocytes could mimic microenvironments in native cartilage and that it might prevent dedifferentiation of chondrocytes. The microenvironment in 3D hydrogel culture might mimic the cell-to-matrix interactions or the cell-to-cell contacts of chondrocytes in native cartilage. Thus it may enable firm embedding of the chondrocytes and supports chondrocyte proliferation and matrix secretion. The scaffold architecture, material composition and porosity can be controlled through design and fabrication, and this ability is crucial to future success in tissue engineering.

1.8. FABRICATION METHODOLOGIES

A number of fabrication technologies have been applied to process biodegradable and bioresorbable materials into 3 D polymeric scaffolds of high porosity and surface area. The techniques vary from the conventional techniques for scaffold fabrication like fiber bonding, solvent casting, particulate leaching, membrane lamination and melt molding to the more advanced rapid prototyping. (*Yang et al 2001*) From the scaffold design and function view point, each processing methodology has its pro and cons. At present the challenge in tissue engineering of bone and cartilage is not only to design 3 D structures, but also to fabricate reproducible bioresorbable 3 D scaffolds, which are able to function for a certain period of time. (*Hutmacher 2000*)

1.8.1. NON WOVEN MESHES

These techniques use textile technologies for the preparation of non-woven meshes of different polymers.(*Sittinger et al 1996*) In particular non-woven polyglycolide structures have been tested for tissue engineering applications. Non woven fibres made of PLA and PGA has been used for cartilage tissue engineering but had insufficient mechanical properties. The principal drawbacks are related to the difficulties of obtaining high porosity and regular pore size. In general, non-woven constructs can be only used for soft tissue regeneration since their physical properties do not allow load-bearing applications

1.8.2. SOLVENT CASTING & PARTICULATE LEACHING

This approach allows the preparation of porous structures with regular porosity, but with a limited thickness. In this method the polymer dissolved in a suitable organic solvent is cast into a mold filled with porogen particles. Porogens can be an inorganic salt like sodium chloride, crystals of saccharose, gelatin spheres or paraffin spheres. Further the solvent is allowed to fully evaporate, and the composite structure in the mold is immersed in a bath of a liquid suitable for

dissolving the porogen to obtain the porous structure. The size of the porogen particles will affect the size of the scaffold pores, while the polymer to porogen ratio is directly correlated to the amount of porosity of the final structure. The solvent casting and particle leaching produce scaffolds with low porosity of 20-50 % and the residual salt particles that leach out may cause toxicity. This technique hence works only for thin membranes or 3-D specimens with very thin wall sections. Moreover most of the pores remain closed. (*Mikos et al 2003*)

1.8.3. GAS FOAMING

This technique uses gas as a porogen and the eleminates the need to use organic solvents and solid porogens. First disc shaped structures made of the desired polymer are prepared by means of compression molding using a heated mold. The discs are then placed in a chamber where they are exposed to high pressure CO_2 for several days. The pressure inside the chamber is gradually restored to atmospheric levels. During this procedure the pores are formed by the carbon dioxide molecules that leave the polymer, resulting in a sponge like structure. (*Mooney et al 1996*) The main problems related to such a technique are caused by the excessive heat used during compression molding. This prohibits the incorporation of any temperature labile material into the polymer matrix and the underlying pores do not form an interconnected structure

1.8.4. THERMALLY INDUCED PHASE SEPARATION

The phase separation procedure requires the use of a solvent with a low melting point that is easy to sublime. (*Nam et al 1999*)Then phase separation is induced through the addition of a small quantity of water to form a polymer-rich and a polymer-poor phase. Following cooling below the solvent melting point and some days of vacuum-drying to sublime the solvent, a porous scaffold is obtained. Liquid-liquid phase separation presents the same drawbacks of emulsification/

freeze-drying. In thermally induced phase-separation technology only, pore sizes of up to 100 μm can be reproducibly fabricated.

1.8.5. EMULSIFICATION/FREEZE-DRYING

This technique does not require the use of a solid porogen. First a polymer is dissolved into a suitable solvent. Then water is added to the polymeric solution and the two liquids are mixed in order to obtain an emulsion. Before the two phases can separate, the emulsion is cast into a mold and quickly frozen. The frozen emulsion is subsequently freeze-dried to remove the dispersed water and the solvent, thus leaving a solidified, porous polymeric structure. The emulsification and freeze-drying allows a faster preparation compared to solvent casting and particle leaching. It does not require a time consuming leaching step. The emulsion freeze-drying method fabricate scaffolds with porosity greater than 90%, median pore sizes ranging from 15 to 35 μm and larger pores greater than 200 μm were fabricated. The scaffold pore architecture was found to be highly interconnected which is necessary for tissue ingrowth and regeneration (*Whang et al 1995).* Fabrication of a truly interconnecting pore structure depends on the processing method and parameters as well as on the used equipment.

1.8.6. CAD/CAM TECHNOLOGIES

Since most of the above described approaches have limitations when it comes to the control of porosity and pore size, computer assisted design and manufacturing techniques have been introduced to tissue engineering. First a three-dimensional structure is designed using CAD software, then the scaffold is realized by using ink-jet printing of polymer powders or through fused deposition modeling of a polymer melt. (*Yang et al 2002*) Solid freeform fabrication systems create scaffolds with controlled internal micro architecture, which should increase the mass transport of oxygen and nutrients deep into the scaffold. However the limitations are difficulty in support powder removal, use of toxic organic binders,

high temperature processing preventing the use of biological molecules and poor mechanical strength. Optimizing the scaffold is essential if tissue engineering is going to be successful in replacing or repairing damaged human tissues. (*Sachlos et al 2003*)

1.9. CELL SOURCE

In order to generate a tissue for transplantation, the cells have to be isolated from a suitable source, expanded and stimulated to secrete cartilage specific molecules. In the invitro culture, they should be able to respond to their environment, differentiate, form new tissue and integrate with native tissue in vivo. Production of engineered tissue necessitates the use of large quantities of cells, but this requirement can be difficult to satisfy with differentiated cells that exhibit limited doublings in culture. Engineering tissues essentially requires an appropriate cell source. Current research has been focused more on stem cells, and their differentiation to appropriate tissue.

1.9.1. CHONDROCYTES

Autologous chondrocytes have been isolated from healthy articular cartilage sites using tissue biopsy methods (*Ringe et al 2002*). Human articular chondrocytes derived from articular cartilage biopsies have only a limited proliferative potential and the number of cell divisions they undergo in vitro decreases with age (*Dozin et al, 2002).* Chondrocytes grown as a monolayer dedifferentiate with passaging and loose both the chondrogenic phenotype, and their redifferentiation potential. Redifferentiation *in vitro* is possible in presence of appropriate stimuli. (*Cancedda et al, 2003).* The use of specific growth factors, such as basic fibroblast growth factor (FGF-2) and transforming growth factor β1 (TGFβ1) is found to increase the rate of cell proliferation, and maintains the ability of cells to redifferentiate upon transfer into a 3D environment. A cartilage biopsy from the joint to obtain differentiated chondrocytes for cell therapy represents an additional injury to the cartilage surface. This may be possibly

detrimental to the surrounding healthy articular cartilage in a damaged joint. This can be further affected by donor age and health status (*Dozin et al 2002*). The use of stem cells avoids this problem altogether. Differentiated chondrocytes can be obtained also from non-articular, hyaline cartilage tissues such as nasal or rib cartilage. However, more studies need to be carried out to assess whether cells of non-articular origin can be successfully used for articular cartilage repair.

1.9.2. STEM CELLS

Stem cells are undifferentiated cells that have high replicative potential and have multilineage differentiation capacity. Plasticity is the ability of stem cells to differentiate across the embryonic germ line boundaries of mesoderm, ectoderm, and endoderm. Their ability to differentiate along one lineage is called unipotent, into multiple cell types is multipotent or into cells derived from all 3 embryonic germ layers is called pluripotent. (*Stemple et al 1992, Young et al 2004*) Stem cells are also categorized into embryonic or adult stem cells cells according to their source. Embryonic stem cells are derived from the early stage of an embryo and can be differentiate into any type of cells. Although embryonic stem cells research have much greater potential than adult stem cells, several ethical and legal controversies still exist concerning their use for humans. Problems with teratoma formation and immune rejection must be overcome before plouripotent embryonic stem cells are practical for therapeutic use. (*Winkler et al 2005, Swijnenburg et al 2005*) The benefits of using adult instead of embryonic stem cells to prepare living tissue substitutes include immuno-compatibility of autologous cells, ease of inducing differentiation to a specific lineage, and availability. (*Prockop et al 2003*) In case of primary cells, the issue of cell age is a major concern, and there exist a weight of evidence that older chondrocytes may be less responsive to anabolic cytokines and other bioactive or physical stimuli. The relative simplicity of bone marrow harvesting procedure, the higher biosynthetic activity of

bone marrow stromal cells obtained from older individuals is other advantages of MSCs.

1.9.3. ADULT STEM CELLS

Multipotent adult stem cells have been found in many tissues, including bone marrow, adipose, skeletal muscle and placenta. (*Jiang et al 2002, Zuk et al 2001, Sinanan et al 2004)* It is also present in umbilical cord blood, amniotic fluid, synovium, brain, blood vessels and blood. (*Anker et al 2003, Conget et al 2000, Bari et al 2001)* The potential advantage of using adult stem cells is that the patient's own cells could be easily isolated, expanded in culture, and then transplanted into the patient's body where tissue regeneration is required. By this approach, there is no (or very minimal) chance of immune rejection, and certain ethical and legal issues can also be conquered. Previous reports have shown that MSCs are immunoregulatory and do not elicit immune response in vitro (*Sotiropoulou et al 2006, Aggarwal et al 2005)* or in vivo (*Bartholomew et al, 2002, Blanc et al 2004, Maitra et al 2004*)

1.9.4. MESENCHYMAL STEM CELLS

Bone marrow has been identified to be a reliable source of mesenchymal stem cells. The harvest of a limited bone marrow sample is an easy and relatively safe procedure. Bone marrow mesenchymal stem cells (BMSCs) were first recognized by Friedenstein et al in the 1960s, where they noticed that an adherent, fibroblast-like cell population has the ability to regenerate the essentials of normal bone. (Bianco et al 2001) Several people have reported mesenchymal stem cells (MSCs) to be fibroblastoid-like cells that differentiate to osteoblasts, adipocytes, and chondrocytes in vitro. (*Ominicini et al 2006,*) These pluripotent bone marrow cells are very rare as one in 10^7 to 10^8 cells. (*Reyes et al 2001*) Stem cells are defined by their behavior and by a definitive set of surface markers that can clearly distinguish between a stem cell and its differentiated neighbors. (*Vogel et al 2003*) Despite their functional heterogeneity, MSC populations obtained from most

tissues commonly express a number of surface receptors including CD29, CD44, CD49a-f, CD51,CD73, CD105, CD106, CD166, and Stro1 and lack expression of definitive hematopoietic lineage markers including CD11b, CD14, and CD45. *(Donald et al 2007)* To differentiate to cells of different linieage, MSCs have to respond to appropriate cell signals and bioactive phenotype specific growth factors. These cells after implantation to specific sites have been able to respond to their environment to generate the required extracellular matrix. . MSCs have already been used for the repair of several damaged tissues. (*Caplan et al 2001, Caterson et al 2001, 2002, Gao et al 2001 Naumann et al 2002, Thesleff et al 2003*) The properties of BMSCs are deeply influenced by the microenvironmental conditions. Effectively replicating the stem-cell microenvironment or ''niche'' could allow adult stem cells to more fully reach their regenerative potential during tissue engineering

1.10. 3D SCAFFOLDS FOR STEM CELL DIFFERENTIATION TO CARTILAGE

Several studies have been conducted to prove the capability of BMSCs to differentiate to cartilage. (*Risbud et al 2002*) Studies have proved that undifferentiated adult bone marrow derived mesenchymal stem cells (MSCs) are capable of giving rise to chondrocytes when maintained in a three-dimensional culture and treated with members of the transforming growth factor-β (TGF-β) family of growth factors. Earlier differentiation was carried out in pellet culture in presence of growth factors. Isolated MSCs from rabbit and human bone marrow were first shown to be capable of in vitro chondrogenic differentiation in micro mass pellet cultures. *(Johnstone et al 1998, Yoo et al 1998)* Later on studies have been conducted to differentiate in presence of appropriate 3 D scaffolds. 3D hyaluronic acid scaffolds were found to promote the differentiation in presence of growth factors. (*Solchaga et al 1999, Angele et al 1999, Radice et al 2000)* Several natural and synthetic scaffolds like gelatin, alginate, silk, polycaprolactone,

PLLA and collagen type II have been used for the mesenchymal stem cell differentiation. (Wang *Y et al 2005, Diduch et al 2000, Li et al 2005, Ponticello et al 2000, Caterson et al 2001, Bosnakovski et al 2006 Richardson et al 2006)* The scaffolds fabricated in the form of porous matrix, nanofibres and hydrogels have been used for the differentiation process. *(Xin et al 2004)* The signalling molecules used for chondrogenic differentiation varied in the different polymeric scaffolds. *(Bosnakovski et al 2006, Wang et al 2005 , Mauck et al 2006)* Some of the recent literature also points out that the biomaterials may also play an immunoprotective role by minimizing host immunoreactivity toward transplanted cells or engineered grafts. (*Chai et al 2007*) The material composition, the structure of the 3D scaffold and the signalling molecules used differ in these studies. All these studies reports the occurrence of chondrogenesis, but the amount of the forming matrix are lower than that produced by chondrocytes. These findings suggest that while MSCs do generate constructs with substantial cartilaginous properties, further optimization of culture conditions need to be carried out in each scaffolds to achieve levels similar to those produced by chondrocytes.

1.11. GROWTH FACTORS FOR DIFFERENTIATION OF STEM CELLS

1.11.1. TRANSFORMING GROWTH FACTOR BETA (TGFβ)

The members of transforming growth factor beta superfamily are multifunctional peptides that controls proliferation, differentiation, and other functions in many cell types. The TGF-βs possess three major activities: they can stimulate the growth of some mesenchymal cells; they exert immunosuppressive effects and can enhance the formation of extracellular matrix. The TGFβ super family consists of more than 40 polypeptide growth factors that share a high degree of homology in having conserved cysteine residues in their C-terminal region *(Wozney et al 1998)*. Besides the various isoforms of TGF β *(Iwasak et al 1993, Monroy et al 1997, Awad et al 2003)* and bone morphogenetic proteins (BMP) (*Shea et al 2003, Chen et al 1992, Kramer et al 2000*), other members of the TGF-

super family include activin (Jiang et al 2003), osteogenic protein-1 (*Asahina et al 1993*), and GDF-5 (*Spiro et al 2005*). They each have an N-terminal signal peptide for secretion from a cell, a pro-region (called latency associated peptide or LAP), and a C-terminal region that becomes the *mature* TGF-β molecule. The mature molecule is formed from the pro-region by proteolytic cleavage. The mature TGF-β protein dimerizes to produce a 25 KDa active molecule with many conserved structural motifs. TGF-β has nine cysteine residues that are conserved among its family.The eight cysteine residuies form disulphide bonds within the molecule to create a cysteine knot structure characteristic of the TGF-β superfamily while the ninth cysteine forms a bond with the ninth cysteine of another TGF-β molecule to produce the dimer. The region between the fifth and sixth conserved cysteines houses the most divergent area of TGF-β molecules that is exposed at the surface of the molecule and is implicated in receptor binding and specificity of TGF-β. The SMAD pathway is the classical signaling pathway that TGF beta family members signal through as represented in Fig 4.

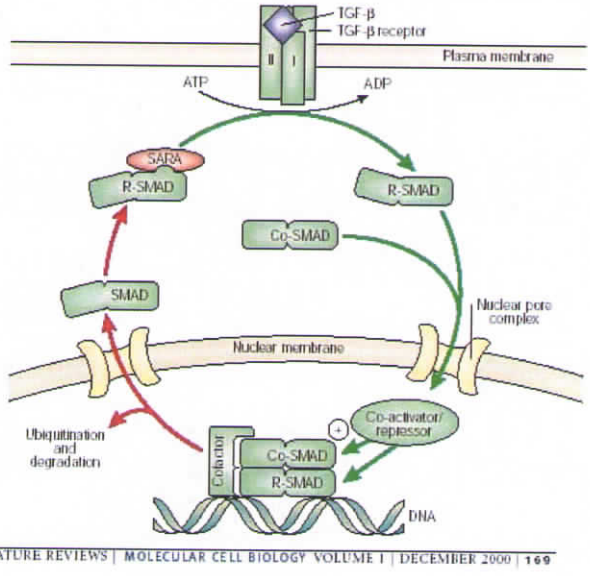


Fig 4. Classical Smad pathway of TGF β signalling

super family include activin (Jiang et al 2003), osteogenic protein-1 (*Asahina et al 1993*), and GDF-5 (*Spiro et al 2005*). They each have an N-terminal signal peptide for secretion from a cell, a pro-region (called latency associated peptide or LAP), and a C-terminal region that becomes the *mature* TGF-β molecule. The mature molecule is formed from the pro-region by proteolytic cleavage. The mature TGF-β protein dimerizes to produce a 25 KDa active molecule with many conserved structural motifs. TGF-β has nine cysteine residues that are conserved among its family.The eight cysteine residuies form disulphide bonds within the molecule to create a cysteine knot structure characteristic of the TGF-β superfamily while the ninth cysteine forms a bond with the ninth cysteine of another TGF-β molecule to produce the dimer. The region between the fifth and sixth conserved cysteines houses the most divergent area of TGF-β molecules that is exposed at the surface of the molecule and is implicated in receptor binding and specificity of TGF-β. The SMAD pathway is the classical signaling pathway that TGF beta family members signal through as represented in Fig 4.

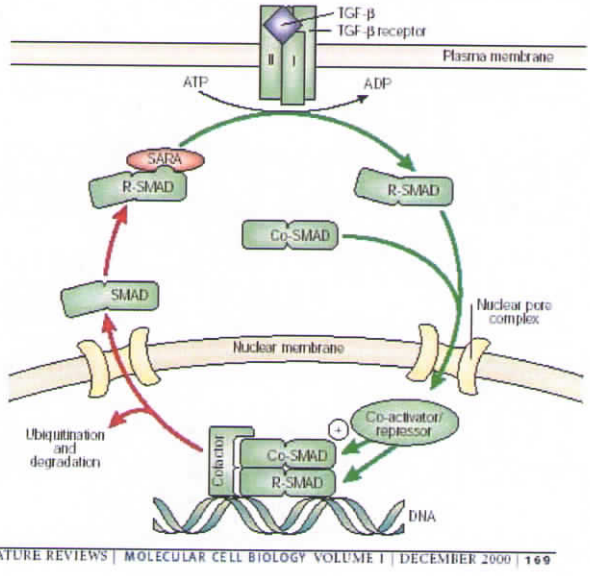


Fig 4. Classical Smad pathway of TGF β signalling

TGF-β exists in at least five isoforms, known as TGF- β1, TGF- β2, TGF-β3, TGF- β4, TGF- β5. The peptide structures of the members of the TGF-β family are highly similar. They are all encoded as large protein precursors. TGF-β1 contains 390 amino acids and TGF-β2 and TGF-β3 each contain 412 amino acids. The three members of the transforming growth factor beta (TGF-β) super family, TGF-beta 1, beta 2 and beta 3 are secreted by most cell types and are most potent inducers of chondrogenic differentiation. (*Lawrence 1996, Barry et al 2001*) In bovine cartilage organ cultures, TGFβ regulates the metabolism of proteoglycans and stimulates collagen and glycosaminoglycan synthesis. (*Morales et al 1983, Redini et al 1988*)

Bone morphogenetic proteins (BMPs) are considered to be among the most potent anabolic factors for articular cartilage. They were discovered by Urist and Wozney (*Wozney et al 1988*) as factors, which can induce ectopic bone formation. They are mainly identified as cytokines that induce bone and cartilage tissues *in vivo* (*Rosen & Wozney 2002).* Multiple functions of BMPs in foetal development of the bone, the cartilage, the kidneys and the neural system have been identified. They play an important role in chondrogenic differentiation of mesenchymal stem cells (*Schmitt et al 2003, Sekiya et al 2005)* BMPs are not only important for the development of the articular cartilages, but are also present and active in the adult articular cartilage. The members of TGFβ, together with other growth factors, co-ordinate the differentiation process. (*Longobardi et al 2006, Zhou et al 2004*)

1.12. MOLECULAR SIGNALLING OF TGFβ BY SMAD PATHWAY

Two major pathways involving Smad proteins are activated by members of the TGFβ super family. (*Miyazono et al 2001, 2002*) The activated ligand binds to two types of receptors, type II and type I receptors. Both the receptors are required for signal transduction. In addition, some cell surface proteins, including betaglycan (also known as TGFβ type III receptor), endoglin also have role in the

ligand binding. The type II and type I receptors are transmembrane proteins with serine/ threonine kinase domains in their intracellular domains. The type II receptor kinases are activated upon ligand binding and form hetero-tetrameric complexes composed of two molecules each of type II and type I receptors. In the tetrameric receptor complexes, type II receptor kinases transphosphorylate the intracellular domain of type I receptors. Following phosphorylation, type I receptor kinases are activated and phosphorylate the downstream intracellular substrates.

Smad proteins are major signalling molecules acting downstream of the serine/threonine kinase receptors. Smads are classified into three subclasses, i.e. receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads). R-Smads are further divided into two subclasses; Smad2 and Smad3 (referred to as activin/TGFβ activated R-Smads) which are activated by TGFβ type I receptors. Smad1, Smad5 and Smad8 (referred to as BMP activated R-Smads) are activated by BMP type I receptors.

The R-Smad-Co-Smad complexes translocate into the nucleus and regulate the transcription of target genes. In the nucleus, R-Smads and Co-Smad interact with various DNA-binding proteins which bind to promoter regions of target genes together with the Smads. In addition, R-Smads and Co-Smad bind to transcriptional co-activators and co-repressors, which induce the acetylation and de-acetylation of histones, respectively, and play important roles in transcriptional regulation. Studies have proved that Smads 2 and 3 are specifically activated by activin/nodal and TGF-β type I receptors, whereas Smads 1, 5 and 8 are activated by BMP type I receptors. Nearly 30 proteins have been identified as members of the TGFβ super family in mammals, and can be classified based on whether they activate activin/TGFβ-specific R-Smads or BMP-specific R-Smads. (*Miyazawa et al 2002*) Understanding the mechanisms of TGF-β super family signalling is thus important for the developing of new growth factor cocktails to treat various clinical diseases in which TGF-β super family signalling is involved. Other studies have

reported that BMP-2 induces neochondrogenesis of rat periosteum-derived cells, and that TGF-β1 modulates the terminal differentiation in BMP-2 induced chondrogenesis. (*Hanada et al 2001*) TGF-β3 appears to be most effective on cells which have not yet undergone cell condensation, a critical event in early cartilage differentiation, whereas BMP-2 is most effective after cells have condensed or differentiated. These observations suggest that TGF-β3 and BMP-2 may be acting in a sequential manner to regulate chick limb mesenchymal cells through the different stages of cartilage differentiation. (*Roark et al 1984)*

1.13. AIM AND SCOPE OF THIS THESIS

Hyaline cartilage at the articular joint helps in the friction free movement of the joints by distributing and transferring the load applied at the joints and resisting compression. Diseases such as osteoarthritis, trauma associated with injury, chondrosarcoma and other congenital diseases form a defective cartilage where the complex metabolic balance of the cartilage is disrupted leading to the loss in integrity of tissue and biomechanical function. These disease conditions affect almost all people above the age of sixty and according to the world health organization, the prevalence of a damaged joint is going to increase drastically in the coming years. Currently available treatment options provide short term relief with poor tissue regeneration. Tissue engineering is an emerging alternative treatment using autologous cells, isolated from patients, expanded and cultured in vitro in three dimensional (3D) scaffolds that can be implanted back into the patient. Appropriate combination of 3D polymeric scaffolds, cells and signaling molecules are required for successful invitro regeneration of the tissue.

Tissue engineering of cartilage generally utilizes a natural or synthetic matrix to serve as a 3 D template for growing cells. The two dimensional cultures are usually inadequate to model the complex cellular interactions compared to the 3 D nature of tissue. 3 D culture in scaffolds on the other hand, promotes high cell-cell and cell-matrix interaction, provides more surface area and is a better representation of actual tissue. It serves as an artificial extra cellular matrix, providing support, for the cells during invitro culture. The scaffolds serve as an analogue of extracellular matrix and generate the microenvironment required for tissue regeneration. Several 3 dimensional scaffolds made of both natural and synthetic polymers have been used for chondrocyte culture for invitro regeneration of cartilage tissue. Both natural and synthetic materials have been found to be favorable for chondrocyte culture. At the same time, both of them have certain limitations. The chondrogenic response varied widely in these scaffolds. So far

there is no consensus regarding the selection of an appropriate scaffolding material for cartilage regeneration.

Considering the major role of 3D scaffolds in invitro engineering of tissue, it is likely that a single polymeric material cannot serve as an artificial substitute for extracellular matrix. Only a hybrid material composed of more than one polymer can mimic the properties of native extra cellular matrix, and can provide the required physico-chemical and molecular cues for, tissue regeneration. The properties of the scaffold depend on its chemical composition, 3 dimensional pore characteristics, mechanical properties, nutrient uptake ability and biodegradation. The physico-chemical properties of the 3 dimensional scaffolds play a significant role in regulating the chondrogenic response. The functional properties of the cartilage mainly depend on the nature of extra cellular matrix molecules present in the tissue. The chemical composition, pore morphology, nutrient uptake ability and rate of degradation of the scaffolds are likely to influence the cell morphology, viability, extracellular matrix synthesis, and their distribution to form a tissue like structure. Hence the first hypothesis is

The physicochemical properties of the scaffolding material may significantly influence the behavior of chondrocytes during invitro regeneration of cartilage tissue.

One of the major challenges of tissue engineering of cartilage is the availability of cells in sufficient number for invitro tissue regeneration. Chondrocytes have a well characterized lack of expansion potential or loss of phenotype on long term culture in monolayer. Mesenchymal progenitor cells serve as an alternative source with high expansion potential capable of supplying large quantities of cells to form a 3D tissue like structure. The differentiation of stem cells to chondrocytes is cued by signaling molecules. Most of the previous studies on chondrogenesis employed a single growth factor to evaluate the invitro differentiation process. In

native tissue the formation of a proper chondrogenic phenotype is regulated by the combined action of several growth factors. Hence the second hypothesis is

The supplement of a single growth factor may not elicit all the necessary molecular signals for the differentiation process during invitro tissue regeneration.

A combination of growth factors along with a chosen 3 D scaffold may be appropriate to attain appropriate differentiation. The effect of 3 D scaffolds and role of signaling molecules in the differentiation of mesenchymal stem cells is investigated in this study, by supplementing growth factors individually and in various combinations.

The Scope of this study is limited to

- Fabrication of a hybrid 3 dimensional scaffold designed using synthetic and natural polymers.
- Evaluation of physico-chemical properties of these 3 D scaffolds.
- Standardizing the isolation and culture of chondrocytes in monolayer and in 3 D scaffolds.
- Evaluating the effect of the two, 3 D scaffolds on chondrocyte morphology, viability, extracellular matrix synthesis and distribution, to form a 3 D cartilage like tissue.
- Standardizing the isolation and culture of mesenchymal stem cells in monolayer and in 3 D scaffolds.
- Evaluating the effect of supplementing the growth factors individually and in combinations for chondrogenic differentiation of mesenchymal stem cells in 3 D scaffolds.
- Evaluating the effect of the two 3 D scaffolds on the chondrogenic differentiation of mesenchymal stem cells in presence of signalling molecules.

Chapter 2
Materials and Methods

2.1. FABRICATION OF 3 DIMENSIONAL (3D) SCAFFOLDS

MATERIALS

Poly(vinyl alcohol) (PVA) (Typical Mw 13,000-23,0000, Sigma-Aldrich, MP-200°C), Poly(caprolactone) (PCL) (Typical Mw 65000, Sigma-Aldrich, MP-60°C), Chloroform ($CHCl_3$), Gluteraldehyde (Merck), Isopropanol (Merck), Gelatin (Porcine skin, Type A, ~300 blooms, Sigma), Egg albumen flakes (Spectrochem), (1-ethyl-3(3-dimethyl aminopropyl) carbodiimide hydrochloride) (EDAC) (Sigma), Acetone (Merck). All other chemicals were of analytical grade. Double distilled water (DDW) was used for preparing aqueous solutions.

METHODS

2.1.1. SCAFFOLD FABRICATION METHODOLOGY

A uniform method of fabrication of scaffold was followed for all the compositions employing the following steps.

a. Preparation of solutions of polymer.
b. Preparation of a uniform foam and lyophilization
c. Crosslinking and lyophilization

2.1.2. 3D SEMI INTERPENETRATING POLYMER NETWORK (SEMI IPN) OF POLY (VINYL ALCOHOL) – POLY(CAPROLACTONE) (PVA-PCL) SCAFFOLD

3D PVA-PCL scaffolds were fabricated by uniform blending and freeze drying process.

COMPOSITIONS

Three compositions of PVA-PCL scaffolds were produced by blending different weight percentages of PVA and PCL as represented in table 1.

Table 1. Different compositions of PVA-PCL

Code	PVA (% weight ratio)	PCL (% weight ratio)
PVA-PCL 30:70	30	70
PVA-PCL 50:50	50	50
PVA-PCL 70:30	70	30

2.1.2. a. PREPARATION OF SOLUTIONS OF PVA-PCL

A solution of poly (vinyl alcohol) was prepared by dissolving the required amount of PVA as per the compositions in table 1. in 50 ml DDW with continuous stirring at 90°C for 15 minutes. A solution of required concentration of poly (caprolactone) was prepared in $CHCl_3$ with continuous stirring at 37°C for 2 hours.

2.1.2. b. PREPARATION OF A UNIFORM FOAM AND LYOPHILISATION

To produce a uniform blend of poly (vinyl alcohol) and poly (caprolactone), the $CHCl_3$ in the poly (caprolactone) solution was evaporated so as to get a viscous paste to which solution of poly (vinyl alcohol) was added with continuous stirring using a mechanical stirrer at 3000 rotations per minute (rpm) for 30 minutes. When the foam occupied thrice the initial volume it was immediately poured into precooled polypropylene vials and frozen at -70°C (Ultra low Sanyo, MDF-44086S) for 48 hours. The frozen samples were lyophilized at -70°C for 48 hours (Christ, Alpha 2-4D) to obtain 3D porous scaffolds.

2.1.2.c. CROSSLINKING AND LYOPHILISATION

The stability of 3D freeze dried samples were improved by crosslinking the free hydroxyl (-OH) groups of poly (vinyl alcohol) in the scaffold using gluteraldehyde. A single cross linking methodology was used for all the compositions. For crosslinking, about 1500 mg of the freeze dried samples were immersed in 50 ml of 0.5% gluteraldehyde solution prepared in a mixture of

isopropanol: water in the ratio (90:10) in presence of HCl as a catalyst. Crosslinking was carried out at 37°C with continuous shaking at 100 rpm for 3 hours. The scaffolds were further washed in a mixture of isopropanol: water in the ratio (50:50) for 2-3 hours with continuous shaking at 100 rpm and subsequent washes with two to three changes of double distilled H_2O for 18 hours so as to remove the excess of gluteraldehyde. The crosslinked scaffolds were frozen at -70 °C for 24 hours and lyophilized in the same condition for 48 hours to obtain the Semi IPN 3 D scaffolds of PVA-PCL.

2.1.3. 3D GELATIN-ALBUMIN SCAFFOLD (GA)

3 D Gelatin-Albumin scaffolds (GA) were fabricated by uniform blending and freeze drying technique. The scaffold fabrication process was similar to that of PVA-PCL

2.1.3.a. PREPARATION OF SOLUTIONS OF GELATIN-ALBUMIN

. A uniform blend of Gelatin-Albumin was prepared by dissolving gelatin and albumin in percentage weight ratio (90:10) in 100 ml DDW at 37°C with continuous stirring for 1 hour.

2.1.3.b. PREPARATION OF A UNIFORM FOAM AND LYOPHILISATION

Uniform foam of the blend was obtained by continuous stirring using a mechanical stirrer at 2000 rpm for 30 minutes. This was immediately poured into precooled polypropylene vials and frozen at -70°C (Ultra low Sanyo, MDF-44086S) for 48 hours. After freezing for the specific period the foam was lyophilized at -70°C for 24 hours (Christ, Alpha 2-4D).

2.1.3. c. CROSSLINKING AND LYOPHILISATION

The stability of 3 D freeze dried samples in aqueous solution was improved by forming stable covalent peptide bonds between the free carboxyl (-COOH) and

amino (-NH2) groups present in gelatin and albumin. Crosslinking was carried out using EDAC.

For crosslinking about 200 mg of the freeze dried samples were immersed in 5 mg EDAC dissolved in acetone: water mixture in the ratio (90:10) and incubated at 37°C with continuous shaking at 100 rpm for 24 hours. The scaffolds were further washed in a mixture of acetone: water in the ratio (50:50) for 2-3 hours with continuous shaking at 100 rpm and subsequently with two to three changes of DDW for 18 hours to remove the unreacted crosslinker. The crosslinked scaffolds were frozen at -70°C for 24 hours and lyophilized at -70°C for 24 hours to obtain the crosslinked 3D porous scaffolds of gelatin and albumin.

2.2. PHYSICOCHEMICAL CHARACTERIZATION OF 3 D SCAFFOLDS

The 3D scaffolds were characterized for their physicochemical properties like thermal analysis, chemical composition, surface hydrophilicity, surface morphology, pore characteristics, mechanical property and swelling ability.

2.2.1. THERMAL ANALYSIS

The thermal stability and the mass loss pattern of the hybrid scaffolds were compared to the parent materials using thermogravimetric analysis (TGA). Phase transitions in the scaffold materials were assessed using differential scanning calorimeter (DSC).

2.2.1. a. THERMOGRAVIMETRIC ANALYSIS (TGA)

Thermal stability of the scaffolds was studied using SDT 2960 (Simultaneous TGA-DTA, TA Instruments Inc) and standard protocol of (ASTM 1131-03). For TGA analysis, about 10-12 mg of scaffold samples were taken in a platinum cup and heated under nitrogen atmosphere at a rate of 10°C/minute from 0°C to 600°C. The weight loss of different materials heated to different

temperatures is compared in TGA. The decomposition temperature at initial, 50 wt % and final weight loss were noted. The thermogram is a plot of % weight loss against temperature. Different stages of degradation were confirmed from the derivative curve of (wt % / °C) against temperature.

2.2.1. b. DIFFERENTIAL SCANNING CALORIMETER (DSC)

The phase transitions of the scaffolds were evaluated using differential scanning calorimetry using (DSC 2920 Differential scanning Calorimeter, TA Instruments Inc) with TA400 controller and standard protocol of (ASTM E 1356-03). For DSC analysis about 4-6 mg of samples were crimped inside aluminium sample pans and heated under nitrogen atmosphere at a rate of 10°C/minute from -20°C for PVA, PCL and PVA-PCL and from -50°C for gelatin, albumin and GA to the desired temperature. The data is a plot of heat flow against temperature.

2.2.2. FOURIER TRANSFORM IR SPECTROSCOPY (FTIR)

FTIR spectra of the parent materials and the scaffolds were analyzed for the determination of identity, functional groups and for the evidence of crosslinking. FTIR spectrum of the samples were obtained from Nicolet 5700 FTIR spectrophotometer with DTGS (Deutrated triglycine sulfate) detector (Thermo electron corporation) using diamond attenuated total reflectance (ATR) accessory in reflectance mode with a resolution 4. Fifty scans were recorded per collection in the range of 4000 – 400 cm^{-1} and the spectra was analyzed using Omnic software.

2.2.3. MEDIUM UPTAKE ABILITY

Scaffolds were cut in the form of discs of 10 mm diameter and 3 mm thickness. Preweighed samples were immersed in fresh phosphate buffer saline (PBS) at 37°C at pH 7.4 in preweighed containers for known intervals of time. The medium was carefully and completely withdrawn at specific intervals. The scaffolds were blotted with tissue paper, uniformly to remove the excess of water.

The wet weights of the scaffolds were measured until equilibrium in weight was attained. The percentage swelling was calculated using the formula given below.

$$\text{Percentage swelling} = \frac{[\text{Final wet weight} - \text{Initial dry weight}]}{\{\text{Initial dry weight}\}} \times 100$$

2.2.4. MECHANICAL PROPERTY

The compression modulus of the dry 3D porous scaffolds were tested using (Instron Series IX automated Material Testing system 8.30.00.) with a 5 kN load cell. The sample size and the testing speed were as per the ASTM standards, (ASTMD 895-96). Cylindrical samples with height twice that of the diameter was used for testing. Six samples of each composition were compressed at a speed of 1.3 mm/minute to half height and at this point the maximum load, stress, % strain and modulus was calculated.

2.2.5. WATER CONTACT ANGLE MEASUREMENTS

The surface property of the hybrid compositions were evaluated using sessile drop method. Contact angles of the crosslinked films of the hybrid compositions equilibrated with water were compared to the parent materials. The water contact angle was measured using video contact analyzer (Data Physics, OCA 15 Plus, Germany) and imaged using SCA 20 software.

Deionized water (3 µl) was automatically dropped on to the film using a gastight Hamilton precision syringe. Images of the droplets were captured within 5 seconds. The baseline and the tangent were drawn using software and the contact angles from both the sides of water droplet were measured. Contact angles were measured from seven different portions in the same film with four replicates. The left and right contact angle was measured and the data was represented as an average of the two values for each observation.

2.2.6. SCANNING ELECTRON MICROSCOPY

The 3D porous structure, pore size and the pore morphology was evaluated using scanning electron microscope (Hitachi, Model S-2400, Japan). The lyophilized porous samples were mounted on to aluminium stubs and coated with ultra thin 300A° layer of gold in a coating apparatus and observed under microscope. SEM images were analyzed using image analysis software. (Optima's TM 6.1, West Ford, MA)

2.2.7. MICRO CT (μCT)

The overall porous structure of the scaffolds was imaged using a high-resolution micro computed tomography (μCT 40 Scan Co Medical, Bassersdorf, Switzerland). Cylindrical samples were irradiated using X-rays of power 45 kvp and data analyzed using μCT evaluation program V 6.0. An optimized threshold was used to isolate the scaffolds from the background. For PVA-PCL scaffold the threshold was 10 and for GA scaffold was 3. About two hundred 2D slices (2048 x 2048 pixels) were scanned for every sample. The resulting gray-scale images were segmented and a threshold was applied to extract the polymer architecture and then inverted to extract the pore volume architecture. This allowed 3D reconstruction from stacked 2D images. The reconstruction algorithm used was cone beam. 3 D structures were reconstructed at different angles to examine the over all porous nature of the scaffolds.

2.2.8. LIQUID EXTRUSION POROSIMETRY

The presence of continuous pores in the 3D scaffold was evaluated using liquid extrusion porosimeter (LEP –Model 1100A, PMI Porous Materials, Inc.USA). The pore characteristics were evaluated in dry state and wet state of the sample using two solvents Galwick and water respectively. LEP is a mercury free technique for characterization of pore structure using very low pressures. For measurement of pores in the dry state of the sample, the scaffold was prewetted

with Galwick (propene 1,1,2,3,3,3-hexa fluoro oxidized and polymerized). This liquid spontaneously filled in the pores of the sample with negligible or minimal swelling of the material. Further the pressure of non reacting gas (N_2) was slowly increased over the sample to displace the liquid from the pores of the sample. The sample weight, bulk density and surface tension of the solvent was calculated and input in the software. Using the software the total pore volume, % porosity and distribution of continuous pores were calculated. The experiment was repeated using water as the solvent which swells the polymer and gave the measurement of the pores of sample in wet state.

2.2.9. BIO-DEGRADATION PROFILE OF 3D POROUS SCAFFOLDS

The bio-degradation pattern of the sample was evaluated using a stimulated physiological condition of PBS at pH 7.4 at 37°C. Preweighed scaffolds were incubated in PBS for specific period with continuous stirring at 60 rpm. 0.02 % sodium azide was added to the PBS to avoid bacterial contamination. At intervals the samples were withdrawn and washed with distilled water, and lyophilized. The weight of the lyophilized samples was measured. The % degradation was calculated using the formula given below.

$$\text{Percentage degradation} = \frac{\text{Initial weight-weight after degradation}}{\{\text{initial dry weight}\}} \times 100$$

2.3 MODIFICATION OF 3D POROUS PVA-PCL SCAFFOLD

MATERIALS

Dicyclohexylcarbodiimide (DCC), Arginine (Sigma), Arginine-glycine-aspartic acid (RGD) peptide, Acetone (Merck), Tetrahydrofuran (THF) (Merck), Triethylamine (Sigma).

METHODS

The feasibility of modifying the synthetic PVA-PCL scaffolds with peptides was examined by coupling a model amino acid arginine, to free carboxyl (–COOH) groups of PVA and PCL using DCC.

The coupling was between the amino ($-NH_2$) groups of arginine and the carboxyl (–COOH) groups of PVA and PCL. Briefly the scaffolds (500 mg) were immersed in DCC (10 mg) in 20 ml THF in presence of triethylamine and incubated at 37°C for 2 hrs. Further the scaffolds were transferred to 2 ml of arginine solution (10mg/ml) and incubated for 24 hours. Further coupling with RGD peptide DCC was also carried out. For coupling 60 mg scaffold was incubated in DCC (30 mg) in 6 ml THF and triethylamine at 37°C for a period of 4 hrs. After the activation procedure, 15 mg of the activated sample is incubated with RGD peptide 0.5 ml (2mg/ml) for 24 hrs at 37°C. After the coupling procedure the samples were washed well in DDW and dried in hot air oven at 50°C. Further the modified samples were analyzed using FTIR spectroscopy.

2.3. CYTOTOXICITY EVALUATION WITH L929 CELLS

MATERIALS

Dulbecco's modified eagles medium (high glucose) (DMEM-HG) (Gibco), Foetal bovine serum (FBS) (Gibco), Polyvinyl chloride (PVC), High density polyethylene (HDPE), (3-(4, 5-dimethylthiazol-2yl) 2, 5 diphenyl tetrazolium bromide) (MTT), Dimethyl sulfoxide (DMSO), Antibiotic-antimycotic solution (Gibco).

METHODS

2.4.1. DIRECT CONTACT TEST

The cytotoxicity of ethylene oxide sterilized PCL film, PVA scaffold, PVA-PCL and GA scaffolds were tested in vitro, using a mammalian mouse

fibroblast cell line L929 (commonly used cell line to evaluate the toxicity of a biomaterial) by direct contact test according to ISO standards. (ISO 10993-5, 1992)

Briefly L929 cells were subcultured from stock culture (National Centre for Cell Sciences, India) by trypsinization and seeded on to multiwell tissue culture plates. Fibroblast cells were fed with DMEM (Gibco) supplemented with 10% FBS (Gibco) and incubated at 37°C in a 5% CO_2 atmosphere. When the cells attained semi confluence to form a monolayer, scaffolds of size (0.75 cm^2) were kept in contact with cells, in triplicate, for the direct contact test for 24 hrs and 10 day period. After the incubation time, cells surrounding the material were examined under microscope for cellular response. The morphology of the cells in contact with the biomaterials were qualitatively compared with the morphology of cells in direct contact with discs of negative nontoxic material like HDPE and positive cytotoxic material like PVC control.

2.4.2. MTT ASSAY

The cytotoxic effect of the scaffold materials on cells were evaluated quantitatively by test on extract method. The cells were incubated with the extracts of the scaffold for 10 day period. The metabolically active cells after treating with extracts of the material were quantified using MTT assay and compared to the untreated control.

The extract was prepared by incubating each of 3D Semi IPN PVA-PCL scaffolds (10 mm diameter, 2-3 mm thickness) in 5 ml of medium for 72 hrs at 37°C with continuous stirring at 60 rpm. The L929 cells were seeded on to multiwell tissue culture plates at different seeding density so that they attain confluence only during the end of the incubation period. The cells were cultured in DMEM with 10 % FBS and after 24 hours of seeding, 50% of the medium of cells was replaced with the extracts and incubated at 37°C in a 5% CO_2 atmosphere for 10 day period. Cells supplied with normal culture media served as control for each

samples. After the incubation period the metabolically active cells were quantified using MTT assay and compared with untreated control.

For MTT assay the medium was removed and the cells were treated with 20 µl of MTT dye (5mg/ml in PBS) and incubated for 3 hrs at 37°C. The assay is based on metabolic reduction of soluble MTT by mitochondrial dehydrogenase to insoluble coloured formazan product. The optical density was measured spectrophotometrically after dissolution in DMSO and measured at 540 nm (UVM 340, Asys, Digired software). Analysis was performed in triplicates for each sample. The data was represented graphically and compared to untreated control. Statistical analysis was performed using one way ANOVA test. The values were expressed as the mean ± of four different replicates. Values of $p<0.05$ were considered significant and $p<0.0001$ were considered very significant.

2.4. ISOLATION AND CULTURE OF CHONDROCYTES

MATERIALS

DMEM-HG (Gibco), FBS (Gibco), Antibiotic-antimycotic solution (Gibco), Glutamine (Gibco), Gentamycin sulphate (Gibco), Non essential amino acids (NEAA) (Gibco), Sodium pyruvate (Gibco), L-proline (Gibco), Ascorbic acid (Gibco), Dexamethasone (Sigma), Trypsin-EDTA (Gibco), , Collagenase type II (Sigma), Live dead assay kit (Molecular probes), Gluteraldehyde (Merck), ter-butyl alcohol (Merck), Papain (Wako), EDTA (Gibco), Trypan blue (Gibco), N-acetyl cysteine (Sigma), ((1, 9- dimethyl)methylene blue) (DMMB) (Sigma), Sodium formate (Sigma), Formic acid (Merck), Chondroitin-4 sulphate (Sigma), xylene (Merck), Mayer's haematoxylin (Sigma), Fast green FCF (Sigma), Ultra Tech HRP (DAB) Streptavidin-Biotin Detection System kit (Beckman Coulter), anti collagen type II rabbit monoclonal antibody (Abcam), Anti aggrecan rabbit polyclonal antibody (Santacruz Biotechnology, Inc), Dulbecco's PBS (PBS), Safranin O.

METHODS

2.5.1. ISOLATION OF CARTILAGE FROM PORCINE JOINT

Cartilage tissue was isolated from articular joint of pigs (Desi breed) *(Sus domesticus)* 3-4 months old, weighing 10-15 kg. All protocols followed were with the consent of Institutional animal ethical committee. Chondrocytes were isolated from articular cartilage in the hind joint of these pigs by a sterile procedure as per the accepted protocol.(*kuettner et al, 1982)* Briefly an incision was made across the upper surface of the joint and the skin was removed. The whole articular joint was separated with intact capsule and immediately transferred to PBS containing antibiotic-antimycotic solution (10X) and kept for 15 minutes.

Majority of connective tissue was removed from the joint capsule without exposing the hyaline cartilage. The capsule surface was washed well with sterile PBS. Using sterile blade a cut was made into joint capsule, the patellar ligament was transected and the patella was pulled from the joint avoiding contact between the inner capsule and muscle. The patella was removed from the surrounding tissue and joint was washed with PBS to keep it moist. The knee joint was exposed by cutting sagitally on medial and lateral sides of the knee. The lateral ligament was transected and the leg was flexed. Further the anterior crucial ligament and the posterior crucial ligament were transected.

With new sterile blades the surface of the cartilage was scraped to obtain the cartilage. The pieces of cartilage were placed in petri dishes with PBS to keep it moist. Scraping was continued till the bone is exposed. The isolated cartilage pieces were washed 3-4 times with fresh PBS and diced into 1-3 mm^3 cubes using sterile blades. The cartilage pieces were further transferred to preweighed petri dishes and the wet weight of the tissue was recorded.

2.5.2. ISOLATION OF CHONDROCYTES

The cartilage pieces were transferred to conical flask using sterile spatula. 10 ml of the digestion media containing 0.2 % collagenase type II was added per gram of tissue and placed in shaker at 37°C for 18 hours at a frequency of 100 rpm. After complete digestion, cool resuspension media was added to prevent further digestion. The entire solution was passed through the cheese cloth to remove the debri and undigested particles. The solution was centrifuged at 1200 rpm for 10 minutes at 4°C. The supernatant was decanted and the cell pellet was resuspended in fresh resuspension media and again centrifuged to remove the traces of collagenase. The pellet was suspended in 1 ml of fresh resuspension media and an aliquot of this solution was used to determine the cell number and viability.

2.5.3. DETERMINATION OF CELL NUMBER AND VIABILITY

10 µl of cell suspension is mixed with 40 µl of PBS. This cell suspension was mixed with 50 µl of trypan blue dye and mixed well. 10 µl of this was used to determine the concentration of cells. The cells loaded in hemocytometre were observed under microscope. The total cell number per milliliter and the % viability of cells was determined using the formula

$$\text{Cell concentration} = \text{Na} \times 10^4 \times \text{Dilution factor (D)}$$

$$\text{Na} = \text{Number of cells counted in the hemocytometre}$$

$$\%\ \text{Viability} = \frac{\text{No. of unstained Cells}}{\text{Total number of cells}} \times 100$$

Cells with viability greater than 90 % were used for all seeding experiments.

2.5.4. CULTURE OF CHONDROCYTES IN MONOLAYER

After determining the viability, cells were seeded on to a 25 cm^2 flask at a concentration of 5 x 10^5 cells/ml and 5 ml of chondrocyte culture media was added

to the flask. The cells were cultured in an incubator at 37°C with 5% CO_2. When the cells attained confluence they were trypsinized and frozen for future use.

2.5.5. CHARACTERIZATION OF CHONDROCYTES IN MONOLAYER

The production of glycosaminoglycans by chondrocytes in monolayer was identified by safranin O staining. For this, cells were cultured on top of cover slips to attain confluence. For safranin O staining, the cells fixed with 3.7% formalin in PBS and incubated with 0.1% of aqueous safranin O dye for 5 minutes. Further washed with PBS to remove the traces of unbound dye and mounted using DPX. The slides were observed under microscope. (Nikon 4500).

2.5.6. EFFECT OF DEXAMETHASONE IN MAINTAINING THE CHONDROCYTE MORPHOLOGY

Dexamethasone is reported to have some role in maintaining the chondrocyte phenotype by retaining the spherical morphology of cells in monolayer culture. This was examined by culturing the cells in monolayer in presence and absence of dexamethasone for a period of 2 months. The cells from passage 1 were seeded on to a 25 cm^2 flask at a density of $3x10^5$ cells/ml. The cells were cultured in chondrogenic medium supplemented with dexamethasone at a concentration of 10^{-7} M. Cells cultured in normal chondrogenic medium served as a control. The changes in morphology of cells were observed under phase contrast microscope (Nikon 4500).

2.6. CYTOTOXIC EVALUATION OF SCAFFOLDS WITH CHONDROCYTES

The cytotoxicity of 3D scaffolds to chondrocytes is evaluated by direct contact test. The chondrocytes from first passage were seeded on to 6-well plates and cultured to semi confluence. Scaffold samples of size 0.75 cm^2 in triplicates

were placed on to monolayer of chondrocytes and incubated at 37°C for 24 hrs and for a period of 10 days. Cells were examined microscopically for cellular morphology around test material and compared to control negative nontoxic material like HDPE and positive toxic material like PVC control discs.

2.7. MORPHOLOGY & VIABILITY OF CHONDROCYTES IN 3D SCAFFOLDS

The scaffold composition and the pore structure play a significant role in regulating the morphology of cells and in maintaining the viability of cells seeded on to 3D scaffolds. This was examined by seeding chondrocytes on to 3D PVA-PCL and GA scaffolds at a low density of 1x 10^5 cells by static seeding method using a pipette. The one week constructs were retrieved from culture and the constructs were cut into two pieces. For comparison of data, one half of the construct was used to study cell morphology and the deposition of extra cellular matrix components using scanning electron microscopy and the other half was used to determine the cell viability using Live dead assay kit .

2.7.1. SCANNING ELECTRON MICROSCOPY

For scanning electron microscopy observation the cell seeded constructs were washed in PBS and fixed with 2.5% gluteraldehyde overnight at 4°C. Samples were dehydrated with a series of alcohol (50%, 70%, 80%, 90% and 100%) and finally in ter-butyl alcohol for 10 minutes. Further they were subjected to critical point drying for 5 hrs (Hitachi HCP-2), gold sputtered in vaccum (E1010-Hitachi) and observed on scanning electron microscope (S-2380-N, Hitachi, Japan).

2.7.2. VIABILITY OF CHONDROCYTES IN 3D SCAFFOLDS

The viability of cells in the construct was evaluated using Live Dead assay kit and the constructs were further imaged using confocal microscopy (Carl Zeiss

510 Meta). The constructs were washed with PBS to remove the traces of serum that may interfere with the staining procedure. Further they were incubated for 10 minutes in the staining solution of live dead kit containing 2μM Calcein AM and 4μM ethidium bromide (EtBr). After incubation the constructs were washed well in PBS to remove the excess amount of dye and observed under confocal microscopy (Carl Zeiss 510 Meta) at ex/em 495/515 nm (green) and ex / em 495/635 nm (red). Images were taken at several planes by z stacking and using software (LSM image examiner) they were combined to obtain a single image.

2.8. CULTURE OF CHONDROCYTES IN 3D SCAFFOLDS

The results of direct contact tests revealed that the material is nontoxic and chondrocytes remain viable in the 3D scaffold. Further chondrocytes were cultured in both the 3D PVA-PCL Semi IPN and Gelatin-Albumin scaffolds to evaluate the effect of scaffold composition in promoting the production of extracellular matrix components.

Scaffolds of dimension 15-mm diameter and 3-mm thickness were seeded with porcine chondrocytes from passage 0 at a density of $1x10^6$ cells/0.5 ml/ scaffold by static seeding method using a pipette. Further the construct is centrifuged at 1200 rpm for 2 minutes to promote the movement of cells into the pores of the scaffold. The constructs were incubated in 5% CO_2 at 37°C for 3hrs for cell adhesion. After 3 hours they were incubated with fresh chondrogenic media supplemented with dexamethasone (10^{-7} M). Chondrogenic media was replaced every 3 days and constructs were cultured for a period of 2 months and 4 months with six replicates. Unseeded scaffolds served as the control. The constructs were retrieved after 2 months and 4 months. The 2 month and the 4 month old constructs were processed for evaluation of overall cell and extra cellular matrix distribution using scanning electron microscopy. The secretion of typical chondrogenic markers were evaluated by biochemical analysis and by staining of sections of the constructs for GAG, type II collagen and aggrecan link protein.

2.8.1. CELL MORPHOLOGY & DISTRIBUTION ON 28 DAY CONSTRUCTS

The over all distribution of cells and deposition of extra cellular matrix components on the surface were examined using Scanning electron microscopy. For this the constructs were retrieved from culture and washed well with PBS and observed using environmental SEM (ESEM) (FEI Quanta 200, Netherlands) under ESEM mode using GSED detector.

2.8.2. BIOCHEMICAL ANALYSIS

2.8.2.1. DIGESTION OF THE CONSTRUCT

Glycosaminoglycans and collagen are the major components of extra cellular matrix of the cartilage. These chondrogenic markers were estimated after digesting the construct with papain solution (pH 6.5). Briefly the constructs were washed well with PBS and digested using papain and incubated at 65°C for 2.5 hours to completely digest the constructs. After digestion, this was centrifuged and the supernatant was used for the estimation of total collagen and glycosaminoglycans (GAG).

2.8.2.2. ESTIMATION OF TOTAL GLYCOSAMINOGLYCANS (DMMB ASSAY)

Total glycosaminoglycan was estimated using DMMB assay as per the accepted protocol. (Farndale et al 1986) DMMB is a cationic dye, which binds to sulphate and carboxylate groups within GAGs, producing a concentration dependent metachromatic change (Enobakhare *et al* 1996). The magnitude of this change can be quantified by the measurement of optical density.

To generate a calibration curve of GAG concentration versus optical density, a 200µg/ml solution of chondroitin-4-sulphate was prepared in DDW to give standard solutions of following concentrations. 0, 12.5, 50, 100, 150 and 200

μg/ml. 150 μl of the calibration standard solutions and samples of papain-digested constructs and control were treated with 1350 μl of DMMB dye (1X). 200 μl of this solution was immediately transferred to 96 well plates and optical density is measured at 535 nm using a colorimetric plate reader (UVM 340 Asys, Digired software). The results were calculated from the standard calibration curve. The total glycosaminoglycans in PVA-PCL and the GA constructs for 2 and 4 months were represented graphically. Statistical analysis was performed using one way ANOVA test. The values were expressed as the mean of four different replicates.

2.8.3. STAINING OF THE SECTIONS OF CONSTRUCTS FOR CHONDROGENIC MARKERS

The staining of sections of the construct for cartilage specific molecules allowed the identification of the deposition of extra cellular matrix components through out the construct. Glycosaminoglycan deposition was identified by safranin O staining, and typical chondrogenic markers like type II collagen and aggrecan link protein was identified by immunostaining.

2.8.3.1. PROCESSING, PARAFFIN EMBEDDING AND SECTIONING OF THE CONSTRUCT

The 3D constructs were fixed in 10% neutral buffered formalin for 24 hrs and dehydrated by passing through an increasing series of ethanol (50%, 70%, 90%, 100%) and in xylene. Processed tissue was orientated and embedded in paraffin wax for 60 minutes. Sections of 5 μm thickness were cut using a microtome. The sections were stretched out on a water bath at 50°C, mounted on Superfrost™ microscope slides and heated to 56°C on a hot plate. The sections were deparaffinised in xylene for 5 minutes (3X) and rehydrated by passing through a decreasing series of alcohol (100%, 90%, 80% and 70%) and finally in DDW.

2.8.3.2 STAINING OF THE SECTIONS OF CONSTRUCT FOR GLYCOSAMINOGLYCANS.

For glycosaminoglycan staining, sections were incubated in Mayer's haematoxylin for 10 minutes at room temperature, washed in DDW and incubated with acid alcohol for 10 seconds. Further dipped in DDW, tap water for 5 minutes, incubated in 0.02% (*w/v*) aqueous fast green FCF for 3 minutes, incubated in 1% (*v/v*) acetic acid for 30 seconds and placed in 0.1% (*v/v*) aqueous safranin O for 10 minutes. Tissue sections were then dehydrated through an increasing series of ethanol (50%, 70%, 90%, and 100%) and xylene, before mounting in DPX and viewing under microscope (Leica DM 4000 M).

2.8.3.3. IMMUNOSTAINING FOR TYPE II COLLAGEN AND AGGRECAN LINK PROTEIN

The secretion of type II collagen and aggrecan link protein in the construct was estimated by immunostaining using specific antibodies and visualized using Ultra Tech HRP (DAB) Streptavidin-Biotin Detection System kit. One set of sections were stained for Type II collagen and another set for aggrecan link protein following the same immunostaining protocol except for the use of specific primary antibodies. The staining procedure was as per the protocol of the manufacturer in the kit. Briefly the sections were deparaffinised using xylene, rehydrated using ethanol, dipped in tap water and DDW. For antigen retrieval the sections were treated with trypsin (0.005% in 0.05 M Tris HCl pH 7.6) and incubated at 37°C for 30 minutes. Endogenous peroxidase activity was blocked using 3% H_2O_2 in (Ultra Tech HRP (DAB) kit and incubated for 5 minutes at RT. The sections were further washed with PBS for 5 minutes and treated with protein blocking agent (Ultratech HRP (DAB) kit) for 5 minutes at RT. One set of the sections were incubated with primary rabbit anti collagen type II monoclonal antibody (1:100) dilution in 2% BSA and another set of sections were incubated with primary anti aggrecan rabbit

polyclonal antibody (1:100) dilution in PBS containing 1% BSA for 1 hr in humidity chamber . Washed with PBS 2X, incubated with Streptavidin-peroxidase solution for 10 minutes and treated with DAB chromogen working solution for 5 minutes. The slides were further washed in water for 2 minutes, counterstained using haematoxylin and again washed in running water to remove the traces of unbound dye. Finally the stained sections were mounted in mounting medium (DPX) and the images were obtained from (Leica DM 4000 M) microscope using Leica application suite software.

2.9. ISOLATION AND CULTURE OF MESENCHYMAL STEM CELLS

MATERIALS

α Minimum essential medium (αMEM) (Gibco), FBS (Gibco), DMEM-HG (Gibco), Antibiotic-antimycotic solution (Gibco), Glutamine (Gibco), Gentamycin sulphate (Gibco), Non essential amino acids (NEAA) (Gibco), Sodium pyruvate (Gibco), L-proline (Gibco), Ascorbic acid (Gibco), Dexamethasone (Sigma), Anti Vimentin-FITC mouse monoclonal antibody (Santacruz Biotechnology Inc), Anti Endoglin (CD105) mouse monoclonal-phycoerythrin (PE) conjugated antibody (Santacruz Biotechnology Inc), Hoechst 33258 (Sigma), Recombinant human transforming growth factor β1 (TGFβ1) (Genzyme), Recombinant human Transforming growth factor β3 (R&D Systems), Bone morphogenetic protein (BMP2), Protease (Wako, Japan), ((1, 9 dimethyl)methylene blue) (DMMB) (Sigma), Sodium formate (Sigma), Formic acid (Merck), Chondroitin-4 sulphate (Sigma), Sircoll (Sirius Red kit) (R & D systems), Safranin O, (SAB PO(R) kit, (Histofine, Japan), mouse monoclonal anti aggrecan antibody (Abcam, UK) ,Antibody diluent (Dako cytomation), Midi kit Qiagen column (Qiagen), β-mercaptoethanol (Sigma), (Superscript II reverse transcriptase (superscript RT), oligodT, 5X buffer, DTT, dNTP Mix, RNA out, RNaseH (Ecoli), (Invitrogen)), SYBR@Green PCR Mix (Applied Biosystems),

2.9.1. ISOLATION OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells were isolated from 2 weeks old male Wistar rats as per the accepted protocol. (Majumdar et al 2000) Briefly the rats were sacrificed by CO_2 asphyxiation using diethylether and the rats were wiped with alcohol. The skin was removed and the bone was cut at the joint where the femur is connected to the pelvic girdle. The femur and tibia was made muscle free and placed in sterile culture plates. The entire marrow was flushed into an eppendorff tube using 1 ml PBS using a 21 gauge needle and flushed repeatedly to get uniform cell suspension. The whole suspension was transferred to 25 cm^2 containing 5 ml of culture medium (αMEM (Gibco) supplemented 15 % FBS (Sigma) and 1% antibiotic solution. The cells were incubated at 37°C with 5% CO_2 for 3 days. After 3 days the supernatant was removed and the attached cells with mesenchymal phenotype were cultured in the medium to attain confluence. When the cells attained confluence they were trypsinized and subcultured.

2.9.2. SUBCULTURE OF MESENCHYMAL STEM CELLS

The medium was removed and the cells were washed with PBS. They were incubated with trypsin-EDTA solution (1X) and incubated for 5 minutes. After the time period 5 ml of fresh culture medium was added and the whole cell suspension from a 25 cm^2 flask is equally transferred to two 225 cm^2 flask and supplemented with 45 ml culture medium. On attaining confluence the cells were trypsinized and used for all the experiments.

2.9.3. CHARACTERIZATION OF MESENCHYMAL STEM CELLS

The mesenchymal stem cells were characterized for their typical markers like vimentin and CD105 by immunocytochemistry and imaged using fluorescent microscopy. Briefly the mesenchymal stem cells from passage 1 was seeded on to two sets of cover slips and cultured to semi confluence in αMEM. One set of cells was used for immunostaining for vimentin and the other set was used for CD105

following the same immunostaining protocol except for the use of different primary antibodies.

The medium was removed and fixed in 3.7% formaldehyde in PBS and incubated for 15 minutes at RT and permeabilized with ice cold methanol for 20 minutes at -20°C. After blocking the nonspecific sites with 2% BSA, cells were stained with primary anti Vimentin-FITC mouse monoclonal antibody (1: 100) dilution in antibody diluent for 2 hours at 37°C in dark. The second set of cells were incubated with primary anti Endoglin (CD105) mouse monoclonal-Phycoerythrin (PE) conjugated antibody (1: 100) dilution in antibody diluent for 2 hours at 37°C in dark and further washed with 2% BSA. To visualize nuclei and DNA, cells were stained with Hoechst (0.5μg/ml) for 15 minutes in the dark. Cells were washed with 1X PBS after all incubations. All the cover slips were mounted with fluorescent mounting media and were examined under a flourescent microscope (Leica DM 4000 M). The images analyzed with Leica application suite software.

2.9.4. EFFECT OF SEEDING TECHNIQUE IN DISTRIBUTION OF CELLS ON 3D SCAFFOLDS

An appropriate seeding methodology has to be adopted depending on the 3D morphology of the scaffolds. The seeding methodology plays a very important role in providing a uniform deposition of cells and secretion of extra cellular matrix molecules in the 3D scaffolds. This was evaluated by seeding mesenchymal stem cells on to PVA-PCL and GA scaffolds at a density of $1x10^5$ cells/ scaffold using two different techniques viz, static and dynamic seeding.

In static seeding method, the cells were resuspended in 0.5 ml of culture media and the cell suspension was dropped on to upper and lower surfaces of the scaffold in equal volumes with 3 hours incubation in between after seeding on each

surface. In dynamic seeding method, the scaffolds were placed in polypropylene tubes. The cells are resuspended at a density of 1x 10^5 cells /ml of culture medium (αMEM) containing 15 % FBS and 1% antibiotic solution. The cell suspension was added on to the scaffold. Further the tubes were placed on to shaker and continuously stirred at 300 rpm for 6 hours with incubation at 37°C and 5% CO_2.

After 6 hours of incubation the cells seeded constructs by static and dynamic methods were retrieved and washed with PBS to remove the unattached cells and processed for scanning electron microscopy, live dead assay and Hoechst staining.

2.9.4.1 EVALUATION OF PATTERN OF CELL DISTRIBUTION BY SEM

For scanning electron microscopy analysis, the cell seeded constructs were washed in PBS and fixed with 2.5% gluteraldehyde overnight at 4°C, followed by dehydration with a series of alcohol (50%, 70%, 80%, 90% and 100%) and finally in ter-butyl alcohol for 10 minutes. The samples were subjected to critical point drying and coated with Palladium for 30 seconds. After the coating the constructs were observed on scanning electron microscope (S-2380-N, Hitachi, Japan).

2.9.4.2. VIABILITY OF MSCs ON 3D SCAFFOLDS

The viability of cells in the construct was evaluated using Live Dead assay kit (Molecular probes) and further imaged using confocal microscopy. The constructs were washed with PBS and incubated for 10 minutes with staining solution in the kit containing 2μM Calcein AM and 4μM ethidium bromide (EtBr) in dark. After incubation the constructs were washed in PBS to remove the excess amount of dye and observed under confocal microscopy (Olympus) at ex/em 495/515nm (green) and ex/em 495/635 nm (red). The confocal images were taken at different planes are combined using z stacking and represented as a single image.

2.9.4.3. CELL DISTRIBUTION BY HOECHST STAINING

The over all distribution of cells seeded by dynamic method was detected using Hoechst dye. The constructs were washed with PBS and incubated in 0.5 ml of staining solution (0.5μg/ml) for 5 minutes. The constructs were washed with PBS to remove the traces of unbound dye and observed under confocal microscopy at an excitation wavelength (λ) at 365 nm and emission (λ) at 465 nm.

2.10. EFFECT OF GROWTH FACTORS ON STEM CELL DIFFERENTIATION TO CHONDROCYTES IN 3D SCAFFOLDS

The role of growth factors in the differentiation of adult bone marrow mesenchymal stem cells to chondrocytes in 3D scaffolds was evaluated by seeding mesenchymal stem cells on to both PVA-PCL and GA scaffolds and culturing them in presence of growth factors supplied individually and in combinations. The differentiation process was initiated in presence of growth factors belonging to TGF beta super family such as TGFβ1, TGFβ3 and BMP2 supplemented individually and in two different combinations. The constructs were cultured for a period of one month and evaluated for the presence of chondrogenic markers.

2.10.1.SEEDING OF MESENCHYMAL STEM CELLS ON 3D SCAFFOLDS

Mesenchymal cells were isolated from bone marrow of 2 week old male Wistar rats as described previously in section (2.9.1.) .The cells were seeded on to 3D PVA-PCL and GA scaffolds of 10 mm diameter and 3 mm thickness at a density of 5 x 10^5 cells /scaffold. For obtaining sufficient number of mesenchymal stem cells for the whole experiments, cells were isolated from 6 rats simultaneously and cultured. On attaining confluence these cells are pooled together and used for seeding using dynamic seeding protocol. For seeding, the scaffolds were placed in polypropylene tubes and the cell suspension in αMEM

containing 15 % FBS was added and placed in orbital shaker at 300 rpm for 6 hours. After 6 hours the constructs were supplied with fresh culture medium for 24 hours. Further the medium was replaced with chondrogenic medium containing respective growth factors. Chondrogenic medium consisted of DMEM, 10 % FBS, antibiotic-antimycotic solution (1X), 50μM Proline, 37μg/ml ascorbic acid, 1% NEAA and 10^{-7} M dexamethasone. The constructs were grown in different growth factor conditions supplemented with Recombinant Human transforming growth factor β1 (TGFβ1) (20ng/ml), Recombinant Human Transforming growth factor β3 (TGFβ3) (10ng/ml), Bone morphogenetic protein (BMP2) (25ng/ml), TGFβ1+TGFβ3 (20ng/ml, 10ng/ml), TGFβ3+BMP2 (10ng/ml, 25ng/ml) and control without any growth factors respectively. The constructs were maintained in dynamic culture at 50 rpm in different growth factor supplemented conditions for 28 days. Medium was replaced every 3 days. There were 9 samples per group.

2.10.2. EVALUATION OF CHONDROGENESIS

After 28 days the constructs were retrieved from culture and processed for various analyses. Three samples were used separately each for biochemical and gene expression analysis. The other three samples from each group were used for evaluating overall cell distribution and morphology with scanning electron microscopy. The viability of cells in PVA-PCL scaffold was evaluated using Live dead assay and imaged using confocal microscopy. For comparison of data from scanning electron microscopy and live dead assay the construct was cut into two pieces with one half used for SEM and the other half processed for live dead assay. The presence of glycosaminoglycan and collagen type II secretion through out the construct was estimated by staining the sections of construct using safranin O staining and immunostaining using DAB respectively.

2.10.3.MORPHOLOGY & DISTRIBUTION OF CELLS IN DIFFERENTIATED CONSTRUCTS

For scanning electron microscopy observations, the constructs cultured for 28 days were washed in PBS and fixed by 2.5% gluteraldehyde solution, followed by dehydration, with a series of alcohol and critical point dried. After gold coating the constructs were observed on scanning electron microscope (S-2380-N, Hitachi, Japan).

2.10.4. VIABILITY OFCELLS

The viability of cells in the construct was evaluated using Live dead assay. The constructs were processed for live dead assay as described previously in section (2.7.2) and further imaged using confocal microscopy. The confocal images were taken at different planes are combined using z stacking and represented as a single image.

2.10.5.ESTIMATION OF TOTAL GLYCOSAMINOGLYCANS

For biochemical evaluations the constructs were digested using papain as described in section (2.8.2.1) and the supernatant was used for the estimation of total glycosaminoglycans. The total glycosaminoglycan content by DMMB assay as described in section (2.8.2.2) and the data was represented graphically.

2.10.6.ESTIMATION OF TOTAL COLLAGEN

The total collagen, content was estimated using Sircoll (Sirius Red kit) (R & D systems) as per the protocol provided by the manufacturer. Briefly standards were prepared in 0.5 M acetic acid to generate a calibration curve of collagen concentration versus optical density using the collagen standard available in the kit. To 200 µl of standards and samples in 0.5 M acetic acid, 500 µl of Sircoll reagent was added and vortexed for 30 minutes. The solution was centrifuged at 10,000 rpm for 10 minutes at 4°C and supernatant was decanted completely. The

pellet was dissolved in 0.5 ml of the alkali reagent and optical density was measured at 540 nm. The concentration of total collagen in the sample was calculated from the calibration plot of the standards. The results of total collagen content in different growth factor supplemented conditions were represented graphically.

2.10.7. DETECTION OF GLYCOSAMINOGLYCAN DEPOSITION IN SECTIONS OF THE CONSTRUCTS

The deposition of glycosaminoglycans in the sections of constructs grown in different growth factor supplemented conditions was evaluated by Safranin O staining. The constructs were fixed, embedded in paraffin and processed for staining as discussed in section (2.8.3.1) and (2.8.3.2).

2.10.8.IMMUNOSTAINING FOR TYPE II COLLAGEN

For immunostaining the paraffin sections of the constructs were processed as per protocol described in section (2.8.3.1). Immunostaining was performed using (SAB PO(R) kit, histofine, Japan) as per manufacturer's protocol. Briefly for antigen retrieval, the sections were treated with 0.005% protease (pronase from Streptomyces griseus, Wako) in 0.5 M Tris HCL, pH 7.6 and incubated at 37°C for 30 minutes. After blocking the internal peroxidase using 30% H_2O_2 in methanol for 30 minutes, sections were washed with PBS. Sections were further blocked with blocking solution in the kit for 10 minutes and incubated with mouse monoclonal anti collagen type II antibody (1:250) dilution overnight at 4°C. Unbound antibodies were washed with PBS and treated with secondary antibody for 30 minutes at RT. After PBS wash the sections were incubated with Streptavidin-HRP for 30 minutes at RT. The sections were further treated with DAB chromogen working solution, counterstained using hematoxylin, dehydrated and mounted. The collagen type II deposition in the sections of different growth factor supplemented constructs was imaged using microscope. (Leica)

2.10.9. GENE EXPRESSION ANALYSIS BY REAL TIME PCR

The collagen type II gene expression in different growth factor supplemented constructs was estimated by Real Time PCR. The gene expression analysis involves 3 stages as described below.

a) Isolation of total RNA

b) Synthesis of cDNA

c) Amplification of cDNA using PCR

a) Isolation of total RNA

The total RNA was first isolated from the constructs using a Midi kit Qiagen column as per the manufacture's protocol. The 28 day constructs were processed as per the following steps. Briefly constructs were incubated in 1 ml RLT buffer containing 10 µl β-mercaptoethanol, homogenized at 7000 rpm using ultrasonicator and centrifuged at 5000g at 4°C for 10 minutes. 2 ml 70% ethanol to supernatant was added and mixed well. The whole contents were transferred to Midikit column & centrifuged at 5000g at 20°C for 5 minutes. Further 4 ml of RW1 buffer was added & centrifuged at 5000g at 20°C for 5 minutes. Added 2.5 ml of RPE buffer & centrifuged at 5000g at 20°C for 5 minutes. Finally the RNA bound to the column was eluted using RNase free water. An aliquot of RNA solution was used to determine the concentration and the purity of RNA spectrophotometrically. The RNA was stored at -90°C and converted to cDNA.

b) First cDNA Strand Synthesis

The RNA was reverse transcribed to cDNA using standard conditions. Total RNA was reverse transcribed in a 20µl reaction volume using Superscript II reverse transcriptase. The reaction mix contained RNA, oligodT, dNTP were incubated at 65°C for 5 minutes. Further after the addition of 5Xbuffer, DTT and

RNase out the reaction mixture was incubated at 42°C for 2 minutes. After the addition of superscript II reverse transcriptase, the incubation was carried out at 42°C for 50 minutes and 70°C for 15 minutes. Further the newly formed strand is separated from the RNA by incubating with RNase H at 37°C for 20 minutes. The reaction volume and the incubation steps were as follows.

Step 1

RNA	10µl
OligodT	1µl
dNTP Mix (10mM)	1µl
Heat at 65°C for 5 minutes	

Step 2

5X buffer	4µl
DTT	2µl
RNase out (40U)	1µl
Heat at 42°C for 2 minutes	

Step 3

Superscript II RT (200U)	1µl
Incubate at 42°C for 50 minutes	
Incubate at 70°C for 15 minutes	

Step 4

RNase H (2U)	1µl
Incubate at 37°C for 20 minutes	
Stored at -20°C	

c) Amplification of cDNA using PCR

For cDNA amplification, the cDNA and primers were incubated in presence of SYBR@Green PCR Master Mix (Applied Biosystems). Samples were amplified using specific primers to type II collagen. The GADPH was used as the control gene. The specific primers for rat type II collagen and glyceraldehyde phosphate dehydrogenase (GADPH) (control gene) were designed using AB express software. The primer sequences are represented in table 2.The amplification was carried out in real time PCR machine (Applied Biosystems 7500).The reaction volume and incubation temperatures were as follows

PCR Master Mix (25µl)

SYBR Green PCR mix	12.5µl
DDW	7.5µl
Forward Primer (10µM)	1µl
Reverse Primer (10µM)	1µl

Reaction Conditions

Incubate at 50°C for 2 minutes

Incubate at 95°C for 10 minutes

Incubate at 94°C for 15 seconds

Incubate at 57°C for 15 seconds

Incubate at 72°C for 1minute

Number of cycles 40

The relative gene expression for type II collagen in various samples was calculated by normalizing the values to the corresponding GADPH values which

was further normalized to the control sample (construct grown in chondrogenic medium without the growth factors).

Table 2. Primer Sequences

GADPH	
Forward Primers	**5' TGACTCTACCCACGGCAAGTT3'**
Reverse Primers	**5'ATGGCATGGACTGTGGTCAT3'**
Collagen Type II	
Forward Primers	**5'TCCTCCGTCTACTGTCCACTGA3'**
Reverse Primers	**5'GTCCACACCAAATTCCTGATCA3'**

Chapter 3
Results & Discussion

3.1. SECTION 1

FABRICATION AND CHARACTERIZATION OF 3D POROUS SCAFFOLDS

3.1.1. EXTRA CELLULAR MATRIX AND 3 DIMENSIONAL SCAFFOLDS

Extra cellular matrix (ECM) is the natural medium in which cells proliferate, differentiate and migrate, and therefore is the gold standard for tissue regeneration. (*Meredith et al 1993, Bosman et al 2003*) Cells continuously interact with ECM and the crosstalk between cells and ECM is essential for tissue formation, organ development and repair. Natural ECM is a condensed matrix mainly composed of locally secreted proteins and polysaccharides, arranged as a molecular network or an agglomerate of gels interconnecting cells with matrix proteins. Tissue engineering aims to create biological substitutes that might restore, maintain or improve tissue functions. For a successful tissue engineering approach, an appropriate cell source must be identified, isolated and amplified on 3D-scaffolds. A suitable synthetic or natural material to be used as a substrate should be chosen and fabricated in the desired shape and dimension. (*Hutmacher et al 2001*) A tissue engineering scaffold serves as a temporary ECM, which support cells and enhance the subsequent 3D tissue regeneration. Thus, a critical step in this process is the mimicking of some ECM characteristics, to provide cells, with an adequate mechanical stability and biological environment for tissue growth and integration. (*Langer 2000*).

3.1.2. SELECTION OF POLYMERS

The 3D scaffolds play a very important role in regulating cell adhesion and the production of extra cellular matrix molecules in invitro regeneration of cartilage. The quality of the invitro developed construct is partly determined by the structure and the chemical composition of the supporting matrix on which the

chondrocytes are cultured. The viability of cells seeded on to the scaffold and its chondrogenic response is influenced by the cell-material interactions. Natural polymers are widely explored to serve as 3D scaffolds due to their biomimetic nature which provides an environment favorable for cell adhesion and subsequent events. Even though cell friendly, there are some concerns regarding their animal origin and risk of disease transmission. Synthetic polymers have the advantages that they have reproducible properties which can be tailored according to our requirements. The physico-chemical properties like mechanical stability, swelling behavior and rate of degradation can be varied according to the applications. Moreover the synthetic polymers show less antigenic properties when compared to natural polymers and the risk of disease transmission can be avoided completely. Even though less biomimetic, they may be functionally modified with suitable ligands to enhance the biomimeticity. Concerns about cartilage tissue viability, long term durability, retention of chondrocyte morphology, and mechanical stability have prompted the present work to look into the effect of scaffold in invitro regeneration of cartilage tissue. The effect of chemical composition of the scaffolding material on chondrogenic response is evaluated in scaffolds fabricated from both natural and synthetic polymers in this study. The scaffold made from natural polymers used for the study is a blend of gelatin and albumin (GA) and synthetic scaffold is a semi Inter penetrating polymer network of poly (vinyl alcohol) and poly (caprolactone) (Semi IPN of PVA-PCL).

3.1.3. POLY (VINYL ALCOHOL) AND POLY (CAPROLACTONE)

The synthetic polymer used to fabricate 3D scaffold is a Semi IPN of synthetic polymers, poly (vinyl alcohol) and poly (caprolactone). The synthetic polymers have the advantage that their properties are finely tunable according to our requirements. The selection of synthetic polymers was made with a view of mimicking the properties of native components of the cartilage. The extra cellular matrix of cartilage consists of collagen and glycosaminoglycans with the collagen

providing mechanical strength to cartilage, while the glycosaminoglycans with a larger amount of water retention capacity helping to resist compression. The PCL component of the scaffold is expected to provide the mechanical stability to the scaffold while PVA with its high water retention capacity is expected to behave like the glycosaminoglycans. These are biodegradable and biocompatible polymers and have nontoxic degradation products.

PCL is an FDA approved polymer and is now one of the most widely preferred polyester for scaffold fabrication in tissue regeneration, particularly for load bearing applications. Polyesters like poly (lactides), poly (glycolic acid) and their blends are widely used for scaffold fabrication. Even though they are found to be good for many cells, use of these materials for long term applications like cartilage regeneration is not advisable. Studies have shown that local accumulation of acidic degradation products around the implant site produce remarkable change in pH and may affect the viability of cells surrounding the implant. (*Athanasiou et al 1996*) The low melting point of PCL allows for easy processing. Pure PCL shows a degradation time of 2 years. The long degradation time of PCL is advantageous as it provides sufficient time for the cells to secrete the new extracellular matrix. One of the limiting factors of PCL is its extreme hydrophobicity and its inability to absorb large amount of medium. The limitations of PCL are overcome with the addition of polyvinyl alcohol, a highly hydrophilic polymer.

PVA is also an FDA approved polymer that has been widely used for many biomedical applications. Hydrogels of PVA have been used as an artificial cartilage. (*Noguchi et al 2004*) A two year study on use of PVA hydrogels as artificial meniscus proves its excellent biocompatibility. (*Kobayashi et al 2005*) Another study reports the use of polyvinyl alcohol hydrogel as a biocompatible viscoelastic material with mechanical property comparable to that of native cartilage (*Grant et al 2006*). Moreover PVA has a larger number of hydroxyl (-

OH) groups that can form hydrogen bonds both in inter and intra chains to form a mesh like structure. Free hydroxyl groups are also available for modification with peptides if required. PVA retain properties of a hdrogel that provide numerous advantages like high tissue like water content, promotes efficient transport of nutrients and waste and have powerful ability to effectively and uniformly encapsulate cells. (*Hoffman et al 2002*) A low molecular weight PVA with Mw 12,000-23,000 is used for this study. Several previous studies on animals have shown that low molecular weight of PVA is completely excreted from body through urine and there is no accumulation of the degradation products in any internal organs that could produce any detrimental effects.

3.1.4. GELATIN AND ALBUMIN

A novel 3D Gelatin-Albumin scaffold is fabricated to recreate an artificial structure analogous to extracellular matrix of cartilage. Gelatin has a structure similar to collagen and retains many of the biomimetic features to promote cell adhesion and secretion of extra cellular matrix production. Gelatin is highly hydrophilic and can hold a large amount of water similar to the proteoglycans in cartilage. Gelatin also serves as a nontoxic biodegradable substrate for invitro regeneration of tissue. (*Kang et al 1999*) Gelatin and their blends have been widely used for drug delivery (*Adhirajan et al 2007, Liu et al 2007, Pal et al 2007*) and as cell carriers for tissue engineering applications of soft tissues (*Liu et al 2007, Mao et al 2003*) . Hydrogels of gelatin are not suitable for long term applications due to its extensive swelling and lack of stability in culture medium.

Albumin is proposed to serve as a nontoxic foaming agent and helped to attain a hydrophobic-hydrophilic balance. Albumin from egg white has been reported earlier to serve as a foaming and consolidating agent for the design of smart porous bioceramics. (*Berthold et al 2007, Bhattacharjee et al 2007*) The addition of small amount of albumin is expected to reduce the extensive hydrophilicity of gelatin and is likely to influence the physicochemical properties

of the hybrid scaffold. There are previous reports that hydrophobic interactions govern the protein adsorption and very high hydrophilic surfaces favour biocompatibility due to preferential adsorption of albumin, which firmly binds to hydrophilic surfaces in high concentration. (*Alves et al 2007*). A common assumption in biomaterials research is that cellular interactions with natural and artificial surfaces are mediated through adsorbed proteins. The extensive hydrophilic nature of gelatin may not promote attachment of cell adhesion proteins like fibronectin, vitronectin and laminin available in serum, which plays a major role in promoting cell attachment and gene expression. Thus addition of albumin and subsequent crosslinking during fabrication of porous scaffold is expected to enhance the overall stability of the gelatin scaffold by reducing the extensive hydrophilicity of native gelatin. Addition of albumin promotes better attachment of, cell adhesion promoting components, from serum on to the scaffold. The hybrid scaffold is expected to retain the biomimetic nature of gelatin, and albumin in the blend is expected to overcome the limitations of gelatin.

3.1.5. FABRICATION OF 3D POROUS SCAFFOLDS

In this study both PVA-PCL and Gelatin-Albumin, 3D porous scaffolds were fabricated by a similar process of uniform phase mixing, freeze drying and crosslinking technique. Freeze drying technique is the most commonly employed, conventional and cost effective technique used to produce 3D scaffolds with open interconnected pores. Several fabrication methods are currently employed to process synthetic and natural scaffold materials into porous structures. They include conventional fabrication techniques like solvent-casting and particulate leaching (*Mikos et al 1993a, 1994, 1996,* fibre bonding *(Mikos et al 1993b*), gas foaming *(Mooney et al 1996*) and the recent rapid prototyping techniques. (*Yeong et al, 2004*) Each of these techniques have several advantages and as well as limitations. The particulate leaching technique is suitable for controlling pore sizes by varying the particle size. Some of the limitations of this technique are formation

interstitial spaces. The ice crystals were then removed by freeze drying. The scaffolds were further stabilized by chemical crosslinking. Addition of crosslinker solution during the initial step of foaming may cause non uniform distribution of crosslinker solution and may result in uneven crosslinking of the scaffold. Hence crosslinking was carried out after forming the 3D porous structure. The simple freeze drying technique allowed fabrication of scaffold in any required shape and structure according to the application. The general scheme of fabrication methodology is represented in Fig 5.

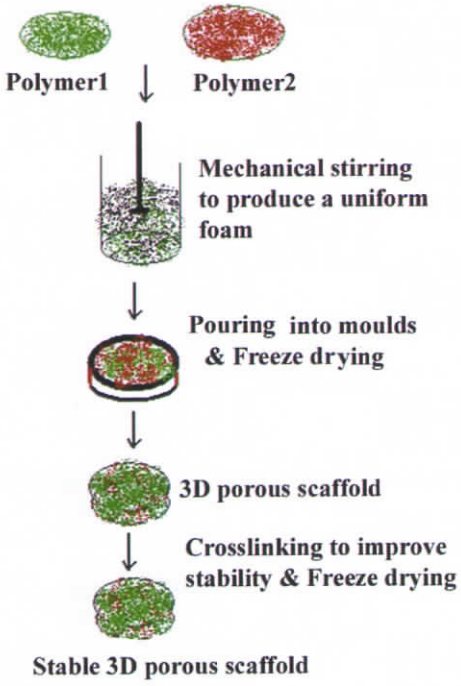


Fig 5. Schematic representation of the fabrication methodology

3.1.6. FABRICATION OF 3D POROUS SEMI IPN OF PVA-PCL

A Semi IPN of the two polymers was fabricated to produce a structure similar to the extracellular matrix of cartilage, which is a Semi interpenetrating polymer network of collagen and glycosaminoglycans. More over the Semi IPN

of closed pores without any interconnectivity and retention of residual particles/salts which are harmful to the cells. Further more, the formation of skin layer and agglomeration of salt particles makes controlling of pore size difficult. *(Hutmacher, 2001)* The gas foaming technique also results in closed cellular structures with only 10-30% of interconnected pores (*Mooney et al 1996*) in the scaffold which is inadequate for cell seeding and diffusion of metabolites. Bonding of nonwoven fibre meshes to form porous structures have poor mechanical integrity. The more advanced techniques of scaffold fabrication like fused deposition modeling employ higher temperatures ($\geq$ 120°C), which exclude the incorporation of biological molecules during the process.(*Sachlos et al 2003*) The freeze drying technique on the other hand permits incorporation of some biological active system into the scaffold during the fabrication process. Among the conventional techniques, freeze drying is hence the more appropriate and widely employed method for scaffold fabrication. Even though the freeze drying method of fabrication does not permit precise control over the pore structure as in rapid prototying and solid freeform fabrication techniques, the porosity and pore size can be controlled to a certain extent by varying some of the parameters like stirring speed, freezing temperature and crosslinking methodologies.

In this study scaffolds were fabricated by conventional simple process of foaming and freeze drying technique. A uniform foam with phase mixing of the two components in the hybrid scaffolds were produced by high speed stirring technique at specific rpm using mechanical stirrers. The shear caused by high speed stirring also introduces air bubbles in the emulsion which results in a porous structure. Sodium dodecyl sulphate (SDS), sodium lauryl sulphate (SLS) and Tween are the commonly used foaming agents/emulsifiers, but they are likely to be toxic to cells if they are not completely washed out from the system. Nontoxic emulsifiers, which are part of the scaffold components, served as emulsifiers in both the scaffolds used in this study. The immediate freezing of the uniform foam results in the formation of ice crystals that force and aggregate the polymer into the

interstitial spaces. The ice crystals were then removed by freeze drying. The scaffolds were further stabilized by chemical crosslinking. Addition of crosslinker solution during the initial step of foaming may cause non uniform distribution of crosslinker solution and may result in uneven crosslinking of the scaffold. Hence crosslinking was carried out after forming the 3D porous structure. The simple freeze drying technique allowed fabrication of scaffold in any required shape and structure according to the application. The general scheme of fabrication methodology is represented in Fig 5.

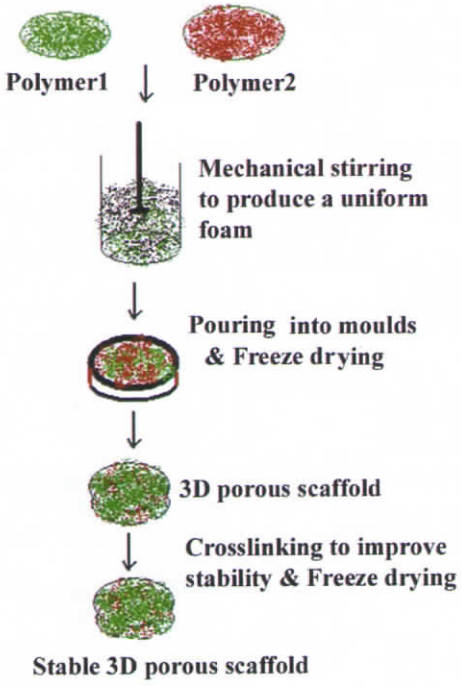


Fig 5. Schematic representation of the fabrication methodology

3.1.6. FABRICATION OF 3D POROUS SEMI IPN OF PVA-PCL

A Semi IPN of the two polymers was fabricated to produce a structure similar to the extracellular matrix of cartilage, which is a Semi interpenetrating polymer network of collagen and glycosaminoglycans. More over the Semi IPN

structure would keep the chains of both the polymers intermingled and this also is expected to provide mechanical stability to the system. The fabrication process involved three steps like formation of uniform foam, lyophilization and crosslinking to form the 3D porous Semi IPN scaffold.

Three different compositions were fabricated as discussed in section (2.1.2), in order to study the effect of concentration of parent material on the physicochemical properties of the scaffold as well as to select a suitable composition that would be appropriate for this application. The compositions were prepared by mixing the two polymers in the % wt ratio of PVA: PCL (30:70), (50:50) and (70:30).The compositions were selected within the range where visible phase separation was not observed.

The two polymers used in this study have widely differing polarities. PCL was dissolved in chloroform and PVA in water. To produce uniform foam with adequate phase mixing, chloroform in PCL solution was evaporated to produce a paste of PCL. The use of chloroform as a solvent enabled this process due to its high vaporizing ability. Further PVA solution was added to PCL and continuously stirred at 3000 rpm using a mechanical stirrer to produce the foam. PVA also served as a foaming agent and this helped to avoid the use of any toxic foaming agents like SLS or SDS in the system. The high speed stirring and technique of chloroform evaporation produced uniform foam with phase mixing which is immediately frozen to -70°C. The frozen structure was lyophilized to produce the 3D porous network of PVA and PCL. The freeze drying technique allowed fabrication of scaffold in different shapes and sizes. (Fig 6)

Further to make the system stable in aqueous solution and suitable for cell seeding, PVA was crosslinked. Hydrogels of PVA have been fabricated by cryogel method using freeze thaw cycles (*Hassan et al 2000*) which resulted in physical crosslinking of PVA, but this method will not yield a scaffolding structure which

can be used for load bearing applications. So a stable chemical crosslinking method using gluteraldehyde was adopted for our studies. Gluteraldehyde is an efficient chemical cross linker and is the only commercially viable process that has received wide spread acceptance. (*Khor et al 1997*)The concentration of gluteraldehyde was standardized to attain a balance between the stability and the size of porous structure of the scaffold as well as to avoid any toxicity. The crosslinking was carried in a mixture of water and isopropanol to avoid fast dissolution of polyvinyl alcohol in the crosslinking solution with HCl as a catalyst. Hydroxyl groups of PVA reacts with the aldehyde groups of the gluteraldehyde, only with the aid of protons (H^+) as catalyst. There are also previous reports that reducing the equilibrium water content of PVA hydrogels would improve the creep resistance, which can be attained by soaking in solvents like isopropyl alcohol. (*Choi et al 2007*) Gluteraldehyde crosslinking in isopropanol is expected to improve the mechanical stability of the Semi IPN. The scaffolds were washed with several changes of DDW to completely remove the traces of gluteraldehyde as well as isopropanol from the system and to avoid any toxic effects. Moreover being volatile, traces of isopropanol are easily removed from the scaffold during the next lyophilization process.

In the semi IPN, PCL is retained as the linear polymer while the PVA chains are partially crosslinked with gluteraldehyde resulting in chain entanglement. The semi interpenetrating polymer network of PVA-PCL is schematically represented in Fig 7.The crosslinking resulted in inter and intra chain bonding by covalent linkage of PVA by gluteraldehyde, within which the PCL chains are entangled. The crosslinking also prevented the phase separation of the two polymers resulting in a semi interpenetrating polymer network. The crosslinking with gluteraldehyde was optimized, so that all the free hydroxyl groups are not involved in crosslinking and the free hydroxyl (-OH) groups are available for holding the water molecules and chemical modification with peptides. The degree of crosslinking may affect the mechanical strength and hydrophilicity

of scaffold. Swelling characteristics and surface properties of PVA-PCL scaffold could be varied according to the degree of crosslinking. The gluteraldehyde cross linking of hydroxyl (-OH) group of PVA is illustrated in Fig 8.

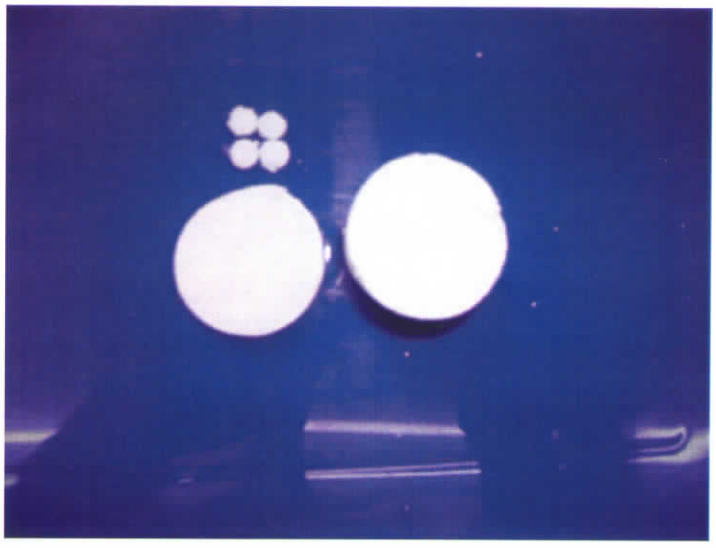


Fig 6. 3D porous discs of PVA-PCL scaffolds in different sizes

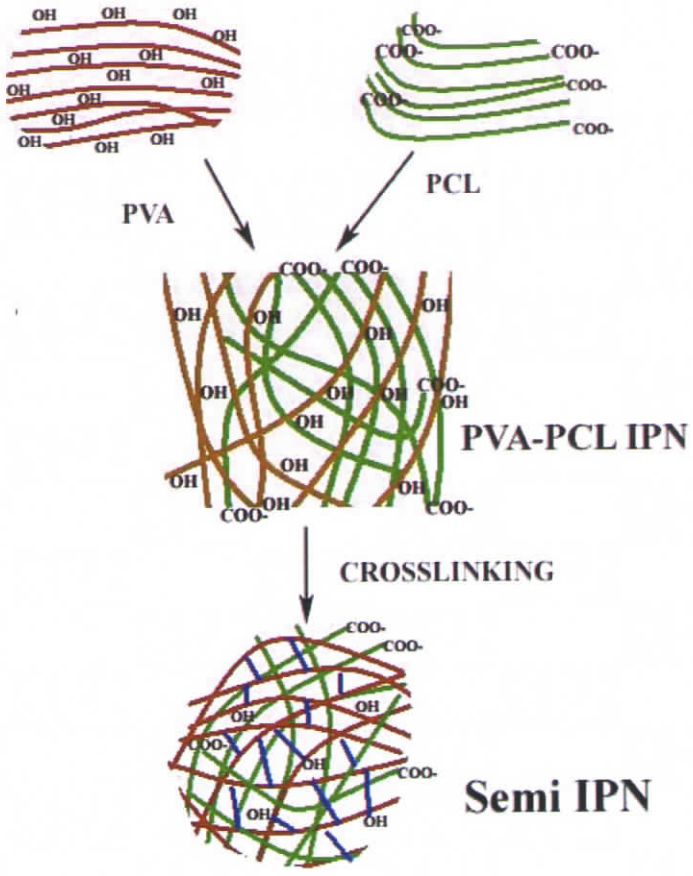


Fig 7. Schematic representation of PVA-PCL Semi IPN

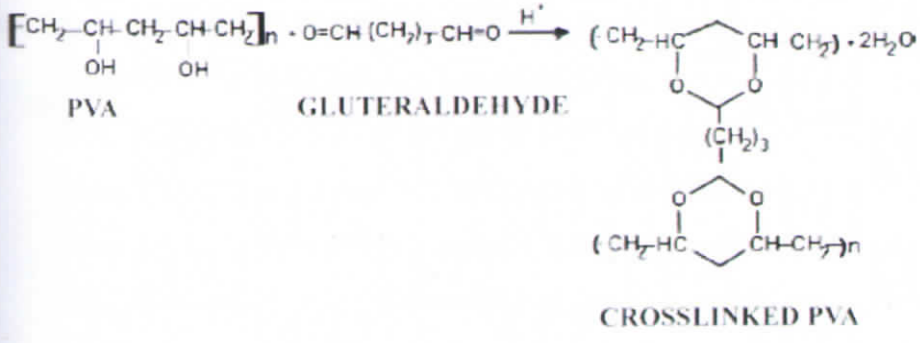


Fig 8. Gluteraldehyde crosslinking of PVA in the scaffold to form the Semi IPN

3.1.7. FABRICATION OF 3 D POROUS GELATIN-ALBUMIN SCAFFOLD

Porous 3D Gelatin-Albumin scaffolds were fabricated by freeze drying a uniform blend solution of natural polymers, gelatin and albumin in % weight ratio of 90:10. Albumin was added only at a low concentration, to serve as a nontoxic foaming agent and to produce a slight reduction in the hydrophilicity of gelatin. The high speed mechanical stirring with albumin, serving as a foaming agent helped to produce stable foam. Immediate freezing at low temperature and lyophilization at -70°C helped to produce 3D porous scaffolds. The scaffold was further processed to remain stable in culture medium for long period by crosslinking the free amino ($-NH_2$) and carboxyl (-COOH) groups in both gelatin and albumin using EDAC to form stable covalent peptide bonds. The choice of crosslinking agent was based on the reaction groups present on the amino acid side chain and appropriate ambient conditions that do not negatively affect the scaffold and its application. The protonated carbodiimide reacts with carboxylic functional groups of amino acids, aspartic acid and glutamic acid and forms an intermediate O-isoacylurea, which then undergoes nucleophilic attack by amine functional groups of lysine and hydroxylysine amino acids in the adjascent gelatin chains or the amino groups of albumin. The covalent peptide linkage was similar to that found in collagen fibres of cartilage. The crosslinking reaction is represented in Fig 9. Even though water-soluble EDC has been very useful as a crosslinking agent in biomedical application, it is known to easily lose its activity in aqueous solution. EDC crosslinking was carried out in acetone: water (90:10) mixture, to prevent the deactivation or eliminate the hydrolysis of carbodiimide activated derivative and there by to improve the crosslinking efficiency. Studies have also shown that EDAC crosslinking in organic solvent can be done effectively which minimize or inhibit the shrinkage of collagen scaffolds. (*Gordon et al 2004*) (Barnes et al 2007) Extensive washing was carried out with distilled water to remove any trace of unreacted EDAC, isourea byproduct and acetone in the crosslinking solution to avoid toxicity. The schematic representation of gelatin–albumin blend is

represented in Fig 10. The blending process, fabrication technique and the appropriate crosslinking methodology was expected to produce a 3D scaffold with adequate porosity, uniform pore morphology and enhanced stability. The scaffold was characterized for its physico-chemical properties and initial cytotoxicity. Further the suitability of the scaffold to serve as a matrix for chondrocyte culture and stem cell differentiation to chondrocytes was explored in this work.

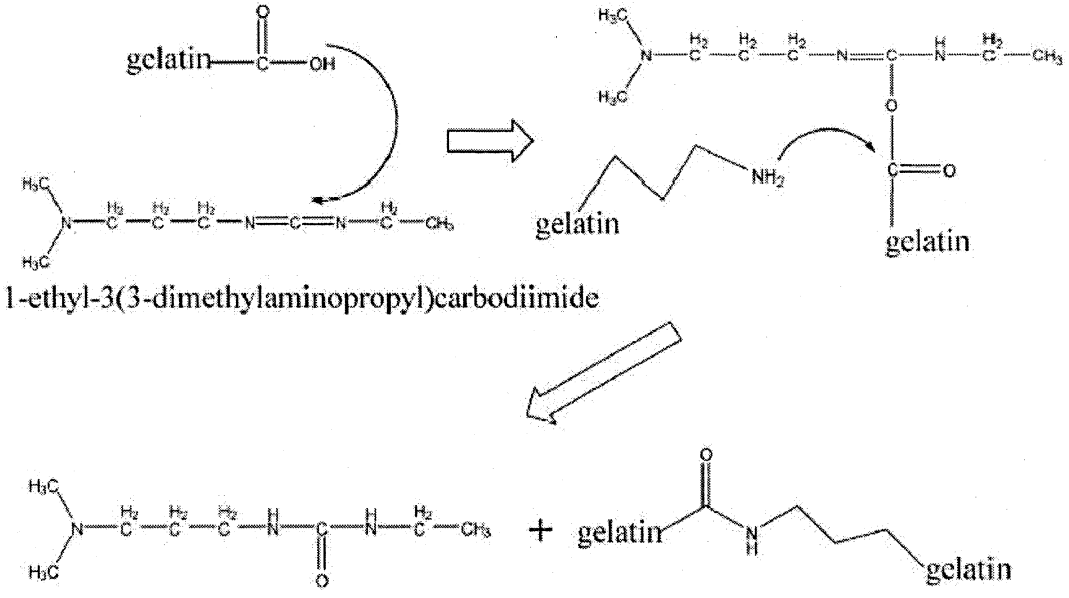


Fig 9. Peptide bond formation using EDAC crosslinking

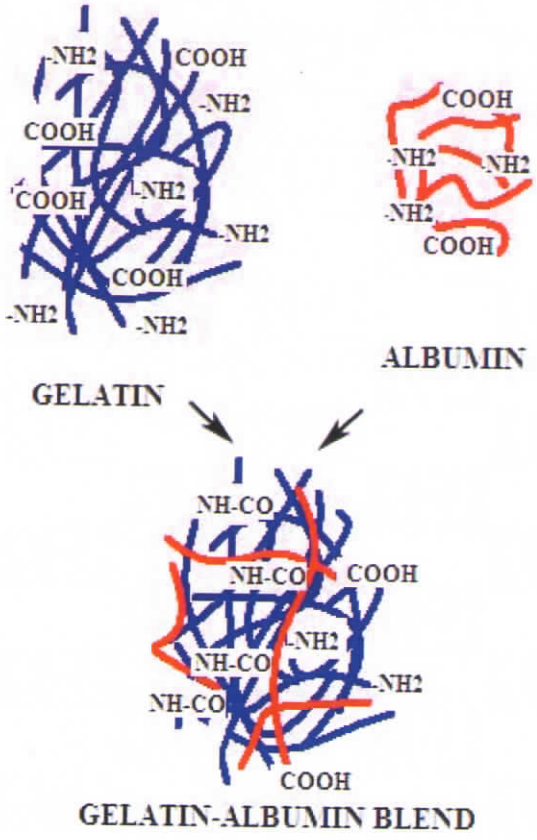


Fig 10. Schematic representation of gelatin and albumin in the blend

3.1.8. PHYSICOCHEMICAL CHARACTERIZATIONS OF 3D SCAFFOLDS

The 3D scaffolds were characterized for the physicochemical properties like thermal analysis, mechanical property, swelling ability, chemical composition, surface hydrophilicity, surface morphology and pore characteristics using various techniques.

3.1.8.1.THERMAL ANALYSIS

Thermal analysis comprises techniques in which the physical property of a substance is measured as a function of temperature when the substance is subjected

to a controlled temperature program. Whenever a material undergoes a change in physical state, such as melting or transitions, or whenever it reacts chemically, heat is absorbed or liberated. Temperature and energy transitions can be measured using differential scanning analysis (DSC) while the weight loss can be measured using thermogravimetric analysis (TGA).

3.1.8.1.1. PVA-PCL SEMI IPN

The TGA of virgin PVA, PCL, and the three hybrid scaffolds were compared in Fig 11. The parent materials and the three hybrid compositions were thermally stable at 37°C and degradation starts only at higher temperature. The thermal degradation is only 10% even at 200°C for PVA: PCL (50:50) and PVA: PCL (70:30) compositions. The degradation pattern is clearly observed in the derivative of TGA curve (DTG) shown in Fig 12. PVA have undergone a two step degradation process. The first step is initial dehydration, accompanied by the formation of some volatile products with polyene structures. The second step of degradation in PVA is due to further degradation of polyene residues to yield hydrocarbons. PCL showed single step degradation which appeared as a single peak at 401.90°C corresponding to polymer pyrolysis. All the hybrid compositions showed degradation patterns of both PVA and PCL which appeared as some additional peaks or shoulders in the DTG curve. As the concentration of PCL increased from 30 to 70% from PVA: PCL (70:30) to PVA: PCL (30:70) composition, the thermal degradation pattern also became similar to PCL. The single step degradation process of the entire hybrid scaffolds indicates that it is a miscible blend with co-continuous phases as is expected of the Semi-IPN. The results of this study also indicate the thermal stability of the material at 37°C, the temperature of end use of the scaffolding material.

The DSC curve (Fig 13) of PVA showed a melting peak at 230°C and that of PCL at 60°C. All the hybrid scaffolds showed two melting peaks in the DSC, indicating the presence of both PVA and PCL in these scaffolds. The intensity of

the melting peaks varied in different hybrid compositions. As the ratio of PVA to PCL varied from 30:70 to 70:30 in the hybrid compositions there was a variation in the intensity of melting endotherms of PCL and PVA which confirm the inversion of phases in the miscible blend. The thermograms also indicate that initially the PVA is the continuous phase with the PCL as the filler up to a composition of PVA:PCL (50:50), however further increase in PVA concentration causes an inversion of phases with the PCL tending to become the cocontinuous phase and PVA acting as the filler. The results of the thermal studies hence reveal that though there are different continuity of phases in specific compositions, the hybrid scaffolds are miscible blends of both the polymers at all their specific compositions. This inversion of phases was reflected in all the physicochemical properties measured.

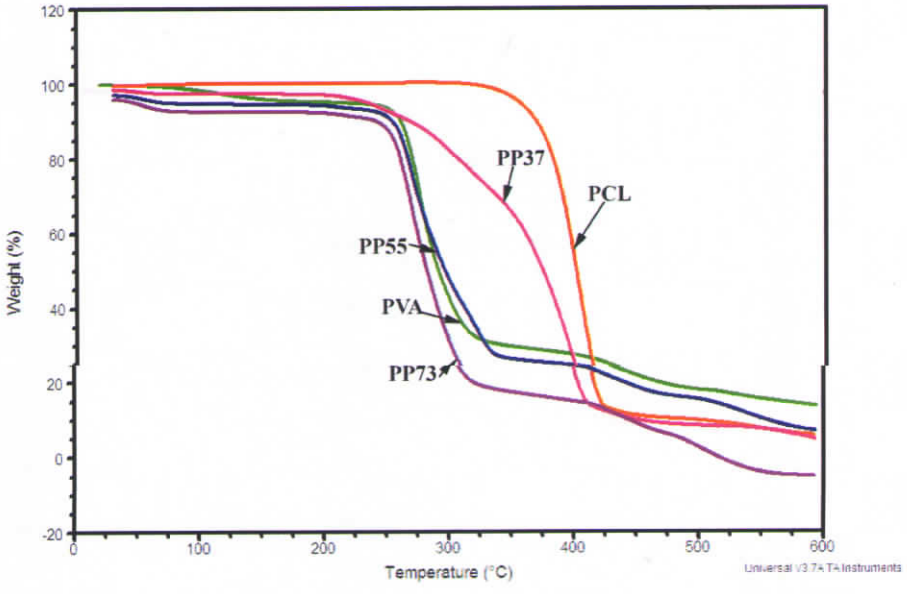


Fig 11. TGA of hybrid Semi IPN scaffolds compared to PVA and PCL

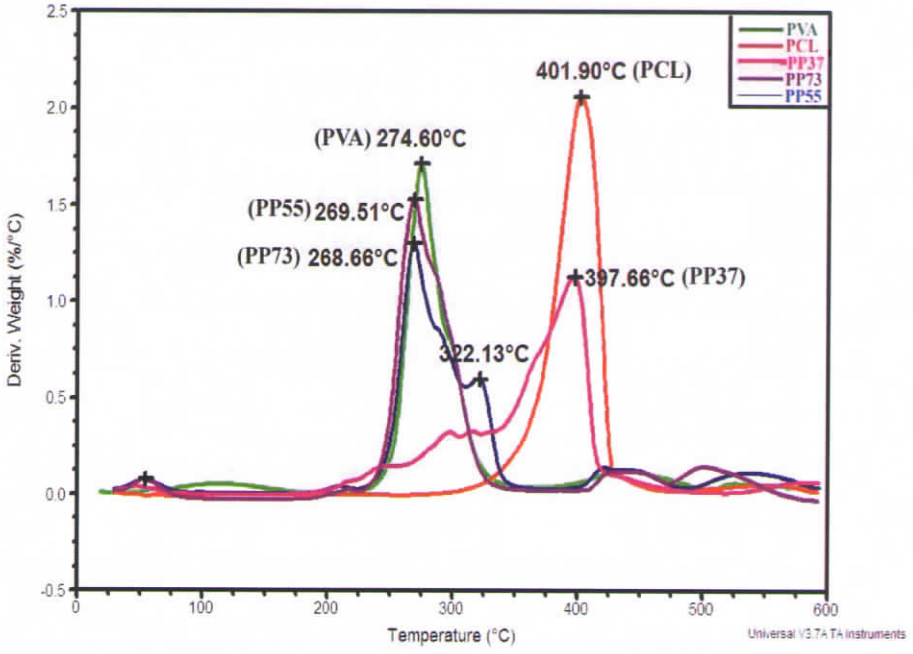


Fig 12. DTG curve of the Semi IPN scaffolds compared to PVA and PCL

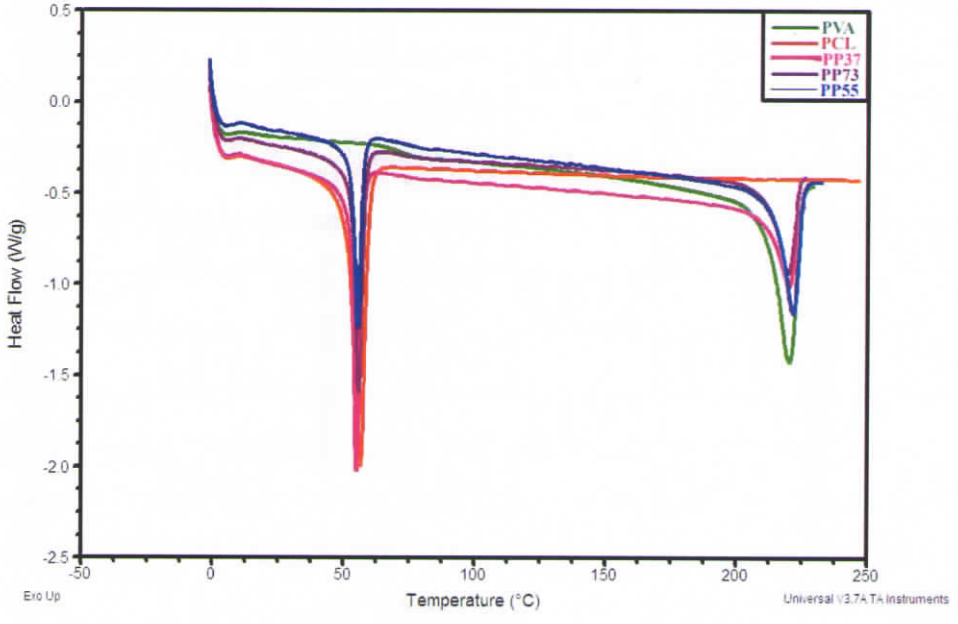


Fig 13. DSC curve of hybrid Semi IPN scaffolds compared to PVA and PCL

3.1.8.1.2. GELATIN-ALBUMIN

The thermal degradation pattern of the Gelatin-Albumin blend was compared to the parent materials in Fig 14. The hybrid scaffold as well as the parent materials was thermally stable at 37°C and showed a two step degradation as observed in DTG (Fig 15). The initial degradation in the hybrid composition at around 52.26°C is due to the loss of water bound to the polar groups in protein molecule. The second degradation is due to the break down of peptide bond. The thermal degradation pattern of hybrid scaffold was similar to that of native gelatin and the addition of little amount of albumin did not bring significant change in the degradation pattern. A similar thermal degradation pattern was observed for gelatin, albumin and the Gelatin–Albumin blend which could be due to the same chemical composition in all the proteins. The data reveals that the thermal degradation pattern of gelatin in the hybrid composition is not much affected with the addition of albumin.

The DSC curve of hybrid GA scaffold is compared to that of parent gelatin and albumin in Fig 16. The DSC curve of gelatin shows two sharp transitions at 12.37°C and 22.07°C. These observations were similar to the transitions previously reported by others. *(Kozlov et al 1983)* A peculiarity of gelatin as a biopolymer is its specific interaction with water which is different to that observed with synthetic hydrophilic polymers. This peculiarity governs the structural and physico-mechanical properties of gelatin in the solid state. (*Kozlov et al 1983*) These thermal transitions observed in gelatin are mainly because, the gelatin-water system is in a non equilibrium state and also due to desorption of water. Studies have shown that gelatin exhibit a large variety of supramolecular structures, from the simplest globular structure, typical of amorphous polymers, to a well developed fibrillar structure with various intermediate states. (*Kozlov et al 1983*) Gelatin exhibits a change in confirmations with varying temperatures. Albumin showed a single transition at 14.27°C. The hybrid compositions exhibited three thermal

transitions at 5.43°C, 16.41°C and 26.88°C respectively. The transitions were similar to normal gelatin samples showing that the gelatin retains many of its inherent physico-chemical properties even in presence of albumin in the blend.

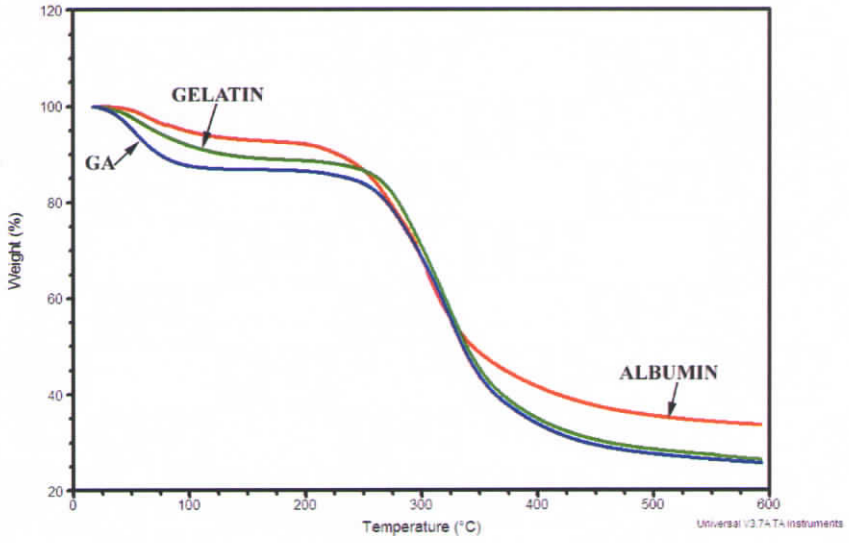


Fig 14. The TGA curve of Gelatin-Albumin compared to parent materials

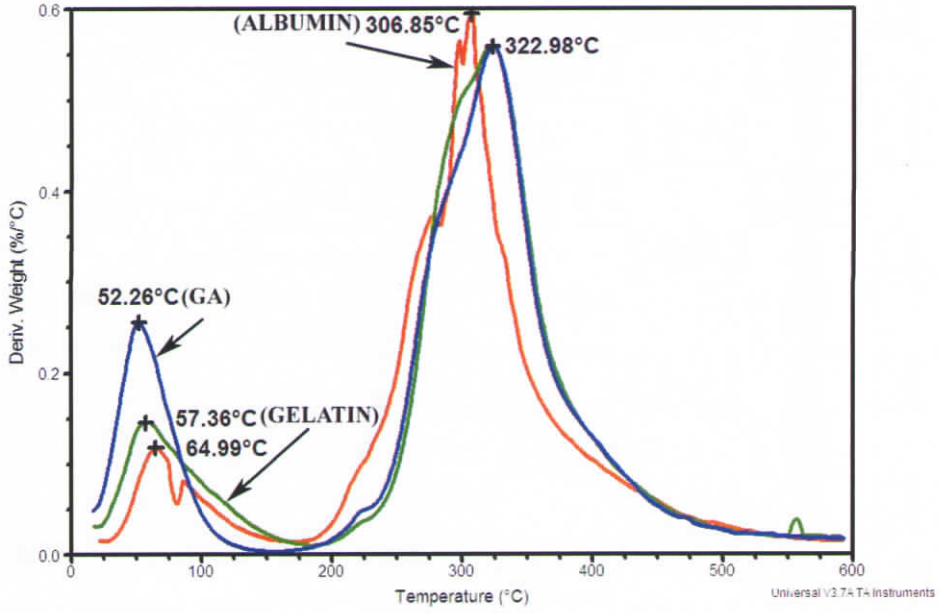


Fig 15. The DTG curve of Gelatin-Albumin compared to parent materials

transitions at 5.43°C, 16.41°C and 26.88°C respectively. The transitions were similar to normal gelatin samples showing that the gelatin retains many of its inherent physico-chemical properties even in presence of albumin in the blend.

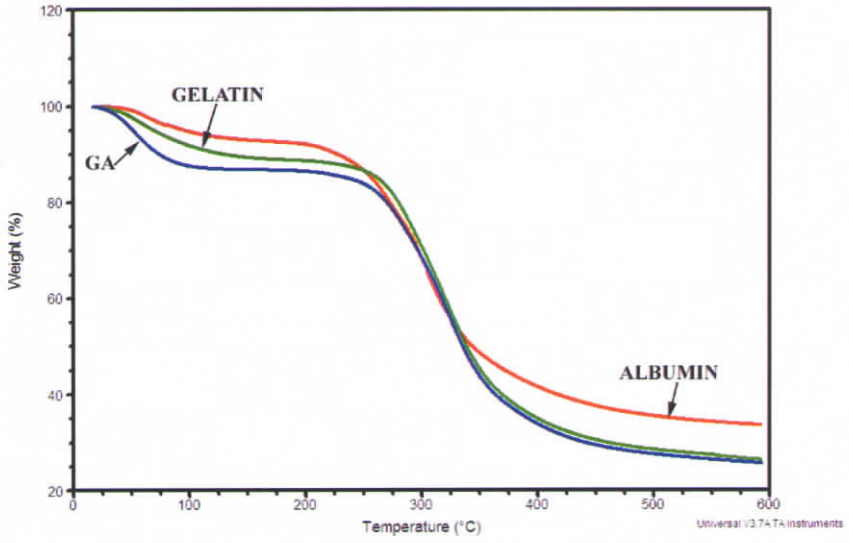


Fig 14. The TGA curve of Gelatin-Albumin compared to parent materials

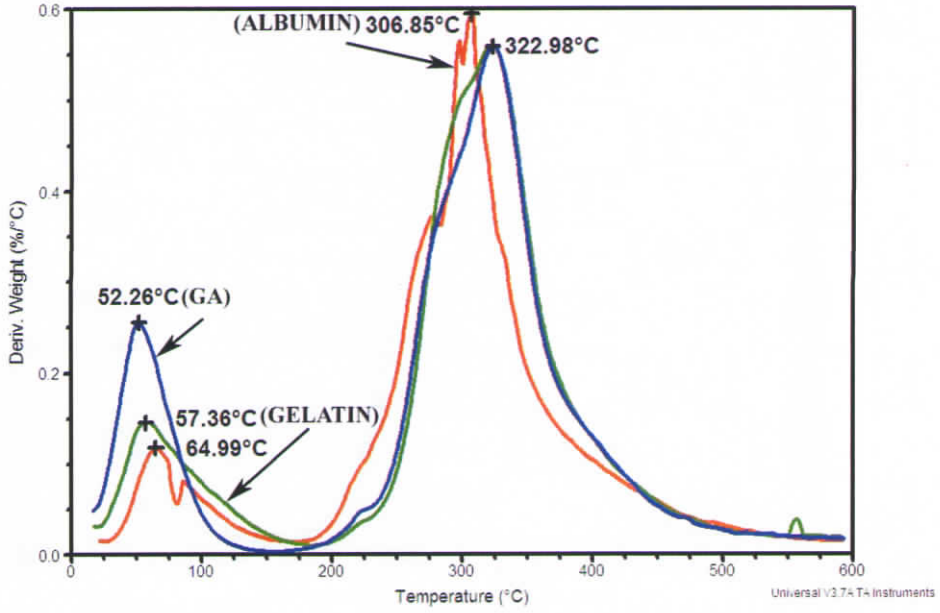


Fig 15. The DTG curve of Gelatin-Albumin compared to parent materials

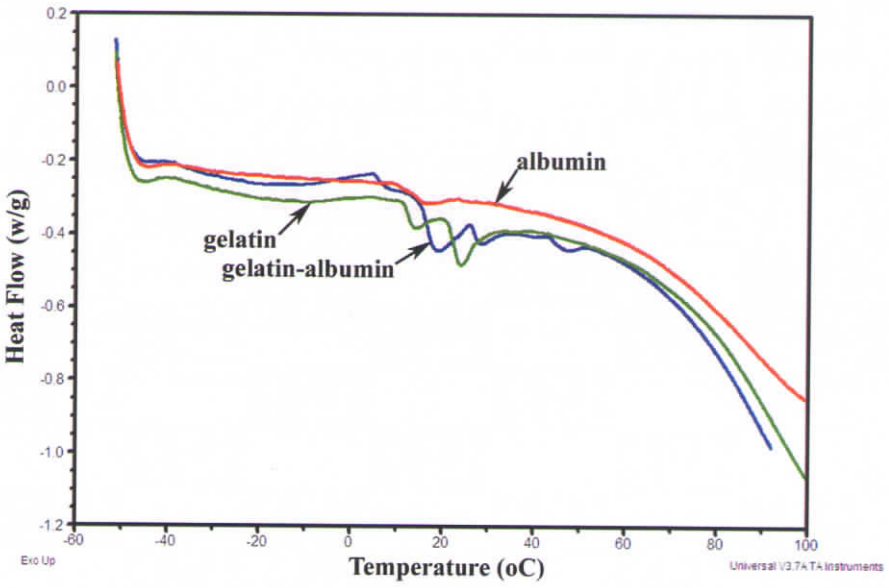


Fig 16. The DSC curve of Gelatin-Albumin compared to parent materials

3.1.8.2. FOURIER TRANSFORM IR SPECTROSCOPY

The FTIR spectrum of the scaffolds give information regarding the chemical composition of the scaffold which can be used to examine the identity of the polymers. Surface infrared spectroscopy couples the analytical method of IR spectroscopy with physical phenomenon of total internal reflection to enable the molecular vibrations within the surface regions of materials to be studied.

3.1.8.2.1. PVA-PCL

FTIR spectroscopic method is employed to understand chemical composition of the Semi IPN scaffolds. The spectra also give information regarding the free functional groups available in the polymer which can be used for functional modification. The FTIR spectrum of PVA and PCL is represented in Fig 17. The FTIR spectrum of PVA exhibits several bands characteristic of stretching and bending vibrations of O–H, C–H, C-C and C–O groups. A broad and strong band was observed at 3267 cm^{-1} corresponding to the hydroxyl (-O-H) stretching frequency, which indicate the presence of hydroxyl groups. The broad nature of the

band results from the hydrogen bonding of hydroxyl groups with PVA chains. The band observed at 2943 cm^{-1} indicates an asymmetry in stretching mode of $-CH_2$ groups. Two strong bands observed at 1413 and 839 cm^{-1} have been attributed to bending and stretching modes of $-CH_2$ groups, respectively. A weak band at 1326 cm^{-1} has been assigned to the combination frequency of (-CH + -OH) groups. The strong band at 1142 cm^{-1} and sharp band at 916 cm^{-1} have been attributed to the stretching mode of -CO and –C-C groups, respectively. The FTIR spectrum of PCL showed typical bands at 2941 cm^{-1} and 2863 cm^{-1} due to $-CH_2$ stretching vibrations and 1720 cm^{-1} is associated with carbonyl adsorption.

FTIR spectrum of the all the three compositions of 3D scaffolds showed the presence of characteristic peaks of poly (caprolactone) and poly (vinyl alcohol) (Fig 18). The absorption band at 1724 cm^{-1} corresponds to characteristic carbonyl stretching of polycaprolactone. The presence of an absorption band at 3330 cm^{-1} indicates the presence of free hydroxyl (-OH) groups, which is characteristic peak of PVA. These two characteristic peaks of polycaprolactone and PVA were present in all the three compositions of Semi IPN, confirming the presence of both the polymers in the Semi IPN. The presence of band at 3330 cm^{-1} corresponding to the hydroxyl (-OH) groups of PVA indicates that these functional groups were not completely masked by polycaprolactone and are available for further modification with amino acids that will favor better adhesion of chondrocytes to the scaffold. The broad nature of these peaks also suggests that the hydroxyl groups are extensively hydrogen bonded. The intensity of the peak at 1724 cm^{-1} due to carbonyl stretching of PCL increased with increasing concentration of PCL in different Semi IPN compositions.

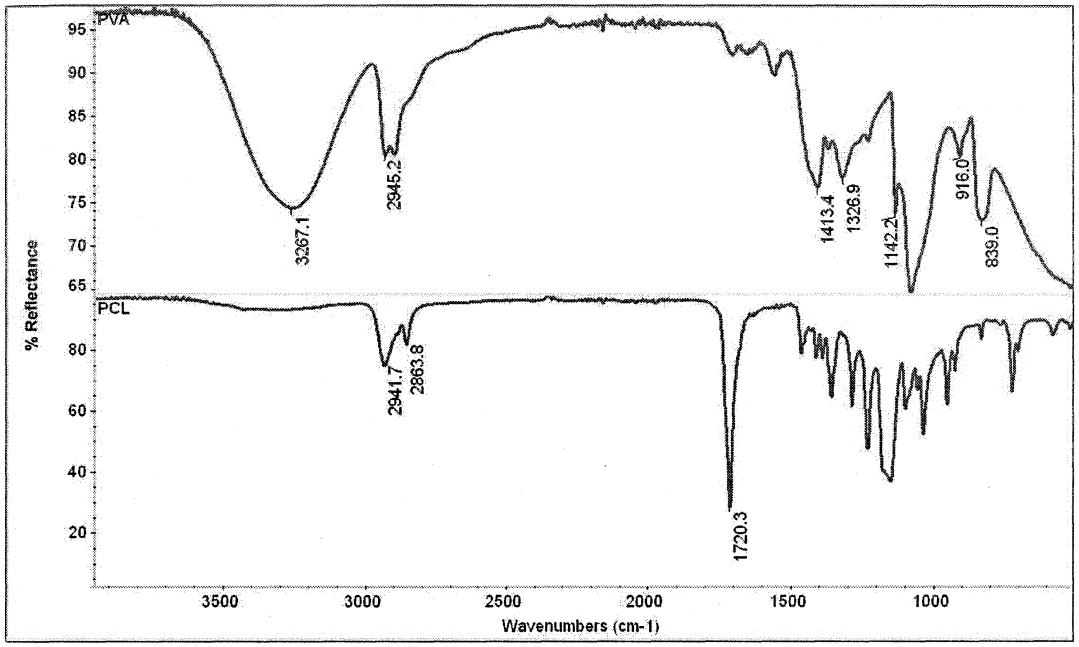


Fig 17. FTIR spectrum of PVA and PCL

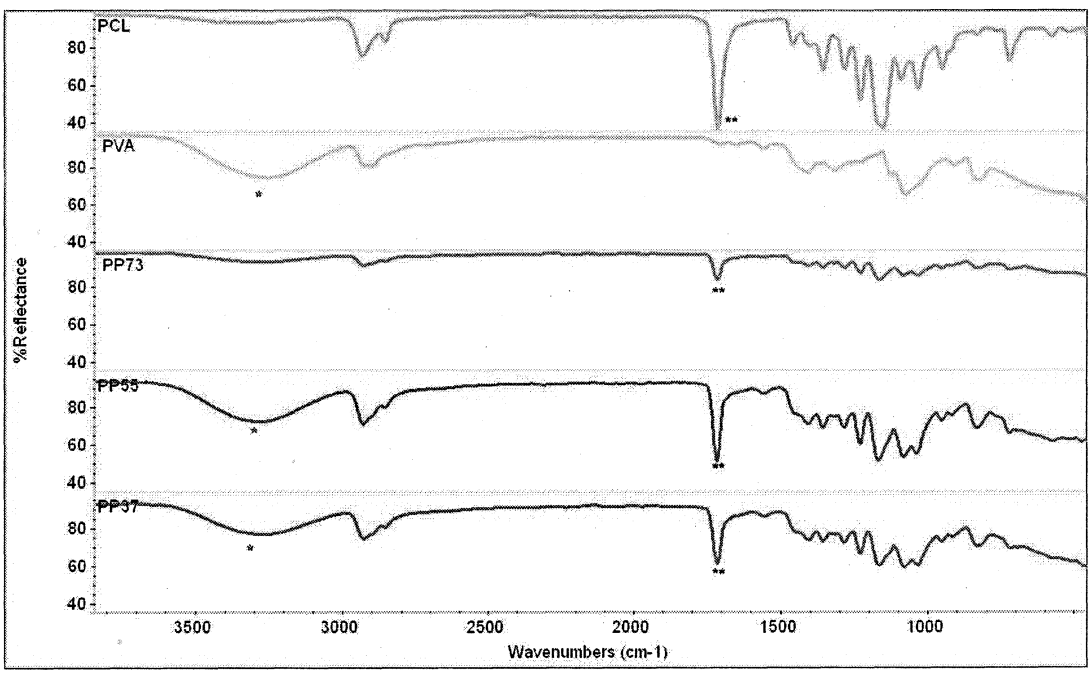


Fig 18. FTIR spectrum of different hybrid compositions of Semi IPN compared to PVA and PCL. ** indicates the carbonyl (-C=O) stretching of PCL at 1724 cm-^{1} and * indicates 3330 cm-^{1} the (-OH) groups of PVA.

3.1.8.2.1. GELATIN-ALBUMIN

The hybrid Gelatin-Albumin scaffold retains the characteristic bands of the protein molecule. The FTIR spectrum of gelatin and albumin is represented in Fig 19 and that of hybrid scaffold in Fig 20. The broad absorption within the range of 3200-3600 cm^{-1} correspond to amino ($-NH_2$) stretching vibrations of 1^{o} amide groups. The absorption bands in the range of 2950-2750 cm^{-1} corresponds to –CH, $-CH_2$ and $-CH_3$ carbon to hydrogen stretching vibrations. A strong band at around 1630 cm^{-1} is an indication of amide carbonyl group which is a representative of all proteins. The chemical composition of the hybrid scaffold was analyzed using FTIR spectrum and compared to that of native gelatin and albumin. The hybrid composition retained the carbonyl (-C=O) stretching peak (amide I) and N-H bending peak (amide II) band of peptide bond at 1630 cm^{-1} and 1539 cm^{-1} with a slight shift towards intermediate region from that of native gelatin. Since the hybrid composition is just a blend of gelatin and albumin no additional peaks were observed rather than a shift in some of the bands as expected. The spectrum of the crosslinked Gelatin-Albumin samples showed appearance of two smaller peaks at 1539 cm^{-1} and 1521 cm^{-1} in amide I band of peptide bond. (Fig 21) Formation of additional inter and intra chain peptide bonds within and between the two polymers due to crosslinking with EDC might have resulted in the appearance of this smaller peaks. The spectra of the hybrid composition also proved that neither the fabrication methodology nor the crosslinking procedures bring any change in the chemical structure of the proteins.

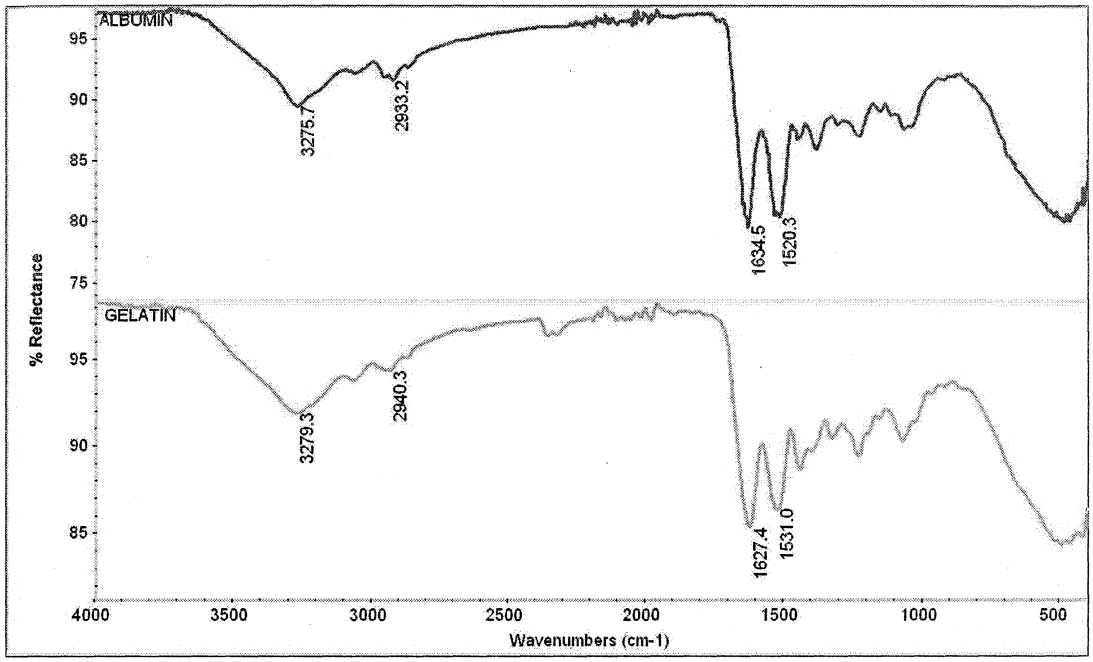


Fig 19. FTIR spectrum of Gelatin and Albumin.

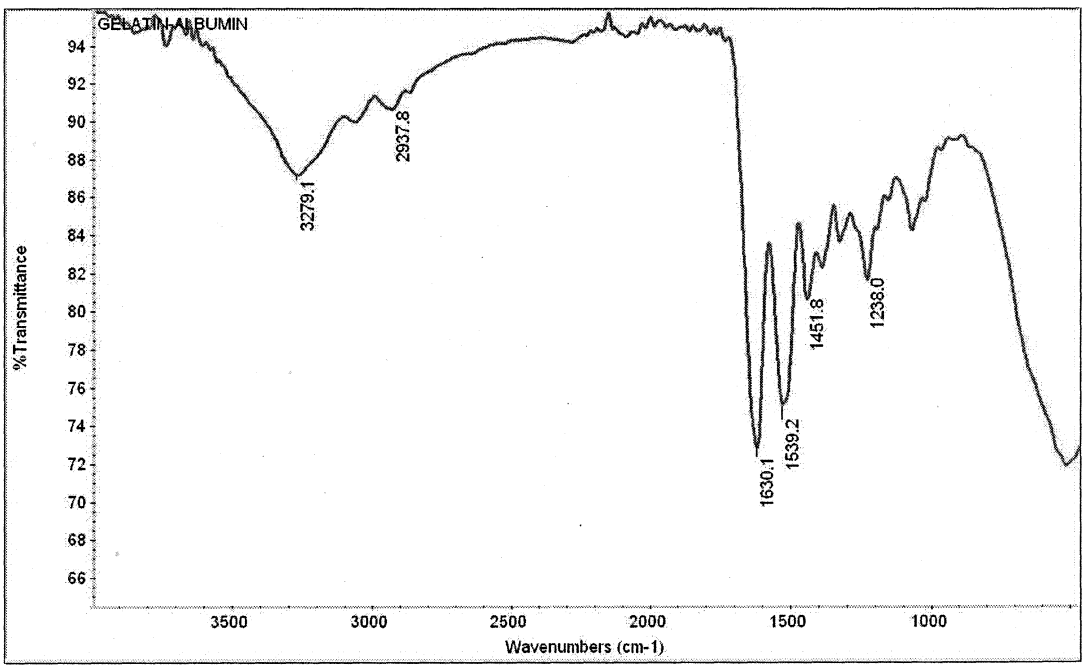


Fig 20. FTIR spectrum of Gelatin-Albumin hybrid composition

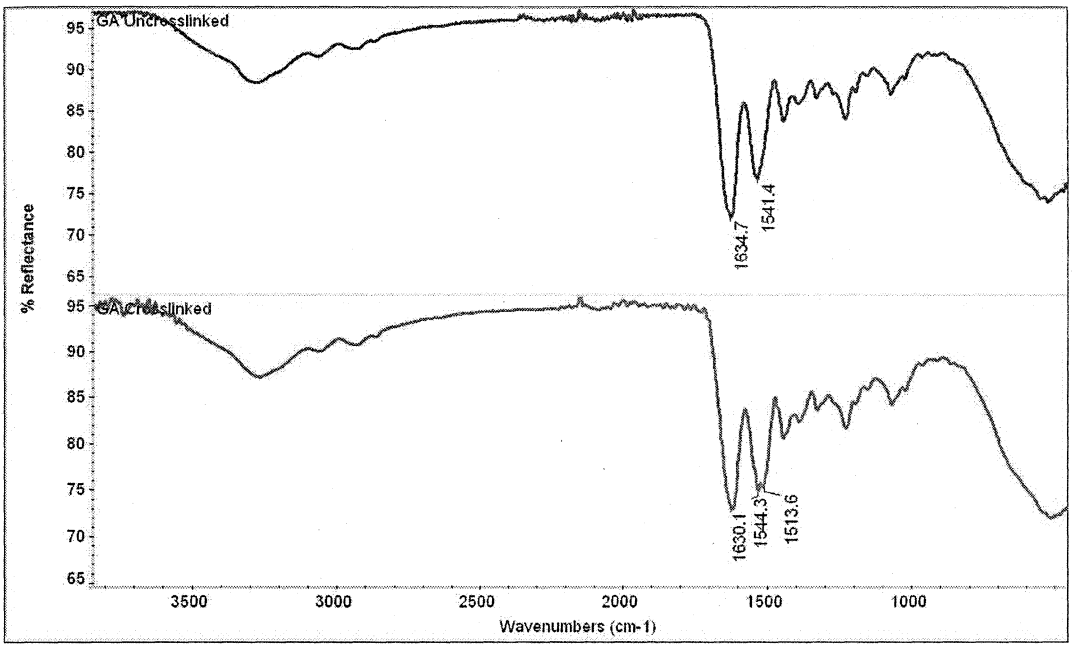


Fig 21. FTIR spectrum of Gelatin-Albumin scaffold before and after crosslinking.

3.1.8.3. MECHANICAL PROPERTY

The mechanical properties of the porous scaffolds were measured as per the ASTMD 895-96 by applying a constant load and compressing at a constant rate. The scaffolds were compressed to half height (Fig 22) and the load, stress, % strain were calculated.

A comparison of the compressive properties of the three compositions of the semi IPN, with that of 3 dimensional PVA scaffold, showed that the maximum load at half height increases with increase in concentration of PCL. The maximum load of all the Semi IPN compositions was significantly higher when compared to 3D PVA scaffold. Comparison was not made with PCL as 3D porous PCL scaffold could not be fabricated by freeze drying technique. The compressive mechanical loads, stress, strain and modulus of the scaffolds were calculated and the data is represented in Table 3 .The compressive load of PVA-PCL (50:50) was almost twice when compared to PVA-PCL (70:30) composition. However a substantial

increase has not been observed in (30:70) composition. Stress at maximum load was significantly increased in all compositions of Semi IPN. On initial addition of up to 30% of PCL the stress was higher than in comparison to PVA alone. The strain or % elongation was also increased in all the Semi IPN compositions but the strain decreases at greater than 50% PCL. Toughness of the semi IPN compositions were significantly increased in comparison to the control PVA. The toughness of the individual Semi IPN compositions increased up to 30% addition of PCL and decreased with further addition of PCL. The mechanical properties of Load at maximum load, stress at maximum load, % strain and toughness for all the Semi IPN were significantly increased in comparison to that of PVA alone. All the semi IPN compositions had a compressive stress-strain graph similar to that of a viscoelastic polymer. (Fig 23) On initial increase in the concentration of PCL in the Semi IPN, PCL acts as reinforcing filler, and the PVA as a continuous phase. Increased addition of PCL in PVA hence brings enhancement of properties in comparison to that of pure PVA. However the properties did not increase linearly with increased concentration of PCL, and maximum increase were seen on addition up to 30%. Further increase in concentration of PCL tends to cause an inversion of phases in the Semi IPN, with PCL trying to form the continuous phase. Such behavior of inversion of phases has also been reported by in rubber filled latex and the particle filled blends. These data also correlate well with the conclusion of thermal studies. The compressive stress of our Semi IPN compositions was similar to PLGA scaffolds and substantially higher than alginate scaffolds reported in literature.

The mechanical loads, compressive stress and % strain of the Gelatin-Albumin scaffolds were evaluated and the data was represented in Table 4 and the stress strain graph in Fig 24. The compressive stress was 0.251 MPa which was in the range of the compressive stress of several porous scaffolds reported in literature. The modulus of the PVA-PCL scaffold was low when compared to the modulus of the cartilage (8-16 MPa) (*Frank et al 1987*) but was similar or higher than porous

foams fabricated from PLA (*Ma et al 2001*) and agarose gels (M*auck et al 2003*). The scaffolds had better crosslinking and mechanical stability than the conventional hydrogels.

Fig 22. Compressive mechanical test of 3D porous scaffolds

Table 3. Compressive mechanical properties of PVA-PCL Semi IPN scaffolds

Compositions	Load at Max load (KN)	Stress at Max Load (MPa)	% Strain at Max Load	Modulus (MPa)	Toughness (MPa)
PVA	0.013±0.004	0.142±0.043	22.5 ±6.8	1.47	150
PVA:PCL(30:70)	0.070 ±0.036	0.342±0.032	39.18 ±3.38	1.52	330
PVA:PCL(50:50)	0.081 ±0.010	0.403±0.066	46.7 ±2.88	1.78	540
PVA:PCL(70:30)	0.045 ±0.007	0.303±0.050	33.4± 1.013	3.51	720

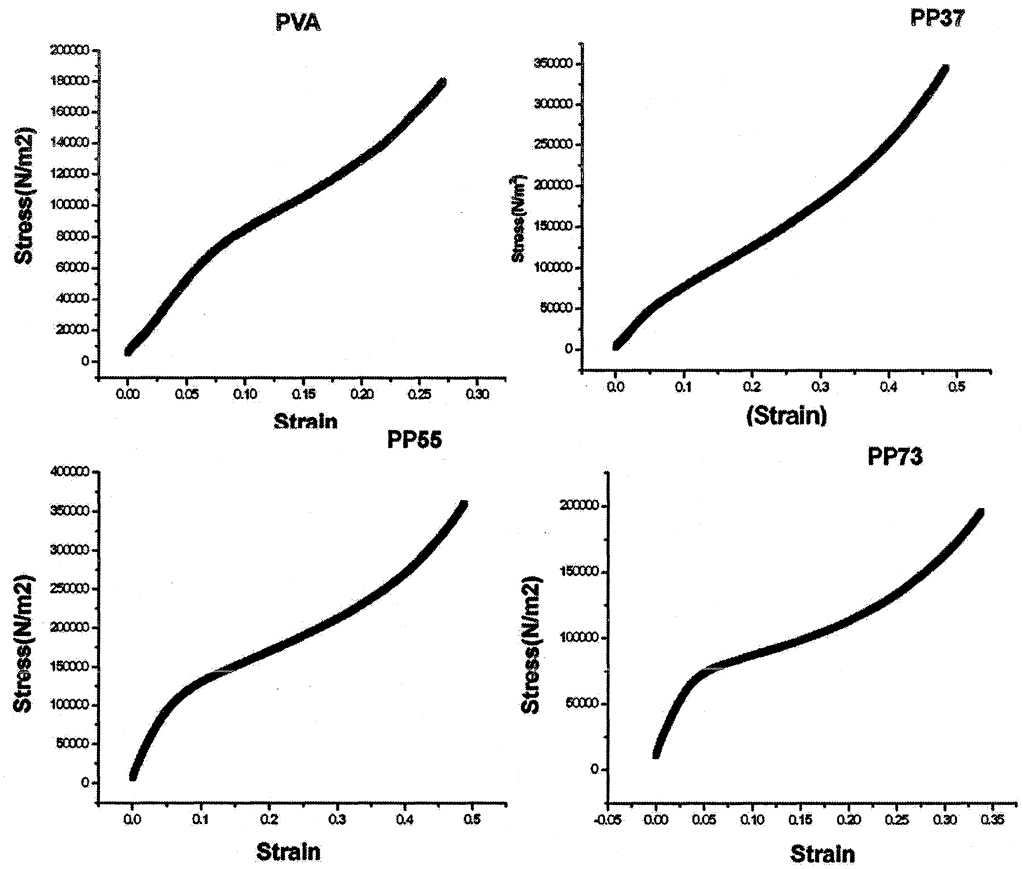


Fig 23. The stress-strain graph of the hybrid PVA-PCL Semi IPN scaffolds

Table 4. Compressive mechanical properties of Gelatin-Albumin scaffold

composition	Load at Max Load (KN)	Stress at Max Load (MPa)	% strain at Max Load (MPa)
GA (90:10)	0.075±0.005	0.251 ±0.021	53.665 ± 6.3

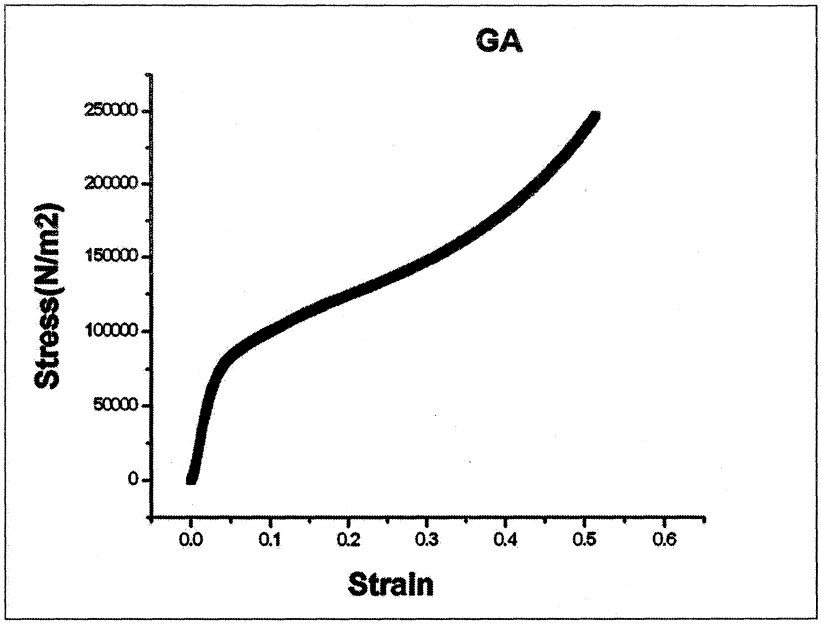


Fig 24 . The stress-strain graph of the Gelatin-Albumin scaffolds

3.1.8.4. PERCENTAGE SWELLING AND WATER CONTACT ANGLE

3.1.8.4.1. PVA-PCL

The high swelling behavior is a requirement for the 3D scaffolds to serve as a matrix for tissue regeneration. The percentage swelling of the three compositions and PVA scaffold as a function of time is represented in Fig 25. PVA scaffold alone showed very high swelling percentage above 1200. The high swelling nature is due to hydration of free hydroxyl (-OH) groups of PVA, as well as retention of some water molecules within the porous structure. The medium uptake ability of the Semi IPN scaffold decreased with the addition of polycaprolactone, when compared to parent PVA scaffold. This decreases in swelling behaviour in the hybrid compositions when compared to parent PVA scaffold is due to the decrease in PVA content in the hybrid compositions, as well as the introduction of hydrophobic nature by PCL. In the hybrid compositions, an increase in swelling was observed with increase in concentration of PVA upto a composition of 50:50. Further increase in concentration of PVA from PVA: PCL (50:50) to PVA: PCL

(70:30) did not bring any significant increase in swelling. This change in swelling is explained in later sections of this chapter along with similar observations in other physicochemical properties. The PP 50:50 compositions showed the highest increase in swelling when compared to all other compositions. We infer that this could be due to optimum blending and an ordered arrangement between the polymer chains within this composition as observed in other physicochemical properties. The high medium uptake ability of the scaffold even in the first two minutes suggests the potential ability of the scaffold to absorb and supply nutrients to all the cells that are seeded within the porous structure of the scaffold. This is an essential requirement for the scaffolding material particularly for chondrocyte culture, as the cells entirely depend on the nutrients that are diffused into the matrix from the surrounding area. Cartilage is an avascular tissue and chondrocytes live entirely on the nutrients that move into the area just by the process of diffusion. Moreover it also points out the inherent capacity of the scaffolding material to hold large amount of water similar to the glycosaminoglycans in the native extra cellular matrix of the cartilage. The high water holding capacity of PVA may also help to resist compression as observed in the glycosaminoglycans in the native cartilage tissue.

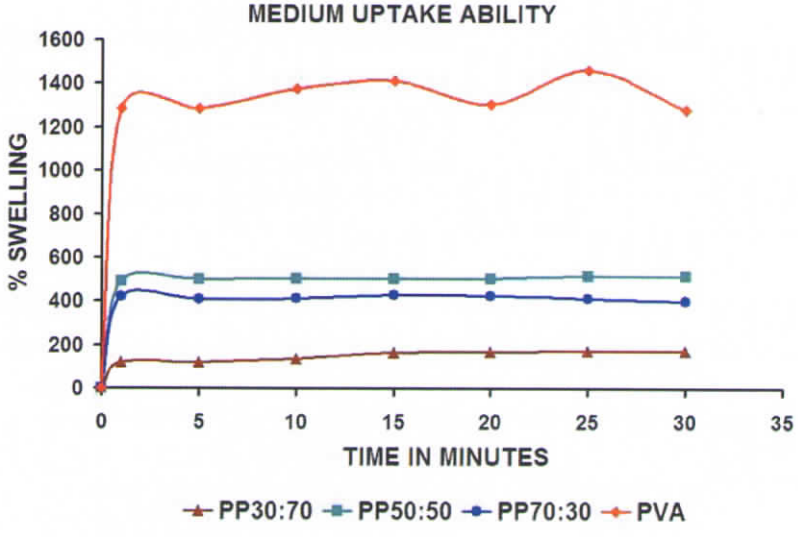


Fig 25. Swelling behavior of hybrid Semi IPN scaffold compared to PVA

The water contact angle values of Semi IPN hybrid scaffolds was compared to PVA and PCL in Table 5 and Fig 26 is a representative image of the video contact angle of the Semi IPN compositions. The water contact angle of the 3D PVA film was 35° and that of PCL was 82.3°. This confirms that the PVA is highly hydrophilic while PCL is hydrophobic. With the addition of polycaprolactone in the Semi IPN the water contact angle increased and the material became more hydrophobic than PVA. However the contact angle did not directly increase with increase in PCL composition. The water contact angle increased from 57° to 79° as the composition of PCL is increased from 30-70%, but the PVA: PCL (50:50) composition showed a more hydrophilic nature with water contact angle of 55.2°. The PVA: PCL (50:50) composition tends to be more hydrophilic than the (70:30) composition. This again suggests that a more favorable blending and arrangement may be observed in PP55 which resulted in altered surface properties, and high swelling. The PVA: PCL (50:50) composition had a hydrophilic-hydrophobic balance with contact angle value of 55.2. Previous reports have shown that hydrophilic-hydrophobic balance in contact angle values (54-61) is optimal for cell adhesion. (*Ciardelli et al 2005*)

Table 5. The contact angle values of Semi IPN scaffolds compared to PVA &PCL

Compositions	Mean contact angle
PVA	35 ± 3
PCL	82.3 ± 2
PVA-PCL (30:70)	79.2 ± 2
PVA-PCL (50:50)	55.2 ± 1
PVA-PCL (70:30)	57.8 ± 1

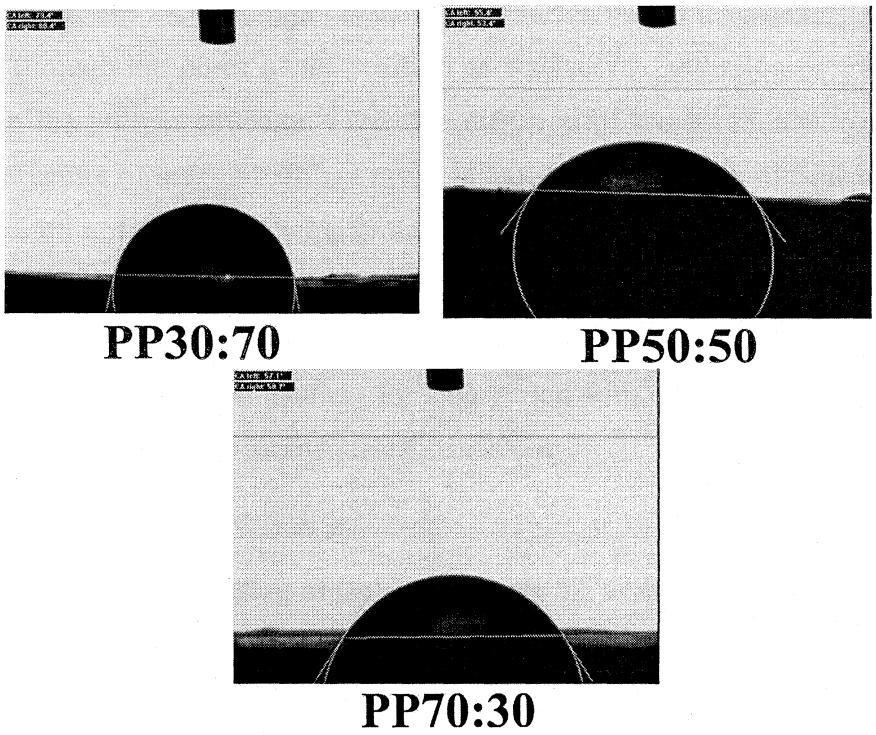


Fig 26. Representative images of water contact angle of Semi IPN PVA-PCL compositions.

Based on the results of physicochemical properties like mechanical property, medium uptake ability, contact angle value and over all stability of the scaffolds in the culture medium of all the Semi IPN PVA-PCL hybrid compositions, it was found that the PP50:50 composition have comparatively higher medium uptake ability, a hydrophilic-hydrophobic balance and is able to withstand high mechanical load. This composition had a higher stability and best handling properties in culture medium. Based on these results, the PVA: PCL (50:50) composition was selected for detailed physico-chemical characterization and cell culture studies.

3.1.8.4.2. GELATIN-ALBUMIN

The Gelatin-Albumin scaffolds showed a very high ~~percentage~~ swelling ratio of 140 even in the first two minutes and attained an equilibrium state as

represented in Fig 27. The high uptake of medium is due to the wicking action of solution through the pores of the scaffold as well as the hydration of free (-OH) groups in the protein structure. The free diffusion of the solution through open porous structure of scaffold did not result in extensive change in dimensions. This high medium uptake ability helps in the fast and uniform distribution of nutrients to all the cells seeded throughout the construct. However the high swelling nature did not result in collapse of the scaffold as observed in many other hydrogels. The scaffold remained stable in medium for long period with a good handling property which was attained due to its adequate crosslinking

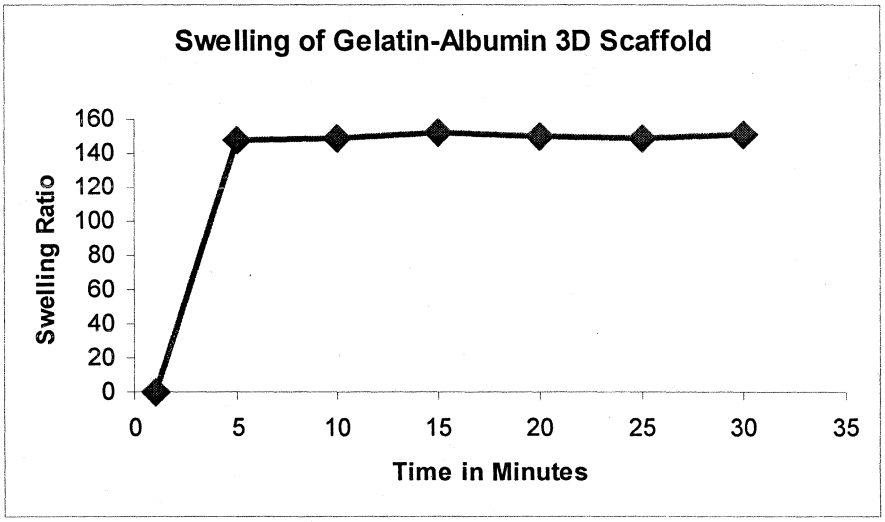


Fig 27. Swelling of 3D Gelatin-Albumin scaffold

The surface property was studied by water contact angle and showed a hydrophobic-hydrophilic balance with a mean contact angle of 65°. Fig 28 is a representative image of water contact angle of Gelatin-Albumin film. This data confirms the above explanation that the high medium uptake of the scaffold is by a wicking action of the porous scaffold and not due to high hydrophilicity. The addition of albumin might have helped to attain the hydrophilic-hydrophobic balance and might have a role in the stability of the scaffold. However a detailed

study has not been conducted in this work to compare this with that of pure gelatin scaffold.

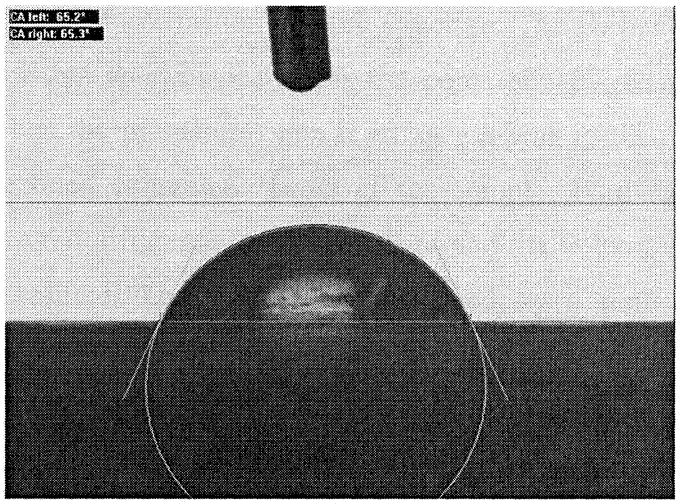


Fig 28. A representative video contact angle image of Gelatin-Albumin film

3.1.8.5. PORE MORPHOLOGY OF THE 3 D SCAFFOLDS

One of the major requirement for 3D scaffold to serve as a supporting matrix for tissue regeneration is that the scaffold should be highly porous with open interconnected pores. The open interconnected porous structure is required for uniform distribution of cells during seeding and helps in the free diffusion of the nutrients and waste materials during the culture. The viability of cells within the scaffold depends on the availability of nutrients to all the cells. The open interconnected porous structure, thus play a role in maintaining the cells viable in the scaffold. The porous structure also helps in the deposition of extracellular matrix secreted by the cells in a 3 dimensional pattern which helps in the formation of a 3 dimensional structure similar to that of the native tissue.

The nature of the pore structure and the over all 3 dimensional structure of the scaffold was obtained from the micro CT images and scanning electron micrographs. High resolution Micro CT can be used to image the scaffold non-

destructively and using computer aided algorithms 3D structure of the scaffold can be reconstructed. (*Moore et al 2004*) The presence of continuous or through pores was evaluated using liquid extrusion porosimetry (LEP). This is a mercury free technique used for the characterization of pore structure. The porous sample was wetted with a liquid that spontaneously fills the pores and placed on top of a membrane filter. When the pressure of non reacting gas was applied it displaces the liquid from the open continuous pores of the sample. Pore diameter was computed from measured differential pressure of gas and the volume of displaced liquid yields pore volume. The data was represented by a pore distribution histogram that displays the percentage of the total number of pores for each range of pore sizes within the sample. The % total pore volume of continuous pores also represented graphically for each pore size range.

3.1.8..5.1. PVA-PCL

The micro CT images of the 3D porous scaffolds taken from sections at different angles indicated that porosity was retained uniformly throughout the entire length of the sample. Fig 29 represents the 3D micro CT images of PVA: PCL (50:50) compositions at different angles showing the porous nature of the scaffold. The scanning electron micrograph of the PVA: PCL (50:50) compositions showed that the scaffold was highly porous with open interconnected pores. The pore sizes were within the range from 30–300 microns. The scanning electron micrograph of the 3 D scaffold is represented in Fig 30. The results reveal crosslinking was also optimized so that the pore sizes were within the limit of accepted range for tissue regeneration.

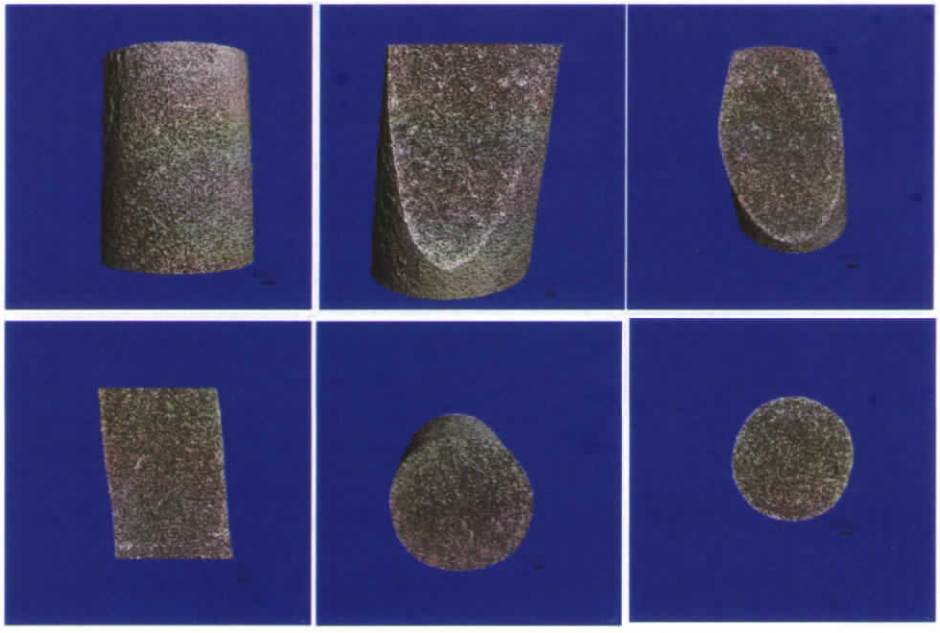


Fig 29. Micro CT images of 3D porous PVA: PCL (50:50) Semi IPN scaffold

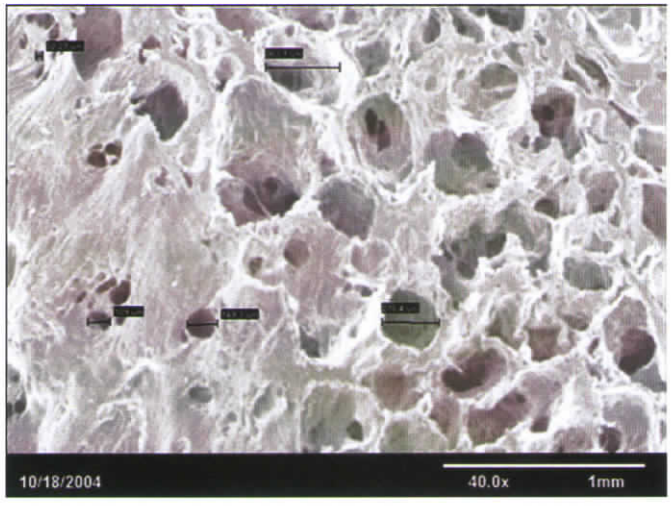


Fig 30. SEM of 3D porous PVA: PCL (50:50) composition with open interconnected pores

The presence of continuous through pores was measured using LEP. The % porosity of continuous pores in different pore size range is represented as a

histogram and % total pore volume for each pore size range is represented graphically in Fig 31 & Fig 32. The pore size of the dry sample was within the range of 7-192 μm. About 40 % of the continuous pores were in the narrow range of 8-10 μm, the porosity of pores in each of the range of 20-30, 30-50, 50-70 and 70-100 μm were about 8-10 %. When the scaffold was placed in culture medium, the pore size increased due to the swelling of PVA. The size of the pores in aqueous solution was depicted graphically in Fig 33 & 34. The pore sizes are subjected to increase in aqueous state due to swelling of PVA. In aqueous solution there was a 5 fold increase in pore size. About 66 % of the total pores were within the range of 100-500 μm.

These results confirm that the freeze drying fabrication methodology and appropriate crosslinking helped to attain scaffolds with adequate porosity and open interconnected porous structure. Increased porosity and interconnected pores permit extensive diffusion of nutrients to the interior of the scaffolds. The swelling of PVA in aqueous solution produced a five fold increase in pore size which is within the previously reported accepted range of pore size suitable for chondrocyte culture. The entanglement of polyvinyl alcohol with polymer chains of PCL in Semi IPN and appropriate crosslinking might have prevented extensive swelling and further increase in pore size.

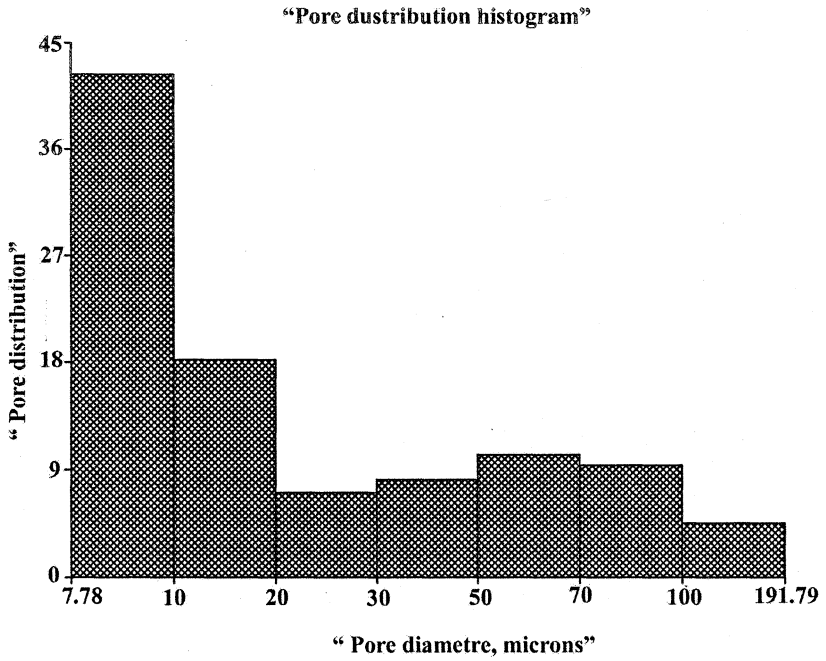


Fig 31. Continuous pores of PVA-PCL scaffold in dry state

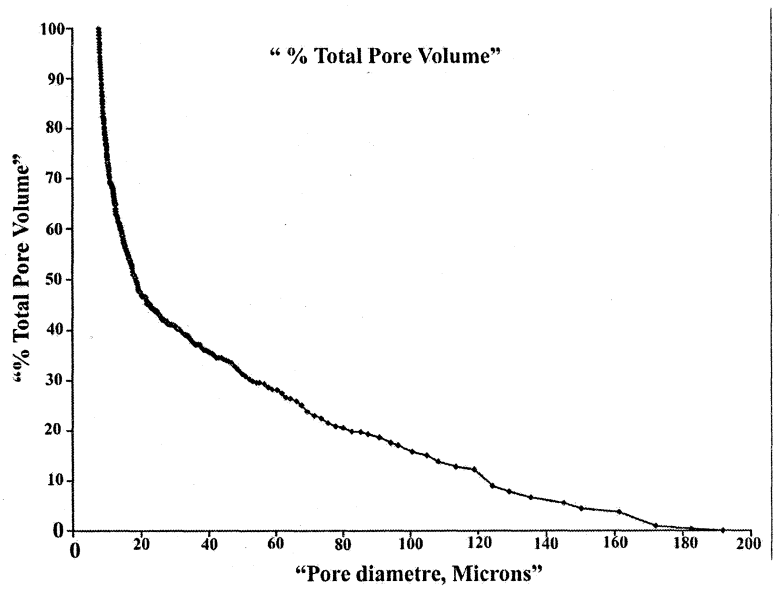


Fig 32. Percentage pore volume of continuous pores in dry PVA-PCL scaffold

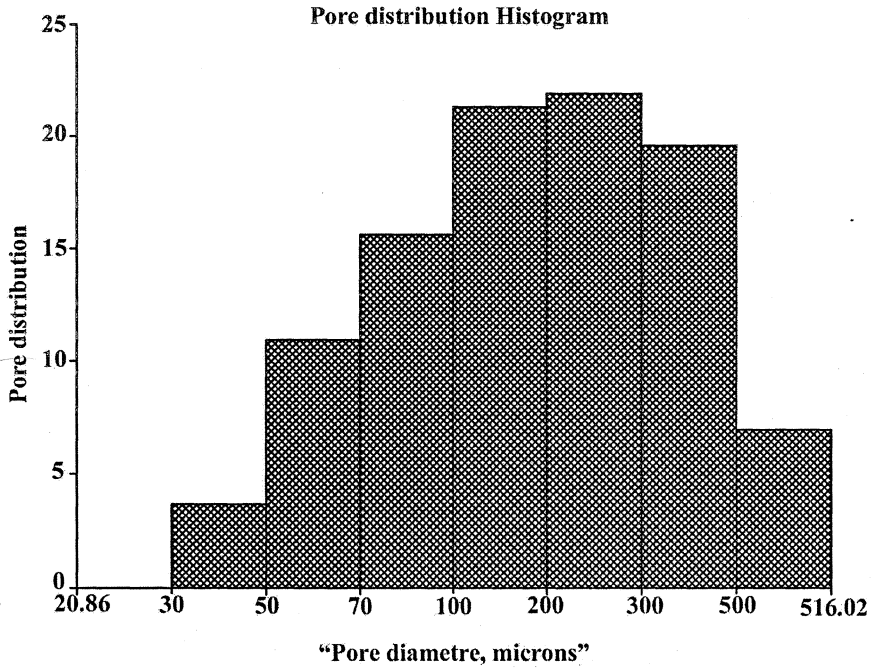


Fig 33. Continuous pores of PVA-PCL scaffold in wet state

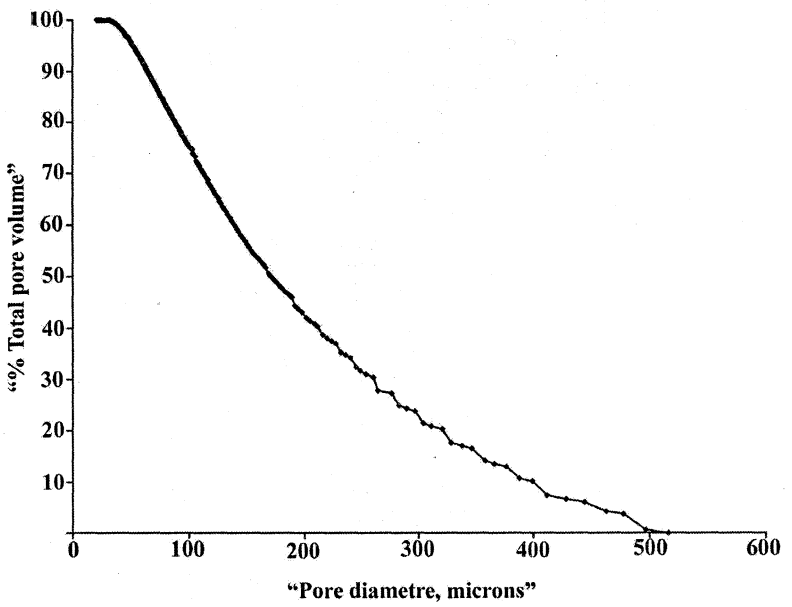


Fig 34. Percentage pore volume of continuous pores in wet PVA-PCL scaffold

SECTION 1

3.1.8.5.2. GELATIN-ALBUMIN

The micro CT images of the 3D porous scaffold taken from sections taken at different angles showed that porosity was retained uniformly throughout the entire length of the sample.(Fig 35) The scanning electron micrograph of Gelatin-Albumin scaffold was highly porous with open interconnected pores. (Fig 36) The continuous pores were estimated using LEP in the dry state of the scaffold. The continuous pores were in a range of 20-70μm with about 95% of pores were uniform in the narrow range of 50-70μm as in Fig 37 and Fig 38. The fabrication methodology and adequate crosslinking helped to attain continuous pores with uniform pore size. When the scaffolds are kept in culture medium, a uniform increase in pore structure is expected due to swelling.

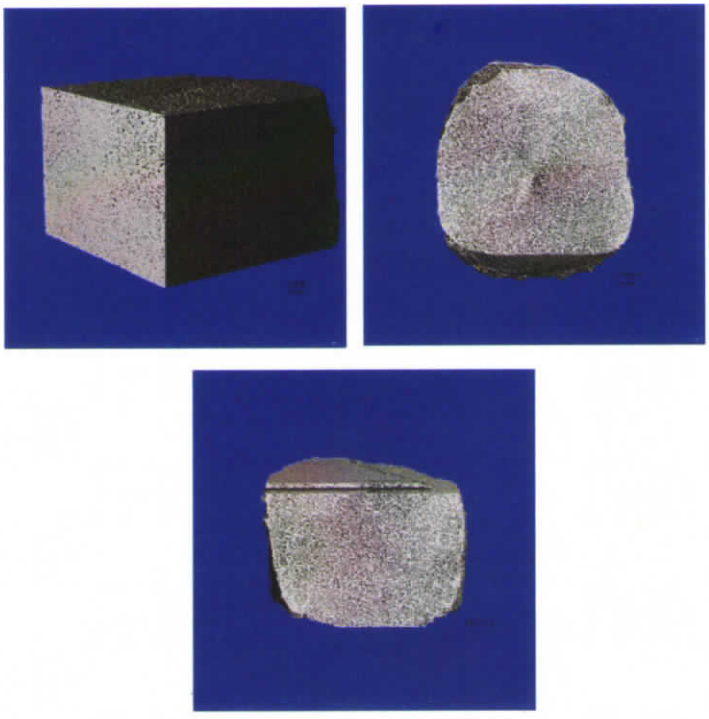


Fig 35. Micro CT images of Gelatin-Albumin scaffolds taken at different cross sections

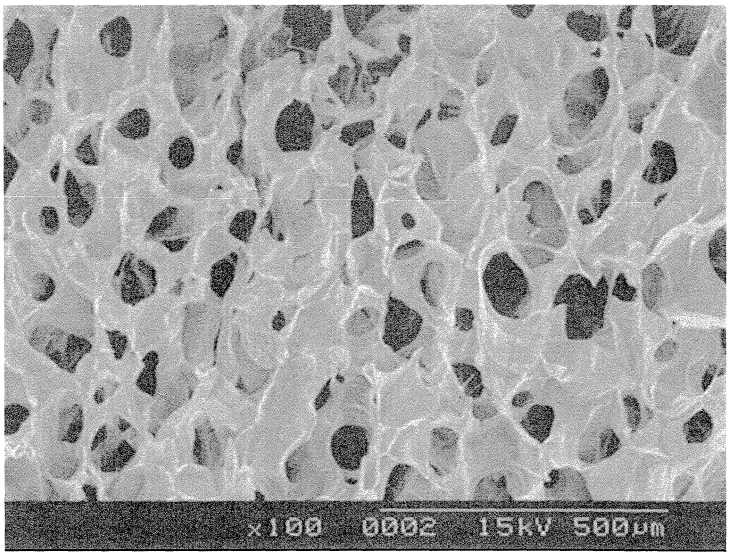


Fig 36. SEM image of Gelatin-Albumin scaffolds showing open interconnected pores.

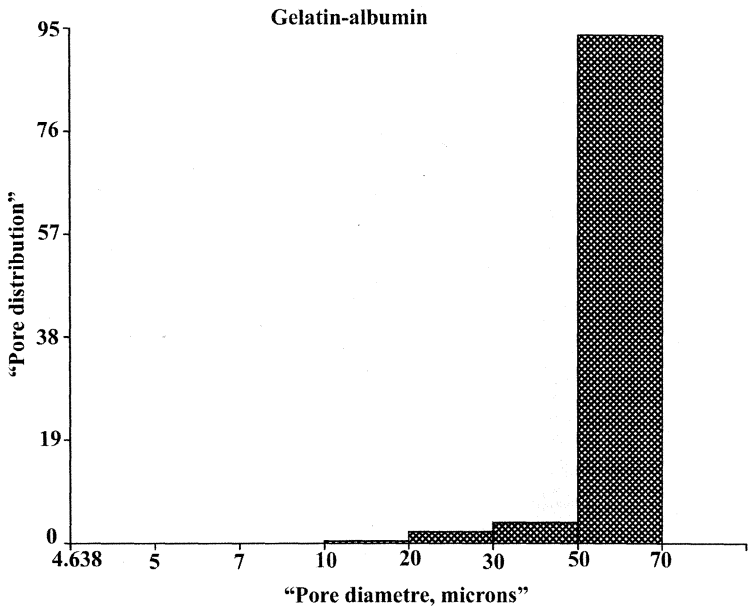


Fig 37. Continuous pores in dry Gelatin-Albumin scaffold.

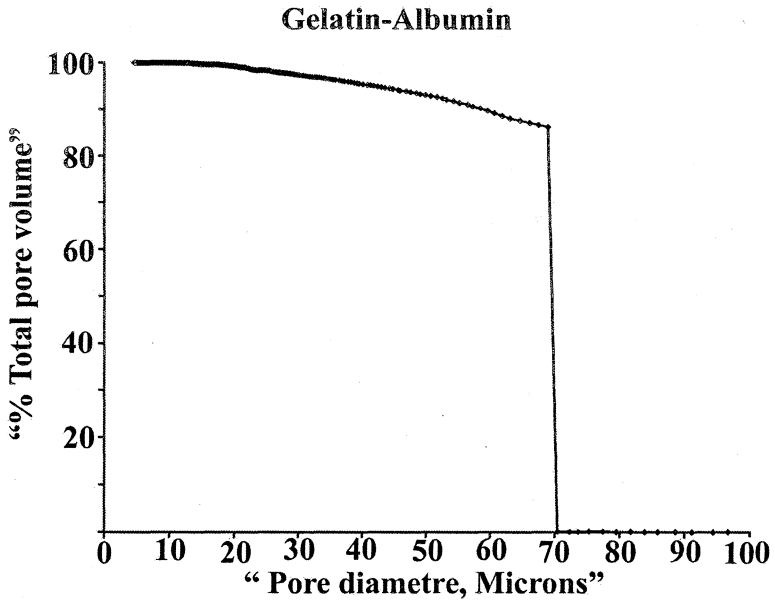


Fig 38. Percentage pore volume of continuous pores in dry Gelatin-Albumin scaffold

It is well recognized that the pore size of the scaffolds play an important role for cell binding, migration, ingrowth (*Tienen et al 2002, Wake et al 1994*), tissue ingrowth and regeneration (*Whang et al, 1999, Zeltinger et al, 2001*). Generally it is reported that larger pore size and porosity can allow effective nutrient supply, gas diffusion and metabolic waste removal. Many researchers have reported optimum pore size range, for different kinds of cells and tissues like fibroblasts, hepatocytes (*Yang et al 2001*), vascular smooth muscle cell binding (*Zeltinger et al 2001*), neovascularization (*Brauker et al 1995*) and bone regeneration (*Bobyn et al 1999, Hulbert et al 1970*).

In case of chondrocyte culture few studies have been reported in different pore size range. In all the reports chondrocyte growth was found to be greater in the largest pore size selected for each study. *Lee et al 2004*, studied in polycarbonate membrane in submicron and micron range of 0.2-8μm and found chondrocytes responded well in 8 micron pore size. *Chia et al 2006* studied the influence of pore size on chondrocyte culture in polyurethane membranes in the

range of <5 μm, 10-20 μm and 40-60 μm and did not observe any difference in the chondrocytes response. *Griffon et al 2006* reported 70-120 μm range suitable for chondrocyte culture. This study conducted on chitosan scaffolds in pore size range <10 μm, 10-50μm, 70-120 μm found chondrocytes proliferation and ECM synthesis to be higher in larger range used for the study. *Yamane et al 2007* studied the effect of pore size on chitosan - hyaluronicacid fibers in 100, 200, 400 micron pore size where 400 microns was found to promote better chondrogenesis. A recent report by *Heang et al 2007* show that the scaffold section with 380–405 μm pore size to be suitable for chondrocytes culture.

Even though many previous reports are available on the effect of pore size on chondrocyte culture, however so far there is no consensus regarding the optimum pore size suitable for chondrocyte culture. In all the previous reports, chondrocytes, proliferated and secreted matrix components in the largest pore size used for each study. Moreover a complete comparison could not be made between all these reports as culture was carried out in different scaffold materials. In general chondrocytes prefer a larger pore size, promoting free diffusion of nutrients that allows sufficient space for the secretion and localization of large aggregating proteoglycans and collagen molecules.

3.1.8.6. INVITRO DEGRADATION OF 3D SCAFFOLDS

3.1.8.6.1. PVA-PCL

One of the general requirements of a scaffold used for tissue regeneration is its biodegradability. The scaffold should gradually degrade creating space for the cells to secrete extracellular matrix. The extra cellular matrix deposited by the cells is expected to take over the functions of the scaffold. The rate of invitro degradation of the PVA-PCL scaffold was estimated from the percentage of weight remaining after incubation in PBS for a specific period of time. The data is represented graphically in Fig 39. The rate of degradation in the first eight weeks

range of <5 μm, 10-20 μm and 40-60 μm and did not observe any difference in the chondrocytes response. *Griffon et al 2006* reported 70-120 μm range suitable for chondrocyte culture. This study conducted on chitosan scaffolds in pore size range <10 μm, 10-50μm, 70-120 μm found chondrocytes proliferation and ECM synthesis to be higher in larger range used for the study. *Yamane et al 2007* studied the effect of pore size on chitosan - hyaluronicacid fibers in 100, 200, 400 micron pore size where 400 microns was found to promote better chondrogenesis. A recent report by *Heang et al 2007* show that the scaffold section with 380–405 μm pore size to be suitable for chondrocytes culture.

Even though many previous reports are available on the effect of pore size on chondrocyte culture, however so far there is no consensus regarding the optimum pore size suitable for chondrocyte culture. In all the previous reports, chondrocytes, proliferated and secreted matrix components in the largest pore size used for each study. Moreover a complete comparison could not be made between all these reports as culture was carried out in different scaffold materials. In general chondrocytes prefer a larger pore size, promoting free diffusion of nutrients that allows sufficient space for the secretion and localization of large aggregating proteoglycans and collagen molecules.

3.1.8.6. INVITRO DEGRADATION OF 3D SCAFFOLDS

3.1.8.6.1. PVA-PCL

One of the general requirements of a scaffold used for tissue regeneration is its biodegradability. The scaffold should gradually degrade creating space for the cells to secrete extracellular matrix. The extra cellular matrix deposited by the cells is expected to take over the functions of the scaffold. The rate of invitro degradation of the PVA-PCL scaffold was estimated from the percentage of weight remaining after incubation in PBS for a specific period of time. The data is represented graphically in Fig 39. The rate of degradation in the first eight weeks

was very negligible. There was a 20 % weight loss in a period of 4 months. In literature, the degradation of pure PCL is found to be very long for a period of 2 years. As PCL is hydrophobic and has high crystalline properties, it does not allow faster water penetration into the PCL matrix. The mechanism of degradation in PCL is by random hydrolytic chain scission of ester linkage like other polyesters, but the degradation rate is lower than other polyesters like PLA and its copolymers. (*Pitt et al 1981*). There are previous reports that the mass loss in polyesters does not start until the molecular chains are reduced to a size that allows them to diffuse out of the polymers. The good mechanical property with a lower degradation rate has been considered as the excellent requirements of PCL that makes it a promising material for tissue engineering of bone and cartilage. (*Gunatillake et al 2003, Kweon et al 2003, Causa et al, 2006*) The degradation pattern of PVA-PCL scaffold in this study was evaluated only for a period of four months and this data cannot be extrapolated till the complete degradation of the sample. However it is expected that this rate of degradation give adequate time for the cells to secrete its own extracellular matrix for the formation and maturation of a partly developed tissue.

There are reports that PCL undergoes a two-stage degradation process: The first lengthy phase involves nonenzymatic hydrolytic cleavage of ester groups, the second phase begins when the polymer is highly crystalline, and of low molecular weight. PCL fragments ultimately are degraded in phagosomes of macrophages and giant cells, the process requiring less than 13 days for completion at some sites. These studies support the intracellular degradation of PCL as the principal pathway of degradation once the molecular weight of the aged polymer is reduced to 3000 or less. (*Woodward et al 1985*) The degradation is well known as a metabolism via tricarboxylicacid cycle. Other studies prove that polycaprolactone is completely eliminated from the body through urine and faeces with no accumulation in the body. (*Song et al 2000*)

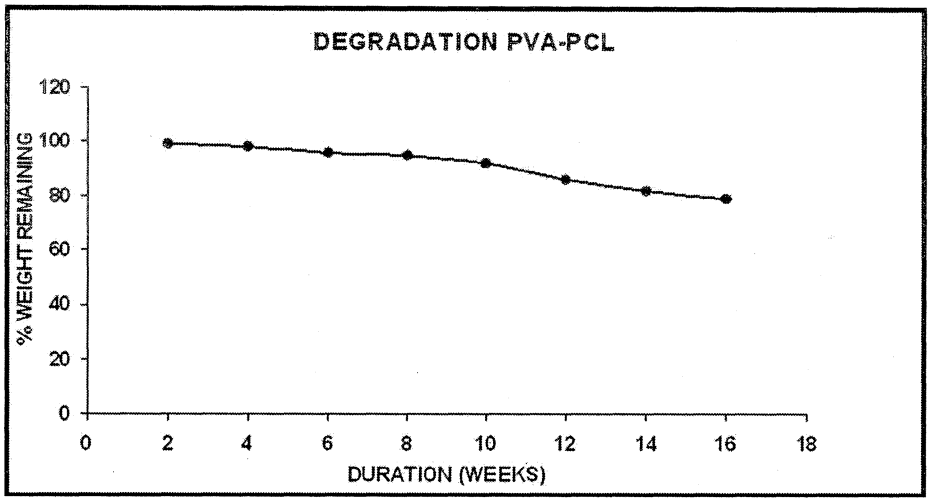


Fig 39.The invitro degradation pattern of 3D PVA-PCL scaffold

Low molecular weight PVA is expected to be completely excreted from the body through urine. Several studies have been conducted to evaluate the toxicity and elimination of PVA. These studies have proved that there was no evidence of PVA in the body and is completely excreted from the body through urine without accumulation in any of the body parts. (*Sanders & Mathews 1990).* Intravenous injection of low relative molecular mass 14,800 in BALB mice was excreted by this route within 30 min of administration. The rate of excretion of high molecular weight 4,34,000 was low due to lower permeability at the renal glomeruli. (*Yamaoka et al 1995*). In a long term study, conducted with molecular mass of 24000, for a period of 104 weeks, in 100 mice, have been reported, to find no evidence of carcinogenicity. (*National Toxicology Program (1998), USA*). *Hall and Hall, 1963* reported that subcutaneous injection of PVA of relative molecular mass, 37,000 for 30 days in rats did not show any abnormalities like hypertension, ascetic fluid accumulation, hemorrhage, glomerulonephritis, enlargement of the heart, liver and spleen. Histological examinations reported no lesions in any organs. PVA and preparations containing PVA were found to be non genotoxic in a range of studies in vivo through mouse bone marrow micronucleus formation studies. (*Huntingdon Life Sciences 2000*). The international program on chemical

safety reported that low molecular weight PVA is nontoxic and showed no evidence of carcinogenicity, however high molecular weight polyvinyl alcohol showed very low toxicity. (*WHO food additives series: 52)*A recent report on the urinary excretion of high molar mass PVA (1,25,000g/mol) shows that despite its higher molar mass, PVA is excreted through kidneys without significant molar mass change.(*Besheer et al 2007*) There is a general consensus that kidney acts as a filter that retains macromolecules with molecular mass equal or more than albumin, but the exact renal cutoff is controversial. The report suggests that apart from the molecular weight, the shape of the molecule also has some role in determining the renal excretion. Pharmacokinetics and biofate of large molar mass PVA upto 1,96,000 g/mol have already been studied earlier by (*Yamaoka et al, 1995a, 1995 b)*

3.1.8.6.2. GELATIN-ALBUMIN

The rate of invitro degradation of the Gelatin-Albumin scaffold was estimated from the percentage of weight remaining after incubation in PBS for a specific period of time. The data is represented graphically in Fig 40. The rate of degradation in the first six weeks was very negligible. There was a 20 % weight loss in a period of 3 and half months. This rate of degradation is comparatively lower than the conventional gelatin hydrogels. The slow degradation may be due to the sufficient crosslinking and the slight hydrophobicity introduced by the albumin in the blend. This degradation rate gives sufficient time for the cells to secrete the extracellular matrix. However the data cannot be extrapolated to predict the entire degradation time of the scaffold. In PBS the degradation may be only due to the hydrolytic attack. In actual site of application within the cartilage, the cells will produce collagenases that are likely to enhance the degradation of this scaffold. So a faster degradation might be expected in the site of application in presence of degradative enzymes. However the scaffold had good handling properties even after 4 months culture with chondrocytes seeded on it as further observed in cell

culture studies. This indicates that there is no drastic change in the stability of the scaffold during chondrocyte culture in these scaffolds. The scaffold is fabricated using natural protein molecules. The degradation produces nontoxic products and can be completely assimilated into the metabolic pathways.

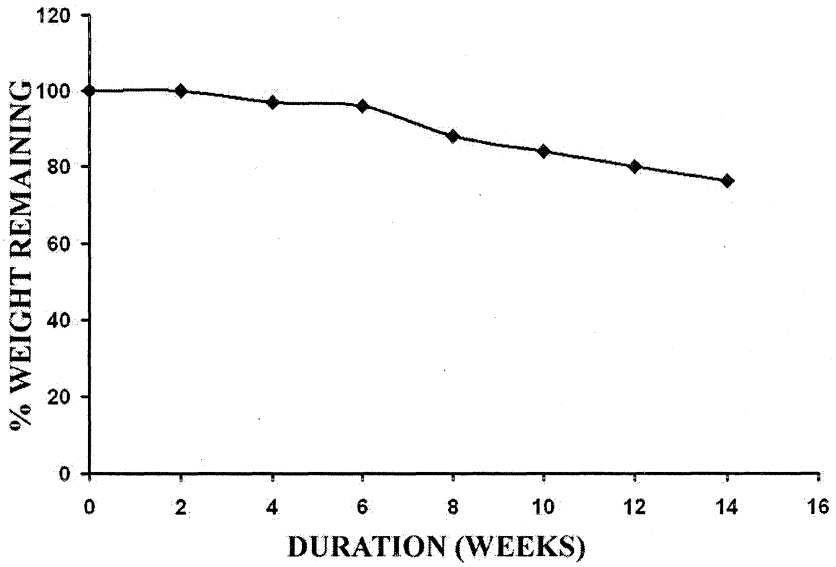


Fig 40. The invitro degradation pattern of 3D Gelatin-Albumin scaffold

3.1.9. MODIFICATION OF THE PVA-PCL SCAFFOLD

The feasibility of modifying the synthetic Semi IPN PVA-PCL scaffold with peptides is also explored in this study. This is examined by coupling RGD peptides, the commonly used peptide sequence found in fibronectin. The coupling reaction was initially tested using a representative amino acid arginine and further with RGD peptide. The reaction is a coupling of carboxyl (-COOH) groups present at the end of PVA and PCL chains as coupled to amino ($-NH_2$) groups in amino acid arginine.

3.1.9.1. COUPLING OF ARGININE USING DCC

The coupling of amino groups of arginine to the -COOH groups in PCL was carried out using DCC as the coupling agent. DCC is a commonly used

coupling agent that has been widely used for peptide synthesis. (*Barany and Merrifield, 1980*) The reaction mechanism of arginine coupling using DCC is represented in Fig 41. DCC reacts with carboxyl (-COOH) groups in the PCL chain to form an O-acylisourea intermediate. Further attack of an amine forms an amide linkage with dicyclohexylisourea as a byproduct. The activation efficiency of DCC is very high since the reaction is carried out in anhydrous organic solvent. The FTIR spectrum of the modified scaffold is represented in Fig 42. The amino acid coupled scaffold showed a sharp decrease in the intensity of the –C=O stretching at 1725 cm^{-1} and the appearance of a new band at 1655 cm^{-1} corresponding to amide I band of peptide linkage formed as a result of the coupling reaction.

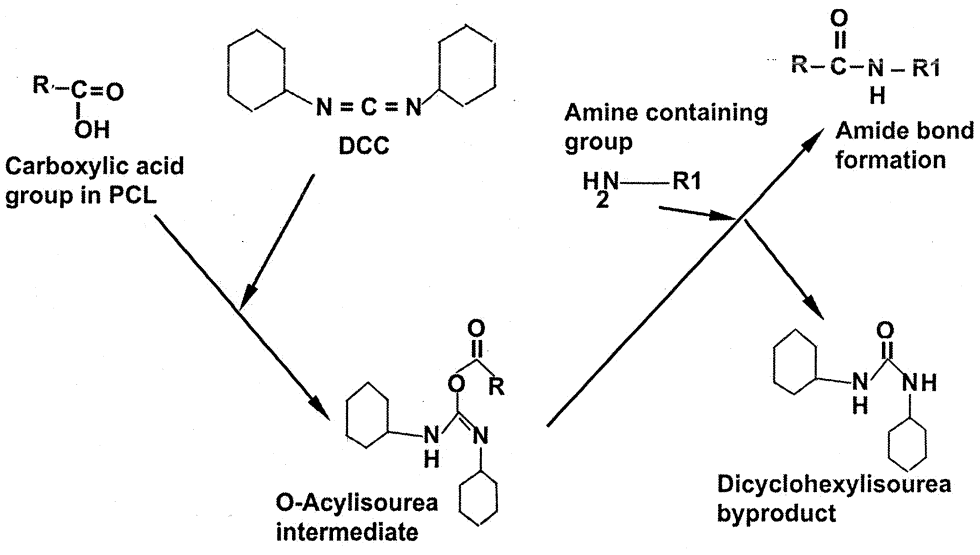


Fig 41. Reaction mechanism of arginine coupling using DCC

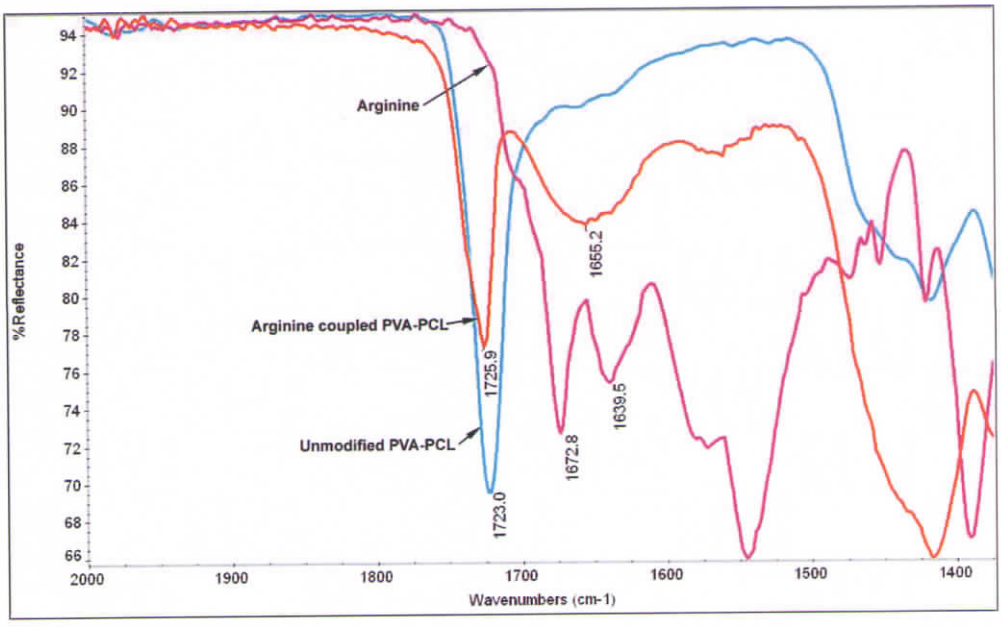


Fig 42. FTIR spectrum of arginine coupled to PVA-PCL using DCC

3.1.9.2. COUPLING OF RGD ON TO PVA-PCL USING DCC

Arginine-glycine-aspartic acid (RGD) is a common oligopeptide ligand in the ECM proteins of collagen and fibronectin, which is known to enhance cell attachment. (*Staatz et al 1991, Pierschbacher et al 1984*) RGD binding induces cell adhesion, spreading, focal contact formation, and cytoskeletal organization. (*Hubbel et al 1999*). Several reports have shown that coupling of RGD to synthetic polymers enhances it biomimeticity.(*Schmedlen et al 2002*) In this work the feasibility of modifying the PVA-PCL with RGD peptide is examined.

The FTIR spectrum of arginine coupling using DCC showed the appearance of a better peak corresponding to the amide I band. So coupling of RGD peptide was attempted using DCC. The FTIR spectrum of RGD coupled PVA-PCL scaffold is compared to that of unmodified PVA-PCL and RGD peptide in Fig 43. The sharp decrease in the –C=O stretching at 1724 cm^{-1} and the

appearance of two new bands at 1624 and 1570 cm^{-1} corresponding to amide I and amide II bands of peptide bond confirms the coupling process.

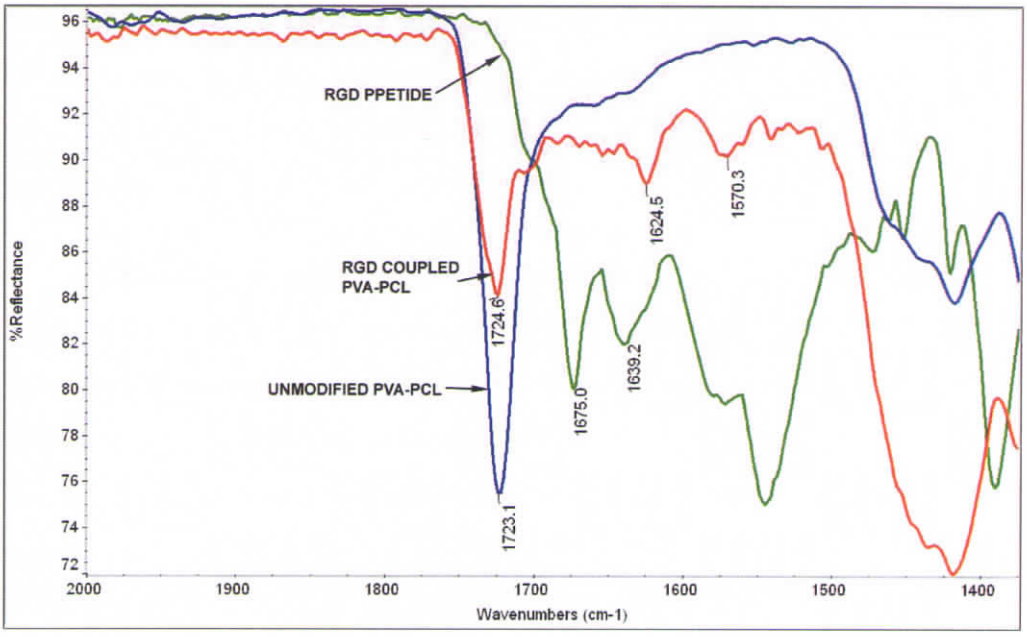


Fig 43. FTIR spectrum of RGD coupled PVA-PCL scaffold.

3.1.10. INITIAL CYTOTOXICITY EVALUATIONS

Cytotoxicity tests are important for screening and evaluation of biocompatibility for implantable materials. Cytotoxicity testing represents initial phase in testing biocompatibility of potential biomaterials. Its purpose is to act as a reliable, convenient and reproducible screening method to detect at an early stage in the testing process, cell death or other serious negative effects on cellular functions. *(Sgouras D et al 1990, Dekker et al 1994)*. Polymers often have low molecular weight leachables (additives, low molecular weight components, crosslinking agents) that can exhibit varying levels of cytological and physiological activity responsible for cell toxicity, alteration of metabolic balances. *(Cascone et al 1993)* The degree of in vitro toxicity of a biomaterial is almost proportional to its invivo compatibility. *(Pizzoferrato et al, 1985)* Mammalian cell

lines are used in the first stage of cytotoxicity evaluations, because in comparison with primary cultures, they are highly sensitive, homogeneous cultures and presents fast and easily detectable morphological observations such as decrease in cell size, reduced adherence and higher nucleus.*(British standard, 1988)* The ideal situation is to use primary cultures that serves as a representative of actual tissue and give more specific results of biocompatibility relevant for final application.

Cytotoxicity assays differ in the manner in which the test material is exposed to the cells. The choice of method varies with characteristic of the test material and the application of data. *(Kirkpatrick et al, 1992)* It is not possible to maintain in vitro cell cultures for indefinite time; thus the need to mimic long term degradation is done through tests on extract. Morphologic changes of cells that are brought in contact with biomaterial or biomaterial extracts indicate toxicity. Metabolically active cells can be quantified using MTT assay. In this study the initial cytotoxicity evaluation is carried out with L929 fibroblasts cell line by direct contact test .Further the test on extract method is conducted and metabolically active cells are quantified by MTT assay. Cytotoxicity using primary porcine chondrocytes is evaluated in the next section.

3.1.10.1. CYTOTOXICITY EVALUATION BY DIRECT CONTACT METHOD USING L929 CELLS

The initial cytotoxicity of PVA-PCL scaffold (50:50) composition, 3D PVA scaffold and PCL film was tested by direct contact method using fibroblast cell line L929. The cells were compared to cells exposed to nontoxic polyethylene (–ve control) and cytotoxic PVC disc (+ve control). Cytotoxicity was evaluated for 24 hours as well as for a period of 10 days. A qualitative evaluation of cell morphology was made using inverted microscope. The L929 cells in direct contact with the PVA, PCL film and the Semi IPN scaffolds retained their characteristic

spindle shaped morphology. The cells were well spread and properly attached similar to the negative nontoxic polyethylene control, where as the cells in direct contact with the positive PVC control (cytotoxic), lyse and assume a round shape due to toxicity. Cytotoxic alterations like shrinking of nucleus, formation of granules was not observed in the samples. Occasional round cells were observed since the cells attained confluence during the time period which is also observed in the –ve control. The phase contrast micrographs of PVA, PCL and PVA-PCL are compared to the control materials in Fig 44.

The initial cytotoxicity studies on Gelatin-Albumin scaffold were also conducted by direct contact tests and compared to the control materials. The result shows that the L929 cells was well attached and retained the spindle shaped morphology as observed in Fig 45. No microscopic observations that were harmful to the cells were observed in the micrographs. The data gives an initial confirmation that the polymeric materials, organic solvents used during the fabrication methodology and the crosslinking reagents do not elicit any harmful effects on the cell viability.

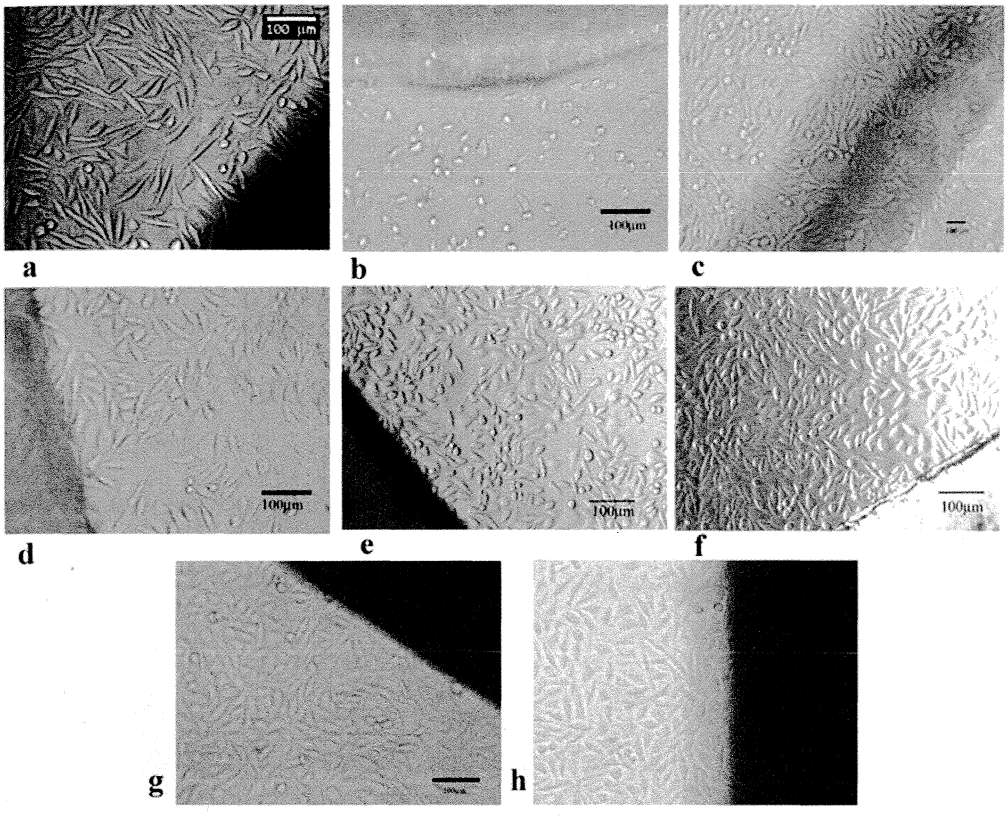


Fig 44. In vitro cytotoxicity studies of PVA-PCL scaffolds and parent materials with L929 cells. a) –ve control (PE), b) +ve control (PVC), c) PVA 24 hrs, d) PVA 10 days, e) PCL 24hrs, f) PCL 10 days, g) PP 50:50 24 hrs, h) PP 50:50 10days

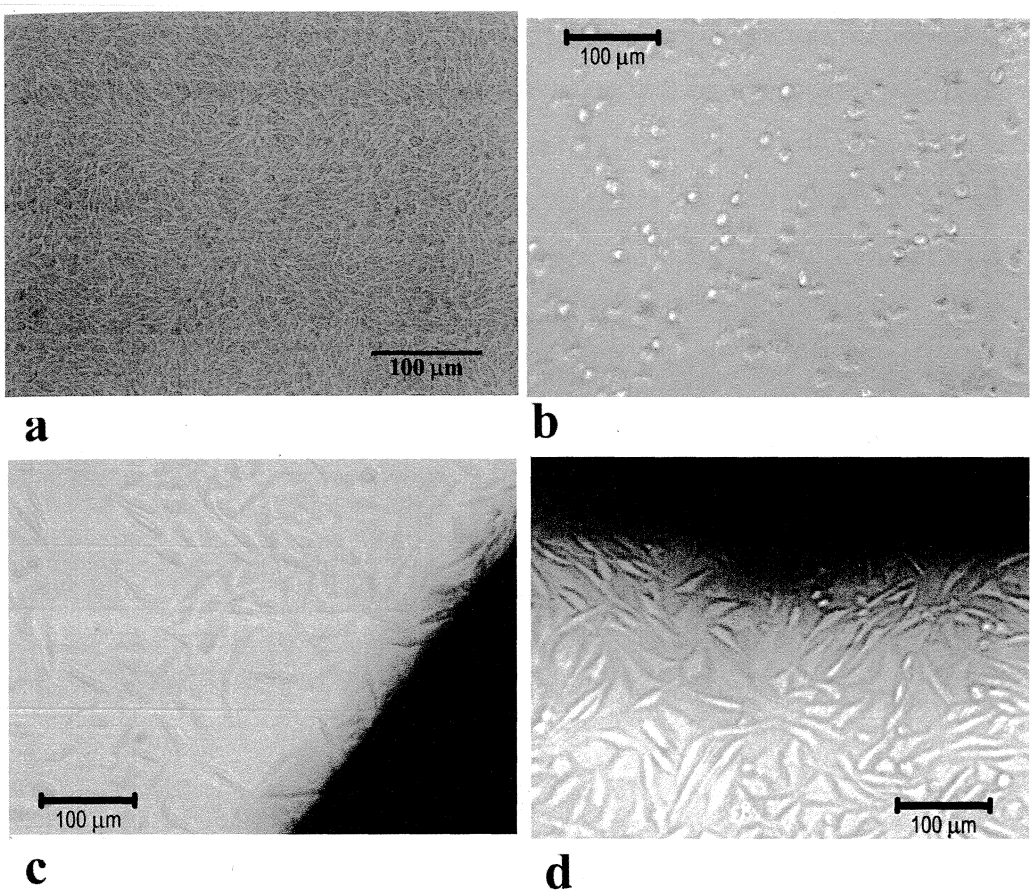


Fig 45. In vitro cytotoxicity studies of Gelatin-Albumin scaffold with L929 cells.
a) –ve control (PE), b) +ve control (PVC), c) GA 24 hrs, d) GA 10 days.

3.1.10.2. MTT ASSAY

The non toxicity of the PVA-PCL scaffold was further confirmed by treating the monolayer of L929 cells with extracts of the scaffold for a period of 1, 2, 4, 6, 8 and 10 days. The untreated cells served as control for each period of time. The metabolically active cells were quantified using MTT assay. The MTT dye is reduced by the mitochondrial dehydrogenases present in the live cells to purple formazan crystals. The soluble dye is quantified to represent the metabolically active cells. The results are graphically represented in Fig 46. No significant

difference was observed between the control untreated and the test samples in a period of 10 days. The data confirms that the material extracts do not produce any harmful effects to the cells. The extracts were prepared by incubating scaffolds in PBS with continuous shaking for 3 days at 37°C. The data indicates that even in an accelerated condition, no harmful components are leached out of the materials that are harmful to cells. MTT assay was not carried out for a longer time because the cell lines become confluent within this time period and may undergo cell death independent of treatment. This may bring a decrease in the OD value. However our further results indicate that the scaffolds do not generate any harmful substances and is suitable for long term cell culture.

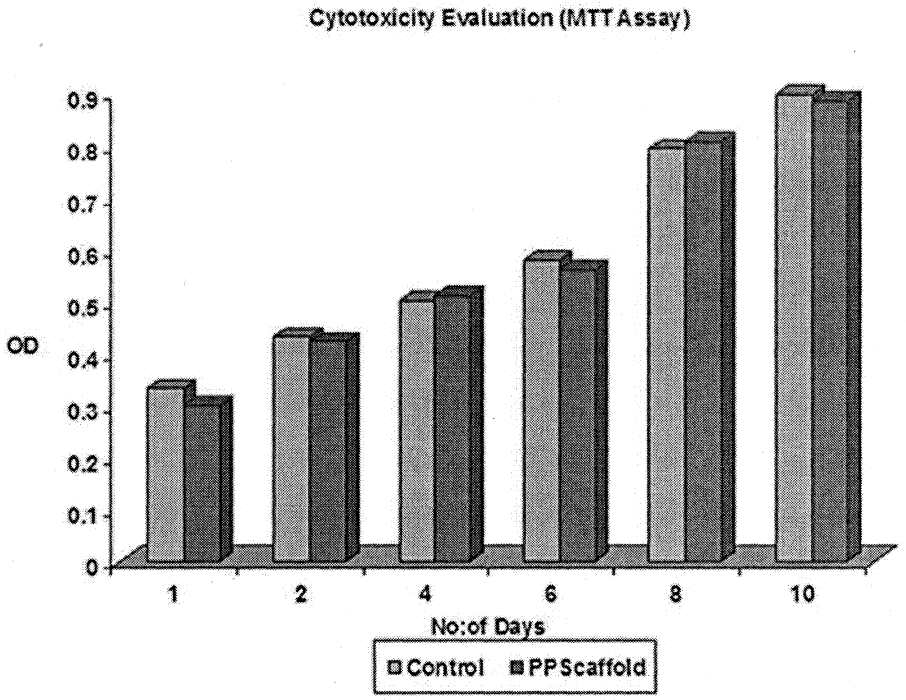


Fig 46. Evaluation of metabolically active cells exposed to extracts of the PVA-PCL (50:50) scaffold by MTT assay.

3.1.11. COMPARISON OF THE TWO 3 D SCAFFOLDS

To evaluate the first hypothesis, 3 dimensional scaffolds made of both natural and synthetic polymers were fabricated in this study. With a view that a 3D scaffold, made of single polymer cannot recreate a microenvironment similar to the extra cellular matrix, a hybrid material made of more than one polymer that mimic the properties of the native extracellular matrix is fabricated. The two hybrid scaffolds fabricated were a Semi IPN of PVA-PCL and a blend of Gelatin-Albumin. The scaffolds were fabricated using the conventional technique of freeze drying. Since synthetic polymers have reproducible properties which can be tailored according to our requirements, three compositions of PVA-PCL was fabricated. The results of the physico-chemical properties of the three compositions of PVA-PCL indicated that the properties vary with the concentration of the parent materials and one appropriate composition can be selected for our application.

The 3 D scaffolds were evaluated for their physico-chemical properties using various techniques. 3D Semi IPN scaffold exhibit synergistic properties of both the polymers. Physicochemical properties of Semi IPN depended on concentration of individual components as well as in proper interaction of polymeric chains. Fabrication process was appropriate to produce 3D scaffolds with a favorable porous structure to serve as a scaffold for TE applications. Both the PVA-PCL Semi IPN and Gelatin-Albumin scaffolds used in this study satisfy the general requirements to serve as a scaffolds for tissue engineering applications ie, cytocompatibility, mechanical stability and adequate 3 D pore morphology. At the same time, the two scaffolds varied in their chemical composition, medium uptake ability, mechanical property and in pore size. The basic structure of the PVA-PCL Semi IPN was that of mechanically stable PCL chains forming interpenetrating network with hydrophilic PVA. This was similar to the network of collagen and glycosaminoglycans in native cartilage. In Gelatin-Albumin scaffold the structure was like that of network proteins. The composition of two polymers

in the Semi IPN and the blend was confirmed from the results of thermal analysis and FTIR spectroscopy. The TGA results indicated the thermal stability of the scaffolds at the temperature of application. The PVA-PCL did not have any inherent functional moiety to favor the cell adhesion like the natural polymers Gelatin-Albumin. Both the scaffolds were highly porous but varied in the pore sizes. Gelatin-Albumin scaffold had uniform pores within a narrow range while the PVA-PCL possessed a wide range of pore size (8-192 microns). Both the scaffolds had high medium uptake ability which will promote efficient transfer of nutrients to all the cells. The medium uptake ability of Gelatin-Albumin was higher than PVA-PCL. The contact angle measurements indicated that both the scaffolds had a contact angle where a hydrophilic-hydrophobic balance is attained. The addition of hydrophobic PCL to PVA in the Semi IPN PVA-PCL and albumin to gelatin in the Gelatin-Albumin blend helped to attain this hydrophilic-hydrophobic balance. The PVA-PCL Semi IPN (50:50) composition which had the highest stability in culture medium along with was selected for the cell culture. Both the scaffolds had adequate mechanical stability to withstand the load imparted by the cells during invitro culture. The degradation rate of the PVA-PCL scaffold was comparatively low than Gelatin-Albumin scaffold. The degradation rate of the scaffold is expected to balance with the synthesis of the extracellular matrix. Both the scaffolds were found to be cell compatible as revealed from the initial cytotoxic tests carried out with L929 cells.

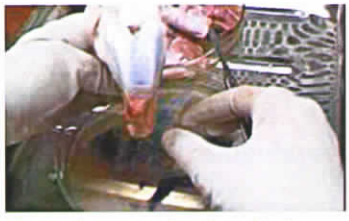
In conclusion two scaffolds that meet the general requirements of tissue engineering applications were fabricated to examine the matrix mediated effects in chondrogenesis. A hybrid material made of more than one polymer that mimics the properties of the native extracellular matrix is fabricated. The polymers and the fabrication methodology were selected to make 3D scaffolds suitable for chondrocyte culture. The two scaffolds varied in their physicochemical properties such as the chemical composition, medium uptake ability, pore characteristics, mechanical property. The chondrogenic response of cells in these scaffolds is further evaluated in this work.

3.2. SECTION 2

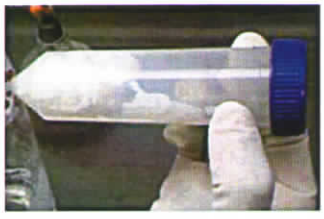
ISOLATION OF CELLS & SEEDING OF CELLS IN 3D SCAFFOLDS

3.2.1. ISOLATION OF CHONDROCYTES FROM PORCINE ARTICULAR JOINT

Hyaline cartilage is isolated from the articular joint of 3-4 months old pigs as per the accepted protocol. (*Kuettner et al 1982)* The isolated tissue had white glistening appearance with a wet weight of around 10-12 grams. The cartilage pieces of 0.5-1mm thickness were diced to small pieces and digested using collagenase type II in sterile conditions. The digestion conditions were optimized to completely digest the extracellular matrix as well as to retain the viability of cells. Soon after digestion cells appeared as single or aggregate and were surrounded by remnants of matrix. Photographs of the porcine articular joint, pieces of articular cartilage and the isolated chondrocytes are represented in Fig 47.



Porcine articular joint



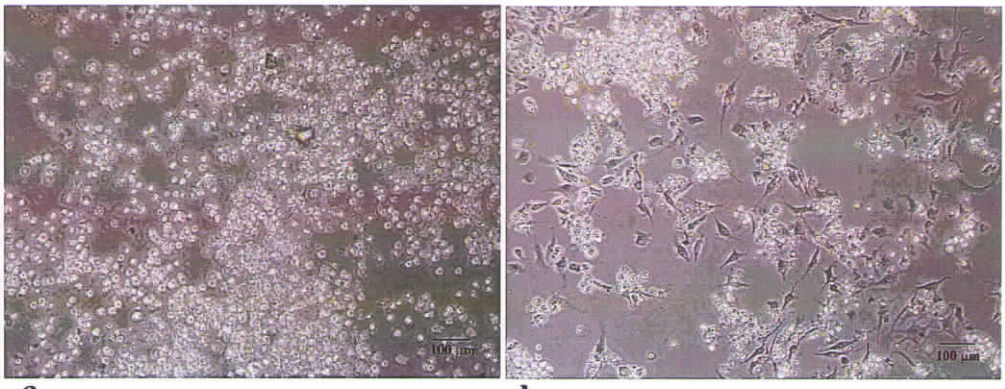
Pieces of cartilage

Porcine chondrocytes

Fig 47. Different stages of chondrocyte isolation

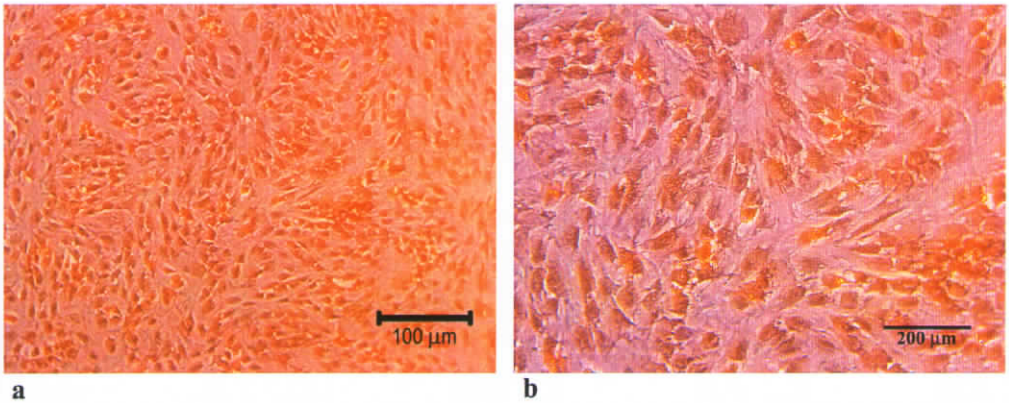
3.2.2. CHONDROCYTE CULTURE IN MONOLAYER

The microscopic gross morphology is usually the first and important characteristic that has to be recorded upon establishment of a cell population in culture. Soon after isolation, chondrocytes had a spherical morphology and remain viable with % viability greater than 90%. Unlike other cells they take longer time to attach to dishes and retain the spherical morphology even after attachment to culture flasks. Further on maintaining the culture they tend to loose the spherical morphology and start spreading on to the culture dishes. With increase in passage numbers, chondrocytes in the monolayer culture became polygonal or flattened and were closely attached to the adjacent cells. These observations were similar to several previous reports that the chondrocytes lose their typical round morphology and form a spindle fibroblast-like shape, and decrease cartilaginous protein synthesis when they are transferred to culture in a monolayer. This phenomenon is called dedifferentiation. (*Mark et al 1977*). The morphology of chondrocytes soon after isolation showing a spherical morphology and cells tending to become fibroblastic in monolayer culture is represented in Fig 48. It is known that the prevention of dedifferentiation or the preservation of chondrocyte quality, during proliferation culture, is one of the major requirements for invitro chondrocyte culture. The cells in monolayer were characterized for the production of glycosaminoglycans using safranin O stain. The red color of the dye indicates the production of glycosaminoglycans. Fig 49 represents the safranin O staining of monolayer culture in different magnifications.



a b

Fig 48. Chondrocytes in monolayer culture. a) Soon after isolation, b) tending to be fibroblastic in monolayer



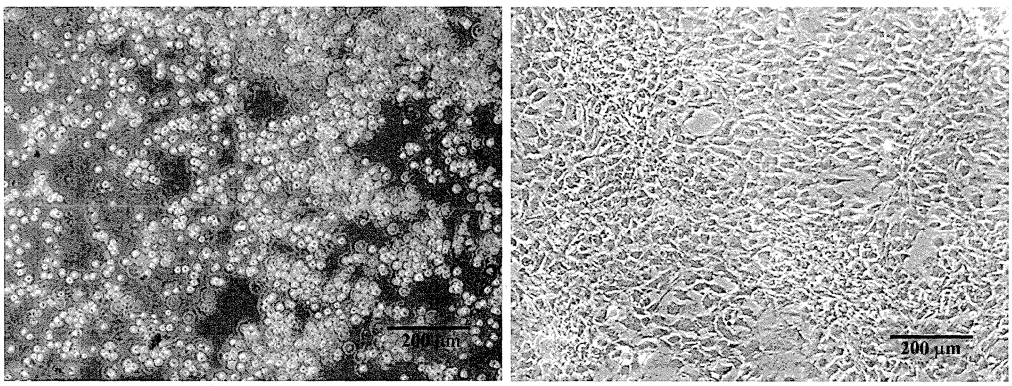
a b

Fig 49. Safranin O staining for production of glycosaminoglycans

3.2.3. EFFECT OF DEXAMETHASONE ON THE CELL MORPHOLOGY IN MONOLAYER CULTURE

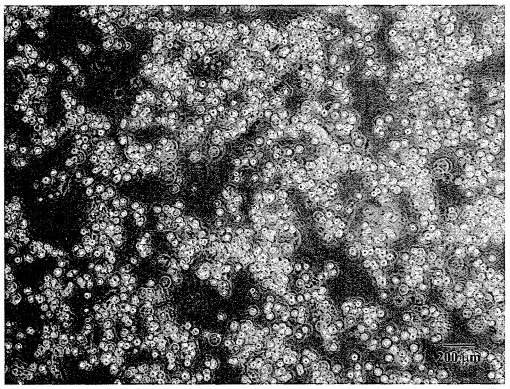
Chondrocytes when cultured in monolayer retain the spherical morphology only for a short period. They tend to spread and attach to the plastic dishes like fibroblasts. *Addition of dexamethasone to the culture medium delayed this* dedifferentiation process. It was found that without the addition of dexamethasone, cells tend to become fibroblastic in a period of 2 weeks. When dexamethasone was

added, the dedifferentiation process was further delayed in monolayer culture. Cells started to become fibroblastic only after a period of 1 month. Clusters of spherical cells could still be observed even in a period of 2 months. The phase contrast images of chondrocytes, without the addition of dexamethasone are represented in Fig 50. Fig 51 represents micrographs of chondrocytes in a period of 2 weeks, 1 month and 2 months cultured in presence of dexamethasone (dexamethasone +ve). Our observation correlates with previous reports that addition of dexamethasone helps to retain the chondrogenic phenotype. *(Derfoul et al 2006, Grigoriadis et al 1988).*

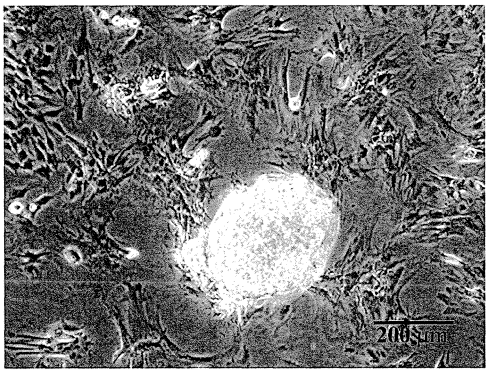


a. 5days (Without dexamethasone) **b. 2 weeks (Without dexamethasone)**

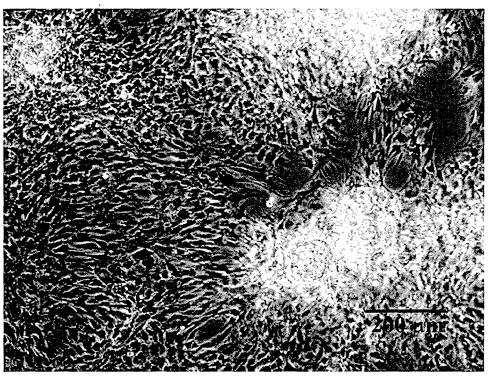
Fig 50. Morphology of chondrocytes in 2D culture, in the absence of dexamethasone



a. 2 weeks (Dexamethasone +ve)



b.1 month (Dexamethasone +ve)



c. 2 months (Dexamethasone +ve)

Fig 51. Morphology of chondrocytes in monolayer, cultured in presence of dexamethasone. a. 2 weeks, b. 1 month, c. 2 months

3.2.4. CYTOTOXICITY OF PVA-PCL AND GELATIN-ALBUMIN SCAFFOLDS WITH CHONDROCYTES

Primary cell cultures derived from enzymatic or mechanical disintegration of the tissue is propagated as an adherent monolayer or as a cell suspension. These cells are representative of the actual tissue. The toxicity of scaffold to primary chondrocytes was evaluated by direct contact test for 24 hrs and was compared to the control cytotoxic (PVC) discs and non toxic (PE) discs. The cells retain the typical characteristic morphology of chondrocytes in monolayer and were similar to the –ve control (PE discs). The cells exposed to + ve toxic material (PVC discs)

became spherical in shape. The results revealed that both the 3D PVA-PCL and Gelatin-Albumin scaffolds were nontoxic to chondrocytes and can be used for chondrocyte culture. Fig 52 represents the direct contact test of PVA-PCL and Gelatin-Albumin scaffold with chondrocytes in monolayer compared to the control materials.

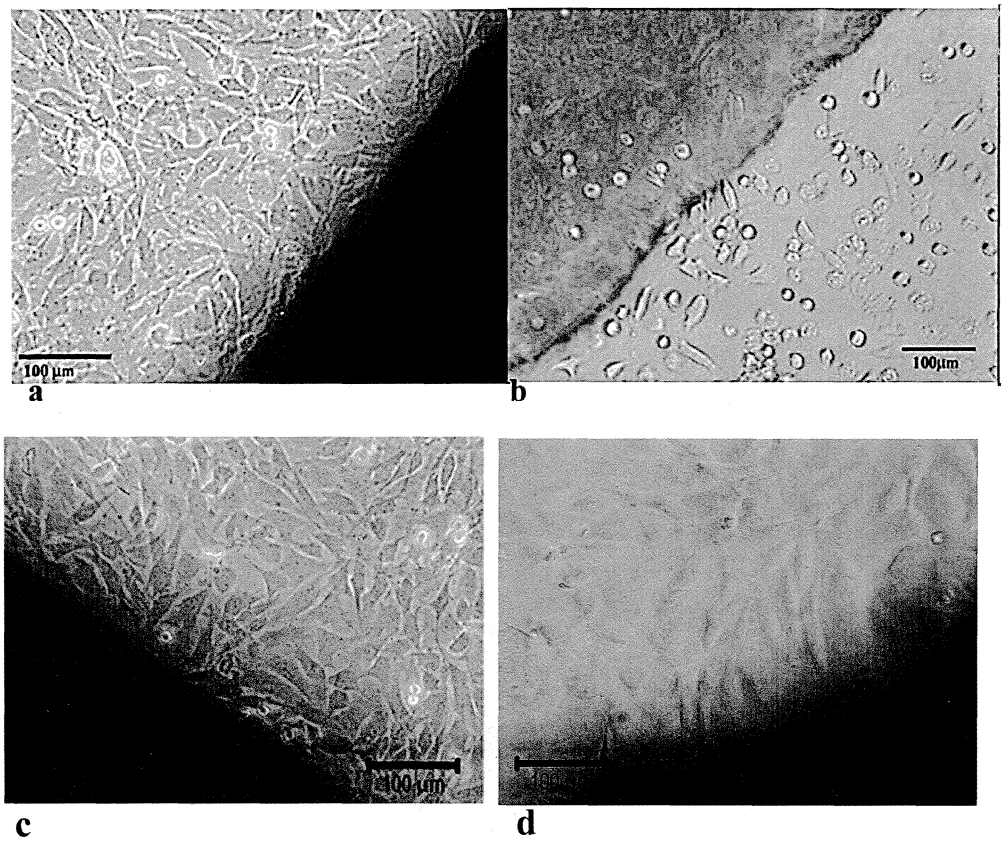


Fig 52. Direct contact test with chondrocytes. a) –ve control (PE), b) +ve control (PVC), c) PVA-PCL, d) Gelatin-Albumin

3.2.5. MORPHOLOGY OF CHONDROCYTES IN 3D SCAFFOLDS

Chondrocytes were seeded at a low density on to both the scaffolds and cultured for a week in order to examine the morphology of cells when seeded onto 3D matrix. The morphology of cells in one week constructs was examined under SEM. The micrographs (Fig 53) showed that all the seeded chondrocytes retain

their characteristic spherical morphology on the 3D PVA-PCL scaffolds. The cells were properly attached to the material and appear in clusters or as individual cells. Higher magnifications also showed the secretion of extracellular matrix like components that appeared as thin strands of fibres in the micrographs. However in Gelatin-Albumin scaffolds, the cells tend to attach and properly spread with an appearance similar to fibroblasts (Fig 54).

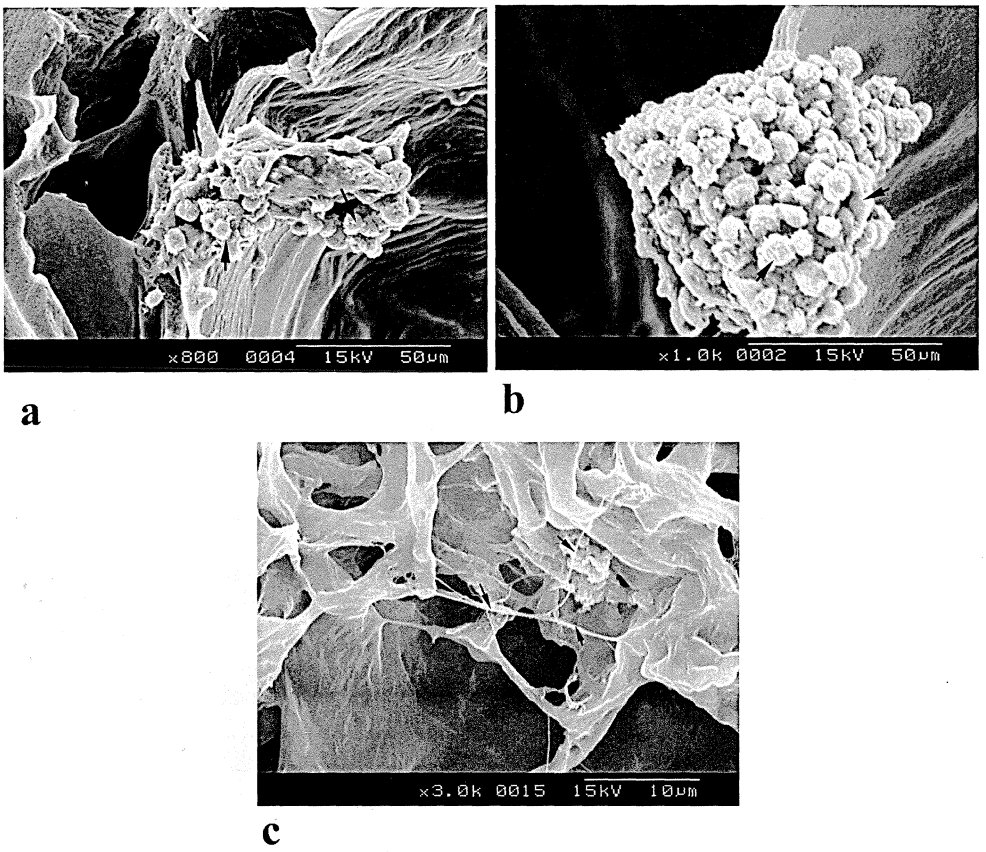


Fig 53. Chondrocytes retaining spherical morphology in 3D PVA-PCL scaffolds a) X 800, b) X 1000, c) cells secreting thin strands of extracellular matrix

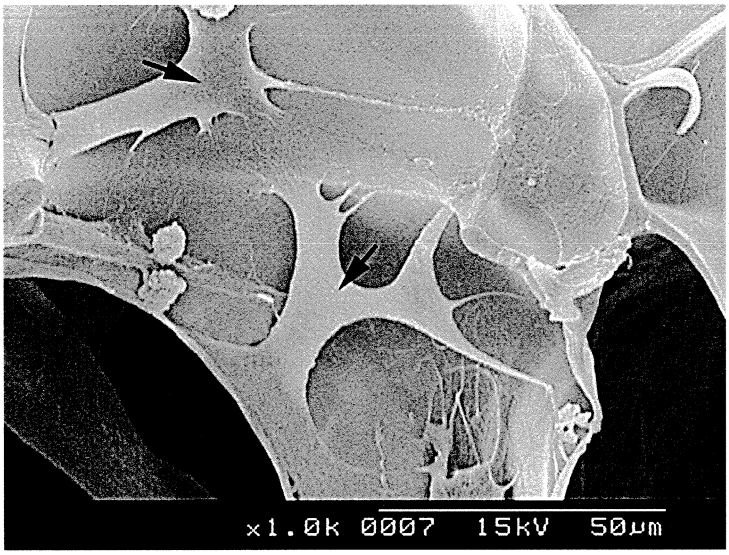


Fig 54. Chondrocytes with elongated morphology in 3D Gelatin-Albumin scaffolds

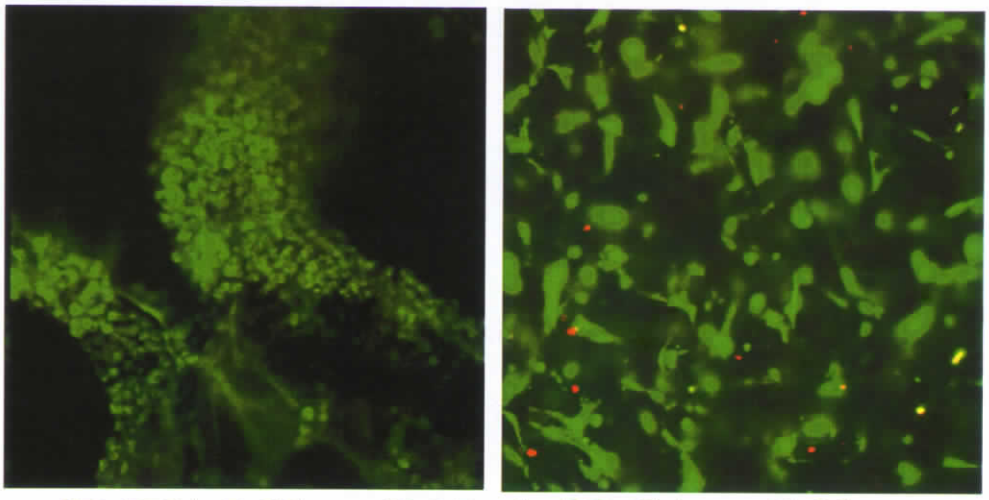
Cell adhesion on 3D scaffolds was markedly influenced by the physical and chemical properties of the material surface, which influence the non-receptor-mediated and receptor mediated attachment mechanisms. Non-receptor-mediated cell adhesion consists of a non specific cell-material interaction via weak chemical bonding (hydrogen bonding, electrostatic, polar, ionic interactions between molecules on cell membrane and functional chemical groups on the polymer substrate). On the contrary, the cells receptor mediated cell adhesion is due to the extra cellular matrix (ECM) molecules, such as fibronectin, vitronectin, collagen, and laminin. Cells bind specific amino acid sequences of these ECM molecules through integrin receptors. *(Bacakova et al 2004)* In synthetic PVA-PCL semi IPN, chondrocytes adhesion may be due to non-receptor-mediated as well as receptor mediated attachment. Adsorption of adhesion promoting proteins like fibronectin, laminin etc from the serum onto the material will help in the receptor mediated attachment. In Gelatin-Albumin scaffold the inbuilt biomimetic sequences stimulated the cell to attach and spread on to the material to form a fibroblastic

morphology. *Awad et al,* 2004 had reported a similar observation of flattening of chondrocytes in gelatin scaffold.

3.2.6. VIABILITY OF CHONDROCYTES SEEDED ON 3D SCAFFOLDS

Viability of chondrocytes seeded on to 3D scaffolds is evaluated by Live Dead assay and imaged using confocal microscopy. The chondrocytes remain viable and appear green in colour in both the 3D PVA-PCL and Gelatin-Albumin scaffolds. Fig 55 represents the confocal micrograph of chondrocytes in 3D PVA-PCL and Gelatin-Albumin scaffolds. The intracellular esterases present in the live cells cleave the Calcein AM dye to green fluorescent Calcein which is retained in the live cells with intact membrane. The Ethidium bromide binds to the DNA of the dead cells with damaged membrane and appears red in colour. The calcein dye is membrane impermeable and is retained within the cells that have an intact membrane. The fluorescent Calcein retained in the live cells therefore give information about the cell morphology. All the chondrocytes seeded in the PVA-PCL scaffolds appear spherical in shape while the chondrocytes in Gelatin-Albumin scaffolds had an elongated morphology. This was similar to the observation of scanning electron micrographs. The Live Dead assay thus confirms that the round cells in the 3D PVA-PCL scaffold are alive and maintain the characteristic chondrocyte morphology and hence this indicate this is a nontoxic material. The open interconnected porous structure of 3D scaffolds hence help to retain the viability of cells seeded in it by providing adequate nutrients. The Gelatin-Albumin scaffold might possess some biomimetic sequences that binds to cell surface receptors which in turn enhance cell attachment and spreading, thus preventing the chondrocytes from retaining its spherical morphology. The confocal microscopy permitted imaging of cells seeded at different planes in the 3D scaffold. The data thus confirms that chondrocytes are viable and able to maintain

the specific morphology in PVA-PCL scaffold, while the morphology is changed on seeding in Gelatin-Albumin scaffold.

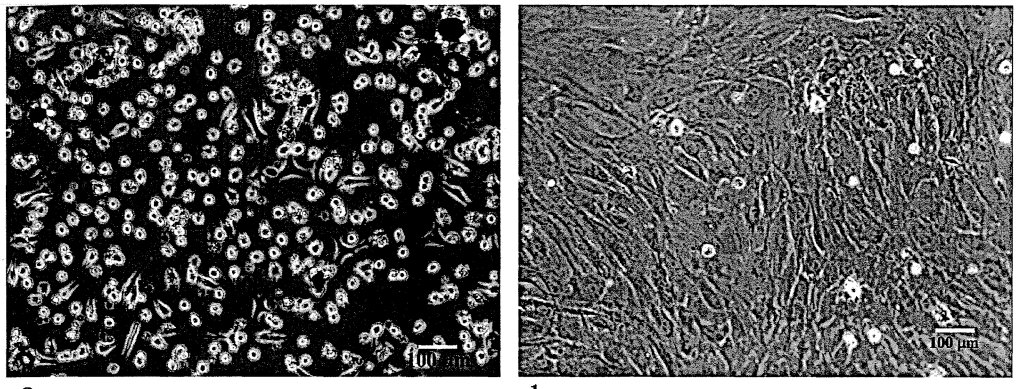


a. 3D PVA-PCL scaffold **b. 3D GA scaffold**

Fig 55. Confocal image of chondrocytes in 3D PVA-PCL and Gelatin-Albumin scaffolds

3.2.7. ISOLATION OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells isolated from 3 week old male wistar rats were maintained in culture for 3 days. The media was removed after 3 days and cultured in fresh media. The cells have attached and started spreading on to culture dishes in days. The mesenchymal stem cells possessed a fibroblastic morphology and attained confluence within 10 days. Fig 56 represents mesenchymal stem cells in 3 day culture (a) and cells in confluence in 10 days (b). Upon confluence the cells were trypsinized and used for experiments.



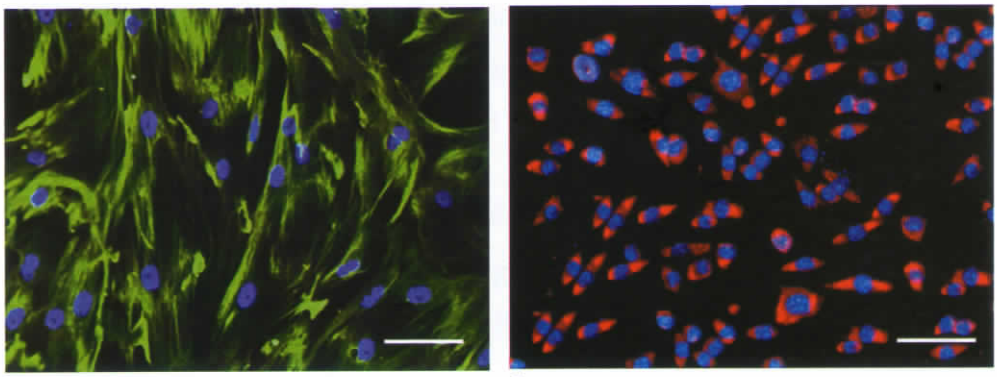
a b

Fig 56. Mesenchymal stem cells in culture. a)) 3 days, b) Cells in confluence.

3.2.8. CHARACTERIZATION OF MESENCHYMAL STEM CELLS

The cells were found positive for markers vimentin and CD105. FITC conjugated vimentin appeared green in colour and the nucleus of the cells were counter stained using hoechst in Fig 57.a. Vimentin is a general marker of cells originating in the mesenchyme. It is a member of the intermediate filament family of proteins. Intermediate filaments are an important structural feature of eukaryotic cells. They, along with microtubules and actin microfilaments, make up the cytoskeleton. Vimentin plays a significant role in supporting and anchoring the position of the organelles in the cytosol. Therefore, it is generally accepted that vimentin is the cytoskeletal component responsible for maintaining cell integrity. Essentially, vimentin is responsible for maintaining cell shape, integrity of the cytoplasm, and stabilizing cytoskeletal interactions. Cytoskeletal intermediate filaments constitute a diverse group of proteins that are expressed in a highly tissue-specific manner. Intermediate filaments are constructed from two-chain, α-helical, coiled-coil molecules arranged on an imperfect helical lattice and have been widely used as markers for distinguishing individual cell types within a tissue.

The cells were found to be positive for phycoerythrin conjugated CD105 marker which appeared red in colour in Fig 57.b. Endoglin (P3D1) (CD105) is another marker of mesenchymal stem cells. Endoglin is a member of TGF β type III receptor super family. It is a 75 kDa receptor that binds ligands of the TGF β super family via association with the respective ligand binding receptors for TGFβ1, TGFβ3, Activin-A, BMP-2 and BMP-7.



a b

Fig 57. Mesenchymal stem cells characterized for (a) vimentin (green), (b) CD 105 (red) and nuclei (blue) in colour

3.2.9. EFFECT OF SEEDING TECHNIQUE IN DISTRIBUTION OF CELLS ON 3D SCAFFOLD

One of the initial important steps in developing a construct is the seeding of cells onto the scaffold. Seeding methodology plays an important role for subsequent tissue development in a 3D structure. There are previous reports that compared to static methods, dynamic seeding methods yields a higher seeding efficiency and more even distribution of cells. Most of the previous reports used bioreactor system. The effect of seeding methodology in cell distribution pattern in our scaffolds was evaluated by employing two seeding methodologies. In static seeding method, cell suspension was dropped on to scaffold using a pipette to both

sides of the scaffold at an interval of 3 hours. In dynamic seeding, a simple technique of incubating scaffold in cell suspension with continuous shaking at 300 rpm for a period of 6 hours was employed. Further the distribution and viability of cells on both the scaffolds was evaluated by scanning electron microscopy and live dead assay respectively.

3.2.9.1. SCANNING ELECTRON MICROSCOPY

The over all distribution pattern of cells on the surface was evaluated by scanning electron microscopy. Micrographs of different magnifications were taken to examine the distribution pattern and cell morphology.

Fig 58 represents mesenchymal stem cells on PVA-PCL scaffold by static seeding methodology in different magnifications. Most of the cells were seen on the surface. The distribution of cells was uniform over the surface. Lower magnifications showed the arrangement of cells in a circular pattern around the pores of the scaffold denoted by white arrows in Fig 58.a and Fig 58.b. The cells were well attached and located along the margins of the pores as observed in Fig 58.c. Not much cells could be seen within the pores.

The pattern of distribution was different in dynamic seeding method compared to the static seeding method. Scanning electron micrographs at lower magnifications showed only few cells on the surface of the scaffold. The cells on the surface were properly attached and spread. Cells did not accumulate around the pores in a circular fashion as observed in static method, but moved into the pores. The scanning electron micrograph of cell seeding by dynamic method is represented in Fig 59. a. Lower magnification, showing over all distribution pattern of cells on surface. b. Properly attached cells on the surface. c. Cells inside the pores.

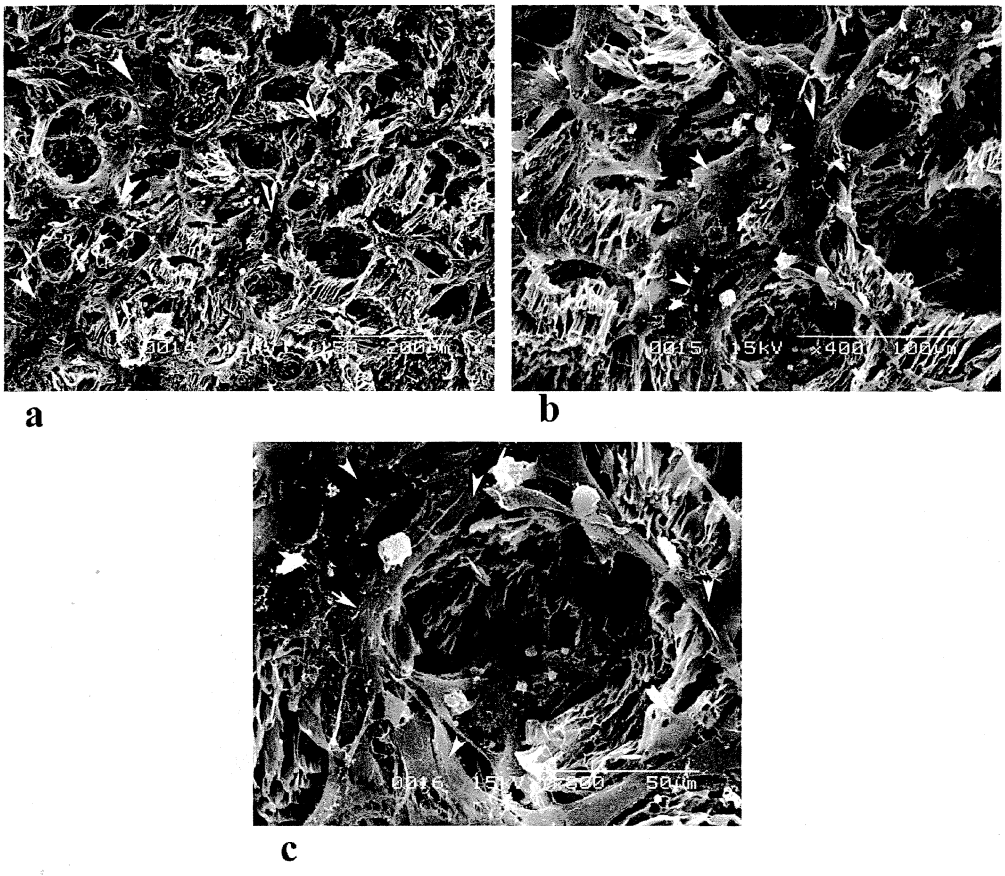


Fig 58. Arrangement of mesenchymal stem cells in a circular pattern, along the margins of the pores of the scaffold, in static seeding methodology, in PVA-PCL scaffold.

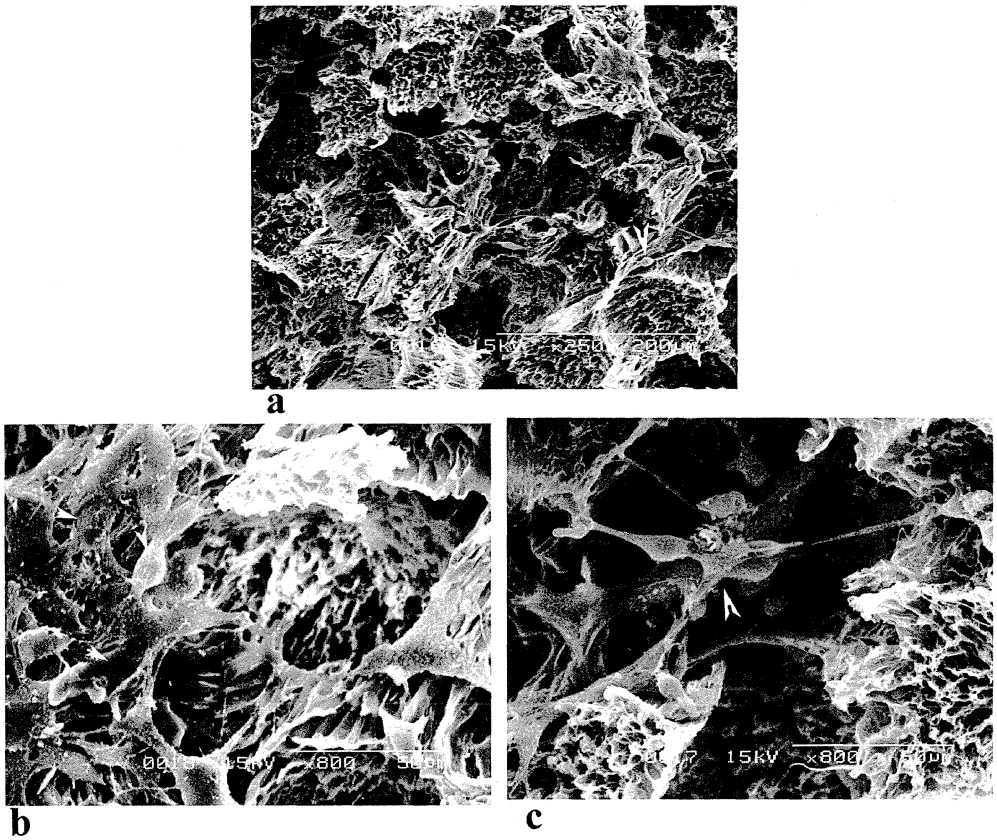
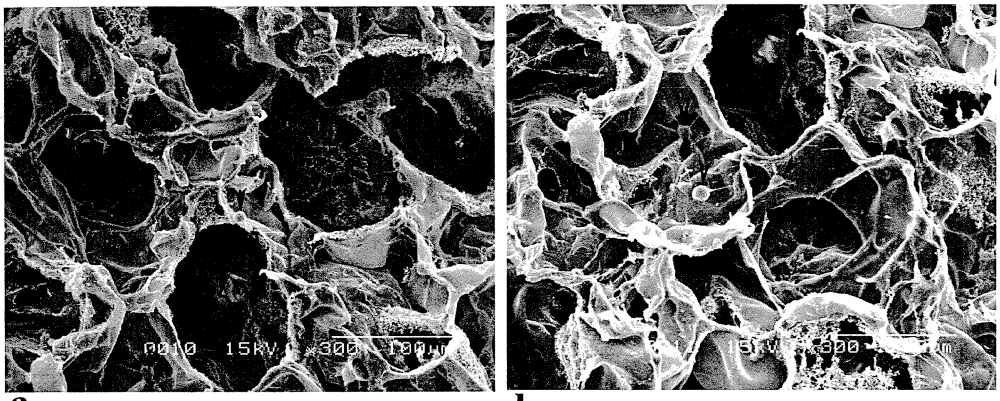


Fig 59. Distribution of mesenchymal stem cells on PVA-PCL scaffold by dynamic seeding methodology. a) Lower magnification, showing over all distribution patterns of cells on the surface of the scaffold. b) Properly attached cells on the surface. c) Cells inside the pores.

The experiment was carried out using less number of cells so the overall number of cells observed was low. Comparing the two methods in PVA-PCL scaffold it was observed that in static seeding method using pipette, cells mostly reside on the surface. Information regarding the cells in the interior of the scaffold could not be obtained using SEM micrographs. However in dynamic seeding method, the continuous stirring caused the cells in suspension, to uniformly distribute over the scaffold. The circular stirring motion also caused the cells to move into the pores. Thus it is inferred that the dynamic seeding method promotes the penetration of cells into the pores for uniform distribution throughout the entire

thickness of the scaffold. Information regarding the distribution of cellular pattern in 3D scaffold was further obtained from the confocal images of live dead assay.



a **b**

Fig 60. Distribution of mesenchymal stem cells on Gelatin-Albumin scaffold by a) static and b) dynamic seeding methodology

The results of the two seeding methodologies in Gelatin-Albumin scaffolds showed a similar pattern of cell distribution on the surface. The scanning electron micrographs of the cells seeded by static and dynamic seeding methods are represented in Fig 60. Less number of cells was observed on the surface of the scaffolds in both the seeding methods. The high uptake of medium by gelatin albumin scaffolds would have promoted the diffusion of cells into the interior of the scaffold.

3.2.9.2. CELL VIABILITY

It is essential to evaluate the viability of cell in the scaffold seeded by two methodologies. For this the cell seeded constructs were stained using Calcein AM and EtBr and imaged using confocal microscopy. This assay not only gave information regarding the viability of the cells but also on the over all distribution pattern of cells in the scaffold. The Calcein dye retained by the live cells with intact membrane showed the morphology of the cells.

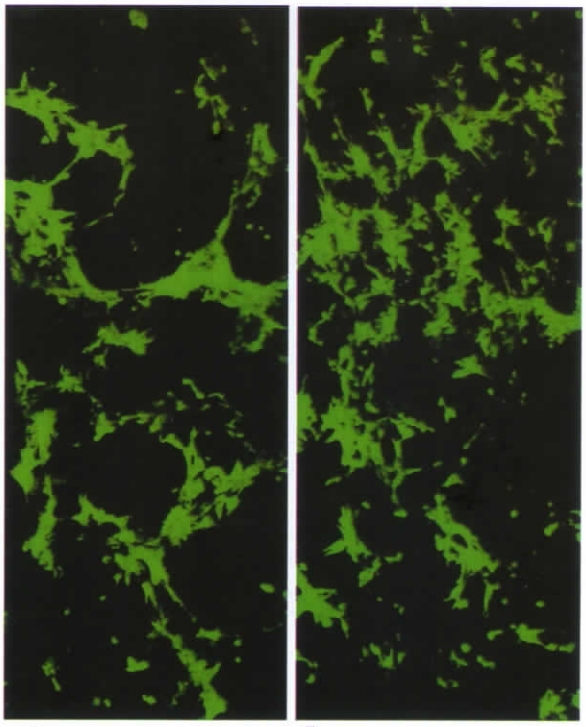
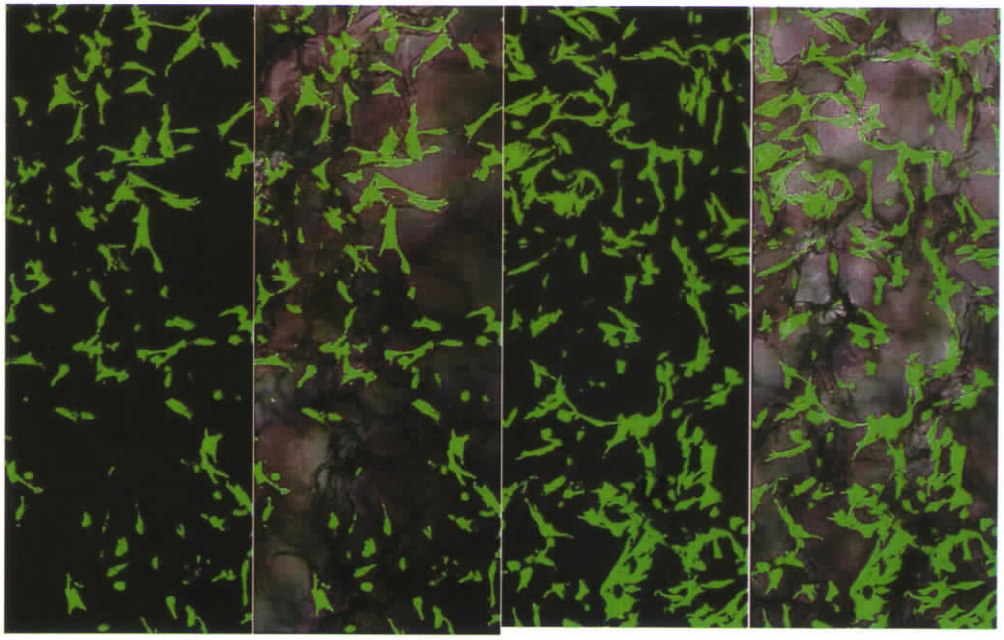


Fig 61.a. Viability of mesenchymal stem cells on PVA-PCL scaffold seeded by a) static and b) dynamic seeding methodology.

The confocal images of the live dead assay indicated the presence of viable cells in both the seeding methodologies. Unlike the scanning electron micrograph, confocal images gave information regarding the cell distribution throughout the thickness of the scaffold. It also gave information regarding the cell morphology. The viable cells in both the seeding methodologies in PVA-PCL scaffold is represented in Fig 61. The cells are well spread in both the seeding techniques. The image also indicated that the stirring speed and shear caused during the dynamic seeding did not affect the viability of the cells. The circular seeding pattern of cells around the pores in static seeding is also evident in the image. The dynamic seeding showed attachment of more number of cells than the static seeding method.



a b

Fig 62.a. Viability of mesenchymal stem cells on Gelatin-Albumin scaffold by a) static and b) dynamic seeding methodology.

The viability of cells in Gelatin-Albumin scaffold seeded by two techniques is represented in Fig 62. The second frame of Fig 62.a and Fig 62. b shows the cells, with the scaffold imaged in gray scale. The cells remain viable in both the seeding techniques in this scaffold. The scanning electron micrographs could not give much information about the distribution pattern of cells in this scaffold. The confocal images showed that the cells were uniformly distributed within the scaffold in both the techniques. There was not much difference in the distribution pattern of cells. However the dynamic seeding method showed comparatively higher number of cell attachment than the static seeding.

On comparing the two seeding methods on each scaffold, it was found that more number of cells attached in the dynamic seeding methodology than the static seeding. Moreover the stirring in dynamic seeding also promoted the penetration of cells to the interior of the scaffolds while in static seeding method, cells resided on the surface of the scaffold. The shear caused by the cells did not the affect the

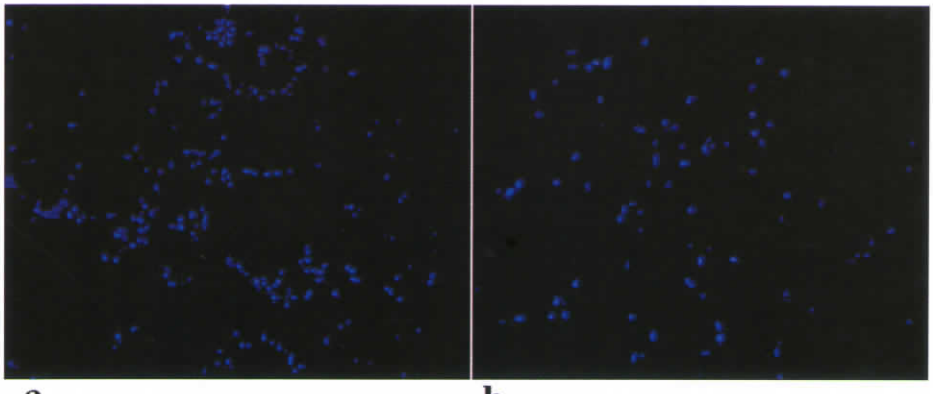
viability of cells. On comparing the two seeding techniques between the two scaffolds it was found that effect of seeding also depends on the pore structure of the 3D scaffold. The dynamic seeding produced a significant difference in the number and the distribution pattern in PVA-PCL scaffold than the Gelatin-Albumin scaffold. The pore characteristic of Gelatin-Albumin scaffold was different from the PVA-PCL scaffold. In Gelatin-Albumin the pore size was comparatively higher than the PVA-PCL in the swollen state in medium. This and the high swelling caused faster penetration of cells deeper into the interior regions of the scaffold even without any stirring. However the dynamic seeding enabled the attachment of more number of cells in this method. The cell distribution was also different from PVA-PCL. These data revealed that the distribution pattern of cells also depends on the pore characteristics of the 3Dscaffolds.

Since the dynamic seeding was found to be better in both the scaffold, this methodology was employed for further seeding of cells during the long term culture. In this study we did not employ any bioreactors for the uniform seeding. Bioreactor culture is the most commonly employed technique for uniform seeding of cells in 3D scaffolds. Dynamic seeding is a simple technique of continuous stirring at 400 rpm in circular motion which provided uniform distribution of cells in our scaffolds retaining the viability of cells. However it is necessary to explore the efficiency cell seeding using bioreactor in our future experiments.

3.2.10. DISTRIBUTION PATTERN OF CELLS IN 3 D SCAFFOLDS

The distribution pattern of cells in the two scaffolds seeded by dynamic seeding method was examined by staining the nuclei of cells with Hoechst dye. The constructs were imaged using confocal microscopy at XYZ planes. Both the scaffolds were scanned by uniform number of planes to compare the distribution pattern. The confocal images of cell seeded PVA-PCL and Gelatin-Albumin scaffold by dynamic method is represented in Fig 63. It was observed that the PVA-PCL showed more fluorescence than the Gelatin- Albumin within the region

of scanning. The scaffold was scanned in 56 different Z sections to confirm that, the cells seeded on the Gelatin-Albumin scaffolds remain within the interior of the pores, and have not been washed off. The images were combined together and represented as a single image in Fig 64. The result indicated that the cells have moved into the interior of the scaffold during the uptake of the medium and remain attached. Taken together it was found that both the scaffolds promote a uniform cell distribution in dynamic seeding. In Gelatin-Albumin the high swelling of the scaffold increased the surface area than PVA-PCL. With the increase in surface area the cell density in a particular area is decreased. Scanning of scaffolds in the Z sections indicated that the cells were not washed off but remain within the scaffold.



a b

Fig 63. The confocal images of cell distribution in PVA-PCL and Gelatin-Albumin scaffold seeded by dynamic method.

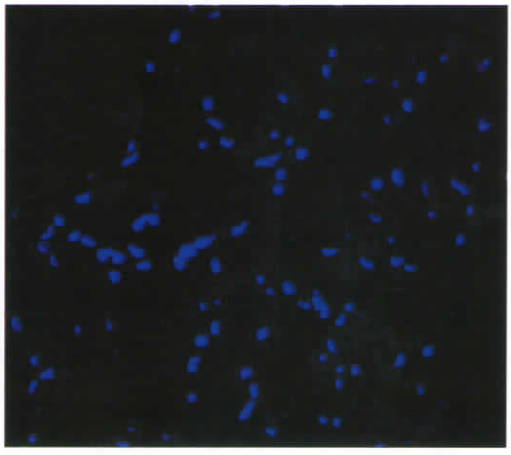


Fig 64. The cells in Gelatin-Albumin scaffolds distributed within the interior of pores.

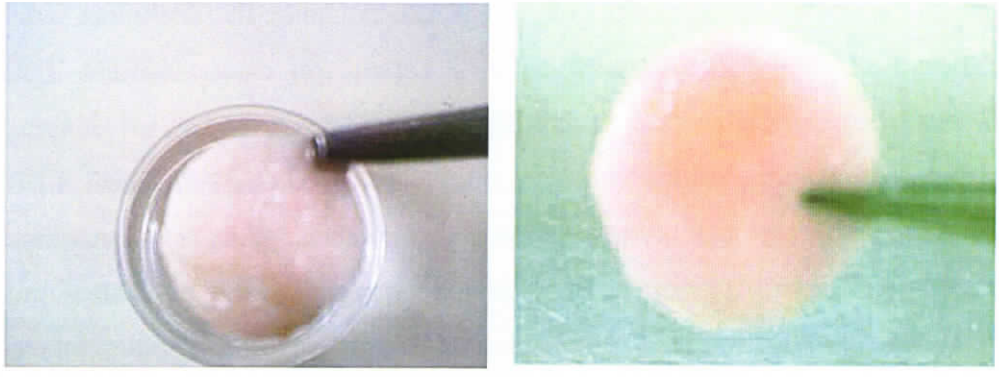
3.3. SECTION 3

CHONDROCYTE CULTURE IN 3D SCAFFOLDS

The PVA-PCL and gelatin albumin scaffolds of size 10 mm diameter and 2-3 mm thickness were seeded with porcine chondrocytes at a density of 1 X 10^6 cells/ scaffold. The cells from passage 0 were used for seeding to ensure its chondrogenic phenotype. The cell seeded scaffolds were placed in polypropylene vials and cultured in chondrogenic medium with dexamethasone for 2 months. At the end of the culture period the constructs were retrieved and processed for evaluation using SEM, biochemical analysis and staining. The culture was continued in one set of constructs for 4 months and was processed for SEM and biochemical analysis.

3.3.1. MACROSCOPIC OBSERVATIONS

The 2 month old PVA-PCL constructs had a white glistening appearance with deposition of matrix like components. The construct was stable in culture and had good handling property. The cell seeded Gelatin-Albumin construct also showed a glistening appearance and was soft on visual observation (Fig 65). The Gelatin-Albumin construct was stable in culture but had less handling properties when compared to the PVA-PCL construct.



a. **b.**

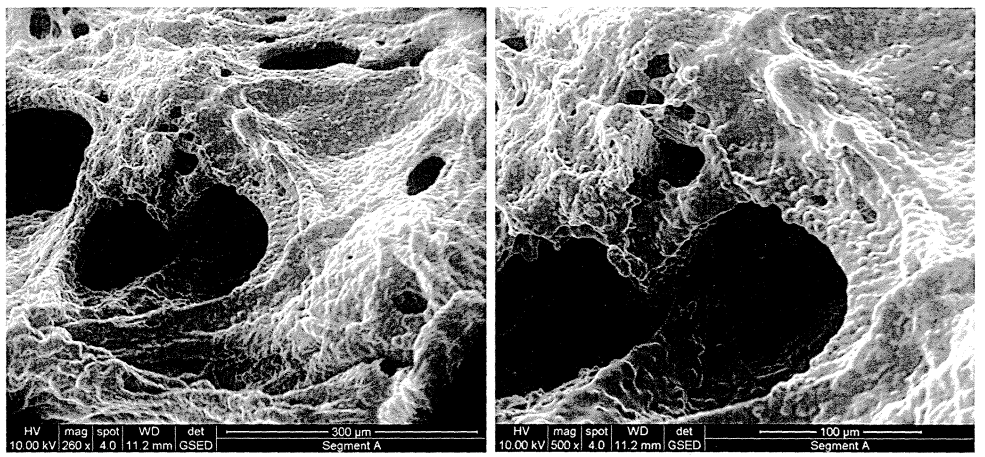
Fig 65. The 2 month old chondrocyte cultured construct a.PVA-PCL & b. GA

3.3.2. SCANNING ELECTRON MICROSCOPY

3.3.2.1. PVA-PCL

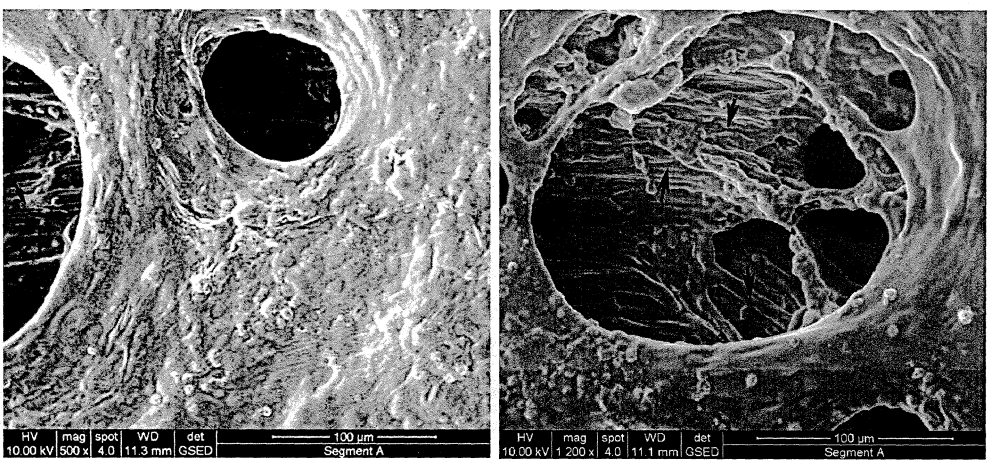
The cell morphology, over all distribution and deposition of extracellular matrix components on the surface of the 2 month and 4 month old constructs, were examined using scanning electron microscopy. The micrographs of 2 month old PVA-PCL constructs revealed, well attached cells, retaining their typical spherical morphology. The cells have secreted ECM-like components and were entrapped in it. The ECM formed a layer on the surface masking the minute pores of the 3D scaffolds. Micrographs also showed some deposition of bundles of fibres that run parallel to each other, with chondrocytes attached to these fibres. This structure was similar to the bundles of collagen fibres in the native cartilage. The 3 dimensional and interconnected pore structure of our Semi IPN scaffold is expected to have helped the chondrocytes, to retain the spherical morphology as in the native tissue.

The 4 month old construct was completely covered with ECM like components and not many cells could be observed on the surface. In higher magnification, some spherical cells were observed in some areas of the scaffold. The surface properties with a balance of hydrophilic-hydrophobic moieties, might have promoted cell adhesion and high deposition of ECM components. The PVA-PCL scaffolds were not coated with cell friendly molecules or modified with peptides for cell adhesion. These results indicated that the hybrid scaffolds of PVA -PCL Semi IPN, provided a favorable environment for the cells to secrete matrix components even in absence of bioactive growth factors. Thus the scaffold is implicated to have immense potential for cartilage tissue engineering. The scanning electron micrograph of chondrocyte seeded scaffold, cultured in vitro for 2 months is represented in Fig 66, in different magnifications. Fig 67 represents deposition of extracellular matrix components that resembles bundles of collagen fibers. Fig 68 shows the SEM images of 4 month old constructs.



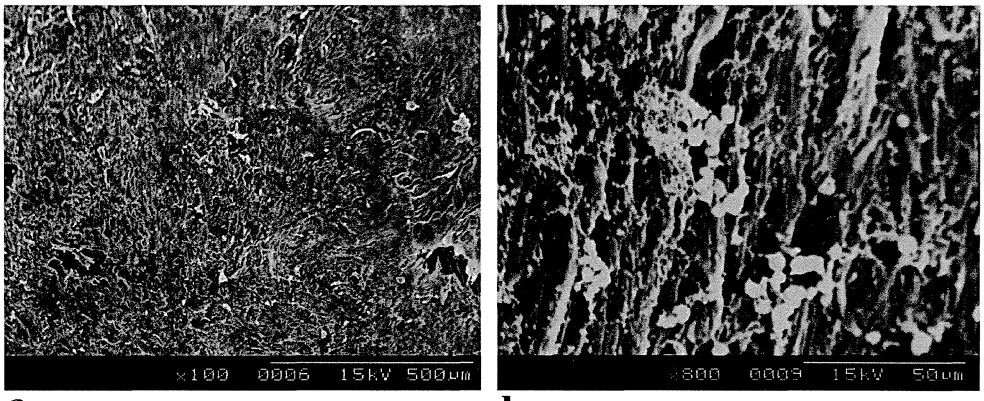
a b

Fig 66. The SEM images of 2 month old PVA-PCL construct showing the uniform distribution of chondrocytes with spherical morphology.



a b

Fig 67. The SEM images of 2 month old PVA-PCL construct at two different magnifications indicating deposition of collagen fibres arranged in the form of bundles.



a b

Fig 68. The SEM images of 4 month old PVA-PCL constructs in two different magnifications.

3.3.2.2. GELATIN-ALBUMIN

The surface analysis of the 2 month old Gelatin-Albumin constructs showed high deposition of extra cellular matrix components, which completely masked the pores of the porous scaffold. The micrographs did not give much information regarding the shape of cells. It was difficult to differentiate between the deposited extra cellular matrix and the well spread cells. However no spherical cells could be observed on the surface. SEM images of 4 month old constructs, at low magnification showed, that most of the pores were covered by matrix, leaving very few pores to be visible. Higher magnification showed a smooth surface with the deposition of these ECM molecules. Not much information could be obtained regarding the shape of the cells, as it was masked by ECM constituents. Fig 69 represents the Gelatin-Albumin constructs covered with extra cellular matrix like components, in different magnifications.

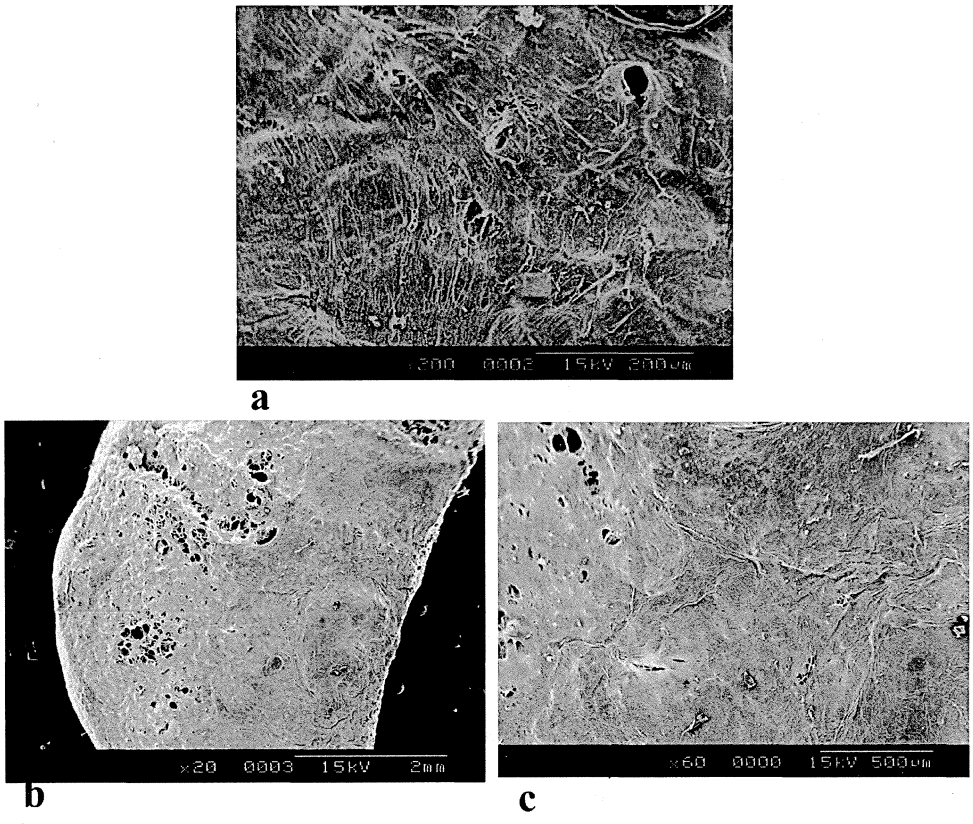


Fig 69. Scanning electron micrographs of 2 months and 4 months old Gelatin-Albumin construct showing high deposition of matrix components that masks the pores of the 3Dscaffold.

3.3.3. TOTAL GLYCOSAMINOGLYCAN CONTENT

The constructs were digested with papain solution and the total glycosaminoglycans were estimated using DMMB assay. The data is represented graphically in Fig 70.There was a significant difference in the total glycosaminoglycans content between the chondrocyte seeded PVA-PCL and the Gelatin-Albumin constructs. A significant increase in the GAG content from 2 months to 4months was observed both in PVA-PCL and Gelatin-Albumin scaffolds. Statistical analysis was performed using one way ANOVA test. The

values were expressed as the mean ± of four different replicates. Values of $p<0.05$ were considered significant (*) and $p<0.0001$ were considered very significant (**). The data suggests that the PVA-PCL scaffolds provide a favorable environment for cell adhesion, maintaining the cell morphology as well as for secreting the glycosaminoglycans. The gelatin albumin scaffold also promoted the secretion of glycosaminoglycans but the total GAG content was less than the Semi IPN.

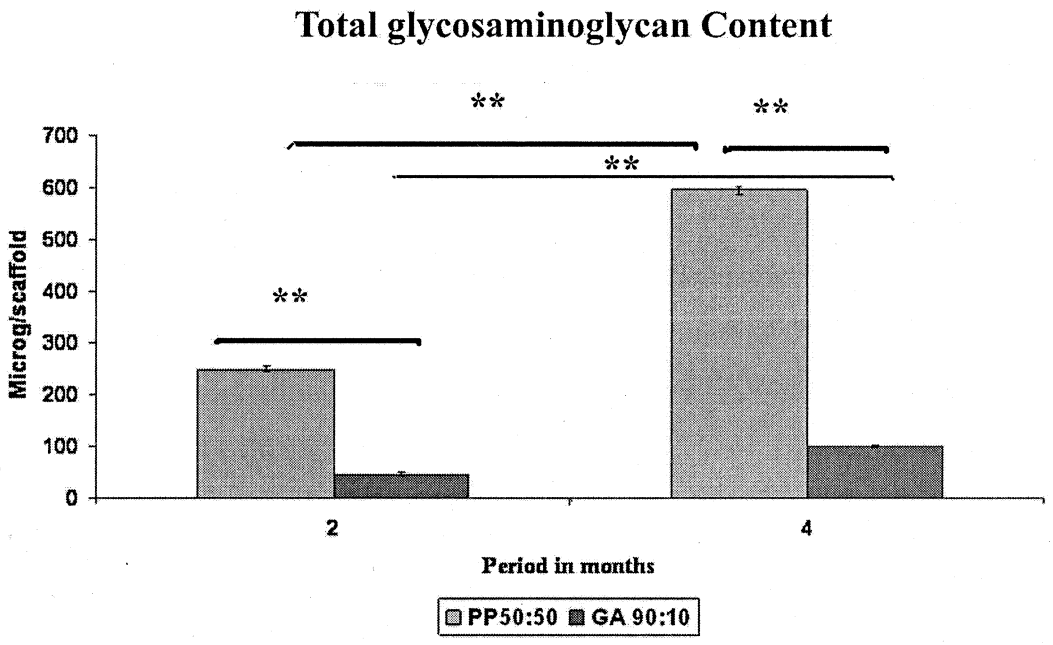


Fig 70. Total glycosaminoglycans content in the 3D constructs in 2 and 4 months

3.3.4. HISTOLOGICAL STAINING OF THE CONSTRUCT

The two most definable elements used to study cartilage regeneration are type II collagen and glycosaminoglycans. Collagen type II is used to assess the differentiation state of resident chondrocytes. Glycosaminoglycan is used to assess many aspects of cartilage status like cartilage quality, chondrogenic capacity and as a guide to extracellular matrix maturity.

3.3.4.1. STAINING FOR GAG USING SAFRANIN O DYE

The deposition of glycosaminoglycans throughout the thickness of the construct was evaluated by Safranin O staining of paraffin sections of the constructs. *Grogan et al 2006* have reported that the quality of an invitro generated cartilage can be graded from Safranin O- Fast green stained sections of the construct. The sections of chondrocyte seeded PVA-PCL constructs showed uniform distribution of glycosaminoglycans. The intense red staining of the sections represents very high deposition of glycosaminoglycans. Cells were also evenly distributed in the matrix. Based on the report by Grogan et al, a high grade section shows uniform, dark, even stain of GAG, extensive matrix, low cell density with moderate distance between cells and cells with round morphology. Studies have reported that in native tissue, chondrocytes are evenly spaced and they are arranged favorably to actively deposit and remodel the extracellular matrix. *(Jadin et al 2007)* These features were very evident in sections of PVA-PCL construct. The Safranin O staining of 2 month old PVA-PCL construct in different magnifications is represented in Fig 71.

The Gelatin-Albumin scaffold also showed deposition of glycosaminoglycans, but the secretion was confined to specific areas on the sections. A uniform distribution was not observed. The GAGs were located mostly at the edges of the sections. However a uniform distribution of ECM was observed on the surface, in scanning electron micrographs. This indicates that most cellular activity was confined to the scaffold periphery, where intense staining was observed. Sections deep within the interior of the scaffolds did not show much accumulation of extracellular matrix components. Fewer cells were observed in this matrix. The formation of a skin layer of cells and ECM on the surface of the scaffold might have prevented the supply of nutrients to the interior of the scaffold, resulting in poor deposition of GAG. Fig 72 represents the Safranin O staining of sections of Gelatin-Albumin construct in different magnifications. Studies have

shown that safranin O intensity positively correlates with the glycosaminoglycans content measured using biochemical analysis.

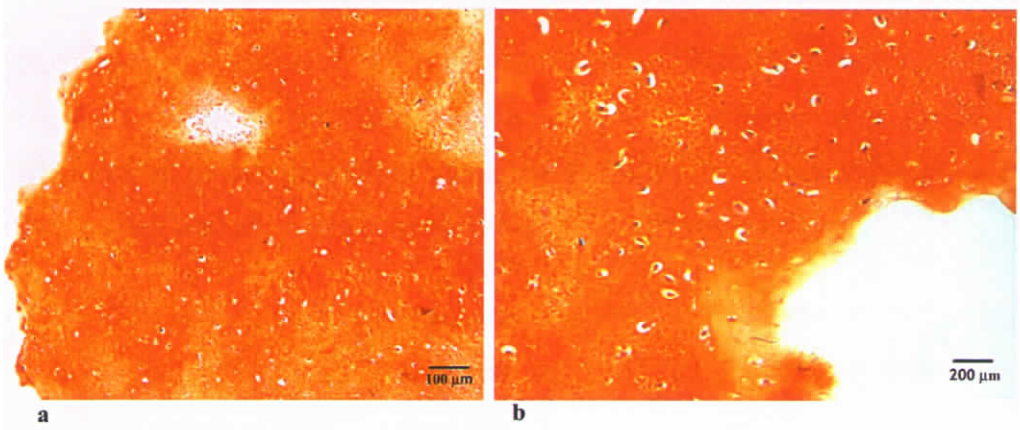


Fig 71. Safranin O staining of sections of PVA-PCL construct in different magnifications

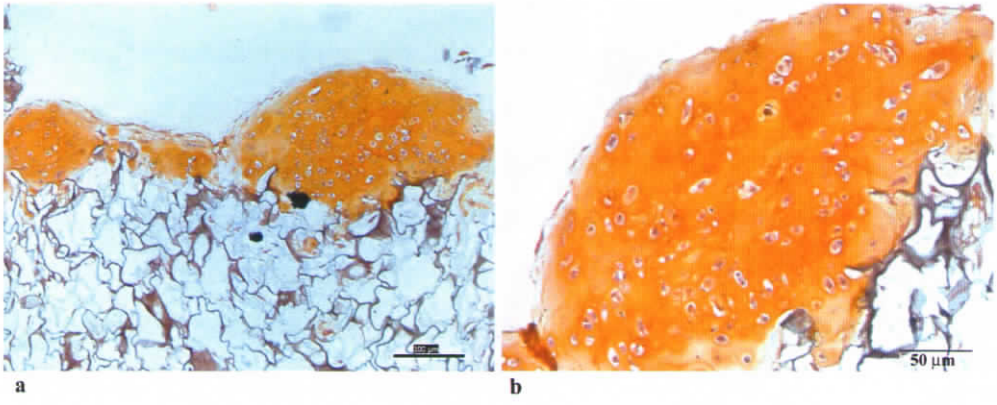


Fig 72. Safranin O staining of sections of Gelatin-Albumin construct in different magnifications

3.3.4.2. IMMUNOSTAINING FOR TYPE II COLLAGEN AND AGGRECAN LINK PROTEIN

The deposition of type II collagen, a typical chondrogenic marker, is a good representation of cartilage regeneration in the scaffolds. The secretion of type II

collagen throughout the thickness of the scaffold was examined by immunostaining using monoclonal anti collagen type II antibody. The brown colour in the sections indicated deposition of collagen type II. The sections of 2 month old PVA-PCL constructs indicated a uniform deposition of type II collagen throughout the construct, while in Gelatin-Albumin scaffolds the deposition was confined to periphery. The location of type II collagen was similar to the glycosaminoglycan deposition along the periphery in Gelatin-Albumin scaffolds. The section also showed accumulation of cells in this region.

The secretion of aggrecan was examined by immunostaining for aggrecan link protein in sections of both the scaffolds. The pattern of distribution was similar to collagen type II, with a uniform deposition in PVA-PCL constructs and confined to the periphery in Gelatin-Albumin scaffolds. Aggrecan is the large aggregating chondroitin sulfate proteoglycan of cartilage. The macromolecular aggregates have potent viscoelastic properties and are responsible for controlling the osmotic pressure of the cartilage matrix. The components like collagen type II and aggrecan form supramolecular complexes and function to generate the load bearing properties of the cartilage. The presence of aggrecan link protein is an indication of the maturing matrix. In the extracellular matrix of native cartilage, aggrecan non covalently associates with hyaluronicacid and link protein to form the proteoglycan aggregates. The link protein is important in stabilizing the interaction between the proteoglycan monomers and hyaluronan. Proteoglycans not stabilized by link protein can easily be dissociated and diffuse out of the matrix. Fig 73 represents the sections of PVA-PCL scaffolds immuno stained for collagen type II and aggrecan using DAB as the visualizing agent. Fig 74 represents the sections of Gelatin-Albumin scaffolds immuno stained for collagen type II and aggrecan using DAB as the visualizing agent.

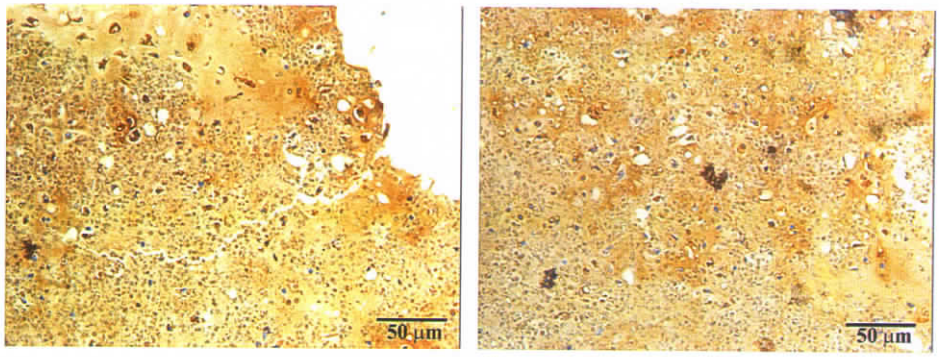


Fig 73. Sections of PVA-PCL scaffolds immuno stained for collagen type II and aggrecan respectively.

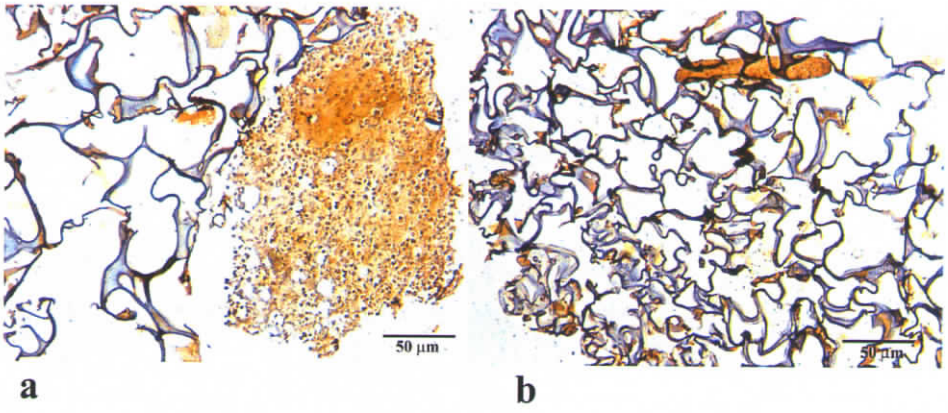


Fig 74. Sections of Gelatin-Albumin scaffolds immuno stained for collagen II and aggrecan link protein respectively.

3.3.5. COMPARISON OF CHONDROGENIC RESPONSE IN 3D PVA-PCL AND GELATIN ALBUMIN SCAFFOLDS

The aim of this chapter was to examine the influence of two different scaffolds on the chondrocyte morphology and matrix synthesis during invitro culture. For this, primary porcine chondrocytes were seeded at an equal density on both the scaffolds and cultured in chondrogenic medium, for a period of 2 months. The constructs were retrieved after the time period, and analyzed for the typical chondrogenic markers. The chondrogenic responses to the two scaffolds were analyzed, in terms of cell morphology, secretion of extracellular and their distribution pattern.

Previous studies have shown that, the chemistry and the topography of biomaterial surface may directly influence the cell behavior. The biomaterial surfaces adsorb the extra cellular matrix molecules and determine the cell-substrate interactions. *(Cannas et al 1998, Luna et al 1994, Uyen et al 1990).* These in turn regulate a wide variety of biological functions like, cell adhesion, migration, differentiation and extra cellular matrix synthesis. In general, the factors like chemical composition, 3D architecture of the scaffold and the swelling characteristics regulate the chondrogenic response. The two scaffolds used for the study, satisfy the basic requirements for serving as a matrix for tissue regeneration. Both the scaffolds were cell compatible, biodegradable, mechanically stable, had 3D porous structure with open interconnected pores. The porosity was within the general accepted range for cartilage regeneration. Even though the basic requirements were met by both the scaffolds, the cells responded differently to each of these scaffolds. The cell response varied during the initial process of attachment, as well as at the end of tissue regeneration in a period of two months. In this study, chondrogenic response was found to be better in PVA-PCL scaffold than Gelatin-Albumin scaffolds. This was analyzed in terms of cell morphology, glycosaminoglycan and collagen type II deposition.

The initial process of cell adhesion varied in the two materials. This might have subsequently regulated the following events of proliferation and extracellular matrix deposition. The cells retained the spherical chondrocyte morphology in PVA-PCL scaffolds, while they spread and had an elongated morphology in the Gelatin-Albumin scaffolds. The two scaffolds had different chemical composition and were characterized by different percent porosity, pore size and mechanical properties. The pores remain open and interconnected in both the scaffolds. The Gelatin-Albumin scaffold had a chemical structure which is similar to native collagen and is expected to be more biomimetic. On the other hand, PVA-PCL is a synthetic scaffold with no inbuilt nature to favour cell response. It is hypothesized that the synthetic PVA-PCL scaffold, promoted cell attachment by adsorbing molecules like fibronectin, laminin and vitronectin on its surface. Several previous reports have shown that synthetic materials promote cell adhesion in this manner. The chemical composition, together with the adequate hydrophobic-hydrophilic balance, of the PVA-PCL scaffold, might have promoted the attachment of adhesion enhancing molecules from serum on to the synthetic scaffold. The basic structure of the PVA-PCL Semi IPN was that of mechanically stable PCL chains, forming interpenetrating network, with hydrophilic PVA. This was similar to the network of collagen and glycosaminoglycans in native cartilage. This structure might have provided a better microenvironment for the chondrocytes to maintain its morphology and to retain the chondrocyte phenotype. Previous studies have reported that, regulation of chondrocyte phenotype by actin cytoskeleton and cell shape is influenced by the extracellular microenvironment.

In gelatin–albumin scaffold, the biomimetic sequences of gelatin and albumin stimulated the cell to attach and spread on to the material to form a fibroblastic morphology. There was no significant difference in the contact angle values between both the scaffolds. The adhesion and growth of cells on the surface are considered to be strongly influenced by the balance of hydrophilicity and hydrophobicity. (*Horbett et al 1985*) Many studies have demonstrated that cells

adhered, spread and proliferated more easily on substrates with moderate hydrophilicity, than hydrophobic or very hydrophilic substrates. (*Wachem et al 1985, Lee et al 2000*) A hydrophilic-hydrophobic balance favoring a moderate hydrophilicity has been attained in the wettability of both the scaffolds. So it can be inferred that, it is the chemical composition of the scaffold that play important role in regulating the cell phenotype in this study. A previous study on chondrocyte response on membranes with a hydrophilic-hydrophobic balance has shown that surface with more hydroxyl groups (-OH) groups show better chondrogenic response. (*Ma et al 2003*) PVA-PCL scaffold have large number of free –OH groups on the surface which might have contributed to the enhanced chondrogenic response.

The two scaffolds were of the same dimensions in the dry state. The pore size was adequate, with open interconnected pores in both the scaffolds. The pore volume was sufficient to hold the aggregating extracellular matrix components. The PVA-PCL had a wide range of pore size while the Gelatin-Albumin had a uniform pore size. The native cartilage is composed of different zones with variation in the structure of matrix assembly. Studies have shown that the chondrocytes favour anisotropy than a uniform ordered structure. The chondrocytes might have favoured wide pore size range of PVA-PCL than the uniform pore size in gelatin.

In culture, the swelling behavior was different in both the scaffolds which widely influenced the quality of invitro generated construct. The PVA-PCL scaffold had adequate swelling which permitted uniform distribution of cells and supply of nutrients. This resulted in accumulation of glycosaminoglycans and collagen. The uniform distribution pattern of matrix, with evenly distributed cells on it, was similar to that of native cartilage. This was evident in the safranin O staining of the sections. Even though PVA is highly hydrophilic, and can swell to a great extent, the PCL component in PVA-PCL Semi IPN, prevented extensive

swelling of PVA thus optimizing the swelling nature of the scaffold. This situation was favorable for cells in creating a suitable 3D environment, which resulted in uniform distribution of extracellular matrix. The semi interpenetrating polymer network structure with PCL, optimized the swelling nature of the scaffold. The PCL in the scaffold provided the mechanical integrity to the construct.

Gelatin-Albumin scaffold had a very high swelling which is more than twice that of PVA-PCL scaffold. Swelling produced an increase in total surface area of the scaffold. This reduced the optimum cell density to produce a uniform distribution of cells throughout the scaffold. It resulted in uneven distribution of ECM, which was confined to the outer surface and edges. There are reports that if the scaffold is incompletely filled with cells, it may result in fibrous ingrowth and this it turn may adversely affect the properties of the tissue to be generated (*Puelacher et al 1994*). The initial events and further the microenvironment, during the course of culture stimulated the cells to produce a skin layer of cells and ECM on the surface of the scaffold. The formation of a skin layer on the surface with spreading of cells and deposition of extra cellular matrix might have prevented the supply of nutrients to the interior of the scaffold. This might have resulted in cell death in the interior of Gelatin-Albumin scaffold. It is also possible that cells are washed out of the scaffolds during medium replenishment. All these factors might have contributed to reduced secretion of chondrogenic markers in the Gelatin-Albumin scaffold. These results showed that chemical composition of the scaffold indirectly have role in regulating the cell phenotype. Subsequently this together with the other physical properties, like swelling and scaffold architecture, might have played an important role in regulating cell viability and secretion of chondrogenic markers.

Since ECM was found to be preferentially deposited at the construct periphery in Gelatin-Albumin scaffolds, further studies should be carried out to investigate the possibility of enhancing the uniformity of the generated tissues by

using bioreactors. The use of bioreactors will provide uniform seeding and will increase mass transfer rates by the application of various regimes of fluid flow. In native tissue, chondrocytes are supplied with oxygen and nutrients by a combination of diffusion and fluid flow during joint loading. The syntheses rate of GAG and collagen in developing tissue invitro depends on gas and nutrient exchange.

The results also points out how the physicochemical properties of the scaffold affects the initial cell response which regulated the whole sequence of events, in the further growth and maturation of the construct. It is essential that the scaffolds need to provide a suitable microenvironment for the cells throughout the course of culture period. The variation in the chondrogenic response between the two scaffolds used in this study is as a result of the combined effect of various physicochemical factors of the scaffold. In this work the role of each of these factors on chondrogenic response have not been examined separately. A single physicochemical parameter cannot be identified as a reason because, to do this, the other parameters must be kept constant in the study.

Taken together, these findings suggest that, in a static in vitro environment, scaffold composition and architectures that retain the chondrogenic phenotype and promote free diffusion of nutrients and removal of waste materials, for all cells that are seeded even deep inside the scaffold, throughout the culture period would serve as an ideal support for cartilage regeneration. Since the properties of the scaffolding material greatly influence the chondrogenic response during invitro culture, it is necessary to give at most importance while selecting the supporting matrix for tissue regeneration. It should be carefully examined how the physicochemical properties of the scaffolding material regulate the chondrocyte behavior during the invitro culture.

3.4. SECTION 4

GROWTH FACTOR MEDIATED INVITRO DIFFERENTIATION OF MESENCHYMAL STEM CELLS TO CHONDROCYTES IN 3 D SCAFFOLDS

Cartilage tissue engineering comprises an appropriate combination of cells, 3D scaffolds and growth factors. One of the major challenges of tissue engineering of cartilage, is the availability of cells in sufficient number for invitro tissue regeneration.*(Dozin et al 2002)* Chondrocytes have a well characterized lack of expansion potential or loss of phenotype on long term culture in monolayer. Differentiation of embryonic stem cells towards chondrocytes has been accomplished, but its clinical application is impractical at present, from the ethical point of view. Mesenchymal progenitor cells serve as an alternative source with high expansion potential capable of supplying large quantities of cells to form a 3D tissue like structure. These stem cells can easily be prepared from the patients without invasive surgery. They also have the differentiation potential to specific lineages upon proper signaling. (*Lorgensen et al 2001, Steinert et al 2003*) The differentiation of stem cells to chondrocytes is cued by signaling molecules like Transforming growth factor (TGFβ). (*Iwasaki et al 1993, Monroy et al 1997, Schofield et al 1990)* and Bone morphogenetic protein (BMP). (*Kramer et al 2000*) Even though several studies have been conducted to differentiate stem cells using signalling molecules, still less is known about the all the signaling cues within the invivo niche and whether these will prove to be useful for invitro tissue engineering.

Most of the previous studies on chondrogenesis employed a single growth factor to evaluate the invitro differentiation process. (*Barry et al 2001*) In native tissue, the formation of a proper chondrogenic phenotype is regulated by the combined action of several growth factors. (*Throp et al 1992*) The use of a single

growth factor may not provide all the necessary signals in the invitro differentiation process. So we decided to evaluate the chondrogenic differentiation of mesenchymal stem cells by supplementing growth factors individually and in combination. Since the members of TGFβ super family are widely known for their chondrogenic potential TGFβ1, TGFβ3 and BMP2 were selected for this study. The combination was selected with some insight into the molecular signaling process which is discussed in detail in the later sections. It would be beneficial to identify and understand the cocktail of soluble signaling factors that influence the stem cell fate in constructing a living tissue substitute. In the concept of tissue engineering it is essential to provide the most favorable condition for the proper and faster differentiation of stem cells to chondrocytes during invitro culture.

Another important factor is the 3 D scaffolds which provide the necessary microenvironment for the maturing phenotype. Studies have shown that MSCs had to be cultured in pellets for differentiation to chondrocytes. The MSCs do not differentiate effectively to chondrocytes, when cultured in monolayer even in presence of growth factors. The 3D culture may provide a microenvironment similar to mesenchymal condensation, which normally takes place on the initiation of chondrogenesis. Although several studies have been conducted invitro, no cartilage tissue has been engineered that fulfills the clinical requirements. This point out the importance of the scaffold, one of key factors in regulating the invitro chondrogenesis. The invitro chondrogenic differentiation of MSCs requires the complex interactions and the three dimensional support to maintain their differentiated phenotype. Poly esters like poly (lactic acid), poly (glycolic acid) and their blends have been used to the study the differentiation process. However these materials are problematic due to acidic degradation products, inconsistent degradation rates and tissue response profiles. Many hydrogels based systems like alginates are compromised by the large gap between the mechanical features of a soft matrix and the need for mechanical robustness of the invitro generated tissue. Many groups have already studied several scaffolding systems for cartilage tissue

engineering. (*Vickers et al 2006, Jun et al 2006, John et al 2003*) As tissue formation takes place in a 3 dimensional pattern, the 3D scaffolding material help in uniform spatial distribution of cells and deposition of extra cellular matrix components during invitro culture. In this study the differentiation of mesenchymal stem cells was evaluated in a 3D Semi interpenetrating network of poly (vinyl alcohol)-poly (caprolactone) and Gelatin-Albumin scaffolds. The scaffolds were found to promote secretion of cartilage specific molecules in our previous studies with chondrocytes. After determining the appropriate seeding conditions, constructs were incubated in the different growth factor supplemented conditions for a four week culture period and chondrogenesis was evaluated qualitatively and quantitatively.

Mesenchymal stem cells from 2 weeks old male Wistar rats were isolated from six animals. The attached cells with mesenchymal phenotype were cultured to attain confluence. The cells were subcultured and the cells from passage one, were trypsinized and pooled together for further seeding experiments. The mesenchymal cells were seeded on to 3D PVA-PCL and Gelatin-Albumin scaffolds of 10 mm diameter and 2-3 mm thickness at a cell density of 5 X 10^5 cells /scaffold. Initial studies were conducted to standardize the seeding conditions to produce a uniform distribution of cells throughout the scaffold. For seeding, the scaffolds were placed in polypropylene tubes and 1 ml cell suspension was added and placed in orbital shaker at 300 rpm for 6 hours. Further the medium was replaced with chondrogenic medium containing respective growth factors. The constructs were supplemented with different growth factors such as like recombinant human transforming growth factor β1 (TGFβ1) (20ng/ml), recombinant human transforming growth factor β3 (TGFβ3) (10ng/ml), bone morphogenetic protein (BMP2) (25ng/ml), TGFβ1+TGFβ3 (20ng/ml, 10ng/ml), TGFβ3+BMP2 (10ng/ml, 25ng/ml) respectively. Control samples were cultured in chondrogenic medium without any growth factors. The constructs were maintained in dynamic culture at

50 rpm in different growth conditions for 28 days. Medium was replaced every 3 days. There were 9 samples per group.

3.4.1. EVALUATION OF THE 28 DAY CONSTRUCTS

After 28 days the constructs were retrieved from culture and processed for various analyses. Three samples were used separately each for biochemical and gene expression analysis. The other three samples, from each group was used for evaluating overall cell distribution and morphology using scanning electron microscopy, viability testing using confocal microscopy and histological analysis, for typical chondrogenic markers respectively.

3.4.1.1. MORPHOLOGY & DISTRIBUTION OF CELLS (PVA-PCL)

SEM images give information regarding the overall distribution of cells and extra cellular matrix molecules. Images were also taken at higher magnification to observe the cell morphology.

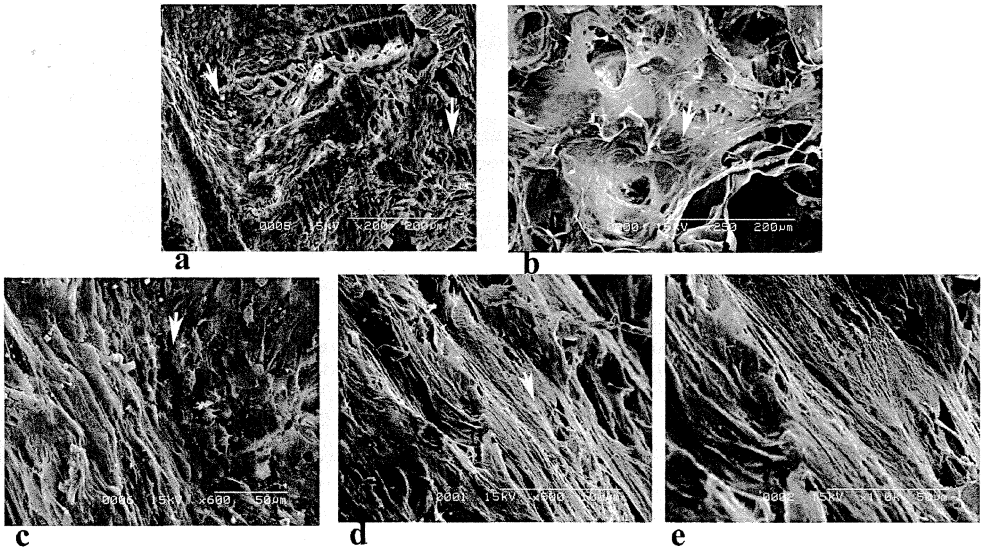


Fig 75. SEM images of 28 days constructs supplemented with TGFβ1 at different magnifications

Fig 75 represents different magnifications of SEM images of 28 days constructs supplemented with TGFβ1. Micrographs at lower magnification shows cells deposited uniformly on the surface of the scaffold.(Fig 75.a) At higher magnifications, extracellular matrix deposition was clearly observed on the surface.(Fig 75.b, d, e) The cells were elongated with typical morphology of stem cells. The cells and ECM covered most of the pores of the scaffold.

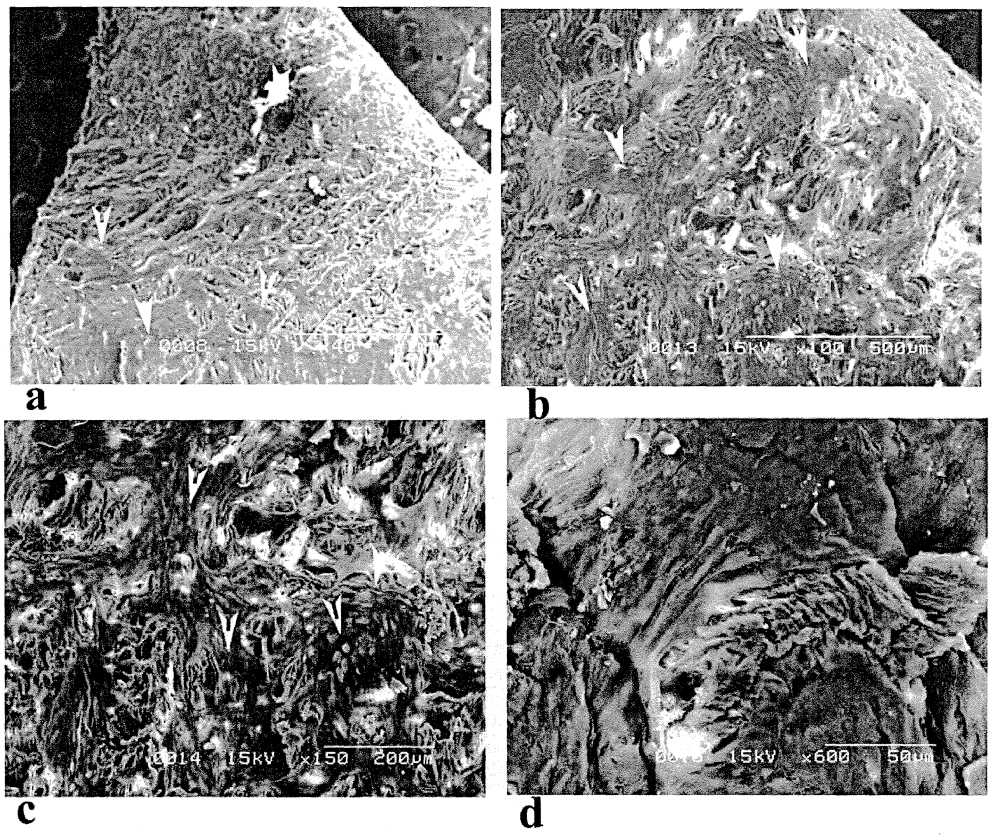


Fig 76. SEM images of 28 days constructs supplemented with TGFβ3 at different magnifications

Fig 76 represents different magnifications of SEM images of 28 days constructs supplemented with TGFβ3. Micrographs at lower magnification shows cells deposited on the surface of scaffold, masking most of the pores of the scaffold. (Fig 76.a) Some areas did not show cell accumulation. At higher

magnifications, the patches of cells were clearly observed. (Fig 76. c, d) The cells had elongated and spherical morphology. The deposition of extracellular matrix was also clearly observed on the surface that covered the pores in that area. (Fig 76.d)

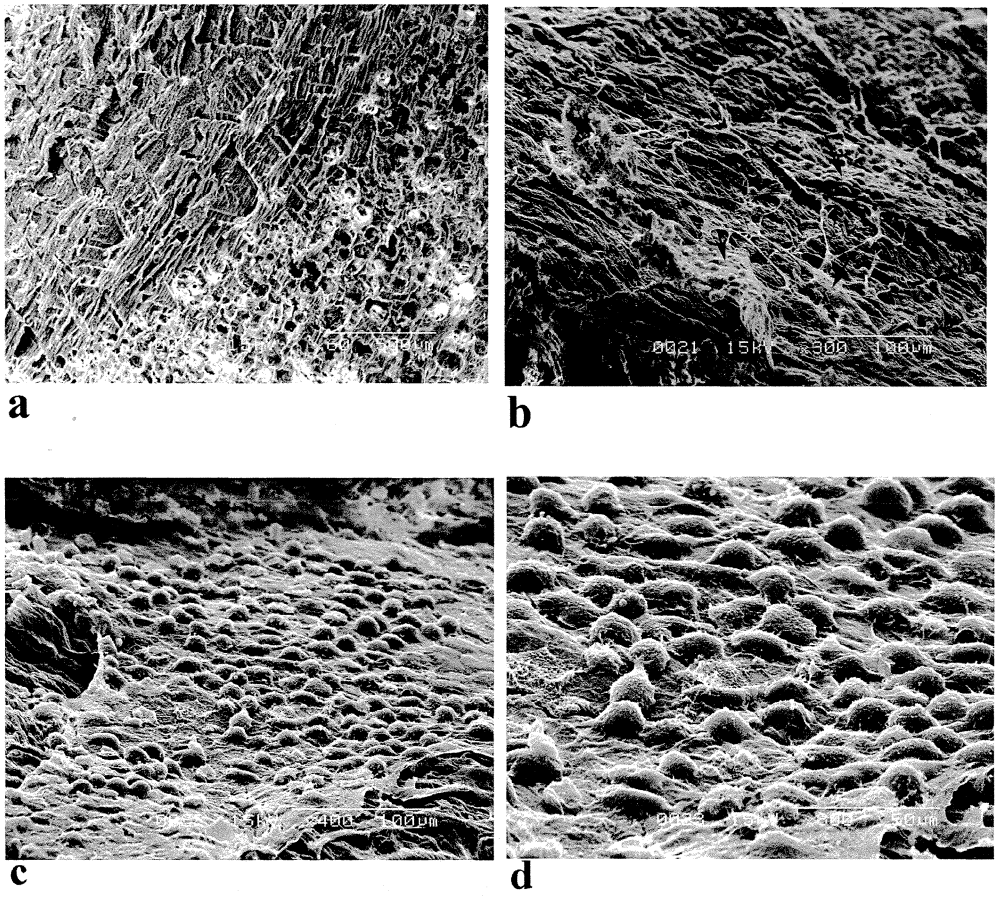


Fig 77. SEM images of 28 days constructs supplemented with BMP2 at different magnifications

SEM images of 28 days constructs supplemented with BMP2 at different magnifications are represented in Fig 77. The BMP2 supplemented constructs showed less number of cells on the surface than TGF β1 and TGF β3. Many areas on the surface were empty with few cell patches sparsely distributed on the surface. (Fig 77.a, b) There was no uniformity in cell distribution. An important observation was that most of the cells on the surface appeared spherical in shape.

(Fig 77.c, d) The spherical shape of the cells was similar to that of chondrocytes on the 3D scaffolds.

Comparing the three different growth factor supplemented groups in 3D PVA-PCL scaffold, TGF β3 supplied constructs showed, more number of cells and extra cellular matrix components on the surface of the scaffold. The BMP2 supplied constructs showed few cells on the surface. The cell morphology was more or less elongated in TGF β1 and TGF β3 supplemented constructs, but in BMP2 supplied constructs, the cells were spherical in shape.

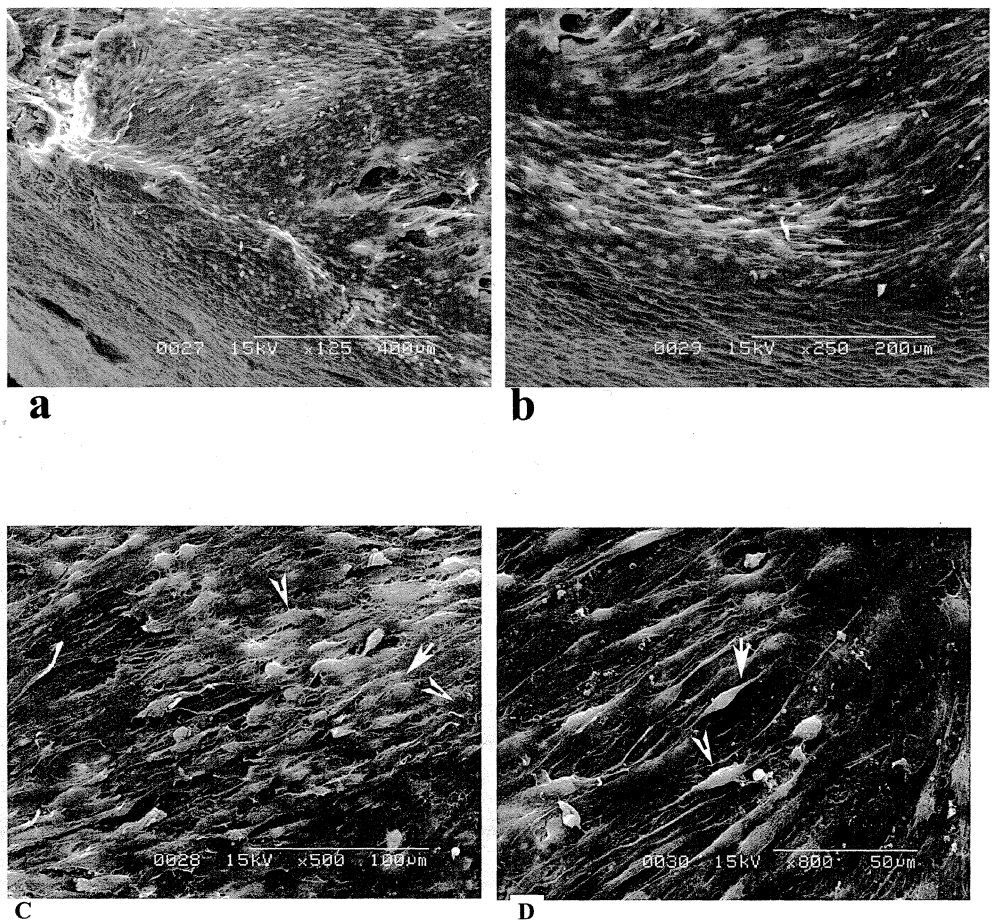


Fig 78. SEM images of 28 days constructs supplemented with TGF β1 + TGFβ3 at different magnifications

SEM images of 28 days constructs supplemented with TGF β1 + TGFβ3 at different magnifications is represented in Fig 78. In the constructs, where a combination of TGFβ1 and TGFβ3 was supplemented, most of the surface of the scaffold was covered with cells and extracellular matrix components. (Fig 78. a, b) This masked most of the pores of the scaffold. The cells were very densely arranged on the matrix. (Fig 78.c) Higher magnifications showed elongated cells residing in an orderly arranged pattern. (Fig 78.d)

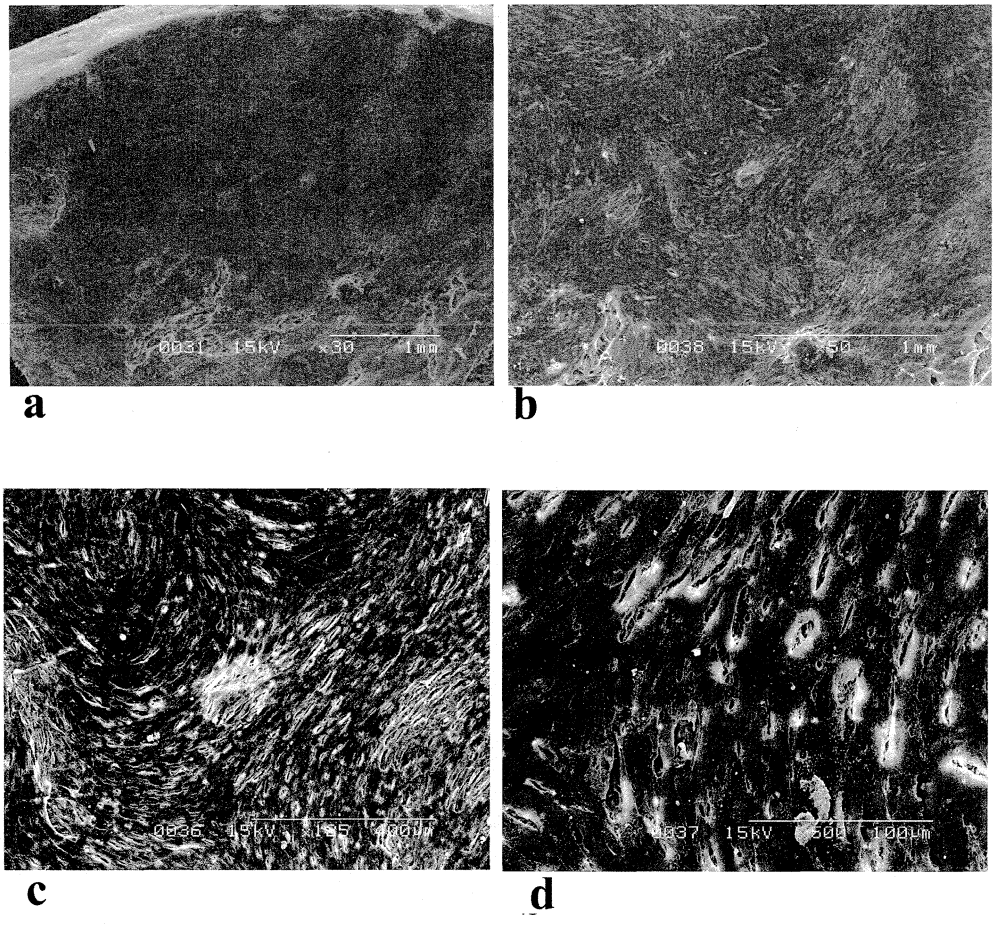


Fig 79. SEM images of 28 days constructs supplemented with TGF β3+ BMP 2 at different magnifications

SEM images of 28 days constructs supplemented with TGF β3+ BMP 2 at different magnifications is represented in Fig 79. The constructs supplemented

with TGFβ3 andBMP2 showed a very smooth surface. (Fig 79.a) The entire surface of the scaffold was covered cells and matrix, completely masking the pores of the scaffold. (Fig 79.b, c, d) The cells were very densely packed on the surface with high deposition of matrix components than all other growth conditions. The higher magnification did not give much information on cell morphology, as the surface was densely covered with matrix.

The constructs supplemented with combination of growth factors showed higher deposition of cells and matrix on the surface than that of individual growth factor supplements. Most of the cells in TGFβ1+ TGFβ3 supplemented groups had an elongated morphology. The TGFβ3 + BMP2 supplemented constructs showed a very smooth surface completely masking the pores of the scaffold. The morphology of cells was not evident in this group as higher magnifications showed regions covered with matrix components.

3.4.1.2. CELL VIABILITY (PVA-PCL)

The viability of cells grown in different growth factor supplemented conditions were estimated by live dead assay using live dead assay kit containing Calcein AM and Ethidium bromide. The cells in the 28 day constructs need to be viable, to secrete extra cellular matrix components, to form the 3D tissue like structure. As the cells proliferate and secrete ECM like components on the surface, they may obstruct the free diffusion of nutrients to the interior of the scaffold. This may affect the viability of cells seeded within the interior of pores. The confocal images indicate that most of the cells remain viable in all the growth conditions. The cell proliferation and matrix deposition on the surface have not affected the viability of cells in the constructs. The images also clearly give information about the morphology and over all distribution of cells in the scaffold.

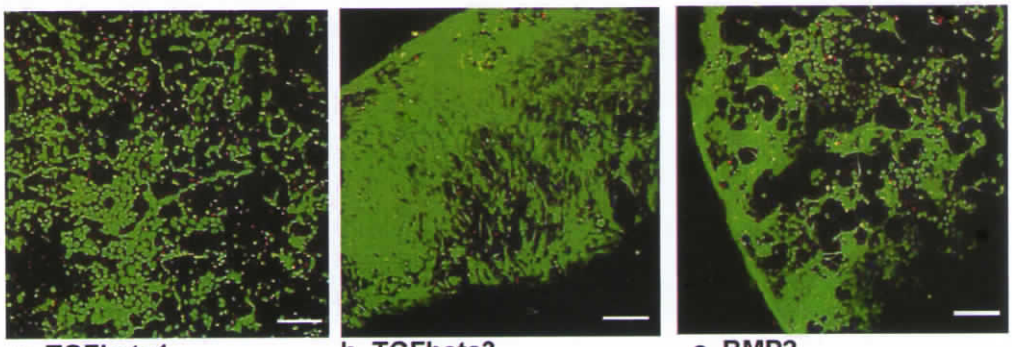


Fig 80. Confocal images of constructs grown in different growth conditions showing the viable cells (green).

The confocal images of the 28 day grown constructs supplemented with individual growth factors is represented in Fig 80. The confocal images gave qualitative information that, the constructs grown in TGFβ3, had more number of cells than the other groups supplemented with individual growth factors. Cells in two other groups appeared in patches as observed in the SEM images. Cells in TGFβ3 supplemented group had an elongated morphology. The cells in BMP2 supplemented constructs showed more spherical cells particularly towards the centre of the scaffold.

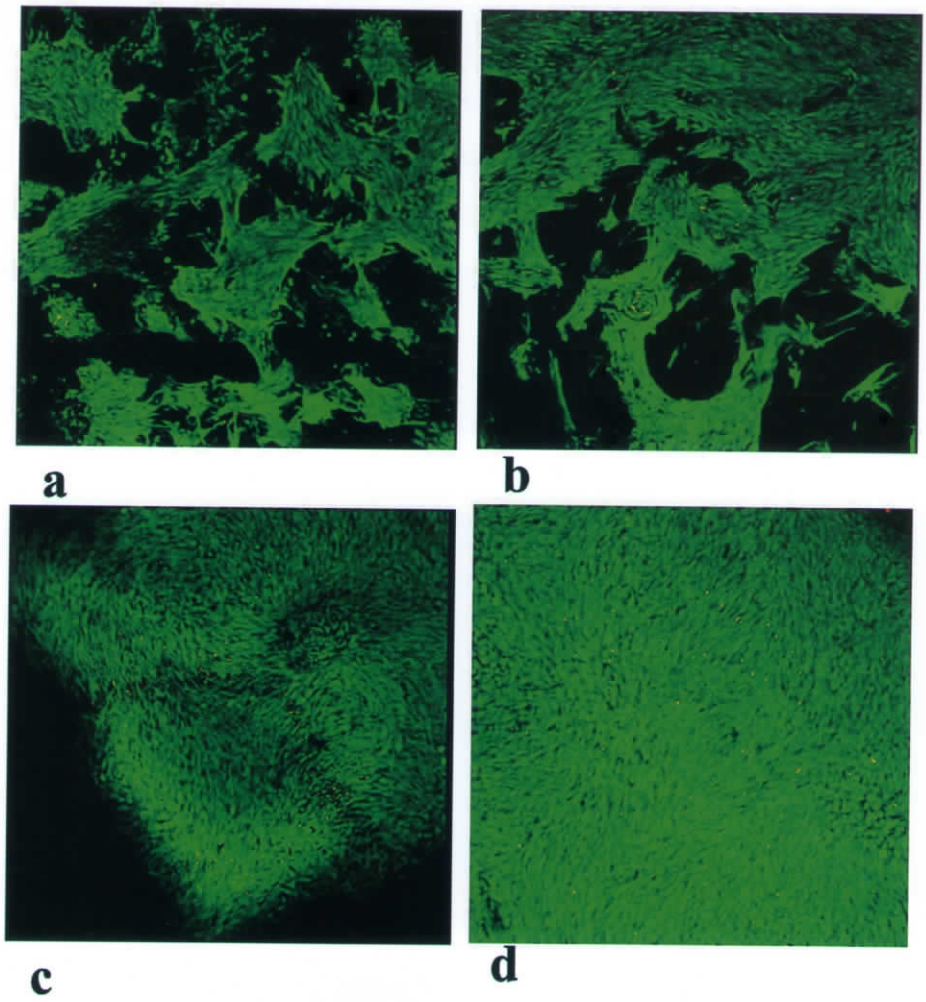


Fig 81. Confocal images of live dead assay of constructs supplemented with combination of growth factors. (a, b) TGF β1+TGFβ3 (c, d) TGFβ3 + BMP2.

Confocal images of live dead assay of constructs supplemented with combination of growth factors are represented in Fig 81. The cells remain viable in both the constructs. The TGFβ1+TGFβ3 supplemented constructs showed patches of cells. The TGFβ3 + BMP2 supplemented constructs had cells, which covered the entire surface of the scaffold. The confocal images also indicate, that the group supplemented with TGFβ3 + BMP2, had the higher number of viable cells that covered the entire scaffold than all other growth conditions. The qualitative

information obtained from the SEM images and the confocal images of live dead assay indicates that the TGFβ3 + BMP2 supplemented group contain more number of cells and extracellular matrix components than all other groups in this study. These results points out that, the combination TGFβ3+BMP2 promotes more cell proliferation, and stimulate the cells to secrete more matrix components in PVA-PCL scaffolds

GELATIN-ALBUMIN

3.4.1.3. MORPHOLOGY & DISTRIBUTION OF CELLS (GA)

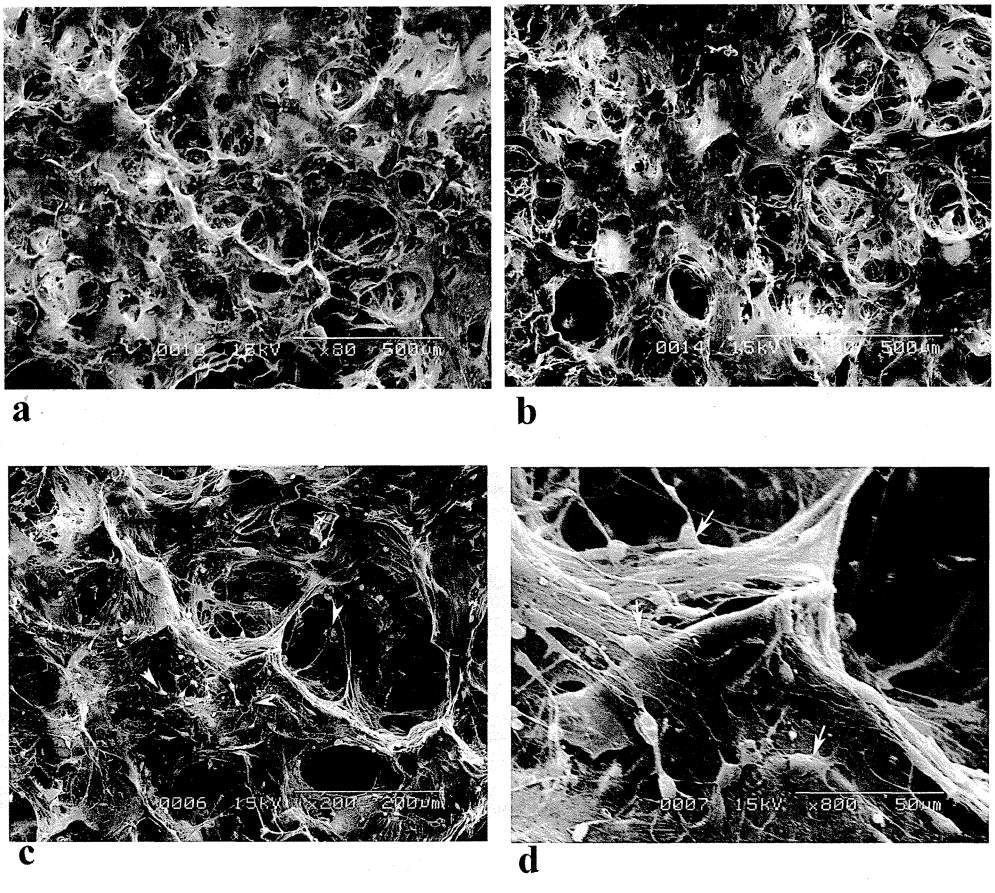


Fig 82. SEM images of 28 days Gelatin-Albumin constructs supplemented with TGFβ1 at different magnifications

Fig 82 represents SEM images of Gelatin-Albumin constructs supplemented with TGFβ1 at different magnifications. The TGF β1 supplemented constructs showed cells and deposition of extracellular matrix components on the scaffold. (Fig 82.a) Most of the pores were partly covered by the deposition of matrix components. (Fig 82.b) Higher magnifications showed cells with elongated morphology. (Fig 82.c, d)

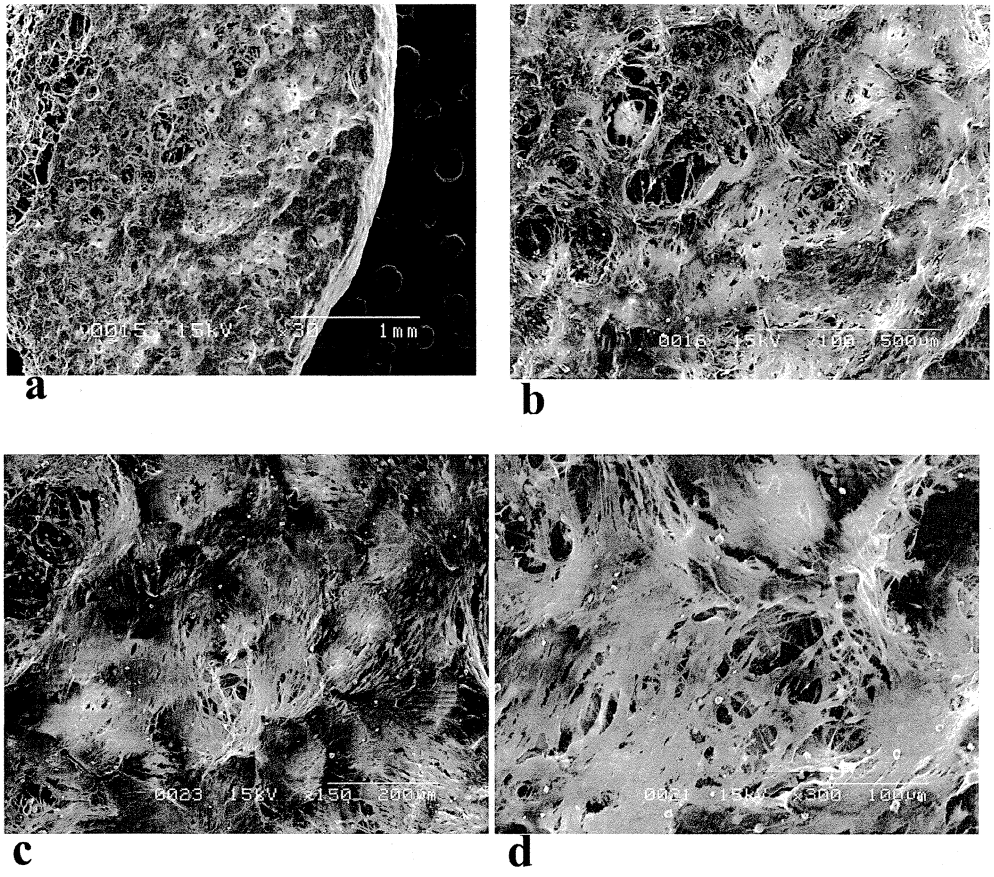
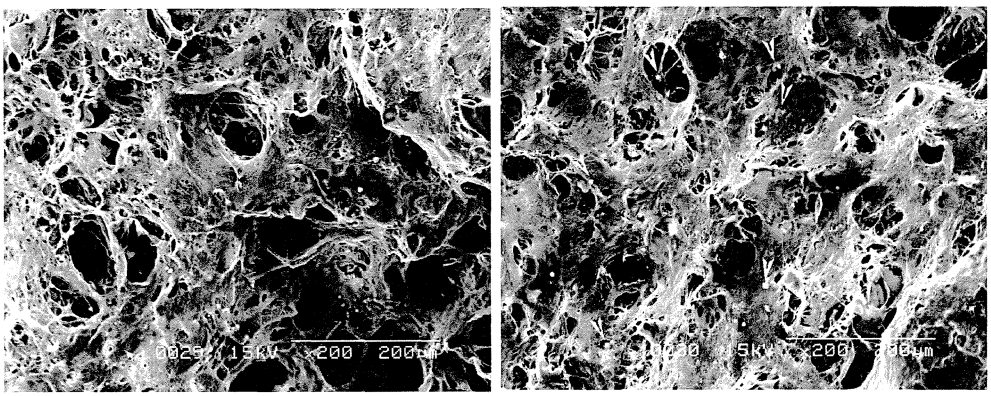


Fig 83. SEM images of 28 day Gelatin-Albumin constructs supplemented with TGFβ3 at different magnifications

Fig 83. Represents SEM images of 28 days Gelatin-Albumin constructs supplemented with TGFβ3 at different magnifications. The TGF β3 supplemented constructs showed comparatively more deposition of cells and matrix components

than TGF β1. (Fig 83.a, b) The cells and extracellular matrix deposition, covered most of the pores of the scaffold. The matrix deposition was uniform through out the scaffold. SEM images showed a well arranged orientation of cells and matrix deposition in the pores. (Fig 83.c, d)



a **b**

Fig 84. SEM images of 28 days grown Gelatin-Albumin constructs supplemented with BMP2 at different magnifications.

Fig 84 represents SEM images of 28 days grown Gelatin-Albumin constructs supplemented with BMP2 at different magnifications. The images indicate less number of cells and more matrices on the surface. The BMP2 supplied constructs showed the deposition of matrix components in an irregular fashion. Occasional round cells could be observed. (Fig 84.b)

Comparing the three groups in Gelatin-Albumin scaffolds, where individual growth factors were supplemented, the TGFβ3 supplemented constructs showed more deposition of cells and matrix on the surface. This constructs showed an orderly arrangement in the deposition of matrix components than the other groups. The BMP2 supplemented constructs showed a random deposition of extracellular matrix with few cells on the surface. Not much information could be obtained regarding the shape of cells. The observations on cell distribution and matrix deposition were more or less similar to that of PVA-PCL scaffolds. TGF β3 showed more number of cells and extra cellular matrix among the individual

growth factors. However the spherical shape of cells in BMP2 supplemented PVA-PCL constructs could not be very clearly observed in Gelatin-Albumin constructs.

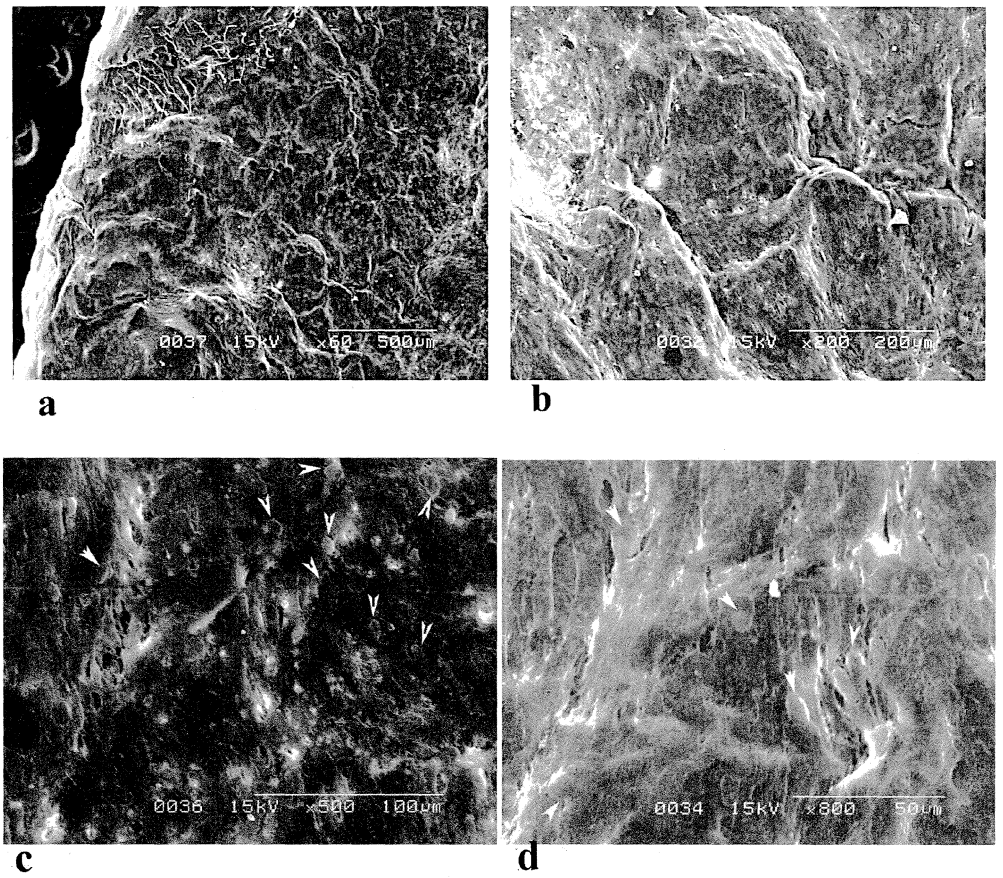


Fig 85. SEM images of 28 days Gelatin-Albumin constructs supplemented with TGFβ1 +TGFβ3, at different magnifications.

Fig 85 and Fig 86 represents SEM images of 28 days Gelatin-Albumin constructs supplemented with TGFβ1+TGFβ3 and TGFβ3+BMP2 at different magnifications respectively. The combination TGFβ1+TGFβ3 showed high deposition of matrix components on the surface, than the individual growth factor supplemented groups. (Fig 85.a, b) The cells were not clearly visible due to the deposition of matrix. Some cells could be seen in between the matrix at higher magnifications. (Fig 85.c, d) The combination TGFβ3+BMP2 showed a very smooth surface with high deposition of extra cellular matrix, which completely

masked the pores of the scaffold. (Fig 86. a, b) The extracellular matrix components deposition showed a proper orientation. Not much cells could be seen on the surface. (Fig 86.c) On comparing the constructs supplemented with combination of growth factors in Gelatin-Albumin scaffolds, the TGFβ3+BMP2 supplemented constructs had a higher deposition of matrix.

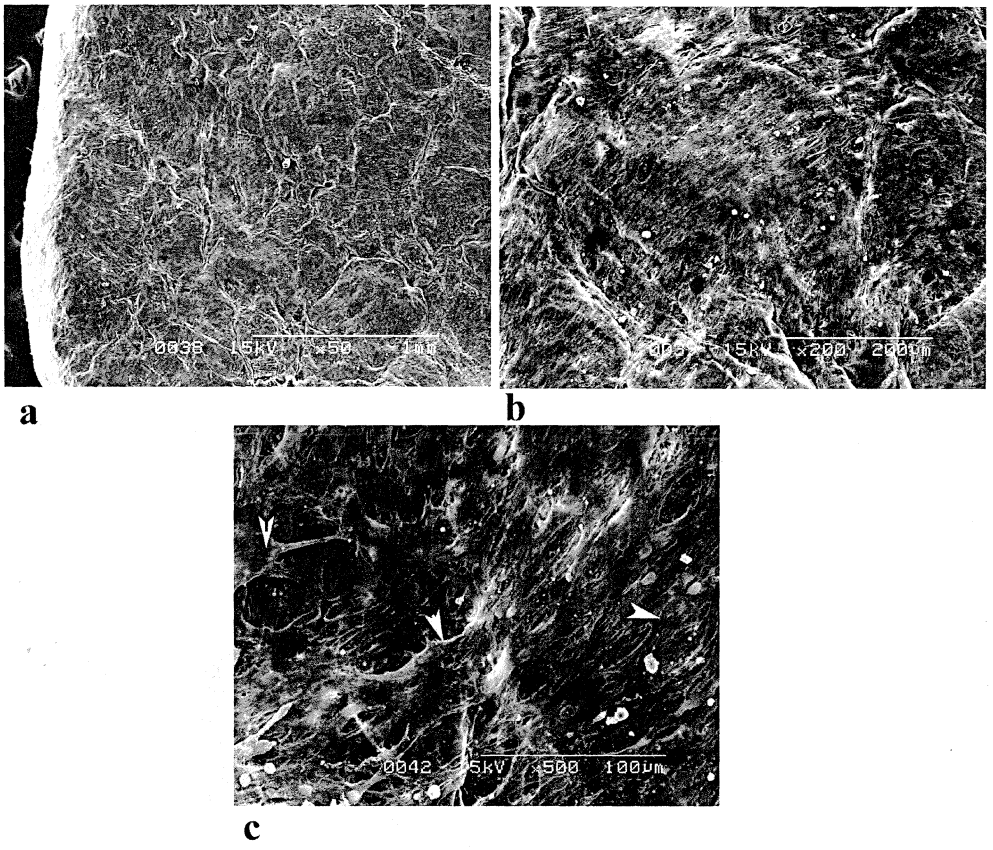


Fig 86. SEM images of 28 days Gelatin-Albumin constructs supplemented with TGFβ3 + BMP2 at different magnifications.

The cells grown in the two different combinations of growth factor supplements in Gelatin-Albumin and in PVA-PCL scaffolds had a similar pattern of distribution. The scanning electron micrographs did not show any substantial difference in the cells and matrix deposition between the two scaffolds. Both the scaffolds show increase in deposition of matrix when the combinations of growth factors were used. In scaffolds supplemented with TGFβ1+ TGFβ3, the PVA-PCL

constructs showed more cells on the surface, while in Gelatin-Albumin scaffolds the surface was covered with more matrix and few cells could be seen on the surface.

3.4.1.4. BIOCHEMICAL ANALYSIS

The total amount of glycosaminoglycans and collagen in each growth factor supplemented constructs were estimated by biochemical analysis. The constructs were digested with papain solution and the total glycosaminoglycans and collagen were estimated using DMMB and Sircoll assay respectively. Statistical analysis was performed using one way ANOVA test. The values were expressed as the mean $\pm$ of three different replicates. Values of $p<0.05$ were considered significant (*) and $p<0.0001$ were considered very significant (**).

3.4.1.4.1. TOTAL GLYCOSAMINOGLYCANS CONTENT

All the growth factor supplemented constructs showed significantly higher amount of glycosaminoglycans than the control.(Fig. 87) The control was grown in chondrogenic medium with ascorbic acid and dexamethasone, but without the addition of any growth factors. On comparing the three groups were individual growth factors were supplemented, it was found that there was a significant increase in glycosaminoglycans content in TGFβ3 than TGFβ1 and BMP2 supplemented constructs. The GAG content in TGF β1 was significantly higher than BMP2.The two combinations of growth factors, had higher amount of GAG than the constructs supplied with individual growth factors. The GAG content in the combination groups was significantly higher than TGFβ3 supplemented constructs, which had the high content of GAG among the individual growth factors. Among the constructs supplemented with combination of growth factors, the combination TGF β3+BMP2 showed a significantly higher amount of GAG than the other combination. The total amount of glycosaminoglycans secreted by MSCs in the scaffold could not be directly compared to previous reports on other 3D scaffolds due to variation in the source of cell, initial seeding density and growth conditions. However the level of GAG production in PVA-PCL scaffold in

response to TGF β1 was comparable to some of the previous studies carried out in scaffolds like collagen, silk etc.(*Hoffmann et al 2006*) This also points out that the growth factor cocktails showed a much higher values of GAG in 3D PVA-PCL scaffold.

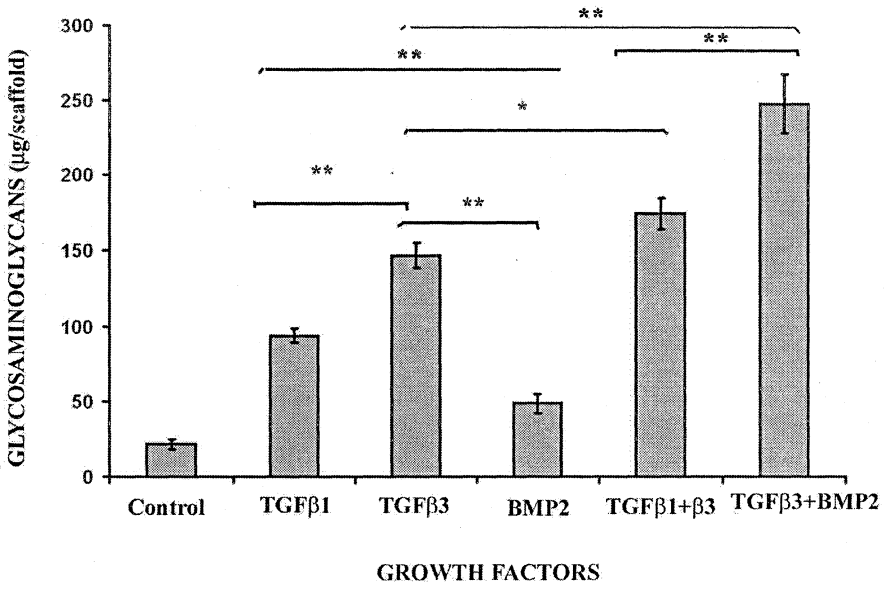


Fig 87. The total glycosaminoglycans in different growth factors supplemented 28 day PVA-PCL constructs

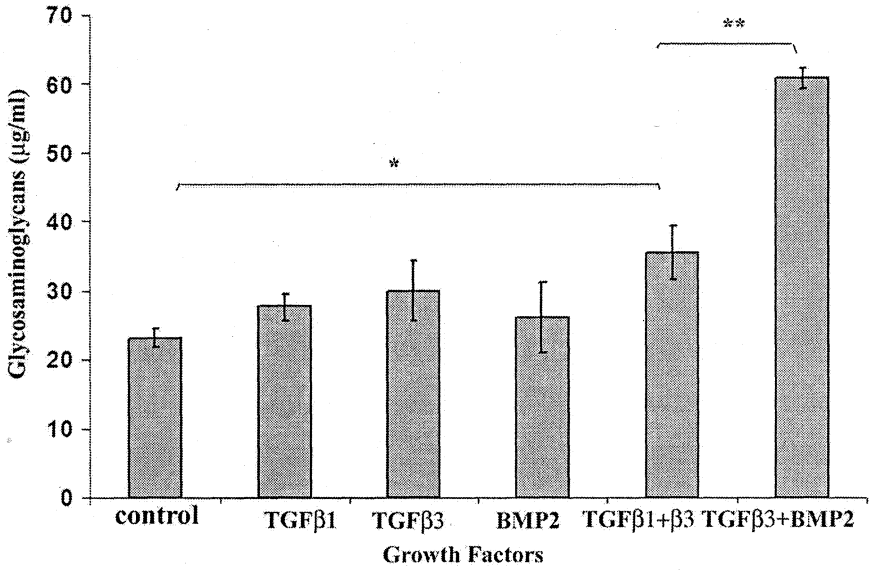


Fig 88. The total glycosaminoglycans in different growth factors supplemented 28 day Gelatin-Albumin constructs

The total glycosaminoglycan content in 28 day constructs in Gelatin-Albumin scaffold is represented in Fig 88. In case of constructs supplemented with individual growth factors, TGFβ3 supplied groups showed the highest GAG content, but this was not significantly different from the other individual growth factors, TGFβ1 and BMP2. Both the combinations showed a significant increase than the control. The combination, TGFβ3+BMP2 showed the highest amount of GAG and was significantly higher than the other combination.

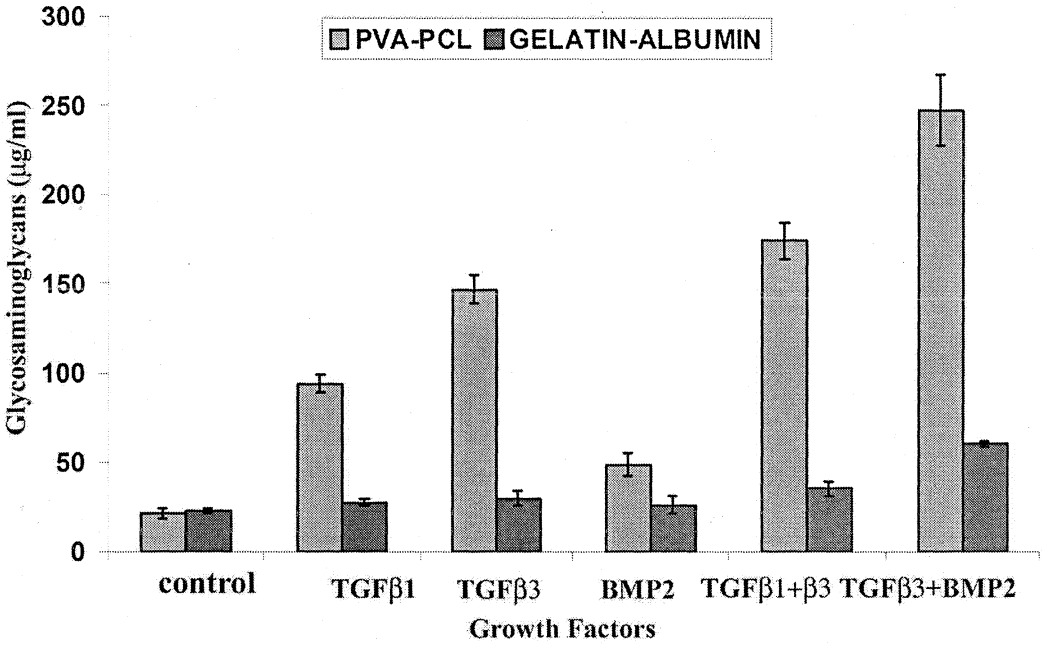


Fig 89. The total glycosaminoglycans in different growth factors supplemented 28 day constructs

The total GaGs in different growth factors supplemented 28 day constructs in the two scaffolds are compared in Fig 89. On comparison, it was found that amount was significantly higher in all growth factor supplemented constructs in PVA-PCL than in Gelatin-Albumin. These results indicate that chemical and physicochemical properties of PVA-PCL scaffold are stimulating the growth factors for more chondrogenic response.

3.4.1.4.2. TOTAL COLLAGEN CONTENT

The total collagen content in different growth factors supplemented 28 day PVA-PCL constructs is represented in Fig 90. All growth factor supplemented constructs showed significantly higher amount of GAG than the control. Among the groups where individual growth factors were supplied, TGFβ3 showed significant increase than TGFβ1 and BMP2. The combination TGFβ3+BMP2 showed significantly high content of collagen than the other combination. This group also had high content of GAG. A significant difference was not observed between the combination TGF β1+TGF β3 and the TGF β3 supplemented group.

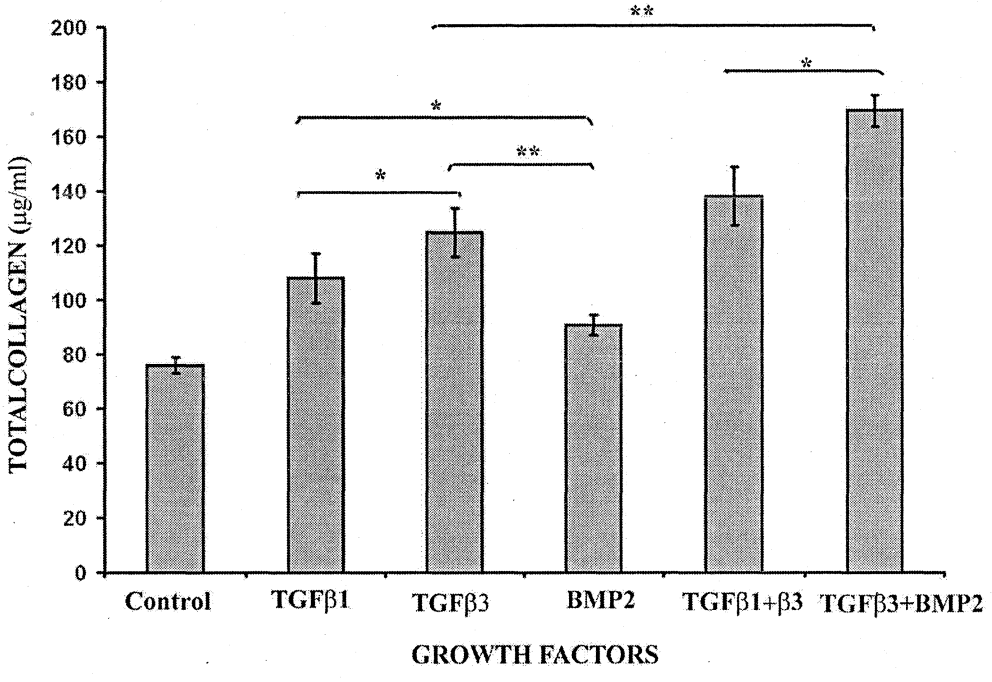


Fig 90. The total collagen content in different growth factors supplemented 28 day PVA-PCL constructs

The total collagen content in Gelatin-Albumin constructs is represented in Fig 91. All the growth factor supplemented constructs showed a significant increase in total collagen content. The TGFβ3+BMP2 supplemented constructs showed the highest amount of collagen than all other growth conditions.

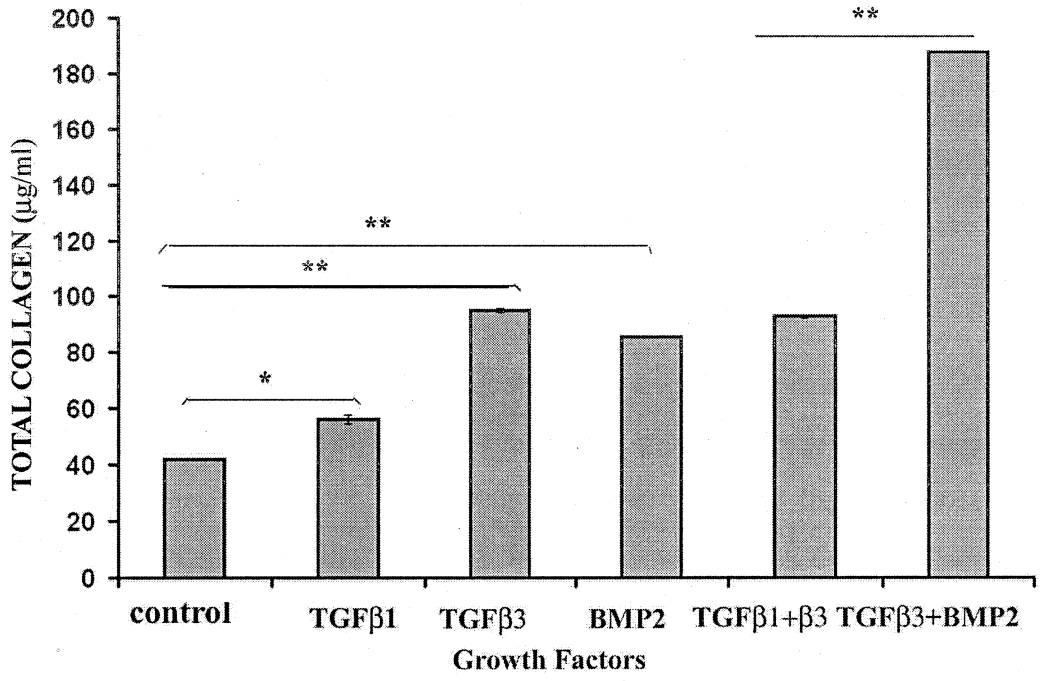


Fig 91. The total collagen content in different growth factors supplemented 28 day Gelatin-Albumin constructs

The results of biochemical analysis revealed that among the individual growth factor supplemented cultures, TGFβ3 supplied groups showed high deposition of the chondrogenic marker, glycosaminoglycans. The glycosaminoglycan deposition significantly increased when a combination of growth factors was used. This indicates that the differentiation process was increased in presence of the two growth factor combinations. The TGFβ3+BMP2 supplemented constructs showed the highest amount of GAG, indicating that this growth factor cocktail is better than the other combination in the in vitro process of chondrogenesis. The total collagen also showed a similar trend as GAGs. However we could not find a significant difference between the TGFβ3 and the combination TGFβ1 + TGFβ3.

3.4.1.5. SAFRANIN O STAINING OF THE 28 DAY CULTURED CONSTRUCTS

The deposition and distribution pattern of glycosaminoglycans within the scaffolds grown in different growth factors supplemented conditions was estimated by Safranin O staining. The paraffin sections of the constructs taken from the interior of the scaffolds were used for the staining procedure. The sections of PVA-PCL constructs stained for glycosaminoglycans are represented in Fig 92. The TGFβ3 supplemented constructs showed more glycosaminoglycans deposition than the TGFβ1 and BMP2. The deposition was higher in these constructs as indicated by the high staining intensity, but was located along the periphery. The pore voids did not show any deposition of GAGs. The TGFβ1 and BMP2 showed a non uniform deposition of GAG that appeared as small strands. The combination of growth factors showed comparatively higher deposition of GAG than the individual growth factors. In the constructs supplemented with the combination TGFβ1+TGFβ3, the GAGs were also deposited in the pore voids. The TGFβ3+BMP2 supplemented constructs showed more deposition of GAG, with deeper Safranin O staining. The deposition was seen on the scaffold margins as well as inside some of the pores.

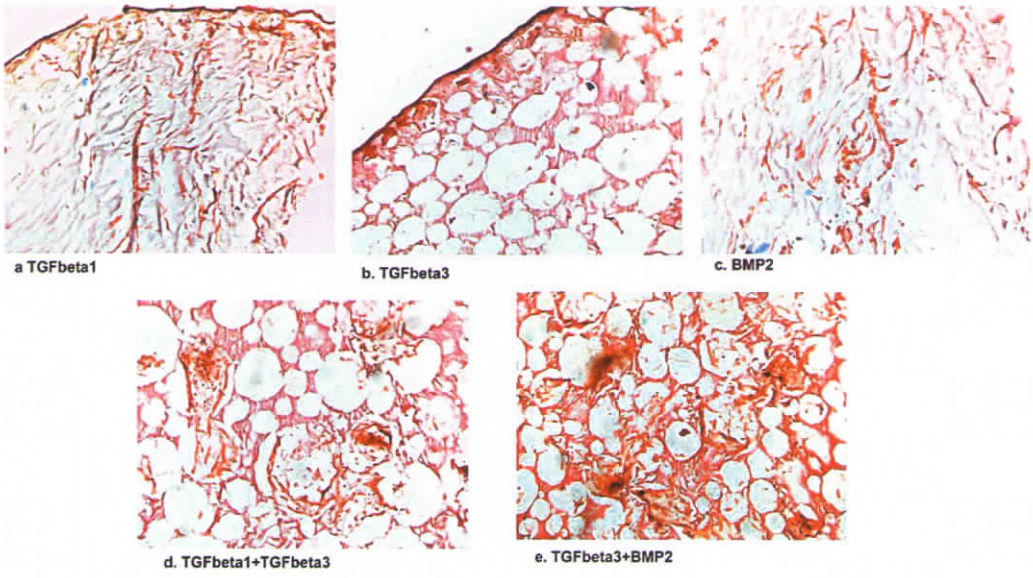


Fig 92. The sections of the 28 days PVA-PCL constructs stained for glycosaminoglycans grown in different growth factor supplemented conditions.

The deposition of GAGs in different growth factors supplemented conditions in Gelatin-Albumin is represented in Fig 93. The sections of TGFβ1 supplemented constructs showed the GAG deposition only on the periphery. Most of the regions in the interior of the scaffold were unstained and appear blue in colour. In TGFβ3 supplemented groups, the GAG deposition was observed throughout the sections with deeper staining towards the periphery. In BMP2 supplemented groups the staining was non uniform with localized accumulation of GAG. The GAG deposition was found to be similar in TGFβ3 supplemented group and in the combination TGFβ1 and TGFβ3. The TGFβ3+BMP2 supplemented constructs showed the highest amount of GAG. The GAG deposition was seen along the scaffold margins as well as on the voids inside some of the pores

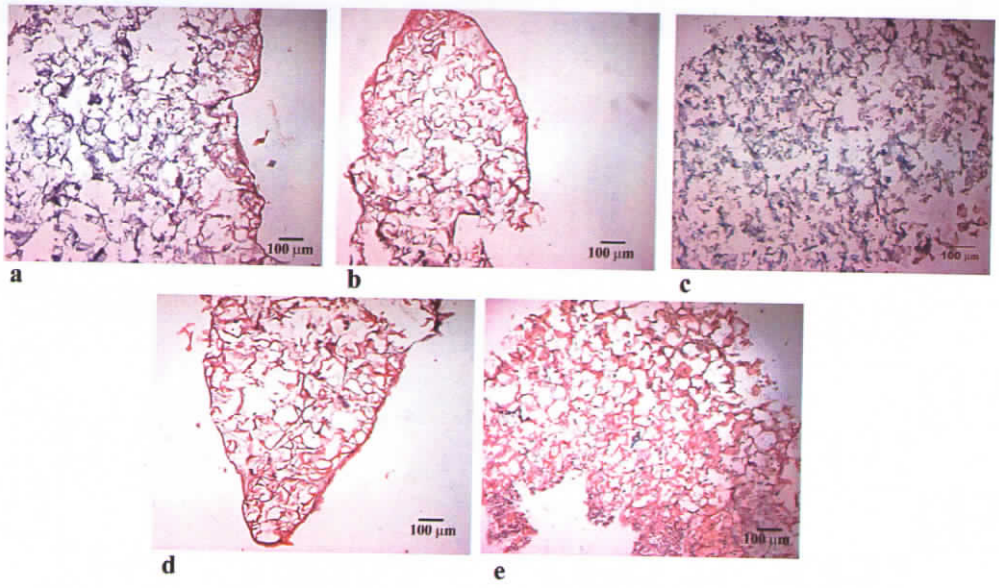


Fig 93. The sections of the Gelatin-Albumin constructs grown indifferent growth factor supplemented conditions stained for glycosaminoglycans. a) TGFβ1, b) TGFβ3, c) BMP2, d) TGFβ1+TGFβ3, e) TGFβ3+BMP2.

In the groups where the individual growth factors were supplied, the deposition was either in the periphery or along the margins of the polymer in the scaffold. This observation was similar in both the scaffolds. Among the groups where individual growth factors were supplied, TGFβ3 showed more deposition of GAG in both the scaffolds. In groups with growth factor combinations, the matrix deposition was also seen inside some of the pores in both the scaffolds. The pattern of glycosaminoglycans secretion in PVA-PCL and Gelatin-Albumin constructs in the different growth conditions was similar in the combination of TGFβ3+BMP2 showing the high deposition of GAG. Even though the deposition pattern was similar in both the scaffolds, deeper staining was observed in PVA-PCL constructs than the Gelatin-Albumin.

3.4.1.6. IMMUNOSTAINING FOR COLLAGEN TYPE II

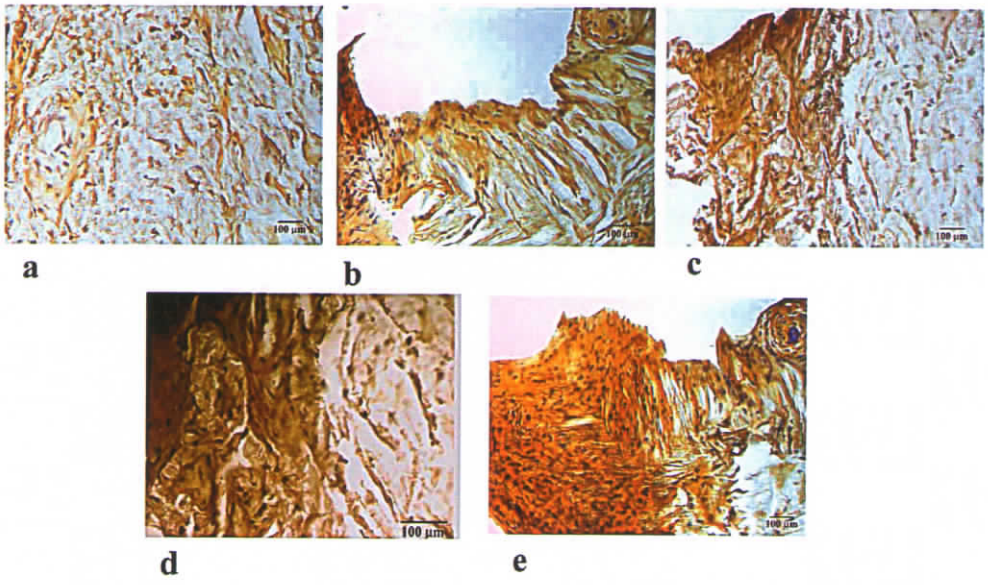


Fig 94. The sections of the PVA-PCL constructs grown indifferent growth factor supplemented conditions immuno stained for collagen type II. a. TGFβ1, b. TGFβ3, c. BMP2, d. TGFβ1+TGFβ3, e. TGFβ3+ BMP2.

The deposition of collagen type II, the typical chondrogenic marker was identified by immunostaining of the sections. The sections of the PVA-PCL constructs grown in different growth factor supplemented conditions immuno stained for collagen type II is represented in Fig 94. The TGFβ1 supplemented constructs showed a uniform deposition of collagen type II, but the staining was very faint showing less deposition. The TGFβ3 supplemented constructs, showed a deeper staining indicating more deposition of collagen type II. In BMP2 supplemented groups, collagen was seen deposited more towards the periphery. The combination TGFβ3+BMP2 showed a deeper staining indicating the secretion of more amount of collagen type II than the other combination.

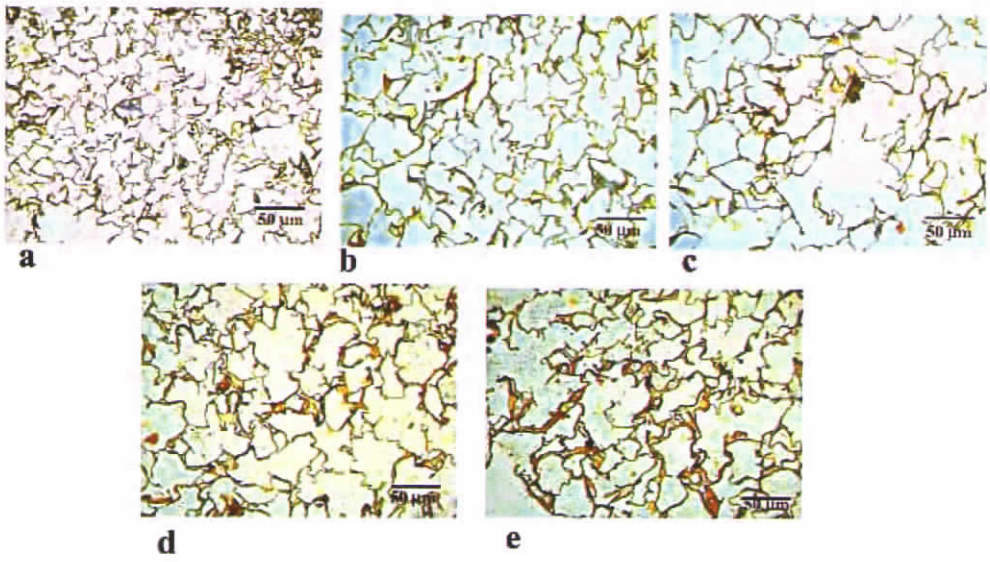


Fig 95. The sections of the Gelatin-Albumin constructs grown in different growth factor supplemented conditions immuno stained for collagen type II. a. TGFβ1, b. TGFβ3, c. BMP2, d. TGFβ1+ TGFβ3, e. TGFβ3 + BMP2.

The sections of the Gelatin-Albumin constructs grown in different growth factor supplemented conditions immuno stained for collagen type II is represented in Fig 95. The immunostaining in Gelatin-Albumin scaffold did not show much deposition of collagen type II. The TGFβ1+TGFβ3 and TGFβ3+BMP2 supplied constructs showed a comparatively higher amount of collagen deposition. The deposition of collagen in Gelatin-Albumin constructs was confined to the margins of the scaffold and was very less when compared to PVA-PCL constructs.

3.4.1.7. GENE EXPRESSION ANALYSIS

The relative expression of collagen type II gene in different growth factor supplemented groups in PVA-PCL scaffold is represented in Fig 96. A significant increase in expression of collagen type II was observed in TGFβ3 than BMP2 and TGFβ1 in PVA-PCL constructs. In the combination the TGFβ+BMP2 showed a significant upregulation when compared to TGFβ1+TGFβ3. But a significant change could not be observed when the expression of TGFβ3 and TGFβ3+BMP2

was compared. In Gelatin-Albumin constructs, reliable results could not be obtained due to low amount of collagen type II expression.

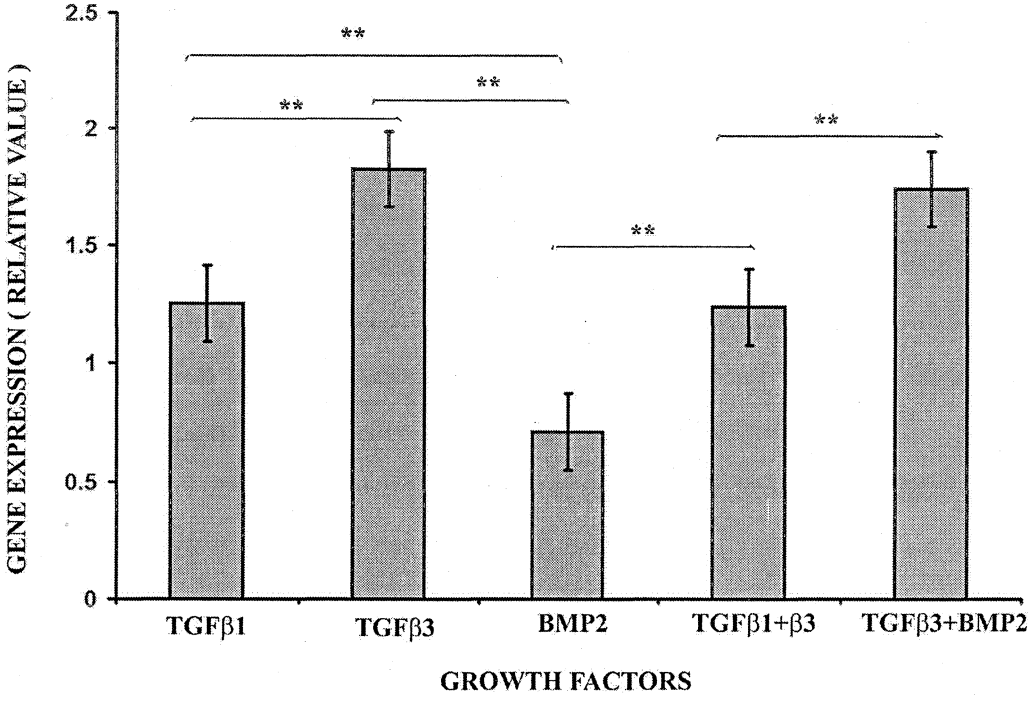


Fig 96. The relative expression of collagen type II gene in PVA-PCL constructs, grown in different growth conditions.

3.4.1.8.COMPARISON OF DIFFERENT GROWTH FACTOR SUPPLEMENTED CONDITIONS IN THE TWO SCAFFOLDS

In this study, both the 3 D scaffolds showed excellent adhesion of mesenchymal stem cells and their high porosity promoted uniform distribution of cells even deep inside the scaffolds. The scaffolds were biocompatible and promoted cell proliferation and deposition of extra cellular matrix as clearly evident from the scanning electron micrographs and other results. This study evaluates the effect of growth factors in mesenchymal stem cell differentiation to chondrocytes, in 3D scaffolds at the end of 28 day culture period. Several studies have been conducted earlier to investigate the effect of individual growth factors in

the differentiation process.(*Huang et al 2002, Miyanishi et al 2006*) However in the native cartilage, the normal process of chondrogenesis is regulated by an array of growth factors, that acts either simultaneously and/or in combination. So it is necessary to study, the effect on chondrogenesis when the growth factors are supplied in combination. Even though some studies have been carried out to see the combined action of growth factors in chondrocyte pellet culture, (IIm et al 2006, Spagnoli et al 2005) few studies have been reported to see this effect of growth factors on differentiation of mesenchymal cells seeded on to 3D scaffolds.

In this context we aimed to look into the effect of growth factors in stem cell differentiation to chondrocytes in two, different 3D scaffolds, when growth factors are supplied individually as well as in combinations. Since the growth factors TGFβ1, TGFβ3 and BMP2 *(Schmitt et al 2003, Williams et al 2003, Wang et al 2005)* were reported to play very important role in chondrogenesis, these growth factors were selected for our study. TGFβ1 was initially used to induce chondrogenesis in most of the earlier studies, but some of the recent studies have shown that TGFβ3 induce a more rapid and thorough expression of chondrogenic markers. (*Bosnakovski et al 2006, Barry et al 2001*) Chondrogenic potential appears to be related to the expression of CD105, a transforming growth factor-β3 (TGFβ3) receptor, present on bone-marrow-derived stromal cells. Reports have shown that chondrocytic phenotype was obtained following exposure of three-dimensional cultures of these cells to TGFβ3 (*Majumdar et al 2000*). So both the isoforms of TGFβ were selected for this study. The concentrations of growth factors were selected based on the widely accepted concentration of each of these signalling molecules for chondrogenesis. (*Mackay et al 1998, Ichiro et al 2005*). TGFβ1 was supplemented at a concentration of 20 ng /ml instead of commonly employed 10 ng/ml. This was based on the report by *Lisignoli et al 2005* that TGFβ1 at a concentration of (20ng/ml) induces better invitro chondrogenesis than 10 ng/ml. Moreover it has been reported that increasing the doses of TGFβ (*Zhou et al 2004)* or a brief exposure to high doses of TGFβ enhance cartilage formation

and limit osteogenesis and adipogenesis. Our studies with chondrocytes have indicated that supplement of dexamethasone would help to retain the chondrogenic phenotype for long time. This and other reports have also shown that supplement of dexamethasone along with growth factors would increase the chondrogenic response of mesenchymal stem cells. (*Bosnakovski et al 2006, Wang et al 2005*) So dexamethasone was included in the chondrogenic medium used in this study.

The constructs supplemented with individual growth factors TGFβ1, TGFβ3 and BMP2 for a period of 28 days and evaluated for chondrogenesis at the end of culture period. The scanning electron microscopy gives information regarding the, overall distribution of cells, cell number, morphology and deposition of matrix components on the scaffolds. The surface morphology of constructs, supplemented with individual growth factors, showed the TGFβ3 supplemented constructs had more number of cells and secretion of extra cellular matrix molecules. The qualitative SEM results thus indicate that among individual growth factors, TGFβ3 promoted the cell proliferation as well as chondrogenesis to a greater extend than others, while BMP2 supplemented constructs showed few cells on the surface. The cells appeared in patches with a non uniform distribution in BMP2. However the cells in these constructs had a more spherical morphology which was similar to nature of chondrocytes cultured in 3D constructs. Several studies have proved that spherical morphology of chondrocytes in culture and in 3D scaffold is an indication of maintaining the chondrogenic phenotype. These results suggest that BMP2 helped to maintain the chondrogenic phenotype rather than promoting cell proliferation. A quantitative data on cell proliferation could not be obtained, as the analysis to quantify the DNA using Hoechst dye, did not give consistent values among the replicates in each group. The TGFβ1 supplied constructs deposited comparatively higher amount of matrix components than BMP2.

Chondrogenesis was evaluated by quantitative estimation of glycosaminoglycans and total collagen. Among the individual growth factors, the total amount of GAG and collagen content were higher in TGFβ3 supplemented constructs. The safranin O staining of sections for GAG and immunostaining for collagen type II was enhanced in TGFβ3 supplied constructs than TGFβ1. Thus our results agree to the previous reports that the TGFβ3 is more responsive than TGFβ1. BMP showed less secretion of these chondrogenic markers.

Chondroinductive effects of TGFβ have been well established in embryonic and adult MSCs. TGFβ enhances cartilage formation and induces cartilage specific gene expression, as well as mitogen activated protein kinases and cytoskeletal molecules. (*Zhou et al 2004, Tuli et al 2003*) TGFβ elicits these effects through the high affinity interactions with two heteromeric complexes (TGFβR1 and TGFβR II) or through additional receptor moieties betaglycan (TGFβR III)/ endoglin highly expressed by MSCs. (*Myazono et al, 1994, Lopez et al*). Endoglin/CD 105, a marker for mesenchymal stem cells is a TGFβR III receptor. The presence of large number of these receptors, on the stem cell surface might have resulted in enhanced chondrogenic response for TGFβ3. Some of the recent studies also support for better chondrogenic response by TGFβ3. (*Bosnakovski et al 2006, Barry et al 2001*) W*ang et al 2005* reported dexamethasone and TGF-β3 were essential for the survival, proliferation and chondrogenesis of MSCs in the silk scaffolds. Differentiation of mesenchymal stem cells is carried out in our study in chondrogenic medium supplemented with dexamethasone. Other Studies have pointed out, that glucocorticoids are required for chondrogenic differentiation of hMSCs in vitro, which enhances the expression of chondrogenic markers. (*Derfoul et al 2006)*

BMPs showed the least amount of chondrogenic markers in our scaffolds at a concentration of 25 ng/ml. BMPs have been described as one of the most potent

factors of chondrocyte differentiation. (*Kramer et al 2000*). However MSCs in pellet culture did not show differentiation to chondrocytes when treated with BMP6 at 10 ng/ml and showed the differentiation only at a higher concentration of 500 ng/ml. (*Sekiya et al, 2002*) Some early studies have shown that a concentration of 1000 ng/ml of BMP2 is required to promote differentiation of chondrogenic ATDC5 cells, suggesting that MSCs and chondrogenic cells are less responsive to BMPs.

The two different culture conditions in our study where growth factors were supplied in combinations are TGFβ1+TGFβ3 and TGFβ3+BMP2. TGFβ3 showed better chondrogenic response than TGFβ1 when supplied individually. So TGFβ3 was selected to form a cocktail with BMP2 instead of TGFβ1. Comparing the effect of growth factors in combinations, the scanning electron micrographs of the constructs supplemented with TGFβ3 and BMP2, showed a very smooth surface covered with cells and extra cellular matrix, completely masking the pores of porous scaffold. The matrix deposition was higher than other group supplemented with TGFβ1+TGFβ3. These results reveal that the growth factor combination, TGFβ3+BMP2 have a better effect on deposition of extra cellular matrix components. Since the extra cellular matrix deposition was so high in these constructs, the morphology of the cells was not very evident. TGFβ3+BMP2 combination had a significant increase in GAG and total collagen content than TGFβ1+ TGFβ3. All the constructs were positive for safranin O staining for GAG and immunostaining for collagen type II, but the staining intensity was higher in the combination TGFβ3+BMP2. The deposition of GAG and collagen type II had a uniform pattern of distribution. Comparing the expression of type II collagen gene, TGFβ3+ BMP2 combinations showed an increase, however we could not find a significant difference with TGFβ3 supplied group.

When the constructs were treated with BMP2, the overall chondrogenic response was very low in both the scaffolds. Our results correlate with previous reports that TGFβ is more responsive than BMP2. But BMP2 showed a better chondrogenesis in presence of TGFβ3. Some of the early studies points out that in limb bud mesodermal cells chondrogenesis is initiated by BMP2 and further modulated by TGFβ. (*Chen et al 1991*) It can also be inferred that signal transduction mediated by TGFβ in the initial step of differentiation may be indispensable for establishment of a response to BMPs, which dramatically accelerate further differentiation. However the close association of TGFβ and BMP2 enhances the chondrogenesis irrespective of the scaffold materials. Based on all these results, on overall distribution of cells and secretion of cartilage specific molecules, it is concluded that, a combination of TGFβ3 and BMP2 promotes better differentiation of mesenchymal stem cells to chondrocytes, in the 3D scaffolds than other growth conditions used in this study. However we have evaluated the chondrogenic response only at the end of 28 day culture. The inferences were made based on the results at the end of 4 weeks time. We did not look into the sequence of events in molecular signalling throughout this period. *Schmit et al 2003* have also observed an increase in the chondrogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) in pellet culture in presence of TGFβ3 and BMP2.

To understand the mechanism of action of growth factors combinations used for our study we looked into molecular signalling process of these growth factors in literature. Studies have shown that two major pathways involving Smad proteins are activated by members of TGFβ super family. (Miyazawa *et al 2002, Hwang et al 2006, Barry et al, 1999*) The isoforms of TGFβ1/β2/β3 bind to TGFβ specific Type I and Type II receptors while bone morphogenetic proteins (BMPs) bind to BMP specific Type I and Type II receptors. Ligand binding further activates the downstream Smad signalling molecules consisting of R Smads, Co

Smads and I Smads. The receptor regulated Smads (R Smads) are of different types- Smad2 and Smad3 which are activated by TGFβ specific receptors while smad1/5/8 are activated by BMP specific receptors. Further the R Smads form complexes with CoSmads and translocate to nucleus and regulate gene expression.

Previous reports have shown that TGFβ and BMP2 act by distinct mechanisms to promote invitro differentiation to cartilage. (Keiji et al, 2002) The molecular mechanism of signalling process very well reveals, two pathways of Smad signalling are simultaneously activated when combination of TGFβ3 and BMP2 is used. A single pathway might have been activated in the case of TGFβ1 and β3 combination. Simultaneous activation of both the pathways in the combination TGFβ3 and BMP2 might have resulted in enhanced chondrogenesis. The probable explanation for enhanced action of TGFβ3 and BMP2 by simultaneous activation of both the molecular pathways is represented in Fig 97. However we have not conducted any further studies to look into this matter in this work. This study also points out the need to understand the molecular signalling process in detail to identify the appropriate growth factor cocktail required for invitro chondrogenesis. Further studies also need to address the effect of these growth factors on chondrogenesis when supplied in a sequential manner rather than combination.

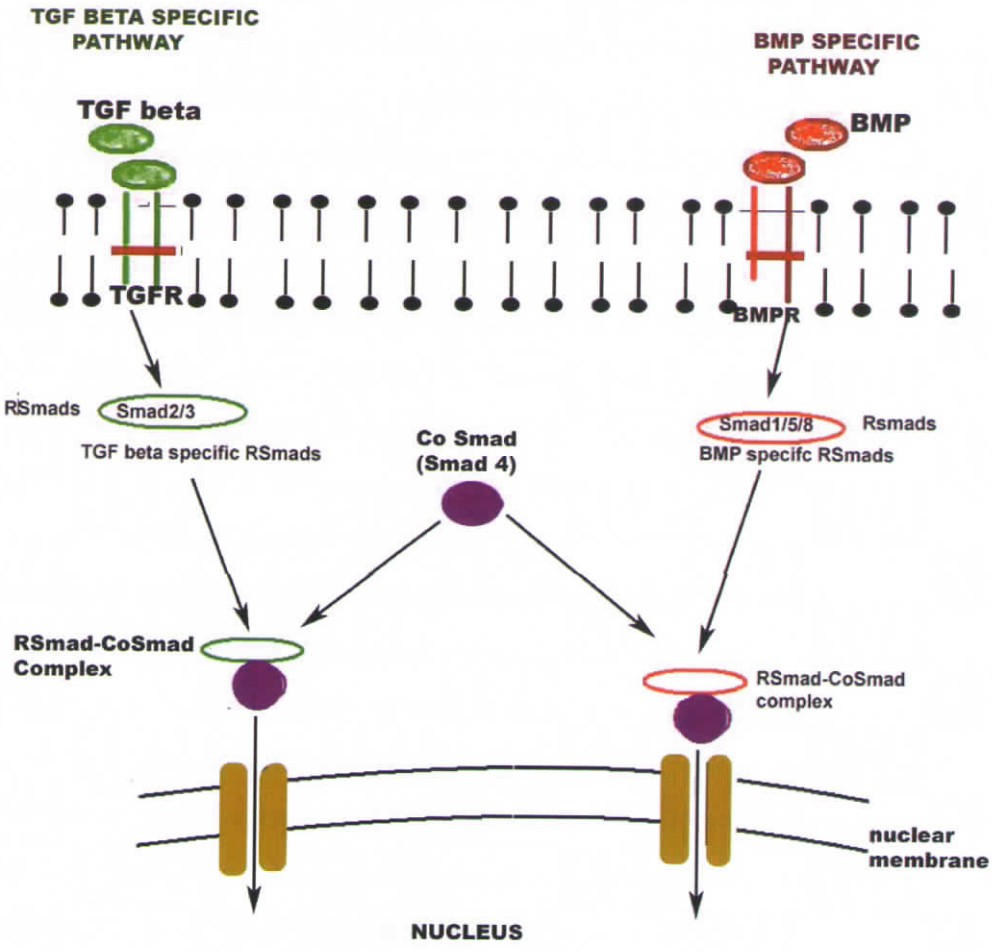


Fig 97. The probable explanation for enhanced action of TGFβ3+BMP2 combination with simultaneous activation of both the molecular pathways.

The induction of chondrogenesis in mesenchymal stem cells depends on the coordinated activities of many factors like cell adhesion, cell density and appropriate signalling of growth factors. The general response of cells to different growth factor supplements was more or less similar in both the scaffolds. The scanning electron micrographs of the two scaffolds did not show much difference in cell and extracellular matrix deposition on the surface. But the amount of chondrogenic markers like GAG and collagen deposition in the interior of the scaffold was higher in PVA-PCL constructs than Gelatin-Albumin. This was

evident from the staining of the sections for GAG and collagen type II. The amount of GAG secretion in PVA-PCL scaffold supplemented with TGFβ is comparable to the values reported in other 3D scaffolds using human MSCs .(*Hofmann et al 2006*) Comparison of invitro differentiation of human and rat MSCs in 3D hyaluronan scaffold have shown that cells acquire a unique phenotype of chondrocytes without significant differences in terms of time of expression of extracellular matrix proteins (*Zavan et al 2007*) It is known that chondrogenesis is initiated when densely packed precursor cells form appropriate cell-cell contact. In Gelatin-Albumin scaffold, the seeding density might have been reduced due to its swelling. This might be one reason for reduced deposition of matrix components in the interior of Gelatin-Albumin construct. We believe that the differentiation of MSCs is favored in PVA-PCL scaffold than the Gelatin-Albumin not only by its three dimensional structure, but also by its composition. A similar response was observed in our studies with chondrocytes in the two scaffolds. The culture with chondrocytes very well shows the inbuilt nature of PVA-PCL promoting more chondrogenesis in terms of cell morphology and extracellular matrix production. The chemical composition and the Semi IPN structure of the PVA-PCL scaffold might have shown a similar influence in mesenchymal stem cell differentiation to chondrocytes. This resulted in enhancement of chondrogenic markers. A change in mesenchymal cell morphology on PVA-PCL scaffold is not observed as in chondrocytes culture. The spherical morphology of cells due to chondrogenic response was seen only in BMP2 supplemented PVA-PCL constructs. It is inferred from the results on the amount and pattern of extracellular matrix deposition, that the biomaterial surfaces may have a substantial effect on the action of growth factors on cellular performance.

Our 3 dimensional Semi IPN poly(vinyl alcohol)-poly(caprolactone) scaffold together with an appropriate combination of growth factors like TGFβ3 and BMP2 promoted better differentiation of mesenchymal stem cells to chondrocytes than other growth conditions used in the study. Future studies are aimed at enhancing the production of extracellular matrix using bioreactors to facilitate better supply of nutrients, gas and metabolites.

Chapter 4
Summary and Conclusions

4.1. RESTATEMENT OF THE PROBLEM

Articular cartilage is a smooth white glistening tissue that helps in the friction free movements of the joints, transfer and distributes the load applied at the joints and resist compression. It is composed of cells and a highly specialized extracellular matrix consisting of collagen and proteoglycans. The functions of the cartilage are performed by these extra cellular matrix molecules, which forms the most important components of cartilage tissue. Cartilage is expected to maintain these functions for six to eight decades but the gradual wear and tear associated with old age, sports injury, arthritis and other congenital diseases damage the healthy cartilage. Once damaged, cartilage has very poor regenerative capacity due to its avascular nature and low mitotic activity of the cells. The currently available treatment options have lot of limitations. Nonsurgical treatment methods only provide a relief to pain at the joints. As the disease progress, surgical treatments methods are often employed depending on the nature, degree of damage and the age of the patients. Treatments like autologous chondrocyte implantation mainly results in the formation of fibroid tissue, with very poor functional properties when compared to native cartilage. Whole joint transplantation is the last option to treat defective cartilage. Even though this has been successful to a certain degree, these artificial implants have a limited life span. The World Health Organization and other statistical survey points out that, the damage to cartilage affect a large number of people and have an increasing tendency in the future. This condition restricts the human activities and creates a huge economic burden to the society. Thus there is a great need for the development of techniques, to stimulate or augment biological healing methods, to restore mobility of damaged articular surfaces.

Tissue engineering is a method of generating biological substitute for damaged organs. The major advantage of tissue engineering approach is that, tissues can be reconstructed to closely match the requirements of the patient and can be transplanted into the patient's body with no immune rejection. This

eventually conquers several limitations encountered in the traditional tissue transplantation approaches. The prime components of tissue engineering are 3 dimensional scaffolds, cells and signalling molecules. An appropriate combination of 3 dimensional scaffolds, cell source and signalling molecules are required for successful invitro regeneration of the cartilage tissue. The three dimensional scaffolds serves as an artificial extracellular matrix, acting as a support for the cells during invitro culture and enabling the complex 3 dimensional cellular interactions, similar to that, in the native tissue.

The first hypothesis evaluated in this thesis is that, the physicochemical properties of the scaffolding material may significantly influence the behavior of chondrocytes during invitro regeneration of cartilage tissue. One of the limitations of tissue engineering of cartilage is to obtain sufficient number of cells for tissue regeneration. Chondrocytes have limited proliferation capacity and they tend to loose its phenotype during invitro culture in monolayer. Mesenchymal progenitor cells, serve as an alternative source with high expansion and differentiation potential. They are capable of supplying large quantities of cells to form a 3D tissue like structure. The differentiation of stem cells to chondrocytes is cued by signaling molecules. Most of the previous studies on chondrogenesis employed a single growth factor to evaluate the invitro differentiation process. In native tissue the formation of a proper chondrogenic phenotype is regulated by the combined action of several growth factors. So in this study the second hypothesis evaluated is that supplement of a single growth factor may not elicit all the necessary molecular signals for the differentiation process during invitro tissue regeneration. A combination of growth factors along with a chosen 3 D scaffold is necessary to attain appropriate differentiation. The effect of 3 D scaffolds and role of signaling molecules in the differentiation of mesenchymal stem cells is investigated in this study, by supplementing growth factors individually and in various combinations. The methodology adopted to evaluate the two hypothesis put forward in the thesis is highlighted below.

4.2. METHODOLOGY

- Fabricate a hybrid 3 dimensional scaffold using synthetic polymers - Semi inter penetrating network (Semi IPN) of poly (vinyl alcohol) and poly (caprolactone).
- Fabricate a hybrid 3 dimensional scaffold using natural polymers – Blend of Gelatin and Albumin.
- Evaluate the physico-chemical properties of these 3 D scaffolds.
- Isolate and standardize the culture of chondrocytes in monolayer and in 3 D scaffolds.
- Evaluate the effect of both the 3 D scaffolds on chondrocyte morphology, viability, extracellular matrix synthesis and distribution, to form a 3 D cartilage like tissue.
- Isolate and standardize the culture of mesenchymal stem cells in monolayer and in 3 D scaffolds used in this study.
- Evaluate the effect of the growth factors supplemented individually, and in combinations for chondrogenic differentiation of mesenchymal stem cells in 3 D scaffolds.
- Evaluate the effect of the two 3 D scaffolds on the chondrogenic differentiation of mesenchymal stem cells in presence of the signalling molecules.

4.3. MAJOR FINDINGS OF THIS STUDY

The inferences and conclusions of the entire study given below were made, relating the cell response to all the physico-chemical properties of the scaffolds instead of concentrating on a single parameter of the 3 D scaffolds.

- The 3 D hybrid scaffolds exhibit synergistic properties of both the polymers.
- The two scaffolds used for the study, satisfy the basic requirements to serve as a matrix for tissue regeneration. The physico-chemical properties of the two scaffolds varied in comparison to each other.
- The seeding methodology was standardized to give a uniform distribution of cells throughout the scaffold.
- The chondrocytes responded differently to each of these scaffolds. The cell response varied during the initial process of attachment as well as the end of tissue regeneration in a period of two months. In this study chondrogenic response was found to be better in PVA-PCL scaffold than Gelatin-Albumin scaffolds.
- The cells retained a spherical morphology in synthetic PVA-PCL scaffolds, while they spread and had an elongated morphology in the Gelatin-Albumin scaffolds. In gelatin–albumin scaffold, the inbuilt biomimetic sequences might have stimulated the cell to attach and spread on to the material to form a fibroblastic morphology The chemical composition, together with the adequate hydrophobic-hydrophilic balance, of the PVA-PCL scaffold, favouring moderate hydrophilicity, might have helped the cell to retain its spherical morphology. This might have subsequently regulated the following events of proliferation and extracellular matrix deposition. A hydrophilic-hydrophobic balance has been attained in the wettability of both the scaffolds. So it can be inferred that, it is the chemical composition that played an important role in regulating the cell phenotype in case of PVA-PCL.
- The PVA-PCL promoted more secretion of the chondrogenic molecules than gelatin and albumin. The basic structure of the PVA-PCL Semi IPN was that of mechanically stable PCL chains, forming interpenetrating

polymer network, with hydrophilic PVA. This was similar to the network of collagen and glycosaminoglycans in native cartilage. This structure might have provided a better microenvironment for the chondrocytes to secrete extracellular matrix. Though biomimetic in nature, such a network structure was not available in Gelatin-Albumin scaffold.

- The PVA-PCL had a wide range of pore size while the Gelatin-Albumin had a uniform pore size. The chondrocytes might have preferred the large difference in pore size of PVA-PCL than the uniform pore size in gelatin.
- In culture, the swelling behavior was different in both the scaffolds which widely influenced the quality of invitro generated construct. The PCL component in PVA-PCL Semi IPN, prevented extensive swelling of PVA thus optimizing the swelling nature of the scaffold. This situation was favorable for cells in creating a suitable 3D environment, which resulted in uniform distribution of extracellular matrix. Gelatin-Albumin scaffold had a very high swelling that produced an increase in total surface area of the scaffold. This reduced the optimum cell density to produce a uniform distribution of cells throughout the scaffold. It resulted in uneven distribution of ECM, which was confined to the outer surface and edges. More over the formation of a skin layer over the surface might have prevented the availability of nutrients to the interior of the scaffold.
- Scaffold structure and the seeding methodology determine the overall cell distribution. The dynamic seeding was appropriate to bring a uniform distribution of mesenchymal cells in both the scaffolds.
- The general response of mesenchymal stem cells to different growth factor supplements was more or less similar in both the scaffolds.
- Different members of TGFβ super family showed significant difference in production of chondrogenic markers. The constructs supplemented

with TGFβ3 showed a higher chondrogenic response than TGFβ1 and BMP2 used in this study.

- The cells had a spherical morphology in BMP2 supplemented PVA-PCL Semi IPN constructs. This indicates that the biomaterial surface influence the growth factors in regulating the chondrogenic phenotype.
- The combination of growth factors promoted more cell proliferation, secretion of glycosaminoglycans and collagen than individual growth factors in our scaffolds.
- Combination of TGFβ3 and BMP2 is a better growth factor cocktail than other growth factor combinations, resulting in enhanced chondrogenesis in this study.
- The PVA-PCL constructs showed a uniform and higher deposition of chondrogenic markers than Gelatin-Albumin.
- In Gelatin-Albumin scaffolds, the cells and extracellular matrix accumulation during the stem cell differentiation was confined to the outer surface and periphery of the scaffolds. These observations were similar to the chondrocyte culture and should be dependent mainly on the physico-chemical properties of the scaffold.
- Proper invitro differentiation of stem cells is cued by appropriate combination of 3D scaffolds, cells & growth factors. 3 dimensional PVA-PCL Semi IPN scaffold, together with an appropriate combination of growth factors, like TGFβ3 and BMP2 promoted better differentiation of mesenchymal stem cells to chondrocytes than other growth conditions used in the study.
- It is inferred from the results on pattern and amount of extracellular matrix deposition, that the 3D biomaterial surfaces may have a substantial effect on the action of growth factors on cellular performance.

4.4. CONCLUSION

These findings points out, how the physicochemical properties of the scaffold affect the initial cell response, which regulates the whole sequence of events, in the further growth and maturation of the construct. It is essential that the scaffolds need to provide a suitable microenvironment for the cells throughout the course of culture period. The variation in the chondrogenic response between the two scaffolds used in this study, is as a result of the combined effect of various physicochemical parameters. The results and the conclusions were made relating all these physico-chemical properties, rather than concentrating on a single parameter of the 3 D scaffolds. Taken together, these findings suggest that, scaffold composition and architecture that retain, the chondrogenic phenotype, cell density and promote free diffusion of nutrients, for all the cells that are seeded even deep inside the scaffold, throughout the culture period would serve as an ideal support for cartilage regeneration.

The TGFβ and BMP2 are the major signalling molecules involved in the differentiation of mesenchymal stem cells to chondrocytes. An appropriate growth factor cocktail is required for providing all the necessary molecular cues for the faster invitro regeneration of cartilage tissue. The microenvironment provided by the 3 dimensional scaffold play a significant role in regulating cell proliferation, differentiation and synthesis of extracellular matrix. It is inferred from the results on the pattern and amount of extracellular matrix deposition, that the 3D biomaterial surfaces may have a substantial effect on the action of growth factors on cellular performance. In conclusion the physico-chemical properties of the scaffolding material greatly influence the chondrogenic response during invitro culture. So it is necessary to give atmost importance while selecting the supporting matrix for tissue regeneration. Appropriate combination of 3 dimensional scaffolds and growth factor signalling is required for differentiation and maintenance of the chondrogenic phenotype during invitro regeneration of cartilage tissue.

FUTURE PROSPECTS

The studies that need to be carried out further to improve the understanding of cartilage regeneration are

- Mimicking the in vivo conditions in constructing tissue engineered cartilage using bioreactor.
- Evaluate the functional properties of the tissue engineered cartilage.
- In vivo toxicological evaluation of PVA-PCL scaffold.
- In vivo evaluation of cell seeded PVA-PCL construct in small defects in rabbits.
- In vivo evaluation of cell seeded PVA-PCL construct in large defects in large animals.

BIBLIOGRAPHY

Adhirajan, N., Shanmugasundaram, N., Babu, M. (2007). Gelatin microspheres cross-linked with EDC as a drug delivery system for doxycyline: Development and characterization. Journal of Microencapsulation. **24:** 659.

Aggarwal, S., Pittenger, M.F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. **105:** 1815.

Aigner, T., Zien, A., Gehrsitz, A., Gebhard, P.M., McKenna, L.A. , (2001). Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology. Arthritis Rheum. **44:** 2777.

Alastair, M., Mackay, Stephen, C., Beck, J., Murphy,M., Barry,F.P.,Chichester,C.O., Pittenger, M.F. (1998). Chondrogenic Differentiation of Cultured Human Mesenchymal Stem Cells from Marrow. Tissue Eng. **4:** 415.

Alves, M. C., Yang, Y., Carnes, D.L., Ong, J., Sylvia, V.L., Dean, D.D., Agrawal, C.M., Reis, R.L. (2007). Modulating bone cells response onto starch-based biomaterials by surface plasma treatment and protein adsorption. Biomaterials. **28:** 307.

Angele, P., Kujat, R., Nerlich, M. (1999). Engineering of osteochondral tissue with bone marrow mesenchymal progenitor cells in a derivatized hyaluronan-gelatin composite sponge. Tissue Eng. **5:** 545.

Anker, P. S., Scherion S.A., Kleijburg-van der Keur. (2003). Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood. **102:** 1548.

Arthritis & Rheumatism. Data Source: 2003 NHIS. (2006), **54:** 226.

Asahina, I., Sampath, T.K., Nishimura. (1993). Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol. **123:** 921.

ASTM, (2003). Standard test method for compositional analysis by thermogravimetry. ASTM International. West Conshohocken, PA.

ASTM, D (1996). Standard test method for compressive properties of rigid plastics. ASTM International. West Conshohocken, PA.

ASTM, E (2003). Standard test method for assignment of the glass transition temperature by differential scanning calorimeter and differential thermal analysis. ASTM International. West Conshohocken, PA.

Athanasiou, K. A., Agrawal, C.M., Barber, F.A., Burkhart, S.S. (1998). Orthopaedic applications for PLA-PGA biodegradable polymers. Arthroscopy. **14:** 726.

Athanasiou, K. A., Niederauer,G.G.,Agrawal,C.M. (1996). Sterilization toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymer. Biomaterials. **17:** 93.

Awad, H. A., Halvorsen, Y.D., Gimble, J.M. (2003). Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. Tissue Eng. **9:** 1301.

Awad, H. A., Wickham, M.Q., Leddy, H.A., Gimble, J.M., Guilak, F. c. (2004). Chondrogenic differentiation of adiposederived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials. **25:** 3211.

Bacáková, L., Filová, E., Rypácek, F., Svorcík, V., Starý, V. (2004). Cell adhesion on artificial materials for tissue engineering. Physiol. Res. **53:** 35.

Bahrami, S., Stratmann, U., Wiesmann ,H.P., Mokrys ,K., Bruckner,P.,Szuwart,T. (2000). Periosteally derived osteoblast-like cells differentiate into chondrocytes in suspension culture in agarose. The Anatomical Record. **259:** 124.

Barany, G., Merrifield,R.B. (1980). The Peptides, Academic press, Newyork.

Bari, C. D., Dell'Accio, F., Tylzanowski, P. (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum. **44:** 1928.

Barnes, P., Pemble, C.W., Brand, D.D., Simpson,D.G., Bowlin,G.L. (2007). "Cross-Linking Electrospun Type II Collagen Tissue Engineering Scaffolds with Carbodiimide in Ethanol." Tissue Eng.

Barry , F., Boynton, R., Murphy, M., Haynesworth, S., Zaia, J. (1999). The monoclonal antibody SH-2 raised against human mesenchymal stem cells, recognizes the epitope on endoglin (CD105). Biochem Biophys Res Commun. **265:** 134.

Barry, F., Boynton, R.E., Liu, B. (2001). Chondrogenic differentiation of mesenchymal stem cells from bone marrow: Differentiation-dependent gene expression of matrix components. Exp Cell Res. **268**:189.

Bartholomew, A., Sturgeon, C., Siatskas, M. (2002). Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. **30:** 42.

Bau, B., McKenna, L.A., Soeder, S., Fan, Z., Pecht, A., Aigner, T., (2004). Hepatocyte growth factor/scatter factor is not a potent regulator of anabolic and catabolic gene expression in adult human articular chondrocytes. Biochem. Biophys. Res. Commun. **316:** 984.

Beaver, R. J., Mahomed, M., Backstein, O., Davis, A., Zukor, D.J., Grass. (1992). Fresh osteochondral allografts for post-traumatic defects in the knee: A survivorship analysis. J Bone Joint Surg. **74B:** 105.

Benslimane, S., Guidoin, R., Roy, P.E., Friede, J., Hébert, J., Domurado, D., Luizard, M.F. (1986). Degradability of crosslinked albumin as an arterial polyester prosthesis coating in in vitro and in vivo rat studies. Biomaterials. **7:** 268.

Benya, P. D., Shaffer, J.D. (1982). Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell. **30:** 215.

Berkland, C., Pollauf, E., Varde, N., Pack, D.W., Kim, K. (2007). Monodisperse liquid-filled biodegradable microcapsules. Pharmaceutical Research. **24:** 1007.

Berthold, A., Haibel, A., Brandes, N., Kroh, L., Gross, U., Uharek, L., Schubert, H. (2007). Biocompatible porous ceramics for the cultivation of hematopoietic cells. J. Mater Sci: Mater in Med **18:** 1333.

Besheer, A., Mäder, K., Kaiser, S., Kressler, J., Weis, C., Odermatt, E.K. (2007). Tracking the urinary excretion of high molar mass poly(vinyl alcohol. J Biomed Mater Res. **82:** 383.

Bhattacharjee, S., Besra, L., Singh, B.P. (2007). Effect of additives on the microstructure of porous alumina. J. Europ Ceramic Soc. **27:** 47.

Bianco, P., Riminucci, M., Gronthos, S. (2001). Bone marrow stromal stem cells: Nature, biology, and potential applications. Stem cells. **19:** 180.

Birkedal, H. H. (1995). Proteolytic remodeling of extracellular matrix. Current Opinion in Cell Biology. **7:** 728.

Blanc, L. K., Rasmusson, I., Sundberg, B. (2004). Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. **363:** 1439 -1441.

Bobyn (1999). Characteristics of bone ingrowth and interface mechanics of a new porous tantalum biomterial. J Bone Joint surg. **81:** 907.

Bosman, F. T., Stamenkovic, I. (2003). Functional structure and composition of extracellular matrix. J Pathol. **200:** 423.

Bosnakovski, D., Mizuno, M., Kim, G., Takagi, S., Okumura, M., Fujinaga, T. (2006). Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. Biotechnol Bioeng. **93:** 1152.

Brauker, J. H., Brendel, V.C.E., Martinson, L.A., Crudele, J., Johnston, W.D.,Johnson,R.C. (1995). Neovascularization of synthetic membranes directed by membrane microarchitecture. J. Biomed Mater Res. **29:** 1517.

Bruckner, M., van der Rest. (1994). Structure and function of cartilage collagens. Microsc. Res. Technol. **28:** 378.

Bryant, S. J., Anseth, K.S. (2001). The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels. Biomaterials. **22:** 619-626.

Buckwalter, J., Hunziker, E., Rosenberg, L., Coutts, R., Adams, M., Eyre, D. (1988). Articular cartilage: composition and structure. Injury and Repair of the Musculoskeletal Soft Tissues. S. Woo, Buckwalter, JA., eds. Park Ridge: 405.

Buckwalter, J. A., Lappin, D.R. (2000). The disproportionate impact of chronic arthralgia and arthritis among women. Clin Orthop. **372:** 159.

Buckwalter, J. A., Mankin, H.J. (1997). Articular cartilage part I: tissue design and chondrocyte-matrix interactions. Journal of Bone and Joint Surgery. **79A:** 600.

Buckwalter, J. A., Martin J.A. (2006). Osteoarthritis. Advanced Drug Delivery ReviewsOsteoarthritis. **58:** 150.

Bunaprasert, T., Thongmarongsri, N., Thanakit, V., Ruangvejvorachai, P., Buranapraditkul, S., Maneesri, S., Kanokpanont, S. (2006). Tissue engineering of cartilage with porous polycarprolactone--alginate scaffold: the first report of tissue engineering in Thailand. Journal of the Medical Association of Thailand. **89:** 108.

Burczak, K., Gamian, E., Kochman, A. (1996). Long-term in vivo performance and biocompatibility of poly(vinyl-alcohol) hydrogel macrocapsules for hybrid-type artificial pancreas. Biomaterials. **17:** 2351.

Buschmann, M. D., Gluzband, Y.A., Grodzinsky, A.J., Kimura, J.H., Hunziker, E.B. (1992). Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix. J Orthop Res. **10:** 745.

Buxton, A. N., Zhu, J.,Marchant,R., West, J.L., Yoo,J.U., Johnstone, B. (2007). Design and Characterization of Poly(Ethylene Glycol) Photopolymerizable Semi-Interpenetrating Networks for Chondrogenesis of Human Mesenchymal Stem Cells. Tissue Eng. **13:** 2549.

Cancedda, R., Dozin, B., Giannon,i P., Quarto, R. (2003). Tissue engineering and cell therapy of cartilage and bone. Matrix Biol. **22:** 81-91.

Cannas, M., Denicolai, F., Webb, L. X., Gristina, A.G. (1988). Bioimplant surfaces: binding of fibronectin and fibroblast adhesion. J orthop res. **6:** 58.

Cao, T., Ho, K.H., Teoh, S.H. (2003). Scaffold design and in vitro study of osteochondral coculture in a three-dimensional porous polycaprolactone scaffold fabricated by fused deposition modeling. Tissue Engineering. **9:** 103.

Capito, R. M. (2003). Scaffold based Articular cartilage repair. IEEE Eng. Med Biol. Mag. **22:** 42.

Caplan, A. I., Bruder, S.P., (2001). Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med. **7:** 259.

Cascone, M. G., Tricoli,M., Cerrai,P., Guerra,R.S.D. (1993). Cell cultures in the biocompatibility study of synthetic materials. Cytotechnology. **11:** 137.

Caterson, E. J., Li, W.J., Nesti, L.J., Albert, T., Danielson, K., Tuan, R.S., (2002). Polymer/alginate amalgam for cartilage-tissue engineering. Ann N Y Acad Sci. **961:** 134.

Domurado, D., Giuidon, R., Marois, M., Martin , L.,Gosselin, C., Awad , J. (1987). Albuminated Dacron prosthesis asimproved blood vessel substitutes. J. Bioeng. **2:** 79.

Donald, G., Phinney, Darwin, J., Prockop. (2007). Mesenchymal Stem/Multipotent Stromal Cells: TheState of Transdifferentiation and Modes of Tissue Repair-CurrentViews. Stem Cells. **25:** 2896.

Dorr, L. D., Takei, G. K., Conaty, J.P. (1983). Total hip arthroplasties in patients less than forty-five years old. J Bone Joint Surg. **65:** 474.

Dozin, B., Malpeli, M., Camardella, L. (2002). Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects. Matrix Biology. **21:** 449.

Drury, J. L., Mooney, D. J. (2003). Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials. **24:** 4337.

Eileen, F., Roark, Greer, k. (1994). Transforming Growth Factor beta and Bone Morphogenetic Protein-2 Act by Distinct Mechanisms to Promote Chick Limb Cartilage Differentiation In Vitro. Developmental Dynamics. **200:** 103.

Eyre, D. R., Weis, M.A.,Wu, J.J. (2006). Articular cartilage collagen: an irreplaceable framework? European Cells and Mater. **12:** 57.

Eyrich, D., Wiese, H., Maier, G., Skodacek, D., Appel, B., Sarhan, H., Tessmar, J.,Blunk, T. (2007). In vitro and in vivo cartilage engineering using a combination of chondrocyte-seeded long-term stable fibrin gels and polycaprolactone-based polyurethane scaffolds. issue Engineering. **13:** 2207-2218.

Farndale, R. W., Buttle, D.J., Barret. (1986). Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue. Biochem Biophys Acta. **883:** 173.

Francois, N. J., Allo, S., Jacobo, S.E., Daraio, M.E. (2007). Composites of polymeric gels and magnetic nanoparticles: Preparation and drug release behavior. J of Appl PolY Sci. **105:** 647.

Frank, E. (1987). Cartilage electro mechanics.1. Electrokinetic transduction and effects of electrolyte pH and ionic strength. J Biomech. **20:** 615.

Chubinskaya, S., Huch, K., Mikecz, K., Cs-Szabo, G., Hasty, K.A., Kuettner, K.E., Cole, A.A., (1996). Chondrocyte matrix metalloproteinase- 8: up-regulation of neutrophil collagenase by interleukin-1 in human cartilage from knee and ankle joints. Lab. Invest. **74:** 232.

Ciardelli, G., Chiono, V., Vozzi, G., Pracella, M., Ahluwalia, A.,Barbani, N.,Cristallini, C., Giusti, P. (2005). Blends of poly-(e-caprolactone) and polysaccharides in tissue engineering applications. Biomacromolecules. **6:** 1961.

Conget, P., Minguell, J.J. (2000). Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol. **109:** 235.

Convery, F. R., Meyers, M.H., Akeson, W.H. (1991). Fresh osteochondral allografting of the femoral condyle. Clin Orthop. **273:** 139.

Cristino, S., Grassi, F., Toneguzzi, S., Piacentini, A., Grigolo, B., Santi, S., Riccio, M., Tognana, E., Facchini, A., Lisignoli, G. (2005). Analysis of mesenchymal stem cells grown on a three-dimensional HYAFF 11-based prototype ligament scaffold. J Biomed Mater Res. **73:** 275.

Dailey, L. A., Jekel, N., Fink, L., Gessler, T., Schmehl, T., Wittmar, M., Kissel, T., Seeger, W. (2006). Investigation of the proinflammatory potential of biodegradable nanoparticle drug delivery systems in the lung. Toxicology and Applied Pharmacology. **215:** 100.

Dekker, A., Panfil, C., Valdor, M., Pennartz, G., Richter, H., Mittermayer, C. H.,Kirkpatrick, C.J. (1994). Quantitative methods for invitro cytotoxicity testing of biomaterials. Cells Mater. **4:** 101.

Derfoul, A., Perkins, G.L., Hall,D.J.,Tuan, R.S. (2006). Glucocorticoids promote chondrogenic differentiation of adult human mesenchymal stem cells by enhancing expression of cartilage extracellular. Genes Stem Cells. **24:** 1487.

Diduch, D. R., Jordan, L.C., Mierisch, C.M. and Balian, G. (2000). Marrow stromal cells embedded in alginate for repair of osteochondral defects. Arthroscopy. **16:** 571.

Dominici, M., Le, Blank, K., Mueller, I. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. **8:** 315.

Domurado, D., Giuidon, R., Marois, M., Martin , L.,Gosselin, C., Awad , J. (1987). Albuminated Dacron prosthesis asimproved blood vessel substitutes. J. Bioeng. **2:** 79.

Donald, G., Phinney, Darwin, J., Prockop. (2007). Mesenchymal Stem/Multipotent Stromal Cells: TheState of Transdifferentiation and Modes of Tissue Repair-CurrentViews. Stem Cells. **25:** 2896.

Dorr, L. D., Takei, G. K., Conaty, J.P. (1983). Total hip arthroplasties in patients less than forty-five years old. J Bone Joint Surg. **65:** 474.

Dozin, B., Malpeli, M., Camardella, L. (2002). Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects. Matrix Biology. **21:** 449.

Drury, J. L., Mooney, D. J. (2003). Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials. **24:** 4337.

Eileen, F., Roark, Greer, k. (1994). Transforming Growth Factor beta and Bone Morphogenetic Protein-2 Act by Distinct Mechanisms to Promote Chick Limb Cartilage Differentiation In Vitro. Developmental Dynamics. **200:** 103.

Eyre, D. R., Weis, M.A.,Wu, J.J. (2006). Articular cartilage collagen: an irreplaceable framework? European Cells and Mater. **12:** 57.

Eyrich, D., Wiese, H., Maier, G., Skodacek, D., Appel, B., Sarhan, H., Tessmar, J.,Blunk, T. (2007). In vitro and in vivo cartilage engineering using a combination of chondrocyte-seeded long-term stable fibrin gels and polycaprolactone-based polyurethane scaffolds. issue Engineering. **13:** 2207-2218.

Farndale, R. W., Buttle, D.J., Barret. (1986). Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue. Biochem Biophys Acta. **883:** 173.

Francois, N. J., Allo, S., Jacobo, S.E., Daraio, M.E. (2007). Composites of polymeric gels and magnetic nanoparticles: Preparation and drug release behavior. J of Appl PolY Sci. **105:** 647.

Frank, E. (1987). Cartilage electro mechanics.1. Electrokinetic transduction and effects of electrolyte pH and ionic strength. J Biomech. **20:** 615.

Freed, L. E., Hollander, A.P., Martin, I., Barry, J.R., Langer, R., Vunjak-Novakovic, G. (1998). "Chondrogenesis in a cell-polymer-bioreactor system." Experimental Cell Research **240**: 58.

Freemont, A. J., Hampson, V.,Tilman, R.,Goupille, P.,Taiwo,Y., Hoyland, J.A. (1997). Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific. Ann. Rheum. Dis. **56:** 542.
Fuchs, J. R., Hannouche, D., Terada, S., Vacanti, J.P., Fauza, D.O. (2003). Fetal tracheal augmentation with cartilage engineered from bone marrow-derived mesenchymal progenitor cells. J Pediatr Surg. **38:** 984.

Fussenegger, M., Meinhart, J., Hobling, W., Kullich, W., Funk, S., Bernatzky, G.*(2003)* Stabilized Autologous Fibrin-Chondrocyte Constructs for Cartilage Repair in Vivo. Ann. Plastic Surg. **51:** 493.

Gan, Z., Liang, Q, Zhang, J.,Jing, X. (1997). Enzymatic degradation of poly(caprolactone) in buffer solution containing lipases. Polym Degrad Stab. **56:** 209.

Gao, J., Dennis, J.E., Solchaga, L.A., Awadallah, A.S., Goldberg, V.M., Caplan, A.I. (2001). Tissue-engineered fabrication of an osteochondral composite graft using rat bone marrow-derived mesenchymal stem cells. Tissue Eng. **7:** 363.

Gerard, C., Catuogno, C., Amargier-Huin, C., Grossin, L., Hubert, P., Gillet, P., Netter, P., Dellacheral E., Payan, E. (2005). The effect of alginate, hyaluronate and hyaluronate derivatives biomaterials on synthesis of non-articular chondrocyte extracellular matrix. J Mater Sci Mater Med. **16:** 541.

Gibbons, D. F. (1992). Tissue response to resorbable synthetic polymers. Degradation Phenomena on Polymeric Biomaterials. D. M. R. M. Plank H, eds. New York, Springer Verlag: 97.

Gobbels, M., Spitnas, M. (1991). Effects of artificial tears on corneal epithelial permeability in dry eyes. Graef arch Clin Exp Opthal. **229:** 345-9.

Gordon, T. D., Schloesser, L., Humphries, D.E., Spector, M. (2004). Effects of the Degradation Rate of Collagen Matrices on Articular Chondrocyte Proliferation and Biosynthesis in Vitro. Tissue Eng. **10:** 1287.

Grande, D. A., Mason, J., Light, E., Dines, D: (2003). Stem cells as platforms for delivery of genes to enhance cartilage repair. J Bone Joint Surg Am. **85:** 111.

Grant, C., Twigg, P., Egan, A.,Moody, A., Smith, A., Eagland, D.,Crowther, N., Britland, S. (2006). "Poly(vinyl alcohol) hydrogel as a biocompatible viscoelastic mimetic for articular cartilage." Biotechnol. Prog. **22**: 1400.

Griffon, D. J., Sedighi, M.R., Schaeffer, D.V., Eurell, J.N., Johnson, A.L. (2006). Chitosan scaffolds: Interconnective pore size and cartilage engineering . Acta Biomaterialia. **2:** 313.

Grigoriadis, A. E., Heersche, J.N.M., Aubin,J.E. (1988). Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J Cell boil. **106:** 2139.

Grisel, Y., Saavedra, L., Mateescu, M., Diana, A., Bates, A., Devisee, F. (2003). Polyvinyl alcohol three-dimensional matrices for improved long-term dynamic culture of hepatocytes. J Biomed Mater Res. **66:** 562.

Grogan, S. P., Barbero,A., Winkelmann,V., Rieser,F., Fitzsimmons, J.S., Driscoll, S., Martin,I.,Varlet,P.M. (2006). Visual Histological Grading System for the Evaluation of in Vitro-Generated Neocartilage. Tissue Eng. **12:** 2141.

Gruber, H. E., Hoelscher, G.L., Leslie, K., Ingram, J.A., Hanley, E.N. (2006). Three-dimensional culture of human disc cells within agarose or a collagen sponge: assessment of proteoglycan production. Biomaterials. **27:** 371.

Gu, Z. Q., Xiao, J.M., Zhang,X.H. (1998). "The development of artificial articular cartilage - PVA-hydrogel." Bio-Medical Materials and Engineering **8**: 75 .

Gunatillake, P. A., Adhikari, R. (2003). Biodegradable synthetic polymers for tissue engineering. Eur cells Mater. **5:** 1.

Hall, C. E., Hall, O. (1963). Polyvinyl alcohol nephrosis: Relationship of degree of polymerization to pathophysiological effects. Proc. Soc. Biol. Med. **112:** 86.

Hanada, K., .Solchaga,L.A. ,Caplan,A., Hering, T.M., Goldberg,V.M., Yoo,J.U., Johnstone,B. (2001). BMP-2 Induction and TGF-b1 Modulation of Rat Periosteal Cell Chondrogenesis. J. Cell. Biochem. **81:** 284.

Hascall, V. C. (1988). Proteoglycans: the chondroitin sulfate/keratan sulfate proteoglycan of cartilage. ISI Atlas Sci Biochem. **1:** 189.

Hassan, C. M., Peppas, N.A. (2000). Structure and applications of polyvinyl alcohol hydrogels produced by conventional crosslinking or by freezing / thawing methods. Adv Polym Sci. **153:** 37.

Heinegard, D. K., Pimentel, E.R., . (1992). Cartilage matrix proteins. Articular Cartilage and Osteoarthritis. R. S. K. Kuettner, J.G. Peyron, V.C. Hascall Eds. Raven Press, New York: 95.

Hoffman, A. (2002). Hydrogels for biomedical applications. Adv. Drug deliv. Rev. **54:** 3.

Hoffmann, S., Knecht,s.,Langer,r.,Kaplan, D.I., Novakovic,G.V., Merkle, H.P., Meinel,L. (2006). Cartilage-like Tissue Engineering Using Silk Scaffolds and Mesenchymal Stem Cells. Tissue Eng. **12:** 2729.

Holland, S. J., Tighe, B.J. (1992). Biodegradable polymers. Advances in Pharmaceutical Science. London, Academic Press. **6:** 101.

Hootman, J. M., Helmick, C.G. (2006). Projections of US prevalence of arthritis and associated activity limitations. Arthritis Rheum. **54:** 226.

Horbett, T. A., Schway, M.B.,Ratner, B.D. (1985). Hydrophilic-hydrophobic copolymers as cell substrates: effect on3T3 cell growth rate. J Colloid Interface Sci. **104:** 28.

Hsu, S.-H., Yen, H. J., Tseng, C.-S., Cheng, C.-S., Tsai, C.L. (2007). "Evaluation of the growth of chondrocytes and osteoblasts seeded into precision scaffolds fabricated by fused deposition manufacturing." Journal of Biomedical Materials Research - Part B Applied Biomaterials **80**: 519.

Htay, A. S., Teoh, S.H., Hutmacher, D.W. (2004). Development of perforated microthin poly(epsilon-caprolactone) films as matrices for membrane tissue engineering. Biomater Sci Polym Ed. **15:** 683.

Huang, Q., Goh, J.C.H., Hutmacher, D.W., Lee, E.H. (2002). Invivo mesenchymal cell recruitment by a scaffold loaded with transforming growth factor L1 and potential for insitu chondrogenesis. Tissue Eng. **8:** 469.

Hubbell, J. A. (1999). Bioactive biomaterials. Curr. Opin. Biotechnol. **10:** 123.

Hulbert, S. F., Young, F. A., Mathews, R. S., Talbert, C. D. , Klawitter, J. J., Stelling, F. H. (1970). Potential of ceramic materials as permanently implantable skeletal prostheses. J. Biomed. Mater. Res. **4:** 433.

Huntingdon, L., Sciences. (2000). Polyvinyl alcohol (PVA): mouse micronucleus test. Eye, Suffolk, England., Unpublished report No. 003/994931 from Huntingdon Life Sciences.

Hutmacher, D. W. (2000). Scaffolds in tissue engineering bone and cartilage. Biomaterials. **21:** 2529.

Hutmacher, D. W. (2001). Scaffold design and fabrication technologies for engineering tissues - state of the art and future perspectives. J Biomater Sci Polymer Edn. **12:** 107.

Hutmacher, D. W., Schantz, T., Zein, I., Ng, K.W., Teoh, S.H., Tan, K.C. (2001). Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling. J Biomed Mater Res. **55:** 203.

Hwang, N. S., Kim, S.M. , Sampattavanich, S., Baek, J. H., Zhang, H., Elisseeff, J. (2006). "Effects of three dimensional culture and growth factors on chondrogenesis differentiation of murine embryonic stem cells." Stem cells **24**: 284.

Im, G. I., Jung, N.H.,Tae ,S.K. and -. Tissue Engineering. (2006). Chondrogenic Differentiation of Mesenchymal Stem Cells Isolated from Patients in Late Adulthood: The Optimal Conditions of Growth Factors. Tissue Eng. **12:** 527.

ISO, -. (1992). International Standardization Organization. Biological evaluation of medical devices. Part 5. Tests for Cytotoxicity: In vitro Methods. International Organization for Standardization. Geneva, Switzerland.

Iwasaki, M., Nakata, K., Nakahara, H. (1993). ransforming growth factor-beta 1 stimulates chondrogenesis and inhibits osteogenesis in high density culture of periosteum-derived cells. Endocrinology. **132:** 1603.

Jadin, K. D., Baea,W.C., Schumacher,B.L., Sah,R.L. (2007). Three-dimensional (3-D) imaging of chondrocytes in articular cartilage: Growth-associated changes in cell organization. Biomaterials. **28:** 230.

Jiang, T. X., Yi, J.R.,Ying, S.Y. (1993). Activin enhances chondrogenesis of limb bud cells: stimulation of precartilaginous mesenchymal condensations and expression of NCAM. Dev Biol. **155:** 545.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., and T. Lund, Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., Verfaillie, C.M. (2002). Pluripotency of mesenchymal stem cells derived from adult bone marrow. Nature. **418:** 41.

Jiankang, H., Dichen, L. , Yaxiong, L. , Bo, Y. , Bingheng, L. , Qin, L. (2007). Fabrication and characterization of chitosan/gelatin porous scaffolds with predefined internal microstructures. Polymer. **48:** 4578.

John, M. M., Pazzano, D., Bonassar, J.L. (2003). Characterization of Poly (lactic acid) - Poly(glycolic acid) composites for cartilage. Tissue eng. **9:** 63.

Johnson, W. E., Wootton, A., El Haj, A., Eisenstein, S.M., Curtis, A.S., Roberts, S. (2006). Topographical guidance of intervertebral disc cell growth in vitro: Towards the development of tissue repair strategies for the anulus fibrosus. European Spine Journal. **15:** 389.

Johnstone, B., Hering, T.M., Caplan, A.I. (1998). In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res. **238:** 265.

Jones, G. S. H., Cartmell, J. (2006). Optimization of cell seeding efficiencies on a hree-dimensional gelatin scaffold for bone tissue engineering. Journal of Applied Biomaterials and Biomechanics. **4:** 172.

Jones, P. L., Schmidhauser, C., Bissell, M.J. (1993). Regulation of gene expression and cell function by extracellular matrix. Crit Rev Eukaryot Gene Expr. **3:** 137.

Juliano, R. L., Haskill, S. (1993). Signal transduction from the extracellular matrix. J Cell Bio. **120:** 57.

Jun, X., Maki, I., Youngmee, J., Keun, K., Soo, H.K., Young, H.K., Takehisa, M. (2006). Mechanoactive scaffold design based on microporous Poly (L-Lactide-co- caprolactone) for articularcartilage tissue engineering: Dependence of porosity on compression force in applied mechanical behaviors. Tissue Eng. **12:** 449.

Kang, H. W., Tabata, Y., Ikada, Y. (1999). Fabrication of porous gelatin scaffolds for tissue engineering. Biomaterials. **20:** 1339.

Keiji, M., Masahiko, S.,Takane, H., Toshio, F., Kohei, M. (2002). Two major Smad pathways in TGF-superfamily signaling. Genes to Cells. **7:** 1191.

Kessel, R. (1998). Basic Medical Histology. Oxford, Oxford University Press.

Khor, E. (1997). Methods for the treatment of collagenous tissues for bioprostheses. Biomaterials. **18:** 95.

Kirkpatrick, C. J., Dekker,A. (1992). Quantitative evaluation of cell interactions with biomaterials invitro,in biomaterial -tissue interfaces. Adv Biomater**:** 31.

Klinkhammer, K., Schnell, E., Dalton, P., Klee, D., Brook, G., Mey, J., Mo¨ller, M. (2006). Electrospun fibres of collagen-blended polycaprolactone for oriented cell growth. DWI Reports. **130**.

Kobayashi, M. (2004). A study of Polyvinyl Alcohol-Hydrogel (PVA-H) artificial meniscus in vivo. Bio-Med Mater and Eng. **14:** 505.

Kobayashi, M., Chang, Y.-S., Oka, M. (2005). A two year in vivo study of polyvinyl alcohol-hydrogel (PVA-H) artificial meniscus. Biomaterials. **26:** 3243.

Kobayashi, M., Oka, M. (2004). Characterization of a polyvinyl alcohol-hydrogel artificial articular cartilage prepared by injection molding. J of Biomater Sci, Poly Edn. **15:** 741.

Kobayashi, M., Toguchida, J., Masanori, O. (2001). Developement of the shields for tendon injury repair using polyvinyl alcohol hydrogel (PVA-H). J Biomed Mater Res. **58:** 344.

Kobayashi, M., Toguchida, J., Oka, M. (2001). Development of the shields for tendon injury repair using polyvinyl alcoholhydrogel (PVA-H). J Biomed Mater Res. **58:** 344.

Kozlov, V., Lomonosov, M.V. , Burdygina, G.I. (1983). The structure and properties of solid gelatin and the principles of their modification. Polymer. **24:** 651.

Kramer, J., Hegert, C., Guan, K. (2000). Embryonic stem cellderived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. Mech Dev. **92:** 193.

Kronenthal, R. L. (1975). Biodegradable polymers in medicine and surgery. Polymer Sci Technol. **8:** 119.

Kuettner, K. E., Pauli, B.U.,Gall, G.A., Memoli, V.A., Schenk,R.K. (1982). Synthesis of Cartilage Matrix by Mammalian Chondrocytes In Vitro . I . Isolation, Culture Characteristics, and Morphology. The J. Cell Biol. **93:** 743.

Kuijpers, A. J., Wachem, P.B.V., Luyn, M.J.A., Plantinga, J.A., Engber, G.H.M.,Krijgsveld,J., Zaat, S.A.J.,Dankert,J., Feijen,J. (2000). In vivo compatibility and degradation of crosslinked gelatin gels incorporated in knitted Dacron. J. Biomed Mater Res. **51:** 136.

Kweon, H., Yoo, M.K., Park, I.K., Kim, T.H., Lee, H.C., Lee, H.S., Oh, J.S., Akaike, T., Cho, C.S. (2003). A novel degradable polycaprolactone networks for tissue engineering. Biomaterials. **24:** 801.

Labow, R. S., Meek, E., Matheson , L.A., Santerre, J.P. (2002). Human macrophage-mediated biodegradation of polyurethanes: assessment of candidate enzyme activities. Biomaterials. **23:** 3969.

Landers, R., Hubner, U., Schmelzeisen, R., Mulhaupt, R. (2002). Rapid prototyping of scaffolds derived from thermoreversible hydrogels and tailored for applications in tissue engineering. Biomaterials. **23:** 4437.

Landers, R., Pfister, A., Hubner, U., John, H., Schmelzeisen, R., Mulhaupt, R. (2002). Fabrication of soft tissue engineering scaffolds by means of rapid prototyping techniques. J Mater Sci. **37:** 3107.

Langer, R. (2000). "Tissue engineering." Mol Ther **1**: 12.

Langer, R., Tirrell, D.A (2004). Designing materials for biology and medicine. Nature. **428:** 487.

Lawrence, D. A. (1996). Transforming growth factor-beta: a general review. Eur Cytokine Netw. **7:** 363.

Lawson, M. A., Barralet, J.E., Wang, L., Shelton, R.M. Triffitt, J.T. (2004). Adhesion and growth of bone marrow stromal cells on modified alginate hydrogels. Tissue Eng. **10:** 1480.

Lee, J. H., Lee,S.J.,Khang,G.,Lee,H.B. (2000). The effect of fluid shear stress on endothelial cell adhesiveness to polymer surfaces with wettability gradient. J colloid Interface Sci. **230:** 84.

Lee, S. J., Lee, Y.M., Han, C.W., Lee, H.B., Khang, G. (2004). Response of human chondrocytes on polymer surfaces with different micropore sizes for tissue-engineered cartilage. J. App Poly Sci. **92:** 2784.

Lemos, A. F., Ferriera, J.M.F. (2004). Designing of bioceramics with bonelike structures tailored for different orthopaedic applications. Key. Eng. Mater. **254:** 1041.

Li, R. H., White, M., Williams, S., Hazlett, T.J. (1998). Poly(vinyl alcohol) synthetic polymer foams as scaffolds for cell encapsulation. Biomater Sci Polym Ed. **33:** 239.

Li, W.-J., Danielson, K.G., Alexander, P.G., Tuan, R.S. (2003). "Biological response of chondrocytes cultured in three-dimensional nanofibrous poly(e-caprolactone) scaffolds." Journal of Biomedical Materials Research - Part A **67**: 1105.

Li, W. J., Tuli, R., Okafor, C., Derfoul, A., Danielson, K.G., Hall, D.J., Tuan ,R.S. (2005). A three dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials. **26:** 599.

Li, W. J., Tuli, R., Okafor, C., Derfoul, A., Danielson, K.G., Hall, D.J., Tuan, R.S. (2005). A threedimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials. **26:** 599.

Lieshout, M., Peters, G., Rutten, M., Baaijens, F. (2006). A knitted, fibrin-covered polycaprolactone scaffold for tissue engineering of the aortic valve. Tissue Eng. **12:** 481.

Lisignoli, G. (2005). Analysis of mesenchymal stem cells grown on a three-dimensional HYAFF 11-based prototype ligament scaffold. J Biomed Mater Res. **73:** 275.

Lisignoli, G., Fini, M., Giavaresi, G., Nicoli, A.N., Toneguzzi, S., Facchini, A. (2002). Osteogenesis of large segmental radius defects enhanced by basic fibroblast growth factor activated bone marrow stromal cells grown on non-woven hyaluronic acid-based polymer scaffold. Biomaterials. **23:** 1043.

Liu, H., Yin, Y., Yao, K. (2007). Construction of chitosan-gelatin-hyaluronic acid artificial skin in vitro. J. Biomater Appl. **21:** 413.

Liu, J., Meisner, D., Kwong, E., Wu, Y.X.,Johnston, M.R. (2007). A novel trans-lymphatic drug delivery system: Implantable gelatin sponge impregnated with PLGA-paclitaxel microspheres. 3236.

Liu, Y., Shu, X.Z., Prestwich, G.D. (2006). Osteochondral Defect Repair with Autologous Bone Marrow–Derived Mesenchymal Stem Cells in an Injectable, In Situ, Cross-Linked Synthetic Extracellular Matrix. Tissue Eng. **12:** 3405.

Longobardi, L., O'Rear, L., Aakula, S. (2006). Effect of IGF-1 in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-beta signaling. J Bone Miner Res 2006. **21:** 626.

Lorgensen, C., Noel, D., Apparailly, F. (2001). Stem cells for repair of cartilage and bone: the next challenge in osteoarthritis and rheumatoid arthritis. Ann Rheum Dis. **60:** 305.

Lu, H. D., Cai, D.-Z., Liu, Q., Wang, Y.J., Zheng, Y.D. (2005). Experimental study of rabbit knee joint articular cartilage defects repaired with transplantation of polyvinyl alcohol/hydroxyapatite composite hydrogel. Chinese Journal of Clinical Rehabilitation. **9:** 184.

Lu, Y., Zhang, G., SUN, D., Zhong, Y. (2007). Preparation and evaluation of biodegradable flubiprofen gelatin micro-spheres for intra-articular administration. J. Microencapsulation. **24:** 515.

Luna, P. V. H., Horbett, T.A., Ratner, B.D. (1994). Developing correlations between fibrinogen adsorption and surface properties using multivariate statistics. J Biomed Mater res. **28:** 1111.

Ma, P. X., Choi,J.W. (2001). Biodegradable polymer scaffolds with well defined interconnected pore netwok. Tissue eng. **7:** 23.

Ma, Z., You,C., Gong, Y., Shen,J. (2003). Chondrocyte behaviors on poly L lactic acid PLLA membranes containing hydroxyl, amide or carboxylgroups,. Biomaterials. **24:** 3725.

Machado, C. B., Ventura, J.M.G., Lemos, A.F., Ferreira, J.M.F., Leite,M.F., Goes, A.M (2007). 3D chitosan-gelatin-chondroitin porous scaffold improves osteogenic differentiation of mesenchymal stem cells. Biomedical Materials. **2:** 124.

Mackay, A. M., Beck, S.C., Murphy, J.M., Barry, F.P.,Chichester, C.O., Pittenger,M.F. (1998). Chondrogenic Differentiation of Cultured Human Mesenchymal Stem Cells from Marrow. Tissue Eng. **4:** 415.

Maitra, B., Szekely, E., Gjini, K. (2004). "Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation." Bone Marrow Transplant **33**: 597.

Majumdar, M. K., Banks, V., Peluso, D.P., Morris, E.A. . (2000). Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. J Cell Physiol. **185:** 98.

Malemud, C. J., Stevenson, S., Mehraban, F., Papay, R.S., Purchio, A.F.,Goldberg, V.M. (1994). The proteoglycan synthesis repertoire of rabbit chondrocytes maintained in type II collagen gels. Osteoarthritis Cartilage. **2:** 29.

Mao, J., Zhao, L.,Yao, D.K., Shang, Q.,Yang, G .,Cao, Y. (2003). Study of novel chitosan-gelatin artificial skin in vitro. Mater Res. **64:** 301.

Mark, K. V. D., Gauss, V., Mark, H.V.D., Muller, P. (1977). Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature. **267:** 531.

Marlovits, S., Zeller, P., Singer, P., Resinger, C.,Vecsei,V. (2006). Cartilage repair: generations of autologous chondrocyte transplantation. Eur J Radiol. **57:** 24.

Mauck, R. L., Nicoll,S.B.,Seyhan, S.L.,Ateshian, G.A., Hung, C.T. (2003). Synergistic action of growth factors and dynamic loading for articularcartilage tissue engineering. Tissue Eng. **9:** 597.

Mauck, R. L., Yuan, X.,Tuan, R.S. (2006). Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. Osteoarthritis and Cartilage. **14:** 179-189.

Mayne, R. (1989). Cartilage collagens-what is their function, and are they involved in articular disease? Arthritis Rheum. **32:** 241.

Mehlhorn, A. T., Schmal, H.,Kaiser, S.,Lepski,G., Finkenzeller, G., Stark,G.B., Südkamp,N.P. (2006). Mesenchymal Stem Cells Maintain TGF-ß-Mediated Chondrogenic Phenotype in Alginate Bead Culture. Tissue Eng. **12:** 1393.

Mendler, M., Eich-Bender, S.G., Vaughan, L., Winterhalter, K.H., Bruckner, P., (1989). Cartilage contains mixed fibrils of collagen types II, IX and XI. J. Cell Biol: 108.

Meredith, J. E., Fateli, B., Schwartz, M.A. (1993). The extracellular matrix as a cell survival factor. Molecular Cellular Biology. **9:** 953.

Middleton, J. C., Tipton, A.J. (2000). Synthetic biodegradable polymers as orthopaedic devices. Biomaterials **21**: 2335- 2346.

Mikos, A. G., Bao, Y., Cima, L.G., Ingber, D.E., Vacanti, J.P., Langer, R. (1993). Preparation of poly (glycolic acid) bonded fibres structures for cell attachment and transplantation. J Biomed Mater Res. **27:** 183.

Mikos, A. G., Sarakinos, G., Leite, S.M., Vacanti, J.P., Langer, R. (1993). Laminated three-dimensional biodegradable foams for use in tissue engineering. Biomaterials. **14:** 323.

Mikos, A. G., Sarakinos, G., Vacanti, J.P., Langer, R., Cima, L.G. (1996). Biocompatible polymer membranes and methods of preparation of three dimensional membrane structures. US Patent Number 5514378.

Mikos, A. G., Thorsen, A.J., Czerwonka, L.A., Bao, Y., Langer, R. (1994). Preparation and characterisation of poly(L-lactic acid) foams. Polymer. **35:** 1068.

Milton, K. (1998). British standards B. Evaluation of medical devices for biological hazards.part 10. Method of test for toxicity ofcells in culture of extracts from medical devices, British standards institution.

Miyanishi, K., Trindade, M.C.D., Lindsey, D.P.,Beaupré, G.S., Carter, D.R., Goodman,S.B.,Schurman, D.J. R., Smith,L. (2006). Effects of Hydrostatic Pressure and Transforming Growth Factor-beta3 on Adult Human Mesenchymal Stem Cell Chondrogenesis In Vitro. Tissue Eng. **12:** 1419.

Miyazawa, k., Shinozaki, M., Hara, T., Furuya, T.,Miyazono, K. (2002). Two major Smad pathways in TGF- beta superfamily signaling. Genes to Cells. **7:** 1191-1204.

Miyazono, K., Kusanagi, K., Inoue, H. (2001). Divergence and convergence of TGF-./BMP signaling. J. Cell. Physiol. **187:** 265.

Monroy, C. J., Diaz de Leon, L. (1997). Differential effects of transforming growth factors beta 1, beta 2, beta 3 and beta 5 on chondrogenesis in mouse limb bud mesenchymal cells. Int J Dev Biol. **41:** 91.

Mooney, D. J., Baldwin, D.F., Suh, N.P., Vacanti, J.P., Langer, R. (1996). Novel approach to fabricate porous sponges of poly(D,L-lactic co-glycolic acid) without the use of organic solvents. Biomaterials. **17:** 1417.

Moore, J. M., Jabbari, E., Ritman, E.L.,Lu,L.,Currier, B.L.,Windebank, A.J.,Yaszemski, M.J. (2004). Quantitative analysis of interconnectivity of porous biodegradable scaffolds with micro-computed tomography. J Biomed Mater res. **71:** 258.

Morales, T. I., Kuettner, K. E., Howell, D. S.,Woessner, J. F. (1983). Characterization of the metalloproteinase inhibitor produced by bovine articular chondrocyte cultures. Biochim. Biophys. Acta. **760:** 221.

Morbidity and Mortality Weekly Report. MMWR. (2006) **55:** 1089.

Morbidity and Mortality Weekly Report. MMWR. (2007) **56:** 4.

Mouw, J. K., Case, N.D., Guldberg, R.E., Plaas, A.H., Levenston, M.E. (2005). Variations in matrix composition and GAG fine structure among scaffolds for cartilage tissue engineering. Osteoarthritis Cartilage. **13:** 828.

Mow, V. R., M. (1988). Articular cartilage: biomechanics. Injury and Repair of the Musculoskeletal Soft Tissues. B. J. Woo SLY, Eds. Park Ridge, American Academy of Orthopaedic Surgeons: 427.

Nakashima, K., Murakami, T., Sawae, Y. (2004). Evaluation of wear property of PVA hydrogel as artifical cartilage and effect of protein film on wear-resistant property. Trans of the Japan Soc of Mech Eng. **70:** 2780.

Nam, Y. S., Park, T.G. (1999). Biodegradable polymeric microcellular foams by modified thermally induced phase separation method. Biomaterials. **20:** 1783.

National Toxicology Program technical report on the toxicology and carcinogenesis studies of polyvinyl alcohol (molecular weight 24 000) (CAS No. 9002-89-5) in female B6C3F1 mice (intravaginal studies). Research Triangle Park, NC, USA, Report No. NTP TR 474 of the National Toxicology Program, (1989).

Naumann, A., Dennis. J., Staudenmaier, R., Rotter, N., Aigner, J., Ziegelaar, B., Happ, T., Rasp, G., and A. I. Caplan (2002). Mesenchymal stem cells--a new pathway for tissue engineering in reconstructive surgery. Laryngorhinootologie. **81:** 521.

Nguyen, T. L. U., Farrugia, B., Davis, T.P., Barner-Kowollik, C., Stenzel, M.H. (2007). Core-shell microspheres with surface grafted poly(vinyl alcohol)

as drug carriers for the treatment of hepatocellular carcinoma. J of PolY Sci, Part A: Poly Chem. **45:** 3256.

Nicoli, S., Penna, E., Padula, C., Colombo, P., Santi, P. (2006). New transdermal bioadhesive film containing oxybutynin: In vitro permeation across rabbit ear skin. International Journal of Pharmaceutics. **325:** 2.

Noguchi, T., Yamamuro,T.,Oka, M., 2, Kumar, P.,KotourA, Y., Hyonyt, S.H., Ikada, Y.J (2004). Poly(vinyl alcohol) hydrogel as an artificial articular cartilage: Evaluation of biocompatibility. Journal of Applied Biomaterials. **2:** 101.

Nuttelman, C. R., Henry, S.M., Anseth, K.S. (2002). Synthesis and characterization of photocrosslinkable, degradable poly(vinyl alcohol)-based tissue engineering scaffolds. Biomaterials. **23:** 3617.

Oh, S. H., Kang, S.G., Kim, E.S., Cho, S.H., Lee, J.H. (2003). Fabrication and characterization of hydrophilic poly (lactic-co-glycolic)/poly(vinyl alcohol) blend cell scaffolds by melt-molding particulate-leaching method. Biomaterials **24**: 4011.

Oh, S. H., Park, I.K., Kim, J.M., Lee, J.H. (2007). In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method. Biomaterials. **28:** 1664.

Ohta, S., Imai, K., Yamashita, K., Matsumoto, T., Azumano, I.,Okad, A. (1998). Expression of matrix metalloproteinase 7 (matrilysin) in human osteoarthritic cartilage. Lab. Invest. **78:** 79.

Oliveira, J. T., Crawford, A., Mundy, J.M., Moreira, A.R., Gomes, M.E., Hatton, P.V., Reis, R.L. (2007). A cartilage tissue engineering approach combining starch-polycaprolactone fibre mesh scaffolds with bovine articular chondrocytes. J of Mater Sci: Mater in Med. **18:** 295.

Pal, K., Banthia, A.K., Majumdar, D.K. (2007). Biomedical evaluation of polyvinyl alcohol-gelatin esterified hydrogel for wound dressing. J. Mater Sci: Mater in Med. **18:** 1889.

Pal, K., Banthia, A.K., Majumdar, D.K. (2007). Preparation and characterization of polyvinyl alcohol-gelatin hydrogel membranes for biomedical applications. AAPS PharmSciTech. **8:** 21.

Paradossi, G., Cavalieri, F., Chiessi, E., Spagnoli, C., Cowman, M.K. (2003). Poly(vinyl alcohol) as versatile biomaterial for potential biomedical applications. J Mater Sci Mater Med. **14:** 687.

Peppas, N. A., Merrill, E.W. (1977). Development of semicrystalline poly(vinyl alcohol) hydrogels for biomedical application. J Biomed Mater Res. **11:** 423.

Pereira, M. M., Jones, J.R., Hench, L.L. (2005). Bioactive glass and hybrid scaffolds prepared by sol-gel method for bone tissue engineering. Adv Appl Ceram. **104:** 35.

Peretti, G. M., Xu, J.W., Bonassar, L.J., Kirchhoff, C.H., Yaremchuk, M.J. and Randolph, M.A. (2006). Review of injectable cartilage engineering using fibrin gel in mice and swine models. Tissue Eng. **12:** 1151.

Pierschbacher, M. D., Ruoslahti, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature. **309:** 30.

Pitt, C. G. G., M. M.; Kimmei, G. L.; Surles, J.; Schindler, A. (1981). Aliphatic polyesters II. The degradation of poly (DL-lactide), poly (-caprolactone), and their copolymers in vivo. Biomaterials. **2**: 215.

Pizzoferrato, A., Vespucci, A., Ciapetti, G., Stea, S. (1985). Biocompatibility testing of prosthetic implant materials by cell cultures. Biomaterials. **6:** 346.

Ponticiello, M. S., Schinagl, R.M., Kadiyala, S., Barry, F.P. (2000). Gelatin-based resorbable sponge as a carrier matrix for human mesenchymal stem cells in cartilage regeneration therapy. J Biomed Mater Res. **52:** 246.

Praemer, A. P., Furner, S., Rice, D.P. (1999). Musculoskeletal Conditions in the United States. Rosemont, Illinois.

Prockop, D. J., Gregory, C.A.,Spees, J.L. (2003). One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. Proc. Natl. Acad. Sci., USA. **100:** 11917.

Puelacher, W. C., Kim, S.W., Vacanti, J.P., Schloo, B., Mooney, D., Vacanti, C.A.. (1994). Tissue-engineered growth of cartilage: the effect of varying the concentration of chondrocytes seeded onto synthetic polymer matrices. Int J Oral Maxillofac Surg. **23:** 49.

Qiu, Z. G., Xiao, J.M., Zhang, X.H. (1998). The development of artificial articular cartilage – PVA-hydrogel. Bio-Med Mater and Eng. **8:** 75.

Radice, M., Brun, P., Cortivo, R., Scapinelli, R., Battaliard, C., Abatangelo, G. (2000). Hyaluronan-based biopolymers as delivery vehicles for bone-marrow-derived mesenchymal progenitors. J Biomed Mater Res. **50:** 101.

Rai, B., Oest, M.E., Dupont, K.M., Ho, K.H., Teoh, S.H., Guldberg, R.E. (2007). Combination of platelet-rich plasma with polycaprolactone-tricalcium phosphate scaffolds for segmental bone defect repair. J. Biomedical Mater Res **81:** 888.

Redini, F., Galera, P., Mauviel, A., Loyau, G., Pujol, J. P. (1988). Transforming growth factor beta stimulates collagen and glycosaminoglycan biosynthesis in cultured rabbit articular chondrocytes. FEBS Lett. **234:** 172.

Rest, V. D. M., Mayne, R., (1988). Type IX collagen proteoglycan from cartilage is covalently cross-linked to type II collagen. J. Biol. Chem. **263**.

Reyes, M., Verfaillie, C.M. (2001). Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann. N. Y. Acad. Sci. **938:** 231.

Richardson, S. M., Curranb, J.M., Chenb, R., Thomasc, A.V., Huntb, J.A., Freemonta, A.J., Hoylanda, J.A. (2006). The differentiation of bone marrow mesenchymal stem cells into chondrocyte-like cells on poly-l-lactic acid (PLLA) scaffolds. Biomaterials. **27:** 4069.

Ringe, J., Kaps, C., Burmester, G.R., Sittinger, M. (2002). Stem cells for regenerative medicine: advances in the engineering of tissues and organs. Nature **89**(89): 338.

Risbud, M. V., Sittinger, M. (2002). Tissue engineering: advances in in vitro cartilage generation. Trends Biotechnol. **20:** 351.

Ro, J., King, M., Synder, R.,Guidoin, R., Martin, L. (1985). Polyester (dacron)arterial prostheses treated with crosslinked and freeze dried albumin. Am, Soc. Artif. Intern. Organs. **8:** 166.

Roark, E. F., Karen. (1994). Transforming growth factor+ and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. Greer Developmental Dynamics. **200:** 103.

Rosen, V., Wozney, J.M. (2002). Bone morphogenetic proteins. Principles of Bone Biology. L. G. R. J.P. Bilezikian, G.A. Rodan, eds. San Diego:, San Diego: Academic Press.: 919.

Roughley, P. J., Rroopuegahn, P.J. (2006). The structure and function of cartilage proteoglycans. Cells and Mater. **12:** 92.

Sachlos, E., Czernuszka, J.T. (2003). Making tissue engineering scaffolds work. review on the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. European Cells and Materials. **5:** 29.

Sanders, J. M. M., H.B. (1990). Vaginal absorption of polyvinyl alcohol in Fischer 344 rats. Human Exp. Toxicol. **9:** 71.

Santiago, L. Y., Nowak, R.W., Rubin, J.P. Marra, K.G (2006). Peptide-surface modification of poly(caprolactone) with laminin-derived sequences for adipose-derived stem cell applications. Biomaterials. **27:** 2962.

Sarasam, A., Madihally, S.V. (2005). Characterization of chitosan-polycaprolactone blends for tissue engineering applications. Biomaterials. **26:** 5500.

Schantz, J. T., Hutmacher, D.W., Lam, C.X., Brinkmann, M., Wong, K.M., Lim, T.C., Chou, N., Guldberg, R.E., Teoh,S.H. (2003). Repair of calvarial defects with customised tissueengineered bone grafts II. Evaluation of cellular efficiency and efficacy in vivo. Tissue Eng. **9:** 127.

Schmedlen, R. H., Masters, K.S., West, J.L. (2002). Photocrosslinkable polyvinyl alcohol hydrogels that can be modified with cell adhesion peptides for use in tissue engineering. Biomaterials **23**: 4325.

Schmitt, B., Ringe, J., Haupl, T. (2003). BMP2 initiates chondrogenic lineage development of adult human mesenchymal stem cells in high density culture. Differentiation. **71:** 567.

Schmitt, B., Ringe, J., Haupl, T. (2003). BMP2 initiates chondrogenic lineage development of adult human mesenchymal stem cells in high density culture. Differentiation. **71:** 567.

Schofield, J. N., Wolpert, L. (1990). Effects of TGF beta1, TGF beta 2 and bFGF on chick cartilage and muscle cell differentiation. Exp Cell Res. **91:** 148.

Sekiya, I., Larson, B.L., Vuoristo, J.T. (2005). Comparison of effect of BMP-2,-4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res. **320:** 269.

Sekiya, I., Vuoristo, J.T.,Larson, B.L., Prockop, D.J. (2002). In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular eventsduring chondrogenesis. PNAS. **99:** 4397.

Sell, S., Barnes, C., Smith, M., McClure, M., Madurantakam, P., Grant, J., McManus, M., Bowlin, G. (2007). Extracellular matrix regenerated: Tissue engineering via electrospun biomimetic nanofibers. Polymer International. **56:**1349.

Sgouras, D., Duncan R. (1990). Methods for evaluation of biocompatibility of soluble synthetic polymers which havepotential for biomedicaluse. 1. Useof tetrazolium basedcolorimetric assay (Mtt)asa preliminary screen for evaluation of invitro cytotoxicity. J. mater.sci. Mater. Med. **1:** 61.

Shao, X., Goh, J. C.H., Hutmacher, D. W., Lee, E. H., Zigang, G. (2006) Repair of Large Articular Osteochondral Defects Using Hybrid Scaffolds and Bone Marrow-Derived Mesenchymal Stem Cells in a Rabbit Model. Tissue Eng. **12:** 1539.

Sharma, B., Elisseeff, J.H. (2004). Engineering structurally organized. cartilage and bone tissues. Ann. Biomed. Eng. **32:** 148.

She, H., Xiao, X., Liu, R. (2007). Preparation and characterization of polycaprolactone-chitosan composites for tissue engineering applications. Journal of Materials Science. **42:** 8113.

Shea, C. M., Edgar, C.M., Einhorn, T.A. (2003). BMP treatment of C3H10T1/2 mesenchymal stem cells induces both chondrogenesis and osteogenesis. J Cell Biochem: 1112.

Sherwood, J. K., Riley, S.L., Palazzolo, R., Brown, S.C., Monkhouse, D.C., Coates, M., Griffith, L.G., Landeen, L.K., Ratcliffe, A. (2002). A three-dimensional osteochondral composite scaffold for articular cartilage repair. Biomaterials. **23:** 4739.

Shlopov, B. V., Lie, W.R., Mainardi, C.L.,Cole,A.A.,Chubinskaya,S.,Hasty, K.A. (1997). Osteoarthritic lesions-involvement of three different collagenases. Arthritis Rheum. **40:** 2065.

Shoichet, M.S., Li, R.H., White M.L., Winn, S.R. (1996) Stability of hydrogels used in cell encapsulation: An in vitro comparison of alginate and Agarose. Biotechnol and Bioeng. **50**: 374.

Shor, L., Gu¨c¸eri, S., Wen, X., Gandhi, M., Sun, W. (2007). Fabrication of three-dimensional polycaprolactone/hydroxyapatite tissue scaffolds and osteoblast-scaffold interactions in vitro. Biomaterials. **28:** 5291.

Sinanan, A. C., Hunt, N.P.,Lewis, M.P. (2004). Human adult craniofacial muscle-derived cells: neural-cell adhesionmolecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells. Biotechnol. Appl. Biochem. **40:** 25.

Sittinger, M., Reitzel, D., Dauner, M., Hierlemann, H., Hammer, C., Kastenbauer, E., Planck, H. (1996). Resorbable polyesters in cartilage engineering: afinity and biocompatibility of polymer fiber structures to chondrocytes. J Biomed Mater Res Appl Biomater. **33:** 57.

Solchaga, L. A., Dennis, J.E., Goldberg, V.M. (1999). Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage. J Orthop Res. **17:** 205.

Song, C., Wang, P., Sun, H., Shi, H., Yang, Q., Cui, X., Shi, R. (2000). The in vivo degradation, adsorption and excretion of poly(epsilon-caprolactone). J Biomed Eng **1:** 25.

Sotiropoulou, P. A., Perez, S.A., Gritzapis, A.D. (2006). Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells. **24:** 74.

Spagnoli, A., Longobardi, L., O'Rear, L., Basel, Karger. (2005). Cartilage Disorders: Potential therapeutic use of mesenchymal stem cells. Endocr Dev. **9:** 23.

Spiro, R. C., Liu, L., Heidaran, M.A. (2000). Inductive activity of recombinant human growth and differentiation factor-5. Biochem Soc Trans. **28:** 362.

Spitzer, R. S., Perka, C., Lindenhayn, K., Zippel, H. (2002). Matrix engineering for osteogenic differentiation of rabbit periosteal cells using alpha-tricalcium phosphate particles in a three-dimensional fibrin culture. J Biomed Mater Res. **59:** 690.

Staatz, W. D., Fok, K.F., Zutter, M.M., Adams, S.P., Roderiguez, B.A.,Santoro, S.A. (1991). Identification of a tetrapeptide recognition sequence for the a1b2 integrin in collagen. J. Biol. Chem. **266:** 7363.

Steinert, A., Weber, M., Dimmler, A. (2003). Chondrogenic differentiation of mesenchymal progenitor cells encapsulated in ultra high viscosity alginate. J Ortho pres. **21:** 1090.

Stemple, D. L., Anderson, D.J. (1992). solation of a stem cell for neurons and glia from the mammalian neural crest. Cell. **71.**

Stevens, M. M., Qanadilo, H.F., Langer, R.,Prasad Shastri, V. (2004). A rapid-curing alginate gel system: utility in periosteum-derived cartilage tissue engineering: utility in periosteum-derived cartilage tissue engineering. Biomaterials. **25:** 887.

Storey, R. F., Taylor, A.E. (1998). Effect of stannous octoate on the composition, molecular weight, and molecular weight distribution of ethyleneglycol-initiated poly(ecaprolactone). J Macromol Sci- Pure Appl Chem**:** 723.

Sun, H., Mei,L., Song , C., Cui, X.,Wang, P. (2006). The in vivo degradation, absorption and excretion of PCL-based implant. Biomaterials **27**: 1735.

Swijnenburg, R. J., Tanaka, M., Vogel, H., Baker, J., Kofidis, T., Gunawan, F., Lebl, D.R., Caffarelli, A.D., de Bruin, J.L., Fedoseyeva, E.V., Robbins, R.C. (2005). Embryonic stem cells immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation. **112:** 1166.

Thesleff, I., Tummers, M. (2003). Stem cells and tissue engineering: prospects for regenerating tissues in dental practice. Med Princ Pract. **12:** 43.

Throp, B. H., Anderson, I., Jakowlew, S B. (1992). Transforming Growth Factor 1, 2 and 3 in cartilage and bone cells during endochondral ossification in the chick. Development. **114:** 907.

Tienen, T. G., Heijkants, R.G., Buma, P., Groot, J.H., Pennings, A.J., Veth, R.P. (2002). Tissue ingrowth and degradation of two biodegradable porous polymers with different porosities and pore sizes. Biomaterials. **23:** 1731.

Ting, V., Sims, C.D., Brecht, L.E., McCarthy, J.G., Kasabian, A.K., Connelly, P.R., Elisseeff, J., Gittes, G.K.,Longaker, M.T. (1998). In vitro prefabrication of human cartilage shapes using fibrin glue and human chondrocytes. Ann Plast Surg. **40:** 413-420.

Tseng, C. L., Wang,T.W., Dong,G.C., Yueh-Hsiu ,W.S.,Young, T.H., Lou, M.J. P.J.,Lin,F.H. (2007). Development of gelatin nanoparticles with biotinylated EGF conjugation for lung cancer targeting. Biomaterials. **28:** 3996.

Tuan, R. S., Boland, G.,Tuli, R. (2003). Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Res Ther. **5:** 32.

Tuli, R., Tuli, S., Nandi, S. (2003). Transforming growth factor beta mediated chondrogenesis of human mesenchymal progenitor cells involves N cadherin and mitogen activated protein kinase and Wnt signaling crosstalk. J Biol Chem. **278:** 227.

Twyman, R. S., Desai, K., Aichroth, P.M. (1991). Osteochondritis dissecans of the knee: a long-term study. J Bone Joint Surg. **73:** 461.

Uematsu, K., Hattori, K., Ishimoto, Y., Yamauchi, J., Habata, T., Takakura, Y., Ohgushi, H., Fukuchi, T., Sato, M. (2005). Cartilage regeneration using mesenchymal stem cells and a three-dimensional poly-lactic-glycolic acid (PLGA) scaffold. Biomaterials. **26:** 4273.

Uhlich, T., Ulbricht, M., Tomadchewski, G. (1996). Immobilization of enzymes in photochemically crosslinked polyvinyl alcohol. Enzyme Microbiol Technol. **19:** 124.

Ulubayram, K., Cakar, A.K.,Korkusuz, P., Ertan, C.,Hasirci, N. (2001). EGF containing gelatin-based wound dressings. **Biomaterials**. **22:** 1345.

Ulubayram, K., Cakar, A.N., Korkusuz, P.,Ertan, C., Hasirci, N. (2001). EGF containing gelatin-based wound dressings. Biomaterials. **22:** 1345.

Uyen , H. M. W., Schakenraad,J. M., Sjollema, J., Noordmans ,J.,Jongebloed, W.L.,Stokroos,I., Busscher,H.J. (1990). Amount and surface structure of albumin adsorbed substrata with different wettabilities in a parallel plate flow cell. J Biomed Mater res. **24:** 1599.

Van Lieshout, M., Peters, G., Rutten, M., Baaijens, F. (2006). A knitted, fibrin-covered polycaprolactone scaffold for tissue engineering of the aortic valve. Tissue Engineering. **12:** 481.

Vasara, A. I., Nieminen, M.T., Jurvelin, J.S., Peterson, L., Lindahl, A., Kiviranta, I. (2005). Indentation stiffness of repair tissue after autologous chondrocyte transplantation. Clin Orthop. **433:** 233.

Vickers, S. M., Squitieri, L.S., Spector,M. (2006). Effects of Cross-linking Type II Collagen-GAG Scaffolds on Chondrogenesis In Vitro: Dynamic Pore Reduction Promotes Cartilage Formation. Tissue Eng. **12:** 1345.

Vogel, G. (2003). Stem cells: 'stemness' genes still elusive. Science. **302:** 371.

Wachem, V. P. B., Beugeling, T., Feijen, J., Bantjes, A., Detmers, J.P., Aken,V. W.G. (1985). Interaction of culturedhuman endothelial cellswith polymeric surfaces on different wettabilities. Biomaterials. **6:** 403.

Wake, M. C., Patrick, C.W., Mikos, A.G. (1994). Pore morphology effects on the fibrovascular tissue growth in porous polymer substrates. Cell Transplant. **3:** 339.

Wang, S., Lu, L., Gruetzmacher, J.A., Currier, B.L., Yaszemski, M.J. (2006). Synthesis and characterizations of biodegradable and crosslinkable poly(e-caprolactone fumarate), poly(ethylene glycol fumarate), and their amphiphilic copolymer. Biomaterials. **27:** 832.

Wang, Y., Kim, U.J., Blasioli, D.J. (2005). In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. Biomaterials. **26:** 7082.

Weber, M., Steinert, A., Jork, A., Dimmler, A., Thurmer, F., Schutze, N., Hendrich, C., Zimmermann, U. (2003). "Formation of cartilage matrix proteins by BMP-transfected murine mesenchymal stem cells encapsulated in a novel class of alginates." Biomaterials **23**: 2013.

Weng, H. H., Fitzgerald, J. (2006). Current issues in joint replacement surgery. Curr Opin Rheumatol. **18:** 163.

Whang, K., Healy, K.E., Elenz, D.R., Nam, E.K., Tsai, D.C., Thomas, C.H., Nuber, G.W., Glorieux, F.H., Travers, R., Sprague, S.M. (1999). Engineering bone regeneration with bioabsorbable scaffolds with novel microarchitecture. Tissue Eng. **5:** 35.

Whang, K., Thomas, C.H., Healy, K.E., Nuber, G. (1995). A novel method to fabricate bioabsorbable scaffolds. Polymers. **36:** 837.

WHO (2001). Global Economic and Health Care Burden of Musculoskeletal Disease, World Health Organization.

WHO, Food additives, series: 52. International Programme on Chemical Safety (IPCS), inter organization programme for soundmanagement of chemicals acoperative agreement among. UNEP,WHO,FAO, OECD, ILO.

Williams, C. G., Kim, T.K., Taboas, A. (2003). In vitro chondrogenesis of bone-marrow derived mesenchymal stem cells in a photopolymerizing hydrogel. Tissue Eng. **9:** 679.

Winkler, J., Hescheler, J.,Sachinidis, A. (2005). Embryonic stem cells for clinical research and potential clinical applications in cardiology. Biochim. Biophys. Acta. **240:** 1740.

Woei, N. k., Hutmacher, D.W., Schantz, J.T., Seng, Ng, C., Too, H.P., Lim, T.C.,Phan, T.T., Teoh, S.H. (2001). Evaluation of Ultra-Thin Poly(Caprolactone) Films for Tissue-Engineered Skin. Tissue Eng. **7:** 441.

Wong (1997). Synthesis and properties of biodegradable polymers used as synthetic matrices in tissue e ngineering. Synthetic biodegradable polymer scaffolds . a. M. D. J. In. Atala A, eds. Boston: Birkhausen**:** 51.

Woodward, S. C., Brewer, P.S., Moatamed, F. (1985). The intracellular degradation of poly(caprolactone). J Biomed Mater Res. **44:** 437.

Wozney, J. M. (1998). The bone morphogenetic protein family: multifunctional cellular regulators in the embryo and adult. Eur J Oral Sci. **106:** 160.

Xia, W., Liu, W., Cui,L.,Liu, Y., Zhong,W., Liu , D., Wu,J., Chua,K., Cao, Y. (2004). Tissue engineering of cartilage with the use of chitosan-gelatin complex scaffolds. J.Biomed Mater Res. **71:** 373.

Xin, X., Borzacchiello, A., Netti, P.A., Ambrosio, L., Nicolais, L. (2004). Hyaluronic-acid-based semi-interpenetrating materials. J Biomater Sci Polym Ed. **15:** 1223.

Yamada, Y., Boo, J.S., Ozawa, R., Nagasaka, T., Okazaki, Y., Hata, K., Ueda, M. (2003). Bone regeneration following injection of mesenchymal stem cells and fibrin glue with a biodegradable scaffold. Craniomaxillofac Surg. **31:** 27.

Yamane, S., Iwasaki, N., Kasahara, Y., Harada, K., Majima, T. Monde, K., Nishimura, S.-I., Minami. J. (2007). Selected Effect of pore size on in vitro cartilage formation using chitosan-based hyaluronic acid hybrid polymer fibers. J. Biomed Mater Res. **81:** 586.

Yamaoka, H., Asato, H., Ogasawara, T., Nishizawa, S., Takahashi, T., Nakatsuka, T., Koshima, I., Nakamura, K.,Kawaguchi, H., Chung, U.I., Takato, T., Hoshi, K. (2006). Cartilage tissue engineering using human auricular chondrocytes embedded in different hydrogel materials. Biomed Mater Res. **78**.

Yamaoka, T., Tabata, Y., Ikada, Y. (1995). Fate of water-soluble polymers administered via different routes. J. pharm. sci **84**: 349.

Yamaoka, T., Tabata, Y.,I kada, Y. (1995). Comparison of body distribution of poly(vinyl alcohol) with other water-soluble polymers after intravenous administration. J. Pharm. Pharmacol. **47:** 479.

Yanaga, H., Yanaga, K., Imai, K., Koga, M., Soejima, C.,Ohmori, K. (2006). Clinical application of cultured autologous human auricular chondrocytes with autologous serum for craniofacial or nasal augmentation and repair. Plast Reconstr Surg. **117:** 2019.

Yang, K. G., Saris, D.B., Geuze, R.E., Helm, Y.J., Rijen, M.H., Verbout, A.J., Dhert, W.J., Creemers, L.B. (2006). Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes. Tissue Eng. **12:** 2435.

Yang, Q., Chen, L., Shen, X., Tan, Z. c Part B: Physics (2006). Preparation of polycaprolactone tissue engineering scaffolds by improved solvent casting/particulate leaching method. Journal of Macromolecular Science, Part B: Physics. **45:** 1171.

Yang, S., Leong, K.F., Du, Z., Chua, C.K. (2001). The Design of Scaffolds for Use in Tissue Engineering. Part I. Traditional Factors. Tissue Eng. **7:** 679.

Yang, S., Leong, K.F.,Du, Z.,Chua, CK. (2002). The Design of Scaffolds for Use in Tissue Engineering. Part II. Rapid Prototyping Techniques. Tissue Engineering. **8:** 1.

Yeong, W. Y., Chua, C.K., Leong, K.F., Chandrasekaran,M. (2004). M. Rapid prototyping in tissue engineering: challenges and potential. Trends Biotechnol. **22:** 643.

Yoo, J. U., Barthel, T.S., Nishimura, K. (1998). The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. J Bone Joint Surg. **80:** 1745.

You, S. J., Ahn, W.S. Jang, H.S., Kang, M.I. , Chun, H.J., Lim, Y.M. , Nho, Y.C. (2007). Preparation and characterization of gelatin-poly(vinyl alcohol) hydrogels for three-dimensional cell culture. J of Indust and Eng Chem. **13:** 116.

Young, H. E. (2004). Existence of reserve quiescent stem cells in adults, from amphibians to humans. Curr. Top. Microbiol.Immunol. **280**.

Zavan, B., Giorgi, C., Bagnara, G.P., Vindigni, V., Abatangelo, G., Cortivo, R. (2007). Osteogenic and chondrogenic differentiation: comparison of human and rat bone marrow mesenchymal stem cells cultured into polymeric scaffolds. European journal of histochemistry. **51:** 1.

Zeltinger, J., Sherwood, J.K., Graham, Ralph Mueller, Linda G. Griffith. (2001). Effect of Pore Size and Void Fraction on Cellular Adhesion, Proliferation, and Matrix Deposition. Tissue Eng **7**: 557.

Zhang, Y. Z., Feng, Y., Huang, Z.-M., Ramakrishna, S., Lim, C.T. (2006). Fabrication of porous electrospun nanofibres. Nanotechnology **17**: 901-908.

Zhao, J., Yuan, X., Cui, Y., Ge, Q., Yao, K. (2004). Preparation and characterization of poly(L-lactide)/poly(e-caprolactone) fibrous scaffolds for cartilage tissue engineering. Journal of Applied Polymer Science. **91:** 1676.

Zheng, Y., Wang, Y.Chen, X., Lu, H., Liu, Q.Key Engineering Materials Volume 330-332 I, 2007, Pages 475-478 . (2007). Bioactive hydroxyapatite/polyvinyl alcohol composite for repair and regeneration of articular cartilage. Key Eng Mater. **330-332:** 475.

Zhou, S., Eid, K., Glowacki, J. (2004). Cooperation between TGF- beta and Wnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. J Bone Miner Res. **19:** 463.

Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., Hedrick, M.H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng **7**: 211.

APPENDIX

APPENDIX-A

1. DIGESTION MEDIA

DMEM HG (Gibco)

0.2% Collagenase type II (Sigma)

Antibiotic-antimycotic solution (1X) (Gibco)

Syringe filtered 0.22 □m (Millipore)

2. RESUSPENSION MEDIA

DMEM HG (Gibco)

10 % FBS (Gibco)

3. CHONDROGENIC MEDIUM

DMEM-HG (Gibco)

10% Foetal bovine serum (FBS) (Gibco)

Antibiotic-antimycotic solution (1X) (Gibco)

Glutamine (2mM) (Gibco)

Non essential amino acids (NEAA) (0.1M) (Gibco)

Sodium pyruvate (1mM) (Gibco)

Lproline (40μg/ml) (Sigma)

Ascorbic acid (50μg/ml) (Sigma)

Dexamethasone (10^{-7}M) (Sigma)

4. PAPAIN BUFFER SOLUTION

Papain (Wako) – 6mg

EDTA – 11.9 mg

N acetyl cysteine – 7.2mg

Phosphate buffer (0.1M) pH 6.5 – 20 ml

Make upto 20 ml

5. (1, 9 DIMETHYLMETHYLENE BLUE) (DMMB) 10X

((1, 9 Dimethyl) methylene blue) (16 mg in 5 ml ethanol)

Sodium formate (2g)

Formic acid (2g)

Made upto 100 ml, filtered and stored at RT

he dye is diluted to 1X using distilled water and used for the assay

APPENDIX-B

LIST OF PUBLICATIONS

Publications in international journals

1. *Neethu Mohan, Prabha D. Nair.* Poly(vinyl alcohol)-Poly(caprolactone) Semi IPN Scaffold with implication for cartilage tissue engineering. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2008, 84B, 2, 584-594.
2. *Neethu Mohan, Prabha D Nair.* A 3 D biodegradable protein based matrix for cartilage tissue engineering and stem cell differentiation to cartilage. (Journal of Material Science: Materials in Medicine) (Accepted).

Publications in conference proceedings

1. *Neethu Mohan, Prabha D Nair.* Role of scaffold composition in regulating the chondrogenic phenotype during invitrotissue regeneration. 8th World Biomaterial Congress, 2008, Amsterdam, Netherlands. (*Accepted for oral presentation*)

2. *Neethu Mohan, Prabha D Nair.* A 3 D biodegradable protein based matrix for cartilage tissue engineering and stem cell differentiation to cartilage. 10 th International conference on advance materials, IUMRS-ICAM, 2007, India.

3. *Neethu Mohan, Prabha D Nair.* Protein based scaffold for cartilage tissue engineering.5 th Annual meeting of ISSCR, 2007, Australia.

4. *Neethu Mohan, Prabha D Nair*. Growth factor mediated mesenchymal stem cell differentiation to chondrocytes in novel 3D scaffolds with implication for cartilage tissue engineering. 19th Kerala science congress, 2007, India.

5. *Neethu Mohan, Prabha D Nair.* Porous 3D scaffolds for Tissue Engineering Applications. Indo australian conference on biomaterials, implantable devices and tissue engineering, BITE, 2005, India.

6. *Neethu Mohan, Prabha D Nair.* Novel porous 3D scaffolds for cartilage tissue engineering applications. Regenerate 2005, USA.

7. *Neethu Mohan, Prabha D Nair.* Potential of Poly(caprolactone)-poly(vinyl alcohol) blend scaffold for tissue engineering. Tissue Engineering Society International annual meeting, Shanghai, China, 2005.

Publications communicated

1. *Neethu Mohan, Prabha D Nair.* Growth factor mediated effects on mesenchymal stem cell differentiation on 3D Semi IPN poly(vinyl alcohol)-poly(caprolactone) scaffolds with implication for cartilage tissue engineering.

2. *Neethu Mohan, Prabha D Nair.* Role of scaffold composition and phenotype in regulating the chondrogenic phenotype during invitrotissue regeneration. (to be communicated)

APPENDIX-C

LIST OF ABBREVIATIONS

αMEM	α Minimum Essential Medium
3D	3 dimensional
BMP	Bone morphogenetic protein
cDNA	Complementary DNA
DAB	Diamino benzidine
DCC	Dicyclohexylcarbodiimide
DMEM	Dulbecco's Modified Eagles Medium
DMMB	((1, 9- dimethyl)methylene blue)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidephosphate
DSC	Differential Scanning Calorimetry
DTG	Differential thermogravimetry
DTT	Dithiothreitol
ECM	Extra cellular matrix
EDAC	1-ethyl-3(3-dimethyl aminopropyl) carbodiimide hydrochloride
EDTA	Ethylene diamine tetraacetic acid
ESEM	Environmental scanning electron microscopy
EtBr	Ethidium bromide
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FITC	Flourescien isothiocyanate
FTIR	Fourier transform Infrared Spectroscopy
GA	Gelatin-Albumin
GADPH	Glyceraldehyde phosphate dehydrogenase
GAG	Glycosaminoglycans

HDPE	High density polyethylene
HRP	Horse raddish peroxidase
LEP	Liquid extrusion porosimetry
MICRO CT	Micro Computed Tomography
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Mw	Molecular weight
NEAA	Non essential amino acids
oligodT	Oligo deoxyribo thymidine
PBS	Phosphate buffer saline
PCL	Poly(caprolactone)
PCR	Polymerase chain reaction
PE	Phycoerythrin
PLGA	Poly(lactide –co-glycolide)
PVA	Poly(vinyl alcohol)
PVC	Polyvinyl chloride
RGD	Arginine-glycine-aspartic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RNaseH	Ribonuclease H
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SDT	Simultaneous TGA-DTA
SEM	Scanning electron microscopy
Semi IPN	Semi Interpenetrating Polymer Network
SLS	Sodium lauryl sulphate
TGA	Thermogravimetric Analysis
TGFβ	Transforming growth factor β
THF	Tetrahydrofuran